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Citric acid as a functional supplement in diets for juvenile turbot, *Scophthalmus maximus* L.: Effects on phosphorus discharge, growth performance, and intestinal health

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ABSTRACT

The present study was conducted to investigate the suitability of citric acid as a feed additive for juvenile turbot (*Scophthalmus maximus*) to reduce phosphorus (P) excretion. A practical diet with similar profile as commercial diet was used as the positive control (C-P). C-P diet had no inorganic P supplementations, which was used as the negative control (C-0). Two citric acid supplemented diets, C-1.5 and C-3.0, were formulated by incorporating 1.5% and 3.0% citric acid into the C-0 diet, respectively. Each diet was fed to triplicate groups of 30 fish. The feeding trial lasted for 12 weeks. Turbot fed different diets showed similar growth performance, feed utilization efficiency and whole-body composition. Compared with group C-0, the pH of stomach contents in group C-3.0 was significantly lower while the pepsin activity and apparent digestibility coefficient of P in this group were notably higher. Diet C-3.0 resulted in the highest P retention, which was significantly higher compared to diet C-P but not to diet C-0. Compared to both control diets, citric acid supplemented diets significantly increased the activity of pepsin. No diet-related difference was observed in the blood biochemistry, nor in the histology and bacterial community profile of distal intestine. In summary, dietary inclusion of 3% citric acid markedly improved the bioavailability of P, without compromising intestinal function and health of fish. Citric acid seemed to be a promising feed additive for aqua-feeds to reduce P discharge into environment.

1. Introduction

Aqua-feed industry has undergone rapid growth in last decades with the rapid growth of aquaculture. However, phosphorus (P) has been a limiting nutrient in either fishmeal-based or plant protein-based fish feed. P exists as tricalcium phosphate and hydroxyapatite in fishmeal (Sarker et al., 2005) and its bioavailability varies considerably from fish to fish, ranging from 0% to 74% (Ogino, 1979; Watanabe et al., 1980; Yone and Toshima, 1979). In plant ingredients, P exists in phytic acid, which dramatically decreased the P bioavailability (Erdman, 1979; Nelson, 1967; Reddy et al., 1982; Ravindran et al., 1994). Moreover, to prevent P deficiency, inorganic P is often supplemented to the commercial diet, but the unavailable dietary P within either fish meal or plant ingredient would inevitably escalates the P emission into environment (Coloso et al., 2003; Hung et al., 2015). For example, a loading of 8.7 g total phosphorus was generated when 1 kg of turbot (*S. maximus*) fed with a commercial diet was produced, and the P retention of turbot was 42% (Mallekh et al., 1999). Therefore, a large amount of the unutilized dietary phosphorus could be excreted considering the huge biomass of farmed turbot industry. Excessive P discharge into the culture waters results in P loss from recycling in food production, in addition to a series of negative ecological impacts such as oxygen deficiencies and eutrophication (Ling et al., 2007). Many feed processing techniques have been developed to deal with the issue such as the addition of phytase and organic acids (Biswas et al., 2007; Li et al., 2015; Liebert and Portz, 2005). Since monogastric or agastric aquatic animals lack intestinal phytases, exogenous phytase can help hydrolyze the indigestible phytate in dietary ingredients to improve P utilization (Cao et al., 2007). Organic acids could lower the pH and subsequently result in higher dissociation of mineral compounds, which aids P absorption

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(Pandey and Satoh, 2008; Sugiura et al., 1998).

The positive effect of organic acids on P bioavailability in feed was well documented in terrestrial animals (Boling et al., 2000; Kemme et al., 1999; Kirchgessner and Roth, 1982). Several possible action modes involved in this effect have been indicated. A lower pH brought about by the organic acids supplementation could directly increase the solubility of phytate and improve P utilization in the intestine (Canibe et al., 2005). Alternatively, organic acids can promote P absorption by chelating calcium (Ca) to reduce the antagonistic interactions between them (Sugiura et al., 1998).

The optimization of protein digestion plays a key role in reaching high fish performances in aquaculture. The main gastric protease, pepsin, is strictly dependent on a low-enough environment pH (Márquez et al., 2012), and the optimum pH for protease activity in the stomach of turbot was 2.2 (Chen et al., 2006), while for the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) the optimum pH was 3 and 1.5, respectively (Hidalgo et al., 1999; Krogdahl et al., 2015a). However, the postprandial pH observed in the stomach of rainbow trout increased by more than two full units and the increased pH maintained for 8 h following feeding (Bucking and Wood, 2009). The increase of pH in stomach negatively affects the activation of pepsin, and possibly decreases the protein digestion capability in fish (Luckstadt, 2008; Márquez et al., 2012). In pig, providing acidifiers in the feed addressed this problem and aided in feed digestion (Thaela et al., 1998). Similarly, citric acid improved gastrointestinal digestive enzyme activities in red drum (*Sciaenops ocellatus*) and tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) (Castillo et al., 2014; Li et al., 2009).

The intestinal microbiota plays critical roles in stimulating intestinal cell proliferation and nutrient uptake, protects gastrointestinal (GI) tract from infectious agents, regulates immune system in the GI tract, and produces enzymes which might have significant roles in digestion (Wang et al., 2017). Organic acid has been used in swine diets for decades and appears to have some antimicrobial effects (Dibner and Buttin, 2002). In aqua-feeds, use of organic acid is expected to significantly increase in the coming years due to global regulatory controls on the use of antibiotics. The most obvious mode of actions of these acidifiers is via acidification of the extracellular pH thorough dissociating into ions and releasing protons to the surrounding mediums, since many pathogens are unable to grow under acidic conditions (Ng and Koh, 2016). However, the general consensus is that the antimicrobial efficacy of organic acid is mainly due to their ability to passively diffuse through a bacterium's semi-permeable membrane and dissociate in the pH neutral cytoplasm (Booth and Stratford, 2003; Cherrington et al., 1991). Accumulation of the protons released by organic acids within cell will lower the cytoplasmic pH, interfere the cell metabolism, suppress transport of nutrients, lead to depletion of cellular ATP, inhibit the synthesis of macromolecules, and eventually result in cell death (Cherrington et al., 1990; Dibner and Buttin, 2002; Ricke, 2003; Warth, 1991). The acidification by organic acids could favor the growth of acidophilic *lactobacilli* and exert an anti-adherence effect on *Escherichia coli* as observed in the gastro-ileal region in pig (Partanen and Mroz, 1999). In aquatic animals, organic acids blend and potassium diformate were able to markedly reduce total bacteria in feces and shift the fecal population of *A. hydrophila* in red hybrid tilapia (*Oreochromis* sp.), potentially preventing diseases caused by *Aeromonas* (Ng et al., 2010). A blend of organic acids and essential oil enhanced the bacteria diversity and richness and beneficially affect intestinal microflora in Pacific white shrimp (*Litopenaeus vannamei*) (He et al., 2017). Among the organic acids, citric acid has been widely used in terrestrial animals for its good flavor and high buffer capacity. In fish, citric acid has been reported to increase the P availability in rainbow trout (Pandey and Satoh, 2008), red sea bream (*Pagrus major*) (Hossain et al., 2007), beluga (*Huso huso*) (Khajepour and Hosseini, 2012), rohu (*Labeo rohita*) (Baruah et al., 2007) and yellowtail (*Seriola quinqueradiata*) (Sarker et al., 2012). It has been proposed as a promising feed additive to reduce P loading in aquaculture. Therefore, the present study was conducted to test the effectiveness of citric acid application in turbot, which is a major aquaculture species all over the world.

