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Citric Acid as a Feed Supplement: Effect on *Labeo rohita* Fingerlings to Promote Nutrient Digestibility, Growth and Hematological Indices

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Key words
Citric acid, *Labero rohita*, Hematological indices, Weight gain, *Moringa oleifera*, Nutrients digestibility

ABSTRACT

Moringa oleifera leaf meal (MOLM) supplemented with citric acid (CA) was observed in this research to promote the digestibility of nutrients, growth and hematology of *Labeo rohita* (rohu) fingerlings. During the 90-day trial, six test diets were created, one of which was a control diet and the other five of which included 1, 2, 3, 4, and 5% CA, respectively. The fingerlings of rohu were fed diets at a rate of 5% of their live body weight. Results of this investigation revealed that the 3% CA level had the greatest weight gain (WG, 23.73), specific growth rate (SGR, 1.41), weight gain percentage (WG%, 255.07), and lowest feed conversion ratio (FCR, 1.28) values. In terms of digestibility of nutrients, the highest values were observed at a 3% supplementation level of CA, which were crude fat (70.51%), crude protein (67.40%) and gross energy (65.67%). The improvement in RBCs (red blood cells), PLT (platelets), WBCs (white blood cells), and Hb (haemoglobin) was also at 3% CA after the hematological examination. It was revealed that the optimal dosage of CA supplementation was 3% to increase digestibility, growth performance, and hematology of rohu fingerlings. Hence, it can be concluded that the significant effect of MOLM based diet supplementing with citric acid was observed to promote nutritional digestibility, growth and hematological indices in *L. rohita* fingerlings.

INTRODUCTION

Despite the fact that the proportion of hungry people has dropped over the past 50 years due to a significant increase in food production (Mondialsee, 2008) but still about 795 million people are extremely undernourished (FAO *et al*., 2015). By 2050, there is an increase in global production up to 9 billion approximately. To fulfill the growing demand of increasing population, there would be about 50% more requirement of food (Diana *et al*., 2013). Aquaculture is a basic component of food security and it provides at least 20% of animal protein intake to 3.1 bisllion people. If supported, aquaculture can help minimize hunger, food insecurity, and malnutrition (FAO, 2016). There were approximately 73 million tonnes of global aquaculture production and there is expectation of increase in its production by 33% in 2025. There are 80 million metric tonnes per year global production of marine fisheries out of which two-thirds are used for consumption by humans and other remaining part is used for the production of fishmeal and fish oil (Smith *et al*., 2011). There is high need to produce some economically suitable

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feed sources of better quality to satisfy the demands of future food production through aquaculture (FAO, 2012).

The industry of aqua-feed is majorly dependent upon fishmeal and fish oil. Fishmeal is either in the form of cake or brown powder. It is a commercial material and is an ideal and exceptional source of some major nutrients i.e., minerals, essential amino acids, vitamins, growth factors and fatty acids which are required for formulation of fish feed (NRC, 2011). It has high amount of nutrients and composed of high protein content which could be stored easily so we use it in diets of many aquatic and terrestrial animals and sometimes as a fertilizer (Pauly *et al*., 2005). From the last 25 years, there is no increase in the overall production of annual fishmeal and fish oil. That's why for the supply of fishmeal and fish oil, commercial aquaculture cannot constantly depend on restricted stocks of marine pelagic fish (Turchini *et al*., 2009). Moreover, in the last three decades, the prices of fish meal have also increased definitely and there is expectation of further increase in its costs due to increasing demand of fishmeal with the passage of time (FAO, 2016). Fishmeal is also used as an important constituent of feed in intensive farming of livestock i.e., poultry and pork, therefore, this issue in not unique to aquaculture. However, fishmeal resources are used widely in the sector of aquaculture globally because there is comparatively large variety of substitutes of poultry and livestock (Bostock *et al*., 2010).

