

Growth Performance Together with Analysis of Haematological and Biochemical Indices of Nile Tilapia Fed Citric Acid and Phytase-supplemented Peas

Jan Mareš, Lucie Všeticková*, Miroslava Palíková, Marija Radojičić, Ondřej Malý, and Eva Poštulková

Mendel University in Brno, Faculty of AgriSciences, Department of Zoology, Fisheries, Hydrobiology and Apiculture, Zemědělská 1665/1, 61300 Brno, Czech Republic

ABSTRACT

At present, fish farmers are searching for cheaper alternatives of fish feed. Here, we assess the suitability of pea meal (*Pisum sativum*), with and without enzyme (phytase)/citric acid supplementation, as a feed for Nile tilapia (*Oreochromis niloticus*). After 90 days, we recorded a significant increase in total length, weight, and weight gain in groups fed 60% pea meal, both with and without citric acid and/or phytase. The number of erythrocytes dropped significantly against controls in all 60% pea meal groups, while leucocytes were higher in all groups fed 30 and 60% pea meal. No significant changes were recorded in plasma biochemical parameters, except for aspartate aminotransferase, triacylglycerol, and chlorine which were significantly lower, and contrarily calcium, sodium and blood plasma iron were significantly higher, than in controls in all 60% groups. Consequently, feed with 30 and 60% pea meal is suitable for feeding farmed tilapia and enrichment with phytase and citric acid is recommended. The results of the study show that peas can be a new source of protein in intensive fish aquaculture nutrition. It therefore has the potential to partially replace soybeans in aqua feeds. Peas may also have the advantage of being a crop easy to grow, widespread throughout the world, and not genetically modified.

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E-mail addresses:

jan.mares@mendelu.cz (Jan Mareš)

lucie.vsetickova@mendelu.cz (Lucie Všeticková)

miroslava.palikova@mendelu.cz (Miroslava Palíková)

marija.radojicic@mendelu.cz (Marija Radojičić)

ondra.malous@gmail.com (Ondřej Malý)

eva.postulkova@mendelu.cz (Eva Poštulková)

*Corresponding author

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INTRODUCTION

Current trends in human nutrition place an emphasis on the production of healthy, high quality raw materials, whether of plant or animal origin. As the world population grows, and individual countries' economies

improve, so the demand for quality plant, and especially animal, foodstuffs increases. Increasingly, therefore, the agricultural sector is focusing on those areas that can produce quality food in the shortest time. In livestock production, this is mainly fish and poultry farming (FAO, 2021).

Quality feed is one of the most important, if not the most important, factors influencing the growth rate of animals and the quality of the final product. At the same time, however, feed is one of the main costs in animal husbandry. Consequently, full knowledge of the impacts of a given nutritional strategy on the quality and marketability of animal products is essential for the profitability of livestock farming (Szollosi et al., 2021), including aquaculture (Watson et al., 2021).

Both animal and plant ingredients can be used as the main source of protein in fish feed; however, not only are these ingredients the most expensive component in the feed but the digestibility of different forms can vary greatly, with subsequent impacts on fish growth (Imsland et al., 2016). The digestion pattern of fish, as cold-blooded animals, differs somewhat to that of warm-blooded animals. From this point of view, ground fishmeal is a particularly suitable animal protein group in fish feed (Luthada-Raswiswi et al., 2021); however, it is both expensive and its use carries certain risks, e.g. contamination with heavy metals (Murthy et al., 2013). Insect meal is beginning to be used in aquaculture (Bullon et al., 2023) but the most commonly used substitute for fishmeal in animal feed is soybean (*Glycine max*) meal. However, soya is the most common genetically modified crop (GMC) (Sieradzki & Kwiatek, 2006), which restricts its use in Europe, and Europe itself is far from self-sufficient in non-GMC soybean production (FAO, 2021). Consequently, efforts are being made to find other high-quality protein sources for animal feed, such as peas (*Pisum sativum*) (Allan et al., 2000), peanuts (*Arachis hypogaea*) (Vo et al., 2020) or lupine (*Lupinus angustifolius*) (Glencross et al., 2011).

Peas are an important legume, grown mainly for their tasty fruits, which are high in vitamins (mainly B vitamins) and minerals, especially phosphorus (P) and potassium (K), but also calcium (C) and magnesium (Mg). Furthermore, Crépon (2007) reported that peas contain around 20 to 25% of protein substances and a high lysine content, though actual content varies depending on the soil quality and length of growing season. Not all the microelements in peas are available to fish in their natural form, however. Indeed, fish receive a significant proportion of their P intake from plant foods in a form that is difficult to utilise as fish do not produce the enzyme phytase, which breaks down the phytate bond to release P. Therefore, fish digest this form of P poorly and most is excreted in the faeces and passes into the aquatic environment without being used. Moreover, phytate readily forms complexes with divalent and trivalent cations of zinc (Zn), C or Mg, which also limits utilisation of these minerals. Some fish species produce low levels of acid in their digestive tract, which can negatively affect digestion and nutrient utilisation and increase the risk of pathogens developing in the digestive tract (Shah et al., 2015).

In this study, we determine the effect of adding i) pea protein, (ii) pea protein with added phytase and (iii) pea protein with added phytase and citric acid on a) haematological and biochemical plasma parameters, and b) production parameters, of Nile tilapia (*Oreochromis niloticus*), a fish commonly bred under intensive aquaculture. In doing so, we determine whether pea protein is a suitable feed additive for intensively reared tilapia, and whether one, or both, of the additives improve utilisation of the pea protein. The percentage of pea inclusion in the feed was determined on the basis of the previous pilot tests. Both doses are high for the reason that they were intended to ensure sufficient protein in the feed. The hypothesis was as follows: the addition of peas as a protein source in the diet will not adversely affect the growth and haematological parameters of tilapia.

