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Choline Chloride Induces Growth Performance of Indian Major Carps and Air-Breathing Fish Species with an Outcome of Quality Food-Fish under a Semi-Intensive Culture System: A Biochemical Investigation

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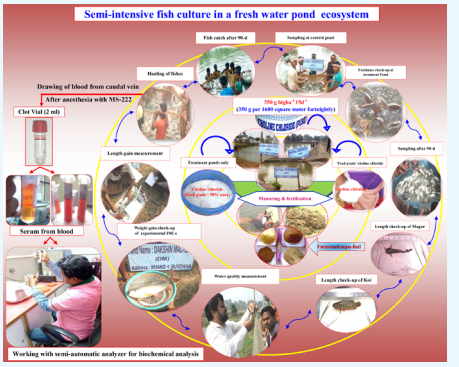
ABSTRACT: The present study was intended to determine the possible influences of direct field application of choline chloride into pond water in addition to farm-made-aqua-feed under a semi-intensive culture system on the growth and biochemical parameters of two Indian major carps (IMCs), *Catla catla* (Catla) and *Labeo rohita* (Rahu), and two air-breathing species, *Clarias batrachus* (Magur) and *Anabas testudineus* (Koi), cultured in a ratio of 2:5:1:1 (Catla/Rahu/Magur/Koi) in three experimental ponds for a period of 90 days during the breeding season (June to August). Results were compared with control (C: fed only with farm-made-aqua-feed) and treatment (T: P1 and P2: farm-made-aqua-feed plus choline chloride into pond water directly at the rate of 350 g bigha^{-1} fortnightly or 350 g per 1600 square meter fortnightly). A significant increasing trend was observed in the growth parameters including total length-final (TLF), standard length-final (SLF), mean weight-final (MWF), % gain of mean total length (MTL), % gain of mean standard length (MSL), % weight gain (WG), specific growth rate (SGR) % per day, and survivability %. However, a reverse pattern was noticed in the food conversion ratio (FCR) both in IMCs and air-breathing fish species under choline supplementation. Serum biochemical responses, *e.g.,* total protein (PRO), lactate dehydrogenase (LDH), glucose (GLU), and calcium (Ca) showed significant enhancement, and alkaline phosphatase (ALP), alanine amino transaminase (ALT), aspartate amino transaminase (AST), cholesterol (CHOLES), and triglycerides (Trig) showed gradual significant reduction during the breeding season under choline exposure. Treated fishes showed prevention from liver dysfunction and fatty liver formation, and increased body crude protein content. Results indicated favorable growth and yield, which may benefit fish farmers during their culture practices, and the output fish species under choline supplementation resulted in quality food-fish for human consumption.



1. INTRODUCTION

Choline chloride ($C_5H_{14}NO{\cdot}Cl$), having a linear formula of $(CH_3)_3N(Cl)CH_2CH_2OH$, with a molecular weight of 139.62 g/mol, and also chemically known as (2-hydroxyethyl) trimethylammonium chloride, is also an quaternary ammonium compound with a choline cation and chloride anion bearing the IUPAC name 2-hydroxyethyl (trimethyl) azanium chloride[1,2] (Figure 1). It is nothing but an organic, thick, and colorless, strongly alkaline substance which is known to be an essential vitamin (grouped under the B-complex family) for humans as well as terrestrial and aquatic animals including fish.[3,4] Moreover, choline and its metabolites induce(s) an emergent role in maintaining the structural integrity and signaling functions of the cell membranes of the animal body; it is recorded as one of the major sources of methyl groups in the diet (*e.g.,* betaine, responsible in methylation of homocysteine to form methionine, also known as renal

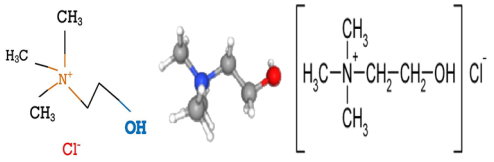


Figure 1. Structure of choline chloride. 3D structure of choline chloride: iStock by Getty images.

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osmolyte), and other cellular functions such as lipid transport or metabolism, cholinergic neurotransmission, and transmembrane signalling are also influenced by the activities of choline.[5] It is also considered to be responsible for apoptotic signaling in neurons and liver cells and the transport of lipoproteins from hepatic cells, thus preventing hepatic carcinogenesis.[6] Other metabolites of choline, *e.g.*, glycerophosphocholine and phosphocholine, act as intracellular storage pools of choline, whereas phosphatidylcholine and sphingomyelin are familiar as necessary building blocks of biomembranes and also trigger lipid and cholesterol transport and metabolism processes.[7,8] As a mandatory item of the human diet, it is essential to as an obligatory food constituent, and it has profuse nutritional value to mammals.[3] Similar in humans, it also acts as a growth promoter in animals (chicks), as well as for fishes when supplemented with a requisite and balanced feeding.[9] Its deficiency leads to muscle damage, liver damage, fatty liver, and elevated liver enzymes in blood and tissues, and its abundance in the animal body may reduce the chances of formation of defective neural tubes, cleft palates, and also increases brain development.[10−12] Efficacy of choline in many animals including fish has been reported by various researchers stating the presence of choline in the diet showed improved growth of spleen and head kidney of *Cyprinus carpio*,[13] while a deficiency of choline showed growth retardation, poor survival rate, poor feed efficiency, and elevated liver lipid concentration in yellow perch (*Perca flavescens*)[14] and juvenile cobia (*Rachycentron canadum*),[15] and poor digestion and less absorption capacities in fishes.[16,17] Moreover, the supplementation of such lipotropic factor in the fish diet showed a higher efficacy with respect to reduced lactate dehydrogenase (LDH), alanine amino transaminase (ALT), and alkaline phosphatase (ALP) activities by mitigating endosulfan-induced stress,[18] and increased body crude protein content of juvenile Atlantic salmon (*S. salar*) and grass shrimp (*P. monodon*) and also in juvenile Jian carp *Cyprinus carpio* respectively.[19−21] Nevertheless, the practice of the application of choline (choline chloride: CC) directly into the pond water for the purpose of fish culture of carps and air-breathing fish in a semi-intensive fish culture system is still unidentified.

Furthermore, traditionally, the fish farmers are used to and habituated to use choline chloride along with fish-feed (mainly floating fish-feed) as their regular practice for enhancement of growth performance and yield. But the application of this type of feed (as formulated and marketed by different manufacturing organizations) as per market observations is costlier for the marginal fish farmers. Therefore, our intention is (i) to study the influences of direct choline administration on growth performance and biochemical parameters, and to assess fish physiology with the outcome of quality food-fish after choline application; and (ii) to minimize the cost of the culture under semi-intensive culture practices for those fish farmers through direct application of choline into the pond without hampering growth performance as well as actual yield, but simultaneously to maintain the sustainability of the pond ecosystem.