To obtain and maintain environment friendly, economically feasible and substainable production, biologists are particularly interested in discovering unconventional protein sources from plants (Abo-State *et al*., 2014). Different plants and their products have been used in different studies as partial or complete replacement of fishmeal (Daniel, 2018). One of the most potential sources of protein which we use for inclusion in different aquaculture feeds is *Moringa oleifera* (Hussain *et al*., 2017). Moringa is a slender tree with softwood and its family is Moringaceae. It is native to the regions of Sub-Himalaya and drum-stick is used to name it commonly. It is well known as tree of life because it has various health benefits and high nutritive values. Leaves of *M. oleifera* are highly nutritious and roots, barks, seeds, seed oils, leaves, fruits, flowers and gums i.e., all parts of this tree are used for different beneficial purposes and they have unique properties (Anwar *et al*., 2007). *M. oleifera* leaf meal (MOLM) is considered as an innovative alternative to fishmeal because it has favorable amino acid profile and it is widely available throughout the whole globe (Tagwireyi *et al*., 2014). Moreover, there are plenty of minerals i.e., calcium, potassium and iron and vitamins in moringa (Kou *et al*., 2018).

Unfortunately, diet formulated by these plants ingredients is composed of many anti-nutritional factors and substances such as inhibitors of protease, lectins, glucosinolates, phytates, tannins, saponins, gossypols, and non-starch polysaccharides, and they all cause the bitter taste of diet which is poorly acceptable by fishes (Francis *et al*., 2001). Studies are underway to tackles this problem by supplementing plant-based diets with some organic acids which can lower fish gut's pH (Baruah *et al*., 2005). Nutrient absorption (Boling *et al*., 2011) and phytate solubility (Shah *et al*., 2015) are increased by lowering the pH of gut and feed. In addition to this, acidification of diet reduces the rate of gastric emptying (Mayer, 1994). Disease resistance is the powerful effect of organic acid and it leads to improved growth and nutrient utilization (NRC, 2011). Citric acid (CA), owing to its distinct/sweet taste and comparatively high absorbing power, they are often employed in aquaculture (Hossain *et al*., 2007). It provides an optimum pH so that's why it also causes increase in the effectiveness of both exogenous and endogenous phytases. Apart from this, it also acts as a feed attractant and immunity booster in fish (Shah *et al*., 2015). Therefore, we planned the current study to reveal the impact of MOLM based diet supplemented with CA on digestibility, growth ratio and hematological indices of *L. rohita* fingerlings.

MATERIALS AND METHODS

Fish trial setup

Fingerlings of *L. rohita* were obtained from the Government Fish Seed Hatchery in Faisalabad. They were immersed in 5g/L NaCl for 2 minutes, prior to the experiment to get rid of ecto-parasites (Rowland and Ingram, 1991). The fingerlings were acclimated for two weeks in the V-shaped lab containers with a volume of 70 L of water and which were especially designed for the collecting of fish feces. Fingerlings were fed a basal diet for their satiation once daily throughout the acclimation phase. Daily monitoring was done for physical factors including temperature, pH, and dissolved oxygen. Air was supplied through an air pump to maintain an optimum dissolved oxygen level. Tap water was used during the whole experiment.

Feed components and experimental diets

Leaves of *M. oleifera* were collected from the botanical garden of University of Agriculture, Faisalabad whereas the rest of the feed components (Table I) were purchased from a commercial market to formulate MOLM based experimental diet. The ingredients of feed were granulated finely so they could fit through a mesh with a 0.5 mm opening. Then in a food mixer, all of the

Table I. Composition (%) of feed ingredients.

Feed ingredients	Dry matter (%)	Total carbohydrate (%)	Gross energy (kcal g^{-1})	Ash (%)	Crude fat (%)	Crude protein (%)	Crude fiber (%)
Fish meal	91.62	17.94	3.69	26.23	7.16	48.15	0.52
Wheat flour	92.45	83.82	2.96	2.08	2.35	10.10	1.65
Rice polish	94.09	64.73	4.33	7.90	12.31	12.35	2.71
Soybean meal	93.8	37.99	3.54	10.83	3.74	41.93	1.97
MOLM	91.83	36.02	3.84	8.91	2.83	28.95	19.45

ingredients were combined for five minutes followed by the addition of fish oil. The total six diets were prepared, one control with 0% CA and five test diets, with 1% (50g), 2%(100g), 3%(150g), 4%(200g) and 5%(250g) CA supplementation, in MOLM based diet. Suitable dough was formed using water then a pelleting machine was used to create pellets from that dough (Lovell, 1989).