MATERIAL AND METHODS

Fish

Three hundred and sixty clinically healthy (without injuries and symptoms of disease) Nile tilapia (originating from our own breeding ponds) were divided into nine groups comprising two replications, with 180 tilapia per replication. The number of tilapia in the experiment was determined taking into account the capacity of the recirculation system and the welfare of the fish. For each test run, 20 weight balanced tilapia (for mean weights, see Table 1) were placed into an 80 L tank fed by a Nexus Easy 210 recirculation system (Evolution Aqua s.r.o., Czech Republic). Prior to each replication, the fish were given a two-week acclimatization period, during which they were fed with a standard KP1 feed mixture.

Table 1
Mean weights (+ standard deviation [SD]) in each treatment tank (two replicates per treatment)

Treatment	Tank 1	Tank 2
C	85.25 ± 15.64 g	87.75 ± 15.52 g
CF	88.65 ± 10.61 g	87.15 ± 14.88 g
CF+	87.55 ± 11.63 g	91.00 ± 14.37 g
P30	85.25 ± 19.06 g	86.30 ± 11.84 g
P30F	78.35 ± 12.20 g	78.45 ± 15.73 g
P30F+	91.20 ± 12.36 g	91.95 ± 11.28 g
P60	88.65 ± 11.95 g	90.70 ± 14.59 g
P60F	77.10 ± 8.50 g	77.85 ± 14.28 g
P60F+	91.35 ± 11.65 g	88.50 ± 10.00 g

Note. Treatments: 30% (P30) and 60% (P60) pea meal, commercial carp feed as control (C) and feeds enriched with the enzyme phytase (CF, P30F, P60F) and phytase and citric acid (CF+, P30F+, P60F+)

Dietary Feed and Feeding During the Experiment

Three diet variants were used in the experiment. The control (C) group were fed a commercially prepared carp feed (KP1, FMP Silver Mountains, Czech republic) containing wheat, wheat flour, rapeseed cake, wheat bran, soybean meal, barley, corn and calcium carbonate (CaCO₃), sodium chloride (NaCL) and soybean oil (see Table 2). In addition, two test variants were prepared by the feed producer, where part of the KP1 was substituted with pea meal in the following proportions: 70:30 KP1/pea meal (group P30) and 40:60 KP1/pea meal (group P60). Each of these three diets was also prepared as a variant with 500 FTU of phytase (CF, P30F and P60F) and 500 FTU of phytase and 3% citric acid (CF+, P30F+ and P60F+). The pea meal

was produced by a local agricultural farm ZD Kojcice using cultivar ESO pea seeds that had previously been analysed in our laboratory to determine protein content (confirmed at 20.30%). The phytase used was commercial Phyzyme XP 10.000 TPT in fine granular form, produced by Danisco Animal Nutrition (United Kingdom), and the citric acid was a commonly available food grade product suitable for human consumption.

All test fish were fed three times a day at 8 a.m., noon and 4 p.m. over the 10-week experiment, with the daily feeding ratio corresponding to 3% of tank stock weight. Control weighing took place every 14 days (i.e. five times per experiment), after which the daily feed ration was recalculated based on the actual weight of the fish. The control days also served for examination of fish health. Mortality caused by aggressive behaviour due to tilapia switching gender during the experiment was as follows: C = 2 ind., CF = 0 ind., CF+ = 2 ind., P30 = 4 ind., P30F = 4 ind., P30F+ = 0 ind., P60 = 2 ind., P60F = 3 ind. and P60F+ = 4 ind.

There were no significant differences in water quality parameters throughout the experiment (for details see Table 3).

Table 2
Analytical composition of the feed mixture (KP1) used in this study

Component	Content
Moisture (%)	11.53
Crude protein (%)	17.84
Crude fibre (%)	4.81
Crude oils and fats (%)	4.51
Crude ash (%)	5.84
Lysin (%)	0.73
Methionine (%)	0.29
Calcium (%)	0.96
Phosphorus (%)	0.57
Sodium (%)	0.18
Butylhydroxyanisol (mg)	1.25
Butylhydroxytoluene (mg)	5.48
Vitamin D3 (IU)	1,500.00
Vitamin A (IU)	8,100.00
Sulphate ferrous monohydrate (mg)	100.26
Iodine calcium (mg)	1.02
Sulphate cupric pentahydrate (mg)	4.95

Table 3
Mean water quality parameters for each test treatment over the course of the experiment

Parameter	Biofilter	Group								
		C	F	CF+	P30	P30F	P30F+	P60	P60F	P60F+
Water temperature (°C)	25.17 ± 0.67	25.10 ± 0.70	25.14 ± 0.68	25.16 ± 0.67	25.15 ± 0.70	25.16 ± 0.66	25.18 ± 0.68	25.17 ± 0.69	25.15 ± 0.70	25.17 ± 0.69
Dissolved oxygen content (mg.L ⁻¹)	5.96 ± 0.66	6.84 ± 0.58	6.59 ± 0.62	6.58 ± 0.62	7.13 ± 0.50	6.54 ± 0.64	6.46 ± 0.67	6.61 ± 0.62	6.59 ± 0.71	6.49 ± 0.64
Oxygen saturation (%)	75.02 ± 7.75	85.48 ± 6.47	82.25 ± 7.21	82.19 ± 7.15	83.24 ± 7.07	81.60 ± 7.39	80.71 ± 7.79	82.40 ± 7.02	82.28 ± 8.00	80.88 ± 7.59
pH	7.81 ± 0.47	7.95 ± 0.51	7.94 ± 0.49	7.92 ± 0.49	7.92 ± 0.50	7.90 ± 0.48	7.90 ± 0.48	7.91 ± 0.49	7.92 ± 0.51	7.89 ± 0.49
N-NH ₄ (mg.L ⁻¹) ¹	0.21 ± 0.08									
N-NO ₂ ⁻ (mg.L ⁻¹) ¹	0.36 ± 0.22									
Cl ⁻ (mg.L ⁻¹) ¹	123.85 ± 16.05									