2. RESULTS AND DISCUSSION

2.1. Growth Performance. After experimental culture periods of 90 days, the growth performance of *L. rohita* and *C. catla* depicted very significant changes under choline supplementation, whereas in *C. batrachus* and *A. testudineus* the change was less (Table 1). In choline treatment conditions, total length-final (TLF), standard length-final (SLF), mean

Table 1. Comparative Analysis of Growth Performance in *L. rohita*, *C. catla*, *C. batrachus*, and *A. testudineus* between Control (Only Farm-Feed-Fed Groups) and Treatment (Farm-Feed Plus Choline-Supplemented) Conditions[a]

		L. rohita		*C. catla*		*C. batrachus*		*A. testudineus*	
Sl no.	parameters	C	T	C	T	C	T	C	T
1	total length (initial) [TLI] (cm)	16.00 ± 1.00	16.00 ± 1.00	17.00 ± 0.80	17.00 ± 0.80	8.80 ± 0.70	8.80 ± 0.70	5.80 ± 0.40	5.80 ± 0.40
2	total length (final) [TLF] (cm)	26.60 ± 0.65^{e}	37.90 ± 1.35^{h}	30.30 ± 0.92^{f}	37.80 ± 1.09^{g,h}	14.00 ± 0.18^{c}	15.20 ± 0.10^{d}	9.80 ± 0.01^{a,b}	10.00 ± 0.02^{b}
3	standard length (initial) [SLI] (cm)	15.00 ± 1.00	15.00 ± 1.00	15.20 ± 0.50	15.20 ± 0.50	7.50 ± 0.80	7.50 ± 0.80	5.00 ± 0.40	5.00 ± 0.40
4	standard length (final) [SLF] (cm)	22.20 ± 1.43^{e}	32.00 ± 0.88^{g,h}	25.40 ± 0.96^{f}	32.20 ± 1.65^{h}	11.50 ± 0.66^{c}	13.70 ± 0.81^{d}	7.90 ± 0.57^{a,b}	8.60 ± 0.62^{b}
5	mean weight (initial) [MWI] (g)	60.00 ± 1.10	60.00 ± 1.10	70.00 ± 0.50	70.00 ± 0.50	18.00 ± 1.00	18.00 ± 1.00	10.00 ± 0.60	10.00 ± 0.60
6	mean weight (final) [MWF] (g)	290.00 ± 2.35^{e}	420.00 ± 3.08^{h}	305.00 ± 2.69^{f}	395.00 ± 2.77^{g}	30.20 ± 0.95^{c}	33.00 ± 0.65^{d}	20.00 ± 0.47^{a}	21.50 ± 0.46^{b}
7	% gain of mean total length (% MTL)	66.25 ± 2.69^{b}	136.88 ± 2.69^{h}	78.24 ± 2.30^{f}	122.35 ± 3.47^{g}	59.09 ± 0.59^{a}	72.73 ± 0.95^{e}	68.97 ± 0.83^{c}	72.41 ± 0.52^{d,e}
8	% gain of mean standard length (% MSL)	48.00 ± 0.83^{a}	113.33 ± 1.44^{h}	67.11 ± 0.84^{d}	111.84 ± 2.83^{g}	53.33 ± 0.87^{b}	82.67 ± 2.58^{f}	58.00 ± 1.07^{c}	72.00 ± 1.11^{e}
9	% weight gain (% WG)	383.33 ± 3.00^{f}	600.00 ± 3.50^{h}	335.71 ± 2.50^{e}	464.29 ± 3.50^{g}	67.78 ± 2.00^{a}	83.33 ± 2.50^{b}	100.00 ± 1.00^{c}	115.00 ± 1.20^{d}
10	specific growth rate (SGR % per day)	1.75 ± 0.02^{f}	2.16 ± 0.01^{h}	1.64 ± 0.02^{e}	1.92 ± 0.04^{g}	0.57 ± 0.05^{a}	0.67 ± 0.06^{b}	0.77 ± 0.02^{c}	0.85 ± 0.03^{d}
11	survivability %	75.00 ± 0.90	90.00 ± 1.52	75.00 ± 0.68	90.00 ± 1.52	70.00 ± 0.47	85.00 ± 0.68	70.00 ± 0.48	85.00 ± 0.52
12	food conversion ratio (FCR)	0.97 ± 0.01^{d}	0.52 ± 0.03^{a,b,c,d}	0.96 ± 0.02 ^{c,d}	0.58 ± 0.07^{b,c,d}	18.74 ± 1.11^{g}	12.55 ± 0.70^{e}	22.86 ± 1.03^{h}	16.37 ± 0.49^{f}

[a]Data are reported as mean ± SD (n = 60). Values with the same superscripts in the same row are not significantly different (p < 0.05); C - control, T - treatment.

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Table 2. Percent Increase (+) or Decrease (−) of Different Growth Parameters in *L. rohita*, *C. catla*, *C. batrachus*, and *A. testudineus* between Treatment (Farm-Feed Plus Choline-Supplemented) and Control (Only Farm-Feed-Fed Groups) Conditions (T vs C)

Sl no.	growth parameters	*L. rohita*	*C. catla*	*C. batrachus*	*A. testudineus*	max	min
1	TLF (cm)	42.48	24.75	8.57	2.04	42.48	2.04
2	SLI (cm)	0.00	0.00	0.00	0.00	0.00	0.00
3	SLF (cm)	44.14	26.77	19.13	8.86	44.14	8.86
4	MWI (g)	0.00	0.00	0.00	0.00	0.00	0.00
5	MWF (g)	44.83	29.51	10.00	7.50	44.83	7.50
6	% MTL	106.61	56.38	23.08	4.99	106.61	4.99
7	% MSL	136.10	66.65	55.02	24.14	136.10	24.14
8	% WG	56.52	38.30	22.94	15.00	56.52	15.00
9	SGR % per day	23.43	17.07	17.54	10.39	23.43	10.39
10	survivability %	20.00	20.00	21.43	21.43	21.43	20.00
11	FCR	−46.39	−39.58	−33.03	−28.39	−28.39	−46.39

