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Kaolin incorporated diet on growth and immune response in *Ctenopharyngodon idellus* against *Aeromonas hydrophila*

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ABSTRACT

The effect of kaolin enriched diet on growth, hematology, and immune response in the grass carp *Ctenopharyngodon idellus* against *Aeromonas hydrophila* is reported. Both the infected and uninfected groups fed with kaolin enriched diet gained better weight, protein efficiency ratio (PER) and feed conversion ratio (FCR). The survival rate was 98.3% in control (C) and uninfected (UI) fed with 2 g kg^{-1} kaolin diet, whereas it was 95.0% in uninfected (UI) fed with 1 g kg^{-1} or infected (I) fed with 1 g kg^{-1} kaolin diets. In both groups the red blood cell (RBC) and white blood cell (WBC) counts, percentage of lymphocytes and eosinophils, level of albumin and globulin increased in uninfected (UI) fed with 1, 2, and 3 g kg^{-1} diets. The serum Ig level significantly increased in both groups when fed with 3 g kg^{-1} diet, whereas the phagocytic activity did not increase significantly. Further the respiratory burst activity in both groups significantly increased with any supplemented diet. The serum superoxide dismutase (SOD) activity increased significantly in the infected group fed with 2 g kg^{-1} diet and the uninfected group fed with 1 g kg^{-1} diet. The complement activity was significantly enhanced in both groups when fed with 1 and 2 g kg^{-1} diets; the lysozyme activity increased with 2 g kg^{-1} diet; besides the group was 5% mortality whereas 10% mortality was observed when fed with 1 or 3 g kg^{-1} diets. The present results suggest that *C. idellus* fed with kaolin enriched diet promotes growth, hematology, innate and adaptive immune response against *Aeromonas hydrophila* infection.

1. Introduction

Among the family Cyprinidae the grass carp *Ctenopharyngodon idellus* (Val. 1844) is distributed worldwide and is one of the most favored species for aquaculture with lucrative commercial value [1] as an affordable aquaprotein. The genus *Ctenopharyngodon* is a native of East Asia from the Amur River especially in eastern Russia and endemic in the rivers from south to West River of China [2,3]. In China, the grass carp was cultivated since time immemorial for food and was introduced in the Northern Hemisphere in countries such as Europe, United States and subsequently in other parts of the world essentially for aquatic weed control; it is eurythermal thriving well from 20 to 30 °C and is amenable for controlled reproduction. The production cost of grass carp is normally about USD 0.50/kg [4]. The global production of farmed grass carp increased from about 10,000 tonnes in 1950 to over 1,00,000 tonnes in 1972; its production exceeded 1 million tons in 1990 shooting to above 3 million tons in 1999. Currently the global production exceeds over 5 million tons per year [5].

Of late along with increasing intensification and diversification of aquaculture practices to maximize production the correspondingly increasing out breaks of diseases pose a major threat resulting in serious economic loss in aquaculture [6]. Indeed high intensification of aquaculture triggers stressful conditions, such as poor water quality and the proliferation of opportunistic pathogens which may amplify the risk of disease [7]. Despite their excellent growth and large-scale breeding the grass carp remains susceptible to various diseases like haemorrhagic disease (Reovirus (GCRV)), bacterial septicemia (*Aeromonas sobria*, *A. hydrophila*, *Yersinia ruckerri*, *Vibrio* sp.), bacterial enteritis (*A. punctata f. intestinalis*), erythroderma (red-skin disease, *Pseudomonas fluorescens*), bothriocephalosis (*Bothriocephalus* sp.), dactylogyriasis (*Dactylogyrus* sp.), *Ichthyophthiriasis* (*Ichthyophthirius multifiliis*), sinergasiliasis (*Sinergasilus*) [4]. In China, one of the serious diseases affecting about 80% of the cultured grass carp is hemorrhagic virus disease (HVD) caused by a retrovirus [8]. In Europe, a rhabdovirus with pathological signs

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similar to the HVD has been isolated from grass carp [9]. Septicemia is another bacterial disease caused by *Aeromonas*; its main symptoms are abdominal dropsy, gill festering, visible optical and cloacal congestion caused by *A. veronii*, *A. hydrophila*, and *A. salmonicida* [10,11]. The outbreaks of *A. hydrophila* bacterium results in high mortality slicing down the production with severe economic loss to grass carp farming industry [12–14].

Generally, antibiotics and chemotherapeutics have been used to control fish diseases resulting in the emergence of drug-resistant pathogens, and associated problems of increased toxicity, residues causing public health and environmental problems [15]. Fish vaccines are effective preventive measures, but the vaccines are often specific to a particular pathogen; besides vaccination of fish is labor-intensive and expensive [16]. Recently, natural clay as feed additive have been reported to enhance growth, immuno-stimulant, conferring disease resistance by boosting immunity in fish and shellfish [17–24]. As an additive the natural clays have become a new line of alternative to antibiotics and chemotherapeutics in the effective prevention of diseases [25–28].

Kaolinite ($Al_2Si_2O_5(OH)_4$) is a layered silicate mineral clay, with one tetrahedral sheet linked through oxygen atoms to one octahedral sheet of alumina and octahedral layers [29]. Rocks rich in kaolinite comprise kaolin which has a low shrink-swell and a low cation exchange capacity. Kaolin, a plastic raw material mostly comprises the clay mineral with kaolinite is classified as phyllosilicate clay. It is a soft, white or yellowish white powder with clay-like taste. Kaolin is considered as a simple effective preventive tool against a number of toxic materials in both in the environment as well as in living organisms due to its adsorption qualities and absence of primary toxicity [30]. Kaolin-based medicaments are commonly used to treat diarrhoeal and digestive disorders in human [30–33]. Supplementation of kaolin in animal diets reduces resorption of harmful toxins present in the feed through intestinal mucosa in animals against diarrhoea [34], aflatoxins [35], pathogenic microorganisms, heavy metals [36,37], and poisons [38]. There is no evidence of side effects on the host health; neither there has been any adverse effect in hematological and biochemical parameters. It has a protective effect against entero-toxigenic strains like *Escherichia coli* (ETEC). The weaned piglets fed with kaolin containing diet at 1% level had a positive effect ($P < 0.05$) on body weight gain (BWG) in both infected and non-infected ETEC piglets; the recommended dose as feed additive is from 1% to 3% [39].

