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Growth performance, intestinal microbiota and immune response of grass carp fed isonitrogenous and isoenergetic diets containing faba bean extracts

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ABSTRACT

Functionally active components of faba bean (*Vicia faba*, FB) are known to improve the muscle texture and growth performance of grass carp (*Ctenopharyngodon idellus*), but this has not yet been proven by feeding trials using isonitrogenous and isoenergetic diets. Here, we formulated isonitrogenous and isoenergetic diets containing four types of FB extracts and examined their effects on the weight gain, textural quality, intestinal microbiota, metabolic enzymes and immune response of grass carp by a 100-day feeding trial. An isonitrogenous and isoenergetic diet with no FB extracts (control) and whole FB were also used. Compared to the control, the FB water extract diet increased weight gain, muscle hardness and chewiness, intestinal microbiota diversity, the activity of metabolic enzymes including lipase (LP), glucose-6-phosphatase (G-6-P), acetyl-CoA and succinate dehydrogenase (SDH) ($P < 0.05$). The FB alcohol extract diet also increased weight gain, intestinal microbiota diversity, but the total cholesterol (TC) and triglycerides (TG) contents were also increased ($P < 0.05$). The FB protein extract and FB residues diets had negative and no effects on weight gain, respectively, and caused intestinal microbiota disorder. FB water and alcohol extracts enhanced intestinal amino acids metabolism, whereas FB water extract and residues enhanced intestinal carbohydrate metabolism. These results further extend previous findings that FB water and alcohol extracts contain most functionally active components of FB, opening up the possibility that these extracts can be used as functional feed additives for freshwater fish.

1. Introduction

Grass carp (*Ctenopharyngodon idellus*) is one of the most crucial freshwater aquaculture species in China, and crisp grass carp (*C. idellus* C.et V) is its high-value commodity product. In the early 1970s, fish farmers accidentally discovered that muscle textural quality (hardness, springiness, etc.) of grass carp was improved significantly after being fed solely with faba bean (*Vicia faba*, FB) for 90–120 days, and this variety was called crisp grass carp (Yu et al., 2017). The muscle textual changes of crisp grass carp were found to associate with the structure of muscle fibers and collagen contents rather than the proximate composition (Wang et al., 2015; Ma et al., 2020a). Because of the high market value, crisp grass carp has been protected as the "China Geographical Indication Product", and its products are exported to North America (Yang et al., 2015). However, a large amount of faba bean intake causes poor palatability, intestinal inflammation, oxidative damage and growth retardation of grass carp (Li et al., 2018; Zhang et al., 2015). To alleviate these negative impacts of faba bean, two previous studies tested the effect of FB extracts on grass carp, demonstrating that FB water extract improved the textural quality with little or no negative effects on grass carp physiology (Ma et al., 2020a, 2020b). FB water and alcohol extracts also promoted growth performance, indicating the possibility that functionally active components of faba bean can be isolated by a simple extraction procedure. However, these studies had a limitation that the

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formulated diets were not isonitrogenous or isoenergetic. This limitation causes the uncertainty about effectiveness and the safety evaluation of FB extracts as aquafeed additives.

The intestinal microbiota plays a crucial role in many important physiological processes of the fish host including growth, metabolism and immunity (Fetissov, 2017). The abundance of beneficial and harmful bacteria in the intestine is closely related to the health of the host (Hornef, 2015). Microbiota regulates intestinal absorption and extraintestinal metabolism of fatty acids in the zebrafish (Semova et al., 2012). In grass carp, intestinal microbiota is known to help digest polysaccharose, contributing to nutritional and physiological homeostasis (Ni et al., 2013). In a study on leopard coral grouper (*Plectropomus leopardus*), Firmicutes and Fusobacteria had been shown to influence the hydrolysis of maltose and trehalose (Mekuchi et al., 2018). Moreover, it was reported in grass carp that *Bacillus subtilis* modulated the bacterial assembly, lipid metabolism and immune response in the intestine (Shi et al., 2020). These findings led to the application of probiotics, in which the modulation of microbiota improved immunity and growth in rainbow trout (*Oncorhynchus mykiss*) (Adel et al., 2017). On the contrary, disruption of intestinal microbial community weakens immunity and increases the risk of disease in fish (Tran et al., 2018). So, assessment of intestinal microbiota is essential for the evaluation of effectiveness and safety of faba bean and their extracts.