weight-final (MWF), % mean total length (MTL), % mean standard length (MSL), % weight gain (WG), and specific growth rate (SGR) % per day were the highest in *L. rohita* and the lowest in *A. testudineus*, while the food conversion ratio (FCR) value decreased significantly ($p < 0.01$) showing a maximum reduction in *L. rohita* (−46.39%) and a minimum in *A. testudineus* (−28.39%) at the end of the experiment. Under choline supplementation, *L. rohita* showed significant ($p < 0.01$) variations in TLF, SLF, MWF, % MTL, % MSL, % WG, and SGR % per day compared to its "farm-feed-fed"groups, while treated *C. catla* showed significant ($p < 0.01$) but comparatively lower values in TLF, SLF, MWF, % MTL, % MSL, % WG, and SGR % per day than *L. rohita*, which finally resulted in a higher influence in *L. rohita*. Interestingly, *C. batrachus* under choline supplementation showed a moderate (19.13 to 55.02%) variation in SLF, % MTL, % MSL, % WG, and SGR % per day and low values (8.57−9.27%) in case of TLF and MWF, while *A. testudineus* under choline induction depicted comparatively low (2.04 to 24.14%) variation in TLF, SLF, MWF, % MTL, % MSL, % WG, and SGR % per day. Moreover, the air-breathing fishes under choline supplementation depicted a higher percentage of survivability than the experimental IMCs compared to its control or "farm-feed-fed" fishes (Table 2). The data on water quality remained within the range of culture, as found during the entire course of experimentation under a semi-intensive pond culture system; so, further discussion of these parameters was avoided.

The growth performance of fish species was attributed to a positive induction under the inclusion of choline into the pond water in the present study. The presence of choline in the diet showed improved growth, and that has been observed by various researchers, *e.g.*, in juvenile red drum (*Sciaenops ocellatus*)[22] and in juvenile hybrid tilapia.[23] It was also been established as a compatible osmolyte and growth promoter in crustaceans.[24,25] In the present study, choline acted as a promoter into the waterbody which induced growth as well as normal physiology of the cultured fish species. Addition of this lipotropic factor into the pond water induced the elevating trends of TLF, SLF, MWF, % MTL, % MSL, % WG, and SGR % per day of choline-supplemented fish species compared to their "farm-feed-fed" groups, and the rate of elevation in all the noted parameters was much higher in choline-supplemented carps (catla and rahu) than the choline-supplemented air-breathing fish species (koi and magur), which may be due to maximum utilization of intake feed, vigorous movability of the fish species over and around the pond, improved digestibility rate, and elevated intestinal growth in the present experiment.[26] This kind of phenomenon was also observed in juvenile Jian carp (*C. carpio*) exposed to choline,[21] in hybrid striped bass (*Morone saxatilis* × *Monrone chrysops*,)[27] and in yellow perch,[14] where choline in the diet of the fish species had a persistent effect in the normal physiology of the fishes which finally triggered the growth performance. Interestingly, decreased FCR was analyzed in all fishes under choline supplementation may be due to a reduction in lipid deposition in the viscera for the presence of phosphatidylcholine,[28,29] resulting in a positive effect in the growth performance as found in *C. carpio*, *var*. Jian, supplemented with exogenous xylanase,[30] in *L. rohita* treated with probiotics (*Bacillus* strains),[31,32] in *Totoaba macdonaldi* fed with high concentration soyabean meal,[33] and in juvenile cobia (*Rachycentron canadum*) treated with choline in its diet.[15]

2.2. Biochemical Analysis. Estimation of different biochemical parameters is recorded in Table 3. In the choline-supplemented condition, total protein (PRO) showed the highest variation in *C. batrachus*, whereas, *C. catla* showed the lowest against the "farm-feed-fed" fishes. The maximum value of lactate dehydrogenase (LDH) was observed in *L. rohita* but decreased significantly ($p < 0.01$) in *A. testudineus* compared to the "farm-feed-fed" condition. *L. rohita* showed the highest value of calcium (Ca) and the lowest levels of glucose (GLU), whereas, *A. testudineus* and *C. batrachus* revealed the highest values of GLU and the lowest of Ca respectively compared to "farm-feed-fed" fishes. In the fishes under choline supplementation, aspartate amino transaminase (AST) and triglycerides (Trig) displayed a drastic reduction, showing a minimum in *C. catla* and maximum in *A. testudineus*. Cholesterol (CHOLES) and alanine amino transaminase (ALT) were also decreased. They showed the highest and lowest trend of reduction in *L. rohita*. On the other hand, *A. testudineus* showed a minimum reduction in CHOLES and a maximum reduction of ALT in *C. batrachus* compared to their "farm-feed-fed" groups. Alkaline phosphatase (ALP) also showed a declining trend and the maximum depletion was observed in *L. rohita* with a minimum in *A. testudineus* under choline influences. *Labeo rohita* showed relatively significant ($p < 0.01$) prominent responses compared to its "farm-feed-fed" condition and ranged from 198.53 to 246.63% in LDH and Ca, but in PRO and GLU it varied from 20.14 to 44.26%; and further, ALP, ALT, AST, CHOLES, and Trig values decreased drastically showing a maximum decline in ALP. In *C. catla* under choline supplementation, LDH and Ca were signifi-

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Table 3. Comparative Analysis of Biochemical Parameters in *L. rohita*, *C. catla*, *C. batrachus*, and *A. testudineus* between Control (Only Farm-Feed-Fed Groups) and Treatment (Farm-Feed Plus Choline-Supplemented) Conditions[a]

