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Research Article

Effect of feeding with different levels of trisodium citrate on growth performance, immunocompetence triats, and digestive enzymes in goldfish (*Carassius auratus*)

Azari A.[1]; Shamsaie Mehrgan M.[1*]; Tatina M.[2]; Rajabi Islami H.[1]

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Abstract

A 56-day feeding trial was conducted to find the potential effects of trisodium citrate (TSC) on the growth performance, immue response, and digestive enzymes of goldfish. The fish (n=150; 2.51±0.11 g) were randomly distributed into five groups with triplicates and fed with a basal diet without TSC (control diet) and four levels of TSC (5, 10, 15, and 20 g kg^{-1}) for eight weeks. Results showed that TSC supplemented diets markedly enhanced the final weight, specific growth rate, feed conversion ratio, and feed intake (p<0.05). Moreover, serum immune parameters such as total immunoglobulin, lysozyme, and complements (C3 and C4) were significantly increased with the supplementation of dietary TSC (p<0.05). Furthermore, protease, lipase, and amylase values were profoundly increased with the increments of dietary TSC compared to the control group (p<0.05) and they were almost doubled in the fish fed with TSC at 20 g kg^{-1} TSC compared to the control. Taken together, the optimum dosage of TSC supplementation was 20 g kg^{-1} in terms of growth efficiency, immune parameters, and digestive enzymes for juvenile goldfish.

Keywords: Acidifier, Digestive enzyme, Growth, Goldfish, Innate immunity, Trisodium citrate

1-Department of Fisheries, Sciene and Research Branch, Islamic Azad University, Tehran, Iran.
2-Department of Fisheries, Astara Branch, Islamic Azad University, Astara, Iran.
* Corresponding author. m.shamsaie@srbiau.ac.ir

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Introduction

The quickly growing aquaculture practice meets sustainability and economic challenges correlated with fish nutrition (Gonçalves *et al.*, 2019). Nowadays, the cost of feed is up to 70% of the total expense in intensive farming; therefore, amelioration in aquatic feed efficiency is already considered as a priority in the aquaculture nutrition (Taghavizadeh *et al*., 2020; Hosseini Shekarabi *et al.*, 2021a,b). Accordingly, researchers turned their attentions to some feed additives that have a significant effect on growth rate and feed efficiency as well as survival in aquaculture.

One of the functional additives used in aquatic animals that can play an imperative role in aquaculture is organic acids and their salts (acidifiers). These compounds contain short-chain fatty acids (SCFAs; C1-C7), weak lipophilic carboxylic acid, and volatile fatty acids that following microbial fermentation (Ng *et al*., 2009; Ng and Koh, 2017; Safari *et al*., 2016). It is a well-established fact that acidifiers in the diet can reduce the diet's antinutrients, whereby increasing mineral bioavailability in the intestine (Khajepour and Hosseini, 2012a). Moreover, by decreasing the chyme's acidity in the alimentary tract, acidifiers can excite the pepsin activity and cause cholecystokinin delivery (Sotoudeh *et al*., 2020). Furthermore, many studies confirmed that acidifiers could improve digestibility by attenuating the intestinal pH, thereby increasing growth performance (Khajepour and Hosseini, 2012b; Kalantarian *et al*., 2020; Mohammadian *et al*., 2020), hinders the growth of Gram-negative bacteria (Lückstädt, 2008), boost up the antioxidant potential (Zhang *et al*., 2016), alleviate the oxidative damages, mitigate the balances in the intestine microbiota (Chen *et al*., 2018), and growth promotants (Ng and Koh, 2017) in various fish species.

Trisodium citrate (TSC) is the sodium salt of citric acid. It is widely used in the food industry as an additive for food preservation, seasoning, and emulsifier agent (Ozcan-Yilsay *et al*., 2007; Chu *et al*., 2020). Moreover, TSC is used as an anticoagulant with potential antimicrobial activity in medicine (Getaneh *et al*., 2020; Weijmer *et al*., 2002).

