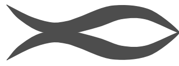

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 17:1039-1048(2017) DOI: 10.4194/1303-2712-v17_5_20

PROOF RESEARCH PAPER

Effect of Replacing Fish Meal with Fermented Mushroom Bran Hydrolysate on the Growth, Digestive Enzyme Activity, and Antioxidant Capacity of Allogynogenetic Crucian Carp (*Carassius auratus gibelio*)

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Received 21 September 2016
Accepted 07 March 2017

Abstract

The objective of this study was to investigate the effects of partially replacing fish meal with FMBH on the growth, digestive enzyme activity, and antioxidant capacity of allogynogenetic crucian carp. Fermented mushroom bran hydrolysate (FMBH) was prepared by enzymatic hydrolysis after the solid fermentation of mushroom bran (MB) inoculated with *Ganodermalucidum* and *Saccharomyces cerevisiae.* Nine hundred fish were allotted to six groups and were fed diets in which the fish meal was replaced with 0% (FMBH-0), 16% (FMBH-16), 32% (FMBH-32), 48% (FMBH-48), 64% (FMBH-64), and 80% (FMBH-80) FMBH. After 71 days, the weight gain ratio and the protein efficiency ratio of FMBH-64 and FMBH-80 were significantly higher than those of the FMBH-0 group ($P<0.05$). No significant differences were noted for the specific growth ratio, feed conversion ratio, or survival rate among all groups ($P>0.05$). The protease activity in the midgut was not significantly different among all groups ($P>0.05$). However, compared with FMBH-0, the protease activity in the fore gut of FMBH-32, FMBH-48, FMBH-64, and FMBH-80 was significantly elevated ($P<0.05$), the amylase activity in the fore gut and midgut of FMBH-32, FMBH-48, FMBH-64, and FMBH-80 was significantly elevated ($P<0.05$), and the lipase activity in the fore gut and midgut of FMBH-48, FMBH-64, and FMBH-80 was significantly elevated ($P<0.05$). Compared with FMBH-0, FMBH-80 demonstrated a significant increase in the serum total antioxidant capacity and anti-superoxide anion activity ($P<0.05$), but a significant decrease in the maleic dialdehyde content ($P<0.05$). FMBH-48 and FMBH-64 also significantly increased the anti-superoxide anion activity ($P<0.05$). Therefore, replacing a 64%-80% proportion of dietary fish meal with FMBH could improve the growth, digestive enzyme activity, and antioxidant capacity of allogynogenetic crucian carp.

Keywords: Fermented mushroom bran hydrolysate, allogynogenetic crucian carp (*Carassius auratus gibelio*), growth, digestive enzyme, antioxidant capacity.

Introduction

In protein metabolism, the demand for feed protein is higher in fish than in livestock. Fish meal is the main protein source of aquatic feed, and given the increasing demand with the worldwide growth of the aquaculture industry, the global yield cannot meet the production demand of the feeding industry. Therefore, replacing highly expensive fish meal with a novel high-quality and highly digestible raw protein material has become an urgent issue.

Several sources of plant protein have recently been tested on various fish to partially or completely replace the more expensive fish meal. Most studies have been conducted on soy proteins (Aksnes, Mundheim, Toppe, & Albrektsen, 2008), but the presence of non-nutritional factors and the lack of essential amino acids have limited the practical application of soy proteins (Gatlin *et al.*, 2007). To date, more attention has been paid in modern biotechnology to the processing of various protein sources in order to obtain a protein hydrolysate with improved functional and nutritional characteristics. This includes hydrolysate products such as soy protein hydrolysate (SPH), fish meal hydrolysate (FMH), and cottonseed meal protein hydrolysate (CMH). Culture experiments have proven that these hydrolysates are ideal nutrient sources and can improve weight gain, feed conversion, immune function, digestive ability, and the intestinal development of fish when added within a suitable range (Kotzamanis, Gisbert, Gatesoupe, Zambonino, & Cahu, 2007). These beneficial effects are attributed to the high proportion of short peptides and free amino acids in the hydrolysates after biotechnological processes, such as enzymatic hydrolysis. Moreover, the smaller peptides and amino acids have independent absorption mechanisms (Chu, Yang, Kim, Kim, & Song, 2012) and can be more quickly and preferentially absorbed by intestinal cells (Onal &

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in cooperation with Japan International Cooperation Agency (JICA), Japan

Langdon, 2009), thereby effectively promoting protein absorption and utilization (Webb, 1990).

Mushroom bran (MB) is the used solid medium after the harvest of mushroom fruit bodies. Approximately 5 kg of MB remains after the cultivation of 1 kg of mushroom fruit bodies (Paredes *et al*., 2009). MB is produced in huge amounts every year, worldwide. For example, the amount of MB produced per year was 3.4×10^6 M^3 in United States, 2.1×10^6 M^3 in the Netherlands, 1.9×10^6 tons in Ireland, and 7.0×10^6 tons in China (Williams, McMullan, & McCahey, 2001). Failing to utilize MB resources will lead to a large amount of waste and may even cause environmental pollution to some degree. MB is rich in nutrients (Lu *et al*., 2008). After the biological fermentation of MB with microbial agents, the protein content of the obtained fermented mushroom bran (FMB) can be improved (Kwak, Jung, & Kim, 2008). To date, some studies have reported the application of MB and FMB in animal culture, however, they are mainly used for livestock and poultry (Chu *et al*., 2012).