Because of its delicious meat, high disease resistance, and rapid growth, turbot is of high economic value, and has been widely farmed in Europe and East Asia. In China, especially, turbot has been a dominant farming species in the northern part. To our knowledge, there has not yet studies evaluating the P utilization efficiency for turbot. The present study aimed at evaluating the P retention in turbot fed a practical diet similar to commercial diet in composition, and the suitability of citric acid as a feed additive to improve P utilization efficiency in this fish.

2. Materials and methods

2.1. Experimental diets

A total of 4 isonitrogenous and isolipidic experimental diets differing only in P and citric acid supplementations were made. The first diet was a positive control diet without citric acid but was supplemented with phosphorus (C-P), which was similar to commercial formulations. The second diet was a negative control without citric acid or P supplementations (C-0). The third and fourth diets had no P supplementations but had citric acid levels of 1.5 (C-1.5) and 3.0% (C-3.0), respectively. The dietary formulations (basal diet) among the diets were the same, with the exception of P and citric acid supplementations. Additionally, 1 g kg^{-1} yttrium oxide (Y_2O_3) was used as an inert tracer in each diet for determination of apparent digestibility coefficient of nutrients.

Diet ingredients were ground into fine powder to pass through 320 μm mesh. All ingredients were thoroughly mixed with fish oil and soybean oil, and water was added to produce a stiff dough. The dough was then extruded with an experimental single-screw feed granulater. The die diameter was 3 mm and the screw-speed 90 rpm. Product temperature at the end of the barrel was 50–55 °C (F-26, South China University of Technology, China). After being pelleted, the feeds were dried for about 12 h in a ventilated oven at 55 °C and stored in a freezer at −20 °C.

2.2. Fish husbandry

Apparently healthy juvenile turbot were obtained from a commercial fish farm in Laizhou, China. Before the start of feeding trial, they were fed a commercial diet (Great seven Bio-Tech, Qingdao, China) for 2 weeks to acclimate to the experimental conditions. Then fish with similar sizes (initial mean weight 8.2 ± 0.22 g) were randomly distributed to 12 fiberglass tanks (300 L) with 30 fish per tank. Each diet was randomly assigned to triplicate groups of fish. They were fed to apparent satiation twice daily (08:00 and 17:00) for 12 weeks. Uneaten feeds were collected from the tank outlets and dried for the calculation of feed intake. Sea water was pumped from the adjacent coast to the experiment base, passed through sand filters, froth separator and biofilter in turn, and finally flowed into each tank at a rate of 2 L min^{-1}. This system water was exchanged at 50% each day with new water. Continuous aeration was supplied with a single air-stone connected to a central air blower. During the feeding trial, the water temperature ranged from 15 °C to 18 °C; salinity varied from 30‰ to 33‰; dissolved oxygen was higher than 7 mg L^{-1}; and NH_4-N was lower than 0.4 mg L^{-1}.

2.3. Sample collection

Two hours after feeding, apparently intact feces were siphoned into a string bag made of three layers of gauze, then artificially scraped in to a 10 mL tube, stored at −20 °C before analysis. At day 84, the biomass in each tank was measured before the evening feeding. At day 85, 2 h

after the morning feeding, 6 fish per tank were randomly selected to collect contents in the stomach, which were then immediately snapped frozen in liquid nitrogen. Five hours after the morning feeding, guts of 6 fish per tank were excised and divided into proximal and distal gut using a clamp, as suggested by Li et al. (2016). The digesta in each gut compartment was collected into 2 mL tubes and snapped frozen in liquid nitrogen right away. For the analysis of intestinal microflora, hindguts with contents of 5 fish per tank were collected. In brief, the abdomen surface of fish was decontaminated with 70% ethanol, after which the hindgut was dissected with sterile anatomic tools near an alcohol burner, transferred to 2 mL sterile tubes, and immersed in liquid N_2. Before dissection, blood was withdrawn from the caudal vein using 1 mL syringe from the above fish and allowed to stay at 4 °C until it clotted. Serum was obtained after centrifugation (3000*g* for 10 min at 4 °C) and frozen at once. The carcasses were kept to obtain skeleton samples. The feeding was stopped after the above sampling events to empty the gut contents. The next morning, five fish were randomly selected from each tank and stored at −20 °C for the analysis of proximate composition. A short segment (about 1 cm in length) of the hindgut of another five fish per tank were cut and immersed in the Bouin's fixative solution. After fixation for 24 h, the gut tissues were transferred to 70% ethanol awaiting further process. All fish were anesthetized with eugenol (1:10000) before handling and euthanized by a sharp blow to the head before sampling.

2.4. Chemical analysis

Proximate composition of feed ingredients, diets, feces and whole fish were analyzed following the standard methods (AOAC, 1995): dry matter (DM) was measured by drying samples to a constant weight at 105 °C; crude protein by the Kjeldahl method (2300- Auto-analyzer, FOSS Tecator, Höganäs, Sweden); crude lipid by the Soxhlet method (B-811, BUCHI, Flawil, Switzerland) and ash by combustion at 550 °C.

The concentration of yttrium (Y), P and Ca in the diets and feces, and P content of the carcass and skeleton were determined using an inductively coupled plasma-atomic emission spectrophotometer (ICP-OES; VISTA-MPX, Varian, America) after perchloric acid digestion (Mai et al., 2006). To measure bone P content, the whole fish was boiled for 20 min, then flesh was stripped off from vertebrae and the remaining was removed by slightly brushing the bone. Rinsed clean by deionized water, the vertebrae was dried at 110 °C for 6 h, immersed into ethyl ether for 7 h, pulverized and dried again (Baruah et al., 2005). Afterwards, the whole skeleton was ground into fine powder for perchloric acid digestion. For the determination of water-soluble P in the feeds and stomach contents, 0.2 g freeze-dried material was weighed accurately and homogenized with 20 mL 2 mol L^{-1} KCl. The homogenate was then shaken for 18 h at 37 °C and the supernatant containing soluble P was collected after centrifugation (5000*g* for 10 min) (Lu et al., 2014). Soluble P was determined via its reaction with molybdic acid to form molybdenum blue, which has an absorption maximum at 660 nm, using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; Catalog NO., C006-3). The concentration of soluble P was calculated as follows: P content in samples = [(OD of supernatants − OD of blank control) / (OD of P standard − OD of blank control)] × P concentration of the standard (0.5 mmol L^{-1}) × dilution factor.

