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Research Article

Evaluation of *Yarrowia lipolytica* lipase 2 on growth performance, digestive enzyme activity and nutritional components of Russian sturgeon (*Acipenser gueldenstaedtii*)

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Abstract:
The effects of *Yarrowia lipolytica* lipase 2 (YLL2) on growth performance, digestive enzyme activity and muscle nutritional components of Russian sturgeon (*Acipenser gueldenstaedtii*) were evaluated in a 56-day feeding experiment. Four experimental groups of fish with mean weight of 4.465 g were used in the study: Control group (0-control), Group 1 (1.0 g/kg YLL2), Group 2 (1.5 g/kg YLL2) and Group 3 (2.0 g/kg YLL2), respectively, with three repetitions. Fish fed diets with YLL2 at 1.0 g/kg showed the highest growth compared with that of the other groups (p<0.05). For nutrient apparent, docosahexenoic acid (DHA) and eicosapentenoic acid (EPA) concentrations in fish muscle of group which fed with 1.0 g/kg YLL2 increased 5.05% and 7.45% respectively, compared with that of control group (p<0.05). Considering the digestive enzyme activity, lipase and protease activity in liver, spleen and intestine of G1 fish was also significantly enhanced compared with that of control group (p<0.05). While, significant increase of amylase activity in intestine was only observed in fish treated with 2.0 g/kg YLL2. The present results suggested that YLL2 (1.0 g/kg) could be used as potential diet additives for aquaculture Russian sturgeon.

Keywords: *Yarrowia lipolytica* lipase, Sturgeon, Nutritional components, Growth performance, Digestibility

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Introduction

Since 2000, China has been the world's largest developing country, the world's largest sturgeon culture region (Wei *et al*., 2010). Previous report has shown that the dominant cultured Russian sturgeon with high market value, accounting for 10% of total production in China (Shen *et al*., 2014). The nutritional requirements of protein (Stuart *et al*., 1989), trace elements (Wang *et al*., 2015), carbohydrates (Hung *et al*., 1989) and lipids (Şener *et al*., 2005) have been studied in a variety of sturgeons. However, the available information on sturgeon about dietary lipid is limited, which is an important factor in intensive fish farming and successful mass production (Luo *et al*., 2015). In recent years, dietary administration of probiotics and enzymes aresuggested as an environmental friendly alternative approach to enhance immune response and increase the growth performance of fish (Luo *et al*., 2010; Cerezuela *et al*., 2012; Zheng *et al*., 2019a).

Yarrowia lipolytica lipase 2 is stable and highly active in test meals and increases fat absorption in an animal model of pancreatic exocrine insufficiency. It is also widely used in wastewater treatment, food processing, medicine and other industrial fields (Schmid and Verger, 1980). YLL2 has been reported to have various beneficial properties when supplementing in animal, including promoting the secretion of digestive enzymes (Yu *et al*., 2010; Zheng *et al*., 2019b). More interestingly, YLL2 could efficiently hydrolyze the crude fish oil to produce polyunsaturated fatty acids, especially docosahexenoic acid (DHA), which would improve growth performance, nutritional components and overall quality of fish (Luo *et al*., 2015; Li *et al*., 2013). Interestingly, previous reports has shown that a positive effect of Artemia DHA proportions on growth and survival of the Persian sturgeon, and demonstrated that larvae of this species require a high ratio of dietary DHA to EPA (Hafezieh *et al*., 2010). Luo *et al*. (2017) has demonstrated that n-3 LC-PUFA can improve reproductive performance of male and female broodstock of Siberian sturgeon and the quality of their offspring, but n-3 LC-PUFA enrichment of the diet is more critical for female broodstock than for male.

