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Original Research Article

An emerging role of arecoline on growth performance, intestinal digestion and absorption capacities and intestinal structural integrity of adult grass carp (*Ctenopharyngodon idella*)

Na Yao [a], Lin Feng [a, b, c], Weidan Jiang [a, b, c], Pei Wu [a, b, c], Hongmei Ren [a], Hequn Shi [d], Ling Tang [e], Shuwei Li [e], Caimei Wu [a], Hua Li [a], Yang Liu [a, b, c, *], Xiaoqiu Zhou [a, b, c, *]

[a] *Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, 611130, China*
[b] *Fish Nutrition and Safety Production University Key Laboratory of Sichuan Province, Sichuan Agricultural University, Chengdu, 611130, Sichuan, China*
[c] *Key Laboratory of Animal Disease-Resistance Nutrition, Ministry of Education, Ministry of Agriculture and Rural Affairs, Key Laboratory of Sichuan Province, Sichuan, 611130, China*
[d] *Guangzhou Cohoo Biotech Co., Ltd., Guangzhou, 510663, China*
[e] *Animal Nutrition Institute, Sichuan Academy of Animal Science, Sichuan Animtech Feed Co. Ltd, Chengdu, 610066, Sichuan, China*

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ABSTRACT

Arecoline is an alkaloid with important pharmacological effects in the plant areca nut, which has been demonstrated to be an agonist of muscarinic receptors (M receptor). This study explored the influences of dietary arecoline on growth performance, intestinal digestion and absorption abilities, antioxidant capacity, and the apical junction complex (AJC) of adult grass carp (*Ctenopharyngodon idella*). Adult grass carp (608 to 1512 g) were fed at 6 graded levels of dietary arecoline (0, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/kg diet) for 9 weeks. The results suggested that appropriate dietary supplementation of arecoline (1.0 mg/kg) increased growth parameters and intestinal growth in adult grass carp ($P < 0.05$), enhanced digestion and absorption capacities ($P < 0.05$), up-regulated muscarinic receptor 3 (*M3*) mRNA level ($P < 0.05$), increased the content of neuropeptide fish substance P ($P < 0.05$), improved antioxidant capacity by activating the Keap1a/Nrf2 signaling pathway ($P < 0.05$), reduced intestinal mucosal permeability ($P < 0.05$), and increased mRNA levels of tight junction (TJ) and adherent junction AJ-related proteins in fish by inhibiting the RhoA/ROCK signaling pathway (RhoA/ROCK/MLCK/NMII) ($P < 0.05$). In addition, the appropriate arecoline supplementation for adult grass carp was determined to be 1.20, 1.21, 1.07, and 1.19 mg/kg based on percentage weight gain, lipase activity, serum diamine oxidase, and protein carbonyl, respectively. Overall, to the best of our knowledge, we investigated for the first time the effects and possible mechanisms of dietary arecoline on intestinal digestive and absorptive capacities and structural integrity in fish and evaluated the appropriate level of supplementation.

1. Introduction

Arecoline (N-methyl-1,2,5,6-tetrahydropyridine-3-carboxylic acid methyl ester) is a colorless oily alkaline liquid that is soluble in water, ethanol, ether, and chloroform (Jahns, 1891). The chemical structure of arecoline ($C_8H_{13}NO_2$) has a double bond at the exact position of the ring and contains an ester bond and a nitrogen methyl functional group. It is a significant alkaloid that was identified and extracted from the areca nut plant and is one of the key medicinal components of the areca nut (Volgin et al., 2019). Studies in terrestrial animals have shown that the accumulation of arecoline in excess in the body produces toxicological effects; however, the addition of appropriate doses of arecoline plays a role in promoting digestion, antioxidation, anti-parasite, anti-inflammatory, anti-thrombosis, and anti-atherosclerosis effects in animals (Liu et al., 2016). Currently, only one study has been conducted on arecoline in animal production. This study on Wenchang broiler

* Corresponding authors.
E-mail addresses: auldKgk@sicau.edu.cn (Y. Liu), zhouxq@sicau.edu.cn (X. Zhou).
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chicks showed that supplementation with 100 to 300 mg/kg areca nut extract (the main active ingredient is arecoline) improved feed intake and weight gain (Wang et al., 2018). Animal growth performance was closely correlated with digestion and absorption abilities as well as intestinal structural integrity (Zhao et al., 2019). However, the effect of arecoline on the digestive and absorptive capacities and intestinal structural integrity of animals has not been reported. Arecoline is a muscarinic receptor agonist that improves digestive function in rats by stimulating muscarinic receptors (such as M1 and M3) (Si et al., 2004). M1 and M3 receptor single knockout or M1/M3 receptor double knockout reduced amylase secretion in mouse pancreatic acini cells (Gautam et al., 2005). These results suggest that arecoline may perform its digestive role through stimulation of the muscarinic receptor, but it has not been studied in fish, which is worth investigating. Fish absorption capacity is reflected by the activities of intestinal brush border enzymes such as Na^+,K^+-ATPase (García-Gasca et al., 2006). A study on rat brain tissue found that arecoline treatment increased Na^+,K^+-ATPase activity (Von Schwarzenfeld et al., 1976). However, there is no study on the influence of arecoline on Na^+,K^+-ATPase activity in the intestine of animals. These data indicate that arecoline may be associated with intestinal absorptive capacity, which requires further investigation.

Fish intestinal function depends on the structural integrity of the intestine, which is influenced by the antioxidant capacity of the cells (Jutfelt, 2011). The antioxidant capacity of animals is crucial to maintain structural integrity (Li et al., 2017). Exogenous arecoline increased 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) free radical scavenging activity, indicating that arecoline had a strong antioxidant capacity (Yin et al., 2020). However, the effect of arecoline on the antioxidant capacity of fish has not been reported. A previous study in the subcortical structure of mice showed that a higher concentration of arecoline increased the level of acetylcholine (Molinengo et al., 1988), and acetylcholine increased the protein expression of manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (CuZnSOD) in the cytoplasm of human laryngeal cancer cell lines (Sun et al., 2014). Animal antioxidants are influenced by nonenzymatic and enzymatic antioxidant defense systems and are regulated by nuclear erythroid 2-related factor 2 (Nrf2) (Kohen and Nyska, 2002). A low concentration of arecoline increased the NO content in endothelial cells (Wu et al., 2020), and NO promoted Nrf2 protein expression in HCT116 cells (Li et al., 2009). These findings suggest that arecoline may affect the antioxidant capacity and the related regulator Nrf2 in animals, which warrants a detailed study.

In addition to intestinal antioxidant capacity, intercellular structural integrity also plays a very important role in maintaining the structural integrity of the intestine. A growing number of studies have shown that the apical junction complex (AJC), which includes tight junctions (TJs) and adherent junctions (AJs), has a significant impact on the structural integrity of the intestine (Ivanov et al., 2007; Sahai and Marshall, 2002). The integrity of TJs and AJs in mammalian enterocytes may be regulated by the RhoA/ROCK/MLCK/NMII signaling pathway (Chen et al., 2014). At present, no studies have explored the influences of arecoline on the structural integrity of intercellular cells and related signaling molecules in the animal intestine. Previous studies showed that arecoline promoted the secretion of catecholamine in rat chromaffin cells (Chu, 2001), and catecholamine increased the expression of TJ protein (zonula occluden-1 [ZO-1]) and AJ protein (E-cadherin) in cEND mouse brain microvascular endothelial cells (Ittner et al., 2020). Furthermore, arecoline reduced the gene expression of interleukin-6 (IL-6) in a stable basal cell carcinoma BCC-1/KMC cell line (Huang et al., 2012), and IL-6 activated the RhoA/ROCK signaling pathway in human gastric cancer cells (Lin et al., 2007). These studies suggest that arecoline may affect AJCs and the related signaling molecules RhoA/ROCK/MLCK/NMII, which deserves further investigation.

In conclusion, the current study was the first to examine the effects and possible mechanisms of dietary arecoline on the digestive and absorptive capacities and structural integrity of the animal intestine, which provided a basic theory for animal intestinal health. Grass carp (*Ctenopharyngodon idella*) is the most popular economic freshwater cultured species (Liu et al., 2013). To the best of our knowledge, no studies have evaluated the optimal level of dietary arecoline supplementation to date. Therefore, this study was the first to assess the appropriate level of arecoline supplementation in grass carp, which provided an essential guide to improve fish growth.

2. Material and methods

2.1. Animal ethical statement

All protocols in this study were approved by the Animal Care Advisory Committee of Sichuan Agricultural University (No. YN-2020214026) and were conducted according to the requirements of the Care and Use of Laboratory Animals in China.

2.2. Experimental design and diets

Table 1 shows the approximate composition and formulation of the basal diet. In this study, six isonitrogenous (26.14% crude protein) and isolipidic (5.16% crude lipid) diets were formulated. Protein sources included fish meal, soybean meal, cottonseed meal, and rapeseed meal. Fish oil and soybean oil were utilized in this experiment as lipid sources. The arecoline (product purity was

Table 1
Ingredients and nutrient composition of the basal diet (as-fed basis, %).