In order to achieve comprehensive evaluation of effectiveness and safety of FB extracts as aquafeed additives, this study used four types of FB extracts including FB water extract (FBW), FB alcohol extract (FBA), FB protein (FBP), and FB residues (FBR) prepared according to our previous report (Ma et al., 2020a). The active components of these four faba bean extracts were firstly analyzed, and then these extracts were used to formulate isonitrogenous and isoenergetic diets for a feeding trial to examine their effects on growth performance, muscle quality, intestinal microbiota, metabolism, immunity and oxidative responses in grass carp.

2. Materials and methods

2.1. Experimental diets

According to our previous study (Ma et al., 2020b), four faba bean (FB) extracts were isolated, including FB alcohol extract, FB water extract, FB protein and FB residues. Content and extraction rate of active components in faba bean and its extracts are listed in Table 1. Briefly, shelled faba bean were firstly ground into 60-um power, and then were suspended in 12 times (volume) of distilled water with pH adjusted to 9.0 using 1 M calcium hydroxide. After ultrasonic processing for 1 h at 45 °C followed by centrifugation, FB water extract was isolated by concentrating the supernatant at 55 °C. The supernatant was incubated for 24 h after adjusting pH to 5.0 using 4 M hydrochloric acid, and FB proteins were obtained from the resulting precipitate after drying. Then the residues were resuspended in 8 times (volume) of 80% alcohol to isolate FB alcohol extract by concentrating at 45 °C. The FB residues were obtained by drying the last precipitate after ethanol extraction at room temperature. Altogether, 7.2 g of FB alcohol extract, 136.2 g of FB water extract, 320.8 g of FB proteins and 335.8 g of FB residues were obtained from 1000 g of faba bean (200 g of faba bean hull).

Isonitrogenous and isoenergetic diets were formulated using the four FB extracts of the above amount for the feeding trial. The base diet containing no FB extracts and the whole faba bean were also used as control. The composition of diet ingredients is shown in Table 2. The diets were formulated as follows: 1) all feed ingredients were thoroughly mixed with one of the four FB extracts or no extracts; 2) the mixture was made into a 6 mm-diameter soft pellet using a pellet feed mill (Taibao, China); 3) the pellet was further cut into 3 mm-length, air-dried at 50 °C, and stored in sealed polythene bags at 4 °C. Moderate temperature (< 70 °C) was used throughout the formulating process to avoid the loss of extract activities. The formulation and composition of the experiment diets are listed in Table 3. Because the nitrogen contents and energy of the four FB extracts were different, inclusion levels of other ingredients needed to be adjusted to formulate the isonitrogenous and isoenergetic diets.

Table 1
Extraction rate and nutritional composition of active substances in faba bean and its extracts.[a]

	FB	FB alcohol extract	FB water extract	FB protein	FB residues
Extract rate (%)	0	8.70	16.32	30.08	44.40
Vicine (%)	1.56 ± 0.003[a]	0.001 ± 0.0002[c]	1.49 ± 0.247[a]	0.02 ± 0.004[b]	0.01 ± 0.010[b]
Saponin (mg/g)	7.01 ± 0.02[b]	25.74 ± 0.04[a]	7.170 ± 0.05[b]	4.24 ± 0.01[c]	1.36 ± 0.01[d]
Flavonoid (mg/g)	4.41 ± 0.18[a]	1.40 ± 0.02[c]	2.50 ± 0.04[b]	1.27 ± 0.03[c]	0.22 ± 0.001[d]
total polyphenol (mg/g)	3.48 ± 0.09[c]	28.23 ± 0.04[a]	10.30 ± 0.05[b]	1.25 ± 0.01[d]	0.09 ± 0.002[e]
Anthocyanin (nmol/g)	13.40 ± 0.24[b]	20.14 ± 0.03[a]	9.39 ± 0.19[c]	9.46 ± 0.06[c]	1.65 ± 0.06[d]
Tannin (mg/g)	6.95 ± 0.10[c]	30.48 ± 0.17[a]	8.61 ± 0.01[b]	1.25 ± 0.01[d]	0.28 ± 0.002[e]
Crude Protein (g/100 g)	28	< 0.5	37.4	58.6	6.6
Crude Fat (g/100 g)	1.4	10.5	< 0.5	2.8	0.65
Carbohydrate (g/100 g)	52.1	< 0.5	33.8	24.6	72.9
Moisture (g/100 g)	14.4	10.4	10.2	10.1	12.2
Crude Ash (g/100 g)	4.1	< 0.1	3.9	3.9	7.7

[a] Note: Values of the same column with different letters are significantly different ($P < 0.05$).