Materials and methods

Russian sturgeons

Russian sturgeon (average weight 4.465 g) purchased from Amur Caviar Company Ltd. Yun Nan province, China, was raised in an 12 indoor plastic tanks (volume: 880 L; radius: 0.75 m; height: 0.5 m). 1200 juvenile fish were randomly allocated into 4 groups with 100 fish in each group with three repetitions. The average stocking density was 0.5 kg/m^3. The water was supplied from Qiantang River (Hangzhou, China) and filtered before used. During the growth period, water temperature ranged from 12ºC and 14ºC, Ammonia-N was<0.40 mg L^{-1},

dissolved oxygen was above 5.0 mg L^{-1}, and pH was around 7.5. Fish were fed to apparent satiation by hand three times (08:00, 12:00, 18:00) under the natural photoperiod for 56 days. The water was allowed to flow into the each pond at a rate of 36 L h^{-1}.

Yarrowia lipolytica lipase 2(YLL2)

YLL2 was prepared in our lab (Qiao *et al*., 2018; Yan *et al*., 2018). The yeast strain was cultured with reinforced medium (10 g/L Yeast Extract, 20 g/L Peptone, 50 g/L Sucrose) in 1000 mL conical flask for 84 h at 28ºC. After centrifugation at 5000×g for 30 min at 4°C, the supernatant was dried with corn starch as carrier material ($W_{products}$: W_{starch} =1:5) in spray drier (input temperature of 150ºC, output temperature of 80ºC, evaporation capacity 2 L/h). The activity of YYL2 dried powder was 4125 U/mg, assayed according to published method (Qiao *et al*., 2018; Yan *et al*., 2018; Zheng *et al*., 2019b).

Diet

Experimental diets were prepared by combining commercial sturgeon feed with different concentration of YYL2 dried powder. The ingredients of sturgeon feed are presented in Table 1. Four basal diet was supplemented with different levels of YLL2 as follows: Control group (Control), Group 1 (1.0 g/kg), Group 2 (1.5 g/kg) and Group 3 (2.0 g/kg). Briefly, the feed was ground into powder, and desired concentration of YLL2 dried powder was added, then mixed with tap water. After pre-incubation for 1 h, the mixture powder was made again into pellets.

Table 1: Ingredients and proximate analysis of experimental diets.

Ingredients (% dry matter)	Control	Group 1	Group 2	Group 3
Fish meal	47	47	47	47
Soybean meal	20	20	20	20
Fish oil	4	4	4	4
Wheat flour	16	15.9	15.85	15.8
Vitamin premix	0.5	0.5	0.5	0.5
Mineral premix	0.5	0.5	0.5	0.5
Ascorbyl-2-polyphosphate	1	1	1	1
HJ-1 Binder	2	2	2	2
Sodium propionate	0.01	0.01	0.01	0.01
Cellulose	8.99	8.99	8.99	8.99
YYL2	0	0.1	0.15	0.2
Chemical analyses (%)				
Moisture	10.98	10.64	10.71	10.47
Crude protein	37.21	37.09	37.25	37.49
Crude fat	9.11	9.32	9.41	9.47
Ash	8.74	9.01	8.93	9.05

Fish meal: Fu Shen Fish meal Co., Qingdao, China; Soybean meal: Yi Hai Kerry Investment Company Limited, Shandong, China; Fish oil: Heng Feng Feed Co., Ltd., Cangzhou, China; Wheat flour: Gu Chan Group, Beijing, China; Vitamin premix (mg or IU per kg diet): retinylacetate 10,000 IU; cholecalciferol 1000 IU; all-rac-a-tocopheryl acetate 30 IU; menadione nicotinamide bisulfite 7; Thiamine hydrochloride 6; riboflavin 3; pyridoxine hydrochloride 12; D-calcium pantothenate 30; niacin 50; biotin 1; folic acid 6; cyanocobalamine 0.03; Mineral mixture (mg per kg diet): $Ca(H_2PO_3)_2{\cdot}H_2O$, 1000; $FeSO_4{\cdot}7H_2O$ 40; $ZnSO_4{\cdot}H_2O$ 100; $MnSO_4{\cdot}H_2O$ 40; $CuSO_4{\cdot}5H_2O$ 2; $CaIO_3{\cdot}6H_2O$ 3; Na_2SeO_3 0.05; $CoSO_4$ 0.05.Ascorbyl-2-polyphosphate: Yi meng si chemical technology Co., Ltd., Shanghai, China; Betaine: Sigma Aldrich, USA; HJ-1 Binder: Jia He material Co., LTD, Shandong, China; Sodium propionate: Sangon Biotech Co., Ltd., Shanghai, China; Cellulose: Sangon Biotech Co., Ltd., Shanghai, China.

