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Effects of Dietary Proanthocyanidins Supplementation on Growth Performance, Digestive Enzymes Activities and Microbiota in the Intestine of Juvenile American Eels (*Anguilla rostrata*) Cultured in Cement Tanks

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Key words: proanthocyanidins; American eels; growth; digestive enzymes; intestinal microbiota

Abstract

This trial was conducted to investigate the effects of proanthocyanidins (PACs) on the growth performance, digestive enzymes activities and microbiota in the intestines of juvenile American eels (*Anguilla rostrata*) cultured in cement tanks. Six cement tanks stocked with similar fish size and weight (about 120 g/fish and 2116 kg/tank) were randomly divided into a control group fed with a commercial diet and a PACs group fed with a commercial diet supplemented 400 mg/kg PACs, with three replicates in each group. The trial period lasted 60 days. The finial fish weight and weight gain rate were significantly improved by PACs supplementation ($P<0.05$). There were no significant differences in feed efficiency and feeding rate between the control group and PACs group ($P>0.05$). Activities of protease in intestines were significantly enhanced by PACs supplementation ($P<0.05$), however dietary PACs supplementation had no significant effect on the activities of amylase and lipase ($P>0.05$). Furthermore, dietary PACs supplementation increased the richness and decrease the diversity of intestinal microbiota. At the phylum level, the higher relative abundance of Proteobacteria and the lower relative abundances of Firmicutes and Actinobacteria were found in PACs group compared to control group. At the genus level, PACs supplemented in diet significantly increased the relative abundances of *Candidatus Arthromitus*, *Paracoccus* and *Eubacterium hallii* ($P>0.05$). In conclusion, dietary 400 mg/kg PACs supplementation improved growth performance and activity of protease in intestine, and modulate intestinal microbiota of juvenile American eel cultured in cement tanks.

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Introduction

Proanthocyanidins (PACs), as the oligomers or polymers of flavan-3-ol, are a group of polyphenolic bioflavonoids diverse in chemical structures, pharmacology and characteristics and widely available in fruits, vegetables, nuts, seeds, flowers and bark (Fine, 2000; Bashir et al., 2014; Tao et al., 2017). PACs are well known for their antioxidant activity which is about 20 times greater than vitamin E and 50 times greater than vitamin C (Fine, 2000; Bashir et al., 2014). Furthermore, PACs have biochemical effects including antibacterial, anti-carcinogenic, anti-inflammatory, and anti-allergic (Fine, 2000; Gao et al., 2020), and they are absorbed in the guts and exert various physiological and biological functions in *vivo* (Yamakoshi et al., 2002). In fact, the absorption and metabolism of PACs is limited due to their polymeric nature and high structural complexity, most of the intake PACs need gut microbiota for further fermentation (Cires et al., 2017; Tao et al., 2019). In addition, the microbial composition could be modulated by PACs administration with suppressing pathogens proliferation and increasing the relative abundances of beneficial bacteria species (Hassan et al., 2019).

In aquaculture, the growth promotion effects of dietary PACs supplementation were reported in European eel (*Anguilla anguilla*) (He et al., 2019), hybrid crucian carp (Huang et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Arslan et al., 2018; Kesbiç and Yigit, 2019), and tilapia (*Oreochromis niloticus*) (Lu and Zhai, 2014; Zhai et al., 2014; Deng, 2015). Additionally, dietary PACs could alleviate the retarded growth of some fish species exposed to different sources of stress (Wang et al., 2018; Zhai et al., 2018; Zhai et al., 2020). The PACs supplementation levels in those fish diets were mainly from 200 mg/kg to 800 mg/kg. However, the previous researches on PACs supplemented in fish diets were conducted under laboratory condition, little information is available regarding effects of dietary PACs on fish species under practical culture condition.

Eel is an economically important fish species for freshwater aquaculture in Southeast Asia, and cultured in China since 1993 (Fan et al., 2016). The American eel (*Anguilla rostrata*) is a facultatively catadromous fish that is cultured in China since 1994, and became the main eel species in Fujian Province of China especially after a drastic decrease of natural stocks of European and Japanese eels (*Anguilla japonica*) (Fan et al., 2016; Zhai et al., 2018). Usually, the American eels are intensively cultured in cement tanks in Southern China. The purpose of the present study was conducted to evaluate the effects of 400 mg/kg PACs supplemented in diet on growth performance, digestive enzyme activities and microbiota in intestine of American eels cultured in intensive cement tanks.