2.5. pH measurement

The pH of experimental diets and gastrointestinal contents was measured following the method of Castillo et al. (2014). Briefly, 1 g freeze-dried feeds or digesta were homogenized with 5 mL cold ddH_2O (1:5, w v^{-1}) on ice. Then pH of the homogenates was measured by a pH Meter (PHS-3C, Leici, Shanghai).

2.6. Digestive enzyme activity

After pH measurement, the homogenate of digesta was further diluted (1:20, v v^{-1}) with cold ddH_2O and shaken gently for 10 min at 4 °C to extract digestive enzyme. The enzyme extract was obtained after centrifugation (13,000*g* for 10 min at 4 °C), and stored as aliquots at −80 °C until analysis. Enzyme assays were performed using commercial kits (Nanjing Jiancheng Bioengineering, Nanjing, China), and the enzyme activity was normalized to the weight of digesta on a DM basis. Pepsin activity was analyzed using casein as substrate and one unit of activity is defined as 1 μg tyrosine liberated by hydrolyzing protein at 37 °C for 1 min (catalog No., A080-1) (Cupp-Enyard, 2008). Trypsin activity was measured using N-benzoyl-L-arginine-ethylester (BAEE) as substrate and one unit of activity is defined as producing ΔA_{253} of 0.003 per min at 37 °C (catalog No., A080-2) (Erlanger et al., 1961). Amylase activity was determined using starch as substrate and one unit of activity is defined as the amount of enzyme that decomposes 10 mg of starch in 30 min at 37 °C (catalog No., C016-1) (Bernfeld, 1951).

2.7. Histology

Distal intestine tissue was dehydrated in ethanol, equilibrated in xylene and embedded in paraffin wax according to standard histological procedures. Tissue sections with thickness of 7 μm were prepared for hematoxylin and eosin (H&E) staining and examined blindly to the reviewer under a microscope equipped with a camera (E600, Nikon, Tokyo, Japan) and an image acquiring software (CellSens Standard, Olympus, Tokyo, Japan), following the criteria suggested by Krogdahl et al. (2015b). Additionally, micrographs were analyzed using the image analysis software Image Pro Plus® (Media Cybernetics, Silver Spring, MD, USA) to determine the perimeter ratio (PR, arbitrary units AU) between the internal and external perimeter of the gut lumen (Dimitroglou et al., 2009). A higher PR value indicates larger absorptive surface area resulting from increased villi height and/or mucosal folding.

2.8. Blood biochemistry

Total protein, total cholesterol, triglyceride, glucose, alanine amino transferase, aspartate transaminase, alkaline phosphatase, urea and creatinine in serum were determined by an automatic biochemical analyzer (HITACHI 7600, Tokyo, Japan) at the hematology center of Affiliated Hospital of Medical College, Qingdao University (Qingdao, China).

2.9. Intestinal microbiota profiling

The bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) as described by Li et al. (2017) except that both intestinal mucosal tissue and contents were collected, mixed and homogenized for the DNA extraction. The library construction and sequencing of 16s rRNA V_4 region on Illumina Hiseq 2500 were performed by Beijing Novogene Genomics Technology Co. Ltd. (Tianjin, China) as described by Yao et al. (2016).

The raw sequence data was deposited in the BIG Data Center (BIGD, Beijing Institute of Genomics) under the accession id CRA000567. QIIME 2 (version 2017.8) pipeline was used for the data analysis and visualization (Caporaso et al., 2010). The pair-ended reads were truncated to a constant length of 150 bp based on the sequence quality plot and denoised by DADA2 to infer exact sequence variants (SVs) (Callahan et al., 2016). The result was a feature table with SVs instead of OTUs that cluster sequences based on certain sequence similarity cut off value (usually 97%). The representative sequences were then aligned by MAFFT (Katoh and Standley, 2013) and a phylogenetic tree was generated from the masked alignment using FastTree (Price et al., 2010). The taxonomic assignment was performed by a pre-trained

Naive Bayes classifier (available at the Data resources of QIIME2 website) trained on the Greengenes 13_8 99% OTUs, which have been trimmed to only include the V4 region. Taxa identified as Cyanobacteria were excluded from the analysis (Baldo et al., 2015). Additionally, features accounting for < 0.005% of the total sequences or only present in one sample were filtered as well, due to technical, statistical and biological considerations (Bokulich et al., 2013; Dhariwal et al., 2017). The resulting count data was normalized by rarefying the total sequence number of each sample to 11,755, which was the minimum count number found in the samples after data filtering (Weiss et al., 2017). Afterwards, Faith's phylogenetic diversity (PD), observed OTUs, Shannon's index and Pielou's evenness were computed for the alpha diversity estimation. The dissimilarity between microbial communities was evaluated by unweighted-UniFrac distance (Lozupone and Knight, 2005) based principal coordinate analysis (PCoA) and visualized by EMPeror (Vazquez-Baeza et al., 2013), followed by permutational multivariate analysis of variance (PERMANOVA, 999 times of permutations) (Anderson, 2001). ANCOM (analysis of composition of microbiomes) (Mandal et al., 2015), which accounts for compositional constraints of microbiome data, was employed to identify differentially abundant taxa between the negative control group and citric acid supplemented groups. The differential abundance testing was restricted to taxa observed in at least 25% of the samples as low frequency OTUs are difficult to interpret statistically.

2.10. Calculations

The following variables were calculated:

$$\text{Survival (\%)} = 100 \times \text{final number of fish/initial number of fish}$$

$$\text{Specific growth rate (SGR,\%day}^{-1}\text{)} = 100 \times (\text{Ln final weight} - \text{Ln initial weight})/\text{days}$$

$$\text{Feed efficiency (FE)} = (\text{final weight} - \text{initial weight})/\text{feed consumed}$$

$$\text{Feed intake (FI,\%day}^{-1}\text{)} = 100 \times \text{feed intake} /[(\text{initial weight} + \text{final weight})/2]/\text{days}$$

$$\text{Apparent digestibility coefficient (ADC) of nutrients (\%)} = 100 \times [1-(\%\text{Y in diet} \times \%\text{nutrient in feces}) /(\%\text{Y in feces} \times \%\text{nutrient in diet})]$$

$$\text{Available P (\%diet, DM basis)} = \text{ADC of P} \times \text{total P in the diet}$$

$$\text{Fecal P (\%diet, DM basis)} = \text{total P in the diet} - \text{available P in the diet}$$

$$\text{P liberated via digestion (\%diet, DM basis)} = \text{available P of the diet} - \text{soluble P in the diet}$$

$$\text{Nutrients retention (\%)} = [(\text{Final body nutrient content} - \text{initial body nutrient content})] /\text{nutrient intake}] \times 100$$

$$\text{Nutrients excretion (kg t}^{-1}\text{)} = [(\text{FCR} \times \text{nutrient in diet (g)} - \text{nutrient retained in fish (g)}) /\text{production (t)}] \times 1000$$

2.11. Statistical analysis

Statistical software SPSS 22.0 for Windows (IBM SPSS corporation, Chicago,USA) was used for the data analysis. All data were subjected to normality test (Shapiro-Wilk test) and Levene's test for equal variance before the one-way ANOVA was performed. Tukey's multiple-range test was used for the multiple comparisons of group means. Alternatively, Kruskal-Wallis H test was used when needed. Pearson's correlation analysis was carried out to quantify the strength of any relationship between the measured parameters and dietary treatments. When necessary, multiple comparisons were corrected by the Benjamini-Hochberg procedure. Differences were regarded as significant when $p < 0.05$.

