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Research Article

Increasing Phosphorus Digestibility in Common Carp (*Cyprinus carpio* L.) Farming Using Phytase and Citric Acid

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The presence of antinutritional substances, such as phytate, in fish feed affects the digestibility and absorption of minerals and nutrients by fish, while reduced availability of phosphorus (P) in wheat-based feeds used in fish farming can increase pollution in the aquatic environment. Phosphorus digestibility can be effectively increased in aquaculture through the addition of both phytase and citric acid. The aim of our study was to investigate the effects of phytase enzyme and citric acid addition on P digestibility, production parameters and blood parameters in farmed common carp (*Cyprinus carpio* L.). Two trials were undertaken using the following experimental diets: control with no additives (C), low enzyme content (500 FTU/kg; L), high enzyme content (1,000 FTU/kg; H), low enzyme contents with 3% citric acid (LA), high enzyme contents with 3% citric acid (HA). Initial results showed that LA increased P digestibility by 27% and HA by 26%, with no increase detected using L and H. In the second trial, in which production and blood parameters were examined, use of LA and HA resulted in a 20% decreased feed conversion ratio and 11% higher specific growth rate. Furthermore, acidified diets resulted in an increased blood plasma calcium and inorganic P, without negative effects on any parameter. Addition of phytase and citric acid to *C. carpio* granulated feeds also has a positive influence on the environment by reducing excreted P.

1. Introduction

There is an ongoing effort to replace animal components in fish feed with more easily available plant proteins. An important factor affecting utilisation of such plant proteins is the presence of antinutritional factors, such as phytic acid ($C_6H_{18}O_{24}P_6$), which can store up to 80% of total phosphorus (P) [1]. Furthermore, phytate complexes may be formed with sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), manganese (Mn), and iron (Fe) cations, as well as enzymes or vitamins, with negative effects on protein and lipid utilisation [2]. Phytases are catalysts in the hydrolytic decomposition of phytate [3]; however, owing to the negligible amount of intestinal phytase and its very low activity, fish are unable to effectively utilise P deposited in the phytate form [4]. Though endogenous phytases occur in plant seeds, they are inactive in dry seeds, with activity only increasing during the germination period when they provide sufficient amounts of P for plant growth. Even then, its utilisation by animals remains almost negligible. Microbial phytases have a much higher efficacy, however, and it is these that are utilised by ruminant animals [5].

Phytases are divided into two groups based on the location of the first hydrolysed phosphate group within the phytin molecule. The first group comprises 3-phytases, which begin to hydrolyse at the third carbon atom, while the second group comprises 6-phytases, which begin hydrolysis at the sixth carbon. Most microbial phytases are 3-phytases, while endogenous phytases form the second group [6]. Use of phytase in farmed fish feed, such as that for *C. carpio*, can improve the digestibility of dry matter, crude protein, carbohydrates, energy, ash, P, and Ca [7]; however, functioning of the enzyme is highly dependent on pH (acid phytases have an optimum pH of 5, and alkaline phytases around 8). Most microbial phytases show highest activity (expressed in phytase units (FTU/kg), where 1 FTU/kg is defined as the amount of enzyme that liberates 1 μmol of inorganic P per minute from 0.0051 mol/l of sodium phytate at 37°C and pH 5.5 [8]) within

Table 1: Composition of experimental diets.

Component	C	L	H	LA	HA
KP1 (18% CP[1]) (g/kg)	900	900	900	870	870
Soybean meal (45% CP[1]) (g/kg)	100	100	100	100	100
Citric acid[2] (g/kg)	0	0	0	30	30
Phytase[3] (FTU/kg)	0	500	1,000	500	1000
Pellet-dur (g/kg)	5	5	5	5	5
Dry matter (g/kg)	925.60	923.30	924.30	925.60	928.40
Crude protein (g/kg)	247.30	246.30	243.80	242.80	241.60
Crude fat (g/kg)	57.40	59.70	53.90	50.70	53.40
Crude fiber (g/kg)	56.00	50.80	49.80	50.00	51.90
Digestible energy (MJ/kg)	17.32	17.14	17.18	17.12	17.08
Total P (g/kg)	6.50	6.10	6.10	6.10	5.90
Calcium[4] (mg/kg)			9,600		
Sodium[4] (mg/kg)			1,800		
Iron[4] (mg/kg)			100.26		
Iodine[4] (mg/kg)			1.02		
Copper[4] (mg/kg)			4.95		
Manganese[4] (mg/kg)			20.10		
Zinc[4] (mg/kg)			86.64		
Selenium[4] (mg/kg)			0.41		
Vitamin A[4] (IU/kg)			8100		
Vitamin D3[4] (IU/kg)			1,500		
Vitamin E[4] (mg/kg)			59.51		

[1]Crude protein. [2]99.8%–100.5% $C_3H_4(OH)x(COOH)_3xH_2O$, CAS: 5949-29-1, E330. [3]Phyzyme XP 10.000 TPT, 6-phytase (EC 3.1.3.26). [4]Declared by the KP1 mixture manufactured by VKS Stříbrné Hory, αCZ 800181-01. C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA; high enzyme concentration with citric acid.

a range of 2.5–5.5 [9]. Owing to unfavourable pH levels (ca. pH 6) in the gastrointestinal tract of carp, however, they are unable to use phytase effectively [10]. Nevertheless, recent studies have shown that addition of an organic acid to fish feed can positively influence phytase activity, thereby increasing phytate digestibility [11, 12]. Furthermore, addition of both phytase and citric acid positively affects the digestibility and absorption of minerals and their deposition into muscles and bones. In the case of fish with stomachs, the stomach acid helps lower the intestinal pH, and thus promotes phytase activity, with a subsequent increase in the utilisation of minerals, including P [2]. FTU/kg level can also influence phytase efficiency, with some studies obtaining better results at an FTU/kg of about 1,000 [13, 14], while others observed optimal diets at 8,000 FTU/kg [15] or at higher levels, such as 2,000 FTU/kg [16].