Materials and Methods

Experimental fish and design

The experimental fish were American eels intensively cultured in Hexagon cement tanks (Water surface area 143 m^2 with 1.2 m height and 0.9m water depth) in a commercial eel farm (Fuyuan Fishery Development Co., Ltd., Fujian Province, China) for eight months. The individual growth rates are substantially different during eel culture, and grading eel juveniles about every two months necessary in order to reach a high overall growth performance. After the grading of eels, six cement tanks with similar fish weight (about 120 g/fish and 2116 kg/tank) were randomly divided into two treatment groups (control group and PACs group) with three tanks per group. The control group was fed a commercial diet, and the PACs group was fed commercial diet supplemented with 400 mg/kg PACs. The trial lasted for 60 days.

Experimental diet and fish management

The commercial diet (provided by Fenghua Aquatic Feed Co., Ltd, Fuzhou, China) contained 46.8% crude protein, 4.7% lipids, 0.6% crude fiber, 14.2% ash. PACs were extracted from grape seed (content >98%, purchased from Nanjing Zelang Medical Technology Co., Ltd., Nanjing, China) and well mixed with the commercial diet.

In the commercial eel farm, all fish were cultured in cement tanks and fed to apparent satiation twice daily (at 5:00 h and 17:00 h). The powder diet was mixed with water 1.2 times the diet weight to form dough, the dough was placed on a feeding table and served to the eels. The uneaten feed was taken out with a net 30 min after feeding and dried. The consumption of diet was recorded daily. The water temperature was kept at 25-30°C. Water quality variables were pH 7-8.5, dissolved oxygen 6-12 mg/L, total ammonia nitrogen 0.4-0.9 mg/L, nitrite nitrogen levels <1.0 mg/L. A water exchange approximately 50 m^3/tank was done twice daily (at 7:00 h and 19:00 h). The fish and water managements of all tanks for trial were same for all the other tanks and the dead fish were recorded and weighted daily.

Sample collection and analysis

On the final day of the trial, twelve fish from each group were sampled randomly after 24 h fasting, and anesthetized with 2 g/L eugenol oil suspension. The fish intestines were dissected on ice and kept frozen at –80 °C for further measurements. Two intestine samples from each tank were pooled for the measurement of amylase, lipase, and protease activity in intestine according to the methods of Zhai and Liu (2014). Another six fish were dissected with sterile scissors, and the intestine samples were separated from the abdominal cavity, and flushed with PBS buffer to remove feces for high throughput sequencing analysis. Total bacterial DNA was extracted and bacterial 16S rDNA sequences spanning the variable regions V3–V4 were amplified according to the method described previously (Shi et al., 2020) The high-throughput sequencing analysis was performed on the Illumina Hiseq2500 PE250 platform (Illumina, San Diego, CA, USA) at Biomarker Technologies Corporation (Beijing, China). The raw data were first spliced by FLASH (version 1.2.11) and sequences were filtered by Trimmomatic (version 0.33), and the sequences were obtained for further analysis.

Data calculation

At the end of the trial, fish weight was measured in each tank after 24 h of feed deprivation. The sum of this fish weight and dead fish weight of each tank was considered as final fish weight (FFW) to calculate the following growth performance parameters.

Weight gain rate (WGR), feed efficiency (FE), and feeding rate (FR) were calculated as follows:

WGR (%) = [final fish weight (kg/tank) - initial fish weight (kg/tank)]/ initial fish weight (kg/tank) ×100%;

FE (%) = [final fish weight (kg/tank) - initial fish weight (kg/tank)]/ feed consumption (kg/tank) ×100%;

FR (%) = feed consumption (kg/tank)/average fish weight of fish (kg/tank) ×100%;

Statistical analyses

The results of growth performance parameters and intestinal activities of digestive enzymes were presented as means ± SD. Statistical analysis was performed with SPSS 22.0 statistical software (SPSS, Chicago, IL, USA). Data from each treatment group were subjected to T-tests and $P<0.05$ provided significant difference. Linear discriminant analysis (LDA) effect size (LEfSe) was performed to present biomarkers of gut microbial communities at genus level between control group and PACs group discriminated by nonparametric factorial Kruskal–Wallis test and pairwise Wilcoxon test with the P value <0.05. The LDA score threshold was 3.5.

