TOPIC AREA

SUSTAINABLE FEED TECHNOLOGY AND NUTRIENT INPUT SYSTEMS

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PELLET FEED IMPROVEMENTS THROUGH VITAMIN C SUPPLEMENTATION FOR SNAKEHEAD CULTURE

Sustainable Feed Technology and Nutrient Input Systems/Experiment/16SFT01UC

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ABSTRACT

Vitamin C, or ascorbic acid (AA), is important in growth and physiological functions of fish. Six soybean meal-based (SBM) diets containing 0, 125, 250, 500, 1000 and 2000 mg.kg^{-1} of AA were fed to snakehead (6.6-6.7 g) for eight weeks in the laboratory. Survival, final weight, and specific growth rate (SGR) of snakehead in the 250 and 500 mg.kg^{-1} treatments were significantly greater than those in the control (0 mg.kg^{-1}). Feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER) also differed significantly. The requirement of AA for snakehead was calculated to be 277 mg.kg^{-1}. No abnormal backbones were observed during the experimental period. Erythrocyte count was significantly higher at 1000 and 2000 mg.kg^{-1} than at 0 and 125 mg.kg^{-1}. Fish in 250 mg.kg^{-1} had significantly higher leukocyte count than 0, 1000, and 2000 mg.kg^{-1}. A bacterial challenge test with *Aeromonas hydrophila* revealed that 500 and 1000 mg.kg^{-1} had the lowest cumulative mortality. In an on-farm trial, SBM-based and commercial diets augmented with 0, 500, 750, or 1000 mg.kg^{-1} AA were fed to *C. striata* for 23 weeks. Optimal results were obtained with SBM-based diets supplemented with 500 mg.kg^{-1} AA, in terms of survival, yield, FCR, production cost and profit. The result was also confirmed via on-farm trial in Cambodia.

INTRODUCTION

Aquaculture of freshwater carnivorous and omnivorous fish species in Cambodia and Vietnam has been highly dependent on inland fisheries of small-size fish (SSF) for sourcing key dietary nutrient inputs (Hien et al. 2015a). Adequate pelleted diets, with minimal content of fish meal (FM), have been developed to overcome the use of SSF harvested from the Mekong for aquaculture (Hien et al. 2015a; 2016a). In 2015, more than 90% of snakehead farmers (who produce 99% of the total production of snakehead) in 13 provinces in the southern region of Vietnam, including the Mekong Delta, were using these diets instead of SSF, thereby reducing fishing pressure on the small-scale fish in the Lower Mekong Delta (Hien et al. 2016a). However, in a commercial-scale farm trial in An Giang province, about 20% of fish fed this diet (vs. an SSF diet) developed vertebral column abnormalities (Hien et al. 2016b). Anecdotal reports from farmers in the region also indicate that this "hunchback" condition is a problem for them. Pictures and X-rays of the fish suggest that this condition (technically, lordosis and scoliosis) is a classic case of vitamin C (ascorbic acid or AA) deficiency in the diet.

Several benefits have been attributed to AA supplementation in fish such as growth, survival, reduction of skeletal deformities, immunoactivity and stress response (Ai et al. 2006). Dietary AA can enhance resistance to bacterial infection in fish, but AA requirements may depend on species and their physiological conditions (Darias et al. 2011). Fish health is an important issue for snakehead culture; bacterial disease is a serious problem (Duc at al. 2012; 2013). Farmers also indicate that the hunchback problem is greatest when fish are fed to satiation; reduced rations appear to lead to less incidence of the condition. We therefore hypothesize that the condition is due to ascorbic acid (AA) deficiency in the diets of fully fed, fast-growing fish, but that AA levels are sufficient for the slower growth of poorly fed fish. Although we have not found any literature on the specific dietary requirements for AA of our research species, *Channa striata* (or any other member of the family Channidae for that matter), the requirement for most fish species is considered to be 50 $mg.kg^{-1}$ (NRC, 2011). One study of the related *Channa punctata* (Bloch, 1793) indicated that 2000 $mg.kg^{-1}$ of AA provided greater resistance to toxicity of the pesticide endosulfan than did 1000 $mg.kg^{-1}$ (Sarma et al. 2009). Hien et al. (2015a; 2016b) used 80-150 $mg.kg^{-1}$ in laboratory tanks and farm trials, considered to be more than sufficient given the NRC standard cited above, but saw a few abnormal fish in the laboratory experiment and pond trials. In the commercial farm trials, abnormal fish were only seen at the farm in An Giang province, where the fish were grown for 6 months to a size of about 400 g, not at the farm in Dong Thap province, where fish were only grown for 4 months to about 200 g and where farmers added additional vitamin premix to the diet (Hien et al. 2016b). In any case, it appears that additional research on AA requirements for snakehead is necessary to solve this issue of abnormal fish. It may be that the stress on a fast-growing fish like snakehead in a densely stocked pond demands higher levels of AA than were previously anticipated. Cambodia banned snakehead culture in 2005, because the use of SSF by snakehead farmers caused a user conflict with the human population who used SSF as a source of protein. A major rationale for previous research (Hien et al. 2015a,b; 2016a,b; 2017) has been to demonstrate to the Cambodian government that snakehead can be raised profitably on pellet feeds, that snakehead culture methods based on SSF can be discarded, and that the ban could be lifted (which it was in June 2016). The objective of this study was to improve cost-effective feeds for snakehead aquaculture in Vietnam and Cambodia, specifically by determining optimal vitamin C requirement in practical diets in laboratory and pond trials.

OBJECTIVE

To improve cost-effective feeds for snakehead aquaculture in Vietnam and Cambodia, specifically by: (i) determining optimal vitamin C requirement in practical diets in laboratory and pond trials; and (ii) evaluating cost-effectiveness of pellet diets with optimal vitamin C for hapa grow out.