The reduced availability of P in plant-based fish feeds used in fish farming can also potentially increase pollution of the aquatic environment, with elevated P levels leading to cyanobacterial blooms [17]. Thus, use of phytase in fish feed has the potential to reduce the discharge of minerals and nutrients into open waters [18] and to contribute to the economic and environmental sustainability of aquaculture production [19]. In this study, we focused on increasing the P digestibility from plant-based feed in carp farming while maintaining the production and fish health indicators. Digestibility was altered using phytase enzyme combined with citric acid.

In this study, we monitored the effects of adding different mixtures of phytase enzyme and citric acid to aquaculture feed on (a) P digestibility, (b) fish production parameters, and (c) haematological and biochemical parameters of fish blood.

2. Materials and Methods

For the purposes of this study, we tested the industrially produced phytase Phyzyme XP 10.000 TPT (Danisco Animal Nutrition, United Kingdom) in fine granular form. Phyzyme phytase, which is produced by *Escherichia coli*, was mainly selected because of its high thermostability (up to 95°C). As the optimum pH for this enzyme ranges from 4 to 4.5, experimental diet formulae were prepared with and without citric acid. A standard commercially used carp breeding feed (KP1; VKS Stříbrné Hory, Czech Republic) was used as a base for all diets. The feed is composed of wheat, wheat flour, rapeseed expellers, wheat bran, extracted soybean meal, barley, maize, calcium carbonate ($CaCO_3$), sodium chloride (NaCl), and soybean oil and has a protein content of 18%. Owing to the low-protein content in KP1, all experimental diets were enriched with 100 g/kg extracted soybean meal. Five experimental diets were prepared (see Table 1): a control with no additions (C), two mixtures enriched with 500 FTU/kg (low enzyme content, L) and 1,000 FTU/kg (high enzyme content, H) phytase, and two mixtures containing 500 and 1,000 FTU/kg with the addition of 30 g kg^{-1} of citric acid ($C_6H_8O_7$; LA and HA, respectively), the latter being food grade crystalline citric acid (CAS: 5949-29-1; Laiwu Taihe

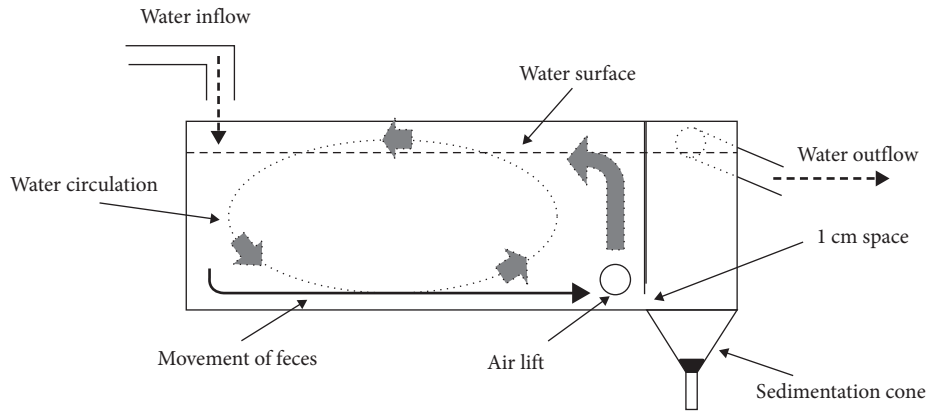

FIGURE 1: Schematic diagram of the tank for collection of excreta.

Biochemistry Co., Ltd.). Stabilisation was achieved using pellet-dur granules.

As a prerequisite for achieving positive results was the accurate detection of phytase activity in the diets used, all analyses were carried out at the Central Institute for Supervising and Testing in Agriculture, according to Standard EN ISO 30024 [20]. The diets used were further assessed for digestible energy content, using the calorimetric method, to prevent different dietary energy values influencing the experiment.

Advanced common carp fry (Amur mirror carp—line Pohořelice [21]) were obtained from Rybníkářství Pohořelice (Czech Republic) and reared at Mendel University in Brno (Czech Republic) in a 3,890 L indoor recirculating system consisting of three 1,000 L tanks and a NEXUS 310 biofilter (Evolution Aqua, United Kingdom) until the beginning of the experiment.

The experiment was divided into two parts aimed at determination of (i) P digestibility, and (ii) production parameters and fish blood parameters.

2.1. Phosphorus Digestibility. Seven fish with an average weight of 85.0 ± 19.8 g were stocked in each of the six 106 L tanks connected to a recirculation system and adapted for the collection of fish excreta (Figure 1). The fish were fed with diet C and left to acclimatise to the experimental conditions for 2 weeks prior to the beginning of the experiment (see Supporting Information for a scheme of the experimental setup). After 2 weeks, the diet in two of the tanks was switched to either the L or H variant for 2 weeks, followed by the LA or HA variant over the next 2 weeks. In both cases two of the tanks remained on the control diet.