Table 1
Formulations and chemical analyses of the experimental diets (% dry matter).

Ingredients (%)	C-P	C-0	C-1.5	C-3.0
Fishmeal[a]	36.00	36.00	36.00	36.00
Soybean meal[a]	15.68	15.68	15.68	15.68
Corn gluten[a]	8.00	8.00	8.00	8.00
Wheat gluten[a]	5.12	5.12	5.12	5.12
Peanut meal[a]	3.20	3.20	3.20	3.20
Bear yeast[a]	2.50	2.50	2.50	2.50
Wheat flour[a]	15.63	16.13	14.63	13.13
Taurine	1.00	1.00	1.00	1.00
DL-Methionine	0.26	0.26	0.26	0.26
L-Threonine	0.18	0.18	0.18	0.18
L-Histidine	0.19	0.19	0.19	0.19
L-Lysine	0.74	0.74	0.74	0.74
Fish oil	8.00	8.00	8.00	8.00
Soybean lecithin	1.00	1.00	1.00	1.00
Vitamin premix[b]	1.00	1.00	1.00	1.00
Mineral premix[c]	0.50	0.50	0.50	0.50
Choline chloride	0.25	0.25	0.25	0.25
Ethoxyquin	0.05	0.05	0.05	0.05
Calcium propionate	0.10	0.10	0.10	0.10
Y_2O_3	0.10	0.10	0.10	0.10
$Ca(H_2PO_4)_2$[d]	0.50	0.00	0.00	0.00
Citric acid[e]	0.00	0.00	1.50	3.00
Chemical analysis				
Crude protein (CP)	52.22	52.30	52.05	51.80
Crude lipid (CL)	14.18	14.19	14.16	14.13
pH	5.94	6.07	5.34	4.90
Total P	1.66	1.46	1.47	1.44
Total Ca	1.61	1.54	1.49	1.44

Formulations and chemical analyses of the experimental diets (% DM).

[a] Fishmeal (DM, %): CP 72.94, CL 10.85; soybean meal (DM, %): CP 53.67, CL 2.81; corn gluten (DM, %): CP 64.92, CL 3.03; wheat gluten (DM, %): CP 83.94, CL 1.34; peanut meal (DM, %): CP 55.62, CL 3.69; beer yeast (DM, %): CP 51.02, CL 2.90; wheat flour (DM, %): CP 16.82, CL 2.11.

[b] Vitamin premix (mg kg^{-1} diet): retinyl acetate, 32; vitamin D_3, 5; DL-α-tocopherol acetate, 240; vitamin K_3, 10; thiamin, 25; riboflavin (80%), 45; pyridoxine hydrochloride, 20; vitamin B_{12} (1%), 10; L-ascorbyl-2-monophosphate-Na (35%), 2000; calcium pantothenate, 60; nicotinic acid, 200; inositol, 800; biotin (2%), 60; folic acid, 20; choline chloride (50%), 2500; cellulose, 6740; antioxidants, 3.

[c] Mineral premix (mg kg^{-1} diet): $FeSO_4 \cdot H_2O$, 80; $ZnSO_4 \cdot H_2O$, 50; $CuSO_4 \cdot 5H_2O$, 10; $MnSO_4 \cdot H_2O$, 45; KI, 60; $CoCl_2 \cdot 6H_2O$ (1%), 50; Na_2SeO_3 (1%), 20; $MgSO_4 \cdot 7H_2O$, 1200; calcium propionate, 1000; zeolite, 2485.

[d] Calcium phosphate: analytical grade (Fulin Biochemical Co., Ltd., Qingdao, Shandong, China).

[e] Citric acid: food grade (Ensign Industry Co., Ltd., Weifang, Shandong, China).

3. Results

3.1. Growth performance and feed utilization

No significant difference was found in the survival, specific growth rate, feed efficiency or feed intake among all dietary groups ($P > 0.05$, Table 2). Turbot fed different diets showed similar whole-body proximate composition (Table S1).

3.2. pH of the diets and gastrointestinal contents

Diets C-0 and C-3.0 reduced pH of the negative control diet from

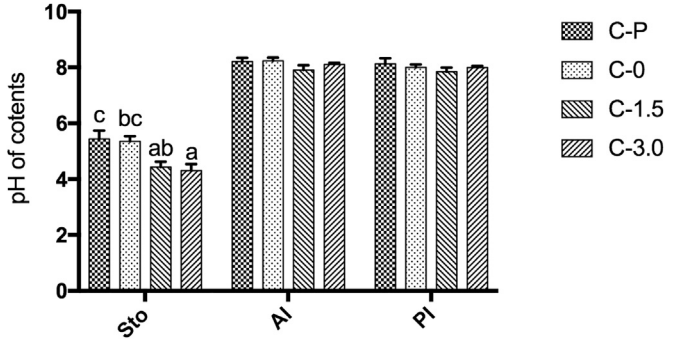

Fig. 1. Effects of phosphorus and citric acid supplementations on pH of gastrointestinal contents of juvenile turbot. Sto, stomach; AI, anterior intestine; PI, posterior intestine. Error bars of columns indicate standard error of means (n = 3) and different letters within each variable denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$).

Table 2
Effects of phosphorus and citric acid supplementations on growth performance and feed utilization in turbot.[a]

	C-P	C-0	C-1.5	C-3.0	Pooled SEM	P
Initial body weight (g)	8.25	8.22	8.24	8.22	0.01	0.17
Final body weight (g)	68.14	66.74	67.93	63.38	3.52	0.76
Survival (%)	97.78	96.67	97.78	100.00	1.94	0.65
SGR (% day^{-1})	2.13	2.10	2.13	2.05	0.06	0.76
FE	1.02	1.04	1.03	1.06	0.06	0.98
FI (% bw day^{-1})	1.49	1.46	1.47	1.41	0.08	0.89

[a] Values represent means of three replicate tanks.

6.07 to 5.34 and 4.90, respectively (Table 1). The reduction of pH was also observed in the stomach contents in but not in the digesta from the anterior and posterior intestine of the citric acid supplemented groups, (Fig. 1). The pH of experimental diets was positively correlated with that of stomach contents ($P = 0.04$, $R^2 = 0.96$) and was negatively correlated with soluble P content in the diets ($P = 0.03$, $R^2 = -1.0$).

3.3. Nutrient digestibility

Turbot fed the C-3.0 diet showed the highest apparent digestibility coefficient (ADC) of DM, P and Ca, which were significantly different from those in groups C-0 and C-1.5 ($P < 0.05$, Fig. 2). ADC of protein was not affected by the addition of citric acid ($P > 0.05$). The ADC of P was positively correlated with that of Ca ($P = 0.01$, $R^2 = 0.99$).