Growth performance

All fish were counted at the beginning and at the end of the experiment. Dead fish were removed and recorded daily. At the end of feeding trial, 10 fish from each tank were randomly selected and anaesthetized with 0.1 g L^{-1} MS-222. Five fish from each plastic tank were used for the analysis of DHA and EPA concentrations and the essential amino acid content.

The growth performance of sturgeons fed with different experimental diets was calculated at the end of the feeding trial, based on the following formulae:

Determination of WG, SGR, FCR

Total number of fish was counted and mean body weight of fish was measured. Based on recording the weight of each fish and feed cost, specific growth rate (SGR), feed conversion ratio (FCR), and weight gain (WG) were calculated using the following equations:

SGR (specific growth rate, % d^{-1})=100×[ln (final body weight)- ln (initial body weight)]/days

FCR (feed conversion ratio)= FI/(FBW–IBW)

where IBW is the initial body weight (g/fish), and FBW is the final body weight (g/fish). FI is feed intake and WG is the weight gain (Li *et al*., 2018).

Concentration of DHA and EPA

Concentrations of DHA and EPA in total lipid extract of Russian sturgeon muscle were determined in present study. The total lipid was extracted by dichloromethane method (Folch *et al*., 1957; Liu *et al*., 2017) and the content of total lipid was determined in the fresh fish muscle. Fatty acid methyl ester was synthesized from boron trifluoride-methanol (Shantha *et al*., 1990) by acid-catalyzed total lipid methylation and analyzed by Thermo-Science Trace GC-UV gas chromatograph with Agilent INNOWax quartz capillary column (30 m, coating thickness 0.2 mm). High purity helium was used as carrier gas in constant pressure mode, and the pressure was 54 kPa, and the shunt ratio was 25:1. The injection temperature was 230ºC, the column temperature was raised from 140ºC to 210 ºC at 3 ºC /min, and the whole analysis process was maintained at 210ºC for 10 min, detector temperature was 250ºC (Shantha *et al*., 1990). The fatty acid content in Russian sturgeon muscle was determined by using artificial fatty acid as standard.

Content of essential and flavor amino acids

To evaluate the content of essential and flavor amino acids in Russian sturgeon muscle after 56 days feeding with YYL2 , the fish muscle was lyophilyzed and defatted to determine amino acids composition according to previous method (Badiani *et al*., 1996).

Measurement of digestive enzyme activity

At the end of the feeding experiment (56 days), the activities of digestive enzymes (lipase, protease and amylase) were measured by ELISA kit (Mlbio®, China). Five fish were dissected randomly from each group, and their liver, spleen and large intestine were completely homogenized in 0.05 m PBS (pH 7.4) solution and centrifuged at 4 ºC for 45 min at 15000×g. The supernatant was used as crude enzyme source for enzyme-linked immunosorbent assay (ELISA) of lipase, protease and amylase activity, respectively. The absorbance (OD) of each hole was measured sequentially by blank zeroing and 450 nm wavelength. The standard curve was prepared, and the corresponding enzyme activity was determined from the standard curve according to the OD value of the sample (Kiszonas *et al*., 2018).

Statistical analysis

The statistical analysis was performed using Statistical Product and Service Solution (SPSS) software (Version 20.0; SPSS, Inc). The variances were analyzed by applying One-way analysis of variance (ANOVA), and the differences were analyzed with Duncan's multiple range test (DMRT). The results were given as mean ± S.D., and differences were considered significant when $*p<0.05$ and $**p<0.01$.

Results

Growth performance

The growth performance of Russian sturgeon fed the diets containing alternative supplementation over a period of 56 days are shown in Table 2. There was a statistically significant increase of Group 1 in the specific growth rate (SGR), weight gain (WG), final weight (FW) compared with that of control ($p<0.05$; Table 2). The highest SGR and WG values were observed in fish fed diet containing 1.0 g YLL2 /kg. However, no significant differences in these parameters were observed in fish fed 2.0 g YLL2 /kg feed.