In fish the innate immune response constitutes a major defense mechanism; it also plays a primary role in improving the acquired immune response and homeostasis. Treatment with natural clay as an immune stimulant is deemed safe in both the fish health and environment in aquaculture. Therefore, the present study was undertaken to investigate the effects of dietary kaolin on growth, immune response, and disease resistance in *C. idellus* against *A. hydrophila* for the first time.

2. Material and methods

2.1. Diet

Dietary ingredients with corn flour, rice polish, and wheat flour as carbohydrate source; fish meal, and gluten, and cellulose as protein source; cod liver oil as lipid source along with premix vitamins and minerals (Table 1); supplementation diet with four different levels of kaolin was prepared as: 0 (basal control diet without kaolin), 1, 2, and 3 g kg^{-1} kaolin supplementation in basal diet by thoroughly mixing the ingredients. The experimental diets were passed separately through a grinder and paste extruder (1 mm diameter) mesh sieve. The prepared diets were air dried and stored at −20 °C until used. The proximate composition such as crude protein, crude lipid, crude carbohydrate, crude ash, and crude fiber of the diets were quantified by following AOAC [40].

Table 1
Feed ingredients and proximate composition (g/kg dry matter) of experimental diets for *C. idellus*.

Ingredient	0 g kg^{-1}	1 g kg^{-1}	2 g kg^{-1}	3 g kg^{-1}
Fish meal	25.0	25.0	25.0	25.0
Rice polish	10.0	10.0	10.0	10.0
Corn flour	10.0	10.0	10.0	10.0
Wheat flour	47.5	47.5	47.5	47.5
Gluten	0.5	0 5	0.5	0.5
Cellulose	0.5	0 5	0.5	0.5
Ascorbic acid	0.5	0 5	0.5	0.5
Carboxymethyl cellulose	3.0	2.0	1.0	0.0
Vitamin premix*	1.0	1.0	1.0	1.0
Cod liver oil	2.0	2.0	2.0	2.0
Kaolin	0.0	1.0	2.0	3.0
Total	100.0	100.0	100.0	100.0
Proximate composition (%)				
Crude protein (%)	21.2	21.8	22.6	23.3
Crude lipid (%)	8.4	8.6	8.9	8.5
Crude carbohydrate (%)	28.8	29.6	32.4	31.2
Crude ash (%)	6.5	6.8	7.5	7.8
Crude fiber (%)	1.6	1.9	2.2	2.5

*1 g vitamin premix containing choline chloride 500 mg; thiamine HCl 5 mg; riboflavin 20 mg; pyridoxine HCl 5 mg; nicotinic acid 75 mg; calcium pantothenate, 50 mg; inositol, 200 mg; biotin 0.50 mg; folic acid 1.50 mg; ascorbic acid 100 mg; menadione (K) 4 mg; a-tocopheryl acetate (E) 40 mg; cyanocobalamine (B12) 0.0001 mg.

2.2. Pathogen

Aeromonas hydrophila was isolated from the kidney of infected fish showing clinical signs like fin rot and gill necrosis, body lesion, scale protrusion, severe deep ulcerative lesions together with swollen kidney and liver. The collected sample was grown on tryptic soy agar (TSA) agar plates for 24 h at 30 °C. The bacterial cells were subculture, purified, and resuspensions with 0.85% NaCl or PBS (physiological saline or phosphate buffered saline) and the resultant bacterial cell suspension were determined by using a Neubauer haemocytometer at a concentration of 4.5×10^7 cfu ml^{-1} [41]. The biochemical characteristics of the bacterial cells were carried out by microbial biochemical identification tubes (Hangzhou Microbial Reagent Co. Ltd) following the manufacture's instruction. Genomic DNA sample was extracted from the bacterial cells and performed polymerase chain reaction (PCR) using universal primers (ABI 3730), forward primer 27F: 5′-agagtttgatcctggctcag-3′ and reverse primer 1492R: 5′-tacggctaccttgttacgactt-3’ (Sangon Biotech, Shanghai Co. Ltd.) were used to amplify 16S genomic DNA gene sequences [42,43] obtained based on BLAST search in GeneBank database [44].

2.3. Fish and experimental design

Healthy *Ctenopharyngodon idellus* (34.8 ± 1.4 g) were purchased from a local fish farm. The fish were examined their health status immediately upon arrival. Then fish were dip treated with 1% $KMnO_4$ solution and acclimated for 2 weeks in 80 L aerated fiber tanks prior to experiment. After acclimation, the fish were divided into eight groups of 25 each in triplicate (8 × 25 x 3 = 600 fish) as: Group I: uninfected fish fed with basal control (C) diet without kaolin, Group II: uninfected fish fed with diet containing: 1 g kg^{-1} (UI-1 g kg^{-1}), Group III: 2 g kg^{-1} (UI-2 g kg^{-1}), Group IV: 3 g kg^{-1} (UI-3 g kg^{-1}) kaolin; Group V: infected fish fed with control diet (I-C), Group VI: infected fish fed diet containing: 1 g kg^{-1} (I-1 g kg^{-1}) diet, Group VII: infected fish fed diet containing 2 g kg^{-1} (I-2 g kg^{-1}), and Group VIII: infected fish fed with diet containing 3 g kg^{-1} (I-3 g kg^{-1}) kaolin at the rate of 5% of their body weight twice a day at 10.00 a.m. and 01:00 p.m. The respective diets were continued till the end of experiment and the unfed feed was carefully collected to quantify the feed utilization and conversion.

About 50% of water was exchanged daily. After 30 days of feeding, Groups V to VIII were challenged intraperitoneally (i.p.) with 50 μl PBS containing *A. hydrophila* at 4.5×10^7 cfu ml^{-1} whereas the other groups (Groups I to IV) were injected 50 μl i.p. with PBS alone. After post-challenge at the end of weeks 1, 2 4, 6, and 8, six fish were randomly collected from each experimental group to collect blood samples by the caudal venipuncture with a 27 gauge needle fitted 1 ml syringe for hematological and immunological assays after fish anaesthetizing with MS-222 ($NaHCO_3$ and tricaine methane sulphonate; Sigma Chemicals, USA) 1:4000 in dechlorinated water for 2 min.