With MB as the raw material, we prepared FMB for the present study with a crude protein content of 15.75% (% dry matter) after the inoculation of *Ganodermalucidum* and *Saccharomyces cerevisiae* by solid fermentation. Enzymatic hydrolysis and extraction successfully produced the fermented mushroom bran hydrolysate (FMBH), with 53.96% of the dry matter attributed to short peptides and free amino acids. Nevertheless, the effects of FMBH on the animals have not yet been reported. In our study, after a partial replacement of dietary fish meal with FMBH, we investigated the effects on the growth, digestive enzyme activity, and antioxidant capacity of allogynogenetic crucian carp. Our results provide a theoretical basis for the application of FMBH in aquaculture feed.

Materials and Methods

FMBH Preparation

FMBH was produced by the methods described by Xu *et al*. (2015) and Ke *et al*. (2016). Specifically, approximately 1000 g of *Pleurotuseryngii* bran was broken into pieces and dried at 50 °C until its moisture content was less than 5%. The seed culture fluid of *G. lucidum* was inoculated into MB solid medium and cultured for 7 days at 28 °C, at 50%–60% humidity. The *S. cerevisiae* seed culture fluid was inoculated and cultured for 4 days under the same conditions. After the culture, the prepared FMB was dried at 50 °C and crushed into powder. Next, 10000 mL of water was added. The mixture was thoroughly stirred in a reaction vessel, which was equipped with the stirring device and an automatic temperature control unit.

The mixture was first adjusted to pH 4.8 with a hydrochloric acid solution, 7 g of cellulose was added, and the solution was hydrolyzed for 1 h at 50 °C. The mixture was adjusted to pH 9.0 with a sodium hydroxide solution, 5g of alkaline protease was added, and it was hydrolyzed for 3 h at 45 °C. Finally, the mixture was adjusted to pH 7.0 with a hydrochloric acid solution, 5 g of neutral protease was added, and it was hydrolyzed for 3 h at 45 °C.

The mixture was then heated to 85 °C for 10 min to inactivate the enzyme and centrifuged for 15 min at 4000 rpm. After removing the solid residue, the supernatant was harvested and concentrated in a vacuum at 55 °C, whereupon it was dried by spray technology. In total, 180 g of FMBH was obtained.

Analysis of Different Diets, MB and the Hydrolysate

The proximate compositions of the MB, FMB, and FMBH diets were measured. The amounts of moisture, crude protein, crude fat, and ash were determined according to the AOAC methods (2003). The dry matter content was determined by drying in an oven at 105 °C. The ash content was determined by combustion at 550 °C in a muffle furnace for 3h. The crude protein content was determined using the Kjeldahl method (Kjeldahl apparatus, Gerhardt, Germany). The crude fat content was determined using dichloromethane extraction (Soxtec System HT6, FOSS TECATOR, Sweden) and the protein solubility was determined according to the nitrogen solubility index (NSI), which was calculated according to the procedure of Morr (1985):

$$\text{NSI (\%)} = 100\% \times \text{Protein content in supernatant} / \text{Total protein content in sample.}$$

The molecular weight of the FMBH soluble protein fractions was measured by placing 20 mg of FMBH in a 10 mL volumetric flask and then diluting it with the mobile phase (acetonitrile: water: trifluoroacetic acid=45:55:0.1, volume ratio) to volume. After being fully dissolved by ultrasonic oscillation, the sample solution was passed through a 0.22 μm nylon membrane, and the obtained filtrate was analyzed with gel permeation chromatography (GPC). The measured data were introduced into the GPC data processing software and calculated with its calibration curve equation before the relative molecular weights and the sample peptide distribution chart were obtained. The sum of the peak area percentage for each relative molecular weight range was calculated with the peak area normalization method and then multiplied by the crude protein content of FMBH, thereby obtaining the percentage dry matter of each relative molecular weight range.

Experimental Diets

Six isonitrogenous and isoenergetic experimental diets (32% crude protein and 6% crude

fat) were formulated (Table 1). Graded levels of FMBH (FMBH-0, FMBH-16, FMBH-32, FMBH-48, FMBH-64, and FMBH-80) were included in the diets to replace 0, 16, 32, 48, 64, and 80% of dietary fish meal. FMBH was incorporated at different ratios into the microbound diets of fish meal to hydrolysate. Firstly, $Ca(H_2PO_4)_2$, NaCl, inositol, Vitamin C, choline chloride, ethoxyquin, and premix were combined. The feed materials were then added and thoroughly mixed and the water was finally added. The dough was pelletized to form 2.0 mm-diameter wet pellets and then air dried at room temperature.