Feeding schedule and collection of samples

The *L. rohita* fingerlings were given 5% of their respective diets at live body weight twice a day. Two hours of feeding were followed by the removal of any uneaten feed from each tank for feed conversion ratio (FCR) analysis. Drainage system was installed for changing of water from tanks, to remove remaining diet particles. The collection of feces from each tank were drawn by fecal collecting tubes, three hours after feeding. These feces were dried at 60°C in oven, then it was entirely ground and kept for further analysis.

Chemical analysis

The homogenization of MOLM based diet samples and feces were separately analyzed by some standard techniques provided by AOAC (2005). Gross energy was estimated using an oxygen bomb calorimeter and crude protein and crude fat were estimated by using micro Kjeldahl apparatus and Soxtec system (HT2 1045), respectively. In order to evaluate the nutrient digestibility, chromic oxide was introduced to test diets (inert marker).

Study of digestibility

The apparent digestibility of crude fat, gross energy and crude protein was analyzed indirectly by using an inert marker (NRC, 1993).

$$\text{ADC (\%)} = 100 - 100 \times \frac{\text{Percent marker in diet} \times \text{Percent nutrient in feces}}{\text{Percent marker in feces} \times \text{Percent nutrient in diet}}$$

Study of growth performance

Standard formulae were used to find growth performance (initial and final weights) of these fingerlings.

$$\text{Weight Gain \%} = \frac{\text{Final Weight} - \text{Initial Weight}}{\text{Initial Weight}} \times 100$$

$$\text{FCR} = \frac{\text{Total dry feed intake (g)}}{\text{Wet weight gain}}$$

Hematological analysis

Tricane methanesulfonate (150 mg/L) solution was used to tranquiliz the fish after the completion of experimental period of 90 days (Wagner *et al*., 1997). For hematology analysis, the collected samples of blood were sent to Molcare Lab, Department of Biochemistry, University of Agriculture, Faisalabad. Haemocytometer was used to analyze the number of RBCs and WBCs (Blaxhall and Daisley, 1973) while haemoglobin (Hb) concentrations were evaluated following Wedemeyer and Yastuke (1977). Wintrobe and Westergreen method was used to evaluate packed cell volume (PCV) (Blaxhall and Daisley, 1973). Mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated by the following formulae.

$$\text{MCH} = \text{Hb/RBC} \times 10$$
$$\text{MCV} = \text{PCV/RBC} \times 10$$
$$\text{MCHC} = \text{Hb/PCV} \times 100$$

Statistical analysis

One-way analysis of variance (ANOVA) was performed on collected data (nutritional digestibility, hematology and growth performance) (Steel *et al*., 1996). To evaluate variations in means, Tukey's honestly significant difference test was applied (Snedecor and Cochran, 1991). The statistical analysis was conducted using the CoStat computer software (Version 6.303, PMB 320, Monterey, CA, USA).

RESULTS

Nutrient digestibility

Table I shows the percentage of nutrients present in MOLM based test diets and the percentage of nutrients available in feces of fingerlings. Fingerlings excreted significantly lower ($p<0.05$) amounts of crude fat (1.39%), crude protein (10.27%), and gross energy (1.06 Kcal g^{-1}) when they were fed diet with 3% CA supplementation.

Moreover, maximum gross energy (65.67%), crude fat (70.51%) and crude protein (67.40%) digestibility was noticed at the same level of CA supplementation (Table II). Next best values of these nutrients digestibility were recorded at 2% CA supplementation level. Relationship between CA and nutrient digestibility is shown in Figure 1. The estimated curves show that the digestibility of nutrients increased by increasing the CA level in MOLM based diets up to 3%, but with further increase in CA level, digestibility of nutrients started to decrease. Values of R^2 for crude fat (0.839), crude protein (0.931) and gross energy (0.920) indicates addition of CA in diets causes more than 80% change in digestibility of all above nutrients.