2.4. Sample collection

The blood samples were pooled from a random sample of six fish in each experimental tank separately and the samples were divided into nonheparinized and heparinized (K3EDTA vacuum) tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) consecutively to obtain the serum and whole blood, respectively.

2.5. Growth performance, nutrient utilization, and survival rate

At the end of weeks 1, 2, 4, 6, and 8 post-challenge, the fish were fasted for 24 h before and moderately anaesthetized with MS 222 than the fish were harvest in each group for weighed. Based on recording the weight of each fish and counting the number of fish, mean weight gain (MWG), specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), protein intake (PI), and fish survival rates (SR %) were calculated according to Harikrishnan et al. [45] using the following equations:

WG = [initial body weight] – [final body weight]

SGR = [in final body weight - in initial body weight] / number of days x 100

PER = [% protein in diet] x [weight of diet consumed] / 100

PI = [fish wet weight gain] / [fish consumed protein] x 100

FCR = [feed consumed] / [weight gain]

Survival (%) = [final number of fish / initial number of fish] x 100.

2.6. Hematology

Hematological parameters such red blood corpuscle (RBC) and white blood corpuscle (WBC) were counted by Neubaur's improved haematocytometer (Superior, Marienfeld, Germany) using Hyem's and Turk's as a diluting fluid, respectively. The differential leucocytes was count after selecting 100 leucocytes from each smear under oil immersion microscope and the percentages (%) of lymphocytes, monocytes, neutrophils, and eosinophils were calculated by counting 100 cells [46,47]. The serum total protein concentration was estimated by Biuret colourimetric reactions [48] while the serum albumin and globulin concentrations were estimated by bromocresol green colourimetric reaction [49,50].

2.7. Immunological assays

2.7.1. Preparation of head-kidney leukocyte

The nonheparinized whole blood samples were allowed to clot at room temperature for 4 h and the samples were centrifugation (3000 × g) at 4 °C for 10 min, than collect blood serum separately and stored at −80 °C until used for immunological assay. The head-kidney (HK) was removed, cut into small pieces (fragments), and transferred to 8 ml of sRPMI [51]. The cell suspensions were obtained by forcing of the fragments through a nylon mesh (100 mm size), washed twice by centrifugation at 400*g* for 10 min, count and adjusted to 10^7 cells ml^{-1} in sRPMI by trypan blue exclusion test.

2.7.2. Immunological assay

The complement activity was determined by using sheep red blood cells (SRBC, Biomedics) as targets [52] and total serum IgM level analysis by enzymatic method utilizing an automatic biochemical analyser (Hitachi 7180, Tokyo, Japan) expressed as unit mg ml^{-1} [53]. The respiratory burst activity of grass carp HK leucocytes was measured by a chemiluminescence method by using phorbol myristate acetate (PMA, Sigma) and luminol (Sigma) [54] and the phagocytic activity of HK leucocytes was calculated through flow cytometry at 488 nm [55]. The lysozyme activity of all serum were measured by turbidimetric assay [56] and the superoxide dismutase (SOD) activity was calculated by its ability to inhibit superoxide anion generated by xanthine and xanthine oxidase reaction system using an SOD detection kit (Nanjing Jiancheng Bioengineering Institute, China) [57].

2.8. Mortality, reisolation, and identification of the bacteria

A group of 20 fish (20 × 8 = 160) were used in each experimental group (Groups I to VIII) separately for mortality. The challenge study, bacterial preparation and concentration, and the experimental conditions were same as mentioned previous section. The mortalities were recorded for 30 days and the cause of mortality was determined and confirmed by reisolation of bacteria from infected fish kidney tissues [58,59] streaked onto TSA agar plate. The identity of *A. hydrophila* was confirmed by morphological, pictorial, biochemical characteristics, and PCR method [42,43]. The cumulative mortality (CM) and relative percent survival (RPS) values in each group were calculated over 30 days as follows:

CM (%) = 100 - [(treatment mortality / control mortality)] x 100

RPS (%) = 1–[(% mortality of challenged group)] / [(% mortality of unchallenged group)] x 100

2.9. Statistical analysis

The data are expressed as standard error of means (means ± SE) and the differences between the means were calculated by Tukey's multiple range test (SPSS 11.0 statistical software). The p values < 0.05 is considered significant.

3. Results

3.1. Growth

Grass carp fed on enriched diets registered improved growth, feed and protein utilization efficacy (Table 2). An overall weight gain was obtained in uninfected and infected fish fed with any enriched diet (1, 2 and 3 g kg^{-1}) fed for 8 weeks. However, weight of the infected fish fed with control diet (without kaolin) declined. The feed intake on being fed with1 and 2 g kg^{-1} diets did not differ significantly from the control whereas with I-3 g kg^{-1} diet or I-C it decreased significantly. The ratios of protein percentage efficiency and feed conversion did not significantly differ from control. The survival rate was 98.3% in C and UI-2 g kg^{-1} groups; it was 95.0% in UI-1 g kg^{-1} and I-1 g kg^{-1} diet fed groups. The survival was 96.7% in UI-3 g kg^{-1} and I-2 g kg^{-1} fed groups while the least survival was (93.3%) obtained in I-C group.

3.2. Hematology

Though the RBC counts increased in uninfected and infected fish fed with 1, 2, and 3 g kg^{-1} diets the increase was not significantly different as compared with control. A similar result prevailed with WBC counts.

Table 2
Growth performance and feed utilization of *C. idellus* fed diet containing kaolin against *A. hydrophila* for 8 weeks.