Ingredients	Content	Nutrients content [2]	Content
Fish meal[1]	5.00	Dry matter	89.04
Cottonseed meal[1]	10.58	Crude protein	26.14
Rapeseed meal[1]	8.40	Crude lipid	5.16
Soybean meal[1]	20.00	Crude ash	9.95
Fish oil	2.45	GE, MJ/kg	16.96
Soybean oil	1.51	n-3 PUFAs	1.02
Butylated hydroxyanisole (99%)	0.02	n-6 PUFAs	0.94
Ca $(H_2PO_4)_2$	1.38	Available P[7], %	0.40
Flour	45.58		
Mineral premix[3]	2.00		
Vitamin premix[4]	1.00		
Choline chloride premix[5]	1.00		
Thr (98.5%)	0.08		
Arecoline premix[6]	1.00		

PUFA = polyunsaturated fatty acid.
[1] Fish meal: Sichuan Huayu Trading Co., Ltd; Cottonseed meal: Sichuan Sanjiu Runyi Trading Co., Ltd; Rapeseed meal: Chengdu Xinxing Grain and Oil Co., Ltd; Soybean meal: Sichuan Hongdabo Feed Co., Ltd.
[2] The analytical methods of the test diets were evaluated according to the standard methods described (AOAC, 2005).
[3] Provided the following per kilogram of mineral premix (g): $MnSO_4 \cdot H_2O$, 2.66; $MgSO_4 \cdot H_2O$, 256.79; $FeSO_4 \cdot H_2O$, 12.61; $ZnSO_4 \cdot H_2O$, 8.87; $CuSO_4 \cdot 5H_2O$, 0.95; Ca $(IO_3)_2$, 1.56; yeast selenium, 13.65; all ingredients were diluted with corn starch to 1 kg.
[4] Provided the following per kilogram of vitamin premix (g): retinyl acetate, 0.193; vitamin D_3, 0.204; DL-A tocopherol acetate, 23.20; vitamin K_3, 0.38; thiamine nitrate, 0.1137; riboflavin, 0.731; vitamin B_6, 0.452; calcium-D-pantothenate, 4.203; niacin, 3.44; meso-inositol, 28.5; vitamin B_{12}, 0.94; D-biotin, 1.05; folic acid, 0.168; vitamin C acetate, 9.77; all ingredients were diluted with corn starch to 1 kg.
[5] Provided choline chloride at 261.95 g/kg premix, and the rest was diluted with corn starch to 1 kg.
[6] Provided arecoline at 0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/kg premix for the six treatment groups, and the rest was diluted with microcrystalline cellulose.
[7] Available P of test diets was calculated according to NRC (2011).

0.23%, and the active ingredient was arecoline) used in this experiment was provided by Guangzhou Cohoo Biotech Co., Ltd., Guangzhou, China. Based on sanguinarine, an analog of arecoline (both have similar functional groups), we designed the gradient of this test with reference to studies on sanguinarine in juvenile grass carp (Liu et al., 2020). Therefore, arecoline was supplemented to the basal diet at 0.0 (unsupplemented), 0.5, 1.0, 1.5, 2.0, and 2.5 mg/kg diet. Different additions of arecoline and microcrystalline cellulose were premixed to obtain six premixes and ensure a homogeneous mixture. Protein ingredients (cottonseed meal, rapeseed meal, and soybean meal) were ground with a grinder (Nanjing, China). As described by Zhao et al. (2014), after thorough mixing, the ingredients were extruded into pellets. The prepared particles were dired and stored at −20 °C for later use (Wang et al., 2014).

2.3. Experimental fish and feeding management

The experiment was conducted in the Dayi research base of the animal nutrition institution, Sichuan Agricultural University. According to the method of Jiang et al. (2014), the fish underwent a 4-week adaptation period after being purchased from a local fishery in Sichuan, China. A total of 450 healthy grass carp (607.87 ± 1.82 g) were randomly assigned to 18 net cages, with 25 fish in each cage. The experiment was divided into 6 treatment groups (25 fish per duplicate, 3 repetitions per treatment). Experimental fish were fed four times daily (08:00, 11:00, 15:00, and 19:00) until satisfied as stated by Deng et al. (2014). After feeding for 30 min, each cage was equipped with a 100-cm^2 disk to collect the feed residue, and then the feed residue was dried and weighed to calculate feed intake (FI) as described by Cai et al. (2005). All nets were located in outdoor freshwater ponds, and microporous aeration was used throughout the experiment. During the 9-week experiment, water was changed regularly and quantitatively every day. The water parameters were as follows: temperature at 26.75 ± 2.3 °C, pH from 7.5 to 8.0, and dissolved oxygen >6 mg/L. All fish were subjected to natural light conditions, which consisted of approximately 12 h light and 12 h darkness (Zhang et al., 2022).

2.4. Sample gathering

At the end of the growth experiment, the fish were starved for 24 h, weighed and counted, and then the growth performance of the fish was calculated according to previous research in our laboratory (Wang et al., 2019). Nine grass carp were randomly selected from each group and anesthetized with benzocaine (50 mg/L) according to the method of Geraylou et al. (2013). Blood was drawn from the caudal vein using a syringe (5.0 mL), centrifuged at 3,000 × *g* at 4 °C for 10 min to obtain serum and stored at −20 °C, and the serum was collected by centrifugation to measure the D-lactate concentration and diamine oxidase (DAO) activity, according to the methods of Feng et al. (2023) and Zheng et al. (2018). As described by Zeng et al. (2016), the intestines and hepatopancreas from the fish were quickly separated, and the intestinal weight and length were measured, immediately frozen in liquid nitrogen and stored at −80 °C.

2.5. Histological examination

Intestinal samples were fixed using 4% paraformaldehyde solution for later histological examination as described by Song et al. (2021). The intestinal samples were first fixed in paraffin, dehydrated in an ethanol solution, dissected into 4-μm slides, and then dyed with hematoxylin and eosin (H&E) staining as stated by Tsertou et al. (2020). The morphological structure was examined using an optical microscope (TS100, Nikon Corporation, Tokyo, Japan).

2.6. Biochemical analysis

The moisture, crude protein, crude lipid, and ash of the feed were assayed according to the Association of Official Analytical Chemists (AOAC, 2005). These indicators are operationalized as follows: the determination of moisture was done by drying at 105 °C, crude protein by Kjeldahl method, crude lipid by Soxhlet extraction, and crude ash by searing at 550 °C in a muffle furnace. Serum diamine oxidase activity (DAO; Lot#QS48580) and D-lactate concentration (Lot#QS48279) were measured according to ELISA kits (Beijing Jason Bio-Technology Co., Ltd., Beijing, China). As described by Hummel et al. (1959), trypsin (Lot#A080-2) catalyzed the hydrolysis of the ester chain of the substrate ethyl arginate, and its absorbance value at 253 nm increased. The enzyme activity was calculated based on the change in absorbance value. Chymotrypsin (Lot#A080-3-1) hydrolyzed protein was used to produce phenol-containing amino acids, the phenol reagent was reduced to a blue substance by the phenol-containing amino acids, and its activity was determined colorimetrically. The activity of lipase (Lot#A054-2-1) was determined by the methylurethane substrate method, and the amount of hydrolyzed starch was determined by iodine colorimetry to calculate amylase (Lot#C016-1-1) activity based on Furne et al. (2005). Alkaline phosphatase (AKP; Lot#A059-2) activity was determined by the reaction of phenol with 4-aminoantipyrine to produce a red quinone derivative as described by Bessey et al. (1946). Gamma glutamyl transpeptidase (γ-GT; Lot#C017-2-1) enzyme activity was determined by the GPNA substrate method based on Furne et al. (2005). Creatine kinase (CK; Lot#A032-1-1) enzyme activity was calculated by measuring the amount of inorganic phosphorus produced in the reduction reaction, and the activity of Na^+,K^+-ATPase (Lot#A070-2) was evaluated by measuring the content of inorganic phosphorus as described by Weng et al. (2002). Malondialdehyde (MDA; Lot#A003-1) was assayed using the thiobarbituric acid reaction as previously described by Livingstone et al. (1990). Protein carbonyl (PC; Lot#A087-1) was determined by the formation of protein hydrazones using 2,4-dinitrophenylhydrazine as described by Chen et al. (2009). Reactive oxygen species (ROS; Lot#S0033S) production was performed by determining 2′,7′-dichlorofluorescein in oxidation according to the method of LeBel et al. (1992). The activities of antioxidant enzymes, including superoxide dismutase (SOD; Lot#A001-1), catalase (CAT; Lot#A007-1-1), and glutathione peroxidase (GPx: A005-1), and the content of glutathione (GSH; Lot#A006-1-1) in the intestine were measured using a commercial kit (Jiancheng Bioengineering Research Institute, Nanjing, China) according to kit instructions as described by Yang et al. (2019). In addition, the intestine fish substance P (SP) content (Lot#YJ716283) was determined according to an ELISA kit (Shanghai Enzyme Linked Biotechnology Co., Ltd., Shanghai, China).

2.7. Quantitative real-time PCR (RT-qPCR)

Following the procedures indicated in the research of Fang et al. (2021), total RNA was extracted using the RNAiso Plus reagent kit (TaKaRa, Dalian, China) and then reverse-transcribed into cDNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). Based on our preliminary experimental results on the assessment of internal control genes, β-actin was used as a reference gene (data not shown). After confirming that the primers were approximately 100% effective, the $2^{-\Delta\Delta CT}$ technique was used to assess gene expression. Table S1 lists the primer sequences.

2.8. Western blot analysis

In accordance with the method stated in our earlier reports (Huang et al., 2021), SDS–PAGE was used to separate the protein samples, which were then transferred to a membrane made of polyvinylidene difluoride (PVDF). PVDF membranes were treated with primary antibody at 4 °C overnight after blocking with blocking solution for 2 h. Primary antibody information is presented in Table S2. The secondary antibody was then applied to the membrane and incubated for 2 h. In addition, a pull-down assay (Cytoskeleton, Inc.) was used to evaluate the activity of the RhoA protein according to the study of Wei et al. (2020). Total RhoA was used with GTP-RhoA as a control protein. Finally, electrochemiluminescence imaging and the quantitative results of Western blotting were observed using Image J 1.53k software.