		L. rohita		*C. catla*		*C. batrachus*		*A. testudineus*	
Sl no.	parameter	C	T	C	T	C	T	C	T
1	PRO (g/dL)	2.93 ± 0.01^{b}	3.52 ± 0.01^{d}	3.26 ± 0.04^{c}	3.86 ± 0.06^{f}	2.24 ± 0.08^{a}	4.59 ± 0.10^{g}	3.65 ± 0.04^{e}	4.85 ± 0.06^{h}
2	ALT (U/L)	$41.00 \pm 2.81^{d,e}$	$29.00 \pm 4.31^{a,b}$	87.66 ± 2.64^{h}	44.66 ± 4.16^{e}	72.33 ± 3.39^{g}	$30.00 \pm 3.59^{b,c}$	54.00 ± 2.35^{f}	34.66 ± 1.86^{c}
3	AST (U/L)	437.66 ± 3.66^{h}	297.00 ± 3.35^{d}	338.66 ± 1.93^{f}	329.33 ± 4.27^{e}	267.66 ± 2.21^{c}	185.33 ± 2.08^{a}	400.66 ± 2.65^{g}	259.00 ± 2.95^{b}
4	ALP (U/L)	$3.33 \pm 0.07^{f,g}$	0.66 ± 0.02^{a}	4.33 ± 0.17^{h}	$1.66.00 \pm 0.03^{c}$	3.00 ± 0.47^{d}	$1.33 \pm 0.03^{b,c}$	3.66 ± 0.34^{g}	$3.33 \pm 0.03^{e,f,g}$
5	LDH (IU/L)	452.66 ± 1.48^{b}	1351.33 ± 1.91^{h}	343.33 ± 2.40^{a}	978.33 ± 2.71^{d}	707.66 ± 1.94^{c}	1146.66 ± 3.70^{e}	1243.33 ± 2.57^{f}	1291.66 ± 2.92^{g}
6	GLU (mg/dL)	145.33 ± 3.50^{f}	209.66 ± 3.65^{g}	137.00 ± 1.71^{e}	221.66 ± 4.32^{h}	19.33 ± 3.84^{a}	60.33 ± 1.31^{c}	29.66 ± 1.35^{b}	101.00 ± 2.47^{d}
7	CHOLES (mg/dL)	217.66 ± 2.81^{d}	71.66 ± 3.36^{a}	248.00 ± 3.32^{e}	179.00 ± 1.54^{c}	260.00 ± 5.58^{f}	120.66 ± 2.61^{b}	619.33 ± 0.94^{h}	498.00 ± 2.36^{g}
8	Trig (mg/dL)	203.00 ± 4.33^{g}	145.00 ± 3.91^{d}	181.33 ± 1.82^{f}	154.66 ± 3.05^{e}	111.66 ± 2.51^{b}	44.66 ± 4.33^{a}	505.00 ± 6.17^{h}	129.66 ± 0.96^{c}
9	Ca (mg/dL)	3.56 ± 0.03^{d}	12.34 ± 1.89^{h}	$3.26 \pm 0.04^{c,d}$	$6.52 \pm 0.02^{e,f}$	$2.60 \pm 0.03^{a,b,c,d}$	$2.65 \pm 0.05^{b,c,d}$	7.11 ± 0.06^{f}	$11.42 \pm 0.18^{g,h}$

[a]PRO - total protein, ALT - alanine amino transaminase, AST - aspartate amino transaminase, ALP - alkaline phosphatase, LDH - lactate dehydrogenase, GLU - glucose, CHOLES - cholesterol, Trig - triglycerides, Ca - calcium, C - contol, T - treatment. Data are reported as mean ± SD ($n = 9$). Values with the same superscripts in the same row are not significantly different ($p < 0.05$).

cantly ($p < 0.01$) increased manifold (100.00 to 184.95%), but GLU changed moderately, and a little change was observed in PRO, whereas, ALP, ALT, AST, CHOLES, and Trig values decreased significantly ($p < 0.01$) showing the highest reduction in ALP compared to its "farm-feed-fed" fishes. In *Clarias batrachus*, PRO and GLU showed significant ($p < 0.01$) higher elevation (104.91 to 212.11%) but moderate in LDH and no marked change in Ca ($p < 0.05$). ALP, ALT, AST, CHOLES, and Trig values declined significantly ($p < 0.01$), showing the highest reduction in Trig compared to its "farm-feed-fed" group. In *A. testudineus*, the changes were significantly ($p < 0.01$) higher in GLU, moderate in PRO and Ca, and less in LDH in the choline-supplemented condition. Values of ALP, ALT, AST, CHOLES, and Trig decreased significantly ($p < 0.01$) showing the highest reduction in Trig (−74.32%) compared to its control or "farm-feed-fed" group (Table 4).

On the other hand, it is stated that accumulation of protein (PRO) content is determined by a balance between protein synthesis and its degradation, regulated by interactions among nutritional, physiological, and other influences through a cellular signaling pathway.[34] In the breeding season, the fishes under choline supplementation showed higher PRO levels compared to the control or "farm-feed-fed" fishes endorsing better growth of fishes and fish health.[35,36] Therefore, in the breeding season, the rate of metabolism was higher, causing the breakage of tissue protein released into the plasma to maintain the plasma protein to combat protein deficiency.[37] Here, the application of supplemented choline resulted in enhanced body crude protein content showing the highest and lowest protein gain in *C. batrachus* and *C. catla* respectively. So, the protein content of the choline-supplemented fish species was higher compared to their control groups. Similar results were also recorded in juvenile Atlantic salmon (*S. salar*), in grass shrimp (*P. monodon*), and in juvenile *C. carpio* when choline was added to the diet.[19−21]

The ALT and AST are the indicators for the diagnosis of liver function and to identify the cellular damage of the liver.[38−40] ALT plays a major role in making a connection between the metabolism of protein and carbohydrate, and it causes stress to catalyze the transference of the amino group from alanine to α-ketoglutarate to generate glutamate and pyruvate.[41] Decreased AST and ALT activity in serum indicated that the oxaloacetate and glutamate are not present in the Kreb's cycle through its root of transmission which has been found in the present study during the breeding season in the fishes under choline supplementation, where ALT decreased manifold in *C. batrachus* and *C. catla*; and the AST level was reduced maximally in *A. testudineus* among all and *L. rohita* between IMCs,[42] whereas, the farm-feed-fed fish species showed increased ALT and AST in the blood due to the absence of such lipotropic factor as found by previous researchers in a different study.[43] Moreover, the increased ALT and AST levels in liver and muscle of *L. rohita* was also noticed when exposed to endosulfan and subsequently recovered by choline and its metabolites.[18] Furthermore, in an another study, it was shown that the plasma ALT and AST levels remained within the normal limit in blunt snout bream (*Megalobrama amblycephala*) after feeding with 1800 mg kg^{-1} choline in a diet containing high fat (11%), which indicated that the liver was healed from steatosis and injury.[44,45] But, the higher ALT and AST activity in plasma was recorded in *C. carpio* when treated with diazinon,[44,46] in eel exposed to

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Table 4. Percent Increase (+) or Decrease (−) of Different Biochemical Parameters in *L. rohita*, *C. catla*, *C. batrachus*, and *A. testudineus* between Treatment (Farm-Feed Plus Choline-Supplemented) and Control (Only Farm-Feed-Fed Groups) Conditions (T vs C)

Sl no.	biochemical parameters	*L. rohita*	*C. catla*	*C. batrachus*	*A. testudineus*	max	min
1	PRO (g/dL)	20.14	18.40	104.91	32.88	104.91	18.40
2	ALT (U/L)	−29.27	−49.05	−58.52	−35.81	−29.27	−58.52
3	AST (U/L)	−32.14	−2.75	−30.76	−35.36	−2.75	−35.36
4	ALP (U/L)	−80.18	−61.66	−55.67	−9.02	−9.02	−80.18
5	LDH (IU/L)	198.53	184.95	62.04	3.89	198.53	3.89
6	GLU (mg/dL)	44.26	61.80	212.11	240.53	240.53	44.26
7	CHOLES (mg/dL)	−67.08	−27.82	−53.59	−19.59	−19.59	−67.08
8	Trig (mg/dL)	−28.57	−14.71	−60.00	−74.32	−14.71	−74.32
9	Ca (mg/dL)	246.63	100.00	1.92	60.62	246.63	1.92

deltamethrin,[47] and in the plasma of blunt snout bream (*Megalobrama amblycephala*), fed with 11% lipid and 1200 mg kg^{-1} choline in its diet.[45]