Table 2
The composition of experimental diet ingredients (g/kg).

	Crude protein (%)	Crude fat (%)	Soluble carbohydrates (%)	Moisture (%)	Crude Ash (%)
Fish meal	61	4	1	24	10
Rapeseed meal	38	1.4	28	7.3	12
Cottonseed meal	42	0.7	28.2	0.5	11
Soybean meal	43	1.9	31	6	12
Extruded-soybean	34	16	29	19	7
Wheat middling	13.6	2.1	66.7	1.8	13
Rice bran	12.8	16.5	44.5	7.5	13
Wheat	13.9	1.7	67.6	1.9	13
$Ca(H_2PO_4)_2$	0	0	0	0	0
Bentonite	0	0	0	0	0
Zeolite powder	0	0	0	0	0
Premix	0	0	0	0	0
Soybean oil	0	167	0	0	0
FBW extract	37.4	0.5	33.8	3.9	10.2
FBA extract	0.56	10.5	0.5	0.1	10.4
FB protein	58.8	2.8	24.6	3.9	10.1
FB resides	6.6	0.65	72.9	7.7	12
Whole FB	28	1.4	52.1	4.1	14.4

Note: Control, formulated diet with no FB extract; FB, whole faba bean; FBA, FB alcohol extract; FBW, FB water extract; FBP, FB protein; FBR, FB residues.

2.2. Feeding trail

The feeding trial was performed for 100 days (d) in the Pearl River Fisheries Research Institute. The fish were cultured in the eighteen

Table 3
Formulation and composition of the experimental diets.

	control	FB group	FBA group	FBW group	FBP group	FBR group
Fish meal	50.5	0	55	44.8	3.9	53
Rapeseed meal	176.5	0	192.4	156.7	13.8	80
Cottonseed meal	176.5	0	192.4	156.7	13.8	200
Soybean meal	83.6	0	88.4	67.2	5.9	200
Extruded-soybean	25.2	0	27.5	22.4	1.9	25
Wheat middling	105.1	0	92	85.3	141.2	8.9
Rice bran	147.3	0	128.8	119.3	197.8	10.4
Wheat	178.3	0	157.3	154.4	243.9	12.4
$Ca(H_2PO_4)_2$	15	0	15	15	15	15
Bentonite	12.5	0	12.5	12.5	12.5	12.5
Zeolite powder	12.5	0	12.5	12.5	12.5	12.5
Premix	10	0	10	10	10	10
Soybean oil	7	0	9	7	7	21.5
FBW extract	0	0	0	136.2	0	0
FBA extract	0	0	7.2	0	0	0
FB protein	0	0	0	0	320.8	0
FB resides	0	0	0	0	0	335.8
Whole FB	0	1000	0	0	0	0
Gelatin	0	0	0	0	0	3
Total (g)	1000	1000	1000	1000	1000	1000
Crude protein (g/kg)	2.744	2.8	2.821	2.884	2.83	2.808
Crude fat (g/kg)	0.525	0.14	0.532	0.526	0.523	0.527
Carbohydrate (g/kg)	37.98	52.1	36.05	36.4	42.4	39.88
Moisture (g/kg)	5.25	4.1	4.96	3.69	6.19	0.2
Crude Ash (g/kg)	10.84	14.4	11.02	10.48	0.3	0.02

Note: Control, formulated diet with no FB extract; FB, whole faba bean; FBA, FB alcohol extract; FBW, FB water extract; FBP, FB protein; FBR, FB residues.

cement pools (2 m × 2 m × 1 m) (three pools for each group, six experimental groups) with 10 fish per pool. There was no significant difference in the initial individual weight (838 ± 40 g, mean ± SD) among all groups ($P > 0.05$). The control group was fed with the formulated diet with no FB extracts, the FB group was fed with whole FB, and each of the four experimental groups was fed with one of the isonitrogenous and isoenergetic diets (FBA, FBW, FBP and FBR groups). The fish was fed at 9:00 and 16:00 every day. The amount of daily feeding was 3~5% of fish weight. Culture conditions were the same in all pools: water temperature 25~30 °C, pH 6.5~7.5, and the dissolved oxygen > 5.0 mg/L.