Note. Treatments: 30% (P30) and 60% (P60) pea meal, commercial carp feed as control (C) and feeds enriched with the enzyme phytase (CF, P30F, P60F) and phytase plus citric acid (CF+, P30F+, P60F+). ¹ parameters measured only on the biofilter not in each fish tank

Fish Body Parameters

At the end of the experiment, the fish were removed from the water, stunned by a blow to the head and, after collecting blood (see below), they were killed by cutting the branchial venae. The fish were treated in accordance with current legislative rules and approved by the Ethics Committee of the Central Commission for Animal Welfare at the Ministry of Education of the Czech Republic. Ethical approval number is MSMT-6675/2018-3. Each fish was measured for total length, standard length, body height, body width, body weight, liver weight, pancreas weight, gonad weight and eviscerated body weight. Three fish were randomly selected from each group and ground whole for analysis of ash, fat and protein content, while filleted muscle only was taken from another three randomly selected fish for the same analysis. All analyses were undertaken at the laboratory of the Department of Chemistry at Mendel University in Brno (Czech Republic). Fulton's condition factor, Clark's condition factor and the highbackedness and widebackedness index were then calculated from these basic data (Gela & Linhart, 2000), along with the feed conversion ratio, specific growth rate, weight gain, hepatosomatic index, viscerosomatic index, splenosomatic index and gonadosomatic index. (Note that not all parameters are listed in the tables as some were only used for the calculation of the indices.)

Blood Examination

Blood samples (2 ml) were taken from eight stunned fish in each tank at the end of the experiment by puncturing the *vena caudalis* and storing the sample in a heparinised syringe, rinsed out with heparin sodium salt to avoid coagulation. Each blood sample was divided into two parts, one being used for haematological examination (haemoglobin, number of erythrocytes, number of leukocytes, haematocrit), while the other part was centrifuged (1500 rpm, 5 minutes) using a MPW 140 350R cooling centrifuge (MPW Med. Instruments, Poland), the plasma obtained being stored in a freezer (Arctiko ULTF 80, Denmark) at -75°C until further analysis. Blood smears, haemoglobin and haematocrit determination (Svobodová et al., 2012) were undertaken immediately after blood collection. Blood smears were stained using the Hemacolor Rapid staining kit (Merck, Darmstadt, Germany). The blood plasma biochemical profile (alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), albumin (ALB), cholesterol (CHOL), creatine (CREA), glucose (GLUC), urea (UREA), total protein (TP), triacylglycerol (TAG), calcium (Ca) and inorganic phosphorous (PI), sodium (Na), potassium (K), chloride (Cl), iron (Fe) and magnesium (Mg)) was measured using the Konelab 20i kit and other commercially available kits (BioVendor, Czech Republic).

Statistical Analysis

Analysis of variance (ANOVA), with subsequent Tukey post-hoc tests, were used to determine significant differences between experimental variants, with all data being first $\log(x+1)$ transformed to meet the assumptions of the parametric test - normality of data and homogeneity of variances. In all cases, each fish was considered as an individual replicate and differences were considered significant at $P < 0.05$. All analyses were performed in Statistica 14 (TIBCO Software Inc., 2020).

RESULTS

Fish Body Parameters

Both tilapia body length and weight increased as the proportion of pea meal in the feed increased (i.e. P60 > P30), with the addition of both phytase and phytase with citric acid also having a positive impact. Tilapia in groups P60, P60F and P60F+ all had a significantly greater body length than those in group C (Table 4). While there was also a significant increase in body weight in group P60, a similar increase in body weight in groups P60F and P60F+ was not significant (Table 4). Similarly, while values for Fulton's condition factor were significantly higher than C in groups P30 and P60, the increase resulting from the addition of the two additives was non-significant. In comparison, Clark's condition factor tended to remain relatively static, with no discernible trend between groups. Hepatosomatic, viscerosomatic and gonadosomatic indices also showed no discernible trend in relation to pea meal content, except for group P30F, where the addition of phytase appeared to have a significant effect (Table 4). While the widebackedness index showed a significant increasing trend in groups P30, P30F and P60F+ compared to C and all P60 groups, the highbackedness index showed the opposite trend, with values in P60, P60F and P60F+ all being significantly higher than those in C and all P30 groups (Table 4).

Production Parameters

None of the fat, ash or protein parameters examined displayed statistically significant changes against C, whether in relation to pea meal concentration or addition of phytase or citric acid (Table 5). Overall trends were variable, with fat content in whole fish similar between P30 and P60, but showing a decreasing trend in groups P30F and P60F and P30F+ and P60F+. While fat in muscle tended to decrease with increasing proportion of pea meal, addition of both additives had little or no further effect. Only minor changes were noted in ash content, with no visible trend among groups. Protein levels in both whole fish and muscle showed little change, the only notable difference being between group P60F+ and CF+ (Table 5). Weight gain increased with increasing proportion of pea meal, with the addition of phytase having a significant positive effect. Feed conversion ratio was highest

Table 4
Fish body parameters at the end of the experiment (mean ± SD)