2.9. Immunohistochemistry

According to the method of Zhao et al. (2022), the immunohistochemistry experiment was performed as follows: The slice was rehydrated, and 3% hydrogen peroxide was used to shift the endogenous enzyme, following the streptavidin biotin-peroxidase complex method (SABC kit) (BOSTER, Wuhan, China). The microwave oven then repaired the heat antigen. Primary antibodies against ZO-1 (A0659), occludin (A12621), and claudin-3 (A2946) were diluted 1:100 in phosphate-buffered saline (PBS) and incubated overnight at 4 °C before being washed three times with PBS. After secondary antibody incubation, PBS was rinsed, diaminobenzidine was colored, and hematoxylin staining was performed. Section staining at magnifications of 200× and 400× was imaged using a digital camera (Nikon TS100). Image-Pro Plus was used to measure the immunohistochemistry's cumulative optical density (Version 5.0, Rockville, MD, USA).

2.10. Calculating and statistical analysis

The formulas for growth performance and intestinal growth-related indicators are presented in Table 2. Statistical analysis of the data was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the means ± SD. We analyzed the samples using one-way ANOVA and Duncan's multiple range test to assess significant differences between treatments ($P < 0.05$), and if the data did not satisfy a normal distribution, we normalized using the nonparametric Kruskal–Wallis test. We performed a comparison of one-dimensional regression, quadratic regression, and triple regression. Based on the results of the *P*-value, it is more reasonable to choose a suitable regression model for evaluation. The correlation analysis between the data was obtained by Pearson's correlation procedure.

3. Result

3.1. Effects of arecoline on the growth performance and intestinal morphology

As shown in Table 3, in comparison with the control group, the final body weight (FBW), percent weight gain (PWG), specific growth rate (SGR), feed intake (FI), and feed efficiency (FE) of the optimum arecoline supplementation group (1.0 mg/kg) were significantly elevated, along with significantly increased intestinal length index (ILI) and intestinal somatic index (ISI) ($P < 0.05$). Arecoline was not detected in the mid intestine of grass carp (detection limit 0.05 mg/kg), and the test report is presented in Fig. S1. The addition of arecoline had no significant effect on muscle water content. Compared with the untreated group, dietary arecoline supplementation (1.0 mg/kg) tended to increase the crude protein content in adult grass carp. Crude lipid content of muscle in adult grass carp at first significantly increased with increasing dietary arecoline supplementation ($P < 0.05$), reaching a plateau at supplementation level at 1.0 to 1.5 mg/kg group, and then decreased with further increasing supplementation level ($P < 0.05$). Moreover, as shown in Fig. 1A and B, in the proximal intestine (PI), mid intestine (MI), and distal intestine (DI), as the dietary arecoline supplementation level increased, the maximum level of intestinal fold height was reached at 1.0 mg/kg compared to the unsupplemented group ($P < 0.05$).

Table 2
Index formula for growth performance of adult grass carp (*Ctenopharyngodon idella*).

Item	Formulas
PWG	PWG (%) = [(FBW, g/fish) − (IBW, g/fish)]/(IBW, g/fish) × 100
SGR	SGR (%/day) = [ln (FBW, g/fish) − ln (IBW, g/fish)]/days × 100
FE	FE (%) = [(FBW, g/fish) − (IBW, g/fish)]/(FI, g/fish) × 100
ILI	ILI (%) = [(Intestine length, cm)/(body length, cm)] × 100
ISI	ISI (%) = [(Intestine weight, g)/(body weight, g)] × 100

PWG = percent weight gain; SGR = specific growth rate; FE = feed efficiency; ILI = intestinal length index; ISI = intestinal somatic index.

3.2. Influences of arecoline on the serum activity of DAO and concentration of D-lactate

As shown in Fig. 2, as the dietary arecoline supplementation level increased, DAO activity in fish serum reached its minimum level at 1.5 mg/kg ($P < 0.05$). An increase in arecoline supplementation beyond that resulted in an increase. When the levels of arecoline in the diet reached 1.0 mg/kg, there was a tendency for the serum D-lactate concentration to decrease and subsequently increase.

3.3. Influences of arecoline on the intestinal digestion and absorption capacities

As illustrated in Table 4, in comparison with the control group, trypsin, chymotrypsin, lipase, and amylase activities in the hepatopancreas of the optimum arecoline supplementation group (1.0 mg/kg) were significantly elevated ($P < 0.05$). The activities of AKP, CK, Na^+,K^+-ATPase, and γ-GT and the content of SP in the intestine of fish fed the dietary arecoline supplementation of 1.0 mg/kg were significantly increased compared with those of the unsupplemented group ($P < 0.05$). As presented in Fig. 1C, the *M3* mRNA levels in the intestine were significantly increased in the group with the dietary arecoline supplementation level at 1.0 mg/kg compared with the unsupplemented group ($P < 0.05$).

3.4. Influences of arecoline on antioxidant and oxidative indicators in the intestine

The intestinal antioxidant parameters are illustrated in Table 5. In comparison with the control group, the content of MDA and PC and ROS production in the intestine of the optimum arecoline supplementation group (1.0 mg/kg) were significantly reduced ($P < 0.05$). In the intestine, as the dietary arecoline supplementation level increased, intestinal SOD and CAT activities and GSH content reached their maximum levels compared to the control at 1.0 mg/kg ($P < 0.05$). GPx activity reached maximum levels compared to the control at 1.5 mg/kg in fish ($P < 0.05$).

Table 3
Effects of arecoline supplementation on the growth performance and intestinal growth of adult grass carp.

Item	Dietary arecoline levels, mg/kg diet						*F*-value	*P*-value
	0.0	0.5	1.0	1.5	2.0	2.5		
IBW[1], g/fish	606.93 ± 1.85	609.07 ± 1.22	608.40 ± 2.62	608.53 ± 1.85	606.67 ± 2.44	607.60 ± 0.40	0.77	0.590
FBW[1], g/fish	1,285.87 ± 8.17^{a}	1,389.33 ± 32.40^{b}	1,512.53 ± 22.49^{d}	1,437.33 ± 2.01^{c}	1,390.4 ± 21.78^{b}	1,255.2 ± 21.48^{a}	63.89	0.000
PWG[1], %	111.87 ± 1.99^{a}	128.12 ± 5.71^{b}	148.61 ± 3.61^{d}	136.20 ± 0.44^{c}	129.18 ± 3.08^{b}	106.58 ± 3.54^{a}	60.27	0.000
SGR[1], %/day	1.19 ± 0.02^{a}	1.31 ± 0.04^{b}	1.45 ± 0.02^{d}	1.36 ± 0.01^{c}	1.32 ± 0.02^{b}	1.15 ± 0.03^{a}	61.65	0.000
FI[1], g/fish	1,197.12 ± 1.76^{b}	1,338.65 ± 1.04^{d}	1,510.11 ± 1.05^{f}	1,435.85 ± 1.17^{e}	1,318.79 ± 1.36^{c}	1,178.02 ± 1.65^{a}	2715.04	0.000
FE[1], %	56.71 ± 0.76^{ab}	58.29 ± 2.53^{bc}	59.87 ± 1.45^{c}	57.72 ± 0.09^{abc}	59.43 ± 1.48^{bc}	54.98 ± 1.89^{a}	3.96	0.024
ILI[2], %	157.45 ± 12.98^{a}	176.62 ± 7.27^{a}	195.33 ± 24.61^{b}	169.93 ± 11.52^{a}	167.49 ± 10.40^{a}	166.35 ± 15.81^{a}	4.55	0.003
ISI[2], %	1.37 ± 0.21^{a}	1.78 ± 0.13^{b}	1.98 ± 0.23^{c}	1.69 ± 0.11^{b}	1.27 ± 0.07^{a}	1.42 ± 0.10^{a}	19.10	0.000
Muscle								
Moisture[2], %	75.08 ± 0.68	75.08 ± 0.58	75.35 ± 0.44	75.29 ± 0.49	75.53 ± 0.44	75.03 ± 0.36	0.88	0.507
Crude protein[2], %	17.43 ± 2.22	17.48 ± 1.58	18.68 ± 1.29	18.29 ± 1.51	16.76 ± 1.74	16.08 ± 1.10	1.90	0.124
Crude lipid[2], %	3.73 ± 0.11^{a}	4.35 ± 0.46^{b}	4.32 ± 0.41^{b}	4.50 ± 0.22^{b}	4.33 ± 0.30^{b}	3.82 ± 0.41^{a}	5.12	0.002

IBW = initial body weight; FBW = final body weight; PWG = percent weight gain; SGR = specific growth rate; FI = feed intake; FE = feed efficiency; ILI = intestinal length index; ISI = intestinal somatic index.
[1] Values are means ± SD for three replicate groups, with 25 fish in each group, and mean values within the same row with different superscripts are significantly different ($P < 0.05$).
[2] Values are means ± SD ($n = 6$); mean values within the same row with different superscripts are significantly different ($P < 0.05$).