2.2. Sampling and Analysis of Excreta. Prior to the first feeding each morning, excreta were collected from each tank using a pipette and subsequently filtered through a 99 μm porosity filter paper, which was then placed in a sample container and stored in a freezer at −18°C. Digestibility of P was determined using endogenous fibre contained in the dietary components as an indicator, according to Liu et al. [22]. P digestibility was calculated as:

$$\text{P digestibility}\,(\%) = 100 \times [1 - (\%\text{EF}^{*}\ \text{in diets}/\%\ \text{EF in faeces}) \times (\%\text{nutrient in faeces}/\%\text{nutrient in diets})] \quad (1)$$

*EF − endogenous fibre.

The experimental diets and faeces samples were both analysed for total P using the photometric method, after which the fibre content was determined using the Henneberg–Stohmann method (weak acid and alkaline digestion) and digestibility of P subsequently determined.

2.3. Effect of Diet on Production Parameters and Fish Body Condition. Fifteen fish (same origin as Part 1) with an average weight of 134.4 ± 32.8 g were stocked in each of 10 160 L tanks and allowed to acclimatise on diet C for 1 week prior to the beginning of the experiment. After 1 week, the fish in each tank were fed one of the five experimental diets (C, L, H, LA, and HA), in two repetitions, for a period of 72 days (see Supporting Information for a scheme of the experimental setup). The fish were fed three times a day at a daily feeding ratio corresponding to 3% of the tank stock weight.

2.4. Water Quality. Basic water quality parameters were monitored throughout the experiment, with water temperature (mean = $26°C \pm 0.31°C$), dissolved oxygen content (6.06 ± 0.87 mg L^{-1}), oxygen saturation ($76.1\% \pm 7.32\%$), and pH (7.83 ± 1.22) measured in each tank twice a day using a HACH HQ40D multiparameter (HACH, Germany). In addition, nitrogen as ammonium ($N\text{-}NH^{4+}$; 0.10 ± 0.09 mg L^{-1}), nitrogen as nitrites ($N\text{-}NO^{2-}$; 0.10 ± 0.05 mg L^{-1}), and chlorides (Cl^{-}; 128.09 ± 33.43 mg L^{-1}) were determined once a day using a PhotoLab 6600 UV–Vis spectrophotometer (WTW, Germany).

2.5. Length–Weight Relationship and Fish Condition Parameters. Fish were measured (±1 mm) for total length (TL), standard length (SL), body height (BH), and body width (BW) and weighed (±1 g; body weight (W)) at the

Table 2: Fish length–weight and condition parameters.

Parameter	C	L	H	LA	HA
TL (mm)	249.07 ± 15.98	248.33 ± 20.35	250.93 ± 28.14	255.07 ± 19.90	258.6 ± 25.40
SL (mm)	189.73 ± 14.09	190.13 ± 15.68	192.13 ± 22.76	194.53 ± 18.46	196.6 ± 20.41
BH (mm)	75.93 ± 7.77	72.4 ± 6.37	72.27 ± 9.66	75.13 ± 7.35	76.4 ± 9.27
BW (mm)	38.2 ± 4.14	37.33 ± 3.39	37.67 ± 3.89	39.33 ± 3.64	39.4 ± 3.07
W (g)	268.07 ± 95.39	259.13 ± 118.89	240.27 ± 94.79	306.93 ± 116.55	331.46 ± 87.45
F_C	3.86 ± 0.48	3.57 ± 0.28	3.74 ± 0.75	3.66 ± 0.42	3.67 ± 0.67
C_C	3.46 ± 0.43	3.21 ± 0.27	3.2 ± 0.38	3.32 ± 0.40	3.27 ± 0.61
I_W	20.12 ± 1.31	19.65 ± 1.00	19.67 ± 1.11	20.26 ± 1.39	20.16 ± 1.73
I_H	2.51 ± 0.17	2.63 ± 0.14	2.67 ± 0.17	2.59 ± 0.14	2.59 ± 0.24

Data represent mean ± SD, $n = 15$ (C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid). TL, total length; SL, standard length; BH, body height; BW, body width; W, body weight; F_C, Fulton's condition factor; CC, Clark's condition factor; IW, widebackedness index; IH, highbackedness index.

beginning and end of the experiment. These data were also used to calculate Fulton's condition factor (F_C), Clark's condition factor (C_C), highbackedness index (I_H), and widebackedness index (I_W) [23].

2.6. Production Parameters. Production parameters determined included the feed conversion ratio (FCR), specific growth rate (SGR), weight gain (WG), fish growth increment during the experiment, the protein efficiency ratio (PER), apparent net protein utilisation (aNPU), lipids retained (LR), and the hepatosomatic (HSI) and viscerosomatic (VSI) indices [24]. No fish died during the experiment.

2.7. Chemical Composition of Fish Tissue. At the end of the experiment, from each treatment, three randomly chosen fish were used to analyse whole-body chemical composition, and three to analyse muscle composition. Likewise, from each treatment, seven fish were used to obtain mixed gut samples, and seven to obtain mixed hepatopancreas samples. Dry matter was determined from homogenised samples by oven drying at 105°C for 24 hr, after which the lipid content was determined according to the Soxhlet method (10 hr extraction by diethyl ether using Soxhlet apparatus) and proteins according to the Kjeldahl method (Kjeldahl apparatus, content of nitrogen × 6.25). In each case, the values were recalculated to reflect the content of components in whole fish body, based on dry matter content.