Experimental Procedure

A total of 900 fish with an average body weight of 12.02±0.07 g, were randomly allotted to six groups and fed with fish meal replaced with 0% (FMBH-0), 16% (FMBH-16), 32% (FMBH-32), 48% (FMBH-48), 64% (FMBH-64), or 80% (FMBH-80) FMBH. Each group had five replicates and 30 fish for each replicate were stocked in 30 round fiberglass tanks (φ820×700mm). The fish were manually fed four times a day (08:00–08:30, 11:00–11:30, 14:00–14:30, and 17:00–17:30). The leftover feed particles in the fish tank were removed by the automatic recirculating system after 1 h and food consumption was recorded every day. The consumed quantity of feed was until apparent satiation on the basis of visual observation of fish feeding behavior. The water temperature was kept constant at 25.0 °C±1.5 °C. The feeding experiment lasted for 71 days.

Sampling and Dissection

At the beginning of the experiment, the weights of each crucian carp were measured. At the end of the experiment, all fish were starved for 24 h to enter a basic metabolic state and eliminate the dietary effect. All of the fish from each tank were weighed, in order to calculate the growth indices and the feed conversion ratios. At the same time, the survival rate was determined by counting the living individuals in each tank. A total of three fish from each replicate were selected and deeply anesthetized before quickly placing samples from each replicate into a 3-ethyl 3-aminobenzoate methane sulfonic acid solution (200 mg L^{-1}) after 24 h of starvation. Blood was sampled from the fish caudal vein with an injector (2 mL specification) wetted with heparin and a sampling volume of approximately 1.5 mL per tube. After being kept at 4 °C for 1–2 h, the blood samples were centrifuged at 3000 rpm for 10 min. The obtained serum was stored at −20 °C for the determination of

Table 1. Formulation and proximate composition of the experimental diets (%)

Ingredients	Diets					
	FMBH-0	FMBH-16	FMBH-32	FMBH-48	FMBH-64	FMBH-80
Fish meal[1]	10	8.4	6.8	5.2	3.6	2
Fermented mushroom bran hydrolysate	0	1.6	3.2	4.8	6.4	8
Chicken meal[2]	5	5	5	5	5	5
Methionine+Cystine	0	0.02	0.04	0.06	0.08	0.09
Lysine	0	0.03	0.06	0.08	0.09	0.12
Soybean meal	22	22	22	22	22	22
Rapeseed meal	23	23	23	23	23	23
Cottonseed meal	10	10	10	10	10	10
Middlings	10	10	11	12	12.5	12.8
Rice bran	7	7	7	7	7	7
Soybean oil	3.1	3.1	3.2	3.3	3.4	3.57
Phospholipid power	1	1	1	1	1	1
Choline chloride (50%)	0.25	0.25	0.25	0.25	0.25	0.25
Premix (vitamins and minerals)[3]	2	2	2	2	2	2
$Ca(H_2PO_4)_2$	1.2	1.4	1.6	1.75	1.95	2.1
NaCl	0.5	0.5	0.5	0.5	0.5	0.5
Bentonite clay	4.38	4.13	2.78	1.49	0.66	0
Vitamin C	0.04	0.04	0.04	0.04	0.04	0.04
Inositol	0.03	0.03	0.03	0.03	0.03	0.03
Ethoxyquin	0.5	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100	100
Proximate composition						
Dry matter (%)	91.37	91.35	91.21	91.08	91.02	90.97
Crude protein (%)	32.64	32.49	32.46	32.43	32.32	32.21
Crude fat (%)	6.04	5.92	5.92	5.93	5.92	5.97
Available Phosphorus	0.76	0.76	0.77	0.76	0.76	0.75
Lysine	1.86	1.86	1.87	1.87	1.86	1.86
Methionine+Cystine	0.84	0.84	0.84	0.85	0.85	0.84

[1]Fish meal (crude protein 66%) was provided by Coprinca Ltd (Lima, Peru).
[2]Chicken meal (crude protein 62%) was provided by MengzhouXindongfang Feed Co., Ltd. (Henan, China).
[3]Premix (vitamins and minerals) was provided by Wuxi Hanove Animal Health Products Co., Ltd. (Jiangsu, China).

serum antioxidant indices. After blood sampling, the fish were dissected and samples of the liver and viscera were collected. The gut samples were stored at –80 °C for subsequent digestive enzyme assays. The fish gut was divided into three sections, namely the fore gut, midgut and hindgut. The fore gut and midgut of 9 individuals from each group (3 fish/ tank) was carefully weighed and homogenized in 0.01M Tris buffer at pH 7.4, a 1:9 tissue: buffer ratio, and with a Teflon pestle of a motor-driven tissue-cell disruptor under an ice-bath. The extract was centrifuged at 5,000 rpm for 30 min and the supernatant was used to analyze digestive enzyme activity within 24 h.