Parameter	Group										
	C	CF	CF+	P30	P30F	P30F+	P60	P60F	P60F+	CA	F
TL (mm)	184.15 ± 9.69 ^a	202.30 ± 10.97 ^a	205.00 ± 15.75 ^a	190.85 ± 9.30 ^{a,b}	198.25 ± 11.88 ^{a,b}	208.45 ± 11.95 ^{a,b}	195.25 ± 8.29 ^b	203.55 ± 11.19 ^b	207.70 ± 11.43 ^b	↑	↑
BW (g)	117.36 ± 18.77 ^a	153.80 ± 26.91 ^a	164.95 ± 37.42 ^a	131.16 ± 19.07 ^{a,b}	149.53 ± 29.70 ^{a,b}	172.79 ± 28.05 ^{a,b}	138.69 ± 13.95 ^b	158.07 ± 24.91 ^b	167.44 ± 26.24 ^b		
F _C	3.42 ± 0.19 ^a	3.28 ± 0.15 ^a	3.38 ± 0.24 ^a	3.42 ± 0.15 ^b	3.47 ± 0.15 ^b	3.45 ± 0.15 ^b	3.37 ± 0.20 ^{a,b}	3.40 ± 0.22 ^{a,b}	3.33 ± 0.20 ^{a,b}		
C _C	3.08 ± 0.17	2.97 ± 0.14	3.02 ± 0.19	3.07 ± 0.11	3.07 ± 0.13	3.08 ± 0.14	3.04 ± 0.17	3.03 ± 0.18	3.00 ± 0.17		
HBI (%)	2.58 ± 0.07 ^a	2.62 ± 0.09 ^a	2.57 ± 0.10 ^a	2.58 ± 0.07 ^a	2.59 ± 0.07 ^a	2.56 ± 0.06 ^a	2.65 ± 0.08 ^b	2.63 ± 0.08 ^b	2.63 ± 0.10 ^b		
WBI (%)	18.09 ± 0.56 ^a	17.70 ± 0.50 ^a	17.72 ± 0.59 ^a	18.09 ± 0.48 ^b	18.31 ± 0.57 ^b	18.29 ± 0.45 ^b	17.83 ± 0.64 ^a	18.14 ± 0.62 ^a	17.99 ± 0.47 ^a		
HSI (%)	2.68 ± 0.59	3.00 ± 0.58	2.99 ± 0.71	2.63 ± 0.58	3.40 ± 1.13	2.72 ± 0.64	2.84 ± 0.62	2.44 ± 0.58	2.63 ± 0.48		
VSI (%)	10.14 ± 1.56	9.67 ± 1.01	10.53 ± 2.20	9.96 ± 1.77	11.27 ± 1.93	10.60 ± 1.79	9.73 ± 1.59	10.9 ± 1.18	10.10 ± 1.53	↑	↑
GSI (%)	1.77 ± 1.52	1.06 ± 1.02	2.13 ± 1.63	1.65 ± 1.69	1.56 ± 1.56	2.35 ± 2.27	1.40 ± 1.34	1.35 ± 1.29	1.27 ± 1.62		

Note. Treatments: 30% (P30) and 60% (P60) pea meal, commercial carp feed as control (C) and feeds enriched with the enzyme phytase (CF, P30F, P60F) and phytase plus citric acid (CF+, P30F+, P60F+). Significant effect of pea meal is shown by superscript letters (same letters represents no effect); significant effect of citric acid (CA) and F addition is indicated by arrows: ↑ = parameter higher, ↓ = parameter lower. CA = citric acid, F = phytase, TL = total length, BW = body weight, F_C = Fulton's condition factor, C_C = Clark's condition factor, HBI = highbackedness index, WBI = widebackedness index, HIS = hepatosomatic index, VSI = viscerosomatic index, GSI = gonadosomatic index

in group C, but fell sharply in groups CF and CF+. There was also a significant reduction in group P30F, and less so in P60F, following addition of phytase. On the other hand, specific growth rate increased significantly compared to C in all experimental groups following addition of phytase. Finally, the FCR:SGR ratio was significantly lower in the P60, P60F and P60F+ groups than the C, CF and CF+ groups, with a similar but non-significant decreasing trend for the P30, P30F and P30F+ groups (Table 5).

Haematological and Biochemical Parameters

The number of erythrocytes was significantly lower than C in groups P60, P60F and P60F+, and slightly lower in P30F and P30F+ (Table 6). In comparison, while the number of leucocytes showed an increasing trend in the P30, P30F+, P60 and P60F+ groups, there was a significant decrease in P30F and P60F, i.e. the groups with added phytase. Haemoglobin and haematocrit values in the P60, P60F and P60F+ groups were significantly lower than those in the other groups, with the highest contrast between groups CF+ and P60F+ (Table 6).

Values for AST, Cl and TAG decreased in all experimental groups compared to the controls, with the decrease being significant in P60, P60F and P60F+. Noticeably, TAG values for P60F and P60F+ were around half those of the CF, CF+, P30F and P30F+ groups, and those for P60 around two thirds. Likewise, Cl values differed significantly at P60, P60F and P60F+, with phytase and citric acid having a significant impact, while Ca levels differed significantly at P30 and P60, with addition of citric acid having a significant impact. Values for Fe values were increasing increased significantly in all six experimental groups, while Na values were significantly different in P30, P30F and P30F+ comparing to both the C and P60 groups, with addition of phytase having a significant impact which can be viewed in Table 6.

While all other parameters showed increasing or decreasing trends, none of the differences were significant (Table 6). Both ALP and CHOL in P60, P60F and P60F+, for example, tended to drop compared the C (ALP) and P30 groups (CHOL), while ALB values dropped in groups P30F+ and P60F+ compared with CF+, and GLUC and TP (phytase significant) showed decreasing values in groups P30F, P60F and P60F+, with values for the other groups tending to fluctuate at the same levels. While non-significant, there was a clear impact from the addition of phytase on TP, and LDH values were lower in all experimental groups compared with the controls. Values for Mg and K showed no significant change in any of the groups, despite the addition of phytase having a significant positive effect on Mg and addition of citric acid having a significant decreasing effect on Mg and K. Likewise, CREA, UREA and IP values fluctuated only slightly between groups, despite the significant positive impacts of phytase on IP and citric acid on UREA.