2.3. Sample collection and measurement of muscle textural parameters

This fish study has a waiver of approval from the ethics committee in conformity with Chinese law. After being cultured for 100 d, each group was fasted for 24 h, and three fish were randomly sampled from each pool (n = 9 per experimental group). The fish were individually euthanized in pH-buffered tricaine methanesulfonate (250 mg/L). The body weight, body length, the indexes of viscera, hepatopancreas and mesenteric were measured. Growth-related parameters were calculated as follows:

Weight gain (WG, %) = (final weight - initial weight)/initial weight × 100%
Condition factor (CF, %) = body weight/length^3 × 100%
Hepatopancreas somatic index (HSI, %) = hepatopancreas weight/ body weight × 100%
Visceral somatic index (VSI, %) =visceral weight/body weight × 100%
Abdominal fat index (AFI, %) = abdominal fat weight/body weight × 100%
Feed conversion ratio (FCR) = Total feed intake/ (final weight - initial weight)
Survival rate (SR, %) = the number of survival fish/total initial number of fish × 100%

Blood samples were collected immediately from the tail before euthanizing fish, and then centrifuged at 3500 r/min for 10 min at 4 °C. The plasma samples were subsequently separated and stored at − 80 °C for measurement of biochemical parameters. The muscle, intestine, kidney and hepatopancreas were sampled for measurement of the activity of metabolic enzymes. The intestinal contents were collected from foregut, midgut and hindgut, and then these contents were mixed for 16 S rDNA analysis of intestinal microbiota.

The textural parameters (hardness, springiness) were measured using the Universal TA texture analyzer (Shanghai Tengba instrument technology, Shanghai, China).

2.4. DNA extraction and high-throughput sequencing of intestinal microbiology

The microbiome DNA was extracted from intestine using the MN NucleoSpin-96 DNA extraction kit (Omega, Shanghai, China), and the agarose gel electrophoresis was performed for the rough estimation of the concentration and purity. The DNA samples were stored at − 80 °C. The V3-V4 region of 16 S rDNA was PCR-amplified using universal primers 338 F (5′-ACTCCTACGGGAG GCAGCAG-3′) and 806 R (5′-GGACTACCVGGGTATCTAAT-3′). The conditions were: predenaturation at 95 °C for 3 min; denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, 40 cycles; and final extension at 72 °C for 7 min. All amplification products were analyzed by library sequencing on Illumina MiSeq platform (Illumina, San Diego, CA, USA).

2.5. Bioinformatics analysis

Based on the raw data from the Illumina HiSeq sequencing platform, the sequencing data quality control (Clean Tags) was performed for PE Reads (the number of double ended Reads obtained by sequencing, PE Length (Length of the original sequencing data) and Q30 (%) (Percentage of the total base with a mass value greater than 30 in the original data). OTUs (Operational Taxonomic Units) were obtained using the Usearch software, and OTUs were taxonomically annotated based on Silva (Bacteria) taxonomic database. Based on the results of OTU analysis, taxonomic analysis of samples at each taxonomic level was performed to obtain the community structure map of each sample at the taxonomic levels of phylum, order, family, genus and species. Species

diversity within individual samples was analyzed by alpha diversity, and alpha diversity indexes included reads. Chao1, Shannon and Simpson indexes were obtained, as well as sample Rank-abundance was plotted. To find statistical differences among groups, the LEfSe analysis were used for the characteristics of significant abundance differences. The species abundance data among groups were subjected to *t*-tests using Metastats software, and then screened for species causing differences in sample composition among these groups. Functional gene prediction analysis (KEGG and COG) was used to predict gene function and calculate the abundance of functional genes. RDA/CCA analysis was used to study the relationship between microflora and serum indexes.

2.6. Measurement of biochemical parameter and enzymes activity

The assay kits (Jiancheng biotechnology, Nanjing, China) were used to determine the indexes including total cholesterol content (TC) (Kit NO. A111-1-1), triglycerides content (TG) (Kit NO. A110-1-1), the activity of aspartate aminotransaminase (AST) (Kit NO. C010-2-1), alanine aminotransaminase (ALT) (Kit NO. C009-3-1), glucose-6-phosphatase (G-6-P) (Kit NO. A027-1-1), acetyl-CoA (Acyl-CoA) (Kit NO. H331-1) , hexokinase (HK) (Kit NO. A077-3-1), citrate synthase (CS) (Kit NO. A108-1-2), pyruvate kinase (PK) (Kit NO. M003) and succinate dehydrogenase (SDH) (Kit NO. A022-1-1) in hepatopancreas. In the intestine, the activity of amylase (AMS) (Kit NO. C016-1-1), lipase (LP) (Kit NO. A054-2-1) and trypsin (Tryp) (Kit NO. A080-2-2) were measured by using assay kits (Jiancheng biotechnology, Nanjing, China).