Analytical Methods

Growth performance was measured by the methods described by Liao *et al*. (2014) as below.

Weight gain ratio (WGR, %)=100×[(final body weight−initial body weight)/initial body weight]

Specific growth ratio (SGR, %/day)=100×(ln final body weight−ln initial body weight) / experimental days

Protein efficiency ratio (PER, %)=100×(final body weight− initial body weight)/protein intake

Feed conversion ratio (FCR)=dry feed/(final body weight−initial body weight)

Survival rate (SR, %) =100×[(initial number of fish −number of dead fish)/ initial number of fish]

Digestive Enzyme Measurement

The protein content of the supernatant solutions was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA, A-2153, Sigma) as the standard.

The overall protease activity was determined using the Cupp-Enyard (2008) method with casein as the substrate (0.65% w/v casein solution, 50 mM potassium phosphate buffer). The protease activity in the intestine was assayed in the same way, following the Forint phenol-reagent method in 0.01M Tris-HCl (pH 7.4) buffer using 2% casein as a substrate. Reactions were carried out at 30 °C for 10 min, stopped with 0.1M trichloroacetic acid (TCA), centrifuged at 4 °C and 3,000×g for 5 min. Next, 0.5 mL of the supernatant was added to 2.5 mL of 0.4M $NaHCO_3$ and 0.5 mL of 50% Folin's phenol reagent and the optical density was read at 680 nm against a tyrosine standard. Both a substrate-free control and an enzyme-free control were run with the experimental samples. The specific protease activity is expressed in micromole of hydrolyzed tyrosine per minute per milligram of protein (U/mg tissue protein).

The lipase activity was determined by evaluating the degradation of triacylglycerols, diacylglycerols, and monoacylglycerols to free fatty acids, following the method presented by Furné *et al*. (2005). For the emulsion, a 1% solution of 1 L polyvinyl alcohol (PVA) in distilled water was used. Then, 5 mL of 0.1 N HCl were added, heated to 75–85 ℃ for 1 h, followed by cooling, filtering, and an adjustment of the pH to 8.0 with 0.1 N NaOH. To an aliquot of the above solution, virgin olive oil was added to achieve a substrate concentration of 0.1M and the mixture was emulsified for 5 min. The reaction mixture was composed of a PVA solution-emulsified substrate (1 mL), McIlvaine buffer at pH 8 (0.5 mL), and digestive extract (0.5 mL). The McIlvaine buffer was prepared from 0.1 M citric acid and 0.2M bisodium phosphate. The reaction mixture was incubated for 4 h at 37 ℃. After incubation, 3 mL of a 1:1 ethanol–acetone solution were added to stop the reaction and break the emulsion. A few drops of 1% phenolphthaleine in ethanol were then added to the reaction mixture and were titrated with 0.01 M NaOH. For the blank tubes, the same procedure was followed, but with boiled enzyme. Porcine type-II pancreatic lipase (Sigma L3126) was used as a standard. One unit of lipase activity was defined as the hydrolysis of 1.0 microequivalent of fatty acids from triacylglycerols in 1 h at pH 7.7 and 37 ℃.

The amylase activity was determined by the starch-hydrolysis method, according to Furné *et al.* (2005). The enzymatic reaction mixture consisted of 2% (w/v) starch solution (0.125 mL), 0.1 M citrate–phosphate buffer at pH 7.5 (0.125 mL), and a digestive extract (0.05 mL). The reaction mixture was incubated for 1 h at 37 ℃. Absorbance was determined at 600 nm. For the blank tubes, the same procedure was followed, except that the extract was added just after incubation. Maltose was used as a standard, and the activity unit of amylase was defined as the quantity of enzyme that produced one mmol of maltose mL^{-1} min^{-1}.

Serum Antioxidant Indices Measurement

Serum total antioxidant capacity (T-AOC), serum anti-superoxide anion (ASA) activity, and serum maleic dialdehyde (MDA) content were determined using the methods described by Rice-Evans and Miller (1994), Granelli, Bjorck and Appelqvist (1995), and Draper *et al*. (1993), respectively. All of the test kits were purchased from Mindray Bio medical Co., Ltd. (Shenzhen, China). The indices were measured with the BS-400 Mindray automated biochemical analyzer according to the manufacturer's instructions. The serum protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA, A-2153, Sigma) as the standard.

Statistical Analysis

Statistical analyses were performed with SPSS

16.0 (SPSS Inc., Chicago, IL, USA). All experimental data were subjected to one-way ANOVA. The various lower-case letters show significant differences (P<0.05) in different dosage groups in Duncan's multiple range tests. All results were expressed as the mean±standard error of the mean (SEM).

Results

Analysis of Different MB and the Hydrolysate

As shown in Table 2, only MB had a decreased protein content of 9.34% (% dry matter). Nevertheless, after the solid step fermentation of MB by *G. lucidum* and *S. cerevisiae*, the obtained FMB had a higher protein content of 15.75%. The extra protein (6.41%, dry matter) was produced by bacteria that fed on the MB. Furthermore, after enzymatic hydrolysis and extraction of FMB, the obtained FMBH had a protein content of 53.96%. The protein solubility of FMBH was close to 100%.