Table 5
Production parameters at the end of the experiment (mean ± SD)

Parameter	Group											CA	F
	C	CF	CF+	P30	P30F	P30F+	P60	P60F	P60F+	P60F+	F		
FCR	6.56 ± 0.03	2.69 ± 0.10	2.59 ± 0.25	4.66 ± 0.51	3.16 ± 1.15	2.29 ± 0.07	3.87 ± 0.53	2.47 ± 0.02	2.93 ± 0.72	2.93 ± 0.72			↓
SGR (%.d-1)	0.33 ± 0.00 ^a	0.69 ± 0.01 ^a	0.78 ± 0.07 ^a	0.52 ± 0.07 ^b	0.79 ± 0.00 ^b	0.81 ± 0.03 ^b	0.55 ± 0.05 ^b	0.84 ± 0.06 ^b	0.83 ± 0.06 ^b	0.83 ± 0.06 ^b			↑
	20.20 ± 0.20 ^a	3.90 ± 0.20 ^a	3.30 ± 0.00 ^a	9.00 ± 0.30 ^{ab}	4.00 ± 1.50 ^{ab}	2.80 ± 0.20 ^{ab}	7.00 ± 0.40 ^b	2.90 ± 0.20 ^b	3.60 ± 1.10 ^b	3.60 ± 1.10 ^b			↓
Weight gain (%)	119.30 ± 0.20	161.90 ± 1.20	164.10 ± 4.60	129.20 ± 3.60	156.30 ± 24.50	176.60 ± 3.60	139.90 ± 5.80	166.70 ± 0.30	160.90 ± 19.00	160.90 ± 19.00			↑
Fat in whole fish (%)	34.89 ± 0.17	34.87 ± 0.14	36.61 ± 0.39	34.20 ± 0.11	32.69 ± 0.43	41.12 ± 5.14	36.05 ± 0.17	30.04 ± 5.18	37.52 ± 0.17	37.52 ± 0.17			
Ash in whole fish (%)	12.93 ± 1.07	14.25 ± 0.09	14.25 ± 0.10	13.33 ± 0.27	13.28 ± 0.56	14.95 ± 0.06	14.59 ± 0.08	16.49 ± 1.08	13.40 ± 0.70	13.40 ± 0.70			
Protein in whole fish (%)	52.21 ± 1.56	55.32 ± 0.50	53.38 ± 0.59	52.30 ± 0.48	53.98 ± 0.57	50.83 ± 4.45	53.73 ± 0.64	52.44 ± 0.62	55.44 ± 0.47	55.44 ± 0.47			
Fat in muscle (%)	24.69 ± 0.59	25.71 ± 0.58	19.74 ± 4.71	14.25 ± 8.58	19.93 ± 4.13	18.34 ± 5.64	12.71 ± 10.62	22.28 ± 1.58	22.35 ± 1.48	22.35 ± 1.48			
Ash in muscle (%)	5.54 ± 0.03	5.57 ± 0.10	5.47 ± 0.25	5.56 ± 0.51	5.95 ± 0.75	5.77 ± 0.07	5.69 ± 0.53	5.51 ± 0.02	5.83 ± 0.72	5.83 ± 0.72			
Protein in muscle (%)	79.13 ± 2.00	83.84 ± 2.01	77.29 ± 4.07	79.85 ± 0.97	82.05 ± 0.50	82.89 ± 0.03	81.67 ± 0.25	82.24 ± 0.06	83.97 ± 0.86	83.97 ± 0.86			

Note. Treatments: 30% (P30) and 60% (P60) pea meal, commercial carp feed as control (C) and feeds enriched with the enzyme phytase (CF, P30F, P60F) and phytase plus citric acid (CF+, P30F+, P60F+). Significant effect of pea meal is shown by superscript letters (same letters represents no effect); significant effect of citric acid (CA) and F addition is indicated by arrows: ↑ = parameter higher, ↓ = parameter lower. FCR = feed conversion ratio, SGR = specific growth rate

Table 6
Haematological and biochemical parameters at the end of the experiment (mean ± SD)