Growth performance

Table III described different parameters of growth of rohu fingerlings which were fed with MOLM based diets. Fingerlings indicated non-significant differences in feed intake against graded levels of CA supplementation (0%, 1%, 2%, 3%, 4% and 5%). However, the results showed that *L. rohita* fingerlings fed diets supplemented with CA performed better in terms of growth than those fed with a control diet. Maximum WG (23.73 g), WG% (255.07%), SGR (1.41) and minimum FCR (1.28) was observed in fingerlings which were fed with 3% CA supplemented diet. The estimated curves in quadratic regression analysis (Fig. 2) shows variation in many growth parameters with change in percentage of CA in diets. The R^2 values for WG% (0.819) WG (0.815), SGR (0.897) and FCR (0.842) show the considerable change i.e., 80% in the parameters of growth is due to supplementation of CA in different percentages. Optimum calculated values of CA supplementation for these parameters were 3.12%, 3.12%, 3.23% and 3.15%, respectively.

Table II. Ingredient composition (%) of experimental diets, nutrient composition of test diets and feces of fish fingerlings and nutrient digestibility of fish fingerlings fed MOLM based diets.

Ingredients	Test diet-I (control)	Test diet-II	Test diet-III	Test diet-IV	Test diet-V	Test diet-VI	PSE	p
MOLM/MOSM	35	35	35	35	35	35		
Fish meal	15	15	15	15	15	15		
Soybean meal	15	15	15	15	15	15		
Wheat flour	17	16	15	14	13	12		
Rice polish	8	8	8	8	8	8		
Fish oil	6	6	6	6	6	6		
Vitamin premix*	1	1	1	1	1	1		
Mineral premix**	1	1	1	1	1	1		
Ascorbic acid	1	1	1	1	1	1		
Chromic oxide	1	1	1	1	1	1		
CA level*	0%	1%	2%	3%	4%	5%		
Nutrient composition of test diets								
Crude protein (%)	29.81	29.81	29.81	29.81	29.81	29.82	0.05251095	0.0055071
Crude fat (%)	4.47	4.48	4.48	4.47	4.48	4.48	0.04554192	0.0107143
Gross energy (Kcal/g)	2.92	2.91	2.91	2.92	2.91	2.92	0.02711457	0.9978
Nutrient composition of feces of fingerlings fed based diets								
Crude protein (%)	17.06^{a}	15.30^{b}	11.24^{d}	10.27^{e}	11.50^{cd}	12.20^{c}	0.15382165	0.0000
Crude fat (%)	2.27^{a}	2.14^{a}	1.46^{c}	1.39^{c}	1.64^{b}	1.65^{b}	0.03318735	0.0000
Gross energy (Kcal/g)	1.73^{a}	1.57^{b}	1.11^{d}	1.06^{d}	1.13^{d}	1.27^{c}	0.02425696	0.0000
Nutrient digestibility of fingerlings fed test diets								
Crude protein (%)	46.66^{e}	52.35^{d}	64.41^{b}	67.40^{a}	63.72^{bc}	61.89^{c}	0.44193902	0.0000
Crude fat (%)	52.60^{d}	55.77^{c}	69.15^{a}	70.51^{a}	65.49^{b}	65.73^{b}	0.50223703	0.0000
Gross energy (%)	44.74^{d}	49.78^{c}	63.84^{a}	65.67^{a}	63.54^{a}	59.58^{b}	0.53155106	0.0000

*CA was utilized at the cost of wheat flour. Values are means of triplicates. Values along the columns vary significantly (p<0.05) if superscripts are different.

