Effect of fish meal replacement by *Chlorella* meal with dietary cellulase addition on growth performance, digestive enzymatic activities, histology and myogenic genes' expression for crucian carp *Carassius auratus*

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Abstract

This study was conducted to investigate the effects of fish meal (FM) replacement by *Chlorella* meal (CM) with dietary cellulase supplementation on growth performance, digestive enzymatic activities, histology and myogenic genes' expression in crucian carp *Carassius auratus* (initial body weight: 2.90 ± 0.02 g, mean $\pm$ SEM). Six isonitrogenous diets were formulated at two cellulase levels (0 and 2 g kg^{-1}). At each cellulase level, CM was added at three levels of 0, 533.1 and 710.8 g kg^{-1} to substitute 0, 75 and 100% of dietary FM respectively. Each experimental diet was randomly assigned to triplicate groups with 25 juvenile fish per fibreglass tank for 8 weeks. Dietary CM substitution significantly increased growth, feed utilization, amylase activity and the expression of *Myod*, *Mrf4* and *Myf5*, but reduced the *Myog* expression. Dietary cellulase addition increased hepatosomatic and viscerosomatic index, lipase activity and the expression of *Mrf4*, but reduced trypsin activity and the expression of *Myog* and *Myf5*. Dietary CM substitution enlarged the cell size and also caused some karyopyknosis in liver. Our results showed that CM could totally replace FM in diets; dietary cellulase supplementation at the level of 2 g kg^{-1} played a subtle role in improving growth and feed utilization for crucian carp.

Introduction

Traditionally, fish meal (FM) has been used as the most common source of protein in commercial aquafeeds (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke, Lubchenco, Mooney & Troell 2000; Luo, Tan, Chen, Wang & Zhou 2008; Cai, Song, Wang, Wu, Ye, Zhang & Yang 2013). Nevertheless, as the demand for FM is increasing with the development of the aquaculture industry, the worldwide yield of FM cannot meet the production demand of the feed industry (Gui, Liu, Shao & Xu 2010). Thus, searching for FM substitutes has been a major challenge (Luo *et al.* 2008; Hardy 2010). Nowadays, some plant protein sources, such as cottonseed meal (Gui *et al.* 2010; Cai, Li, Ye, Krogdahl, Jiang, Wang & Chen 2011), soya bean meal (Cai, Wang, Ye, Krogdahl, Wang, Xia & Yang 2012), rapeseed meal and peanut meal (Cai *et al.* 2013), potato protein concentrate (Xie, Zhu, Cui & Yang 2001), have been used to partially or totally substitute FM in diets of crucian carp. However, most plant-based feedstuffs have a wide variety of anti-nutritional factors such as non-starch polysaccharides (NSP), phytin and protease inhibitors, which may impair fish

performance, nutrient utilization, enzymatic activities or histology of digestive tract (Francis, Makkar & Becker 2001; Castillo & Gatlin 2015). Therefore, protein sources of these plant origins do not represent the ultimate alternatives to FM, and hence, the need to find new aquafeed ingredients currently remains a challenging goal (Vizcaíno, López, Sáez, Jiménez, Barros, Hidalgo, Camacho-Rodríguez, Martínez, Cerón-García & Alarcón 2014).

Microalgae, a new single-cell plant protein source, has received considerable attention in the aquafeeds industry due to its richness in protein, vitamins, polysaccharides, polyunsaturated fatty acids (PUFA) and microelements (Spolaore, Joannis-Cassan, Duran & Isambert 2006; Hemaiswarya, Raja, Ravi, Ganesan & Anbazhagan 2011). Among the microalgae, *Chlorella* is widely distributed and easily cultured in outdoor ponds (Huo, Wang, Zhu, Zhou, Dong & Yuan 2012) and accordingly is a quite promising source of protein. Owing to the high nutritional value, *Chlorella* has been used as dietary protein sources for marine and freshwater fish to improve weight gain and carcass quality (Kim, Bai, Koo & Wang 2002; Badwy, Ibrahim & Zeinhom 2008; Xu, Gao, Qi, Qiu, Peng & Shao 2014). However, its use in diets of fish has been limited by the relatively high level of fibre, which would retard digestion and absorption of nutrients, because fish cannot excrete cellulase and cannot utilize complex fibrous carbohydrates as an energy source efficiently (Lindsay & Haris 1980). The application of exogenous cellulase as supplements to improve the livestock and poultry production performance and feed utilization has been studied extensively (Titi & Tabbaa 2004; Adeola & Cowieson 2011; Kuhad, Gupta & Singh 2011). However, there are few studies on cellulase supplementation in diets for fish (Yigit & Olmez 2011; Zhou, Yuan, Liang, Fang, Li, Guo, Bai & He 2013; Shi, Luo, Chen, Huang, Zhu & Liu 2016).

Crucian carp (*Carassius auratus*), a dominant freshwater fish, is one of the largest aquaculture species in China. As the crucian carp production is increasing in recent years in China, there is a strong need to look for alternative protein sources to totally replace FM currently used in feeds. However, information was very scarce concerning the use of single-cell plant proteins in crucian carp feeds for the substitution of FM. Our recent study indicated that optimal dietary *Chlorella* substitution

levels were between 47% and 50% for best growth performance of crucian carp (Shi, Luo, Huang, Zhu & Liu 2015). In addition, when 50% of FM in diets was replaced by *Chlorella* meal, dietary cellulase supplementation showed positive effects on growth and feed utilization in crucian carp (Shi et al. 2016). Thus, on the basis of our previous study, the objective of this study was to evaluate the effects of higher cellulase levels in diets with higher fish meal substitution by *Chlorella* meal on growth performance, digestive enzyme activities, histology and mRNA levels of myogenic factors in crucian carp. As a representative of the omnivorous fish, the information of *Chlorella* meal and cellulase application in crucian carp will provide references for other fish.