Content of DHA/EPA and essential amino acids in fish muscle

After 56 days of feeding trial, the contents of DHA/EPA and essential amino acids in fish muscle from each group were determined. The concentration of DHA of Russian sturgeon muscle has increased significantly in dietary supplementation with YYL2 at 1.0g/kg. However, no significant difference in EPA concentration was observed between all treatment groups. The essential and flavor amino acids of fish muscle in all groups exhibited insignificant difference, which means YYL2 does not work in the process of protein metabolism (Table 3).

Table 2: Effect of different experimental diets on growth performance and feed utilization of Russian sturgeon after 56 days.

Index	Control	Group1	Group2	Group3
IWB (g)	4.63±0.15	4.35±0.12	4.34±0.08	4.54±0.11
FWB (g)	10.12±1.54	12.37±0.55*	11.90±0.62	10.98±0.94
WG (g)	5.49±0.84	8.02±0.27 **	7.56±0.38 **	6.43±0.61
SGR (%)	1.40±0.13	1.87±0.13 **	1.80±0.21*	1.58±0.09
FCR	1.53±0.10	1.13±0.07 **	1.15±0.12 **	1.39±0.14

IW=initial weigh fish; FW=Final weight fish; SGR =Specific growth rate fish; FCR = Feed conversion ratio. Data are mean ± SE(n=15). Different stars above bars indicate significant differences between groups (*p<0.05, **p<0.01).

Table 3: Effect of different experimental diets on contents of DHA/EPA and essential amino acids in muscle of Russian sturgeon after 56 days.

Index		*Control*	*Group1*	*Group2*	*Group3*
Unsaturated fatty acids （% /Lipid）	DHA	7.77±0.23	8.35±0.17*	8.27±0.19*	8.24±0.21*
	EPA	4.08±0.17	4.28±0.21	4.26±0.08	4.08±0.17
Essential amino acids (g/100 g fresh muscle)	Lys	1 .60±0.23	1.69±0.15	1.64±0.24	1.62±0.19
	Try	0.13±0.02	0.15±0.02	0.16±0.03	0.13±0.01
	Phe	0.64±0.07	0.67±0.05	0.65±0.06	0.69±0.03
	Met	0.49±0.02	0.52±0.03	0.49±0.03	0.50±0.04
	Thr	0.82±0.11	0.86±0.09	0.79±0.13	0.84±0.07
	Ile	0.69±0.08	0.74±0.11	0.68±0.10	0.72±0.09
	Leu	1.01±0.07	1.12±0.13	1.07±0.19	1.04±0.13
	Val	0.94±0.11	0.97±0.12	0.94±0.09	0.96±0.13
Flavor amino acids	Glu	2.98±0.35	3.12±0.29	3.08±0.23	3.10±0.31
	Asp	1.94±0.06	2.15±0.12	2.12±0.11	2.08±0.17
	Ala	1.09±0.14	1.21±0.19	1.14±0.17	1.11±0.12
	Gly	0.74±0.08	0.88±0.10	0.84±0.13	0.82±0.14
	Tyr	0.37±0.04	0.45±0.03	0.40±0.02	0.39±0.03

Data are mean ± SE(n=15). Different stars above bars indicate significant differences between groups(*p<0.05).

Digestive enzymes (amylase, lipase and protease) activities

After 8 weeks of experiment, the effects of different diets on digestive enzymes activities of Russian sturgeon were determined. The results showed that the amylase activity in the digestive tract of fish supplemented with YLL2 increased after 8 weeks of feeding, compared with the control group (Fig. 1). Fish fed with 1.5 g/kg YLL2 (G2) and 2.0 g/kg YLL2 (G3) exhibited the highest amylase activity in liver compared with that of fish in control group (p<0.05). While, fish fed with 1.0g/kg YLL2(G1) exhibited insignificant increase in spleen and liver compared with that of the other groups (p<0.05). Interestingly, amylase activity in intestines of G1,G2 and G3 increased significantly compared with that of fish in control group (p<0.01), and rose up to a similar level.