Results

Parameters of growth performance

Parameters of growth performance of American eels between the control and PACs group were shown in **Table 1**. There were significant differences of FFW and WGR between control group and PACs group ($P<0.05$), FE and FR differences were not significant between

the two groups (P>0.05).

Table 1 Growth performance parameters of American eels between the control and PACs groups

Item	Control group	PACs group
IFW (kg/tank)	2149.6±55.5[a]	2118.2±63.3[a]
FFW (kg/tank)	3508.6±147.6[a]	3752.3±126.9[b]
WGR (%)	63.22±5.45[a]	77.14±1.96[b]
FE (%)	74.73±6.97[a]	85.73±3.60[a]
FR (%)	0.64±0.02[a]	0.65±0.02[a]

IFW=initial fish weight; FFW= final fish weight; WGR= weight gain rate; FE= feed efficiency; FR= feeding rate.
[ab]Values within the same column without the same superscript were significantly different at P<0.05 level.

Activities of digestive enzymes in intestine

Digestive enzyme activities in intestine of American eels between the control and PACs group were shown in **Table 2**. Compared with control group, protease activity in intestine of PACs group increased significantly (P<0.05). The activities of Amylase and lipase were not significantly affected by PACs supplementation (P>0.05).

Table 2 Digestive enzyme activities in intestine of American eel between the control and PACs groups

Item	Control group	PACs group
Amylase (U/mg prot)	0.36±0.01	0.40±0.01
Lipase (U/mg prot)	12.41±1.94	12.13±2.88
Protease (U/mg prot)	63.98±0.29[a]	76.81±0.75[b]

[ab]Values within the same row without the same superscript were significantly different at P<0.05 level.

The alpha diversity indexes of the intestinal microbiota

The alpha diversity indexes of the intestinal microbiota of American eels between the control and PACs group are shown in **Table 3**. Results from alpha diversity indexes indicated that the Chao1 index and ACE index in the PACs group were higher compared to the control group. The value of the Shannon index was lower and the Simpson index was higher in the PACs group in comparison with the control group. The value of Coverage was almost 1.00.

Table 3 Alpha diversity indexes of intestinal microbiota of American eels between the control and PACs groups

Item	Chao1	ACE	Shannon	Simpson	Coverage
Control group	180.80	189.37	2.84	0.19	0.9996
PACs group	210.84	214.10	1.98	0.45	0.9996

The composition of Intestinal microbiota at the phylum level

The composition of Intestinal microbiota at the phylum level of American eels in control and PACs groups appear in **Figure 1**. The top three predominant phylum of American eels in the control group were Proteobacteria, Firmicutes and Actinobacteria, as same as PACs group. While in PACs group, there was an increasing trend of relative abundance of Proteobacteria, and a decreasing trend of the relative abundances of Firmicutes and Actinobacteria.

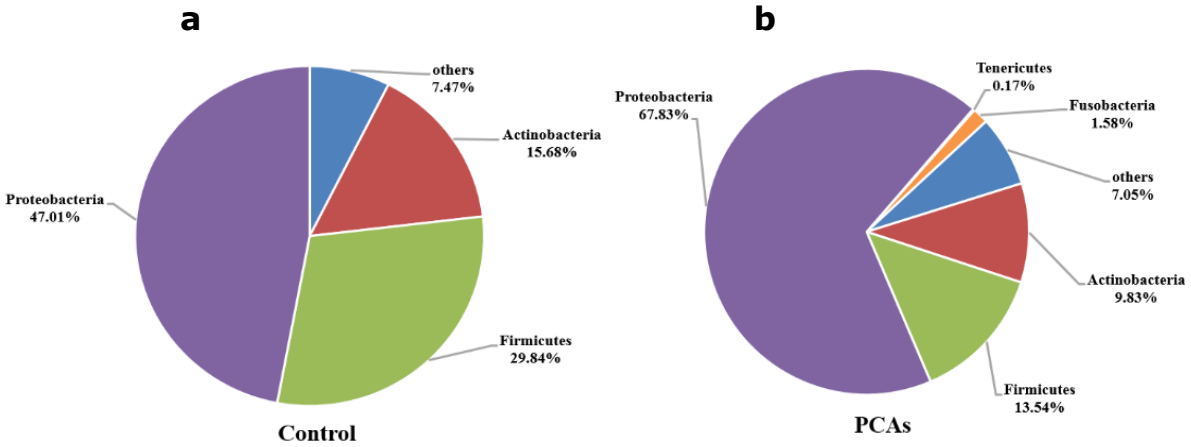

Figure 1 The intestinal microbiota composition at phylum level of American eels in control group (a) and PACs group (b).