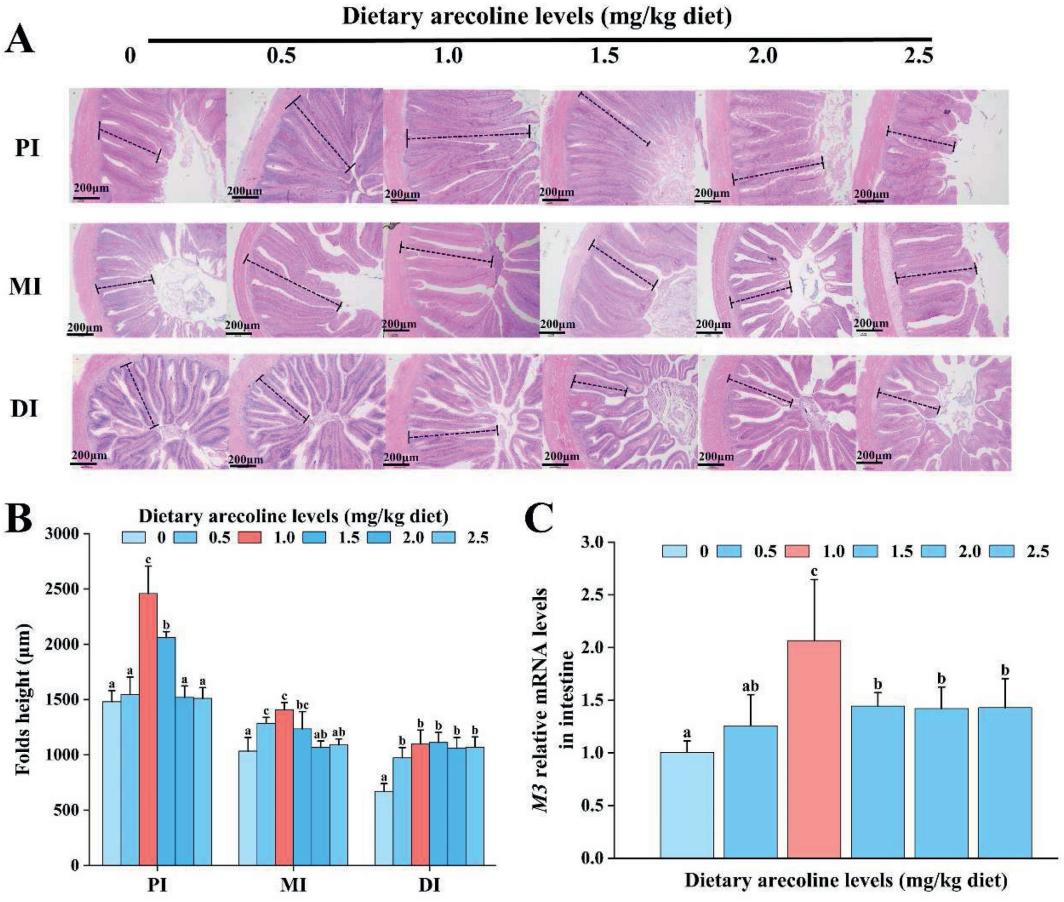

Fig. 1. The histology in the PI, MI and DI of adult grass carp (*Ctenopharyngodon idella*) (H&E staining; scale bar, 200 μm; magnification 40×). (A) The black dotted line indicates the villus height. (B) Intestinal folds height. (C) Relative mRNA levels of muscarinic receptor 3 (*M3*) in the intestine of adult grass carp fed diets containing graded levels of arecoline. Data represent means ($n = 6$) and error bars indicate SD. Different letters above the bars indicate a significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). PI = proximal intestine; MI = mid intestine; DI = distal intestine.

3.5. Influences of arecoline on parameters related to intestinal antioxidants

As displayed in Fig. 3A, in comparison with the control group, the *GPx1a*, *GPx4a*, *CuZnSOD*, *MnSOD*, and *Nrf2* mRNA abundances in the intestine of the optimum arecoline supplementation group (1.0 mg/kg) were significantly increased ($P < 0.05$).

The mRNA abundances of *GPx4b*, *GPx1b*, and *CAT* in the intestine were significantly increased ($P < 0.05$) when dietary arecoline levels rose to 2.0, 1.5, and 1.5 mg/kg diet, respectively. In

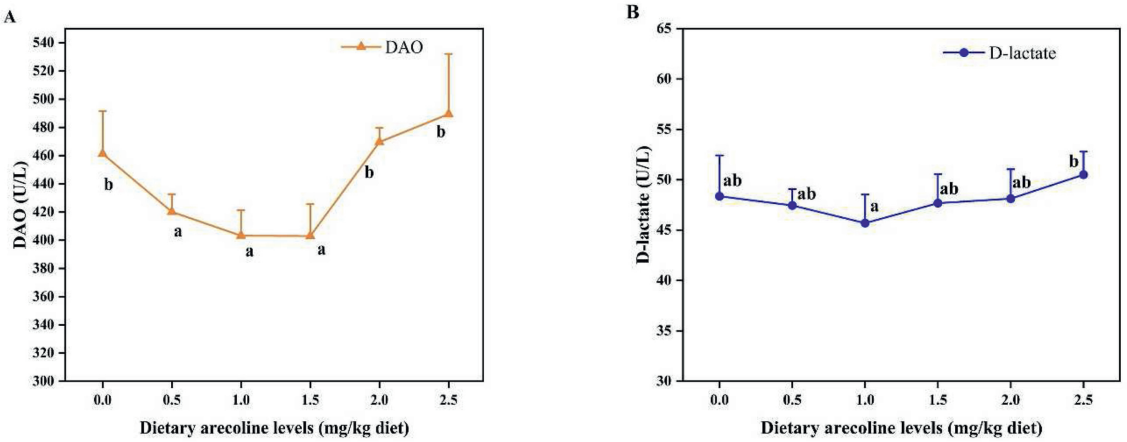

Fig. 2. Effects of dietary supplementation with different levels of arecoline (mg/kg diet) for 9 weeks on indicators of intestinal mucosal permeability in adult grass carp. (A) Diamine oxidase (DAO) activity. (B) D-lactate concentration. Data represent means ($n = 6$) and error bars indicate SD. Different letters indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$).

Table 4
Effects of different levels of arecoline supplementation on the digestion and absorption capacities of adult grass carp.

Item	Dietary arecoline levels, mg/kg diet						*F*-value	*P*-value
	0.0	0.5	1.0	1.5	2.0	2.5		
Hepatopancreas								
Trypsin, U/g tissue	10,960.37 ± 714.77^{ab}	12,045.19 ± 788.49^{c}	14,925.56 ± 412.33^{d}	15,056.48 ± 1082.62^{d}	11,446.67 ± 441.82^{bc}	10,287.04 ± 498.94^{a}	51.78	0.000
Chymotrypsin, U/g tissue	15.91 ± 1.53^{b}	19.63 ± 1.66^{c}	25.72 ± 1.66^{e}	22.00 ± 2.99^{d}	16.58 ± 2.00^{b}	13.54 ± 1.05^{a}	32.86	0.000
Lipase, U/g tissue	1,173.09 ± 83.38^{a}	1,498.17 ± 69.24^{b}	2,218.98 ± 265.02^{c}	2,176.58 ± 276.96^{c}	1,385.10 ± 138.48^{b}	1,060.02 ± 88.94^{a}	48.24	0.000
Amylase, U/g tissue	2,025.89 ± 198.65^{a}	2,504.85 ± 167.94^{b}	3,223.30 ± 162.92^{d}	2,977.35 ± 242.73^{c}	2,828.48 ± 128.61^{c}	1,870.55 ± 130.93^{a}	55.85	0.000
Intestine								
AKP, King unit/g tissue	10.45 ± 0.47^{a}	16.26 ± 0.87^{b}	29.15 ± 0.48^{e}	19.29 ± 0.74^{d}	18.03 ± 1.07^{c}	10.37 ± 0.48^{a}	553.94	0.000
CK, U/g tissue	27.70 ± 2.36^{ab}	33.26 ± 2.37^{c}	35.71 ± 0.77^{c}	34.90 ± 1.62^{c}	29.54 ± 1.73^{b}	25.84 ± 2.73^{a}	23.75	0.000
Na^+,K^+-ATPase, U/g tissue	14.50 ± 0.31^{a}	14.92 ± 0.51^{ab}	17.02 ± 1.11^{c}	15.40 ± 0.18^{b}	14.99 ± 0.24^{ab}	14.39 ± 0.38^{a}	18.25	0.000
γ-GT, U/g tissue	78.42 ± 2.16^{a}	90.08 ± 3.22^{c}	132.92 ± 3.51^{d}	93.00 ± 3.02^{c}	85.51 ± 5.22^{b}	79.65 ± 2.48^{a}	211.02	0.000
SP, ng/g tissue	0.62 ± 0.01^{a}	0.89 ± 0.06^{b}	1.05 ± 0.05^{c}	1.14 ± 0.07^{d}	1.09 ± 0.05^{cd}	0.56 ± 0.02^{a}	166.15	0.000

AKP = alkaline phosphatase; CK = creatine kinase; γ-GT = γ-glutamyl transpeptidase; SP = fish substance P.
Values are means ± SD ($n = 6$); values in the same row with different letter superscripts are significantly different ($P < 0.05$).

Table 5
Effects of graded levels of dietary arecoline on intestinal antioxidant-related indices in adult grass carp.