As shown in Figure 1, the FMBH prepared in our study contained almost 22% free amino acids (<180 Da), 20% short peptides (180–500 Da), and 10% oligopeptides (including 500–1000 Da and 1000–2000 Da), but only contained 1.5% polypeptides (>2000 Da).

Growth Performance

As shown in Table 3, there were no dead fish from any group, throughout the entire feeding period. Compared with FMBH-0, the WGR of FMBH-64 and FMBH-80 increased significantly (P<0.05). Compared with FMBH-0, the PER of the groups in which fish meal was replaced with FMBH increased significantly, as well (P<0.05). No significant differences were observed in the SGR or FCR among all groups (P>0.05). However, as the proportion of replaced dietary fish meal increased, the SGR gradually increased, whereas the FCR gradually decreased.

Activity of Digestive Enzymes in the Intestinal Tract

Amylase activity

As shown in Figure 2, compared with FMBH-0, amylase activities in the fore gut and midgut of

Table 2. Proximate composition (% dry matter) and protein solubility (%) of different MB and hydrolysate

Protein sources	Crude protein	Crude fat	Ash	Moisture	Protein solubility
MB	9.34	0.45	8.63	4.49	13.41
FMB	15.75	0.28	9.98	5.17	18.68
FMBH	53.96	0.22	5.47	5.92	98.96

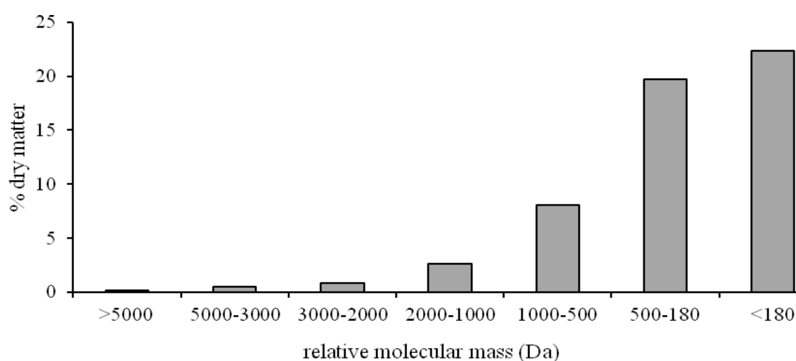

Figure 1. Distribution of molecular weight among peptides in FMBH.

Table 3. Effects of different FMBH levels on the growth performance of allogynogenetic crucian carp

Groups	IBW(g)	WGR (%)	SGR(%/day)	PER (%)	FCR	SR(%)
FMBH-0	12.09±0.04[a]	160.42±5.72[c]	1.35±0.06[a]	142.55±5.32[e]	2.04±0.66[a]	100
FMBH-16	12.02±0.05[a]	169.28±4.06[bc]	1.40±0.04[a]	159.72±3.76[d]	1.91±0.58[a]	100
FMBH-32	12.06±0.07[a]	173.99±6.59[bc]	1.42±0.05[a]	164.90±3.92[cd]	1.85±0.39[a]	100
FMBH-48	12.03±0.05[a]	178.28±6.70[bc]	1.44±0.09[a]	169.48±4.69[bc]	1.80±0.36[a]	100
FMBH-64	12.08±0.02[a]	194.86±5.22[a]	1.50±0.08[a]	172.35±5.85[b]	1.77±0.20[a]	100
FMBH-80	12.01±0.06[a]	200.35±7.88[a]	1.55±0.10[a]	179.45±6.28[a]	1.70±0.29[a]	100

Note: For each column, values followed by different lowercase letters are significantly different (P<0.05).

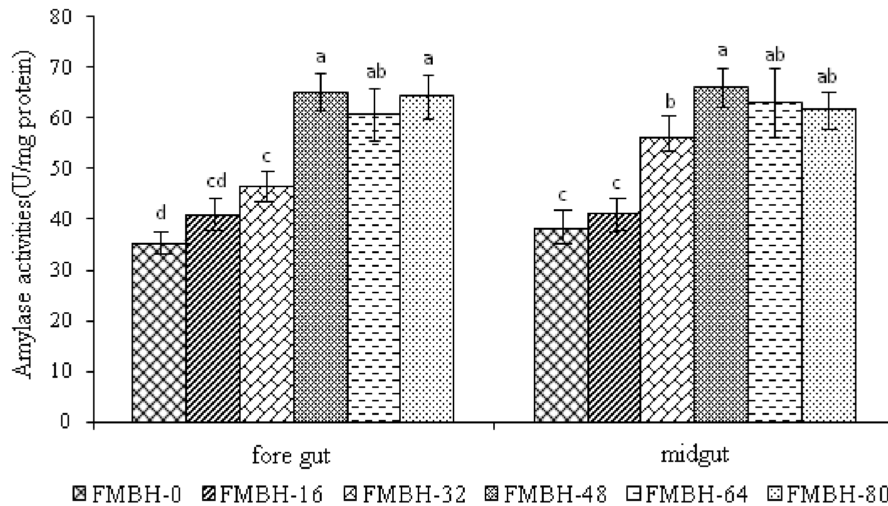

Figure 2. Effects of different FMBH levels on amylase activity in the intestine of allogynogenetic crucian carp.
Note: Data are presented as mean±S.E.M. from five replicate tanks (30 fish per tank) (N=5). Different letters above the bars denote significant differences between diet groups at the P<0.05 level. The vertical bars indicate the size of the data S.E.M.