The assay kits from Solebo Technology (Beijing, China) were used to measure the contents of albumin II (ALBII) (Kit NO. SEKH-0081) and total protein (TP) (Kit NO. A095-1-1) in serum and hepatopancreas. The corresponding assay kits (Jiancheng biotechnology, Nanjing, China) were used to measure the activity of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), peroxisome (POD) and total/CuZn superoxide dismutase (T-SOD/CuZn-SOD) in serum and hepatopancreas. The glutathione peroxidase (GSH-Px) activity was measured with an assay kit NO. BC1195 (Solebo Technology, Beijing, China) in serum and hepatopancreas.

2.7. Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics 23 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test. P value of less than 0.05 was considered as a significant difference. The results were presented as mean ± SE.

3. Results

3.1. Content and extraction rate of active components in faba bean and its extracts

The content and extraction rate of active components in faba bean and faba bean extracts were shown in Table 1. The extraction rate of FB residues (44%) was the highest, followed by FB protein (30.08%), and the extraction rate of FB alcohol extract (8.7%) was the lowest. The content of vicine was the highest in FB water extract (1.49 ± 0.247%) among all of FB extracts. The content of saponin (25.74 ± 0.04 mg/g), total polyphenol (28.23 ± 0.04 mg/g), anthocyanin (20.14 ± 0.03 nmol/g) and tannin (30.48 ± 0.17 mg/g) were the highest in FB alcohol extract, while the content of flavonoid were the highest in FB water extract (2.50 ± 0.04 mg/g).

3.2. Growth performance and textural quality

Overall, the FB water extract group demonstrated favorable aquaculture characteristics, whereas the FB protein and FB residual groups showed the lowest performance. Specifically, after feeding isonitrogenous and isoenergetic diets for 100 d, weight gain (WG) was higher in the FB alcohol and water extract groups, while it was lower in FB and FB protein groups, compared to other groups ($P < 0.05$) (Table 4). The condition factor (CF) was the lowest in the FB residues group, and there was no significant difference in CF among the other five groups ($P > 0.05$). The visceral somatic index (VSI) was high in the FB alcohol extract, FB protein and FB residues groups, and low in the FB water extract and control groups. There was no significant difference in HSI among all groups. The muscle hardness of the whole FB and FB water extract groups were higher than those of other groups ($P < 0.05$).

3.3. Diversity of intestinal microbiota

This study obtained an average of 108,552 PE reads and an average PE length of 247 bp (Fig. 1a). The number of OTUs (Operational Taxonomic Units) for each sample was obtained by clustering the Tags at 97% similarity level using Usearch software, and a total of 1360 OTUs were obtained, with the control having 229 OTUs, FB group having 116 OTUs, FB alcohol extract group having 325 OTUs, FB water extract group having 387 OTUs, FB protein group having 127 OTUs and FB residues group having 176 OTUs (Fig. 1b). The OTU number of intestinal microbiota in FB water and alcohol extract groups were higher than those of other groups, and the abundance curve showed that the abundance of the FB water extract group was the highest among all groups (Fig. 1b, c). The alpha diversity indexes indicated that the reads and Chao1 indexes were higher in FB water extract group (Fig. 1d).

3.4. Microbial composition and function prediction of intestinal microbiota

The difference in relative abundance at Phylum and Genus levels were analyzed (Fig. 2a, b). FB water and alcohol extract groups had more microbial species than other groups, and the FB group had the fewest number of microbial species. At the Phylum level, the Fusobacteria was the most dominant Phylum in all groups, but the relative abundance of Fusobacteria was decreased in FB water and FB alcohol extract groups compared to the control. The relative abundance of Bacteroides was the highest in the FB alcohol extract group. In the FB water extract group, the relative abundance of Acidobacteria was the highest. At the Genus level, Cetabacterium was the most dominant genus in all groups. The abundance of Cetabacterium was decreased in the FB water and alcohol extract groups compared to other groups. The Rhodobacter and Sphingomonas were specific to the FB alcohol and FB water extract groups, respectively. For the LEfSe analysis (LDA score > 4) at the Genus level (Fig. 2c), FB, FB alcohol extract and FB water extract groups had the significant difference of microbial groups. There were 4 different species in the FB group and 8 different species in the FB alcohol extract and FB water extract groups. D-4-Bacteroides and D-5-Rhodobacter had the highest LDA score in the FB alcohol extract group. D-4-Fusobacteriaceae, D-4-Fusobacteriia, D-5-Cetobacterium, D-2-Fusobacteria and D-3-Fusobacteriales had the highest LDA score in the FB water extract group.