3.4. Phosphorus utilization

Compared with the C-0 diet, citric acid supplementation increased the soluble P content in both diets and stomach contents, with a significant increment in the C-3.0 diet ($P < 0.05$) (Table 3). The soluble P content within the stomach was positively correlated with the dietary soluble P content ($P = 0.02$, $R^2 = 0.98$), but there was no correlation with the stomach content pH ($P = 0.17$, $R^2 = -0.97$).

The P utilization efficiency was evaluated in terms of fecal P content, available P content, P retention and P excretion. Compared with the C-0 diet, dietary supplementation of 3% citric acid significantly improved the available P content in the diet and reduced the fecal P content, which is concurrent with significantly higher soluble P content in the feed (Table 3 and Fig. 3A). The available P content in the C-3.0 diet was close to that in diet C-P with inorganic P supplementation in absolute amount. When assessed as the proportion of total dietary P content (Fig. 3B), however, available P content in the C-3.0 diet was the

Table 4
Effects of phosphorus and citric acid supplementations on digestive enzyme activities in turbot.[1]

Parameter	C-P	C-0	C-1.5	C-3.0	Pooled SEM	P
Pepsin (U g^{-1} DM)	214.80^{a}	176.58^{a}	318.36^{b}	328.22^{b}	21.84	0.00
Trypsin (U mg^{-1} DM)	40.06	39.67	40.37	39.87	0.50	0.82
Amylase (U kg^{-1} DM)	97.30	101.20	115.10	96.70	1.01	0.10

[1] Values represent means of three replicate tanks. Different superscript letters within a row denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$). Stomach content was used for the analysis of pepsin, and intestinal content was used for the analysis of trypsin and amylase. DM: dry matter.

Table 5
Effects of phosphorus and citric acid supplementations on the serum biochemistry and enzymes in turbot.[1]

	C-P	C-0	C-1.5	C-3.0	Pooled SEM	P
Total protein (g L^{-1})	26.65	26.41	28.22	25.53	1.14	0.45
Total cholesterol (mmol L^{-1})	2.96	2.51	2.73	2.66	0.16	0.33
Triglyceride (mmol L^{-1})	9.47	8.04	9.07	8.46	0.73	0.55
Glucose (mmol L^{-1})	1.70	1.86	1.72	1.68	0.18	0.90
Alamine amino transferase (IU L^{-1})	2.33	2.66	2.00	2.33	0.29	0.49
Aspartate transaminase (IU L^{-1})	15.50	14.67	8.50	16.33	3.02	0.39
Alkaline phosphatase (IU L^{-1})	12.67	11.67	11.00	12.00	0.85	0.60
Urea (mmol L^{-1})	1.34	1.69	1.45	1.61	0.11	0.16
Creatinine (umol L^{-1})	15.00	16.33	17.33	17.00	2.03	0.85

[1] Values represent means of three replicate tanks. Different superscript letters within a row denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$).

Table 3
Effects of phosphorus and citric acid supplementations on P utilization in turbot.[a]1

	C-P	C-0	C-1.5	C-3.0	Pooled SEM	P
Soluble P in diet (% DM)	0.54^{ab}	0.43^{a}	0.57^{ab}	0.67^{b}	0.01	0.00
Soluble P in stomach contents (% DM)	0.45^{ab}	0.38^{a}	0.45^{ab}	0.49^{b}	0.02	0.02
Available P in diet (% DM)	0.98^{b}	0.83^{a}	0.86^{ab}	0.96^{b}	0.03	0.00
Soluble P in diet/available P in diet (%)	55.27^{a}	52.23^{a}	66.81^{b}	69.67^{b}	2.20	0.00
Available P in diet/total P in diet (%)	59.21^{ab}	56.86^{a}	58.02^{a}	66.97^{b}	2.03	0.04
Fecal P (% DM)	0.68^{b}	0.63^{b}	0.61^{b}	0.48^{a}	0.03	0.00
Total P of whole fish (% DM)	2.43	2.42	2.45	2.53	0.07	0.63
Total P in bone (% DM)	9.92	10.04	10.25	10.60	0.16	0.18
P retention (%)	32.36^{a}	35.66^{ab}	36.81^{ab}	38.95^{b}	1.58	0.03
P excretion (kg t^{-1})	11.04^{b}	9.22^{ab}	9.14^{ab}	8.33^{a}	0.58	0.03

[1] Values represent means of three replicate tanks. Different superscript letters within a row denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$).

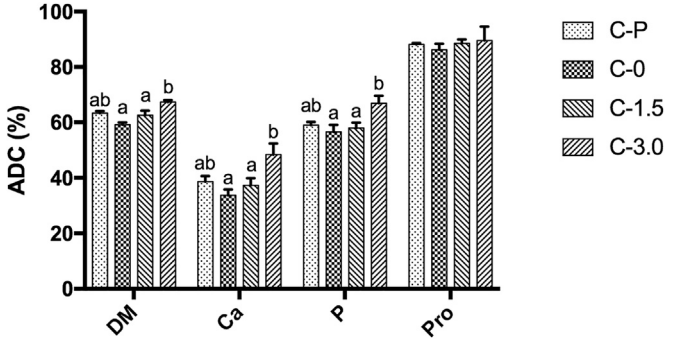

Fig. 2. Effects of phosphorus and citric acid supplementations on apparent digestibility coefficient of nutrients of juvenile turbot. DM, dry matter; Ca, calcium; P, phosphorus; Pro, protein. Error bars of columns indicate standard error of means (n = 3) and different letters within each variable denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$).

highest among the diets, significantly higher than that in diets C-0 and C-1.5. Similar to changes in the available P content, though not significant, increased P retention by citric acid supplementation was also observed (Fig. 3C). In this case, however, the C-P diet resulted in the worst P retention which was significantly lower than that in group C-3.0. P excretion followed an opposite trend to that of P retention as expected.

3.5. Digestive enzyme activity

Compared to the control diets, citric acid supplemented diets significantly increased the pepsin activity ($P < 0.05$) (Table 4), which showed a negative correlation with the pH of stomach contents ($P = 0.03$, $R^2 = -0.97$). In contrast, the activities of trypsin and amylase was similar among all the experimental groups ($P > 0.05$).

3.6. Histology

All intestinal sections examined showed normal intestinal morphology. No typical morphological changes such as widening or shortening of the intestinal folds, widening of the lamina propria within the intestinal folds and a profound infiltration of mixed leucocytes in the lamina propria was observed in all diet groups. Infiltration of inflammatory cells in the submucosa was not observed, nor the loss of the supranuclear vacuolization in the absorptive cells (enterocytes) was observed in the present study (Fig. S2 (ii)). The perimeter ratio was significantly higher in the C-1.5 group compared to the negative control (Fig. S1).

3.7. Blood biochemistry and enzymes

No significant difference was found in serum biochemistry or enzymes ($P > 0.05$) (Table 5).