FMBH-32, FMBH-48, FMBH-64, and FMBH-80 were all significantly elevated (P<0.05). Meanwhile, no significant difference was observed in amylase activity in the fore gut or midgut among the higher proportion replacement groups of FMBH-48, FMBH-64, and FMBH-80 (P>0.05).

Protease Activity

As shown in Figure 3, protease activity in the midgut was not significantly different among any of the groups (P>0.05). Compared with FMBH-0, protease activities in the fore gut of FMBH-32, FMBH-48, FMBH-64, and FMBH-80-fed individuals were all significantly elevated (P<0.05). Meanwhile, there was no significant difference of protease activity in the fore gut among the FMBH-32, FMBH-48, FMBH-64, and FMBH-80 groups (P>0.05).

Lipase Activity

As shown in Figure 4, lipase activities in the fore gut of FMBH-32, FMBH-48, FMBH-64, and FMBH-80-fed individuals were all significantly elevated compared with the FMBH-0 group (P<0.05), whereas lipase activities in the midgut of FMBH-48, FMBH-64, and FMBH-80 were all significantly elevated (P<0.05). Meanwhile, there was no significant difference in lipase activity between the FMBH-64 and FMBH-80 groups (P>0.05). Therefore, by replacing 48%-80% of the dietary fish meal with FMBH, lipase activity increased significantly in the fore gut and midgut. However, in the high proportion replacement (64%–80%), the increase of lipase activity was limited.

Serum Antioxidant Indices

According to Table 4, as the proportion of replaced dietary fish meal increased, T-AOC and ASA activity also gradually increased, whereas the MDA content gradually decreased. Compared with the FMBH-0 group, serum T-AOC and serum ASA activity of the FMBH-80 increased significantly (P<0.05), whereas the serum MDA content decreased significantly (P<0.05). Serum ASA activity of FMBH-48 and FMBH-64 was also shown to increase significantly (P<0.05).

Discussion

The enzymatic hydrolysis of proteins is an effective method to improve the functional and nutritional properties of protein products. The use of multiple enzymes under various conditions can form hydrolysates with beneficial nutritive properties. The peptides and free amino acids weighing less than 3000 Da accounted for 25.5% of the SPH prepared by Song *et al.* (2014), whereas peptides and free amino acids weighing less than 2000 Da accounted for 18.9% of the CMH prepared by Gui, Liu, Shao, and Xu (2010). In contrast, the peptides and free amino acids less than 2000 Da in size accounted for 52% of the FMBH prepared in the present study. These differences might be attributable to the different enzymatic hydrolysis technologies that were employed, as well as the easy hydrolysis of microbial proteins in FMB. The use of fishmeal in aquatic feeds is mainly limited because of the high price and decline of fishery resources. Additionally, excessive use of fish meal in aquatic feeds may cause a series of environmental problems, due to the high phosphorus content (Yang, Wang, Lu, & Li, 2011). Therefore, minimizing the proportion of fish meal in formula feeds is important, both to the economy and the environment. The FMBH produced in the present study had a wide range of low-molecular-weight peptides. Thus, we chose fish meal as the substance to

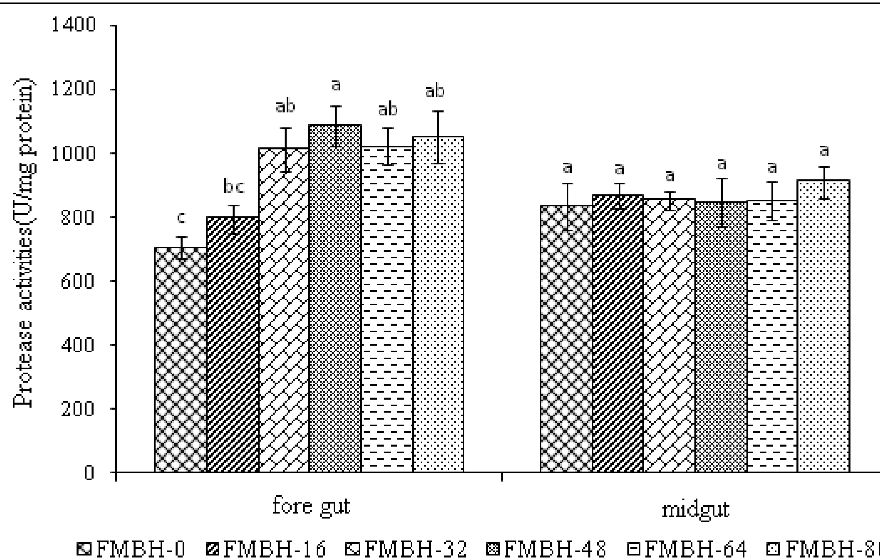

Figure 3. Effects of different FMBH levels on protease activity in the intestine of allogynogenetic crucian carp.
Note: Data are presented as mean±S.E.M. from five replicate tanks (30 fish per tank) (N=5). Different letters above the bars denote significant differences between diet groups at the P<0.05 level. The vertical bars indicate the size of the data S.E.M.