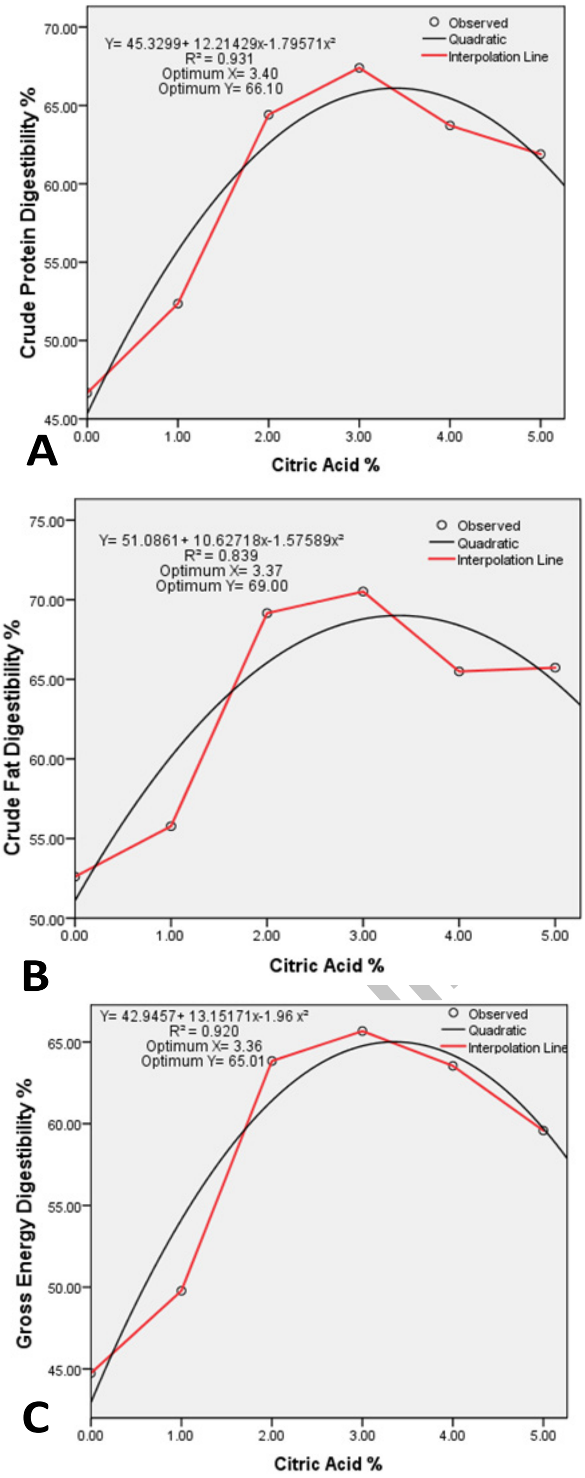

Fig. 1. Effect of CA effects on crude protein digestibility (A), crude fat digestibility (B) and gross energy digestibility (C) of rohu fingerlings.

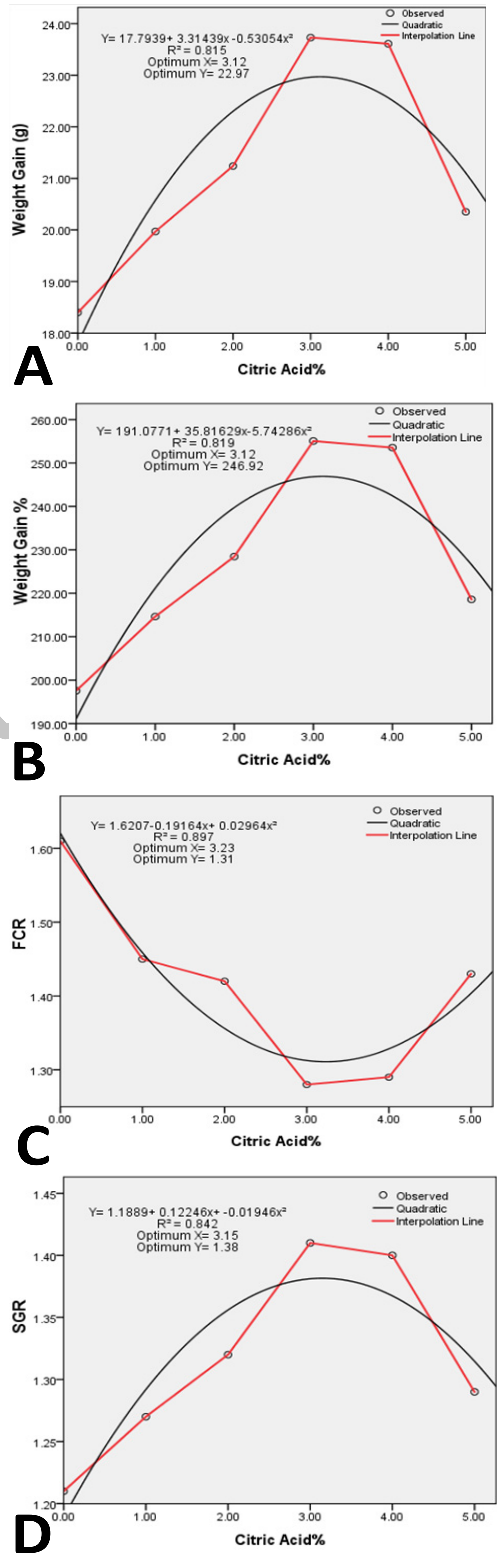

Fig. 2. Effect of CA on weight gain (A); % weight gain (B); FCR (C) and SGR (D) of rohu fingerlings.