2.8. Haematological Parameters. Blood samples were taken from the caudal vessels of 10 fish from each treatment at the end of the experiment using heparinised needles and syringes and immediately cooled to 4°C using a ThermoStat plus (Eppendorf, Germany) and stored in a laboratory refrigerator. Later, the samples were tested for haemoglobin (Hb), red blood cells (RBC), packed cell volume (PCV), mean corpuscular haemoglobin concentration (MCHC), mean cell volume (MCV), mean cell haemoglobin (MCH), and number of leukocytes (WBC) [25].

Part of each blood sample was centrifuged in a refrigerated MPW 350R centrifuge (MPW, Poland) and the plasma separated and stored in a freezer at −75°C (Arctiko ULTF 80, Denmark) until further analysis. Later, the samples were tested for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), albumin (ALB), cholesterol (CHOL), creatine (CREA), glucose (GLUC), lactate (LACT), urea (UREA), total protein (TP), triacylglycerol (TAG), Ca, inorganic phosphate (Pi), Mg, Na, K, and chlorides (Cl−). Biochemical parameters were analysed using an XT20i automatic analyser (Thermo Fisher Scientific, Finland) [26].

2.9. Statistical Analysis. Differences in length–weight, condition indices, and haematological and biochemical parameters between experimental treatments (C, L, H, LA, and LH) were assessed using hierarchical analysis of variance (ANOVA), with the effect of aquarium (two per treatment) nested within the effect of experimental diet and set as a random effect. This design allows for the use of individual fish data per aquarium while diminishing the potential effect of pseudo replication.

As the production parameters were calculated per aquarium, one-way ANOVA was used to determine the parameters on each fish. One-way ANOVA was also used to evaluate dry matter and chemical analyses of fish tissue. The effect of phytase concentration on P digestibility, both with and without the addition of citric acid, was tested using factorial ANOVA, with Tukey HSD post hoc tests used to reveal differences among treatments (including diet C). Data were $\log(x + 1)$ transformed to meet the assumptions of ANOVA. All analyses were performed in Statistica 13 (TIBCO Software Inc.) [27].

3. Results

3.1. Phytase Activity. In diet C, phytase activity was detected at 257 FTU/kg, which corresponds with endogenous phytase. In comparison, diets with the addition of 500 and 1,000 FTU/kg phytase, showed phytase activities of 761 and 1,350 FTU/kg, respectively, indicating higher phytase activity than required, presumably due to the presence of endogenous phytase (Figure 2).

Table 3: Fish production parameters.

Parameter	C	L	H	LA	HA
FCR[1]	2.44 ± 0.01^{a}	2.52 ± 0.09^{a}	2.46 ± 0.13^{a}	1.97 ± 0.06^{b}	1.97 ± 0.08^{b}
SGR[1]	$0.99 \pm 0.02^{a,b}$	0.91 ± 0.05^{a}	0.91 ± 0.03^{a}	$1.09 \pm 0.03^{b,c}$	1.12 ± 0^{c}
PER[1]	1.62 ± 0^{a}	1.67 ± 0.03^{a}	1.72 ± 0.04^{a}	2.17 ± 0.03^{b}	2.23 ± 0.05^{b}
aNPU[1]	$54.71 \pm 0.78^{a,b}$	49.27 ± 2.51^{a}	51.6 ± 1.98^{a}	59.38 ± 1.73^{b}	85.72 ± 1.98^{c}
LR[1]	443.94 ± 3.63^{a}	397.66 ± 5.11^{b}	453.79 ± 8.53^{a}	513.61 ± 4.94^{c}	602.9 ± 6.43^{d}
WG (g)[1]	$2014.5 \pm 118.1^{a,b}$	1807 ± 125.87^{a}	1803 ± 41.01^{a}	$2334.5 \pm 79.9^{b,c}$	2456.5 ± 43.13^{c}
WG (%)[1]	$100.69 \pm 3.63^{a,b}$	90 ± 5.85^{a}	90.52 ± 2.45^{a}	$115.97 \pm 3.4^{b,c}$	120.65 ± 1.28^{c}
HSI[2]	2.15 ± 0.45	2.29 ± 0.41	2.08 ± 0.39	2.05 ± 0.44	2.16 ± 0.24
VSI[2]	10.34 ± 1.38	10.1 ± 1.13	12.85 ± 9.72	9.41 ± 1.36	10.86 ± 2.29

Data represent mean ± SD (C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid, HA, high enzyme concentration with citric acid). FCR, feed conversion ratio; SGR, specific growth rate; PER, protein efficiency ratio; aNPU, apparent net protein utilisation; LR, lipid retained; WG, weight gain; HSI, hepatosomatic index; VSI, viscerosomatic index. Significant differences (one-way ANOVA for all parameters except HSI and VSI, where hierarchical ANOVA was used) are indicated by different indices (a, b, c). [1]$n = 2$. [2]$n = 15$.

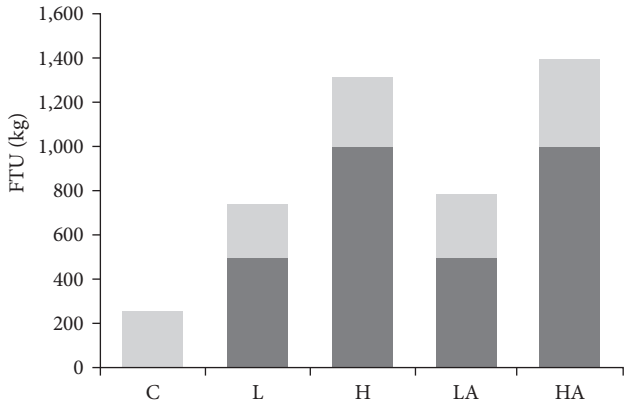

Figure 2: Phytase activity (FT) for each experimental diet. Empty columns, actual; shaded, required; C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid.