Parameter	Group										
	C	CF	CF+	P30	P30F	P30F+	P60	P60F	P60F+	CA	F
Haemoglobin (g.l ⁻¹)	70.62 ± 7.98 ^a	77.38 ± 7.76 ^a	86.45 ± 12.25 ^a	80.86 ± 6.63 ^a	72.33 ± 5.37 ^a	82.79 ± 6.04 ^a	69.86 ± 16.46 ^b	76.29 ± 4.31 ^b	69.28 ± 5.89 ^b		
Erythrocytes (T.l ⁻¹)	1.82 ± 0.18 ^a	2.05 ± 0.34 ^a	2.00 ± 0.28 ^a	2.08 ± 0.25 ^a	1.84 ± 0.18 ^a	1.94 ± 0.15 ^a	1.76 ± 0.25 ^b	1.83 ± 0.20 ^b	1.70 ± 0.13 ^b		
Leukocytes (G.l ⁻¹)	83.10 ± 14.93	79.80 ± 28.24	65.50 ± 13.33	98.00 ± 12.92	70.60 ± 22.14	83.40 ± 20.00	93.50 ± 24.95	71.80 ± 19.72	88.40 ± 9.97		↓
Haematocrit (g.l ⁻¹)	0.28 ± 0.03 ^a	0.31 ± 0.04 ^a	0.31 ± 0.04 ^a	0.30 ± 0.02 ^a	0.27 ± 0.03 ^a	0.31 ± 0.02 ^a	0.27 ± 0.05 ^b	0.27 ± 0.02 ^b	0.23 ± 0.02 ^b		
ALB (g.l ⁻¹)	8.20 ± 1.95	10.04 ± 2.52	12.15 ± 2.35	10.13 ± 1.13	10.38 ± 2.74	9.98 ± 1.72	9.59 ± 1.14	10.19 ± 1.64	10.74 ± 1.97		
ALP (µkat.l ⁻¹)	0.53 ± 0.07	0.51 ± 0.09	0.56 ± 0.12	0.53 ± 0.09	0.63 ± 0.14	0.49 ± 0.07	0.49 ± 0.13	0.46 ± 0.13	0.47 ± 0.08		
ALT(µkat.l ⁻¹)	0.31 ± 0.14	0.32 ± 0.12	0.39 ± 0.32	0.31 ± 0.10	0.31 ± 0.16	0.29 ± 0.12	0.29 ± 0.12	0.23 ± 0.10	0.24 ± 0.11		
AST (µkat.l ⁻¹)	2.19 ± 1.25 ^a	1.96 ± 1.91 ^a	2.06 ± 1.85 ^a	1.27 ± 0.73 ^{a,b}	1.88 ± 1.77 ^{a,b}	1.36 ± 1.14 ^{a,b}	1.11 ± 0.80 ^b	1.06 ± 0.95 ^b	1.20 ± 0.91 ^b		
Ca (mmol.l ⁻¹)	2.65 ± 0.37 ^{a,b}	2.49 ± 0.19 ^{a,b}	3.28 ± 0.46 ^{a,b}	2.98 ± 0.50 ^a	2.84 ± 0.40 ^a	3.27 ± 0.71 ^a	2.73 ± 0.27 ^b	2.76 ± 0.45 ^b	2.70 ± 0.12 ^b	↑	
CHOL (mmol.l ⁻¹)	4.57 ± 1.53	4.67 ± 2.38	5.13 ± 1.81	4.83 ± 1.58	4.81 ± 1.90	5.00 ± 1.78	3.83 ± 1.36	3.74 ± 1.15	4.14 ± 1.14		
CREA (µmol.l ⁻¹)	15.64 ± 5.80	16.76 ± 12.39	12.81 ± 5.52	15.60 ± 3.83	11.72 ± 5.09	12.99 ± 4.71	16.16 ± 5.94	12.82 ± 5.23	15.29 ± 4.72		
GLU (mmol.l ⁻¹)	3.09 ± 0.53	3.21 ± 0.55	3.06 ± 0.36	3.03 ± 0.53	3.20 ± 0.89	2.80 ± 0.28	3.07 ± 0.45	2.74 ± 0.42	2.86 ± 0.44		
Fe (mmol.l ⁻¹)	17.72 ± 4.92 ^a	15.04 ± 5.07 ^a	19.40 ± 4.59 ^a	17.57 ± 3.57 ^b	21.22 ± 7.97 ^b	24.29 ± 4.00 ^b	19.92 ± 8.76 ^b	25.19 ± 5.33 ^b	24.01 ± 5.88 ^b		

Table 6 (continue)

Parameter	Group										
	C	CF	CF+	P30	P30F	P30F+	P60	P60F	P60F+	CA	F
LDH (μkat.l ⁻¹)	15.67 ± 11.08	20.71 ± 20.76	14.46 ± 11.51	9.89 ± 5.18	14.14 ± 14.52	11.71 ± 12.63	10.90 ± 9.89	9.87 ± 10.15	12.53 ± 13.75		
Mg (mmol.l ⁻¹)	0.79 ± 0.13	0.91 ± 0.16	1.02 ± 0.16	0.86 ± 0.12	1.02 ± 0.21	1.07 ± 0.14	0.88 ± 0.08	0.94 ± 0.11	0.95 ± 0.07	↑	
IP (mmol.l ⁻¹)	1.32 ± 0.28	1.48 ± 0.29	1.61 ± 0.21	1.41 ± 0.36	1.60 ± 0.24	1.98 ± 0.50	1.25 ± 0.24	1.82 ± 0.59	1.48 ± 0.15	↑	
TP (g.l ⁻¹)	34.61 ± 4.18	38.01 ± 7.78	40.42 ± 4.24	34.95 ± 3.22	41.77 ± 11.82	38.89 ± 9.50	33.20 ± 4.42	36.65 ± 8.03	34.72 ± 3.80	↑	
TAG (mmol.l ⁻¹)	6.88 ± 3.70 ^a	6.41 ± 3.30 ^a	8.65 ± 4.76 ^a	5.51 ± 2.57 ^a	7.48 ± 4.56 ^a	8.08 ± 3.45 ^a	5.90 ± 4.12 ^b	3.76 ± 2.04 ^b	4.31 ± 2.61 ^b		
UREA (mmol.l ⁻¹)	0.54 ± 0.52	0.30 ± 0.16	0.41 ± 0.28	0.36 ± 0.20	0.32 ± 0.28	0.61 ± 0.60	0.26 ± 0.17	0.25 ± 0.12	0.45 ± 0.16	↑	
Na (mmol.l ⁻¹)	156.27 ± 1.74 ^a	165.37 ± 2.10 ^a	161.44 ± 2.16 ^a	160.97 ± 3.09 ^b	166.62 ± 1.59 ^b	165.96 ± 4.76 ^b	156.61 ± 1.60 ^a	160.90 ± 2.31 ^a	163.32 ± 2.88 ^a	↑	
K (mmol.l ⁻¹)	3.49 ± 0.62	3.74 ± 0.58	3.59 ± 0.82	2.92 ± 0.50	3.73 ± 0.34	3.39 ± 0.26	3.20 ± 0.33	4.00 ± 0.30	3.54 ± 0.38	↓	↑
Cl (mmol.l ⁻¹)	138.20 ± 2.20 ^a	135.19 ± 1.80 ^a	141.74 ± 2.03 ^a	142.37 ± 3.54 ^a	131.45 ± 2.39 ^a	141.56 ± 3.87 ^a	137.79 ± 2.35 ^b	129.95 ± 1.96 ^b	134.34 ± 2.13 ^b	↑	↓