Indices	C	UI-1 g kg^{-1}	UI-2 g kg^{-1}	UI-3 g kg^{-1}	I-C	I-1 g kg^{-1}	I-2 g kg^{-1}	I-3 g kg^{-1}
Initial weight (g)	34.8 ± 1.14	34.6 ± 0.11	34.5 ± 1.21	34.7 ± 1.13	34.4 ± 1.16	34.7 ± 1.15	34.8 ± 1.16	34.9 ± 1.22
Final weight (g)	68.4 ± 3.62	70.4 ± 2.86	71.2 ± 3.32	69.5 ± 2.78	53.7 ± 3.38	67.8 ± 3.44	69.2 ± 2.86	65.8 ± 3.18
Weight gain (g)	33.6 ± 1.78a	35.8 ± 2.16a	36.7 ± 2.48a	34.8 ± 2.66a	19.3 ± 1.98a	33.1 ± 2.74a	34.4 ± 2.46a	30.9 ± 2.68a
Feed intake (g)	44.5 ± 2.1a	42.2 ± 2.2a	43.8 ± 2.2a	41.6 ± 2.6b	35.7 ± 2.0c	42.4 ± 2.2b	43.3 ± 2.8b	39.5 ± 2.2b
SGR (%/day)	0.65 ± 0.034a	0.66 ± 0.04a	0.71 ± 0.03b	0.65 ± 0.04a	0.53.6 ± 0.03c	0.63 ± 0.04a	0.65 ± 0.05a	0.61.4 ± 0.03a
PER	0.79 ± 0.12a	0.82 ± 0.14a	0.86 ± 0.16a	0.81 ± 0.12a	0.67 ± 0.11b	0.80 ± 0.14a	0.83 ± 0.14a	0.79 ± 0.12a
FCR	1.31 ± 0.02a	1.35 ± 0.02a	1.40 ± 0.04b	1.33 ± 0.02a	1.26 ± 0.02c	1.33 ± 0.04a	1.36 ± 0.04a	1.31 ± 0.02a
Survival rate (%)	98.3	95.0	98.3	96.7	93.3	95.0	96.7	95.0

SGR: specific growth rate, PER: protein efficiency ratio, FCR: feed conversion ratio. C: control, I-C: infected control, UI: uninfected, I: infected.

Table 3
Hematological and biochemical profiles of *C. idellus* fed diet containing kaolin against *A. hydrophila*.

Parameter	Weeks	C	UI-1 g kg^{-1}	UI-2 g kg^{-1}	UI-3 g kg^{-1}	I-C	I-1 g kg^{-1}	I-2 g kg^{-1}	I-3 g kg^{-1}
RBC (million/m^3)	1	2.12^a	3.16^d	3.24^d	3.14^d	2.08^c	3.02^c	3.10^c	2.86^b
	2	2.12^a	3.32^d	3.38^d	3.22^d	2.08^c	3.06^c	3.16^c	2.90^b
	4	2.13^a	3.38^d	3.46^d	3.34^d	2.09^a	3.08^c	3.22^d	2.94^b
	6	2.14^a	3.52^d	3.58^d	3.42^d	2.10^a	3.16^c	3.28^d	3.02^b
	8	2.15^a	3.48^d	3.60^d	3.48^d	2.11^a	3.21^b	3.31^d	3.16^b
WBC (Per µl)	1	4325.2^a	4338.8^a	4343.4^a	4331.1^a	4321.5^a	4335.3^a	4339.8^a	4328.6^a
	2	4328.4^a	4352.2^b	4348.8^b	4354.5^b	$4314.4^a 4352.5^b$	4386.4^c	4344.2^b	
	4	4329.6^a	4367.4^b	4455.2^b	4373.8^b	4302.8^a	4374.6^a	4422.6^c	4373.3^b
	6	4330.4^a	4444.4^c	4471.1^c	4388.4^b	4284.2^a	4389.8^b	4451.2^c	4382.5^b
	8	4332.8^a	4498.2^c	4544.5^d	4426.7^c	4262.4^b	4414.8^c	4485.7^c	4396.1^c
Total protein (mg/dl)	1	2.23^a	2.28^a	2.31^a	2.26^a	2.14^b	2.26^a	2.27^a	2.24^a
	2	2.25^a	2.33^a	2.36^a	2.28^a	2.05^b	2.33^a	2.32^a	2.21^a
	4	2.26^a	2.44^b	2.58^b	2.39^a	1.84^c	2.38^a	2.45^b	2.29^a
	6	2.28^a	2.54^b	2.86^b	2.45^a	1.72^c	2.47^a	2.54^c	2.43^a
	8	2.30^a	2.86^b	3.42^c	2.65^b	1.55^d	2.63^b	3.34^c	2.55^b
Albumin (mg/dl)	1	1.32^a	1.36^a	1.46^b	1.34^a	1.26^a	1.39^a	1.56^b	1.32^a
	2	1.34^a	1.52^a	1.71^b	1.43^a	1.21^a	1.48^a	1.62^b	1.38^a
	4	1.38^a	1.74^b	1.93	1.62^b	1.16^c	1.65^b	1.82^b	1.55^a
	6	1.41^a	1.95^b	2.24^c	1.81^b	1.04^d	1.83^b	2.12^c	1.71^a
	8	1.45^a	2.32^c	2.48^c	2.19^c	0.92^d	2.13^c	2.23^c	1.93^b
Globulin (mg/dl)	1	1.36^a	1.44^a	1.52^b	1.39^a	1.32^a	1.41^a	1.46^a	1.36^a
	2	1.38^a	1.71^b	1.76^b	1.65^b	1.27^a	1.66^b	1.62^b	1.61^b
	4	1.40^a	1.87^b	1.91^b	1.77^b	1.21^c	1.75^b	1.79^b	1.68^b
	6	1.42^a	2.05^c	2.11^c	1.88^b	1.17^d	1.89^b	1.84^b	1.75^a
	8	1.45^a	2.23^c	2.38^c	2.16^c	1.10^d	2.15^c	2.14^c	1.98^b
Lymphocytes (%)	1	30.3^a	31.4^a	31.7^a	31.1^a	29.3^a	31.1^a	31.4^a	31.2^a
	2	30.5^a	31.9^a	32.2^b	31.3^a	28.6^c	31.5^a	32.0^b	31.0^a
	4	30.9^a	32.5^b	32.9^b	32.1^b	28.1^c	32.0^b	32.5^b	31.7^a
	6	31.5^a	33.2^c	33.6^c	32.6^b	27.5^d	32.8^b	33.2^c	32.4^b
	8	31.8^a	34.0^c	34.4^c	33.6^b	27.1^d	33.5^b	33.9^b	33.7^b
Monocytes (%)	1	2.2^a	2.3^a	2.5^a	2.4^a	2.2^a	2.2^a	2.4^a	2.3^a.
	2	2.2^a	2.5^a	2.7^a	2.4^a	2.0^a	2.3^a	2.5^a	2.2^a
	4	2.3^a	2.7^a	3.0^b	2.5^a	2.0^a	2.5^a	2.8^a	2.3^a
	6	2.3^a	3.0^b	3.6^b	2.8^a	1.9^a	2.8^a	3.3^b	2.5^a
	8	2.4^a	3.3^b	3.9^b	3.1^b	1.9^a	3.0^b	3.5^b	2.7^a
Eosinophils (%)	1	6.0^a	6.2^a	6.5^a	6.3^a	5.9^a	6.1^a	6.3^a	6.1^a
	2	6.1^a	6.6^a	6.9^a	6.4^a	5.9^a	6.3^a	6.5^a	6.2^a
	4	6.1^a	7.3^b	7.6^b	6.9^a	5.8^a	6.9^a	7.2^b	6.3^a
	6	6.3^a	7.5^b	7.9^a	7.1^a	5.7^a	7.1^b	7.4^b	6.5^a
	8	6.3^a	8.0^c	8.3^c	7.8^b	5.5^a	7.4^b	7.8^b	6.8^a
Neutrophils (%)	1	25.5^a	25.8^a	26.0^a	26.0^a	25.2^a	25.4^a	25.8^a	25.7^a
	2	25.6^a	26.1^a	26.5^a	26.0^a	25.0^a	25.8^a	26.2^a	26.0^a
	4	25.7^a	26.5^a	26.9^b	26.3^a	24.5^a	26.1^a	26.5^a	26.0^a
	6	25.7^a	26.5^a	27.5^b	26.5^a	24.0^b	26.2^a	27.2^b	26.3^a
	8	25.8^a	26.8^a	28.1^c	26.5^a	23.8^c	26.5^a	27.7^b	26.5^a