3.8. Intestinal microbiota

A total number of 951,646 raw pair-ended reads were generated for 12 samples. After sequence quality control and feature filtering, 386,561 merged sequences were retained with a minimum number of sample sequence being 11,755. The rarefaction analysis showed adequate sequencing depth for all the samples (Fig. S3).

At phylum level, the bacterial composition of the positive control group was dominated by Firmicutes (37.85%) and Proteobacteria (31.15%), followed by Bacteroidetes (13.21%) and Actinobacteria (11.31%) (Fig. 4A). At genus level, *Lactobacillus* and *Bacillus* accounted for 16.82% of the total bacterial population, followed by *Mesorhizobium* (2.18%), *Bacteroides* (1.40%), *Pseudomonas* (1.16%), and *Oscillospira* (1.08%) (Fig. 4B). No significant diet-related difference in alpha diversity of intestinal microbiota was observed (Fig. S4). The PCoA plot (Fig. S5) showed no clear clustering of microbial samples under various experimental conditions, indicating that the overall microbial

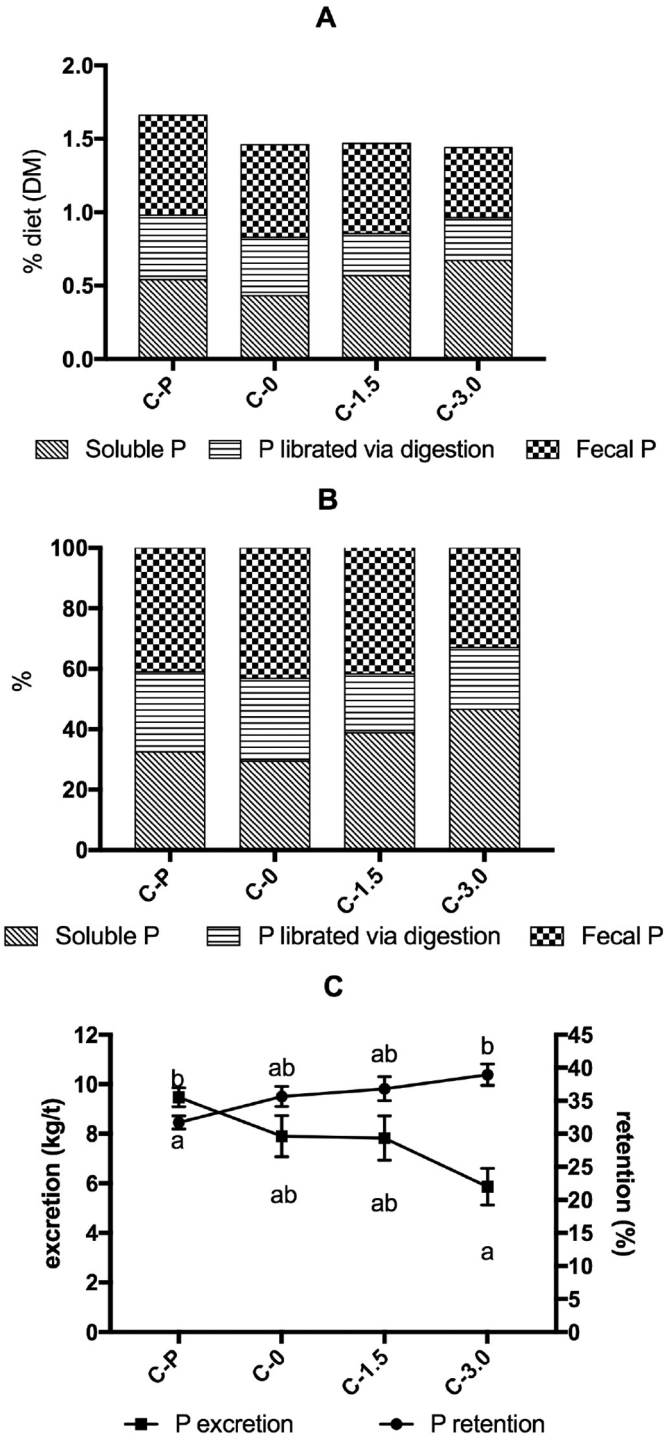

Fig. 3. Effects of phosphorus and citric acid supplementations on P utilization by juvenile turbot. (A) Stacked chart showing the total, soluble, available and fecal P of each diet. (B) 100% stacked chart showing the total, soluble, available and fecal P of each diet, the total dietary P was specified as 1 (100%), the % in the y-axis refers to the proportion of each component of P with respect to total P in diet. (C) P retention and excretion. DM, dry matter. Total P = available P + fecal P; available P = soluble P + P liberated via digestion. Error bars indicate standard error of means (n = 3) and different letters within a series denote significant differences as evaluated by Tukey's HSD test ($P < 0.05$).