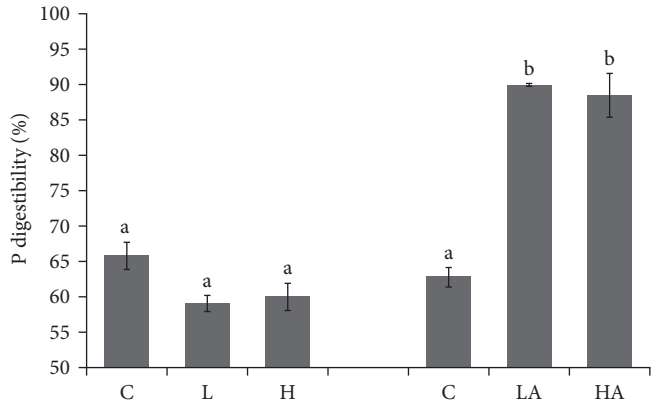

Figure 3: Phosphorus (P) digestibility for each experimental diet (mean ± SD). C, control, L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid. Different indices (a, b) indicate significant differences (factorial ANOVA followed by the post hoc Tukey test, $n = 2$ for each diet).

3.2. Determination of Phosphorous Digestibility from Experimental Diets. There was a significant increase in P digestibility in the LA and HA diets compared with all other diets (Figure 3). While P digestibility in diet C was 64.3%, addition of 3% citric acid to the feed mixture led to a significant increase of 27% and 26% in LA and LH, respectively (Figure 3).

3.3. Effect of Diet on Production Parameters and Fish Body Condition. While the results suggested an increasing trend in TL (hierarchical ANOVA: $F = 1.1$, d.f. = 4, $P = 0.44$) and SL (hierarchical ANOVA: $F = 0.8$, d.f. = 4, $P = 0.6$) in treatments with HA (Table 2), there was no significant difference in length–weight relationships or condition parameters between the experimental groups. On the other hand, production parameters in fish fed the LA and HA diets changed significantly (Table 3), with FCR almost 20% lower (one-way ANOVA: $F = 23.74$, d.f. = 4, $P = 0.002$) and SGR 11% higher (one-way ANOVA: $F = 18.68$, d.f. = 4, $P = 0.003$). Similar significant increases were also observed for PER (one-way ANOVA: $F = 145.0$, d.f. = 4, $P < 0.001$), aNPU (one-way ANOVA: $F = 87.0$, d.f. = 4, $P < 0.001$), LR (one-way ANOVA: $F = 319.0$, d.f. = 4, $P < 0.001$), and weight gain (one-way ANOVA: $F = 20.0$, d.f. = 4, $P < 0.01$) in fish fed the LA and HA diets.

Haematological testing revealed a difference in MCH (hierarchical ANOVA: $F = 5.9$, d.f. = 4, $P = 0.04$) with diet, though it was impossible to distinguish between diet treatments due to high variability (Table 4). While there was no effect of diet on Hb (hierarchical ANOVA: $F = 0.63$, d.f. = 4, $P = 0.66$), aquarium did have a significant effect (hierarchical ANOVA: $F = 2.69$, d.f. = 5, $P < 0.05$; Table 4). All other parameters monitored were unaffected by diet.

Of the 19 biochemical parameters monitored, only three showed significant differences (Table 5). As expected, Pi increased noticeably with both the LA and HA diets (hierarchical ANOVA: $F = 5.99$, d.f. = 4, $P = 0.04$), with levels significantly higher with the HA diet, while CREA was significantly lower with the L, LA, and HA diets (hierarchical ANOVA: $F = 6.42$, d.f. = 4, $P = 0.03$). While there was a significant effect of diet on Mg levels which increased somewhat with the HA diet (hierarchical ANOVA: $F = 9.34$, d.f. = 4, $P = 0.02$), post hoc test was not able to determine specific differences between diets. Other parameters showed either no change or slight nonsignificant changes. For example, Ca content increased with HA and was higher than with the

Table 4: Haematological parameter values.

Parameter	C	L	H	LA	HA
RBC (T/l)	1.67 ± 0.32	1.77 ± 0.34	1.80 ± 0.18	1.63 ± 0.20	1.69 ± 0.22
Hb (g/l)	94.88 ± 10.58	97.53 ± 11.15	94.62 ± 7.03	88.12 ± 7.27	93.43 ± 7.03
PCV (l/l)	0.34 ± 0.04	0.37 ± 0.05	0.36 ± 0.02	0.37 ± 0.99	0.36 ± 0.02
MCV (fl)	207.91 ± 30.22	216.24 ± 31.32	200.91 ± 23.84	233.63 ± 75.92	214.92 ± 25.36
MCHC (l/l)	0.28 ± 0.02	0.26 ± 0.01	0.26 ± 0.02	0.24 ± 0.04	0.25 ± 0.01
MCH (pg)	58.07 ± 9.11^{a}	56.29 ± 7.23^{a}	52.96 ± 6.66^{a}	54.50 ± 5.80^{a}	55.86 ± 7.64^{a}
WBC (G/l)	42.1 ± 14.43	37.6 ± 12.19	28.7 ± 7.54	31.5 ± 12.78	34.7 ± 13.82

Data represent mean $\pm$ SD, $n = 10$ (C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid). RBC, red blood cells; Hb, haemoglobin; PCV, packed cell volume; MCV, mean cell volume; MCHC, mean corpuscular haemoglobin concentration; MCH, mean cell haemoglobin; WBC, number of leukocytes. Significant differences (hierarchical ANOVA) are indicated by different indices, the same index (a) across all experimental diets indicating an overall significant effect of the treatment without the possibility of distinguishing between diets.