Table III. Growth performance of *L. rohita* fingerlings fed MOLM based diets.

Experimental diets	CA %	Initial weight (g)	Final weight (g)	WG (g)	WG %	WG fish^{-1} day^{-1} (g)	Feed intake fish^{-1} day^{-1} (g)	FCR	SGR
Test diet-I (Control diet)	0	9.31	27.71^{d}	18.40^{d}	197.55^{d}	0.20^{d}	0.33^{a}	1.61^{c}	1.21^{d}
Test diet-II	1	9.31	29.28^{c}	19.97^{c}	214.63^{c}	0.22^{c}	0.32^{a}	1.45^{b}	1.27^{c}
Test diet-III	2	9.30	30.53^{b}	21.24^{b}	228.46^{b}	0.24^{b}	0.33^{a}	1.42^{b}	1.32^{b}
Test diet-IV	3	9.30	33.03^{a}	23.73^{a}	255.07^{a}	0.26^{a}	0.34^{a}	1.28^{a}	1.41^{a}
Test diet-V	4	9.31	32.93^{a}	23.61^{a}	253.55^{a}	0.26^{a}	0.34^{a}	1.29^{a}	1.40^{a}
Test diet-VI	5	9.31	29.66^{c}	20.35^{c}	218.59^{c}	0.23^{c}	0.32^{a}	1.43^{b}	1.29^{c}
PSE		0.038562	0.127017	0.136307	1.981647	0.001515	0.005593	0.01675	0.006896
P		0.9995	0.0000	0.0000	0.0000	0.0000	0.1872	0.0000	0.0000

Values are means of triplicates. Values along the columns vary significantly (p<0.05) if superscripts are different

Table IV. Hematology of *L. rohita* fingerlings fed MOLM based diets.

Experimental diets	CA (%)	RBC (10^6mm^{-3})	WBC (10^3mm^{-3})	PLT	Hb (g/100ml)	PCV (%)	MCHC (%)	MCH (pg)	MCV (fl)
Test diet I (Control diet)	0	1.23^{d}	7.17^{bc}	61.48^{c}	6.32^{c}	22.12^{c}	25.82^{e}	37.60^{f}	95.33^{f}
Test diet II	1	1.85^{c}	6.98^{c}	60.17^{d}	7.46^{b}	23.50^{b}	27.40^{d}	39.47^{e}	103.61^{e}
Test diet III	2	2.42^{b}	7.72^{a}	64.31^{b}	7.30^{b}	25.33^{b}	33.60^{b}	41.90^{d}	184.60^{b}
Test diet IV	3	3.30^{a}	7.85^{a}	66.10^{a}	8.46^{a}	25.27^{a}	34.33^{a}	55.40^{a}	186.89^{a}
Test diet V	4	2.26^{b}	7.35^{b}	64.06^{b}	8.21^{a}	23.17^{a}	32.56^{c}	53.36^{b}	183.26^{c}
Test diet VI	5	1.94^{c}	7.03^{c}	61.20^{c}	7.34^{b}	22.31^{b}	28.07^{d}	50.37^{c}	157.53^{d}
PSE		0.052263	0.062198	0.104172	0.09155	0.07734	0.147623	0.195145	0.184822
p		0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Values are means of triplicates. Values along the columns vary significantly (p<0.05) if superscripts are different

Hematological indices

In the current study, fingerlings showed significant (p<0.05) improvement in hematological indices when fed CA supplemented diets in contrast to control diet (Table IV). The greatest levels of RBCs (3.30×106 mm-3), WBCs (7.85×103 mm-3), Hb (8.46 g/100 ml), MCHC (34.33%), PLT (66.10), PCV (25.27%), MCV (186.89 fl), and MCH (55.40 pg) were observed in fingerlings fed a diet of 3% CA, according to comparison of means. However, significantly lower RBC (1.23×10^6 mm^{-3}), Hb (6.32 g/100ml), PCV (22.12%), MCHC (25.82%), MCH (37.60 pg) and MCV (95.33 fl) were observed in fingerlings which were fed with control diet. These results revealed that there were no serious effects of MOLM based diets with CA supplementation on the hematology of fingerlings.