Materials and methods

Experimental diets

Six isonitrogenous (390 g kg^{-1} crude protein) experimental diets were formulated by adding cellulase enzyme at two different levels (0.0 and 2.0 g kg^{-1}). With each cellulose level, *Chlorella* meal (CM) was included at three different levels (0.0, 531.3 and 710.8 g kg^{-1}) to substitute 0%, 75%, 100% of dietary fish meal (FM) respectively. Proximate composition and essential amino acid content of fishmeal and *Chlorella* meal are presented in Table 1. Ingredients and proximate compositions of the experimental diets are presented in Table 2. In the experiment, CM was obtained from Panjin Guanghe Crab (Liaoning, China) and they are the first-class products by frozen-drying. The cellulase enzyme was purchased from Sukahan Bio-Technology Company (Shandong, China), and the enzymatic activity was determined to be 1160 IU g^{-1}. One unit of cellulase activity (IU) was defined as the quantity of enzyme required to release 1 μmol of reducing sugars (expressed as glucose) from substrates per minute at 50°C and pH 4.8. The inclusion level of cellulase in diets was based on the instruction of the producer and our recent study (Shi et al. 2016)

For the formulation of the experimental diets, all dry ingredients were finely ground, weighed, mixed manually for 5 min and then transferred to a mixer for another 15-min mixing. The soya bean oil was then added slowly while mixing was still continuing, and all ingredients were mixed for another 10 min. At last, distilled water was added

Table 1 Proximate composition and essential amino acid (EAA) content of fishmeal and *Chlorella* meal (% dry matter)

Items	Dry matter	Protein	Lipid	Ash	Thr	Val	Met	Ile	Leu	Phe	Lys	His	Arg	EAA
FM	91.25	68.40	6.26	13.87	3.39	3.74	1.39	2.85	5.48	3.17	3.59	1.23	4.46	29.30
CM	92.60	51.20	7.58	15.30	2.21	2.19	0.88	1.63	3.62	2.10	2.10	0.61	2.39	17.73

Table 2 Ingredients and proximate analysis of the experimental diets (% dry matter)

Diets*	C0			C2		
	CM0	CM75	CM100	CM0	CM75	CM100
Ingredients						
Fish meal†	53.20	13.30	0.00	53.20	13.30	0.00
Chlorella meal‡	0.00	53.31	71.08	0.00	53.31	71.08
Maize starch	20.00	20.00	20.00	20.00	20.00	20.00
Binder§	2.00	2.00	2.00	2.00	2.00	2.00
Soybean oil	3.00	3.00	3.00	3.00	3.00	3.00
Vitamin mix¶	0.50	0.50	0.50	0.50	0.50	0.50
Mineral mix**	1.00	1.00	1.00	1.00	1.0.0	1.00
Ca(H$_2$PO$_4$)$_2$	1.00	1.00	1.00	1.00	1.0.0	1.00
Cellulose††	19.30	5.89	1.42	19.10	5.69	1.22
Cellulase	0.00	0.00	0.00	0.20	0.20	0.20
Proximate analysis						
Dry matter	95.30	93.70	93.50	95.00	94.50	93.90
Crude protein	39.90	39.10	38.60	39.70	39.10	38.80
Crude lipid	5.49	5.77	6.41	5.20	5.99	6.72
Ash	8.56	10.52	10.63	8.52	10.79	10.87
Crude fibre	19.74	6.98	2.39	19.89	6.95	2.40
Gross energy (MJ kg^{-1})	19.6	20.3	20.7	19.6	20.2	20.6

*Diets: C0: no cellulase supplementation; C2: 2 g kg^{-1} cellulase supplementation; CM0: no CM; CM75: CM replaced 75% of FM; CM100: CM replaced 100% of FM.
†Fish meal: crude protein 684 g kg^{-1}, steam dried fishmeal, produced by Technologica De Alimentos S.A., Chimbote Plant, Peru.
‡CM: crude protein 512 g kg^{-1}.
§Binder: Henan Jianjie Shiye (Zhengzhou, China).
¶Vitamin mix: Wuhan Huayang Tianle Animal Health Products (Wuhan, China).
**Mineral mix: Dandong Yida Feed Additive Factory (Dandong, China).
††Cellulose: Anhui Sunhere Pharmaceutical Excipients (Anhui, China).

(10%, v/w) to form a dough. A dough of even consistency was passed through a pelletizer with a 2.0-mm-diameter die. The diets were dried at 55°C overnight, and the dry pellets were placed in sealed plastic bags and stored at −20°C until used.

Experimental procedures

The experiment was conducted in an indoor static aquarium system of Panjin Guanghe Crab (Liaoning, China). Nine hundred crucian carp *C. auratus* were obtained from a local fish pond, transported to the laboratory and kept in 20 300-l circular fibreglass tanks for 14-day acclimation. During the acclimation period, the fish were fed the C0-CM0 diet to satiation twice daily to acclimate them to the experimental conditions. Afterwards, 450 uniform-sized fish (mean weight: 2.90 ± 0.02 g, mean ± SEM) were randomly stocked in 18 300-l circular fibreglass tanks, with 25 fish for each tank. Each diet was assigned to three tanks in a completely randomized design. The tanks were continuously aerated to maintain the dissolved oxygen level at saturation. Fish were fed by hand with a rate equalling 4–7% of wet body weight per day, twice daily (0830 and 1600 h), and feeding ration was adjusted according to feeding situation. The amount of feed consumed by the fish in each tank was recorded daily. All rearing water was renewed 30% twice daily with dechlorinated

tap water from a cement tank before feeding to maintain acceptable water quality parameters. Faecal matter was removed twice daily before feeding. Fish were weighed every 2 weeks to monitor growth. Tanks were thoroughly cleaned every 2 weeks when the fish were removed for weighing. Mortality was checked daily. The experiment continued for 8 weeks.