Table 5: Biochemical parameter values for blood plasma.

Parameter	C	L	H	LA	HA
ALB (g/l)	8.16 ± 1.91	7.62 ± 3.10	9.25 ± 1.28	6.97 ± 1.78	7.15 ± 1.64
ALP (μkat/l)	0.92 ± 0.46	1 ± 0.60	0.83 ± 0.66	0.53 ± 0.41	0.82 ± 0.50
ALT (μkat/l)	0.09 ± 0.04	0.07 ± 0.02	0.06 ± 0.03	0.06 ± 0.05	0.06 ± 0.02
AST (μkat/l)	2.6 ± 1.13	2.29 ± 1.32	1.91 ± 0.79	2.67 ± 1.06	2.63 ± 0.63
CHOL (mmol/l)	3.83 ± 0.77	3.81 ± 0.45	4.16 ± 1.06	4.38 ± 0.48	4.3 ± 0.77
CREA (μmol/l)	12.82 ± 11.27^{a}	$9.51 \pm 2.54^{a,b}$	10.47 ± 2.79^{a}	5.2 ± 2.22^{b}	$7.02 \pm 2.45^{a,b}$
UREA (mmol/l)	1.34 ± 0.23	1.4 ± 0.26	1.45 ± 0.26	1.19 ± 0.28	1.13 ± 0.28
GLUC (mmol/l)	4.62 ± 1.17	5.19 ± 1.64	4.75 ± 1.10	4.43 ± 1.37	4.62 ± 0.90
LDH (μkat/l)	7.03 ± 5.53	3.24 ± 3.63	2.52 ± 2.86	3.58 ± 1.90	4.24 ± 3.97
LACT (mmol/l)	3.37 ± 1.16	3.56 ± 1.20	3.57 ± 1.08	3.82 ± 1.48	4.72 ± 1.20
TAG (mmol/l)	3.01 ± 0.95	2.78 ± 0.52	2.8 ± 0.77	2.53 ± 0.58	2.47 ± 0.48
TP (g/l)	28.82 ± 2.3	28.97 ± 1.88	29.32 ± 2.17	26.52 ± 1.47	26.97 ± 1.94
Pi (mmol/l)	2.36 ± 0.56^{a}	2.33 ± 0.33^{a}	2.23 ± 0.47^{a}	$2.68 \pm 0.62^{a,b}$	3.27 ± 0.49^{b}
Ca (mmol/l)	2.37 ± 0.11	2.26 ± 0.19	2.5 ± 0.22	2.41 ± 0.19	2.58 ± 0.24
Mg (mmol/l)	1.07 ± 0.09^{a}	1.06 ± 0.18^{a}	1.04 ± 0.12^{a}	1.04 ± 0.12^{a}	1.16 ± 0.12^{a}
Fe (μmol/l)	68.36 ± 14.56	72.01 ± 12.55	70.86 ± 15.17	54.06 ± 10.42	59.81 ± 18.68
Na (mmol/l)	142.76 ± 2.42	140.36 ± 3.09	142.31 ± 1.79	139.57 ± 1.68	142.19 ± 2.55
K (mmol/l)	4.99 ± 0.76	4.71 ± 0.78	4.61 ± 0.80	4.98 ± 0.94	5.1 ± 0.58
Cl^{-} (mmol/l)	111.51 ± 2.58	110.89 ± 2.04	110.37 ± 3.15	112.5 ± 1.58	111.31 ± 3.23

Data represent mean $\pm$ SD, $n = 10$ (C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid). ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHOL, cholesterol; CREA, creatine; GLUC, glucose; LDH, lactate dehydrogenase; LACT, lactate; TAG, triacylglycerol; TP, total protein; Pi, inorganic phosphate; Ca, calcium; Mg, magnesium; Fe, iron; Na, sodium; K, potassium; Cl^{-}, chlorides. Significant differences (hierarchical ANOVA) are indicated by different indices (a, b), the same index (a) across all the experimental diets indicating an overall significant effect of the treatment without the possibility of distinguishing between diets.

other diets, while Na decreased with LA. In both LA and HA, TP values decreased slightly, with no difference in phytase levels.

Chemical analysis of fish tissues revealed no significant difference between diets (Table 6), though a slight decrease in dry matter was observed with the LA and HA diets, both for muscle and whole-body samples. Protein content analysis indicated a slight increase in whole-body samples with both LA and HA diets and in muscle samples with HA. Greatest differences were observed for fat content, with levels dropping noticeably with the LA and HA diets in muscle samples and especially in hepatopancreatic samples.

4. Discussion

4.1. Phosphorus Digestibility. The results of the present study indicated that the addition of citric acid significantly influenced phytase activity in the four experimental diets used, with the highest P digestibility achieved with the LA diet and only slightly lower readings (−1.5%) for HA. In comparison, the L and H treatments showed P digestibility levels almost 6% lower than C, with no significant difference in L and H phytase levels. The differences in phytase activity between treatments would tend to indicate the presence of endogenous phytase. The dosage of phytase and citric acid addition

Table 6: Dry matter, protein, and fat content in whole body and fish tissue.