DISCUSSION

Presence of high protein contents, low levels of anti-nutritional factors and adequate amount of essential amino acids in moringa leaves make it promising alternative to fishmeal. Moreover, it is being cultivated at commercial bases in several regions of Pakistan. As we found that fish fed diets enriched with 3% CA showed better growth. Improved growth responses to CA supplemented diets have also been reported by Khajepour *et al.* (2012) in *Cyprinus carpio*; Hussain *et al.* (2015) in *C. mrigala*; Zhu *et al.* (2015) in *Pelteobagrus fulvidraco*; and Hisano *et al.* (2017) in *Piaractus mesopotamicus.* This improvement in fish growth performance is may be due to the fact that intestinal pH is lowered by CA which in return increases the efficacy of digestive enzymes. Then it results in higher nutrient digestibility (Hussain *et al*., 2017). Likewise, Zhang *et al.* (2020) concluded that the CA supplementation enhanced the growth of *Carassius auratus* because the activity of the gut enzymes increased the consumption of nutrients.

These results agree with Hussain *et al.* (2017) who found that feeding 3% CA supplemented diets to *C. mrigala* fingerlings substantially (p<0.05) increased the levels of WG, SGR, and FCR. In contrast to our results, Hussain *et al.* (2015) discovered that fish given 2% and

5% CA supplemented diets showed the best results. Sarker *et al*. (2007) analyzed improved WG and FCR in *Pagrus major* fed a 1% CA supplemented plant protein source diet. Hisano *et al*. (2017) reported improved growth in *Piaractus mesopotamicus* fed a 2% CA supplemented diet for 30 days. This disagreement in results of growth parameters may be due to fact that different researchers used different feed ingredients, feed processing methods, species of fish and differences in stomach pH (Wang *et al*., 2009).

Numerous studies have documented the beneficial effects of organic acids on the ability of various fish species to assimilate nutrients. By optimizing the pH of the fish gut, organic acids addition to the feed, enhances the release of digestive enzymes. Optimal gastrointestinal tract pH also improves the growth of beneficial microorganisms which assist in feed digestion (Freitag, 2007; Boling *et al*., 2011). According to the current study, adding CA to diets based on MOLM significantly boosted total digestion of nutrients of rohu fingerlings which increased growth parameters. The outcomes of this study concur with Baruah *et al*. (2007) and Hussain *et al*. (2017), they found that 3% CA supplementation enhanced the nutrient digestibility in *L. rohita* and *C. mrigala* juveniles, respectively. Significant results of CA as an additive, on the digestibility were also observed by Hussain *et al*. (2015), Zhu *et al*. (2015), Rabia *et al*. (2017), and Hisano *et al*. (2017).

Hematological indices are those factors which got least attention for study in fish, however, they are important to access fish overall performance and to evaluate which diet is better (Shahzad *et al*., 2016). The current data are in line with Baruah *et al*. (2009) who also noticed that the hematology and immunological systems of fish were positively affected by nutritional availability. Significant (p<0.001) improvement in blood parameters in fish fed 3% CA added diets was also reported by Khajepour *et al*. (2011). Reda *et al*. (2016) also observed and reported positive impacts of dietary acidification on blood indices in *Oreochromis niloticus*. In contrast to this, Zhang *et al*. (2020) came to the conclusion that the addition of citric acid had no significant results on hematological indices of *C. auratus*. The differences in results of other scientists are due to variation in the strategy, duration, habitat as well as different species of fishes.

CONCLUSION

Finally, CA supplementation at 3% level improves *L. rohita* fingerlings nutritional digestibility, growth, and hematological parameters without having any deleterious effects. It is recommended to consider the impact of CA supplementation in diets on amino acid and fatty acid profiles of fish flesh.

DECLARATIONS

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IRB approval

All applicable institutional, national and international guidelines for the care and use of animals were followed.

Ethical statement

All the procedures and methods used in this study followed the ethical guidelines provided by Government College University Faisalabad.

Statement of conflict of interest

The authors have declared no conflict of interest.

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