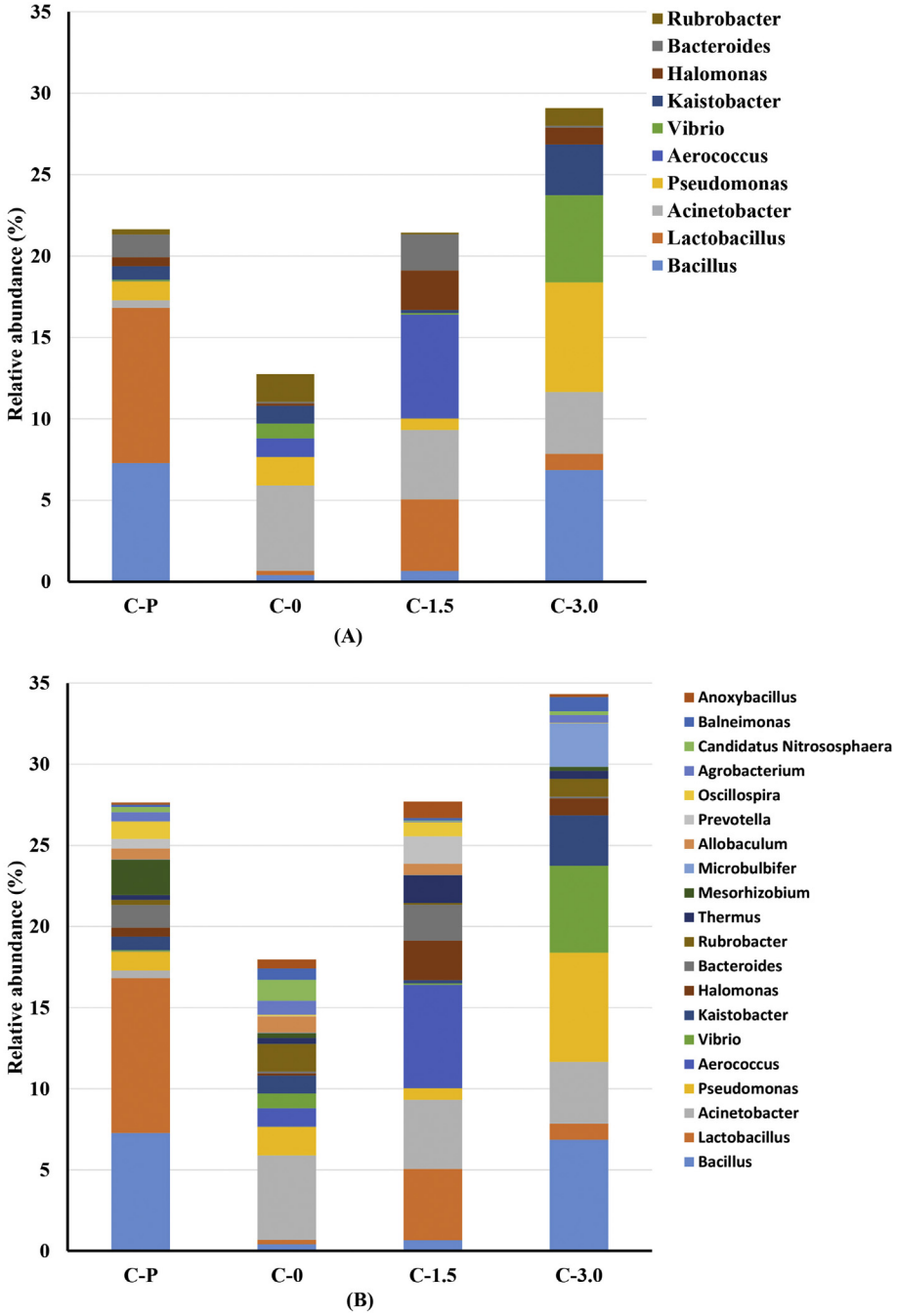

Fig. 4. Effects of phosphorus and citric acid supplementations on bacterial composition in the distal intestine of juvenile turbot. A, top 10 most abundant taxa at phylum level; B, top 20 most abundant taxa at genus level.

community structure may be similar among different treatments. This was confirmed by the PERMANOVA results (Table S2). No differentially abundant taxa were identified at phylum or genus level between the negative control group and citric acid supplemented groups.

4. Discussion

Phosphorus (P) is a major component of hard tissues and is an essential element of organic phosphates, which plays an essential role in fish growth and development. Deficiency in P can result in malformation of skeleton and reduced feed efficiency and fish growth (NRC, 2011). Supplementing citric acid to a low phosphorus fish diet enhanced the P utilization and allowed the growth performance similar to that achieved with an inorganic P supplemented diet in rainbow trout and red sea bream (Hernández et al., 2013; Hossain et al., 2007), suggesting that citric acid has the potential to liberate P from a low-phosphorus diet to boost fish growth. In the present study, however, dietary supplementation of citric acid did not improve the growth of juvenile turbot, despite citric acid increasing soluble dietary P. This was likely due to high levels of already available dietary P that ranged from 8.3–9.7 g kg^{-1} may have exceeded the minimum requirement of the fish for optimal growth. This is also based on the C-P and C-0 diets leading to similar growth, indicating that P supplementations were unnecessary, as well as a similar P content across all the treatment groups. The reported P requirements for optimal growth, feed utilization and bone mineralization has been reported to be 3.3–9.1 g kg^{-1} for channel catfish (*Ictalurus punctatus*), black sea bream (*Acanthopagrus schlegeli*), salmon, haddock (*Melanogrammus aeglefinus*) and yellow

croaker (*Pseudosciaena crocea*) (NRC, 2011).

Compared to the negative control, the ADC of P was significantly improved by the addition of 3% dietary citric acid, which was in line with previous findings in rainbow trout (Hernández et al., 2013), red sea bream (Sarker et al., 2005), beluga (Khajepour and Hosseini, 2012) and rohu (Baruah et al., 2007). However, studies on red sea bream (Hossain et al., 2007) and yellow catfish (*Pelteobagrus fulvidraco*) (Zhu et al., 2015) did not show such effect of dietary citric acid on digestibility of P. Our results indicated that soluble P increased in citric acid supplemented diets and negatively correlated with the diet pH. Although it has been reported acidification induced by citric acid may help solubilizing phosphorus in the phytate (Jongbloed, 1987), this needs further demonstration since citric acid cannot itself degrade phytic acid (Baruah et al., 2007; Romano et al., 2016). The effect of citric acid on availability of P seems more likely due to an acidifying effect which solubilizes bone minerals in fish meal (Sugiura et al., 1998). The increments of soluble P in citric acid supplemented diets seemed to be responsible for the higher level of soluble P in stomach contents. Assuming soluble P in the diet is completely absorbed during the digestion process, we estimated that it accounted for 67.06% and 69.79% of available P in the diet C-1.5 and C-3.0, respectively, as compared to 51.81% in the C-0 diet. Together, these results implied that the primary action mode of citric acid to improve P bioavailability is to increase soluble P in the diet via acidification. Consistent with previous findings in red sea bream (Sarker et al., 2007; Sarker et al., 2005), rainbow trout (Pandey and Satoh, 2008) and yellowtail (Sarker et al., 2012), a significantly higher P retention and correspondingly a significantly lower P excretion was observed in turbot fed 3% citric acid compared with those fed the inorganic P supplemented diet (C-P). Despite having an intermediate P digestibility, turbot fed the C-P diet showed the lowest P retention, most likely due to the excessive P amount in the diet and thereby increased P excretion in the feces. This indicates that the necessity of adding inorganic P to the low fishmeal based diet is questionable when available P in the diet to turbot is unclear. Data on P requirement and its bioavailability in feed ingredients need to be collected in turbot to allow for the optimization of diet formulations and thus avoid excessive dietary P supplementation. On the other hand, the significant improvement in P digestibility by dietary inclusion of 3% citric acid did not result in a significant increase in P retention when compared to the negative control. This might be due to the relatively high P level in the basal diet C-0 which resulted in high P input. A significant increase in P retention might be expected if a basal diet with suboptimal P level was used, which was evidenced by studies on rainbow trout and red sea bream (Hernández et al., 2013; Sarker et al., 2007). Excessive discharge of P can lead to eutrophication of receiving water (Ketola and Harland, 1993). Therefore, dietary supplementing citric acid may be an efficient way to reduce environment pollution in aquaculture.