Note. Treatments: 30% (P30) and 60% (P60) pea meal, commercial carp feed as control (C) and feeds enriched with the enzyme phytase (CF, P30F, P60F) and phytase plus citric acid (CF+, P30F+, P60F+). Significant effect of pea meal is shown by superscript letters (same letters represents no effect); significant effect of citric acid (CA) and F addition is indicated by arrows: ↑ = parameter higher, ↓ = parameter lower. ALB = albumin, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, Ca = calcium, CHOL = cholesterol, CREA = creatine, GLUC = glucose, LDH = lactate dehydrogenase, Na = sodium, K = potassium, Cl = chloride, Fe = iron, Mg = magnesium, IP = inorganic phosphorus, TP = total protein, TAG = triacylglycerol, UREA = urea

DISCUSSION

In this study, addition of pea meal to tilapia feed had a positive impact on fish growth, with fish in the P30 and P60 groups showing an increase in total length of 3.64% and 6.03%, and an increase in weight of 11.76% and 18.17%, respectively, compared to the control group. These figures were increased even further through the addition of phytase and phytase with citric acid. Interestingly, Schulz et al. (2007), working with tilapia fry (body weight 2.25 g), came to the opposite results when partially substituting fishmeal protein with pea meal at 30, 45 and 60%, finding a significant decrease in growth performance at higher inclusion levels. In one of the most recent studies about the use of new protein sources in fish feeds by Iheanacho et al. (2025) is pea protein considered as promising alternative to soy. The difference between this and Schulz's et al. (2007) results is probably explained by the different age categories of fish examined as tilapia of such a small size are probably unable to effectively utilise the pea protein. Furthermore, Schulz et al. (2007) did not enrich the diet with citric acid or phytase. Citric acid has been extensively used as an additive in aquafeeds, both for acidification and to improve nutrient utilisation, the associated decrease in stomach pH being shown to have a positive effect on the efficiency of nutrient utilisation (Sarker et al., 2012). According to Daba and Morris (2021), peas already contain small amounts of citric acid, averaging around 1–4 g/kg, which would also aid digestion. Indeed, the addition of 3% citric acid to our feed had the effect of increasing weight gain in the CF+, P30F+ and P60F+ groups. The same positive results were also obtained by Shah et al. (2015) after adding citric acid to rainbow trout (*Oncorhynchus mykiss*), red seabream (*Pagrus major*) and rohu (*Labeo rohita*) feed. Peas are also a good source of phosphorus and phytase was added to the feed as it is known to improve phosphorous utilisation (Sajjadi & Carter, 2004). As in previous studies (Adeshina et al., 2023), we did indeed observe improved phosphorous utilisation after adding this enzyme. In line with the improved results for fish growth, we also observed improvements in other production parameters (i.e. highbackedness and widebackedness indices) and in Fulton's condition factor in the pea meal supplemented groups, although these parameters showed no additional improvements following addition of citric acid.

No changes were observed in the nutritional composition of the whole fish body or muscles in any of the groups studied, regardless of whether pea meal, citric acid or phytase were added. However, this improvement in muscle composition parameters was not conclusive, as also found in the study of Nascimento et al. (2021), who studied juvenile tambaqui (*Colossoma macropomum*). The tambaqui, a plant-eating piranha, were fed a plant protein-based diet supplemented with citric acid added and, while there was no negative effect on fish nutritional status, health or welfare, retention of nutrients and minerals in the muscle was not improved. Similarly, Hisano et al. (2017) found that acidification of the diet of pacu (*Piaractus mesopotamicus*) did not affect fish muscle composition. The fact that

fish of different species fed a plant protein-based diet with added citric acid did not show any improvement in muscle composition may be due to the length of the experiment, the ideal water temperature of the fish species or the different concentration of citric acid in the diet compared with our own study.

While increasing pea meal content and the addition of citric acid and/or phytase negatively affected FCR, growth parameters (SGR and weight gain) were affected positively by increasing the amount of pea meal in the diet and, more importantly, following the addition of phytase. Djeziri et al. (2020) added 15% of pea meal to tilapia and observed no positive effect on FCR and SGR of tilapia compared to a control group fed a fish meal based diet. In this study, these improved results were clearly influenced by the addition of phytase, which presumably helped to destroy the phosphorus bound in phytic acid. Phytic acid is classified as an anti-nutritional substance that binds minerals (calcium, iron, zinc and magnesium) and forms complexes that are difficult to absorb (Baruah et al., 2004). Ravindran (2000) reported that phytate acid also binds with proteins and amino acids and causes a decrease in digestibility. Conceicao et al. (2023) noted that digestibility and use of nutrients and minerals depend not only on the addition of digestive enzymes but also on breeding condition, the source of protein and the size and age of the fish. Rachmawati et al. (2018) studied tilapia fry (av. wt. 0.62 ± 0.01 g) fed diets with phytase at 500 FTU.kg⁻¹ feed, 1000 FTU.kg⁻¹ feed and 1500 FTU.kg⁻¹ feed, and found that the optimum content of phytase positively affecting SGR, FCR and weight gain ranged from 1060 to 1100 FTU.kg⁻¹. In our study, we added phytase at a lower concentration (500 FTU.kg⁻¹ feed); nevertheless, the positive effect on the SGR, FCR and weight gain was conclusive. Interestingly, if we look purely at the effect of adding pea meal, minus the additives, we find that SGR and weight gain increased with increasing pea protein content while FCR decreased. In terms of fattening economics, fish grew faster in both groups and were able to use the higher pea protein content with no problems. However, according to El-Saidy and Saad (2008), who examined tilapia fry fed a mixture with cow pea (*Vigna sinensis*) meal, the replacement should not exceed 50% otherwise the growth and feed conversion rates would deteriorate. In this case, it is possible that the use of cow pea was to blame for the difference in results. The cow pea is a non-cultivar pea variety, and therefore contains less nutrients and more anti-nutrients than cultivar varieties.