LEfSe analysis of intestinal microbiota at the genus level

The LEfSe analysis showed that the relative abundance of *f_Enterobacteriaceae* and *Cloacibacterium* were significantly higher in control group, while the relative abundances of*_Candidatus Arthromitus*, *Paracoccus* and *Eubacterium hallii* were significantly higher in PACs group (P<0.05)(**Figure 2**).

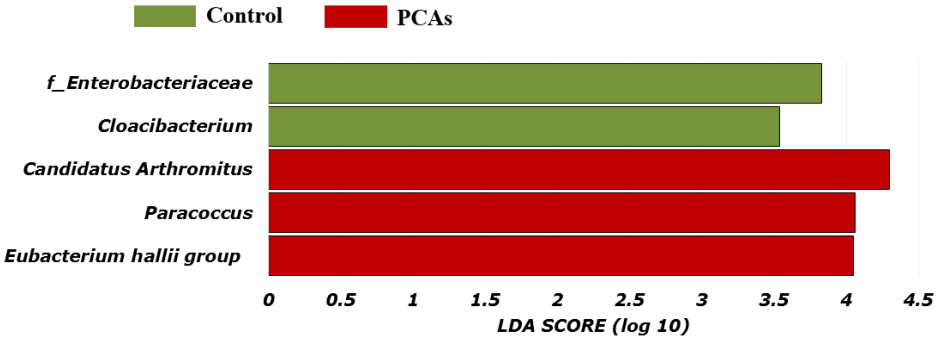

Figure 2 The LEfSe analysis displaying the enriched bacteria in intestine between control group and PACs group.

Discussion

In the present study, the FFW and WGR of American eel were significantly improved by 400 mg/kg PACs supplementation in diets, which were consistent with previous reports of PACs supplemented in some other fish species. The minimum levels of PACs in diets that promote FFW and WGR are 200 mg/kg in tilapia (Lu and Zhai, 2014; Zhai et al., 2014; Deng, 2015), 600 mg/kg in European eel (He et al., 2019), and grape seed extract level of 10 g/kg in diet improve growth rate of hybrid Crucian carp (Huang et al., 2011). In the studies of rainbow trout, the WGR significantly increased by 1000 mg/kg dietary grape seed extract supplement (Arslan et al., 2018; Kesbiç and Yigit, 2019). Furthermore, dietary 300 mg/kg PACs supplementation increased FFW and WGR of American eel exposed to 517 mg/kg dietary histamine (Zhai et al., 2020), 400 mg/kg PACs supplemented in diet effectively improved the WGR of juvenile tilapia exposed to 100 mg/kg dietary cadmium (Zhai et al., 2018), and 800mg/kg PACs ameliorated the WGR in pearl gentian grouper (♀ *Epinephelus fuscoguttatus*× ♂*Epinephelus lanceola*) exposed to 300 mg/kg dietary cadmium (Wang et al., 2018). Even though there were differences in fish species, feeding condition, dietary nutrient levels, and PACs supplementation levels between our study and other reports, the growth promotion effect of PACs was confirmed. The selling price of PCAs extracted from grape seed is about $30 per kilogram in China at present, they are an affordable feed additive used in commercial fish diet.

In this study, 400 mg/kg dietary PACs significantly increased the activity of intestinal protease. Similar results were reported in tilapia fed diet with the minimum level of 200 mg/kg PACs supplemented (Lu and Zhai, 2014; Deng, 2015) and pearl gentian grouper fed diet with 800 mg/kg PACs to counteract the stress of 300mg/kg dietary cadmium (Liu, 2018).Besides, dietary 200 mg/kg PACs could also improve the activity of amylase and lipase in intestine of tilapia (Deng, 2015). The improvement of digestive enzymes activities might be related to the improvements of PACs on intestinal epithelial integrity and barrier function by increasing expression of tight junction proteins (Gao et al., 2020). Furthermore, PACs could alleviate the damage of oxidative stress to intestinal mucosa, and attenuate intestinal inflammation, which might indirectly promote the secretion of digestive enzymes (Deng, 2015; Gao et al., 2020).