The present study indicated that choline induced an elevation of LDH activity in the fishes with the highest elevation in *L. rohita* (column feeder) followed by the surface feeder *C. catla* because of their higher metabolic activities for filling up the energy requirement by synthesizing glycogen for the formation of glucose to produce lactate, mainly in the muscle tissue.[48] The higher movability of *L. rohita* for searching foods from the surface to the bottom resulted in stress to the fish.[49] Stress may induce higher activation of TCA cycle by demanding more energy[50] to combat stress compared to bottom dwellers (*A. testudineus* and *C. batrachus*), unlike higher LDH activity, as found in *A. anguilla*,[51,52] *Puntius conchonius*,[53] *Heteropneustes fossiliz*,[54] and *Channa striatus*[55] due to acute effects of organophosphorus pesticides including diazinon.

ALP, a zinc-containing metallo-enzyme, plays an important role in phosphorus metabolism. It was noticed that in fishes fed a diet containing lecithin and betaine, the ALP activity was normal,[18] which might be due to easy liberation of phosphate ions to combat the stressful condition or a higher metabolism rate and enhanced cell signalling as in consonance with the observations in the present work, where reduction of ALP activity was also noticed, with the highest reduction in *L. rohita* and lowest in *A. testudineus* under choline supplementation. Moreover, decreased ALP activity during the breeding season resulted in a breakdown of glycogen (reserved energy) required for more energy during courtship through glycogenolysis or *de novo* gluconeogenesis to meet the energy demand or otherwise depicting decreased transphosphorylation and uncoupling of oxidative phosphorylation[56] and finally depicting the fall of ALP activity in blood. On the other hand, it was revealed that ALP activity in *L. rohita* was increased under the condition of the diet containing *Microcystis* sp. due to the breakdown of reserved energy (glycogen), required for growth and survivability of the fishes.[57]

A hyperglycemic condition was observed in all fishes under choline supplementation, where *A. testudineus* showed the maximum followed by *C. batrachus*. *L. rohita*, and *C. catla*, which were able to restore the energy in the form of glycogen minimally compared to their "farm-feed-fed" group. In case of *A. testudineus* and *C. batrachus*, the excess glucose was stored as glycogen (glycogenesis) and/or converted to lipid also due to less metabolic activities, and less stress, whereas the activities of *L. rohita* and *C catla* were much higher, resulting in glucose requirements, glycogen degeneration into glucose (glucogenolysis), and/or by *de novo* glucose synthesis through the gluconeogenesis[58] and thus maintaining the balance between glucose storage and glucose production. The fishes under a choline-supplemented condition in the present experiment produced higher glucose levels due to accruing better gonadal maturity, resulting in a higher concentration of glucose in the blood serum,[59,60] whereas, no such significant effect of stress was found in *L. rohita* reared in the tank for 90 day and fed with an optimum level of *Microcystis* sp.[57]

There was a marked decrease of CHOLES in the blood serum of *L. rohita* under choline treatment followed by *C. batrachus*, *C. catla*, and *A. testudineus* compared to their control or "farm-feed-fed" fishes, which indicated the hypocholesteremia[61] due to reduced hepatic lipid contents and thus preventing liver dysfunction in the fishes under choline supplementation.[45,62,63] Similar results were also reported by many authors when the fishes were fed soybean oil,[64−66] and in *C. punctatus* when exposed to monocrotophos,[67] in Atlantic salmon fed with fish-meal-based diets,[68] and in European sea bass fed with plant proteins.[69] On the contrary, an increasing trend was observed in *Anabas testudineus* exposed to anthracene intoxication,[70] in gilthead seabream (*Sparus aurata*) having a deficiency of phosphorus in its diet,[71] and in the plasma of blunt snout bream fed with 11% lipid and 1200 mg kg^{-1} choline in the diet.[45]

Generally, animals fed with a high level of fat and carbohydrates show an increase in triglyceride (Trig) levels in body tissues as well as plasma.[72] Triglycerides are considered as a cause of liver disease because the synthesis of fatty acids leads to an accumulation in fatty tissues in the liver[73] by absorbing from the gut and then transporting to the liver for further enzymatic actions.[74] Other workers also reported lowered Trig levels in the blood after inclusion of certain various diet supplements, *e.g.*, soyabean meal with the complete replacement of fishmeal in the diet of European seabass (*Dicentrarchus labrax*);[75] but interestingly, an increasing trend in the Trig level was also noticed in Japanese flounder (*Paralichthys olivaceus*) after application of dietary soyabean meal in the diet.[76] The Trig level in the breeding season in the present experiment was decreased many fold as induced by choline in *A. testudineus* followed by *C. batrachus*, *L. rohita*, and *C. catla* compared to control or "farm-feed-fed" groups because choline prevented the accumulation of excessive lipid to generate fatty liver[77] and thus resulted into a higher rate of metabolism, and lowered lipid content in the liver and muscles.[78]

Furthermore, it was observed that choline supplementation induced increases in calcium content in fishes during the

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breeding season, where the *L. rohita* showed maximum elevation followed by *C. catla*, *A. testudineus*, and *C. batrachus* due to ovarian development, and it became magnified from pre-spawning to the spawning period during the reproductive cycle for accruing energy.[79] However, interestingly a lowering trend Ca content in *A. testudineus* was also observed due to anthracene exposure caused by enhanced calcium excretion.[70] Moreover, Ca-deficient diets depicted higher ALP activity in Japanese seabass and bighead carp, which is a marker of insufficient bone mineralization processes.[80,81]

3. JUDGMENT OF ECONOMIC GAIN OF DIRECT ADMINISTRATION OF CHOLINE CHLORIDE INTO POND WATER

In a previous experiment (say experiment A), according to Wu et al. (2011),[21] 30 g of choline chloride (of Sigma, St. Louis, MO, USA: Analytical grade) was mixed with 1 kg of the experimental diet of juvenile Jian carp (*Cyprinus carpio* var. Jian), whereas, in the present experiment (say experiment B) the synthetic feed grade crystal choline chloride (of Meden Pharma Pvt. Ltd., Boisar-401506, Maharashtra) was applied directly into the experimental ponds at the rate of 350 g bigha^{-1} 15 day^{-1} (or 350 g per 1600 square meter fortnightly) during the entire experimental tenure of 90 days. A comparative analysis of the economic gain of applying choline chloride into the pond water for the present experiment is depicted in Table 5. However, it is noticed that applying choline chloride into the pond water is more profitable than applying it through the feed route.