MATERIALS AND METHODS

Laboratory feeding trial

Based on the several benefits attributed to AA in other studies of fish nutrition, and the basal diet from previous work with soybean meal (SBM) replacement of FM in diets for snakehead fish, the null hypothesis that AA level does not lead to differences survival, growth, feed conversion ratio (FCR), protein efficiency ratio (PER), and certain blood parameters of snakehead was tested. A feeding trial was conducted, followed by a bacterial challenge, as outlined below. The lowest supplemented AA value was in the mid-range of what Hien et al. (2015a, 2016a) had previously used, the highest supplemented levels were those used by Sarma et al. (2009), and two intermediate values were also included. The feed was analyzed to verify AA according to methods of Nelis at al. (1997). Treatments were: 1) SBM diet + 0 mg $AA.kg^{-1}$ feed; 2) SBM diet + 125 mg $AA.kg^{-1}$ feed; 3) SBM diet + 250 mg $AA.kg^{-1}$ feed; 4) SBM diet + 500 mg $AA.kg^{-1}$ feed; SBM diet + 1000 mg $AA.kg^{-1}$ feed; SBM diet + 2000 mg $AA.kg^{-1}$ feed (Table 1).

The laboratory experiment was conducted in a manner similar to those of Hien et al. (2015a). Experimental units were 500-L tanks, with five replicate tanks per treatment. The stocking density

was 80 fish tank^{-1}. At the beginning of the experiment, fish initial weight (6.56-6.77 g) was determined. Fish were fed to satiation twice a day (0800 and 1600 hrs) and the amount of feed consumed by the fish in each tank was recorded daily by removing and weighing (dry weight) excess feed to ascertain intake. Amounts of feed provided per replicate were recorded so that feed conversion ratio (FCR) and protein efficiency ratio (PER) could be calculated at the end of the experiment. Temperature ranged from 27.1 - 30.1 ºC, dissolved oxygen from 5.3 - 5.9 mg·L^{-1}, pH from 6.9 – 7.3, TAN from 2.1 – 3.2 and NO_2^- < 0.1 mg·L^{-1}, so the water quality parameters in all treatments were in a suitable range for the normal growth and development of this species. Any dead fish were recorded and removed daily.

The experiment lasted eight weeks, at the end of which fish were measured, weighed and then used in a bacterial challenge experiment. Blood samples of a subset of experimental fish were taken at the end of the experiment and examined for leukocyte count, erythrocyte count, and lysozyme activity, using methods of Hien et al. (2016b). Any skeletal disorders were documented by photographs and X-rays. Data from each tank were pooled (i.e., no pseudoreplication) and only one number representing average growth per fish (specific growth rate, SGR) was used per replicate. Data analysis was by one-way ANOVA, following arc-sine square-root transformation of the proportionate data to insure normality. Duncan's multiple range test was used to determine specific differences among means if the ANOVA indicated that significant differences were present. Estimation of the vitamin C requirement for fish growth in this trial was by the broken-line regression method (Robbins et al. 1979).

Bacterial challenge experiment

The null hypothesis that AA levels in the feeding trial above do not lead to significantly different survival in a post-trial bacterial challenge was tested. The bacterial challenge experiment was conducted immediately after the end of the feeding trial. The six treatments in the feeding trial were subdivided, such that fish from five tanks per treatment were intraperitoneally (IP) injected with 0.1 mL of bacterial strain *Aeromonas hydrophila* CD1012 based on the lethal dose (LD_{50}) of 1.16×10^5 CFU.mL^{-1} (Duc et al. 2013). Fish from five other randomly chosen tanks were also IP injected with 0.1 mL of physiological saline (0.85%) as control, following Ward et al. (2016). The bacterial challenge experiment lasted 2 weeks, as in the previous work of Hien et al. (2016a). During the 14-d post-inoculation period, fish continued to be fed their respective diets, and activity and cumulative mortality were noted daily. For moribund fish, clinical signs were observed by gross inspection, and lesions were sampled directly for bacteria. Re-isolation and re-identification of bacteria were carried out according to methods of Barrow and Feltham (1993) and PCR was used to speciate the re-identified bacterial strains. Cumulative mortality was recorded daily. Results of the bacterial challenge experiment were analyzed by one-way ANOVA followed by Duncan's multiple range test at significance level of $p\leq0.05$.

Farm trial in Vietnam

The null hypothesis that source of feed (commercial vs. experimental SBM diet) and levels of added AA do not lead to significant differences in snakehead production to market size in experimental ponds was tested. Based on the results of the laboratory experiment and bacterial challenge, the effects of AA on snakehead in hapas in ponds to simulate farm conditions, was tested using the following treatments: 1) Commercial feed (formulation proprietary, not available); 2) Commercial feed + hand mixed AA at 500 mg.kg^{-1}; 3) Commercial feed + hand-mixed AA at 750 mg AA.kg^{-1}; 4) Commercial feed + hand mixed AA at 1000 mg AA.kg^{-1}; 5) SBM diet (same as Table 1) without AA; 6) SBM diet + 500 mg AA.kg^{-1}; 7) SBM diet + 750 mg AA.kg^{-1}; 8) SBM diet + 1000 mg AA.kg^{-1}. The experiment was conducted in two large experimental ponds at a commercial facility (only SBM diet without AA placed in one pond and the rest in the other pond) with four replicate hapas each. Stocking density was 150 fish.m^2 and culture period was 23 weeks until market size was attained.

Data on water quality parameters were collected daily (as described above) and data on fish survival and growth monthly. Production cost (USD.kg fish^{-1}) included fingerling cost, vitamin C cost (except control treatment), feed cost, hapa cost and labor cost. Feed cost (USD.kg fish^{-1}) was equal to total feed cost (USD) divided by yield (kg). Profit (USD.kg fish^{-1}) was equal to selling price (USD.kg fish^{-1}) minus production cost (USD.kg fish^{-1}). Any skeletal disorders were documented at the end of the experiment by photographs and X-rays. Data on fish survival and growth, FCR, PER and lysozyme were statistically analyzed by two-way analysis of variance.