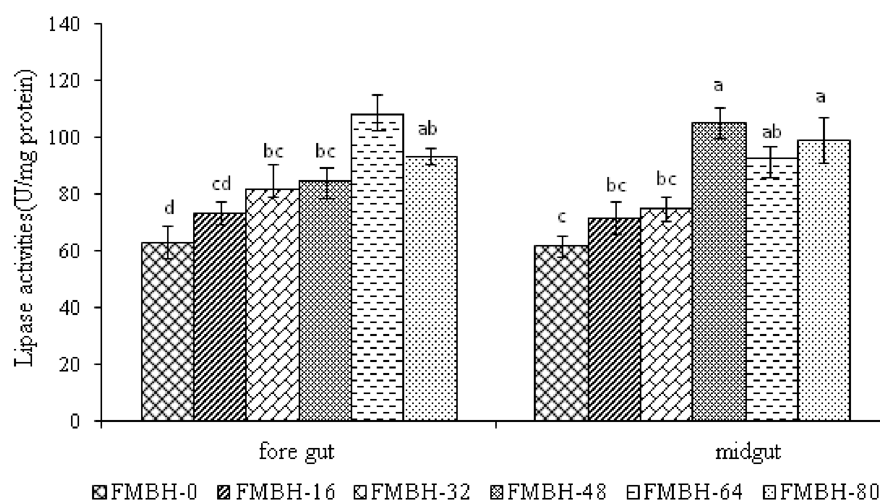

Figure 4. Effects of different FMBH levels on lipase activity in the intestine of allogynogenetic crucian carp.
Note: Data are presented as mean±S.E.M. from five replicate tanks (30 fish per tank) (N=5). Different letters above the bars denote significant differences between diet groups at the P<0.05 level. The vertical bars indicate the size of the data S.E.M.

Table 4. Effects of different FMBH levels on the serum antioxidant indices of allogynogenetic crucian carp

Groups	T-AOC (U/mg protein)	ASA activity(U/mg protein)	MDA content (nmol/mL)
FMBH-0	9.44 ± 0.56^{b}	90.46 ± 2.56^{c}	7.88 ± 0.31^{a}
FMBH-16	12.43 ± 0.84^{ab}	99.96 ± 2.09^{bc}	7.84 ± 0.36^{a}
FMBH-32	13.07 ± 0.63^{ab}	103.82 ± 4.15^{bc}	7.81 ± 0.30^{a}
FMBH-48	13.57 ± 1.05^{ab}	118.63 ± 5.53^{b}	6.49 ± 0.34^{ab}
FMBH-64	15.08 ± 1.44^{ab}	129.84 ± 4.36^{ab}	5.39 ± 0.27^{ab}
FMBH-80	20.69 ± 1.49^{a}	139.88 ± 6.11^{a}	2.71 ± 0.13^{b}

Note: For each column, values followed by different lowercase letters are significantly different (P<0.05).

be replaced with FMBH.

Gui *et al*. (2010) found that, when fed with 50 g/kg of CMH (replacing about 28% of the dietary fish meal), the WGR was much higher and the FCR was lower than was seen in the control in crucian carp. Mamauag *et al*. (2011) reported that the WGR and SGR of Japanese flounder could be improved by replacing 28% of dietary fish meal with SPH, but was

reduced when 43%-59% of their dietary fish meal was replaced with SPH. Our experimental results showed that high proportion replacement of dietary fish meal with FMBH (replacing 64%-80%) could efficiently improve WGR and PER, but had no significant effect on the SGR and FCR of allogynogenetic crucian carp. This difference may be due to the composition of hydrolysates, which were produced by different raw materials and processes. The observed improvements of WGR and PER of FMBH in fish fed with fish meal replaced with high percentages of FMBH may be caused by the elevation of digestive enzyme activity and antioxidant capacity. This would promote nutrient absorption and enhance innate immune function, thereby contributing to the growth of the fish. However, there was no significant difference in WGR between FMBH-64 and FMBH-80. The expected improvement was not observed in these two high proportion replacement groups, highlighting the need for further research.