RBC: red blood cell, WBC: white blood cell, C: control, I-C: infected control, UI: uninfected, I: infected.

Though the total protein slightly increased in uninfected and infected groups fed with 1, 2, and 3 g kg^{-1} diets, in the infected fish (I-C) fed with control diet it declined significantly. The albumin and globulin levels increased significantly in the uninfected and infected groups fed with 1, 2, and 3 g kg^{-1} diets from weeks 1–8 when compared with control. The lymphocytes and eosinophils increased in both groups fed on supplemented diet; however, the difference was significant when fed with 1 and 2 g kg^{-1} diet after week 6. Though the monocytes and neutrophils increased in both groups with any supplemented diet, the difference was significant only when fed with 2 g kg^{-1} diet on week 8

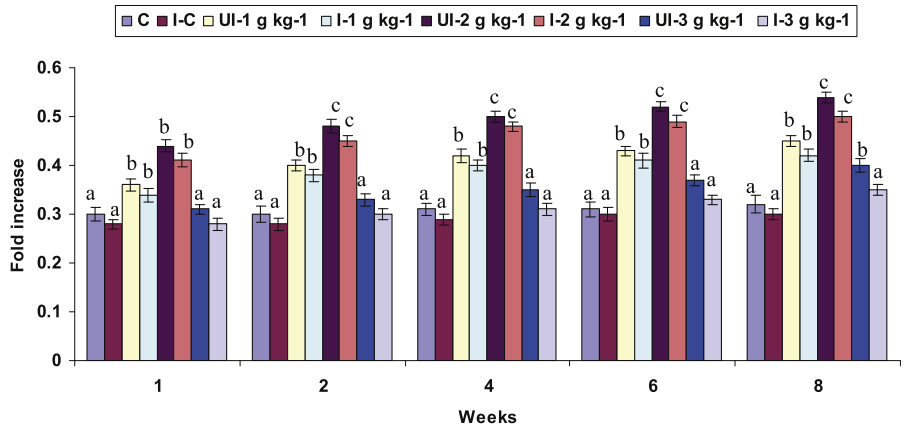

Fig. 1. Phagocytic activity of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical data expressed as mean (SEM) in the same column and same superscript letter did not significantly different as determined by Tukey's test ($p > 0.05$).

(Table 3).

3.3. Immune response

3.3.1. Phagocytic activity

The phagocytic activity increased in the uninfected and infected fish fed with any supplemented diet. However, the difference was significant in the uninfected and infected groups fed with 1 and 2 g kg^{-1} diet after second week. The phagocytic capacity deceased when the infected fish were fed with control diet (Fig. 1).

3.3.2. Serum Ig

The serum Ig level did not increase significantly in uninfected and infected fish fed with 1 or 3 g kg^{-1} supplemented diets on week 1 and 2 whereas it significantly increased in both groups fed with 3 g kg^{-1} diet. The serum Ig level also significantly increased when fed with any supplemented diet after fourth week as compared to control except with 3 g kg^{-1} diet (Fig. 2).

3.3.3. Respiratory burst activity

The respiratory burst activity of uninfected and infected groups fed with any supplemented diet was enhanced significantly during the experimental period. However, it was not significantly enhanced at any time in the infected fed fish with only 3 g kg^{-1} diet (Fig. 3).

3.3.4. Serum superoxide dismutase (SOD) activity

The SOD activity increased significantly in uninfected and infected fish fed with 2 g kg^{-1} diet and in uninfected fish fed with 1 g kg^{-1} diet during the experimental period. However, SOD activity did not increase significantly in other groups such as infected fish fed with 1 g kg^{-1} diet and uninfected and infected fed with 3 g kg^{-1} diet (Fig. 4).

3.3.5. Complement activity

The complement activity increased significantly in both groups fed with 1 and 2 g kg^{-1} diet but the increase was not significant in 3 g kg^{-1} diet fed group (Fig. 5).