Item	Dietary arecoline levels, mg/kg diet						*F*-value	*P*-value
	0.0	0.5	1.0	1.5	2.0	2.5		
ROS, %	100.00 ± 4.64^{d}	82.05 ± 6.45^{b}	77.65 ± 3.03^{ab}	74.58 ± 3.45^{a}	90.17 ± 4.62^{c}	101.82 ± 4.43^{d}	38.06	0.000
MDA, nmol/mg protein	18.36 ± 0.69^{c}	4.92 ± 0.32^{ab}	4.71 ± 0.29^{a}	5.35 ± 0.54^{b}	5.16 ± 0.20^{ab}	18.19 ± 0.35^{c}	1502.94	0.000
PC, nmol/mg protein	3.92 ± 0.42^{c}	3.42 ± 0.22^{ab}	3.33 ± 0.27^{ab}	3.14 ± 0.22^{a}	3.53 ± 0.15^{b}	4.10 ± 0.34^{c}	10.04	0.000
SOD, U/mg protein	19.52 ± 0.23^{b}	21.38 ± 0.16^{c}	22.58 ± 0.18^{d}	19.61 ± 0.11^{b}	19.78 ± 0.17^{b}	17.31 ± 0.45^{a}	331.90	0.000
CAT, U/mg protein	1.38 ± 0.10^{a}	2.01 ± 0.29^{b}	3.21 ± 0.22^{d}	2.52 ± 0.08^{c}	1.99 ± 0.28^{b}	1.28 ± 0.10^{a}	78.12	0.000
GPx, U/mg protein	79.71 ± 9.57^{b}	135.49 ± 17.07^{d}	137.83 ± 18.32^{d}	167.79 ± 15.15^{e}	112.07 ± 10.78^{c}	61.20 ± 8.47^{a}	49.91	0.000
GSH, mg/g protein	4.98 ± 0.41^{b}	7.12 ± 0.26^{c}	8.32 ± 0.33^{d}	8.57 ± 0.46^{d}	7.44 ± 0.49^{c}	4.45 ± 0.48^{a}	103.45	0.000

ROS = reactive oxygen species; MDA = malondialdehyde; PC = protein carbonyl; SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; GSH = glutathione.
Values are means ± SD ($n = 6$); values in the same row with different letter superscripts are significantly different ($P < 0.05$).

comparison with the control group, the *Keap1a* mRNA abundance in the intestine of the arecoline supplementation group (1.5 mg/kg) was significantly reduced ($P < 0.05$), but the mRNA abundance of *Keap1b* in the intestine had no significant change. Fish significantly increased the protein levels of cytosolic-Nrf2 and nuclear-Nrf2 in the intestine when they were supplemented with dietary arecoline at 1.0 mg/kg compared with the unsupplemented group ($P < 0.05$) (Fig. 3B and C). At supplementation levels beyond that, the protein levels of cytosolic-Nrf2 and nuclear-Nrf2 showed a decrease.

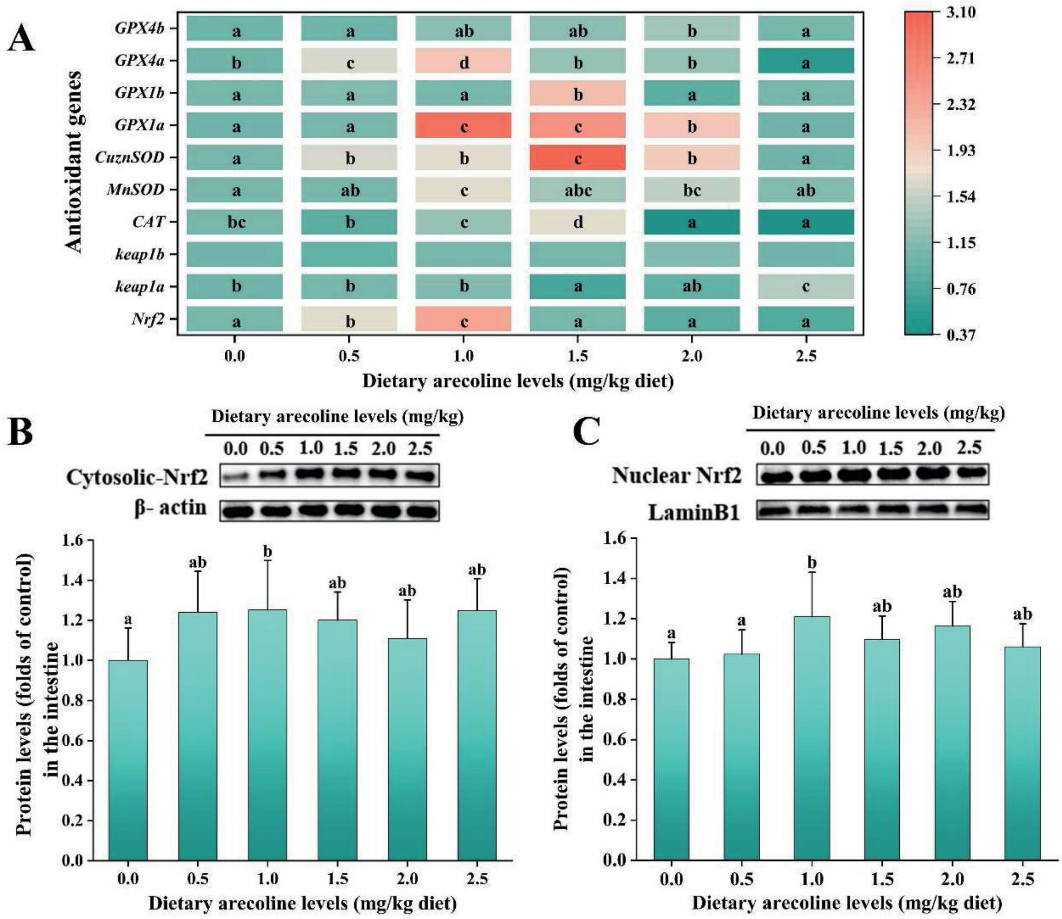

Fig. 3. Effects of dietary supplementation with different levels of arecoline (mg/kg diet) for 9 weeks on the intestinal antioxidant capacity of adult grass carp. (A) Relative expression of genes corresponding to antioxidant enzymes and related signaling factors. (B) Protein expression levels of cytosolic-Nrf2. (C) Protein expression levels of nuclear-Nrf2. Data represent means (n = 6), and error bars indicate SD. Different letters indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). *GPx* = glutathione peroxidase; *CuznSOD* = copper/zinc superoxide dismutase; *MnSOD* = manganese superoxide dismutase; *CAT* = catalase; *Keap1* = Kelch-like ECH-associated protein 1; *Nrf2* = NF-E2-related factor 2.

3.6. Parameters related to intestinal AJC in adult grass carp

3.6.1. Adult grass carp's intestinal TJs relative mRNA levels

The mRNA levels of intestinal TJ proteins are shown in Fig. 4A. In comparison with the control group, *ZO-1*, occludin, claudin-b, claudin-c, claudin-f, claudin-7a, claudin-7b, and claudin-15b mRNA abundances in the intestine of the optimum arecoline supplementation group (1.0 mg/kg) were significantly elevated ($P < 0.05$). The mRNA abundances of claudin-12 and claudin-15a in the intestine were significantly increased ($P < 0.05$) when dietary arecoline levels rose to 2.0 mg/kg diet. However, the mRNA abundance of claudin-11 in the intestine showed no significant change. Arecoline affected the expression of TJ proteins in the intestine, as evidenced by the immunohistochemical results illustrated in Fig. 5A. The immunohistochemistry's cumulative optical density values of ZO-1, occludin, and claudin-3 proteins in the intestine were significantly increased in the group with the dietary arecoline supplementation level of 1.0 mg/kg compared with the unsupplemented group ($P < 0.05$) (Fig. 5B).

3.6.2. Adult grass carp's intestinal AJs relative mRNA levels

The mRNA levels of intestinal AJ proteins are shown in Fig. 4B. The mRNA abundances of E-cadherin, β-catenin, α-catenin, nectin, and afadin in the intestine were significantly increased ($P < 0.05$) when dietary arecoline levels were 1.0, 1.0, 2.0, 2.0, and 2.0 mg/kg diet, respectively.

3.6.3. Influences of arecoline on parameters related to intestinal AJC-relevant signaling molecules

The levels of intestinal AJC-related signaling molecular parameters are presented in Fig. 6A. The mRNA abundances of *RhoA*, *ROCK*, *MLCK*, and *NMII* in the intestine were significantly ($P < 0.05$) reduced when dietary arecoline levels rose to 1.0, 0.5, 0.5, and 1.5 mg/kg diet, respectively. In comparison with the control group, GTP-RhoA, ROCK, and MLCK protein expression levels in the intestine of the optimum arecoline supplementation group (1.0 mg/kg) were significantly decreased ($P < 0.05$), as presented in Fig. 6B and C.

4. Discussion

4.1. Dietary appropriate arecoline supplementation enhanced performance and muscle nutrition composition in fish

This study demonstrated that dietary arecoline supplements at 0.5 to 2.0 mg/kg enhanced the performance (such as PWG, FI, SGR, and FE) of adult grass carp. These results were consistent with the study of Wenchang broiler chicks (Wang et al., 2018). The muscle is arguably the most important edible part of a fish and it is the main