After 8 weeks of feeding trail, lipase activity of Russian sturgeon was determined. The lipase activity in liver,

spleen and intestine of fish in all treatment groups were enhanced (Fig. 2). Sturgeon fed with YLL2 (G1, G2 and G3) exhibited significant higher lipase activity in liver, spleen and intestine compared with that of the control group (p<0.01).

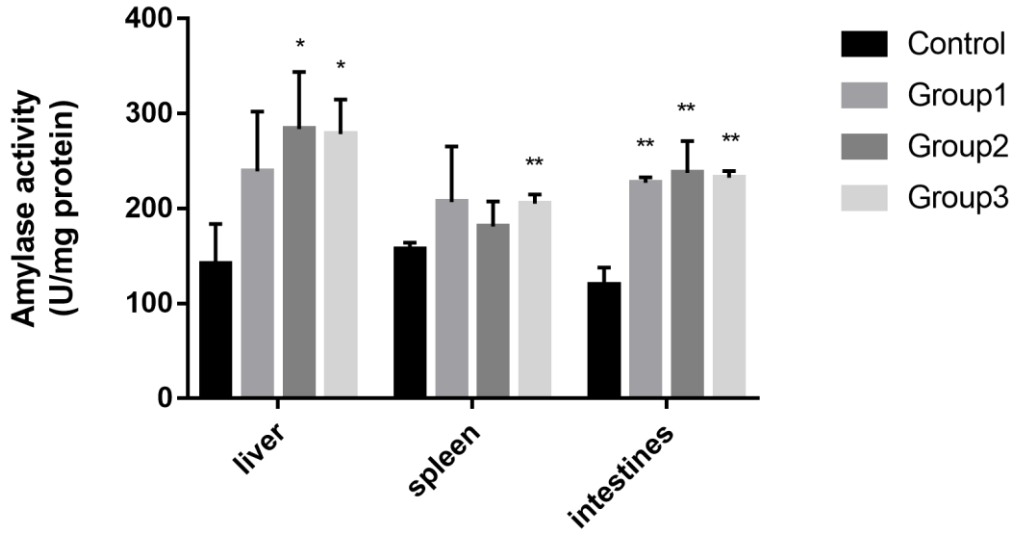

Figure 1: The amylase activity in Russian sturgeon after feeding with additives for 56 days. Control Group (0-control); Group 1 (1.0 g/kg YLL2); Group 2 (1.5 g/kg YLL2); Group 3 (2.0 g/kg YLL2). Values are expressed as mean ± SE(n=15). Different stars above bars indicate significant differences between groups (*p<0.05, **p<0.01).