4. CONCLUSION

Regarding the maximum production among the experimental fishes, *L. rohita* displayed the highest initial of live weight gain, lowest FCR, and highest SGR % per day under choline exposure. Elevated total protein (PRO) gain and Ca content, higher LDH activity, and lower ALP, CHOLES, and Trig values in the breeding season under choline exposure led to maximum weight gain of the fishes. In addition, production of higher quality fish species containing elevated amounts of protein in muscle for consumption as food-flesh was also obtained under choline-supplemented condition. Furthermore, elevation of serum glucose mitigated the stress caused by more physiological activities under choline exposure during the breeding season to reveal an enhanced live weight gain. Moreover, the straight rise in the absorption of nutrients has also been expressed due to less production of cholesterol and triglycerides and decreased ALP levels in the choline-supplemented fish species to combat stressful conditions to catalyze a higher metabolic rate. On the other hand, reduced ALT and AST levels in the blood clearly indicated defattened liver due to exposure of choline like lipotropic factor and also its metabolites. So, this maiden attempt of application of choline chloride directly into the pond water under field trial resulted in the production of good quality fish with high yield under this semi-intensive pond culture system that may support rural, poor fish farmers to a greater extent.

5. MATERIALS AND METHODS

5.1. Experimental Design at the Field and Laboratory Analysis. In the field, the experiment (Figure 2)[82] was set up in Khano village, Purba Bardhaman, West Bengal (geographical location of the three experimental ponds were as

Table 5. Comparative Analysis of Using Both Analytical and Food Grade Choline Chloride Both through Feed Application as Well as Directly into the Pond Water[a]

case no.	type of choline chloride (CC)	application method of choline chloride (via feed application/directly into the pond water)	consumption of choline chloride per month (30 days)	rate/kg (Rs.)	expenditure per month (Rs)	remarks
1	"analytical grade" of Sigma, St. Louis, MO, USA (As per Wu et al., 2011)[21]	feed application	3.60 kg	10511.00	37839.60	So, out of four cases, the application of choline chloride through administration into the pond water directly showed more economic gain and profit to the fish farmers, as observed in our experiment. Moreover, Case No: 4 (like in our experiment) would be the most beneficial, most economical, as well as effective for the fish farmers also.
2		directly into the pond water	0.70 kg	10511.00	7357.70	
3	"feed grade" of Meden Pharma Pvt. Ltd., Boisar-401506, Maharashtra (As per our experiment)	feed application	3.60 kg	80.00	288.00	
4		directly into the pond water	0.70 kg	80.00	56.00	

[a]In a previous experiment, according to Wu et al. (2011),[21] it was seen that 30 g of choline chloride (Sigma, St. Louis, MO, USA: Analytical grade) was mixed with 1 kg of experimental diet of juvenile Jian carp (*Cyprinus carpio* var. Jian). Now, suppose in an experiment (A) if choline chloride is applied *via* feed: Say, 100 kg of fish (initial biomass at day 1) in a unit water area (say 1 bigha or 1600 square meter) has to be reared for one month (30 days). So, 4% per day feeding means consumption of feed is 4 kg per day, so, the choline chloride consumption would be 120 g (4 kg × 30 g) per day. Now, for 30 days it will be (120 g × 30 days) = 3600 g (3.60 kg) of choline chloride. (B) If choline chloride is applied directly into the pond water like in our present experiment: With the same stocking density and biomass, like experiment A, in a unit area (say, 1 bigha or 1600 square meter) consumption of choline chloride would be 700 g [(350 g × 2)] per month (30 days) [as the experimental dose of choline chloride in our experiment is 350 g bigha^{-1} 15 days^{-1} or 350 g per 1600 square meter fortnightly). So, the requirement of choline chloride will be at least five times lower in our experiment, and therefore, it would be highly cost-effective also.

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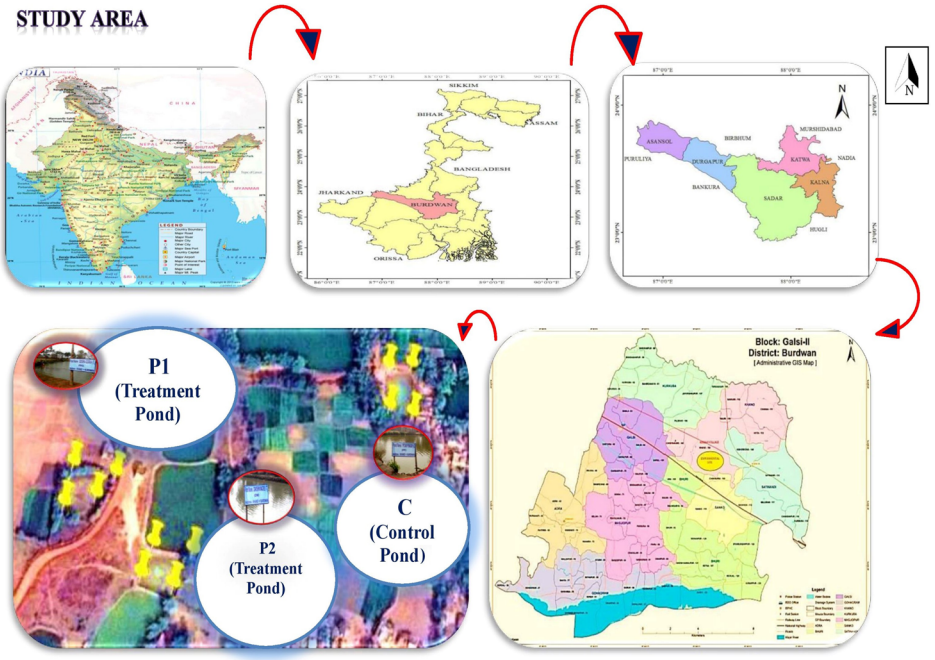

Figure 2. Experimental site[82] (Das et al., 2021).

follows: Control pond (C): lat. and long.: N 23°19′924″ and E 87°43′877″; the other two ponds (P1 and P2) were considered as Treatment (T) with the lat. and long.: N 23°19′872″, E 87°43′702″; and lat. and long.: N 23°19′834″, E 87°43′751″, respectively). Experiment was conducted for a period of 90 days in the breeding season, *i.e.*, from June to August where two Indian major carps (IMCs), *Catla catla* (Catla) and *Labeo rohita* (Rahu), and two air-breathing species, *Clarias batrachus* (Magur) and *Anabas testudineus* (Koi), were selected and introduced. Analysis of different growth and biochemical parameters were done in the Eco-Toxicology Laboratory, Department of Environmental Science, The University of Burdwan, Burdwan, India.