The goldfish, *Carassius auratus*, is a freshwater fish and one of the earliest fish to be domesticated and is an experimental animal model in scientific research (Rashmeei *et al*., 2020; Rashmeei *et al*., 2021). Recently, more investigations have been fulfilled on citric acid and their salts in terrestrial and marine animals. To our knowledge, no investigation has been carried out on TSC in aquatic animals. Therefore, the present study aimed to examine the effects of TSC on growth performance, homural immunological parameters, and digestive enzymes on goldfish.

Materials and methods

Experimental facility and fish husbandry

A total of 150 healthy juvenile goldfish with an average weight of 2.51±0.11 g

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were purchased from a private farm located in Mahalat (Markazi Province, Iran). Fish were transferred to the Fisheries Laboratory Complex of Islamic Azad University, Science and Research Branch (Tehran, Iran). Before the experiment, fish were accommodated to the new culture system and fed six times daily with the basal diet (Kimiagaran Company, Shahrekord, Iran, containing 40.59% protein, 8.11% lipid, 9.32% ash, and 5.46% fiber) by hand to apparent satiation.

Prepared diets received 5, 10, 15, and 20 g kg^{-1} SDF diet TSC (RZBC Co., Ltd, Rizhao, Shandong, China) and each treatment contained three replicates. The additives were sprayed to the basal diet by gelatin (2%). The other basal diet (without TSC or any organic acids or acidifiers) was sprayed with gelatin 2% and applied as the control diet. Before the initiation of the experiment, the juveniles were anesthetized using clove powder (150mg L^{-1}) and weighed by a digital scale (plus 6700, Tehran, Iran). The fish were randomly distributed in the tanks (30×50×37 cm) at a density of 10 fish per container. The trial fish were placed in 15 containers. The water volume of each container was 50 L. About 30% of the isothermal water in the container was exchanged daily. Aeration in the containers was performed continuously. During the 56 days of the feeding trial, water temperature fluctuated from 24-25°C; 7.1 ± 0.2 pH, dissolved oxygen was approximately 7.3-7.5 mg L^{-1}; unionized ammonia <0.05 mg L^{-1}, nitrite <0.1 mg L^{-1}, and photoperiod regime set up as 12 h light: 12 h dark (Rashmeei *et al*., 2020).

Growth parameters and somatic indices

At the end of the feeding experiment, juveniles were starved for 24 h, then sedated with clove powder (150 mg L^{-1}). All fish was weighed separately, and morphometrical indices were recorded. Three fish per treatment were sacrificed by an overdose of anesthetic solution and stored at -20°C for future analysis. The following growth performance and feed indices were calculated following the below formulas:

Weight gain (WG, g)=(W_2-W_1)

Weight gain rate (WGR; %)= 100×(final body weight (g)-initial body weight (g))/ initial body weight (g)

Specific growth rate (SGR, %/day)=100×[(Ln (W_2)-Ln (W_1)/T]

Feed conversion ratio (FCR)=DFI/WG

Condition factor (CF, %)=100× (W_2/FL^3)

Feed intake (FI, g/day)=TFI/T

Survival rate (SR, %) = (initial fish number - dead fish number)/ initial fish number × 100%

where W_1 (g) and W_2 (g) are initial and final weights, respectively; DFI: dry feed intake (g); WG: weight gain (g); FL: final length (cm); TFI: Total feed intake (g); T: time of the feeding trial (day).

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Immunological analysis

Serum lysozyme value was quantified by the turbidimetric method illustrated by Ellis (1990) with slight modifications. In the present study, serum (15 µL) was poured into a well plate including 135 µl of *Micrococcus lysodeikticus* solution (Sigma-Aldrich). The absorbance decrease in absorbance of 0.001 mL^{-1} plasma min^{-1} under the defined conditions of pH 6.2 and at 25°C. Serum immune indices, including total immunoglobulin (Ig) and protein complement 3 and 4 (C_3 and C_4), were detected by an autoanalyzer (Prestige 24i, Tokyo Boeki Medical System, Japan) and determined using the commercial reagent kits (Pars-Azmoon, Iran).

Digestive enzymes assay

At the end of the feeding trial, three fish from each treatment were randomly sampled, sedated, and dissected on ice to achieve the alimentary tract individually. Then, the homogenate suspending was centrifuged at 15000 ×*g* for 30 min at 4°C, and the supernatants were collected and stored at -80°C until future analysis (Hosseini Shekarabi *et al.*, 2021b).