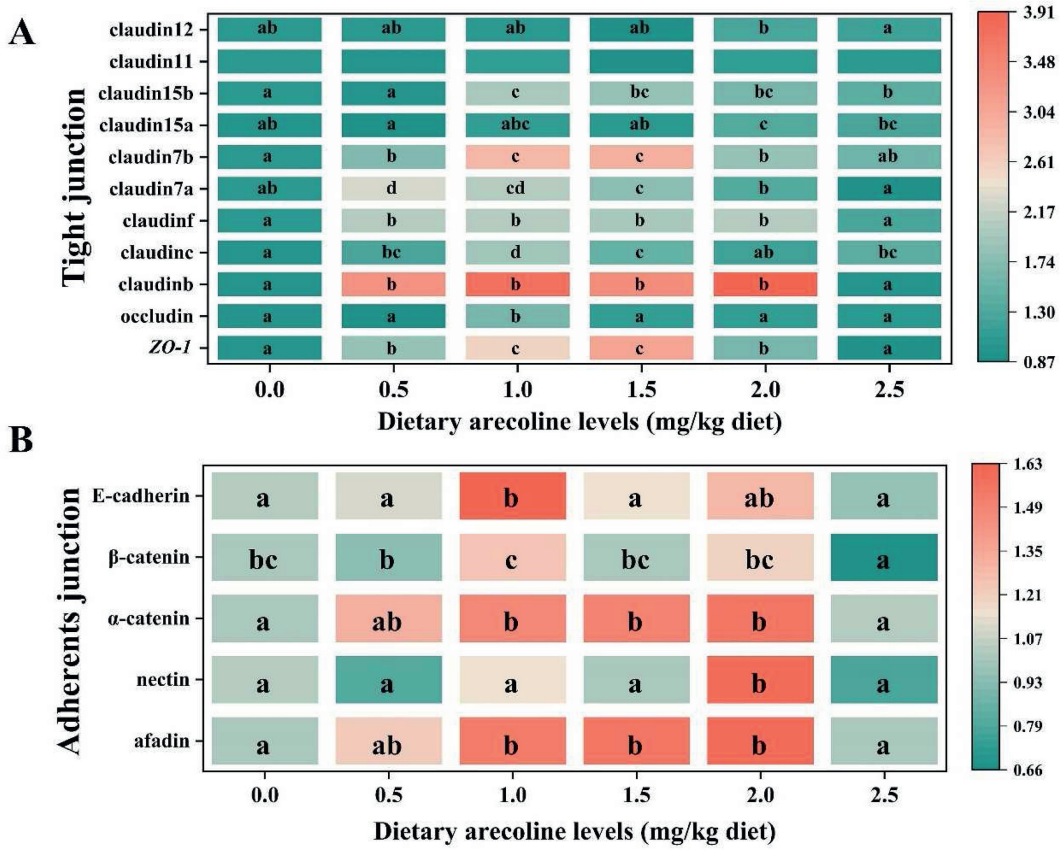

Fig. 4. Effects of dietary supplementation with different levels of arecoline (mg/kg diet) for 9 weeks on the relative mRNA expression of TJ-related and AJ-related proteins in the intestine of adult grass carp. (A) Relative mRNA expression of TJ-related proteins. (B) Relative mRNA expression of AJ-related proteins. Values are means ± SD ($n = 6$). Different letters indicate significant differences (ANOVA and Duncan's multiple-range test, $P < 0.05$). TJ = tight junction; AJ = adherent junction; *ZO-1* = zonula occluden-1.

site of nutritional deposition (Mommsen, 2001), including proteins and lipids. In our study, compared with the untreated group, dietary arecoline supplementation at 1.0 mg/kg or 0.5 to 2.0 mg/kg tended to increase the crude protein content or enhance the crude lipid content of muscle in adult grass carp, suggesting that the appropriate level of arecoline may affect the nutritional value of fish muscle. We speculated that this result may be related to choline. In a mouse brain study, arecoline increased choline levels (Patterson and Kosh, 1994). At the same time, dietary choline increased the protein and lipid contents of fish (Wu et al., 2011). The above evidence supports our hypothesis, but it remains to be tested.

4.2. Dietary appropriate arecoline supplementation improved intestinal function in fish

This study demonstrated that dietary arecoline supplements at 0.5 to 2.0 mg/kg enhanced the intestinal growth (such as ILI, ISI, and fold height) of adult grass carp. In addition, the indices ILI, ISI, and fold height reflect that intestinal development is closely related to fish growth (Wu et al., 2011). In our study, appropriate levels of arecoline supplementation at 1.0 mg/kg increased ILI, ISI, and fold height, suggesting that appropriate levels of arecoline enhanced fish intestinal growth. The intestinal growth of fish is closely related to its digestive capacity. In our study, compared with the control group, dietary arecoline supplements at 0.5 to 2.0 mg/kg enhanced hepatopancreas trypsin, chymotrypsin, lipase, and amylase activities, indicating that the appropriate level of arecoline increased the digestive capacity of fish. Increased digestive enzyme activities by arecoline may be associated with elevated M3 receptor mRNA levels. The G protein-coupled receptor superfamily member M3 receptor increased the release of trypsin and bicarbonate (Gautam et al., 2008; Tolaymat et al., 2019). Our results revealed that dietary arecoline supplements at 1.0 to 2.5 mg/kg increased the mRNA level of the M3 receptor in the intestine. Moreover, the activities of brush boundary enzymes affected the ability of the intestine to absorb nutrients (Zhang et al., 2022). We discovered that dietary arecoline supplementation at 0.5 to 2.0 mg/kg increased the activities of brush border enzymes in the intestine (AKP, Na^+,K^+-ATPase, CK, γ-GT). Increased brush border enzyme activities by arecoline might be associated with increased SP content. Research in the rat intestine showed that SP promoted gastrointestinal peristalsis and enhanced the transport of gastrointestinal substances (Silkoff et al., 1988). We discovered that dietary supplementation with arecoline at 0.5 to 2.0 mg/kg increased the content of fish substance P in the intestine. In addition, fish growth requires a healthy intestine, which is determined by the structural integrity of the intestine (Jutfelt, 2011; Sundh and Sundell, 2015). Therefore, the effect of dietary arecoline on intestinal structural integrity will be the subject of our next study.

4.3. Dietary appropriate arecoline supplementation improved the structural integrity in fish intestine

Intestinal structural integrity was determined by phenotypic indicators such as variations in serum DAO activity and D-lactate concentration in grass carp, which represented the degree of intestinal mucosal permeability (Kong et al., 2017). In our study, compared with the untreated group, dietary arecoline supplementation at 0.5

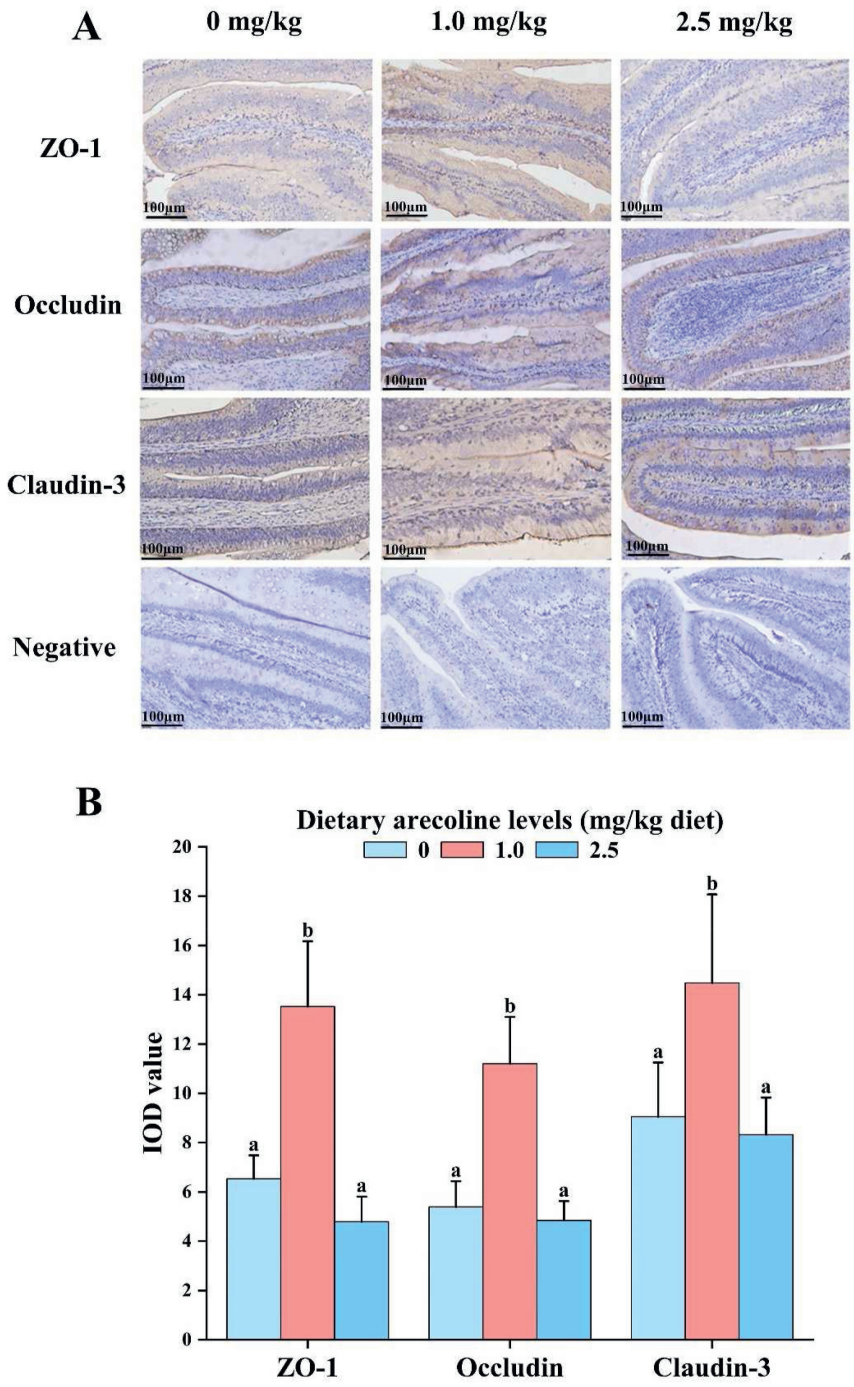

Fig. 5. Immunohistochemical analysis of the effect of dietary supplementation with different levels of arecoline (mg/kg diet) for 9 weeks on the protein expression of tight junction-related proteins in the intestine of adult grass carp (magnification 200×, scale bar = 100 μm). (A) Positive expression maps of ZO-1, occludin and claudin-3 proteins. Negative, the negative control group. (B) Quantitative values of positive regions displayed by Image Pro Plus 5.0. Data represent means ($n = 6$) and error bars indicate SD. Different letters above the bars indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). IOD = immunohistochemistry's cumulative optical density; ZO-1 = zonula occluden-1.

to 1.5 mg/kg or at 1.0 mg/kg reduced serum DAO activity or tended to reduce D-lactate concentration in adult grass carp. The above results demonstrated that dietary supplementation with arecoline enhanced intestinal structural integrity. The structural integrity of the fish intestine, both cellular (e.g., antioxidant capacity) and intercellular (e.g., AJC), determined the intestinal health of the fish. Therefore, we investigated the influences of dietary arecoline supplementation on intestinal antioxidant capacity and its potential mechanism.