The advanced fingerlings, having average age groups of 58−63, 65−70, 68−73, and 45−50 days of IMCs *viz.*, *Catla catla* (Catla), *Labeo rohita* (Rahu), and two air-breathing fish species, *e.g.*, *Clarias batrachus* (Magur) and *Anabas testudineus* (Koi) respectively, were collected from the registered fish farm (Chandimata Fish Farm) as per ratio and acclimatized in a "acclimatization pond" for a period of 15 days. The acclimatized pond contained more than 30% of the total required fish species in the present experiment due to avoiding partial mortality during the time of the acclimatization process and for obtaining the desired stocking ratio as well as for smooth running of the experiment. Before starting the experiment, three selected experimental ponds: one control (C) and two treatment ponds (T) (*i.e.*, P1 and P2) were prepared as per pisciculture protocol,[83,84] maintaining a definite depth of 5 ft. Each experimental pond bears an effective water area (EWA): 0.20 acre (0.50 bigha or 800 square meter). After acclimatization, the fish species were transferred into C, P1, and P2 on the 16th day of acclimation (considered as the first day of culture experiment) as per the desired experimental culture ratio (Catla/Rahu/Magur/Koi = 2:5:1:1). About 900 fishes were stocked in each pond; so, a total of 2700 fishes were finally stocked in all three ponds. The desired length (cm), *e.g.*, total length (initial) (TLI), standard length (initial) (SLI), and weight (g), *e.g.*, mean weight (initial) (MWI), were also maintained (species-wise) during the stocking of fishes in each experimental pond (Table 1). Moreover, considering the field experimentation, here we maintained only one control and duplicate treatment ponds instead of maintaining triplicate conditions, although we critically maintained a significant number of fish species in every pond.

The fish species under the control (C) condition were fed only with locally prepared "farm-made-aqua-feed" (having a crude protein percentage: 35%) (Table 6), in short called "farm-feed", and these fishes were termed "farm-feed-fed" fishes, whereas, the fishes in the treatment ponds (T: P1 and P2) were fed the same feed (farm-made-aqua-feed) plus choline chloride (350 g bigha^{-1} 15d^{-1} or 350 g per 1600 square meter fortnightly) (1 bigha = 0.40 acre = 1600 square meter), and these fishes under the treatment condition were denoted as "fishes under choline supplementation or choline-supplemented fishes" for the convenience of description. Fishes under the acclimation tenure were also fed "farm-feed". As the ponds [both acclimation (one pond) and experimental (three ponds)] were located at adjacent to each other and within the same geographical area of the Chandimata Fish Farm, so 4% feeding per day was maintained in the present experiment as it was found that daily feeding of about 2−5% of the body weight was recommended based on the natural productivity of the fish pond,[85] although another researcher recommended a daily feeding rate of supplementary feed is to some extent higher [5−6% of the body weight up to 500 g sized fish; then 3.5% of body weight from 500 to 1000 g sized fish].[86] It was also noticed that the farmers in the field used to

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Table 6. Ingredients (g kg^{-1}) for Formulation of Farm-Made-Aqua-Feed and Approximate Composition of the Basal Experimental Diet[a]

ingredients	g kg^{-1}	nutritional content (g kg^{-1})	
fish meal[b]	195.00	dry matter	982.00
soya meal[b]	130.00	crude protein	350.00
groundnut oil cake[b]	45.00	crude fat	48.85
yellow corn (maize)[b]	120.00	crude ash	53.53
DO_RB (deoiled rice bran)[b]	230.00	NFE	547.62
broken rice[b]	145.00		
silky bran[b]	45.00		
vitamin premix[c] (Wu et al., 2011)[21]	40.00		
mineral premix[c] (Wu et al., 2011)[21]	40.00		
sodium chloride[b]	10.00		

[a]Crude protein, crude fat, crude ash, and moisture content were measured values.[96] Nitrogen free extract; NFE (%) = 100 − (% crude protein + % total fat + % ash) [b]Local market (Khano, Galsi, Galsi-II Block, Purba Bardhaman, West Bengal, India). [c]Matsya Chas Sahayata Kendra, Tinkonia, Gurudwara, Near Burdwan Municipality, Purba Bardhaman, West Bengal, India.

procure 7.5% (≤500 g of body weight) and 4.60% (>500 g of body weight) daily feeding rate respectively[87,88] during carp culture practice in a semi-intensive culture system in West Bengal of the district East (Purba) Bardhaman and North 24 Parganas.

Pond water was monitored both in control (C) and choline-induced treatment ponds (P1 and P2: T) regularly[89] (in every 15 days of interval) and was expressed in mean value. An average value was also taken into account in the case of treatment ponds also (T as avg. of P1 and P2) (Table 7). Physicochemical parameters of water were maintained as per the standard of pisciculture, and the ranges of each water parameter remained within the permissible limit of aquaculture during the present experiment both in control and treatment conditions.[89] In addition to that, crystal choline chloride of "feed grade" quality (98% pure) of Meden Pharma Pvt. Ltd., Boisar-401506, Maharashtra was administered directly into the P1 and P2 ponds at the rate of 350 g bigha^{-1} 15 day^{-1} (or 350 g per 1600 square meter fortnightly) during the entire experimental tenure of 90 days. Preliminarily, the dose of choline was determined in a previous experiment, consisting of five experimental ponds with five different experimental doses where the ponds were located in the identical geographical area. The experimental fishes and the culture ratio were identical with the present experimental study. The best outcome (*i.e.*, the dose of choline directly into the pond water) with respect to growth performance from the preliminary study was taken into consideration for the present experiment.

5.2. Sampling. *5.2.1. Growth Parameter Analysis.* At the end of the experiment, species-wise fishes were collected randomly on 91st day both from control (C) and treatment (T: P1 and P2) ponds (n = 60; 60 from C, 30 from P1 and 30 from P2) and were handled carefully after anesthetizing with MS 222 for determining the growth performance. Following growth parameters were taken into consideration for determining the growth performance, *viz.*, total length-final (TFL), standard length-final (SLF), mean weight-final (MWF), % gain of mean total length (MTL), % gain of mean standard length (MSL), % weight gain (WG), specific growth rate (SGR) % per day, survivability % and food conversion ratio (FCR).[21,28,30,33,82,90−93] Following, conventional measurements were taken (species-wise) immediately after catch such as live body weight (final), total length of fish (final), and standard length of fish (final).