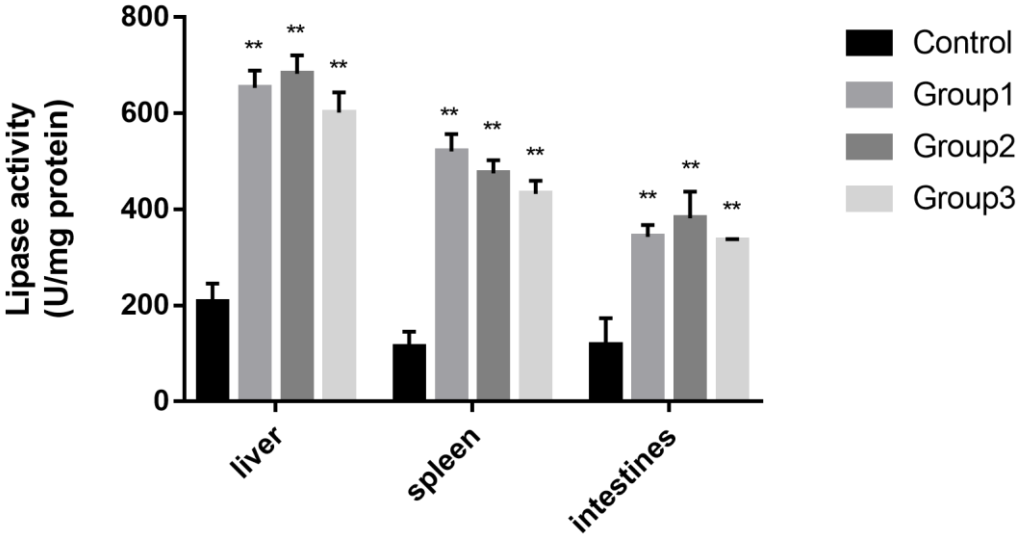

Figure 2: The lipase activity in Russian sturgeon after feeding with additives for 56 days. Control Group (0-control); Group 1 (1.0 g/kg YLL2); Group 2 (1.5 g/kg YLL2); Group 3 (2.0 g/kg YLL2). Values are expressed as mean ± SE(n=15). Different stars above bars indicate significant differences between groups (*p<0.05, **p<0.01).

The effects of different levels of dietary YYL2 on protease activity of Russian sturgeon were assayed after 8 weeks. Compared with the control group, the protease activity of fish in all treatment groups was improved (Fig. 3). Sturgeon fed with dietary YLL2 at 2.0g/kg (G3) exhibited the highest protease activity in liver，spleen and intestine compared with that of control group (p<0.01).

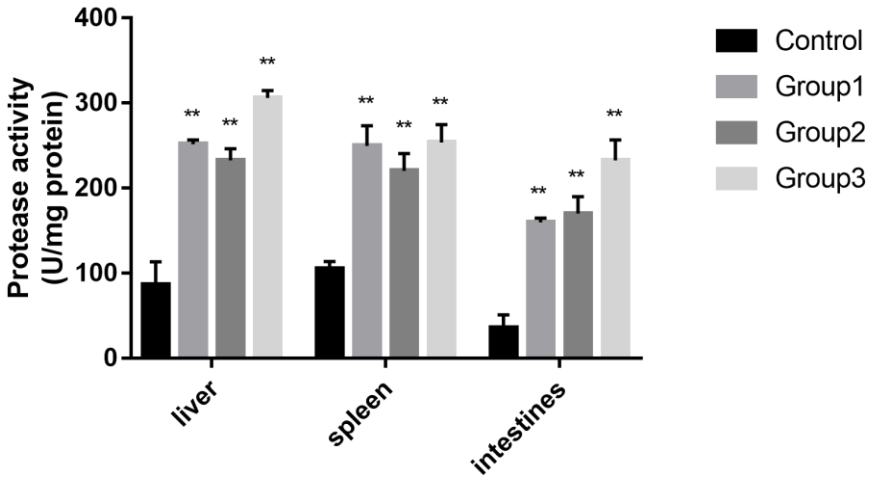

Figure 3: The protease activity in Russian sturgeon after feeding with additives for 56 days. Control Group (0-control); Group 1 (1.0 g/kg YLL2); Group 2 (1.5 g/kg YLL2); Group 3 (2.0 g/kg YLL2). Values are expressed as mean ± SE(n=15). Different stars above bars indicate significant differences between groups (*p<0.05, **p<0.01).

Discussion

It has been reported that supplementing lipase can improve the fat digestion of broilers (Al-Marzooqi *et al*., 2000). Fat digestion can produce fatty acids in the intestine of mammals and fish (Kurtovic *et al*., 2009). Also, dietary medium-chain fatty acids may affect bacterial metabolites and thus affect the intestinal health of weaned piglets (Zentek *et al*., 2012). The previous study demonstrated that the supplementation with porcine lipase showed a distinct effect only in older fish where 45-day-old *Sparus aurata* larvae fed the lipase diet demonstrated a 3.42 times increase in radioactivity in their tissue lipids, indicates that lipase addition might modify the kinetics of lipid absorption and utilization in teleosts (Koven *et al*. 1993). In present study, YYL2 was introduced into the diets for its characteristics of tolerance to the gastrointestinal environment, good biological safety, stable at low pH and superior temperature performance (Aloulou *et al*., 2015). Furthermore, YYL2 was the most effective lipase for DHA purification, which improved discrimination towards DHA, as enzyme selectivity was shown to be mainly based on the position of the double bond closest to the carboxylic group (Casas-Godoy *et al*., 2014). Therefore, exogenous lipase supplementation may affect the fish's

gut by raising fat digestion to improve health, which requires further investigation (Ángeles *et al*., 2012; Xu *et al*., 2016).