Parameter		C	L	H	LA	HA
Dry matter	Whole body[1]	30.4 ± 1.29	29.69 ± 2.60	29.85 ± 1.47	27.87 ± 1.58	28.61 ± 2.41
	Muscle[1]	25 ± 0.72	26.23 ± 1.28	26.95 ± 4.08	23.56 ± 1.23	24.49 ± 1.08
Protein	Whole body[1]	14.83 ± 0.83	13.4 ± 0.54	14.89 ± 0.85	15.29 ± 1.09	15.62 ± 0.88
	Muscle[1]	18.11 ± 1.94	17.89 ± 0.90	18.88 ± 0.94	18.02 ± 0.83	19.55 ± 1.01
Fat	Whole body[1]	14.54 ± 1.07	13.87 ± 2.78	13.88 ± 0.87	11.99 ± 0.83	14.1 ± 2.29
	Muscle[1]	6.57 ± 2.16	7.18 ± 2.03	7.46 ± 3.99	5.32 ± 1.40	4.81 ± 1.69
	Intestine[2]	16.74	14.86	14.88	14.65	13.07
	Hepatopancreas[2]	16.34	20.10	20.73	12.90	11.36

Data represent mean ± SD (C, control; L, low enzyme concentration; H, high enzyme concentration; LA, low enzyme concentration with citric acid; HA, high enzyme concentration with citric acid). Fat in intestine and hepatopancreas was not tested. [1]$n = 3$. [2]$n = 1$.

for enhancing nutrient digestibility in fish differs in many studies. Our results are consistent with those of previous studies, with Phromkunthong et al. [28], for example, presenting similar results for carp, where the digestibility of P in a diet with 2.2% citric acid and 150 FTU/kg enzyme (RONOZYME) was higher by 15.2% compared to a control diet. Similarly, Baruah et al. [2] reported an increase in P digestibility in rohu (*Labeo rohita*), an Asian fish of the carp family, using diets with a 500 FTU/kg enzyme and 3% citric acid, and similar results were for rohu by Bano and Afzal [29] using 3% citric acid and 1,000 FTU/kg enzyme. Hussain et al. [30] reported that a guar meal-based diet with 2.5% of citric acid and 1,000 FTU/kg is optimum for nutrient digestibility in *Cirrhinus mrigala* fingerlings. The addition of 5% citric acid and 500 FTU/kg phytase to corn gluten (30%) meal-based diet was most effective in releasing the chelated minerals *Cirrhinus mrigala* from phytate complexes for *C. mrigala* [31]. Nwanna and Schwarz [10] also recorded significantly higher P digestibility using phytase and citric acid; however, interestingly, their study also reported that higher phytase levels (1,000, 2,000 and 4,000 FTU/kg) in feed had no further significant effect on P digestibility. Our study, where the difference in P digestibility between the 500 and 1,000 FTU/kg diet was not significantly different, would tend to confirm this conclusion. Other comparable results have been obtained for noncyprinid species, including great (beluga) sturgeon (*Huso huso*) [32], and rainbow trout (*Oncorhynchus mykiss*) with 1% of citric acid [33].

4.2. Production Parameters. Our results clearly showed a positive effect on basic fish production parameters through the addition of citric acid and phytase combined, especially on FCR and SGR. In diets containing citric acid (LA and HA), both parameters were significantly improved, with SGR increasing by almost 11% and FCR by almost 20%. While Khajepour et al. [11] also reported that addition of 3% citric acid to carp feed had a positive influence on FCR and SGR values, they failed to provide evidence for the effect of enzyme levels. Debnath et al. [34], on the other hand, recorded an increase in SGR, aNPU and PER in all diets containing phytase fed to Pangas catfish (*Pangasius pangasius*), but found no significant difference between phytase fed at 500, 1,000, and 2,000 FTU/kg. In contrast, Sardar et al. [35] reported no positive effect on FCR or SGR values after addition of 500 FTU/kg phytase to carp feed, which reflects with our own results, where acid-free diets with phytase (L and H) had no observable effect on FCR or SGR. Phromkunthog et al. [28] reported an improvement in both FCR and SGR values in carp diet with 2.2% of citric acid and 150 FTU/kg phytase, finding, as in our own study, an increase in PER and aNPU compared to the control diet. Studies by Nwanna and Schwarz [10, 36], who fed diets with phytase but without addition of organic acid to carp, found no significant influence of phytase addition up to 1,000 FTU/kg on FCR and SGR parameters. To conclude, the use of diets with phytase but without an acidifying agent appear to have no significant effect on production parameters.

While the results of phytase use in carp farming can be reasonably compared with results for other cyprinid species lacking a stomach, comparisons with salmonid farming, or with any other fish with a developed stomach as part of its digestive tract, is almost impossible owing to a completely different physiology of digestion. Baruah et al. [37] studied the effect of adding dietary Natuphos phytase on rohu fingerlings to production parameters and found that best results occurred with 750 FTU/kg phytase, when a significant decrease in FCR values and an increase in PER and aNPU values were observed. In our study, these same values increased after addition of citric acid but not in nonacidified diets. In *Cirrhinus mrigala* fingerlings fed diets containing both phytase and citric acid, Zubair-ul-Hassan Arsalan et al. [12] revealed an increase in SGR and WG when 2.5% citric acid was added, and a further increase in these values with the addition of phytase (750 FTU/kg). Similar results were also obtained in the study of Ahmad et al. [38] on rohu fry. Overall, therefore, addition of citric acid has a positive effect on several production parameters, with the effects further increased by the addition of phytase.