Farm trial in Cambodia

The grow-out experiment was conducted at Freshwater Aquaculture Research and Development (FARDeC). There were two treatments: 1) Commercial feed; and SBM diet + 500 mg AA.kg^{-1} (same as above), each with 3 replicated hapas allocated in 300 m^2 earthen pond. Local Cambodian snakehead fingerlings (ave.11.2 g size) from the hatchery's F_2 generation, at 60 days old after weaning, were stocked at the density of 450 ind. hapa^{-1} (3x1x1.5 m) to corresponding treatments and replicated hapas for the 5-month grow-out study. Fish in each treatment were fed to satiation twice daily at 09:00h and 16:00h. The amount of feed consumed by fish and fish mortality were recorded daily. Water qualities (temperature, pH, dissolved oxygen, NH_3 and NO_2) were monitored weekly. Fish growth was measured monthly with 30 sampled fish. The survival rate and cannibalism rate were determined at the end of experiment.

RESULTS

Laboratory feeding trial

Survival was significantly higher at 250 and 500 mg.kg^{-1} than in the control (0 mg.kg^{-1}), but survival in all treatments with added AA did not differ significantly (Table 2). Final weight and SGR of fish in the 500 mg.kg^{-1} treatments were significantly greater than those in the 125 mg.kg^{-1} treatment, which in turn were significantly greater than those in the control, but there were no differences among the 250, 500, 1000 and 2000 mg.kg^{-1} treatments (Table 2). FI and FCR did not appear to vary in a dose-dependent manner, although some significant differences were seen (Table 2). PER values in all the treatments to which AA had been added were significantly greater than that of the control, but did not differ among themselves (Table 2). Based only on the weight gain data, the vitamin C requirement for the *C. striata* in this experiment was estimated to be approximately 277 mg.kg^{-1} (Figure 1).

Fish in the 1000 and 2000 mg.kg^{-1} treatments had equivalent erythrocyte counts, significantly higher than those in the control and 125 mg.kg^{-1} treatments (Table 3). The highest leukocyte counts were seen in the 125 and 250 mg.kg^{-1} treatments, significantly higher than those in the 1000 and 2000 mg.kg^{-1} treatments, which in turn were significantly higher than those in the control (Table 3). At the end of the feeding trial, lysozyme levels in the control and 2000 mg.kg^{-1} treatments were equivalent, significantly less than those at 250, 500, and 1000 mg.kg^{-1} and the lysozyme levels in the 1000 mg.kg^{-1} treatment were significantly higher than those in any other treatment (Table 3).

No abnormal backbones of snakehead fish were observed during 8 weeks culture in the laboratory trial.

Bacterial challenge experiment

After 14 d of the bacterial challenge, cumulative mortality was significantly greatest, in the control (56%) and 2000 mg.kg^{-1} (54%) treatments, less in the 125 mg.kg^{-1} (30%) and 250 mg.kg^{-1} (34%) and least in the 500 mg.kg^{-1} (20%) and 1000 mg.kg^{-1} (14%) treatments, although the last two listed treatments were not significantly different from each other (Figure 2). Lysozyme levels measured 4 d into the bacterial challenge increased in all treatments compared to those measured prior to the start of the bacterial challenge (Table 3). Lysozyme levels were significantly highest in the 1000 mg.kg^{-1}

treatment, followed by those at 500 mg.kg^{-1}, which in turn were significantly higher than those at 250 and 150 mg.kg^{-1}, which in turn were significantly higher than those at 2000 mg.kg^{-1} and the control (Table 3).

Farm feeding trial in Vietnam

During the 23-week experimental period, all water quality parameters (i.e. temperature, pH, DO, TAN, NO2, and NH3) were similar in the two experimental ponds (Table 5). Results of the two-way ANOVA indicated that diet (commercial vs. SBM) and AA level, as well as their interaction, were significant for all measured variables (final weight, yield, SGR, FI, survival, FCR, lysozyme, hunchback %, production cost, feed cost and profit), with two exceptions. AA level was not significant for hunchback % and diet x AA level interaction was not significant for FCR. More specifically, final weight, yield, survival, FI and lysozyme levels of fish fed SBM with and without AA-supplemented diets were higher than those of the fish fed commercial feed with and without AA-supplemented diets, respectively, and FCR levels were lower (Table 6). The highest values of final weight, yield, and survival were observed in the fish fed with SBM diet + AA 500 mg.kg^{-1}, which were significantly different from the control (fish fed SBM diets + 0 mg.kg^{-1} AA); whereas among the treatment groups of commercial diets + AA, these indices were not significantly different (Table 6). FI was highest in the 1000 mg.kg^{-1} AA-supplemented SBM diet group (FI=6.4% fish^{-1}.day^{-1}, significantly different from the SBM diet + AA 0 mg.kg^{-1}); and the lowest value of FCR, in the 750 mg.kg^{-1} AA-supplemented SBM diet group (FCR=1.27), was significantly different from all commercial diets supplemented with AA ($P<0.05$). Lysozyme levels of the AA-supplemented treatments were higher than those of the control group (no AA supplementation) in both SBM and commercial diets, and the highest lysozyme levels (287.3 and 286.8 µg.mL^{-1}) were found in fish fed diets containing 1000 mg.kg^{-1} AA-supplemented SBM or commercial feed, respectively.

Fish fed an SBM diet + 500 mg.kg^{-1}AA exhibited the lowest percent occurrence of abnormal backbones, production cost, feed cost, and the highest profit (Table 7). The highest percent occurrence of abnormal backbones was found in fish fed commercial diet + 500 mg.kg^{-1} AA. Fish fed the commercial diet + 750 and 1000 mg.kg^{-1} AA exhibited the highest production costs and the lowest profits. The highest percent occurrence of abnormal backbones, in the experimental group of fish fed commercial diet + 750 mg.kg^{-1} AA, was significantly higher than in the control (commercial + 0 mg.kg^{-1} AA).