3.3.6. Lysozyme activity

The lysozyme activity did not significantly vary in both groups with any supplementation diet from weeks 1–4; however, after week 6 it increased in the both groups with 2 g kg^{-1} diet; it was not shown in the uninfected and infected fish fed with 1 and 3 g kg^{-1} diet when compared to control (Fig. 6).

3.4. Disease resistance

There was no cumulative mortality in control and uninfected fish fed with any supplemented diet. The infected fish fed with 2 g kg^{-1} diet suffered 5% mortality whereas infected fish fed with 1 and 3 g kg^{-1} diet suffered 10% mortality. However, the mortality was 85% in infected fish fed with control diet (Fig. 7).

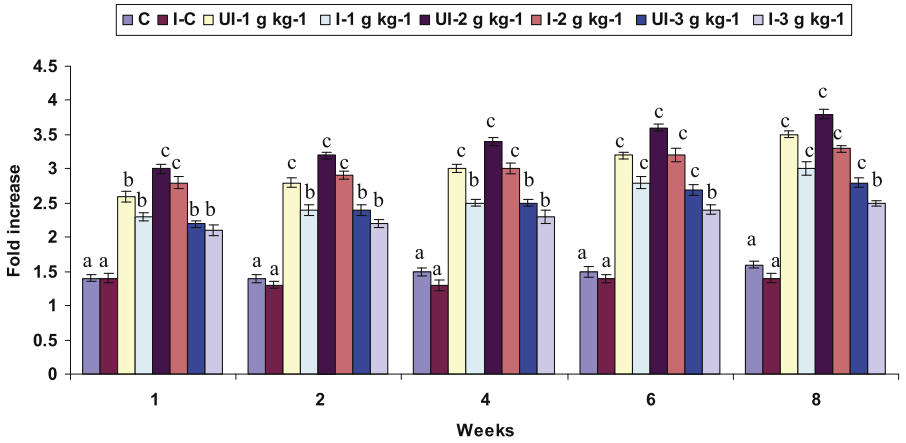

Fig. 2. Serum Ig level of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical information is seen in Fig. 1.

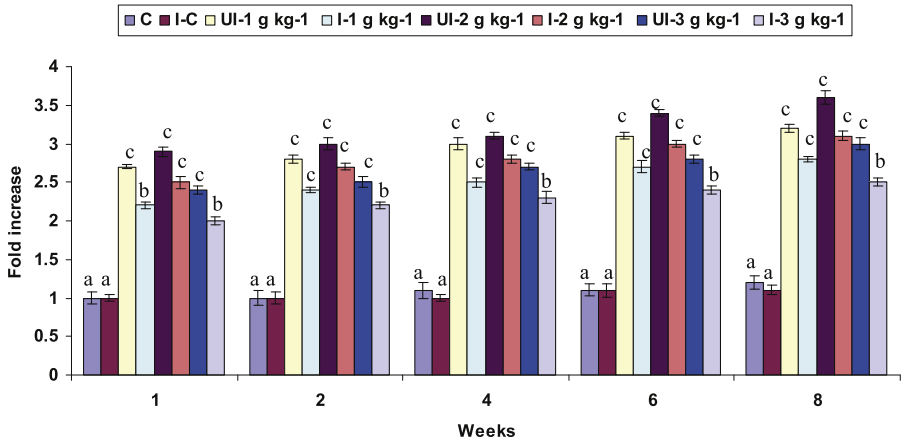

Fig. 3. Respiratory activity of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical information is seen in Fig. 1.

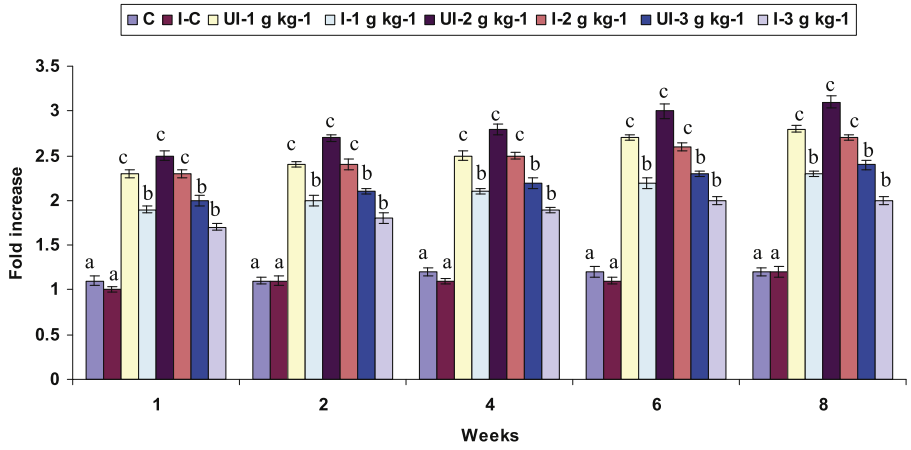

Fig. 4. Serum superoxide dismutase (SOD) activity of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical information is seen in Fig. 1.

4. Discussion

The Gram-negative bacterium, *A. hydrophila* is one of the most common ubiquitous fish pathogens causing diseases like tail rot and epizootic ulcerative syndrome both in the wild and aquaculture. Natural mineral clay immunostimulants, such as azomite, shilajit, and zeolite are known to improve growth and hematological parameters, enhancing the non-specific cellular and humoral defense as well as preventing a number of diseases in several fish and shellfish [17–24].