When receiving a diet that had 25% of the dietary fish meal replaced with protein hydrolysate from fish muscle or squid mantle, the asian seabass showed increased activity of pancreatic enzymes, trypsin, chymotrypsin, and lipase, whereas α-amylase activity was not significantly different (Srichanun, Tantikitti, Kortner, Krogdahl, & Chotikachinda, 2014). The replacement of dietary fish meal with SPH did not affect amylase, pepsin, or lipase activities of the juvenile starry flounder digestive tract, but trypsin activity was significantly elevated (Song *et al*., 2014). In the present study, after replacing dietary fish meal with FMBH, the protease activity in the midgut of the allogynogenetic crucian carp was not affected. However, the fore gut protease activity was significantly elevated in the high-proportion replacement groups (FMBH-48, FMBH-64, and FMBH-80). In addition, the lipase and amylase activities in the fore gut and midgut were both found to be significantly elevated. These results indicate that the high-proportion replacement of dietary fish meal with FMBH could efficiently elevate intestinal digestive enzyme activity. These elevated levels of digestive enzyme activity were probably related to the rich small peptides content of FMBH. The small peptides are absorption substrates of the intestinal lumen, which can efficiently accelerate the growth of villi, promote the proactive development of the intestinal tract, and elevate intestinal digestive enzyme activity (Bamba, Fuse, Obata, Sasaki, & Hosoda, 1993). Meanwhile, the rapid absorption of small peptides and free amino acids can trigger intracellular calcium-mediated signaling events, followed by the release of more digestive enzymes (Liou *et al*., 2011). Nevertheless, the intestinal digestive enzyme activity showed no significant increase with increasing replacement proportions (FMBH48, FMBH64, or FMBH80). This result requires further study.

During the metabolic processes, the animal leukocyte and phagocyte could produce superoxide anions and then transformed them into oxygen free radicals, which might lead to the cell injury (Palace *et al*., 1998). The fish mainly relied on their own antioxidant defense system to remove oxygen free radicals and maintained their biochemical, molecular and physiological homeostasis (Pan *et al*., 2012). T-AOC was the comprehensive index of the body antioxidant function and status, reflecting the body compensatory ability about external stimuli and the body metabolism state of free radicals; ASA activity directly reflected the body ability to remove superoxide anion (Zenteno-Savin, Saldierna, & Ahuejote-Sandoval, 2006). MDA was the characteristic product of unsaturated lipid peroxided by oxygen free radicals, which may hurt organisms (Janero, 1990). Therefore, the detection of T-AOC, ASA activity and MDA could reflect the antioxidant status of fish. The replacement of dietary fishmeal with SPH within a range of 15%–70% was shown to elevate the T-AOC of the juvenile starry flounder (Song *et al*., 2014). Similar results were obtained in our studies. Compared with the control group (FMBH-0), the T-AOC of the FMBH-80 group increased significantly and the ASA activity of the FMBH-48, FMBH-64, and FMBH-80 groups also increased significantly, whereas the serum MDA content of the FMBH-80 group decreased significantly. This demonstrates that a high proportion replacement of dietary fish meal with FMBH could efficiently elevate the antioxidant capacity of allogynogenetic crucian carp. These results were probably related to the rich concentration of small peptides in FMBH. The small peptides including antioxidant peptides could stimulate and activate the antioxidant capacity of fish (Moure, Dominguez, & Parajo, 2006) and promote the activity of antioxidant enzymes (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Antioxidant capacity plays a prominent role in resistance to oxide damage (Lopes, *et al.,* 2001). On the other hand, the activation of non-specific defense mechanisms in fish is evident by increased total antioxidant capacity (Itou, Lida, & Kawatsu, 1996). Taken together, our results suggest that a 48%-80% replacement with FMBH could reduce the potential oxidative stress and enhance the non-specific immunity of allogynogenetic crucian carp.

In conclusion, the present study mainly demonstrates the significant role of FMBH peptides for the nutrition of allogynogenetic crucian carp. When 64%-80% of the dietary fish meal was replaced with FMBH, the weight gain ratio, protein efficiency ratio, digestive enzyme activity, and antioxidant capacity of the fish were shown to significantly improve. This study promotes the application value of mushroom bran in aquaculture feeds. In future, further investigations are needed, in order to clarify possible morphological and proteomic changes in the fish intestine, liver, and muscle in response to dietary

fishmeal replacement with FMBH.

Notes

Abbreviations: MB, mushroom bran; FMB, fermented mushroom bran; FMBH, fermented mushroom bran hydrolysate; SPH, soy protein hydrolysate; FMH, fish meal hydrolysate; CMH, cottonseed meal protein hydrolysate.

Acknowledgements

This work was supported by an open-ended project (KF201305), which was funded by the Key Laboratory of Freshwater Fisheries and Germplasm Resource Utilization of the Ministry of Agriculture at the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences in Wuxi, China, as well as the National Technology System for Conventional Freshwater Fish Industries and the Modern Agriculture Industrial Technology System (CARS-46). The molecular weights of the soluble protein fractions in the FMBH product were analyzed on the State Key Laboratory of Food Science and Technology at Jiangnan University in Wuxi, China.

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