5.2.2. Biochemical Analysis. Species-wise suitable fishes were also hauled from the C, P1, and P2 ponds (n = 9; 9 from C, 5 from P1 and 4 from P2) randomly on 91st day after 90 days of the experiment. The randomly selected fishes (species-wise) were handled carefully, and blood samples were drawn from each fish species from the caudal vein with utmost care after anesthetizing the fishes with MS 222. The blood from

Table 7. Responsive Analysis of Physicochemical Parameters of Water between Control and Treatment Conditions[a]

			T Pond	
parameter	unit	C pond	P1	P2
trans	cm	20.00 ± 0.29^{c}	17.50 ± 0.02^{b}	17.00 ± 0.06^{a}
temp	°C	29.00 ± 0.08^{a}	31.00 ± 0.07^{b}	31.50 ± 0.04^{c}
EC	μS/cm	650.00 ± 3.76^{a}	680.00 ± 1.53^{b,c}	682.00 ± 1.56^{c}
pH		7.55 ± 0.04^{a}	8.20 ± 0.03^{b}	8.30 ± 0.01^{c}
CO_2	mg/L	5.30 ± 0.05^{c}	4.20 ± 0.07^{b}	4.00 ± 0.10^{a}
DO	mg/L	6.00 ± 0.09^{c}	7.50 ± 0.05^{b}	7.40 ± 0.08^{a,b}
TA	mg/L	300.00 ± 3.13^{a}	340.00 ± 2.15^{b}	345.00 ± 1.69^{c}
PO_4^{3-}	mg/L	0.82 ± 0.02^{a}	1.40 ± 0.04^{b}	1.45 ± 0.05^{c}
TH	mg/L	182.00 ± 1.28^{a}	187.00 ± 2.85^{b,c}	190.00 ± 0.95^{c}
Cl^-	mg/L	40.00 ± 2.83^{a}	63.00 ± 1.15^{b,c}	65.00 ± 0.65^{c}
NH_4^+-N	mg/L	0.40 ± 0.04^{a}	0.80 ± 0.20^{b,c}	0.85 ± 0.12^{c}
NO_3^-N	mg/L	0.20 ± 0.01^{a}	0.50 ± 0.04^{c}	0.45 ± 0.02^{b}
Na^+	mg/L	65.00 ± 0.77^{a}	78.00 ± 1.66^{b}	82.00 ± 1.32^{c}
K^+	mg/L	16.00 ± 1.61^{a}	22.00 ± 1.16^{b,c}	24.00 ± 0.52^{c}

[a]Trans - transparency, Temp - temperature, EC - electrical conductivity, CO_2 - carbon dioxide, DO - dissolved oxygen, TH - total hardness, TA - total alkalinity, NO_3^-N - nitrate nitrogen, NH_4^+-N - ammonical nitrogen, Cl - chloride, K - potassium. Data are reported as mean ± SD (n = 6). Values with same superscripts in the same row are not significantly different (p < 0.05); water samples were taken at 15 day intervals (*i.e.*, on D15, D30, D45, D60, D75, and D 90). D = day. Mean value of each datum of D15, D30, D45, D60, D75, and D90 is represented in the table both for C and T (P1 and P2) ponds. C (Control) pond = fed with farm-made-aqua-feed only; T (treatment: P1 and P2) ponds = fed with farm-made-aqua-feed + choline chloride into the pond water.

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each fish species was taken by 2 mL disposable syringes and transferred immediately to individual EDTA-free sterilized red top clotting vial (2 mL). Later, each sample of blood was centrifuged at 2500 rpm for 5 min for separation of serum,[42,94] collected by micropipette, and finally, stored at −20 °C for further analysis. Serum from the blood of each fish species was analyzed by BTS-350 (Bio-systems) semi-automatic analyzer by using the kit method (Table 8).

Table 8. Name of the Kit Used against Each Biochemical Parameter

Sl no.	name of the parameter	kit used
1	total protein (PRO)	ERBA total protein kit (Erba #120231)
2	alanine amino transaminase (ALT)	ERBA SGPT kit (Erba Mannheim #120207)
3	aspartate amino transaminase (AST)	ERBA SGOT kit (Erba Mannheim #120204)
4	alkaline phosphatase (ALP)	Abcam kit (ALP assay kit-colorimetric #ab83369)
5	lactate dehydrogenase(LDH)	Abcam kit (LDH assay kit-colorimetric #ab102526)
6	glucose (GLU)	ERBA glucose kit (Erba #120235)
7	cholesterol (CHOLES)	ERBA cholesterol kit (Erba #120194)
8	triglycerides (Trig)	ERBA triglyceride kit (Erba #120211)
9	calcium (Ca)	ERBA calcium kit (Erba #120225)

5.3. Calculations and Statistical Analysis. *5.3.1. Growth Parameter Calculations.* The following formulas were adopted for calculating different growth parameters:

(a) MWI (g) = TWI/TFS
(b) MWF (g) = TWF/TFS
(c) (% MTL) = 100 [(MTF − MTI)/MTI]
(d) (% MSL) = 100 [MSF − MSI)/MSI]
(e) %WG = 100 [LWF − LWI)/ LWI]
(f) SGR % (per day) = 100 [(ln W_1 − ln W_0/ (T_1 − T_0)]
(g) FCR = WF/WG

where

MWI = mean weight (initial) (g)
TWI = total weight (initial) (g)
MWF = mean weight (final) (g)
TWF = total weight (final) (g)
TFS = total number of fish stocked
% MTL= gain of mean total length (cm)
MTF = mean total length (final) (cm)
MTI = mean total length (initial) (cm)
% MSL = gain of mean standard length (cm)
MSF = mean standard length (final) (cm)
MSI = mean standard length (initial) (cm)
% WG = weight gain or live body weight gain (g)
LWF = mean live body weight (final) (g)
LWI = mean live body weight (initial) (g)
SGR = specific growth rate
FCR = food conversion ratio
WF = dry weight of feed given (g)
W_1 = final live body weight (g) of fish
W_0 = initial live body weight (g) of fish
T_1 = final time of culture (days)
T_0 = initial time of culture (days)

5.3.2. Statistical Analysis. The statistical package for the Social Sciences (SPSS) version 22.0 was taken into consideration for the statistical analysis of data. Analysis of variance (ANOVA) followed by Tukey's test was conducted.[95]

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Notes

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