The experiment was conducted at ambient temperature and subjected to natural photoperiod (approximately 14-h light/10-h dark). Water quality parameters were monitored in the morning twice a week. The ranges of the parameters were as followed: temperature, 22.1–28.5 °C; dissolved oxygen ≥6.0 mg l^{-1}; pH 8.0–8.4; and total ammonia nitrogen 0.040–0.078 mg l^{-1}.

Sampling

At the termination of the experiment, the fish were fasted for 24 h before harvest. All fish were counted and weighed to determine survival, weight gain (WG) and specific growth rate (SGR). After obtaining the final total weight of fish in each aquarium, six fish per tank were randomly selected and dissected on ice. Their anterior intestine, liver and white muscle samples were removed immediately using sterile forceps. Anterior intestine and muscle samples of three fish were frozen in liquid nitrogen and stored at −80°C (not longer than 2 weeks) for subsequent analysis (anterior intestinal samples for enzyme activity determinations and muscle samples for total RNA extraction). Anterior intestine and liver samples of other three fish were obtained for histological analysis. Another ten fish per tank were randomly collected, measured in length and body weight, and condition factor (CF) was calculated. The fish were then dissected on ice to obtain liver and white muscle samples, and viscerosomatic index (VSI) and hepatosomatic index (HSI) were also calculated. Liver and muscle samples were kept at −80°C for subsequent determination of composition values.

Chemical analysis

Moisture content was determined by drying samples to a constant weight in a freeze-dryer (Labconco Corporation, Kansas city, MO, USA) at −50 °C for 48 h and calculated as percentage of water loss. Crude protein, ash, lipid contents of

tissues and experimental diets were determined according to the AOAC (1995) methods. Briefly, crude protein (N × 6.25) was determined by the Kjeldahl method after acid digestion using an auto Kjeldahl System (Hanon K9860 Kjeldahl Auto Analyzer; Jinan, China). Crude lipid was determined by the ether extraction method, and ash was determined using a muffle furnace at 550°C for 8 h. Crude fibre was determined by National Feed Quality Supervision and Inspection Center (Wuhan, China) according to the Chinese standard for crude fibre (GB/T6434-2006). The energy values of the diets were determined using a calorimeter (6200 Isoperibol Calorimeter; Parr Instrument Company, Moline, IL, USA). Amino acid concentrations were analysed using an automatic amino acid analyser (Hitachi Model 835-50; Tokyo, Japan) according to the Chinese standard for amino acid analysis (GB/T 18246-2000, China). Tryptophan could not be measured because of its degradation during acid hydrolysis. All chemical analyses were performed in duplicates.

Assays for digestive enzyme activities

Anterior intestinal samples were homogenized on ice in 10 volumes (w v^{-1}) of ice-cold physiological saline, and homogenates were centrifuged at 12 000 × g for 20 min at 4°C, according to the modified method of Wu, Liu, Li, Xu, He, Li and Jiang (2013) and Vizcaíno *et al.* (2014). The supernatant was separated for enzyme analysis. The activities of amylase, trypsin and lipase were assayed by the commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The enzymatic activities were expressed in units (U); 1 U of amylase was defined as 1 mg of enzyme that hydrolysed 10.0 mg starch in 30 min at 37°C; and 1 U of trypsin was expressed as 1 mg of enzyme that generated an optical density (OD) change of 0.003 in 1 min at 37°C and pH 8.0; 1 U of lipase was defined as 1 mg of enzyme that hydrolysed 1 μmol of substrate in 1 min at 37°C.

mRNA expression analysis (qPCR)

The expression levels of myogenic regulatory factors genes (*MRFs*, including *Myod, Myog, Mrf4, Myf5*) mRNAs were conducted by quantitative real-time PCR (qPCR) method. Total RNA was isolated from muscle samples using TRIzol Reagent (Invitrogen,

Carlsbad, USA) based on the acid guanidinium thiocyanate–phenol–chloroform extraction method. Two micrograms of total RNA was used for reverse transcription with RevertAid™ Reverse Transcriptase (Fermentas, Burlington, Canada) and an oligo-dT primer. qPCR assays were carried out in a quantitative thermal cycler (MyiQ™ 2 Two-color Real-Time PCR Detection System; Bio-Rad, Hercules, CA, USA) with a 20 μl reaction volume containing 2 × SYBR® Premix Ex Taq™ (TaKaRa, Dalian, Japan) 10 μl, 10 mM each of forward and reverse primers 0.4 μl, 1 μl diluted cDNA template (10-fold) and 8.2 μl double distilled H_2O. Primers are given in Table 3. The qPCR parameters consisted of initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 59°C for 10 s and 72°C for 30 s. All reactions were performed in duplicates, and each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. A non-template control and dissociation curve were performed to ensure that only one PCR product was amplified and that stock solutions were not contaminated. Standard curves were constructed for each gene using serial dilutions of stock cDNA. The amplification efficiencies of all genes were approximately equal and ranged from 97.4% to 102.8%. Prior to the analysis, experiments were performed to check the stability of six housekeeping genes (β-*actin, GAPDH, 18SrRNA, Tub-a, EF-1α,*

RPL7). By using GENORM software (Vandesompele, De Preter, Pattyn, Poppe, Van Roy, De Paepe & Speleman 2002), two genes with the most stable level of expression across the experimental conditions were β-*actin* and *RPL7* (M value was 0.44). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) when normalizing to the geometric mean of the best combination of two genes (β-*actin* and *RPL7*) as suggested by geNorm.

Histology of anterior intestine and liver

Histological analyses were performed according to the method described in our previous studies (Chen, Luo, Zheng, Li, Liu, Zhao & Gong 2012; Liu, Luo, Tan & Gong 2013). Briefly, anterior intestine and liver samples were fixed for 24 h in Bouin's solution, and then stored in 70% ethanol. After dehydration in graded concentrations of ethanol, the samples were embedded in paraffin wax. Sagittal sections of 5–7 μm thickness were stained with haematoxylin–eosin (H&E) and then prepared for observation by a Nikon ECLIPSE 80i microscope (Nikon Corporation, Kanagawa, Japan). We randomly examined 10 microscope fields for each sample, and the results from individual observation were then combined for the overall results.