4.3.1. Dietary arecoline supplementation improved the antioxidant capacity of the fish intestine

Some studies have shown that excessive ROS production might result in oxidative damage to biomolecules (e.g., proteins, lipids, and DNA) (Liu et al., 2018; Zhao et al., 2016a). The antioxidant system, consisting of antioxidant enzymes such as SOD, CAT, and GPx and nonenzymatic substances such as GSH (Jiang et al., 2018), can reduce oxidative damage in fish (Martínez-Álvarez et al., 2005). Antioxidant enzyme activities have been demonstrated to be

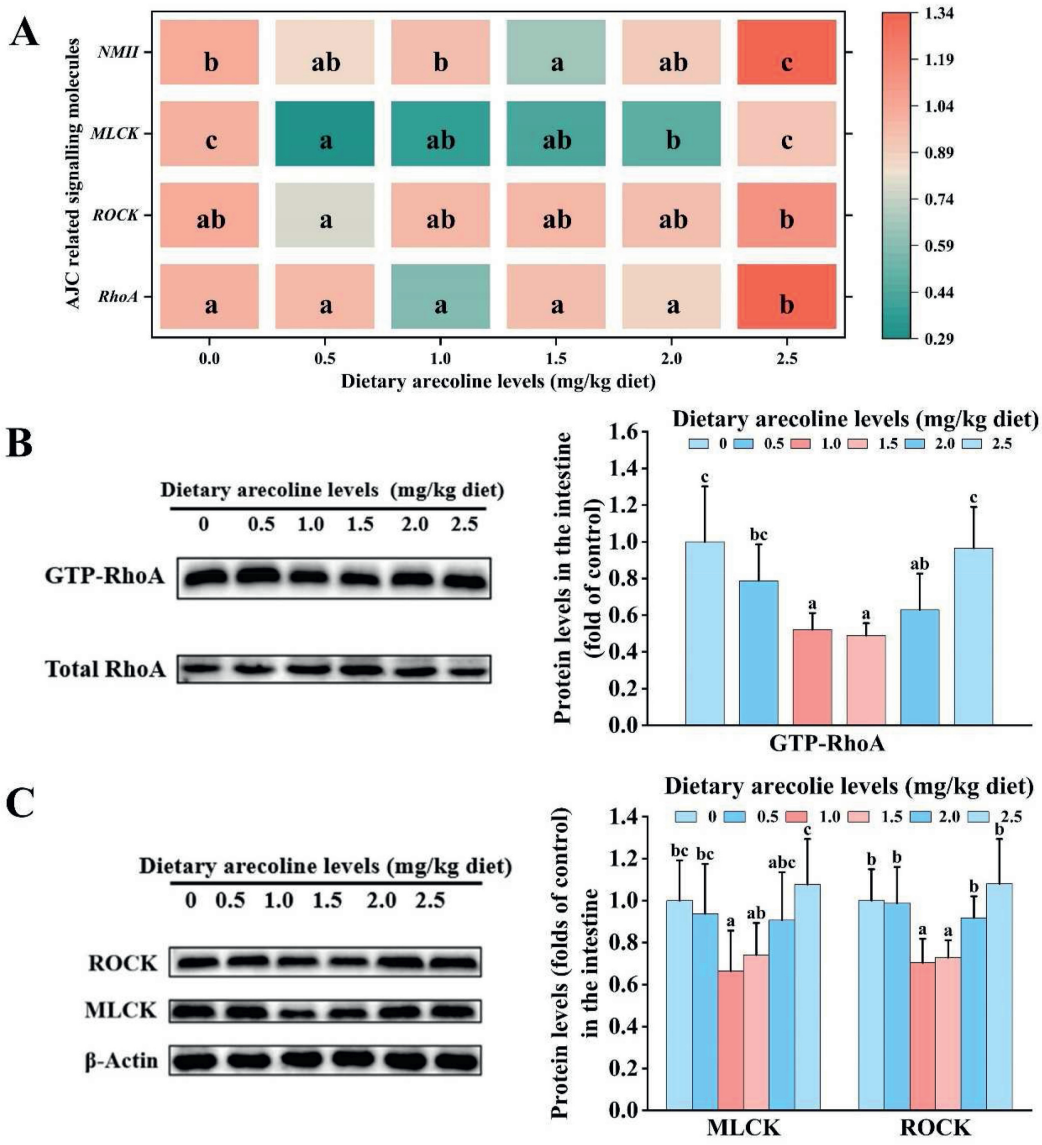

Fig. 6. Effects of dietary supplementation with different levels of arecoline (mg/kg diet) for 9 weeks on the relative expression of AJC-related signaling molecules in the intestine of adult grass carp. (A) Relative mRNA expression of AJC-related signaling molecules. (B) Relative protein expression of GTP-RhoA. (C) Relative protein expression of ROCK and MLCK. Data represent means ($n = 6$) and error bars indicate SD. Different letters indicate significant difference (ANOVA and Duncan's multiple-range test, $P < 0.05$). AJC = apical junction complex; *RhoA* = Rho family GTPase; *ROCK* = Rho associated protein kinase; *MLCK* = myosin light-chain kinase; *NMII* = non-muscle myosin II; GTP-RhoA = GTP-Rho family GTPase.

correlated with their levels of mRNA expression (Lambertucci et al., 2007). This study found that dietary arecoline supplementation at 0.5 to 2.0 mg/kg reduced the contents of ROS, MDA, and PC, increased SOD, CAT, and GPx activities as well as GSH content, and increased the mRNA levels of corresponding antioxidant enzymes compared with the unsupplemented group in the intestine of adult grass carp, suggesting that the appropriate amount of arecoline might prevent oxidative damage, including improved antioxidant enzyme activities and related mRNA levels in fish intestine. Additionally, the transcript levels of antioxidant enzyme genes were partially regulated by the Nrf2 signaling pathway (Na and Surh, 2005). The Nrf2 nuclear translocation level was positively correlated with the activation of the Nrf2 signaling pathway, which might be due to the upregulation of the *Nrf2* mRNA level and the downregulation of the *Keap1* mRNA level (Giudice et al., 2010; Liu et al., 2017). This study found that dietary arecoline supplementation at 0.5 to 1.5 mg/kg increased Nrf2 protein and mRNA levels, and decreased *Keap1a* (non-*Keap1b*) mRNA levels. Therefore, increased mRNA levels of antioxidant enzyme activities in the fish intestine by the appropriate level of arecoline might be associated with the upregulated of the Keap1a/Nrf2 (non Keap1b) signaling pathway.

In contrast, we found that the appropriate level of arecoline downregulated the mRNA levels of *Keap1a* (not *Keap1b*) in the intestine. This result might be related to choline. In a mouse brain study, arecoline increased choline levels (Patterson and Kosh, 1994). Meanwhile, dietary choline reduced the mRNA levels of *Keap1a* (not *Keap1b*) in the intestine of grass carp (Zhao et al., 2016b). The above results support our hypothesis. However, this hypothesis deserves further study.

The above results demonstrated that arecoline increased the antioxidant enzyme activities and the corresponding mRNA levels, as well as the expression of the important regulatory signaling molecule Nrf2. In addition, fish AJCs are crucial for preserving the

structural integrity of the intestine. Consequently, we next investigated the link between fish intestinal AJC and dietary arecoline supplementation as well as any potential mechanisms.

4.3.2. Dietary arecoline enhanced intestinal AJC, partly by inhibiting RhoA/ROCK pathway in fish

ZO-1, occludin, and claudin are three key tight junction proteins, that are important AJC members and essential for maintaining the connection between intestinal epithelial cells (González-Mariscal et al., 2008). We discovered that dietary arecoline supplementation enhanced TJs (*ZO-1*, occludin, claudin-b, -c, -f, -7a, -7b, -15a, -15b, and -12) mRNA levels compared with the unsupplemented group. Meanwhile, the immunohistochemistry results further showed that dietary arecoline supplementation increased the positive expression of ZO-1, occludin, and claudin-3 in the intestine in comparison with the control group. This suggested that appropriate arecoline supplementation might sustain the integrity of the intestinal tight junction structure of fish.

Interestingly, our study found that the effect of dietary arecoline on the mRNA levels of the three TJ proteins was different. Previous research on grass carp demonstrated that intestinal TJs might be improved by lowered mRNA levels of the pore-forming TJs claudin-12 and claudin-15 (Li et al., 2017). However, increased mRNA levels of claudin-12 and claudin-15 were found in our study, which may be explained as follows. First, the mRNA level of claudin-15 was increased by dietary arecoline supplementation at 1.0 to 2.5 mg/kg. Increased claudin-15 mRNA levels induced by arecoline might be associated with the function of claudin-15 itself and increased Na^+ concentration. A study showed that the selective paracellular channel composed of claudin-15 changed from a cation (Na^+) to an anion (Cl^-) (Colegio et al., 2002). Arecoline stimulated the activation of the muscarinic receptor (Liu et al., 2016), which increased the membrane permeability of mouse pancreatic B cells to Na^+, and more Na^+ entered the cells (Gilon and Henquin, 1993). Therefore, we believed that increased levels of claudin-15 mRNA in the fish intestine by arecoline might be connected with the supplemented intracellular Na^+ concentration, but this conjecture needs further verification. Second, the mRNA level of claudin-12 was increased by the appropriate arecoline level at 2.0 mg/kg in this study. Increased claudin-12 mRNA expression by arecoline might be associated with the function of claudin-12 itself and increased Ca^{2+} concentration. It was reported that claudin-12 was mainly used as a Ca^{2+} selective channel to promote the absorption and transport of Ca^{2+} in the intestine (Fujita et al., 2008; Günzel and Yu, 2013). A previous study showed that arecoline increased the Ca^{2+} concentration in the human hepatoma cell line HA22T/VGH (Cheng et al., 2017). Hence, we suggested that the appropriate level of arecoline increased the mRNA level of claudin-12 in the intestine, which may be related to supplementing the intracellular Ca^{2+} concentration, but this conjecture needs further validation. Third, dietary arecoline supplementation did not influence the claudin-11 mRNA level in the fish intestine, which might be connected with the effect of arecoline on Na^+,K^+-ATPase similar to the function of claudin-11 itself. Research in the rat brain showed that arecoline activated Na^+,K^+-ATPase (Von Schwarzenfeld et al., 1976). Na^+,K^+-ATPase regulates the active transport of Na^+ in most higher eukaryotes (Kaplan, 2002). At the same time, claudin-11 selectively reduced the permeability of cations, especially Na^+ (Fromm et al., 2017). The above evidence supports our hypothesis, but it has yet to be verified.