In this study an overall better weight gain was observed in the uninfected grass carp fed with 1, 2, and 3 g kg^{-1} kaolin enriched diets. *Oreochromis mossambicus, Channa striatus, Macrobrachium rosenbergii, Oncorhynchus mykiss*, and *Lithopenaeus vannamei* also gained weight after being fed with azomite, shilajit, and zeolite supplemented diet [17–24]. In the present study when fish fed with 1 or 2 g kg^{-1} kaolin enriched diets the feed intake did not influence the weight gain. The

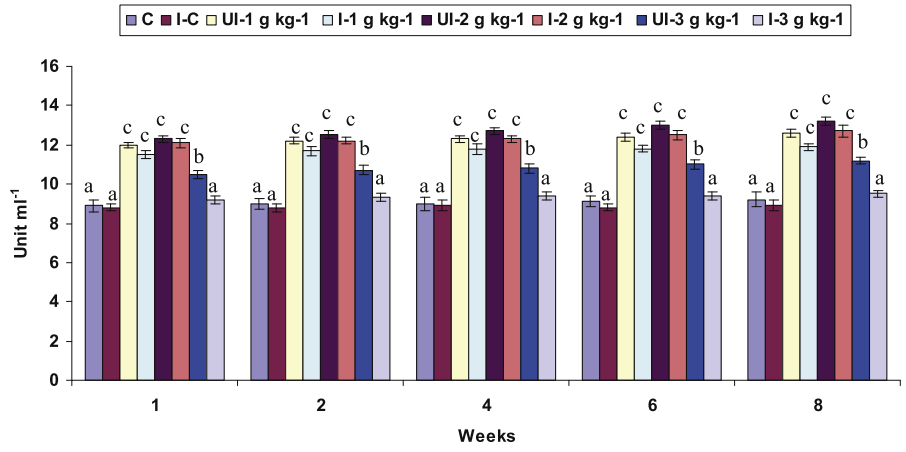

Fig. 5. Complement activity of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical information is seen in Fig. 1.

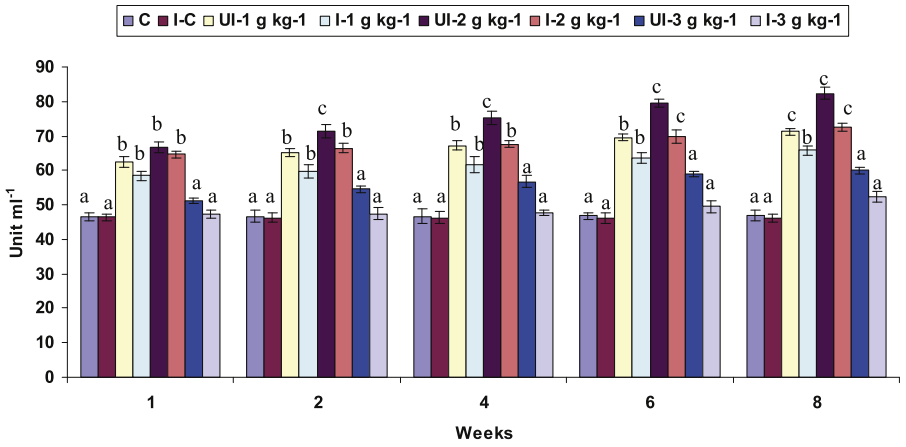

Fig. 6. Lysozyme activity of *C. idellus* (n = 6) fed dietary kaolin against *A. hydrophila*. Statistical information is seen in Fig. 1.

percentage of PEF and FCR also did not differ significantly as reported in previous studies [17–24]. The survival rate was 98.3% in C and UI-2 g kg^{-1} groups, while it was 95.0% in UI-1 g kg^{-1} and I-1 g kg^{-1}diet fed groups. However, a slightly higher survival rate of 96.7% was recorded in UI-3 g kg^{-1} and I-2 g kg^{-1} fed groups followed by the least survival (93.3%) in I-C group.

Hematological parameters provide valuable information in the evaluation of fish health status and stress response. The increase in total WBC count may be due to an initial sign of innate resistance and adaptive immunity [60] which is first line of defense. The RBC and WBC counts increased in uninfected and infected groups fed with 1, 2, and 3 g kg^{-1} diets. Similar results have been documented with zeolite enriched diets in *Sparus aurata* [61] and *C. striatus* [19], *C. idellus and Oreochromis niloticus x O. aureus* [22,23], *O. mykiss* [24]. In the present study the total protein level was slightly high in uninfected and infected fish fed with 1, 2, and 3 g kg^{-1} diets. The albumin and globulin levels also increased in uninfected and infected fish fed with any enriched diet. This result favorably compares with that of *C. striatus* fed with zeolite enriched diet [19]. When the serum albumin level declines the fluid may escape into tissues causing localized oedema reducing the delivery of nutrients to tissues. Decreased serum albumin indicates a diseased condition [62], which is a reliable prognostic indicator for increased risk of morbidity and mortality [63]. The increasing serum globulin values may be due to the stimulation of B lymphocytes differentiation and proliferation by IL-6 and TNF-α [64] that is often implicated in chronic infections by various parasites, viral, and bacterial infections [65]. Differential leukocyte counts indicate the health status of fish; in many cases, they help to evaluate the status of immune system. In *C. idellus*, the differential leucocytes of lymphocytes, monocytes, eosinophils, and neutrophils increased in the uninfected and infected groups on being fed with supplemented diet. Similar findings also have been recorded by Bouic and Etsebeth [66], Nya and Austin [67] on feeding ginger enriched diet to rainbow trout.

The phagocytic activity is the key indicator of non-specific immune response. Higher phagocytic activity might be due to activation of phagocytic cells mostly neutrophils and monocytes in circulation; besides they also activate the complement factors via the alternative pathway, which acts as opsonin enhancing phagocytosis [68]. The phagocytic cells are the most important cellular components of the innate immune system of fish against invading pathogens [69] and constitute the first level of defense [70] along with the proliferation of lymphocytes which is under the influence of gene expression of cytokine [71] or the stimulation by macrophages [72] or an increase in lysozyme activity and other humoral factors [73], neutrophilic granulocytes [74]. The phagocytic activity increased in uninfected and infected fish fed with all supplemented diet but the difference was significant with 1 and 2 g kg^{-1} diet only after second week. The present results are in agreement with *O. mossambicus* fed with azomite enriched diet and *C. striatus* fed with zeolite enriched diet against *A. hydrophila* and *Aphanomyces invadans* [17,19].

Serum Ig levels provide key information on the humoral immune status. Ig as an antibody plays a crucial role in the immune function of mucous membranes greater than all other types of antibody combined [75]. Low Ig levels define some humoral immunodeficiencies while high Ig levels are associated with diseases and hematological disorders [76]. In all gnathostome vertebrates Igs are important in the prevention of bacterial, parasitic, and viral infections and recovery from disease. In the present study the serum Ig level significantly increased in uninfected and infected fish fed with all diet supplemented diets after fourth week.