Table 3 Primers used for qPCR analysis of MRFs and housekeeping genes

Genes	Accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
Myod	KP715154	AACCACCAACGCTGACCG	CTGGTTGGGGTTGTTGGAAG	115
Myog	KP715155	GGACGCACTGCTCCACTCTG	AGGAACATCAGCAGGGAAACC	119
Mrf4	KP715153	CTGCGACGGTCAGTGTCTAATGT	CAGCCTCTGGTTCGGATTGG	169
Myf5	KP715152	GGAACAACTACAACTTTGAAGCACA	TCCCATCGCAACTCCTGTATCT	128
β-*actin*	AB039726	GGCCTCCCTGTCTATCTTCC	TTGAGAGGTTTGGGTTGGTC	156
EF-1α	AB056104	GATTGTTGCTGGTGGTGTTG	GCAGGGTTGTAGCCGATTT	216
GAPDH	AM701793	CAGGGTGGTGCCAAGCGT	AGGGGAGCCAAGCAGTTAGTG	146
18S rRNA	FJ710819	AGAAACGGCTACCACATCC	CACCAGACTTGCCCTCCA	169
Tub-a	JX967534	GACTCGCAAACTGGCTGAT	CGTTCCATTAGAAGAGAGGTGA	105
RPL7	JQ776517	CGCAAGGCAGGCAACTACTAC	TTCAGTTTCACAAAGACACCGTT	152

Statistical analysis

The results were presented as mean ± standard error of means (SEM). Prior to statistical analysis, all data were tested for normality of distribution using the Kolmogorov–Smirnov test. The homogeneity of variances among the different treatments was tested using the Bartlett's test. The data were then analysed by two-way ANOVA, using cellulase and Chlorella meal levels as the two factors (SPSS version 17.0, SPSS, Michigan Avenue, Chicago, IL, USA). If two-way ANOVA showed a significant interaction between the two factors, Turkey's multiple range test was used to identify significant difference among treatments. Differences were considered significant at $P < 0.05$.

Results

Growth performance, feed utilization and morphological parameters

In the present study, survival exceeded 95% and showed no significant differences among the treatments (data not shown). At each cellulase level, dietary CM substitution significantly increased FBW, WG, SGR, FI and PER, but decreased FCR

($P < 0.05$) (Table 4). Dietary cellulase addition had no significant influence on FBW, WG, SGR, FI, PER and FCR ($P > 0.05$). Dietary CM substitution significantly reduced HSI, while CF in the CM75 group tended to be lower than other groups (Table 5). Dietary cellulase addition significantly increased HSI and VSI ($P < 0.05$), but not CF ($P > 0.05$). The interaction of CM and cellulase significantly influenced FI and VSI ($P < 0.05$).

Chemical composition of muscle and liver

Dietary cellulase addition significantly influenced dry matter and lipid contents of muscle ($P < 0.05$) (Table 6). The protein and lipid contents of liver increased with increasing dietary CM levels ($P < 0.05$). Dietary CM substitution did not significantly influence muscle proximate composition ($P > 0.05$). The interaction of CM and cellulase significantly influenced lipid content of muscle ($P < 0.05$).

Intestinal digestive enzyme activities

As shown in Table 7, the activity of amylase increased with increasing dietary CM levels ($P < 0.05$), but the activities of trypsin and lipase

Table 4 Growth performance and feed utilization of crucian carp (C. auratus) fed with each experimental diet

	IBW*	FBW†	WG‡	SGR§	FI¶	FCR**	PER††
C0							
CM0	2.89 ± 0.01	8.5 ± 0.2[a]	195.2 ± 4.7[a]	1.93 ± 0.03[a]	13.4 ± 0.1[a]	2.35 ± 0.06[b]	1.06 ± 0.03[a]
CM75	2.88 ± 0.00	14.0 ± 0.2[b]	384.5 ± 6.9[b]	2.82 ± 0.03[b]	20.8 ± 0.4[c]	1.88 ± 0.02[a]	1.36 ± 0.01[b]
CM100	2.89 ± 0.01	14.8 ± 0.3[b]	412.5 ± 9.7[b]	2.92 ± 0.03[b]	21.5 ± 0.2[c]	1.79 ± 0.04[a]	1.45 ± 0.03[b]
C2							
CM0	2.91 ± 0.01	9.1 ± 0.2[a]	214.6 ± 7.5[a]	2.05 ± 0.04[a]	14.5 ± 0.1[b]	2.33 ± 0.08[b]	1.08 ± 0.03[a]
CM75	2.91 ± 0.01	14.4 ± 0.2[b]	396.5 ± 8.4[b]	2.86 ± 0.03[b]	20.9 ± 0.0[c]	1.81 ± 0.05[a]	1.42 ± 0.03[b]
CM100	2.89 ± 0.01	14.9 ± 0.2[b]	414.0 ± 9.5[b]	2.92 ± 0.03[b]	21.0 ± 0.2[c]	1.77 ± 0.05[a]	1.46 ± 0.04[b]
P-value	NS	**	**	**	**	**	**
Two-way ANOVAS (P value summary)							
Chlorella Meal	NS	**	**	**	**	**	**
Cellulase	NS	NS	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	**	NS	NS

NS, not significant.

Values are means ± SEM of three replicates and values within the same row with different superscript letters are significantly different ($P < 0.05$). The data were subjected to one-way and two-way ANOVA and Tukey's multiple range test (**$P < 0.01$, *$P < 0.05$, NS $P > 0.05$).

*IBW (g fish^{-1}), initial mean body weight.

†FBW (g fish^{-1}), final mean body weight.

‡WG (weight gain, %) = 100 × (FBW − IBW)/IBW.