Farm feeding trial in Cambodia

During the 5-month experiment, growth performance (final weight), daily weight gain and yield of fish in the SBM diet + 500 mg AA.kg^{-1} feed treatment was significantly greater than that in the commercial feed treatment (Figure 3 & 4). Spinal abnormality rate of fish in the SBM diet +500 mg AA.kg^{-1} feed treatment was significantly lower than that in the commercial feed treatment (Figure 5). Survival, cannibalism, and FCR were not significantly different between the two treatments (Table 7). Photographs of the pond trials in Vietnam and Cambodia are provided in Figures 6-10.

DISCUSSION

Our results provide the first estimate of AA requirements in practical diets for *C. striata* grown in ponds in Southeast Asia. Vitamin C is important in multiple processes in the growth, collagen formation, iron metabolism and hematology, reproduction, response to stressors, wound healing and immune response (NRC 2011). Thus, it is not surprising that the diets supplemented with AA promoted better growth of snakehead compared to the control. This result is consistent with the previous studies in hybrid tilapia, *Oreochromis niloticus* (Linnaeus, 1758) × *Oreochromis aureus* (Steindachner, 1864) (Shiau and Hsu, 1995), tilapia, *Oreochromis spilurus* (Günther, 1894) (Al-Amoudi et al. 1992), large yellow croaker, *Pseudosciaena (= Larimichthys) crocea* (Richardson, 1846) (Ai et al. 2006), *O. niloticus* (Ibrahem et al. 2010) and rainbow trout, *Oncorhynchus mykiss*

(Walbaum, 1792) (Adel and Khara, 2016). The finding herein is in agreement with a previous study (Yousefi et al. 2013). Pal and Chakrabarty (2012) and Tewary and Patra (2008), pointed out that supplementation of Vitamin C at 1200 and 1000 $mg.kg^{-1}$ feed yielded better growth of African catfish, *Clarias batrachus* (Linnaeus, 1758) and roho labeo, *Labeo rohita* (Hamilton, 1822), respectively.

Results of experiments on vitamin C may differ due to differences in fish species, size, purity, and sources of ascorbic acid and experimental conditions in different studies. The requirement of vitamin C for snakehead growth in this study was 277 $mg.kg^{-1}$ feed, indicating that the level is higher than that used in previous diet studies with this species (Hien et al. 2015b, 2016b).Vitamin C requirement was 63.37 $mg.kg^{-1}$ feed in *O. niloticus* x *O. aureus* (Shiau and Hsu, 1999), 118 $mg.kg^{-1}$ feed in parrot fish, *Oplegnathus fasciatus* (Temminck & Schlegel, 1844) (Wang et al. 2003) and 43.5 $mg.kg^{-1}$ feed in grouper, *Epinephelus malabaricus* (Bloch & Schneider, 1801) (Lin and Shiau, 2005).

Snakehead in the laboratory experiment fed the SBM diet + 500 mg kg^{-1} AA had the lowest FCR compared to the control, similar to results for African catfish, *Clarias gariepinus* (Burchell, 1822) (Adewolu and Aro, 2009), and significantly greater PER. Vitamin C is known to be positively related to the protein metabolism (Yousefi et al. 2013). Vitamin C is a powerful antioxidant, protecting against oxidative damage to various tissues of fish including red blood cells (Sahoo and Mukherjee, 2003). Red blood cells can act as oxidative status indicators (Pimpimol et al. 2012). In our study, fish fed the diets containing vitamin C had higher total erythrocyte and leukocyte counts than those fed the control diet. Total erythrocyte counts differed significantly between the control and treatments supplemented with AA at 1000 and 2000 $mg.kg^{-1}$. Similar results were observed by Andrade et al. (2007) for pirarucu, *Arapaima gigas* (Schinz, 1822) receiving AA at 800 and 1200 $mg.kg^{-1}$ and by Pimpimol et al. (2012) for Mekong giant catfish, *Pangasianodon gigas* Chevey, 1931 receiving AA at 750 $mg.kg^{-1}$. Moreover, total leukocyte count was significantly increased in snakehead fed AA-supplement diets, which was similar to results in rainbow trout (*O. mykiss*) (Rahimi et al. 2015), tra catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878) (Hang et al. 2015) and Japanese eel, *Anguilla japonica* Temminck & Schlegel, 1846 (Shahkar et al. 2015). Leukocytes reached their maximum level at AA-supplementation of 250 $mg.kg^{-1}$ and then declined as AA levels increased beyond that. Thus, AA supplementation may affect the blood composition of snakehead; however, further studies should be considered to evaluate the relationships between vitamin C and other hematological compositions.

Lysozyme activity is an important index of innate immunity of fish and is ubiquitous in its distribution among living organisms (Saurabh and Sahoo, 2008). Lysozyme is liberated by leukocytes and important in anti-microorganism activity (Pimpimol et al. 2012). In our study, lysozyme activity increased in the serum of snakehead fish fed AA-supplemented diets compared to the control, both before and after challenge with *A. hydrophila*. Lysozyme activity increased with level of AA from 250 to 1000 $mg.kg^{-1}$. This is consistent with the previous reports on *P. gigas* fed the dietary AA supplements at ≥250 $mg.kg^{-1}$ (Pimpimol et al. 2012) and on improved lysozyme activity with AA supplementation in cobia, *Rachycentron canadum* (Linnaeus, 1766) (Zhou et al. 2012). Our study also revealed that lysozyme activity in snakehead fed diet supplemented with AA at 2000 $mg.kg^{-1}$ was not different from the control. This finding is in agreement with previous studies in channel catfish, *Ictalurus punctatus* (Rafinesque, 1818) (Li et al. 1993), Atlantic salmon, *Salmo salar* Linnaeus, 1758 (Thompson et al. 1993), and *R. canadum* (Zhou et al. 2012) showing lower lysozyme activity at higher AA levels.