The protease activity of each extract was detected according to Kunitz (1947), using casein as a substrate. A mixture of 0.1 mL crude extract, 0.5 mL phosphate buffer (100 mM, pH 7.5) and 2 mL casein (1% in phosphate 50 mM, pH 7.5) was incubated at 37°C for 20 min. The reaction was stopped with the addition of 1 mL trichloroacetic acid (30%), and the mixture was clarifed by centrifugation (1200 *g* for 15 min). Absorbance was measured at 440 nm. Additional negative control was generated by replacing crude extract with phosphate buffer. A standard curve of absorbance at 440 nm was established using tyrosine as a standard. One unit of protease activity was considered as the number of micromoles of tyrosine released per minute per gram of protein at 37°C.

The lipase activity in the sample was recognized according to Bülow and Mosbach (1987) method. The reaction mixture was consisted of 350 µL of 0.53 mM p-nitrophenyl myristate in 0.25 mM Tris-HCL, pH 9.0 including 20 µL of crude extract 0.25 mM 2-methoxyethanol and 5 mM sodium cholate. The response was initiated by the blending of the substrate and incubated for 30 min at 30°C. The reaction mixture was energetically agitated and centrifuged at 6080 g for 2 min. One unit of the enzyme extracts was detected as the enzyme's volume required to form 1 µmol of p-nitrophenol per min.

Quantifying of amylase activity was fulfilled using dinitrosalycylic acid based on the method illustrated by Bernfeld (1955), with a trivial alteration. The soluble starch 0.2% (w/v) was solved in 0.02 M Na_2HPO_4 buffer pH 6.9 containing 0.006 M NaCl. At first, 250 µL of the substrate was mixed with 50 µL of buffer solution and 150 µL of the crude extract and incubated at 37°C for 30 min. Then, 100 µL of dinitrosalicylic acid solution (1%) as the color reagent was combined and heated in a warm

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water bath for 5 min. After cooling, 1 mL distilled water was added to the blend, and its absorbance was recorded at 540 nm. Maltose was utilized as a standard draw curve. One unit of amylase activity was manifested as the value of the enzyme-producing 1 μmol maltose per min, which the status mentioned above.

Statistical analysis

All data were expressed as mean±SD. One-way analysis of variance (ANOVA) accompanied by Duncan's test was used for differences between treatments at $p<0.05$, followed by checking the normality and homogeneity of data. All comparisons were carried out by SPSS software version 19.0.

Results

Growth and feeding performance

Table 1 displays growth efficiency and feed utilization of juvenile goldfish fed diets containing different levels of TSC. The WGR and SGR in fish fed diets more than 10 g kg^{-1} TSC were significantly increased compare with the control and 5 g kg^{-1} TSC groups at day 56 ($p<0.05$). Conversely, FCR was notably decreased in fish fed TSC diet groups, so that the inferior FCR was recognized in 20 g kg^{-1} TSC treatment ($p<0.05$). Albeit an increasing trend of CF was observed in juvenile fed the diets of TSC but no significant difference was found across all dietary treatments ($p>0.05$). Nonetheless, fish fed with the basal diet without any inclusion of TSC had significantly lower FI compared to fish fed with different inclusions of TSC ($p<0.05$). In the course of the experiment, no mortality was recognized.

Table 1: Growth performance and feed utilization of juveniles goldfish (*Carassius auratus*) fed with various levels of trisodium citrate (TSC) in 56 days.