§SGR (specific growth rate, % d^{-1}) = 100 × (ln FBW − ln IBW)/days.

¶FI (feed intake, g fish^{-1}): total amount of the dry feed consumed.

**FCR (feed conversion ratio) = (feed intake, g)/(final fish weight − initial fish weight + dead fish, g).

††PER (protein efficiency ratio) = (weigh gain, g)/(protein intake, g).

in the CM75 groups tended to be lower than those in other groups ($P < 0.05$). Dietary cellulase addition significantly increased amylase and lipase

Table 5 Morphological parameters of crucian carp (*C. auratus*) fed with each experimental diet

	HSI*	VSI†	CF‡
C0			
CM0	5.53 ± 0.22^b	14.17 ± 0.27^a	2.95 ± 0.01^b
CM75	5.28 ± 0.06^{ab}	14.80 ± 0.13^{ab}	2.77 ± 0.04^a
CM100	4.69 ± 0.15^a	14.67 ± 0.19^{ab}	2.93 ± 0.04^b
C2			
CM0	6.56 ± 0.13^c	16.03 ± 0.06^c	2.91 ± 0.03^{ab}
CM75	5.77 ± 0.05^{bc}	15.42 ± 0.16^{bc}	2.81 ± 0.02^{ab}
CM100	5.19 ± 0.28^{ab}	15.26 ± 0.28^{bc}	2.87 ± 0.03^{ab}
P-value	**	**	*
Two-way ANOVAS (*P* value summary)			
Chlorella Meal	**	NS	**
Cellulase	**	**	NS
Interaction	NS	*	NS

NS, not significant.

Values are means ± SEM of three replicates and values within the same row with different superscript letters are significantly different ($P < 0.05$). The data were subjected to one-way and two-way ANOVA and Tukey's multiple range test (**$P < 0.01$, *$P < 0.05$, NS $P > 0.05$).

*HSI (hepatosomatic index = $100 \times$ (liver weight, g)/(body weight, g).

†VSI (viscerosomatic index) = $100 \times$ (viscera weight, g)/(body weight, g).

‡CF (condition factor) = $100 \times$ (live weight, g)/(body length, cm)^3.

activities but reduced trypsin activity ($P < 0.05$). The interaction of CM and cellulase significantly influenced the activity of amylase ($P < 0.05$).

mRNA expression levels of *MRFs* genes in the muscle

As shown in Fig. 1, without cellulase addition, dietary CM substitution upregulated the expression of *Myod*, *Mrf4* and *Myf5*, but downregulated *Myog* expression. At the 2% of cellulase group, *Myod* and *Myf5* expression increased in the CM75 group but decreased in the CM100 group. The mRNA levels of *Mrf4* were significantly higher in the CM100 than other groups ($P < 0.05$). With the same CM concentration, dietary cellulase addition significantly increased *Mrf4* expression but decreased *Myf5* and *Myog* expression ($P < 0.05$). The interaction of CM and cellulase significantly influenced the expression levels of *Myod* and *Mrf4* ($P < 0.05$) (Table 8).

Histological observation of anterior intestine and liver

Dietary cellulase addition showed no significant effect on size of hepatic cells and their nuclei (Fig. 2). However, with dietary CM addition, some karyopyknosis were observed (Fig. 2b, c, e, f), and the cell sizes of liver were larger than those in the

Table 6 Proximate composition (%) of muscle and liver in crucian carp (*C. auratus*) fed with each experimental diet

	Muscle				Liver			
	Dry matter	Protein	Lipid	Ash	Dry matter	Protein	Lipid	Ash
C0								
CM0	22.1 ± 0.2	17.5 ± 0.3	2.87 ± 0.02^a	1.19 ± 0.11	32.4 ± 0.6	8.79 ± 0.26^{ab}	0.71 ± 0.04^a	0.74 ± 0.01
CM75	22.8 ± 0.8	17.9 ± 0.7	3.77 ± 0.09^{ab}	1.15 ± 0.06	34.1 ± 0.7	8.50 ± 0.25^{ab}	1.29 ± 0.20^{ab}	0.80 ± 0.02
CM100	23.6 ± 0.8	17.6 ± 0.6	3.96 ± 0.46^{ab}	1.20 ± 0.04	33.9 ± 0.7	9.19 ± 0.41^{ab}	1.65 ± 0.36^b	0.83 ± 0.04
C2								
CM0	24.7 ± 0.3	18.3 ± 0.1	4.09 ± 0.12^b	1.31 ± 0.03	33.7 ± 1.2	7.57 ± 0.38^a	0.75 ± 0.04^a	0.78 ± 0.03
CM75	24.5 ± 0.7	18.3 ± 0.4	4.41 ± 0.20^b	1.25 ± 0.03	33.4 ± 0.6	8.69 ± 0.36^{ab}	1.28 ± 0.03^{ab}	0.82 ± 0.07
CM100	23.7 ± 0.4	18.5 ± 0.2	3.59 ± 0.24^{ab}	1.21 ± 0.03	33.9 ± 0.7	9.36 ± 0.46^b	1.69 ± 0.15^b	0.88 ± 0.04
P-value	NS	NS	*	NS	NS	NS	**	NS
Two-way ANOVAS (*P* value summary)								
Chlorella Meal	NS	NS	NS	NS	NS	*	**	NS
Cellulase	**	NS	*	NS	NS	NS	NS	NS
Interaction	NS	NS	*	NS	NS	NS	NS	NS

NS, not significant.

Values are means ± SEM of three replicates and values within the same row with different superscript letters are significantly different ($P < 0.05$). The data were subjected to one-way and two-way ANOVA and Tukey's multiple range test (**$P < 0.01$, *$P < 0.05$, NS $P > 0.05$).