A total of 33 KEGG gene families and 25 COG gene families were obtained (Fig. 2d, e). It can be seen that there were 12 KEGG gene families and 9 COG gene families of functional difference. More than 27% of the 33 KEGG gene families were metabolism-related pathways, among which the largest proportion was related to carbohydrate metabolism, followed by amino acid metabolism, energy metabolism and lipid metabolism. FB, FB protein and FB residues groups showed higher abundance of carbohydrate metabolism than those of other groups. FB alcohol extract and FB water extracts groups showed higher abundance of amino acid metabolism than those of other groups.

3.5. Immune response in serum and hepatopancreas

After feeding for 100 d, immune-related parameters were analyzed

Table 4
Growth parameters and muscle textual quality of grass carp.

	control	FB group	FBA group	FBW group	FBP group	FBR group
Initial individual weight (g)	835 ± 40^{a}	837 ± 35^{a}	842 ± 44^{a}	838 ± 40^{a}	835 ± 38^{a}	841 ± 43^{a}
Final individual weight (g)	1765 ± 8.0^{b}	1487 ± 4.1^{d}	1804 ± 3.2^{a}	1795 ± 6.5^{a}	1607 ± 7.1^{c}	1774 ± 7.9^{b}
WG (%)	111.41 ± 2.37^{b}	77.68 ± 4.29^{d}	142.59 ± 1.42^{a}	142.31 ± 0.83^{a}	92.55 ± 1.90^{c}	110.17 ± 1.68^{b}
CF (%)	1.99 ± 0.03^{a}	1.56 ± 0.12^{ab}	1.69 ± 0.18^{ab}	1.88 ± 0.10^{ab}	1.60 ± 0.06^{ab}	1.43 ± 0.06^{b}
VSI (%)	5.34 ± 0.64^{c}	7.50 ± 0.55^{ab}	8.00 ± 0.11^{a}	5.53 ± 0.29^{c}	8.06 ± 0.30^{a}	8.03 ± 0.52^{a}
HSI (%)	1.48 ± 0.01^{a}	1.59 ± 0.02^{a}	1.42 ± 0.16^{a}	1.34 ± 0.06^{a}	1.68 ± 0.23^{a}	1.40 ± 0.23^{a}
AFI (%)	1.08 ± 0.01^{a}	1.35 ± 0.21^{a}	2.09 ± 0.84^{a}	0.93 ± 0.14^{a}	1.44 ± 33^{a}	1.60 ± 0.21^{a}
FCR	1.91 ± 0.03^{c}	2.84 ± 0.15^{a}	1.43 ± 0.02^{d}	1.54 ± 0.01^{d}	2.23 ± 0.04^{b}	1.95 ± 0.02^{c}
SR (%)	100	77.8	100	100	88.9	88.9
Hardness (gf)	1103.16 ± 33.99^{c}	1645.42 ± 70.57^{a}	1166.45 ± 22.65^{c}	1438.91 ± 62.48^{b}	834.03 ± 42.82^{d}	945.95 ± 24.49^{d}
Chewiness (gf)	427.00 ± 25.35^{b}	729.61 ± 20.89^{a}	408.26 ± 25.72^{b}	689.25 ± 62.18^{a}	335.42 ± 19.73^{b}	423.45 ± 20.98^{b}

Note: Values of the same column with different letters are significantly different ($P < 0.05$). Control, formulated diet with no FB extract; FB, whole faba bean; FBA, FB alcohol extract; FBW, FB water extract; FBP, FB protein; FBR, FB residues.