	Experimental diets				
Index	**0 (basal diet)**	**0.5**	**1**	**1.5**	**2**
IW (g)	2.55±0.07	2.59±0.03	2.48±0.05	2.52±0.17	2.40±0.10
FW (g)	8.59±1.01^{c}	9.00±0.08^{c}	10.86±0.61^{b}	11.94±0.50^{ab}	12.88±0.29^{a}
WGR (%)	237.20±41.85^{c}	248.19±7.64^{c}	338.92±32.68^{b}	373.73±38.05^{b}	436.62±33.25^{a}
SGR (%/day)	2.16±0.22^{c}	2.23±0.04^{c}	2.64±0.14^{b}	2.78±0.08^{ab}	3.00±0.11^{a}
FCR	1.75±0.11^{a}	1.62±0.02^{b}	1.52±0.05^{bc}	1.44±0.03^{cd}	1.36±0.07^{d}
CF (%)	2.32±0.49	2.39±0.71	2.51±0.64	2.67±0.84	2.91±0.80
FI (g/day)	0.18±0.04^{b}	0.19±0.02^{b}	0.23±0.01^{a}	0.24±0.01^{a}	0.25±0.00^{a}
Survival (%)	98.33±0.58	99.00±1.00	99.33±1.15	98.67±1.53	99.33±1.15

A different superscript in the same row indicates statistically significant differences between treatments ($p<0.05$). The absence of superscript shows no significant variation between treatments. IW, initial weight; FW, final weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; CF, condition factor; FI, feed intake.

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Immune parameters

Lysozyme and C4 were significantly increased using more than 10 g kg^{-1} TSC supplementation in the diet, while this trend was observed in C3 and total Ig in tretaments including more than 5 g kg^{-1} TSC in diet (p<0.05; Table 2), so that the superior and inferior values were observed in 20 g kg^{-1} TSC and basal diet group, respectively.

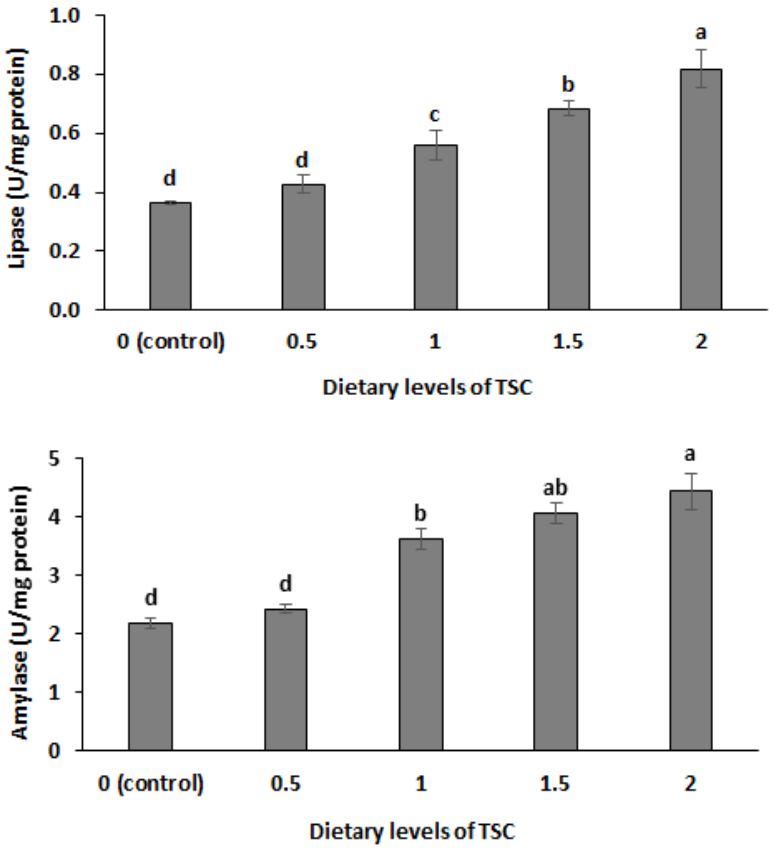
Table 2: Immune parameters of juvenile goldfish fed with trial diets in the course of the experiment

Index	Experimental diets				
	0 (basal diet)	0.5	1	1.5	2
Total Ig (mg dL^{-1})	7.45 ± 0.55^{a}	8.22 ± 0.26^{b}	9.24 ± 0.15^{c}	10.60 ± 0.63^{d}	11.67 ± 0.24^{e}
Lysozyme µg/mL/min)	13.57 ± 2.03^{d}	15.12 ± 1.42^{cd}	16.76 ± 0.82^{bc}	18.41 ± 1.36^{b}	21.83 ± 1.07^{a}
C_3 (mg dL^{-1})	35.51 ± 1.64^{c}	42.94 ± 0.10^{b}	44.06 ± 0.17^{b}	46.28 ± 0.24^{a}	47.42 ± 0.75^{a}
C_4 (mg dL^{-1})	13.11 ± 0.15^{c}	13.64 ± 0.25^{c}	17.49 ± 0.44^{b}	17.78 ± 0.28^{b}	22.45 ± 0.41^{a}

A different superscript in the same row indicates statistically significant differences between treatments (p<0.05). Total Ig, total immunoglobulin; C_3, complement 3; C_4, complement 4.