The bacterial challenge test indicated that cumulative mortality of snakehead decreased with increased AA levels, at least up to 1000 $mg.kg^{-1}$. Moreover, cumulative mortality was negatively correlated with the total erythrocyte and leukocyte counts and lysozyme activity. Similar results were

found in *R. canadum* (Zhou et al. 2012). This finding demonstrates the relationships among dietary AA supplementation, immunological parameters, and disease resistance. However, our highest AA-supplemented diet (2000 $mg.kg^{-1}$) did not appear to be effective for disease resistance, which has been demonstrated previously by Li et al. (1993).

Results of the farm feeding trial were consistent with those of the laboratory feeding trial, demonstrating that dietary vitamin C can significantly improve the normal growth and other physiological functions in snakehead. Fish fed diets with AA supplemented at 500 mg.kg-1 in the farm trial (i.e., the next highest treatment level above the AA requirement calculated from growth results in the laboratory trial) yielded the lowest feed costs and highest profits. Additionally, the farm feeding trial showed that the SBM diet was a viable feed option for use in aquaculture of snakehead compared with the commercial feed. It appears that the diet X AA level interaction in the farm trial was largely due to AA level having much less effect on the commercial diet than on the SBM diet. We speculate that this was caused by hand-mixing of the AA into the commercial diet, rather than fully incorporating it into the diet during production.

CONCLUSIONS

In conclusion, this study demonstrated that dietary AA supplementation is able to improve growth performance, immune responses, and survival of snakehead fish against *A. hydrophila* infection. The requirement of AA by snakehead was determined as 277 $mg.kg^{-1}$ feed in the laboratory study. The diet supplemented with vitamin C at 250 to 1000 $mg.kg^{-1}$ feed is an appropriate concentration range for improving growth performance and immunity of snakehead fish on fish farms. SBM diet was a better diet used in culturing snakehead compared with the commercial diet. Further experiments should be considered to investigate correlations between vitamin C and other immune parameters, as well as disease resistance of snakehead against other pathogens.

QUANTIFIABLE ANTICIPATED BENEFITS

In Vietnam:

1. Development of weaning strategies for snakehead;
2. Development of pellet diets for snakehead in Vietnam;
3. Investigation of vitamin C requirements of snakehead in Vietnam;
4. Transfer of snakehead culture technology to Cambodia;
5. Training snakehead culture to women in An Giang, Dong Thap and Tra Vinh provinces;
6. Two graduate and two under-graduate student were done their thesis research in this investigation.

In Cambodia:

This research provides information on growing out of snakehead fish using vitamin C supplemented feed to for improving growth performance and production on snakehead farm in Cambodia. The following are quantifiable anticipated benefits:

- Scientists, researchers, government fisheries officers/managers and policy makers, extension workers, NGO staffs, private sector and university lecturers and students working on the issues of snakehead aquaculture in Cambodia as well as in other Mekong riparian countries were better informed about research methods and findings,and have better recommended policies and strategies for sustainable snakehead aquaculture.
- One undergraduate student was supported and trained by this investigation through B.Sc. thesis research.

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TABLES AND FIGURES

Table 1. Ingredients used in, and proximate composition of, diets used in the snakehead laboratory feeding trial with varying levels of L-Ascorbate-2-Monophosphate.

Ingredients	**Composition (%)**
Kien Giang fishmeal	35.8
Defatted soybean meal	33.4
Cassava	8.26
Rice bran	15.0
Premix mineral and vitamins	2.0
Oil	3.08
Carboxymethyl cellulose	0.40
Lysine	0.40
Methionine	0.28
Fish solution	1.50
Phytase	0.02
Total	**100**
Crude protein	45.0
Crude lipid	8.91
Ash	12.5
Fiber	2.15
NFE	31.5
Energy ($kJ.g^{-1}$)	19.69

Premix mineral and vitamin ($unit.kg^{-1}$): Vitamin A 2,000,000 IU; Vitamin D 400,000 IU; Vitamin E 6g; Vitamin B_1 800mg; Vitamin B_2 800mg; Vitamin B_{12} 2mg; Calcium D Pantothenate 2g; Folic acid 160mg; Choline Chloride 100g; Iron (Fe^{2+}) 1g; Zinc (Zn^{2+}) 3g; Manganese (Mn^{2+}) 2g; Copper (Cu^{2+}) 100mg; Iodine (I^-) 20mg; Cobalt (Co^{2+}) 10mg. Fishmeal was from Kien Giang. Cassava and rice bran were local products. CMC; methionine and lysine were products of Evonik.

Table 2. Initial and final weights.fish^{-1}, specific growth rate, SGR), feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER) of *Channa striata* fed diets with different levels of vitamin C (as L-ascorbate-2-monophosphate) for 8 weeks. Values (mean±SD in parentheses) in a column sharing a superscript letter are not significantly different (*P>0.05*).

Target vitamin C supplementation (mg kg-1)	Initial weight (g)	Final weight (g)	SGR (%.d^{-1})	Survival rate (%)	FI (%.fish^{-1}.day^{-1})	FCR	PER (%)
0	6.57^{a}	39.06^{a}	2.97^{a}	76.5^{a}	3.99^{ab}	1.18^{c}	1.52^{a}
	(0.09)	(2.63)	(0.13)	(6.3)	(0.27)	(0.04)	(0.14)
125	6.56^{a}	45.45^{b}	3.22^{b}	84.8^{ab}	4.27^{abc}	1.14^{bc}	1.79^{b}
	(0.18)	(3.66)	(0.12)	(3.5)	(0.07)	(0.07)	(0.12)
250	6.61^{a}	48.07^{bc}	3.31^{bc}	88.8^{b}	4.24^{abc}	1.09^{abc}	1.8^{b}
	(0.18)	(1.93)	(0.09)	(7.7)	(0.21)	(0.04)	(0.15)
500	6.60^{a}	51.8^{c}	3.43^{c}	86.3^{b}	4.29^{bc}	1.06^{ab}	1.92^{b}
	(0.10)	(4.17)	(0.14)	(6.7)	(0.16)	(0.09)	(0.11)
1,000	6.68^{a}	49.1^{bc}	3.32^{bc}	83.5^{ab}	3.97^{a}	1.02^{a}	1.9^{b}
	(0.26)	(4.34)	(0.14)	(6.4)	(0.24)	(0.12)	(0.16)