Tilapia are farmed worldwide under intensive aquaculture and, under such conditions, any stress caused by either poor water quality or inadequate nutrition will quickly become apparent. Haematological parameters, such as number of blood cells or changes in plasma mineral composition, are a commonly used method of monitoring for stress in fish (Seibel et al., 2021). In this study, we decided to evaluate whether the changes in diet would impact fish welfare in a way that could be monitored with the help of blood cell count and plasma biochemical parameters. Haemoglobin values decreased slightly in our P30F+ fish group

and significantly so in the P60F+ group, suggesting that the effect of phytase and citric acid was less significant than the increasing concentration of pea meal. According to Palikova et al. (2015), higher levels of haemoglobin and higher erythrocyte counts in fish are most often caused by increased stress. Kesbiç et al. (2024) added pea flour to rainbow trout feed at concentrations of 25, 50, 75 and 100%. None of these concentrations had significantly changed the haematological values of fish, except those fed 100% pea flour diet, which displayed a reduction in erythrocyte counts, haemoglobin content and haematocrit. The slight decrease in haemoglobin and erythrocyte values following addition of phytase (erythrocytes down by 10.5%) and phytase and citric acid (down 9%) in our study, therefore, suggest that the fish were not subject to any further stress. Novák et al. added pea flour to trout feed at concentrations of 25, 50 and 75%. None of these concentrations had any effect. These findings agree with those of Bozorgnia et al. (2011), who recorded increased numbers of red blood cells, and thus an increase in haemoglobin level, in carp under stressful conditions. Leukocyte counts should follow the same trend as they have also been shown to increase in fish under stress (Roberts, 2012). In our study, leukocyte counts were highly variable with no statistically significant change; nevertheless, except for the P30F and P60F groups, levels were slightly higher in all groups compared to the control. While addition of phytase had lowering effect on the reduction in leukocyte count, there was no apparent positive effect from the addition of citric acid.

While studying mrigal carp (*Cirrhinus mrigala*) fingerlings, Hussain et al. (2022) recommended acidification of their seed meal-based diet with 3% citric acid due to its positive effect on mineral absorption, carcass composition and haematological indices. In our own study, however, we observed no significant changes in plasma LDH, ALB, CHOL, UREA, CREA, TP, PI, K or Mg concentrations. Vazirzadeh et al. (2022) suggested that the CHOL levels could be affected by feeding duration, and although this 60-day feeding test is standard in length, no changes in CHOL were observed which may be considered positive. UREA and CREA levels are indicative of feed quality, especially the amount of protein, and if these levels are within the fish's physiological limits, it will ensure healthy excretion by the kidneys and metabolic functioning of the liver (Schrama et al., 2018). Our results show that these values did indeed remain stable and, therefore, it can be assumed that pea meal is a suitable source of protein for tilapia. Blood serum Ca and Fe values increased significantly in our study, especially in the groups with 30% pea meal supplementation, and significantly so following acidification with citric acid. This improvement in Ca and Fe could be attributed to maximum liberation of Ca, P, Fe and Cu from the feed ingredients following acid supplementation (Khajepour & Hosseini, 2012). Levels of GLU in the blood serum remained stable in all study groups, indicating no significant glycemic response, even at higher pea-meal concentrations (60%). In agreement with our results, Affonso et al. (2007) described significantly lower plasma Na and Cl concentrations in matrinxa (*Brycon*

amazonicus) fed a diet acidified with vitamin C, suggesting that matrinxa displayed a lowered metabolism and mineral absorption on the new diet. Plasma ALT, AST and ALP are all cytosolic enzymes found in many tissues; consequently, they are used as blood plasma indicators of tissue health (Haschek et al., 2009). ALT and AST levels fluctuated only slightly in our study and, since these refer to the health of the liver, a highly important organ as regards digestion, we can assume that the addition of pea protein had no effect on digestion and nutrient utilisation by the fish. AST also plays a role in glucose production from amino acids (blood glycemic state is a stress marker; Tejpal et al., 2009) and, in our experiment, AST values decreased proportionally with increasing addition of pea meal (significantly in all three P60 groups). According to our results, therefore, a diet based on pea protein is suitable for tilapia and subsequent acidification with citric acid, together with the addition of phytase, has no adverse effect on the health status of the fish; on the contrary, in most cases these additives had a positive effect on production parameters.

CONCLUSION

This study showed that fish feed based solely on pea protein is suitable for tilapia, with the pea meal having a positive impact on fish production parameters. Furthermore, addition of phytase and citric acid helped decrease any potential negative effects of a plant-protein based diet, and increased utilisation of feed minerals. Consequently, we suggest that a feed containing 30 or 60% pea meal is suitable for feeding tilapia under intensive aquacultural conditions, and its enrichment with phytase and citric acid is recommended.

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