As an effective supplement, dietary YYL2 significantly promoted the SGR value of experimental fish, from 1.40±0.13%/day (control group) to 1.87±0.13%/day (G1, 1.0g/kg diet). Similar trend was recorded in the feeding of *Pseudobagru vachelli* with exogenous lipase after 90 days (Gu *et al*., 2010). The experimental result showed that, growth rate of 300mg/kg lipase additive groups was increased by 9.29%, and the coefficient of feed decreased by 6.35%. (Gu *et al*., 2010). Another novel lipase (LipG1) was evaluated as an aquafeed additive for juvenile common carp (*Cyprinus carpio*). Results showed that dietary supplementation of LipG1 at 6 U/g significantly increased the final body weight and weight gain of common carp compared with negative control after feeding for 4 weeks (Chao *et al*., 2015). In contrast, it had also been reported that lipase supplementation had no effect on growth performance and related parameters of rainbow trout (Samuelsena *et al*., 2001). These contradictory results may due to the characteristics of different lipase, such as substrate specificity, tolerance to the gastrointestinal environment and diversity in catalytic activities. Meanwhile, compared with optimum culture conditions, growth rate (weight gain 5.5-8.02g) of fish in the present study was observably slower, which may be due to the culture temperature (12℃-14℃) and was lower than the suitable temperature (18-25℃) for Russian sturgeon. The ammonia-N content (>0.40 mg L^{-1}) and dissolved oxygen (>5.0 mg L^{-1}) neither had met requirement for the growth of fish (Farabi *et al*., 2011). Thus, the further study should be focused on these parameters, and guaranteed the fish under optimum culture conditions.

As reported, the structure and optimum conditions of exogenous enzymes from microorganisms or plants, are distinguish with that of digestive enzymes *in vivo*. Therefore, there may have synergy function instead of feedback inhibition (Inborr, 1990). In present study, administration of YYL2 to Russian sturgeon resulted in an increase in the specific activity of lipase, protease and amylase in the digestive tract. The lipase activity in liver，spleen and intestine of fish in all treatment groups were enhanced，while it was difficult to distinguish between lipase activity due to enzyme synthesized by the sturgeon and activity due to YYL2 residue. Moreover, the digestive enzyme activity will steep rise with the fish grow up, after 56 days feeding trial, the fish in G1, G2 and G3 were significantly larger than the fish in control group, which resulted in the digestive enzyme activity rose to a higher level compared with that of control group. Interestingly, high level of dietary YYL2 (2.0 g/kg, G3) significantly increased lipase, protease and amylase activities in fish intestine,

and low level of dietary YYL2 (1.0 g/kg, G1) significantly increased lipase and protease activities in fish spleen. The detail mechanism of these result is unclear, warrants further investigation.

For nutritional components variable of experimental Russian sturgeon after 56 days feeding, content of DHA in fish muscle was increased significantly. However, the different level of YYL2 added into the feed resulted in different performance, and evidenced 1.0 g/kg YYL2 was enough to help hydrolyzing the crude lipid in feed. The essential amino acids content was not influenced by dietary YYL2, which means YYL2 does not work in the process of protein metabolism.

In summary, the current research showed that dietary consumption of YLL2 can significantly increase the growth performance and DHA/EPA concentration in Russian sturgeon. These results indicated that optimal exogenous lipase supplementation might be partly through hydrolysis of lipids to produce polyunsaturated fatty acids, which could improve the quality of fish meal, and the environment of the beneficial microorganism in the gastrointestinal tract of fish, and might promote the proliferation of beneficial microorganism, which contributes to intestinal health status in fish. This study may constitute a new strategy for fish diet supplementation of exogenous lipase. However, the precise mechanism of how YLL2 stimulate growth in sturgeon is not clarified as yet and further research on this aspect is needed.

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