Table 3. Effect of Vitamin C (as L-ascorbate-2-monophosphate) supplemented feed on hematology and immune response of *Channa striata.* Pre-challenge lysozyme levels are after 8 weeks of the laboratory feeding trial, whereas post-challenge lysozyme levels are after 8 weeks of the laboratory feeding trial plus four days of the bacterial challenge. Values (mean±SD in parentheses) in a column followed by the same superscript are not significantlhy different.

Target Vitamin C supplementation (mg kg-1)	Total erythrocytes count (x10^{5}cells.mm^{3})	Total leukocytes count (x10^{3}cells.mm^{3})	Pre-challenge[1] lysozyme (µg.mL^{-1})	Post-challenge[2] Lysozyme (µg.mL^{-1})
0	22.8^{a}	108.1^{a}	205.0^{a}	241.1^{a}
	(8.9)	(51.3)	(42.5)	(29.3)
125	31.5^{a}	355.8^{c}	214.5^{ab}	328.2^{b}
	(6.6)	(80.5)	(30.7)	(25.7)
250	32.4^{ab}	385.1^{c}	274.5^{bc}	351.5^{b}
	(5.2)	(53.7)	(31.8)	(17.6)
500	35.1^{ab}	313.2^{bc}	313.0^{c}	413.2^{c}
	(6.7)	(66.2)	(46.6)	(62.5)
1,000	44.7^{b}	263.1^{b}	372.5^{d}	506.5^{d}
	(6.8)	(58.8)	(24.3)	(46.7)
2,000	45.1^{b}	230.8^{b}	212.0^{a}	264.0^{a}
	(16.5)	(83.1)	(41.1)	(15.6)

Table 4. Water quality parameters in two experimental ponds (control and treatment, see text) used in the Vietnam farm trial with *C. striata* during the 23 weeks of culture. Values are mean±SD (in parentheses).

Experimental pond	Temperature (ºC)		pH		DO ($mg.L^{-1}$)		TAN ($mg.L^{-1}$)	NO_2 ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon			
Control	29.3	31.5	8.2	8.3	1.6	3.8	3.48	0.004	0.030
	(0.90)	(0.63)	(0.1)	(0.1)	(0.4)	(0.2)	(0.35)	(0.002)	(0.010)
Treatment	29.2	31.3	8.1	8.2	1.8	3.7	0.55	0.061	0.005
	(0.94)	(0.86)	(0.1)	(0.2)	(0.3)	(0.4)	(0.14)	(0.016)	(0.001)

Table 5. Initial and final weights.fish-1, yield, survival, feed intake (FI), feed conversion ratio (FCR), and lysozyme of *Channa striata* fed soybean meal (SBM) diet and commercial feed (CF) with different levels of Vitamin C (as L-ascorbate-2-monophosphate for 23 weeks in the Vietnam farm trial. Values (mean±SD in parentheses) in a column sharing a superscript letter are not significantly different (*P>0.05*). Results of the two-way ANOVA are given at the bottom.

Diet and target vitamin C supplementation (mg kg-1)	Initial weight (g)	Final weight (g)	Yield (kg/hapa-4m^2)	Survival (%)	FI (%.fish^{-1}.day^{-1})	FCR	Lysozyme (µg.mL^{-1})
SBM-0	9.00	461.7^a	190.6^a	69.1^a	5.40^a	1.40^{bc}	232.9^a
	-	(40.1)	(8.9)	(5.8)	(0.40)	(0.11)	(4.8)
SBM-500	9.00	573.5^c	293.3^c	85.3^c	5.69^{ab}	1.16^a	271.9^b
	-	(34.3)	(10.8)	(3.2)	(0.25)	(0.06)	(17.6)
SBM-750	9.00	556.2^c	268.3^{bc}	80.4^{bc}	6.00^{abc}	1.27^{ab}	283.7^b
	-	(19.2)	(8.6)	(2.2)	(0.05)	(0.01)	(4.9)
SBM-1000	9.00	565.4^c	264.6^b	78.3^{bc}	6.40^c	1.35^{bc}	287.3^b
	-	(44.2)	(7.5)	(4.7)	(0.55)	(0.12)	(9.5)
CF-0	9.00	398.9^{ab}	172.1^a	71.9^{ab}	6.07^{abc}	1.50^c	228.6^a
	-	(41.2)	(18.2)	(3.0)	(0.11)	(0.07)	(10.6)
CF-500	9.00	399.8^{ab}	185.9^a	77.7^{abc}	6.03^{abc}	1.47^c	224.1^a
	-	(28.1)	(9.1)	(4.2)	(0.19)	(0.08)	(16.8)
CF-750	9.00	398.0^{ab}	177.7^a	74.6^{ab}	6.08^{bc}	1.49^c	227.9^a
	-	(38.9)	(13.3)	(3.8)	(0.10)	(0.08)	(13.7)
CF-1000	9.00	376.0^a	173.3^a	76.8^{abc}	6.08^{bc}	1.48^c	286.8^b
	-	(22.6)	(12.0)	(1.8)	(0.05)	(0.02)	(22.9)
P values							
Feed		0.000	0.000	0.033	0.050	0.000	0.000
Vitamin C		0.018	0.000	0.000	0.013	0.017	0.000
*Feed*Vitamin C*		0.006	0.000	0.049	0.020	0.051	0.001