A previous study demonstrated that increasing AJ (such as E-cadherin) mRNA levels enhanced AJ in the intestine of Atlantic salmon (*Salmo salar* L.) (Hu et al., 2016). This study found that dietary arecoline at 1.0 to 2.0 mg/kg increased AJ mRNA levels, including E-cadherin, α-catenin, β-catenin, nectin, and afadin, indicating that appropriate arecoline supplementation might enhance the integrity of AJs in the intestine.

Rho can send signals through the ROCK signaling pathway, and promote the destruction of cell connections by generating contractile force (Sahai and Marshall, 2002). We discovered that dietary arecoline at 0.5 to 1.5 mg/kg decreased the mRNA levels of *RhoA*, *ROCK*, *MLCK*, and *NMII* and the protein levels of GTP-RhoA, ROCK, and MLCK in the intestine. Further correlation analysis revealed that GTP-RhoA protein levels were positively correlated with ROCK and MLCK protein levels (as presented in Table S3), suggesting that inhibition of the ROCK/MLCK signaling pathway by arecoline might be related to reduced GTP-RhoA protein expression.

The above results suggested that dietary arecoline increased the mRNA levels of TJ-related proteins (*ZO-1*, occludin, claudin-b, -c, -f, -7a, -7b, -15a, -15b, and -12) and AJ-related proteins (E-cadherin, α-catenin, β-catenin, nectin, and afadin), which might be associated with partial inhibition of the RhoA/ROCK/MLCK/NMII signaling pathway.

4.4. Excessive arecoline decreased fish feed intake, partial antioxidant capacity and AJC

Arecoline is a pyridine derivative, that has many pharmacological effects and some potential side effects (Liu et al., 2016). This study found that the excessive supplementation of arecoline at 2.5 mg/kg reduced feed intake, partial antioxidant capacity (SOD and GPx activities, GSH content and mRNA expression of *GPx4a*, *CAT*, *Keap1a*) and partial AJC (mRNA expression of β-catenin, *NMII*, and *RhoA*) compared with the no-supplementation group. This result might be explained by the following possible reasons. First, the reduction in feed intake might be connected with the destruction of the intestinal structure. The study of the intestinal structure of rats showed that a high dose of arecoline caused pathological changes (such as degeneration and necrosis) (Wei et al., 2015). The intestine is an important place for animals to digest and absorb nutrients (Jiang et al., 2009). Damage to the intestinal structure may result in decreased digestion and absorption in animals, which ultimately leads to reduced feed intake. Second, the reduced antioxidant capacity may be associated with excess ROS in the intestine. The study showed that high-dose arecoline activated the NADPH enzyme, which increased the ROS content in cells (Shih et al., 2010). Thirdly, high-dose arecoline damaged parts of the AJC, which might be related to the upregulation of the phosphorylation level of proto-oncogene (JunD). It was shown that arecoline upregulated the phosphorylation level of JunD, which inhibited the expression and recruitment of ZO-1 and E-cadherin on the cell membrane, thus destroying the structural integrity of TJs and AJs (Ghosh et al., 2021). The above three reasons might lead to the decline in fish feed intake, partial antioxidant capacity, and AJC, but this speculation needs further verification.

4.5. Appropriate supplementation of arecoline in the adult grass carp diet

Based on PWG (Fig. 7A), lipase activity (Fig. 7B), serum DAO activity (Fig. 7C) and PC (Fig. 7D), the appropriate arecoline supplementation levels were estimated to be 1.20, 1.21, 1.07, and 1.19 mg/kg, respectively. We found that the appropriate supplementation of arecoline determined by the DAO was slightly lower than that determined by the PWG. This reason may be related to the destruction of the intestinal structure by high-dose arecoline. A study showed that high doses of arecoline caused degeneration, necrosis and shedding of intestinal epithelial cells in rats (Wei et al., 2015). The intestinal organs are dose sensitive. A study found that PWG reflected the overall growth of fish, and DAO was a phenotypic

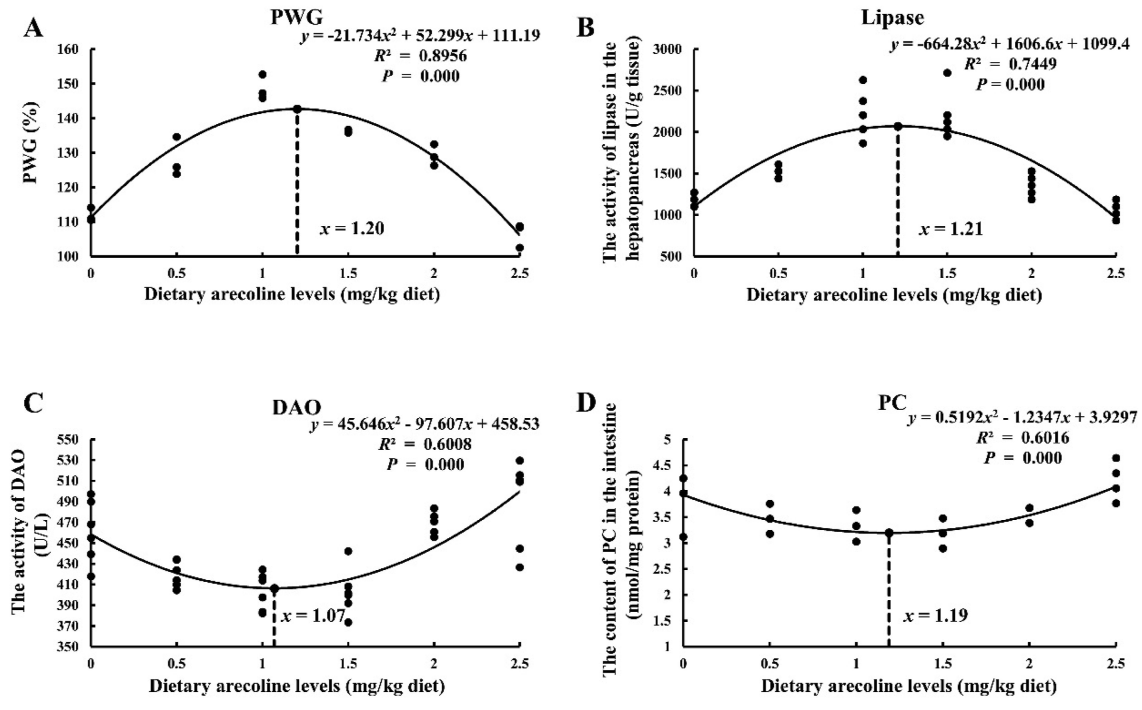

Fig. 7. Quadratic regression analysis for the adult grass carp fed diets with graded levels of arecoline (mg/kg) for 9 weeks. (A) Percent weight gain (PWG), (B) lipase activity, (C) diamine oxidase (DAO) activity, and (D) protein carbonyl (PC) content.

indicator of intestinal structural integrity (Kong et al., 2017). The above evidence suggests that supplementation with low levels of arecoline might protect the structural integrity of the intestine.

5. Conclusions

In summary, the current study found for the first time that dietary appropriate arecoline supplementation enhanced growth performance, digestion and absorption capacities, and improved the intestinal structural integrity of fish. There are some potential links between arecoline and intestinal digestive and absorptive capacities, intestinal antioxidant capacity, and AJC. We discovered that appropriate arecoline supplementation increased the digestion enzyme activities in fish might be connected with the up-regulation of *M3* mRNA level; increased brush border enzyme activities in fish might be related to the increased fish substance P content; increased the antioxidant enzyme activities and corresponding mRNA levels in the fish intestine might be related to the up-regulation of Keap1a/Nrf2 (non Keap1b) signaling pathway; increased the mRNA levels of TJ and AJ related proteins might be associated with the inhibition of RhoA/ROCK signaling pathway (RhoA/ROCK/MLCK/NMII). Differently, dietary arecoline supplementations also increased the mRNA levels of the fish intestinal pore-forming tight junction proteins claudin-12 and claudin-15, and did not have influence on the mRNA levels of claudin-11. Finally, according to the growth indicators (PWG), lipase activity, intestinal mucosal permeability (DAO), and antioxidant indexes (PC), the appropriate arecoline supplementation of adult grass carp (608 to 1512 g) was calculated to be 1.20, 1.21, 1.07, and 1.19 mg/kg, respectively.

Author contributions

Na Yao: Formal analysis, Manuscript writing. **Lin Feng:** performed conceptualization, funding acquisition and supervision. **Weidan Jiang:** performed data curation, validation, project administration and writing-review & editing. **Pei Wu:** performed conceptualization, methodology, validation, data curation and project administration. **Hongmei Ren:** Management. **Hequn Shi, Ling Tang and Shuwei Li:** performed resources. **Caimei Wu** and **Hua Li:** participated in the design and coordination of the study. **Xiaoqiu Zhou:** performed conceptualization, methodology, supervision, funding acquisition and resources. **Yang Liu:** Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

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