CM0 groups (Fig. 2a, d). No noticeable differences were observed in the appearances of the intestines (Fig. 3). Samples had a similar appearance to

intact intestinal mucosal epithelium, well-organized villi. However, the tunica serosa in the C0-CM100 group seemed to be thinner than those from other dietary groups (Fig. 3c).

Discussion

In most studies, decreased growth was observed in fish when a large proportion of the FM was replaced by some plant protein sources in crucian carp (Xie *et al.* 2001). In contrast, in the present work, high and/or even 100% of fish meal replacement by CM significantly promoted the growth and feed utilization in crucian carp, indicating that

Table 7 Activities of digestive enzymes in the intestine of crucian carp (*C. auratus*) fed with each experimental diet

	Amylase (U/mg prot)	Trypsin (U/mg prot)	Lipase (U/g prot)
C0			
CM0	11.9 ± 1.1^a	305.8 ± 11.1^c	175.6 ± 6.7^{ab}
CM75	12.6 ± 0.8^a	287.6 ± 9.0^c	143.3 ± 8.7^a
CM100	20.2 ± 1.6^b	311.1 ± 2.1^c	176.3 ± 11.8^{ab}
C2			
CM0	13.3 ± 0.4^a	159.8 ± 3.4^b	234.1 ± 11.0^c
CM75	19.8 ± 0.9^b	119.4 ± 8.6^a	168.6 ± 9.3^a
CM100	20.5 ± 1.1^b	158.9 ± 4.3^b	219.2 ± 8.5^{bc}
P-value	**	**	**
Two-way ANOVAS (*P* value summary)			
Chlorella Meal	**	**	**
Cellulase	**	**	**
Interaction	*	NS	NS

NS, not significant.
Values are means ± SEM of three replicates and values within the same row with different superscript letters are significantly different (*P* < 0.05). The data were subjected to one-way and two-way ANOVA and Tukey's multiple range test (***P* < 0.01, **P* < 0.05, NS *P* > 0.05).

Table 8 Two-way ANOVAS of mRNA expression levels of *MRFs*

| Two-way ANOVAS (*P* value summary)	*Chlorella* Meal	Cellulase	Interaction
Myod	**	NS	**
Myog	NS	**	NS
Mrf4	**	**	**
Myf5	**	**	NS

NS, not significant.
Different letters and asterisk denote significant differences (***P* < 0.01, NS *P* > 0.05).