Table 6. Hunchback (abnormal backbones), production costs (PC), feed cost, and profit (P) for fish raised on soybean meal (SBM) diet and commercial feed (CF) with different levels of Vitamin C (as L-ascorbate-2-monophosphate for 23 weeks in the Vietnam farm trial. Values (mean±SD in parentheses) in a column sharing a superscript letter are not significantly different (*P>0.05*)

Diet and target vitamin C supplementation (mg kg-1)	Hunchback fish (as % of total)	Production cost (USD.kg fish^{-1})	Feed cost (USD.kg fish^{-1})	Profit (USD.kg fish^{-1})
SBM-0	6.28^{ab}	1.39^{bc}	1.18^{b}	0.12^{ab}
	(0.68)	(0.10)	(0.09)	(0.10)
SBM-500	5.37^{a}	1.12^{a}	0.98^{a}	0.38^{c}
	(0.85)	(0.06)	(0.05)	(0.06)
SBM-750	5.89^{a}	1.23^{ab}	1.07^{ab}	0.27^{bc}
	(0.61)	(0.02)	(0.01)	(0.02)
SBM-1000	6.75^{abc}	1.31^{bc}	1.14^{b}	0.20^{ab}
	(1.08)	(0.10)	(0.10)	(0.10)
CF-0	8.32^{bcd}	1.43^{c}	1.19^{b}	0.08^{a}
	(0.98)	(0.08)	(0.05)	(0.05)
CF-500	9.63^{de}	1.40^{bc}	1.17^{b}	0.11^{a}
	(1.49)	(0.07)	(0.06)	(0.07)
CF-750	11.1^{e}	1.43^{c}	1.19^{b}	0.07^{a}
	(0.81)	(0.06)	(0.06)	(0.05)
CF-1000	8.91^{cde}	1.44^{c}	1.18^{b}	0.07^{a}
	(1.23)	(0.04)	(0.02)	(0.04)
P values				
Feed	0.000	0.000	0.000	0.000
Vitamin C	0.118	0.003	0.017	0.003
*Feed*Vitamin C*	0.009	0.025	0.049	0.015

Table 7. Survival rate (SR), hunchback (abnormal backbones), weight gain (Wg), feed conversion rate (FCR) and yield of *Channa striata* of Cambodian origin during grow-out. Values (mean±SD in parentheses) in a column followed by the same letter are not significantly different (P>0.05)

Treatments	SR (%)	Hunchback (%)	Wg ($g.fish^{-1}$)	FCR	Yield (kg/hapa)
SBM+500mg	45.7^a	21.7^a	322.8^a	1.9^a	65.0^a
$AA.kg^{-1}$ Feed	(1.4)	(1.6)	(11.2)	(0.1)	(3.2)
Commercial	45.7^a	35.0^a	203.8^b	2.8^b	40.3^b
Feed	(13.7)	(14.6)	(10.7)	(0.5)	(10.7)

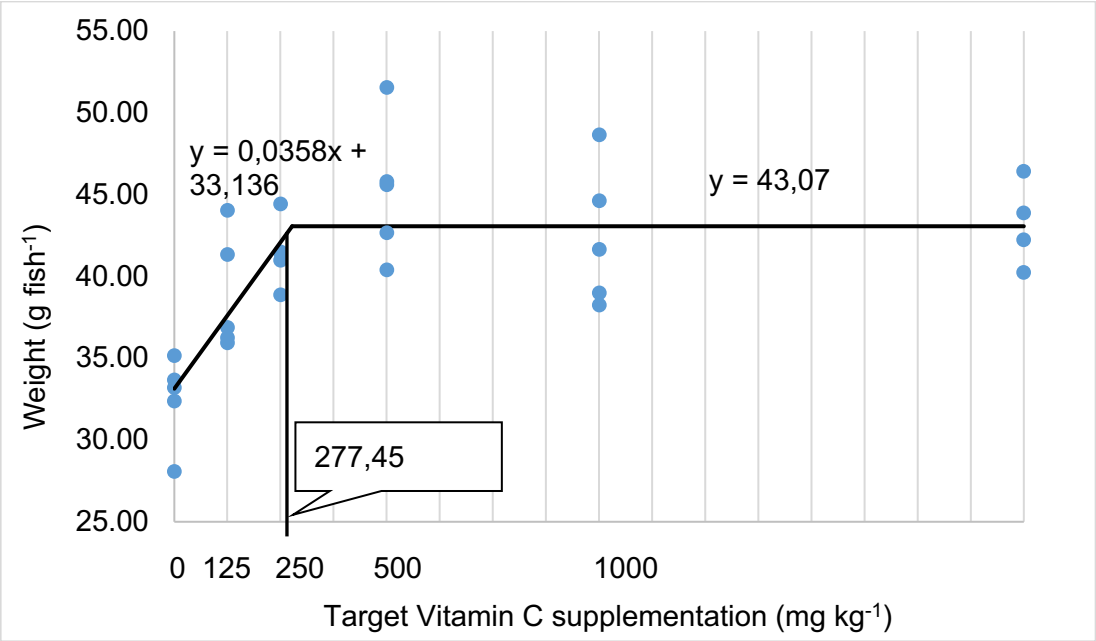

Figure 1. Requirement of dietary vitamin C on growth responses of snakehead fish, C. striata.