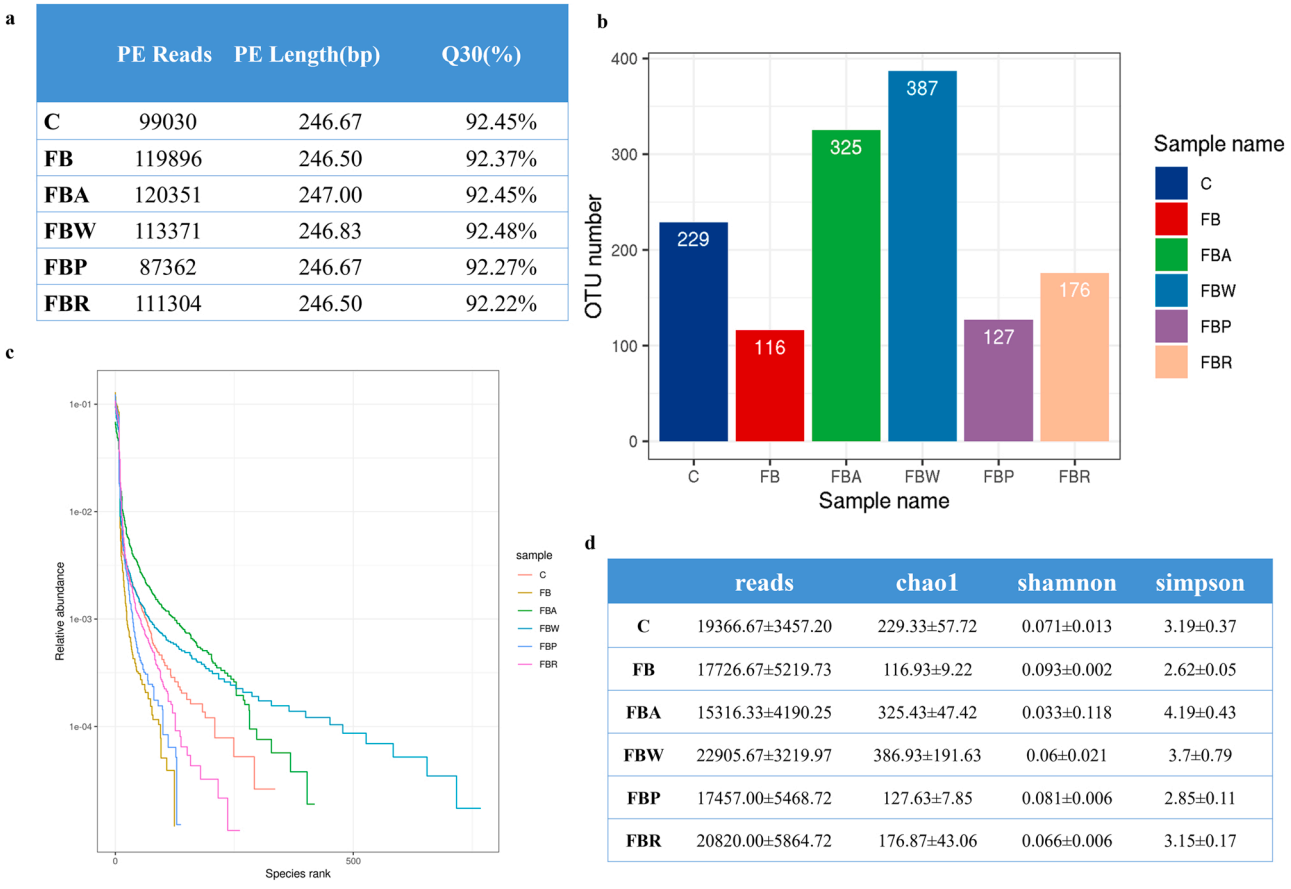

Fig. 1. Diversity of intestinal microbiota in grass carp. **a**, the sequencing data quality control; **b**, the number of OTUs (Operational Taxonomic Units); **c**, Rank-abundance; **d**, the alpha diversity indexes. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group.

(Table 5). The activity of aspartate aminotransaminase (AST) in serum and hepatopancreas was the highest in the FB group, followed by the FB residues group ($P < 0.05$). The activity of alanine aminotransaminase (ALT) was the highest in the FB group in hepatopancreas ($P < 0.05$), but this was not observed in serum. The ALT activity was low in the FB and FB protein groups compared to other groups ($P < 0.05$). The content of albumin II (ALB II) and total protein (TP) in serum and hepatopancreas was the highest in the FB group ($P < 0.05$). The TC contents in the hepatopancreas were generally increased in all FB-fed groups compared to the control group. The FB alcohol extract group showed the highest TC contents, followed by the whole FB and FB protein groups. The FB water extract group showed the lowest TG content ($P < 0.05$). In serum, total TC and TG contents were both highest in the FB group, while those of other groups were not very different ($P < 0.05$).