Figure 1 mRNA expression levels of *MRFs* in the muscle of crucian carp (*C. auratus*) fed with each experimental diet. Values are mean ± SEM (*n* = 3 replicate tanks, three fish were sampled for each tank). mRNA expression values were normalized to *housekeeping gene* (β-actin and *EF-1α*) expressed as a ratio of the control (control = 1). Different superscript letters indicate significant differences (*P* < 0.05). The data were subjected to one-way ANOVA and Tukey's multiple range test.
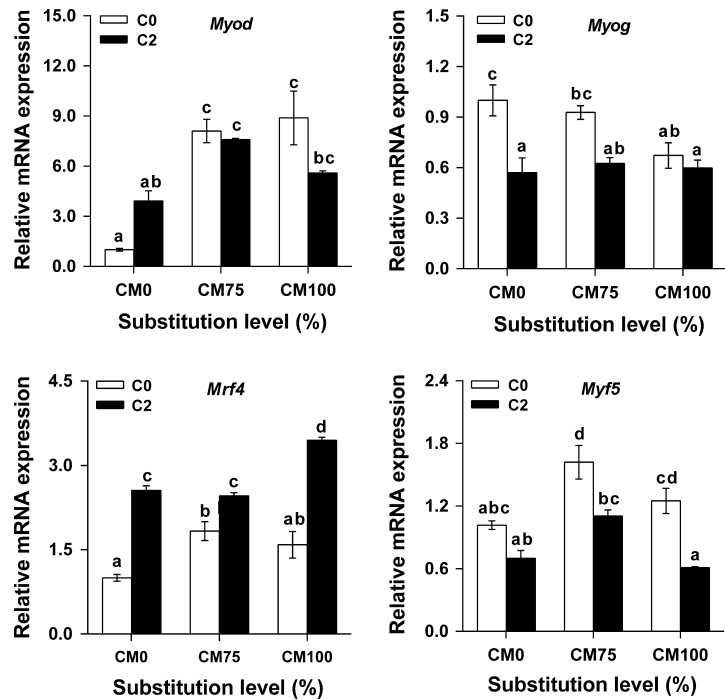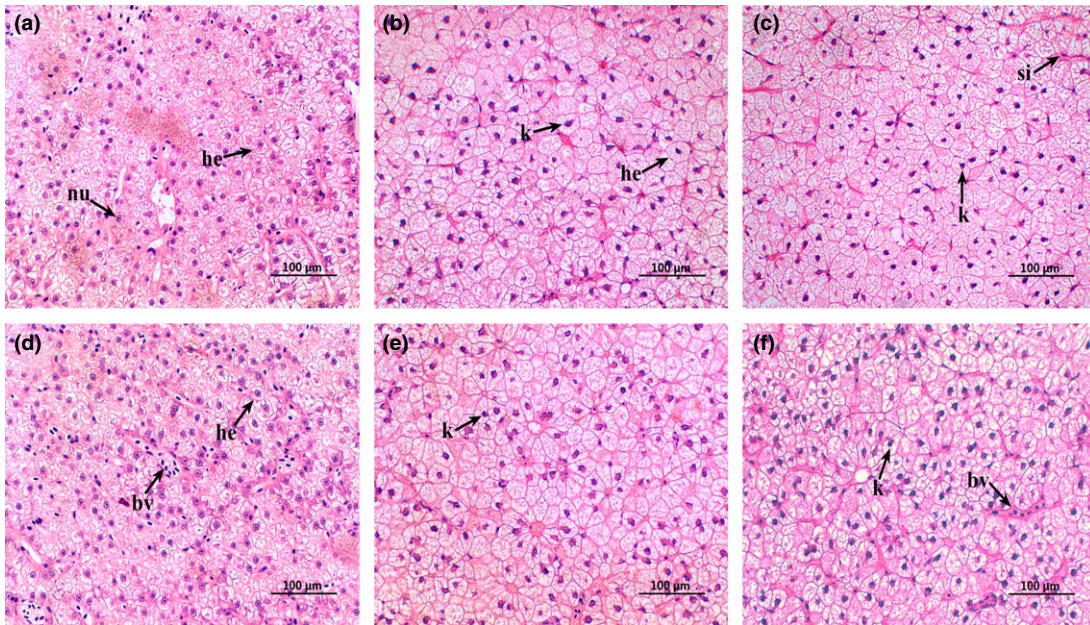
Figure 2 Liver histology of crucian carp *C. auratus* fed with different experiment diets (H&E) (×200). (a–f) represent different liver tissues from C0-CM0, C0-CM75, C0-CM100, C2-CM0, C2-CM75, C2-CM100 respectively. Abbreviation: hepatocytes (he), hepatocyte nucleus (nu), sinusoids (si), blood vessel (bv) and karyopyknosis (k). The cell sizes of livers were larger in the CM substitution groups than the CM0 groups. Some karyopyknosis were observed in the dietary CM groups. [Colour figure can be viewed at wileyonlinelibrary.com].
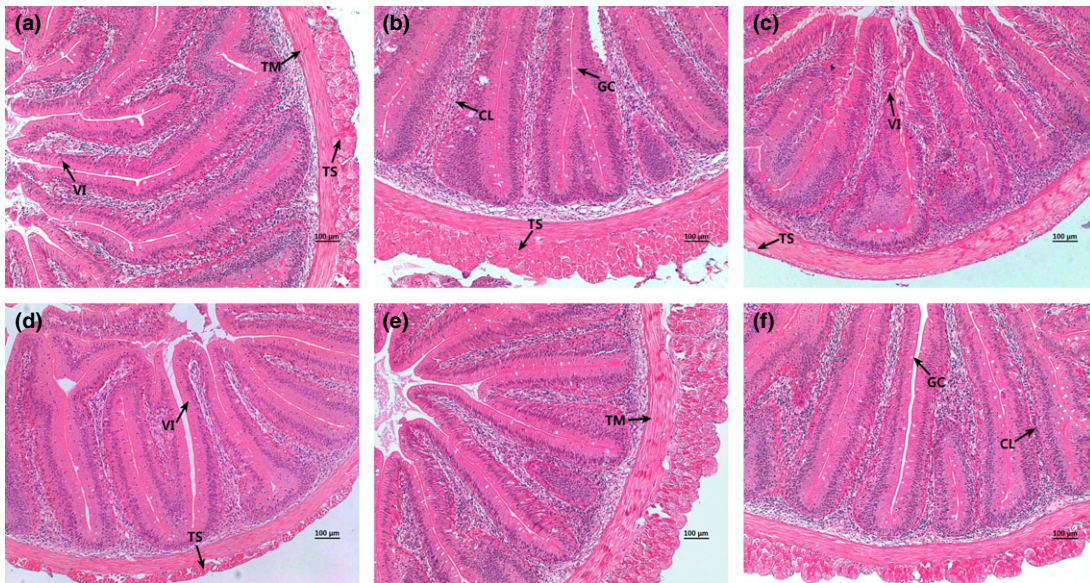
Figure 3 Foregut histology of crucian carp *C. auratus* fed with different experiment diets (H&E) (×100). (a–f) represent different foregut tissues from C0-CM0, C0-CM75, C0-CM100, C2-CM0, C2-CM75, C2-CM100 respectively. Abbreviation: Villi (VI), tunica muscularis (TM), tunica serosa (TS), central lacteal (CL), goblet cell (GC). Samples had a similar appearance to intact intestinal mucosal epithelium, well-organized villi. The density of the goblet cells was not affected by the treatment. Foregut from C0-CM100 showed the thinner tunica serosa. [Colour figure can be viewed at wileyonlinelibrary.com].

the omnivorous crucian carp can well utilize CM-based diet. Studies pointed out that crucian carp, as a typical omnivorous fish, have the relevant digestive system to adapt to the feeding habits, which makes a more efficient use of vegetable protein in the diets. Similarly, our recent study showed that CM replacement could improve growth performance of crucian carp even when the substitution level was up to 87.3% (Shi *et al.* 2015). The optimal dietary *Chlorella* substitution levels were between 47% and 50% for best growth performance in previous study (Shi *et al.* 2015). In contrast, the present study indicated that total FM replacement by CM could be expected in diets for crucian carp. The discrepancy was probably due to the differences in nutrient compositions of CM. The crude protein and EAAs contents of CM are higher in this study, which indicates the better nutritional value. In addition, in the omnivorous Nile tilapia, Badwy *et al.* (2008) also reported that the inclusion of *Scenedesmus* sp. and *Chlorella* sp. up to 50% in diets could improve growth performance. Increased FI and PER, and reduced FCR were observed in fish fed increasing CM diets, which may explain the reason for the increased growth in the present study.

On the other hand, the present study indicated that 2% of cellulase addition did not significantly influence the growth and feed utilization of crucian carp. However, our recent study showed that dietary cellulase addition (1.0–1.5 g kg^{-1}) in diets containing 355.4 g kg^{-1} *Chlorella* powder significantly promotes growth and feed utilization in crucian carp (Shi *et al.* 2016). Different cellulase supplementation levels might contribute to variations in the outcome of different studies. Some authors indicated that less or excessive addition of exogenous enzyme in diets both could not produce positive growth performance (Ai, Mai, Zhang, Xu, Tan, Zhang & Li 2007; Ghomi, Shahriari, Langroudi, Nikoo & von Elert 2012; Qin, Chen, Chang, Liu, Lv & Wang 2014).