The intestinal microbiota plays an important role in fish health by providing vital nutrients and stimulating the innate immune response, the balance of bacteria communities in the intestine for the natural resistance of fish (Abdelhamed et al., 2019). The alpha diversity is a reflection of richness and diversity indices of the bacterial communities, including indexes of Chao1, ACE, Shannon and Simpson (Xu et al., 2019). In the present study, PACs group showed higher values of Chao1 and ACE index compared with control group, demonstrated that dietary PACs could improve the richness of the intestinal microbiota of American eel. And the value of Shannon index was lower and value of Simpson index was higher in PACs group compared with control group, indicated the diversity of bacteria species was decreased due to the PACs supplementation, a decrease in the diversity of the microbiota was also reported after treatment with PACs in rats studies (Gao et al., 2020). The values of coverage indices were above 99%, suggested that in this study the majority of intestinal bacteria were identified (Shi et al., 2020).

In this study, the proportion of Proteobacteria in the American eels of the PACs group was higher than that of the control group. Proteobacteria is the most abundant phylum in many fish species, like Japanese eel (Jian, 2016), European eel (Shi et al., 2020) and turbot (*Scophthalmus maximus* L.) (Yang et al., 2019), and a major group of gram-negative bacteria that prefers proteins as main energy source and produce protease (Liu et al., 2016).The increased abundance of Proteobacteria might be associated with a health metabolic state, and some researchers have verified that some bacteria of Proteobacteria in the gut of healthy fish might significantly contribute to the digestive function, and identified as protease-producing bacteria dominant in carnivorous fish (Liu et al., 2017; Xu et al., 2019; Yang et al., 2019). PACs group had lower abundance of Firmicutes and Actinobacteria compared with control group, which was consistent with a study demonstrated that PACs could significantly reduce the population of some species of Firmicutes and Actinobacteria in healthy rats (Tao et al., 2019). Firmicutes was the main phyla in many fish species, but some of these bacteria could cause the lower growth rate of European eel (Shi et al., 2020). Recently, it was found that some strains belonging to Actinobacteria might be potential pathogen (Jang et al., 2020). In this study, PACs supplementation might decrease the relative abundances of those Potential pathogens in intestine of American eels.

At the genus level, *f_Enterobacteriaceae* and *Cloacibacterium* were significantly higher in control group. Many members of the family *Enterobacteriaceae* are potentially fish pathogens (Abdelhamed et al., 2019), it was also reported as a pathogenic bacterium of marbled eel (*Anguilla marmorata*) (Yang, 2013). *Cloacibacterium* is a gram-negative bacteria that was reported to have greater abundance at inflamed site in the guts of humans (Hirano et al., 2018). These results indicated that American eels in the control group might have a higher risk to be infected by pathogenic species. While the relative abundances of *Candidatus Arthromitus*, *Paracoccus* and *Eubacterium hallii* were significantly higher in PACs group. The *Candidatus arthromitus* is the collective name for gram-positive segmented filamentous bacteria, it has garnered much attention due to their ability to modulate host immune responses and intestinal epithelial cells function (Ladinsky et al., 2019; Zhang et al., 2020). Some members of *Paracoccus* genus demonstrated to have probiotic effects to improve the growth performance of sea cucumber (Yang et al., 2015). *Eubacterium hallii* was considered to be an important microbe in stabilizing the

intestinal metabolic balance (Engels et al., 2016), and *Eubacterium* groups involved in the metabolism of phenolic compounds including PACs, and might trigger A-ring cleavage of PACs (Cires et al., 2017; Tao et al., 2019). In the present study, the change of intestinal microbiota of American eels were caused by dietary PACs supplementation, the modulation of intestinal microbiota by PACs might be related to its acting as substrates by specific bacterial populations and prebiotics to stimulate some probiotics proliferation in intestine (Cires et al., 2017).

In conclusion, under the condition of practical eel culture, 400 mg/kg PACs supplemented in diet could promote the growth performance, and improve the activity of intestinal protease and modulate the microbiota in intestine of American eel.

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