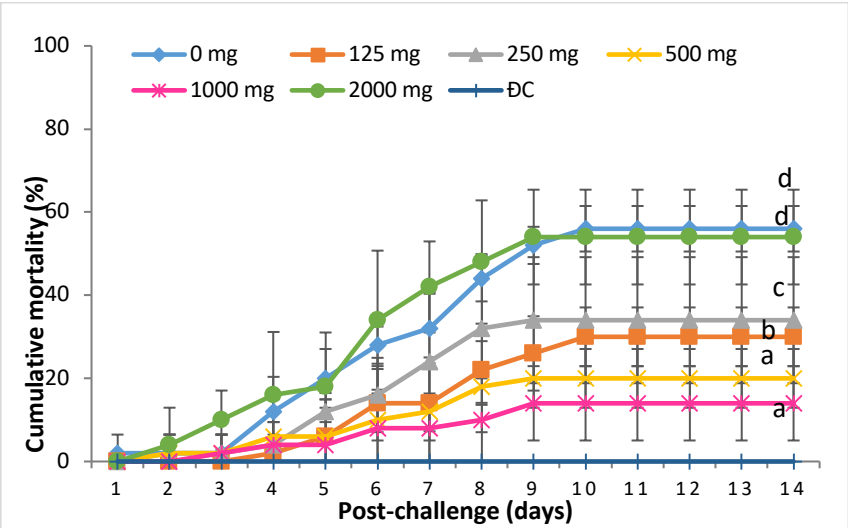

Figure 2. Cumulative mortality over 14 d in snakehead fish, *Channa striata*, fed diets with different levels of Vitamin C (as L-ascorbate-2-monophosphate) for 8 weeks, then inoculated intraperitoneally with *Aeromonas hydrophila* in the bacterial challenge experiment.

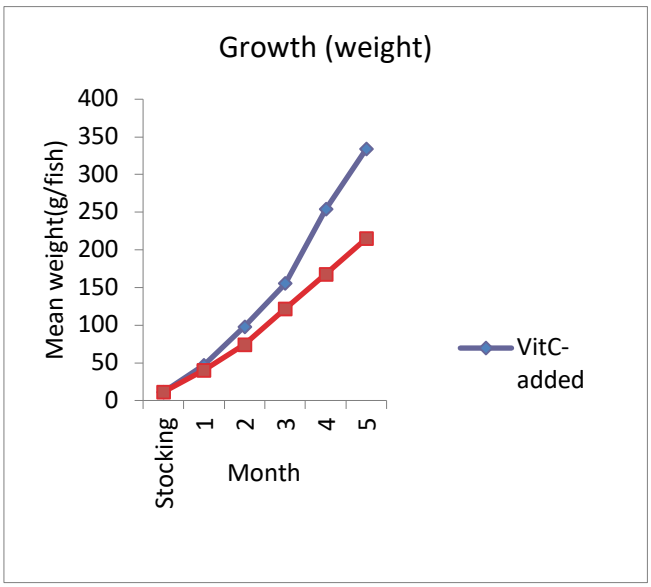

Figure 3. Growth performance of Cambodian snakehead *Channa striata* during 5-month grow-out

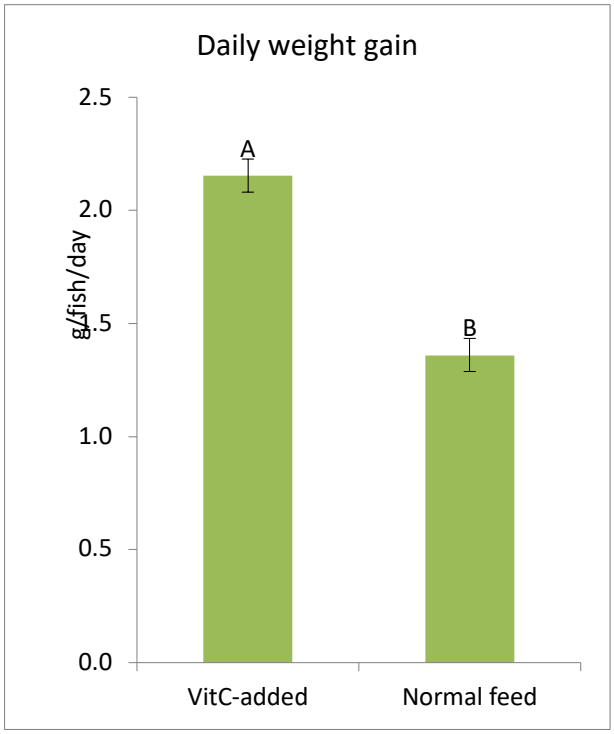

Figure 4. Daily weight gain (g fish^{-1} day^{-1}) of Cambodian snakehead *Channa striata* during 5-month grow-out

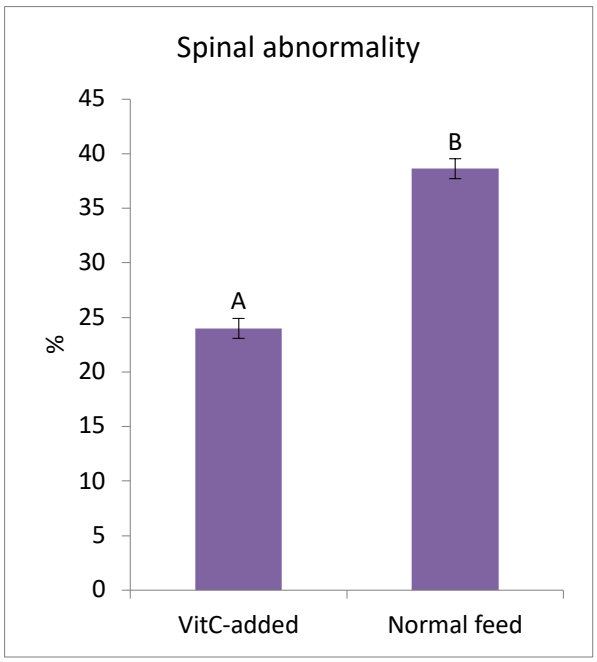

Figure 5. Spinal abnormality rate (%) of Cambodian snakehead *Channa striata* during 5-month grow-out

Figure 6. Grow-out hapas in Vietnam

Figure 7. Harvesting snakehead from grow-out hapas in Vietnam

Figure 8. Grow-out hapas (3x1x1.5m) in Cambodia

Figure 9. Cambodian snakehead fingerling (60 day-old) at stocking

Figure 10. SBM diet (including 500 mgAA.kg^{-1} feed) for hapa study in Cambodia.