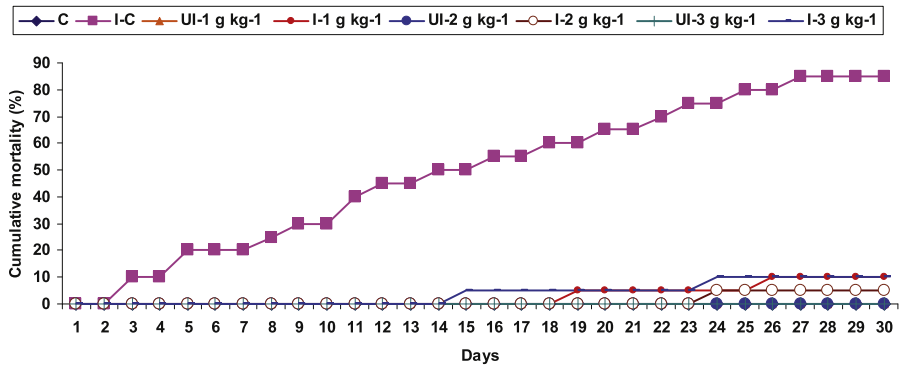

Fig. 7. Cumulative mortality (%) of *C. idellus* (n = 20) fed dietary kaolin against *A. hydrophila* for 30 days.

The respiratory burst sometimes called as 'oxidative burst' is marked by a rapid release of reactive oxygen species (ROS) of superoxide radical and hydrogen peroxide in different types of cells. It plays an important role in the immune system and activates a crucial reaction that occurs in phagocytes to degrade internalized particles and bacteria. In addition, it is an essential component of innate immunity enabling phagocytic cells to eliminate microbes. Excessive amount of ROS production may induce oxidative stress in cells; indeed both pathogenic bacteria and viruses can induce oxidative stress in host cells during infection [77,78]. The respiratory burst of phagocytes is required for the optimal killing of a wide variety of bacteria and fungi [79]. The respiratory burst activity in uninfected and infected groups was significantly enhanced when fed with 1 and 2 g kg^{-1} diets in this study.

Superoxide dismutase (SOD) one of the antioxidant enzymes, protects the cell against the deleterious effects of ROS; it specifically scavenges superoxide by catalyzing dismutation to H_2O_2 and O_2 [80]. The disturbed antioxidant status may be explained on the basis of increased ROS production by phagocytic cells in periodontitis. The strongest evidence to implicate ROS in the pathological destruction of the connective tissue during periodontal disease arises due to the infiltration of polymorpho neutrophils which is a key event of the host response against microbial invasion. Such an infiltration is likely to lead to an increase in the ROS levels [81] causing oxidative stress [82]. Thus, oxidative stress lies at the heart of the periodontal tissue damage that results from host-microbial interactions, either as a direct result of excess ROS activity/antioxidant deficiency or indirectly as a result of the activation of redox-sensitive transcription factors and the creation of a pro-inflammatory state [83]. In this study, SOD activity significantly increased in the uninfected and infected groups fed with 2 g kg^{-1} diet; it was observed in the uninfected group fed with 1 g kg^{-1} diet. Similarly, *C. striatus* fed with zeolite enriched diet had a significantly enhanced SOD activity against *A. invadans* [19]. *O. mossambicus* fed with azomite enriched diet also resulted in enhanced respiratory bust activity of SOD against *A. hydrophila* [17]. *M. rosenbergii* fed with shilajit enriched diet also had enhanced SOD activity against *A. hydrophila* [18].

The complement system consists of over 30 proteins and protein fragments, including serum proteins, serosal proteins, and cell membrane receptors as a part of the immune system that enhances the ability of antibodies and phagocytic cells to clear microbes and damaged cells from an organism, promotes inflammation, and attacks the pathogen's plasma membrane [84,85]. These proteins stimulated by one of several triggers proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages result complement activation or complement fixation cascade that stimulation of phagocytes to clear foreign and damaged material, proxy inflammation to attract additional phagocytes, and activation of the cell-killing membrane attack complex (MAC) [86,87]. The complement activity was significantly enhanced in this study in the uninfected and infected fish fed with 1 and 2 g kg^{-1} diets but it not with 3 g kg^{-1}. The same trends were reported in *C. striatus* fed with zeolite diet against *A. invadans* [19] and *O. mossambicus* fed with azomite enriched diet against *A. hydrophila* [17].

Lysozyme (muramidase or N-acetylmuramide glycanhydrolase) is an antimicrobial enzyme produced by animals and is a major component of Gram-positive bacterial cell wall which constitutes a part of the innate immune system that catalyzes the hydrolysis and breaking of 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan integrity of bacterial cell walls causing lysis of the bacteria [88]. Further the lysozyme activity is an effective tool in attacking the cell wall polysaccharide of different bacterial species, leading to a break down and hydrolyzing a tetrasaccharide found most often in Gram-positive bacteria cell wall and killing them. The lysozyme activity significantly increased in uninfected and infected fish fed with 2 g kg^{-1} diet after week 6 but not with 1 and 3 g kg^{-1} diet in this study. However, it did not significantly increase in uninfected and infected fish fed with any supplementation diet between weeks 1–4. The same was reported recently in *C. striatus* and *O. mossambicus* when fed with azomite and zeolite enriched diets against pathogens [17,19].

There was no cumulative mortality in control and uninfected fish fed with any supplementation diet whereas 5% mortality was observed in infected fish fed with 2 g kg^{-1} diet. However, the mortality was 10% in infected fish fed with 1 and 3 g kg^{-1} diet while it was 85% in infected fish fed with control diet. This result is in agreement with *C. striatus* and *O. mossambicus* when fed with azomite and zeolite enriched diets against pathogens [17,19]. The present study strongly suggests that feeding *C. idellus* with diet containing kaolin at 1 and 2 g kg^{-1} enhances growth, hematological profile, and immunity against *A. hydrophila*. Further detailed molecular studies are needed before incorporating kaolin as a feed supplement in aquaculture.

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