Digestive enzyme activity

The digestive enzyme activity of juvenile goldfish was profoundly influenced by dietary TSC (p<0.05; Fig. 1). Surprisingly, the digestive enzymes increased in the 20 g kg^{-1} TSC group compared to the basal diet reached nearly doubled after 56 days.



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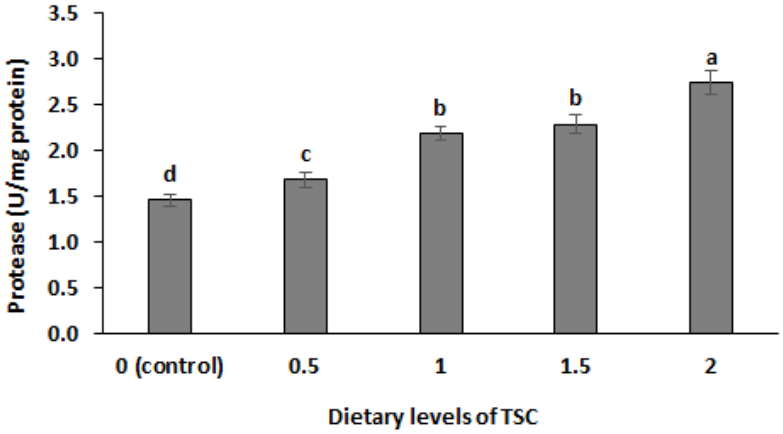


Figure 1: Digestive enzymes activity in the intestine of juvenile goldfish fed with different dietary levels of trisodium citrate (TSC) in the course of the experiment. A different superscript indicates statistically significant differences between the treatments (n=3; $p<0.05$).

Discussion

This study was carried out to find the influences of TSC on the organosomatic efficiency, immunocompetence, and digestive enzymes of juvenile goldfish. The results of current study demonstrated that dietary TSC promoted growth efficiency and feed utilization in juvenile goldfish after 56 days. Numerous studies have been issued on the positive effects of organic salts on the growth indices of different fish species. In line with our result, numerous studies showed that dietary citric acid or their salt derivatives supplementations had a beneficial influence on the zootechnical performance of several fish species (Baruah *et al*., 2007; Khajepour and Hosseini, 2012b; Zhu *et al*., 2015; Zhang *et a*l., 2016; Chen *et al*., 2018; Zhang *et al*., 2020).

Mentioned-above researchers stated contributing factors included minerals solubilization and bioavailability, dephosphorylation of phosphorus from phytate in soybean meal as the main protein source, inactivate or reduction the antinutrient agents (Ng *et al*., 2009; Khajepour and Hosseini, 2012b; Ng and Koh, 2017). Acidifiers also improve protein digestibility, stimulate the pepsin activity by alleviating the acidity of the chime, and provoke appetite by regulating of expression of orexigenic factors such as ghrelin (Hoseinifar *et al*., 2017).

Similarly, Chen *et al*. (2018) indicated that supplemented citric acid in soybean meal diet alleviated the intestinal impairment, leading to narrowed intestinal folds in the lamina propria in juvenile turbot, *Scophthalmus maximus*. Ng *et al*. (2009) stated that organic acid in the diet of red hybrid tilapia, *Oreochromis* sp, leads to a reduction in the total bacteria count in the intestine through acidification extracellular pH. In corroborated with this, Hassaan *et al*. (2018) asserted similar findings in the Nile tilapia, *O. niloticus*.