4.3. Haematological Parameters. As the addition of enzymes as a feed mixture additive is just one of many factors influencing the bodily functions of fish [39], we also analysed the effects of adding phytase and citric acid (diets LA and HA) on haematological and blood plasma biochemical parameters. In the case of haematological parameters, diet LA induced a significant decrease in the level of MCHC, with all other parameters unaffected. Despite this, all MCHC

values remained within the optimal value range for carp [25]. According to Sardar et al. [35] the addition of 500 FTU/kg phytase to carp feed had no effect on RBC, HB, or PCV levels; however, using a feed mixture with the same phytase content (500 FTU/kg) but with reduced amino acids, mineral premix, and dicalcium phosphate ($CaHPO_4$) content led to a decrease in all parameters compared to the control diet and that with 500 FTU/kg but without the reduction in essential components. Consequently, phytase appears to have no effect on the internal functioning of common carp and any potential changes, therefore, are likely to be due to changes in other mixture components. In comparison, Baruah et al. [40] found that the interaction of phytase (500 FTU/kg) with citric acid (3%) resulted in a significant increase in HB and PCV values in rohu, with RBC and WBC values remaining unchanged.

While there was no significant difference in blood plasma biochemical parameters between diets with different enzyme levels (L and H), there was a significant increase in Pi content in both LA and HA diets, with HA also inducing an increase in Ca compared to the C. Sardar et al. [35] reported similar results for the same blood plasma parameters; however, in this case, the diet showing similar results to our study did not contain citric acid, but was instead enriched with the amino acids methionine and lysine along with dicalcium phosphate. In our study, we also recorded a decrease in TP levels with diets LA and HA compared to the other diets, and significantly so in the case of LA versus H, with a similar, but nonsignificant, trend observed for ALB.

In general, the use of phytase and citric acid had no negative impact on any of the haematological or biochemical parameters monitored. This is important as such parameters can be important indicators of the health status of fish, with increases in parameter values indicating the response of the immune system to health or stress factors [40–42]. As we recorded no significant increase in these values, we may assume that the additives had no negative effect on carp health. In a separate study, Baruah et al. [40] recorded increased levels of TP and ALB after feeding diets with phytase and acid to rohu fingerlings, the results indicating the positive effect of acid on phytase functioning, that is, the increase in TP and ALB is likely to have been due to degradation of phytate, and thus an increase in the bioavailability of other substances, especially minerals and amino acids, supporting the immune response through enzymatic reactions. Kubena and McMurray [43] stated that increased nutrient availability potentially influences almost all aspects of the immune system, both in a positive and negative direction.

4.4. Chemical Composition of Fish Tissue. The interaction of phytase and citric acid had no significant effect on dry matter levels in whole-body or muscle samples, although there was a slight decrease of 1% and 2% after feeding LA and HA diets, or on protein content, despite a slight increase in both whole-body and muscle samples after feeding LA and HA diets, and a slight increase following the increase in phytase level in diet H over L. In the HA treatment, there was a slight increase in muscle protein content compared to C, while fat content was slightly reduced in whole-body samples with L and H diets, which increased to a 2.6% drop compared to C following acidification, that is, diet LA. In the HA treatment, the reduction in fat content was not so pronounced, being equal to L and H. There was also a slight reduction in muscle fat content using the LA and HA diets. The most significant differences were related to fat content in hepatopancreatic samples, where both LA and HA diets caused a significant reduction in fat content (−3.4% and −5.0%, respectively) compared to C. Differences were found when comparing LA and HA diets (acidified) with L and H diets (nonacidified), with fat content increasing by 3.8% and 4.4%, respectively, compared to the C. Intestinal samples showed a decreased fat content with all phytase diets. Phromkunthong et al. [28] also reported a reduction in the fat content of whole-body samples in treatments with acid and phytase; however, in the case of acid-free diets, fat content was not significantly affected. In the same study, protein content results were like those from our study, with acidified phytase diets showing a slight increase in protein content in whole-body samples. According to Baruah et al. [37], however, the interaction of citric acid and phytase had no influence on protein and dry matter content in whole-body rohu samples, though fat levels increased slightly compared to the control in diets with added phytase. In the study of Khajepour et al. [11], diets with phytase and citric acid had no influence on dry matter and protein content of common carp, though they obtained similar results for fat as in our study, with a significant reduction in fat content after feeding with acidified phytase diets, and acid-free phytase diets showing no difference from the control. Overall, therefore, it appears that phytase in interaction with citric acid can reduce fat content in muscles, and especially in the intestines and hepatopancreas. However, it has no significant effect on protein content in whole-body or muscle samples.

5. Conclusion

Our results suggest that, while there remain some obstacles, the addition of phytase as an additive in farmed carp nutrition is justified. Addition of phytase to granulated feed also has the potential to positively influence the impacts of carp farming on the environment by reducing P excreted by fish. However, to be truly effective in carp farming, citric acid must be added to the mixture to fully activate the enzyme. Once acidified, addition of phytase to granulated feed has the potential to positively influence the impact of carp farming on the environment by reducing P excreted by fish, improve fish feed utilisation, decrease FCR, increase SGR, and improve breeding production parameters, all without any negative impacts on fish health. Future research should also include a variant containing only citric acid, as the best results were observed in the variants containing acid.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Ethical Approval

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Figure S1: schematic diagram of the Part 1 of the experiment and the system used. Figure S2: schematic diagram of Part 2 of the experiment and the system used. (*Supplementary Materials*)

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