FM replacement by CM in the diets increased the lipid content in liver of crucian carp, in agreement with our previous study (Shi *et al.* 2015). In the present study, dietary CM substitution caused some pathological changes in liver, such as karyopyknosis, enlarged size of hepatic cells, which may damage the function of liver in fat metabolism, resulting in increased hepatic lipid deposition. Dietary CM replacement did not modify muscle proximate composition, which was also observed in other study with microalgae substitution in aquafeeds (Vizcaíno *et al.* 2014). On the other hand, Lin, Mai and Tan (2007) and Yigit and Olmez (2011) stated that enzyme supplementation in the diets did not have significant effect on whole body composition of fish. Nevertheless, in the present study, lipid content of muscle increased with dietary cellulase supplementation, indicating that the lipid metabolism in crucian carp may be influenced by cellulose addition, which was consistent with the results of Farhangi and Carter (2007) and Ghomi *et al.* (2012).

Digestive enzyme activity is often used as an indicator of digestive processes and nutritional condition of fish (Abolfathi, Hajimoradloo, Ghorbani & Zamani 2012). Crucian carp is a stomachless fish. Digestion takes place in the intestine, in which various intestinal enzymes are involved in digestive and absorptive processes, such as amylase, pepsin, trypsin and lipase (Das & Tripathi 1991). This present work indicated that dietary CM substitution could significantly increase the activities of amylase but not activities of trypsin and lipase in intestine of crucian carp. Increased amylase activities may improve the utilization of carbohydrates in diets. Xu *et al.* (2014) reported that dietary *Chlorella* could significantly increase amylase, lipase and protease activities. Vizcaíno *et al.* (2014) found that trypsin activity was significantly increased with algae inclusion in the diets, while α-amylase activity was not affected. In addition, dietary exogenous enzyme addition, with the suitable supplementation levels, can promote the secretion of endogenous enzymes in fish (Lin *et al.* 2007; Zhou *et al.* 2013; Qin *et al.* 2014). Dietary cellulase improved protease and amylase activities in the intestinal tract of grass carp as suggested by Zhou *et al.* (2013). In this study, cellulase supplementation in the diets increased the activities of amylase and lipase, but decreased the trypsin activity. These discrepancies in digestive enzymes' activities among studies were probably due to different dietary cellulase addition levels and nutrient compositions of vegetable protein feedstuffs.

Muscle growth is mainly involved in two processes, hyperplasia (increased fibre number) and hypertrophy (increased fibre size) (Zhu, Wang, Wang, Gul, Yang, Zeng & Wang 2014). The *MRFs* are a family of basic helix–loop–helix (bHLH) transcription factors regulating muscle hyperplasia and hypertrophy, including *Myod*, *Myog*, *Mrf4*, *Myf5* (Watabe 2001). *Myod* and *Myf5*, as the primary

MRFs, direct proliferating myogenic progenitor cells towards a myogenic lineage. *Myog* and *Mrf4*, the secondary *MRFs*, control the differentiation and fusion of myoblasts to form myofibres (Megeney & Rudnicki 1995; Rudnicki & Jaenisch 1995; Watabe 1999). *Myog* and *Mrf4* are activated during myoblast differentiation and probably have cooperative functions with *Myod* and *Myf5* as transcription factor regulators for the activation of muscle contractile protein genes (Lassar, Davis, Wright, Kadesch, Murre, Voronova, Baltimore & Weintraub 1991; Pownall, Gustafsson & Emerson 2002). Alami-Durante, Cluzeaud, Duval, Maunas, Girod-David and Médale (2014) demonstrated that early dietary composition had a long-term effect on the subsequent muscle growth processes of juveniles. In the present study, dietary CM substitution increased the expression of *Myod*, *Mrf4* and *Myf5*, but decreased *Myog* expression. However, *Myod* and *Myog* expression in muscle was not regulated by graded dietary levels of plant protein sources in rainbow trout (Alami-Durante, Médale, Cluzeaud & Kaushik 2010). In this work, the improved expressions of *MRFs* genes were associated with the growth and development of muscle, suggesting that dietary CM substitution may promote the growth in fish. In addition, cellulase addition in the diets downregulated *Myog* expression but increased *Mrf4* expression. The decreased expression of *Myog* may be compensated by the *Mrf4* overexpression, which was supported by Sumariwalla and Klein (2001) who reported that fully differentiated muscle fibres can be generated by overexpression of *Mrf4* in embryonic stem cells lacking *Myog*. The exact mechanism about the effects of dietary plant protein sources and exogenous enzyme addition on *MRFs* expression is not clearly understood in fish, and more studies should be conducted in the future.

Histological change is an important aspect in the understanding of pathological alteration related to nutritional sources in fish. In the present study, dietary CM substitution caused some histopathological changes in liver of crucian carp, such as karyopyknosis and enlarged hepatocyte size, which was in agreement with our previous study (Shi *et al.* 2015). Similarly, a higher vacuolization in liver was presented in seabream with microalgae inclusion diets (Atalah, Cruz, Izquierdo, Rosenlund, Caballero, Valencia & Robaina 2007). Foregut morphology in this study seemed not to be altered by CM substitution and cellulase addition. Some authors, however, reported negative effects on intestinal morphology with dietary microalgae inclusion, such as epithelial degeneration (Atalah *et al.* 2007), oedema and inflammation (Cerezuela, Fumanal, Tapia-Paniagua, Meseguer, Morinigo & Esteban 2012).

In conclusion, the present study suggested that CM could replace 100% of FM protein in the diets for crucian carp based on fish growth and feed utilization. Besides, dietary cellulase supplementation (enzyme activity: 1160 IU g^{-1}) in high CM levels of diets at the level of 2 g kg^{-1} did not produce positive growth and feed utilization in crucian carp. The results from this study provide important information regarding the potential application of CM as a valuable alternative protein for freshwater omnivorous fish.

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Conflict of interest

All the authors stated no conflict of interest.

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