In line with our results, numerous studies in rainbow trout, *Oncorhynchus mykiss* (Vielma *et al*., 1999), yellowtail,

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Seriola quinqueradiata (Sarker *et al*., 2012), juvenile beluga, *Huso huso* (Khajepour and Hosseini, 2012a, 2012b), and sea bream, *Pagrus major* (Hossain *et al*., 2007) have also reinforced that dietary citric acid to diets of plant-based protein is very impressive in ameliorating the growth efficiency, and bioavailability of minerals. Recent researchers suggested that citric acid contributes to the eco-friendly feed formulation for aquatic animals.

Notwithstanding, regardless of improvement in nutrient bioavailability of acidifiers in diets in most literatures, the inconsistent result on organosomatic index of potassium diformate has been announced in the goldfish and hybrid tilapia, *O. niloticus* ♀×*O. aureus* ♂ (Sugiura *et al*., 1998; Zhou *et al*., 2009). These contradictory results may be manifested in different rearing periods, aquatic animal species, dosage and type of organic acid or salt, age of the aquatic animal, diet composition, and culture condition (Sugiura *et al*., 1998; Ng *et al*., 2009; Zhou *et al*., 2009; Hoseinifar *et al*., 2016; Ng and Koh, 2017).

Lysozyme is a part of the innate defense and non-specific immune mechanisms as a natural endogenous antibiotic that directly lytic activity versus Gram-positive bacteria and indirect bactericidal activity against Gram-negative bacteria by exciting complement system by the opsonic effect (Reyshari *et al*., 2019; Ghodrati *et al*., 2021). The present study revealed that incremental supplementation of TSC in the diets up to 20 g kg^{-1} leads to elevation of serum lysozyme, Ig, C_3, and C_4. In agreement with our results, Safari *et al*. (2017) declared that common carp, *Cyprinus carpio,* fed diets supplemented with sodium propionate (20 g kg^{-1}) had a significant increase in lysozyme and Ig in the serum, which was related to up-regulation of immune-related genes expression. Previous study also demonstrated that juvenile loach, *Paramisgurnus dabryanus*, fed diet supplementation of more than 10 g kg^{-1} fulvic acid triggered elevation in immune parameters such as lysozyme and C_3 (Gao *et al*., 2017). Meanwhile, the immune system response varies depending on the species, fish size, type, and purity of the acidifier. In this regard, Reda *et al*. (2016) showed that formic and propionic acid/salt supplementation in low level (10 g kg^{-1}) in the Nile tilapia diet had no reinforcements in immune indices. Therefore, future research is needed to target the effects of acidifiers on the immune system of aquatic animals.

Another aspect of the benefit of the TSC is the improvement in digestive enzymes. Our findings revealed that digestive enzymes markedly increased with concomitant elevating levels of dietary TSC. This could be attributed to superior growth performance and more feed efficiency in diets supplemented with TSC. It has been recommended that acidifiers lead to the secretin excretion by declining pH of digesta and cause cholecystokinin's liberation, then stimulating pancreatic exocrine secretions, including digestive enzymes (Castillo *et al*., 2014; Ng and Koh, 2017).

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The researchers proposed that dietary citric acid and KDF also resulted in higher activities of digestive enzymes in juvenile turbot (Castillo *et al*., 2014). The leucine-aminopeptidase and phosphatases were pronounced to be more in fish fed with organic acid supplementation diets (Ng and Koh, 2017). Similar to our result, it has been revealed that digestive enzymes activities intensified by the addition of dietary acidifiers such as citric acid in red drum, *Sciaenops ocellatus* (Castillo *et al*., 2014) hybrid tilapia (Li *et al*., 2009), sodium diformate in Asian sea bass, *Lates calcarifer* (Reyshari *et al*., 2019). da Silva et al. (2016) claimed that trypsin and chymotrypsin values decreased in whiteleg shrimp (*Litopenaeus vannamei*) fed diet supplemented with butyrate and propionate.

The results of current study revealed that dietary TSC at 20 g kg^{-1} increased the somatic indices by increasing palatability of the diets and feed intake as well as digestive enzymes. In addition, using TSC in the diet was beneficial for the immune parameters of goldfish which lead to consider as a potential feed additive in cultured fish species especially Cyprinids. However, determining the optimal concentration of acidifiers in the diet of any fish need to be elucidated further by future studies.

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