Following the common pattern found in many other vertebrate species, pepsin is an acidic protease that is important in the hydrolysis of proteins (Márquez et al., 2012). It is initially secreted as pepsinogen, an inactive zymogen, and becomes active by the stimulation of HCl. As food accumulates in the stomach, HCl binds to food particles, and when this becomes neutralized, this increases the pH of the chyme that subsequently depresses pepsin activity (Suiryanrayna and Ramana, 2015). In turbot, the optimal pH for pepsin activity was reported to be 1.5–2.5 (Wang, 2004). In the present study, the pepsin activity was negatively correlated with the pH of stomach content, thus the main action by which citric acid increased the pepsin activity was by reducing the pH of the diet and subsequently that of the stomach content. Among many organic acids, because citric acid has the lowest pKa (except for formic acid) - the pH at which the acid is half dissociated (Dibner and Buttin, 2002), it tends to have the greatest effect on dietary pH. Positive effects of citric acid on pepsin activity due to the pH reduction have been reported in juvenile red drum (Castillo et al., 2014) and tilapia (*O. niloticus* × *O. aureus*) (Li et al., 2009) as well, but in the same study on tillapia, citric acid significantly decreased the intestinal proteases activity. For both turbot and tilapia (*O. niloticus*), the optimum intestinal protease activity requires an alkaline pH (Bezerra et al., 2005; Chen et al., 2006). Because there was no change to intestinal pH, this may explain no change to intestinal trypsin or amylase activity. The lack of pH changes in the intestine of turbot fed the C-1.5 and C-3.0 diets could be due to a rapid absorption in the stomach, as observed in rats (Kuether and Smith, 1941). In fact, Kunether and Smith (1941) found that 90% of citric acid in the stomach could be absorbed within 5 h. On the other hand, dietary citric acid at 3% reduced the pH in the intestine of rohu (Baruah et al., 2005), which could be due to rohu being an agastric fish. This is because when acidic chyme from stomach enters the intestine, it is neutralized by bicarbonate in the secretion from the pancreas, liver and intestine, creating an alkaline milieu for digestive enzymes like trypsin, lipase and amylase to work at a pH close to their optimums. This may be the reason for intestinal trypsin and amylase in turbot being unaffected by dietary citric acid.

Microbiota plays an important role in the development of normal gut function and the maintenance of intestinal homeostasis. To date, several studies employing NGS techniques to investigate intestinal microbiota of turbot have been published. Notably, Xing et al. (2013) profiled gastrointestinal microbiome of farmed adult turbot by metagenomic sequencing, which revealed that Proteobacteria (78.8%) and *Vibrio* (72.0%) predominated the microbial community at the phylum and genus level, respectively. In contrast, we found a consortium of more evenly distributed core taxa both at the phylum and genus level, absent of taxa with overweighing abundance. The discrepancy may be due to the difference in life stage, diet composition, rearing conditions, sample type, and sequencing method. Gut microbiota profile in fish is influenced by dietary ingredients such as lipid, protein sources, functional glycomic ingredients, nutraceuticals, antibiotics, dietary iron, chromic oxide, as well as organic acids (Liu et al., 2014; Ringø et al., 2016; Zhou et al., 2009). It is now generally accepted that organic acids have both bacteriostatic and bactericidal effects, and these effects of are mainly due to the ability of organic acids to penetrate bacterial in non-dissociated form and then dissociate within the pH neutral cytoplasm (Booth and Stratford, 2003; Cherrington et al., 1991), resulting in the consumption of its own energy to retain osmotic balance (Salmond et al., 1984), disturbing cell metabolism and enzyme activity, and eventually leading to cell death (Luckstadt, 2008). While organic acids have been reported to be inhibitory to some common pathogens in the fish gut (Gao et al., 2017; Park et al., 2011), no diet-related changes in the intestinal microbiota was observed in the present study. Although some studies has reported the anti-microbial effect of organic acids is largely achieved by creating a low pH environment in the digestive tract (Bosi et al., 1999; Canibe et al., 2005; Gao et al., 2017), citric acid would not be expected to greatly affect gut pH because it is metabolized rapidly before reaching the intestine (Boling et al., 2000; Kuether and Smith, 1941), and this was in accordance with the result that the pH of intestinal digesta was not influenced by the citric acid supplementation in the present study. Further studies are needed to investigate whether the antimicrobial activity of organic acid directly results from the pH reduction in gut lumen, as inconsistent results existed in a number of studies on the effect of dietary organic acid on gastrointestinal tract pH (Castillo et al., 2014; Li et al., 2015). Besides, the variation among individuals should not be ignored because the differences still exist in the intestinal microflora of turbot even fed the same diets under the same experimental condition. Therefore, larger sample size is highly recommended in relevant future studies in order to minimize the variation within groups.

Blood biochemistry is widely used in fish studies as a reflection of general health status of fish (Campbell, 2004). Cholesterol is an important component of cell membranes (Cheng and Hardy, 2004) and the substrate for many substances including bile acid, steroid hormones and vitamin (Hernandez et al., 2004; Holme et al., 2006). Cholesterol and triglycerides have been used to evaluate lipid metabolism and liver

status in fish (Agrahari et al., 2007; Benedito-Palos et al., 2016; Sabzi et al., 2017) and are influenced by the nutritional and physiological states (Babin and Vernier, 1989). No significant difference in cholesterol and triglycerides in the present results indicated that citric acid caused no change to lipid metabolism and liver function of turbot. Serum AST and ALT are sometimes used as an overall health status indicator for the liver in nutritional studies on fish (Molina et al., 2005; Zhang et al., 2008). These enzymes are present at low concentrations in plasma, and increases of their activities in plasma indicate the development of tissue lesions, particularly hepatic lesion (Ebrahimi et al., 2017; Malbrouck et al., 2003). There was no significant difference in serum AST and ALT activity among dietary groups, indicating citric acid has no adverse effect on normal status of turbot liver. In contrast, dietary sodium citrate supplementation at the levels of 1%, 2% and 4%, which were similar to those used in the presents study, elevated the activity of plasma ALT and induced liver damage in tilapia (*Oreochromis* sp.), and this may be due to the concentration of sodium citrate has been excessive to tilapia (Romano et al., 2016). Creatinine is a protein produced by muscle through the breakdown of creatinine phosphate for energy and released into the blood. Elevated creatinine level signifies impaired kidney function (Pakhira et al., 2015). Urea is a by-product of protein metabolism that is formed in the liver and its main function is to remove nitrogen waste from the immune system and to avoid toxicity. The levels of uric acid and creatinine in the serum are useful markers, which indicate the overall health of kidney (Campbell, 2004; Tietz, 1986). The present study shows absence of difference in urea and creatinine among all experimental groups, indicating citric acid had no adverse effects on kidney of turbot. No significant difference was observed in any of the parameters examined, indicating that turbot fed the citric acid supplemented diets were healthy as those fed the positive control diet. This was supported by observing no adverse histopathology of the distal intestine, which detected no noticeable pathological changes in any of the fish examined. Interestingly, citric acid at the lower inclusion level (1.5%) increased the absorptive surface area, but this did not lead to improved digestibility efficiency of the tested nutrients. More comprehensive evaluation about the effects of citric acid on intestine function has to be done in future studies. Together, these results showed that dietary inclusion of citric acid up to 3% in turbot diet induced no detrimental effects on fish health.

5. Conclusions

Results indicate that when available P in low fishmeal-based diets is unclear, the necessity of inorganic dietary P supplementations is questionable to turbot. This is because such a practice would unlikely improve turbot growth and only lead to excessive P excretion. While citric acid supplementations did improve P digestibility, likely due to increased soluble P, it is unclear whether this was released from fishmeal or plant proteins in the diets. There were also no indications of dietary citric acid negatively affecting the overall health of turbot and actually the citric acid supplementation improved the pepsin activity. Thus, citric acid appears to be a promising feed additive to develop more eco-friendly turbot feeds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.04.004.

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