3.6. *Oxidative stress of serum and hepatopancreas and intestinal enzymes*

The content of hydrogen peroxide (H_2O_2) in serum and hepatopancreas was the highest in the FB group among all groups ($P < 0.05$) (Table 6). The FB alcohol extract group had the lowest H_2O_2 content both in serum and hepatopancreas, and FB water extract group had low H_2O_2 content both in serum and hepatopancreas, whereas the FB protein group also had lower H_2O_2 content in hepatopancreas ($P < 0.05$). However, the contents of MDA in serum and hepatopancreas of FB

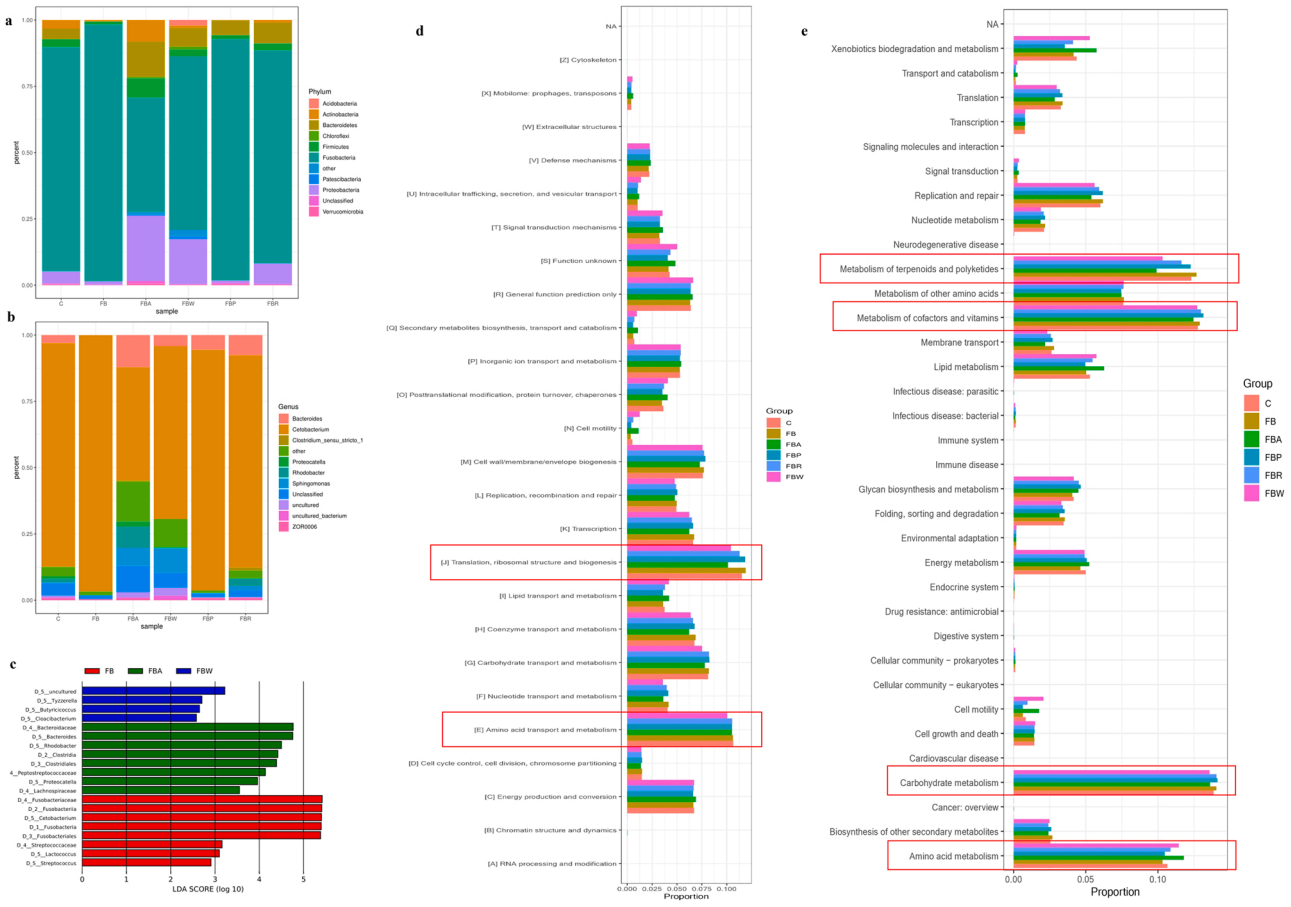

Fig. 2. Microbial composition and function prediction of intestinal microbiota. **a**, The abundance of intestinal microbiota at Phylum level; **b**, The abundance of intestinal microbiota at Genus level; **c**, LEfSe analysis at Genus level; **d**, KEGG pathway; **e**, COG pathway. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group.

Table 5
Immune and metabolic enzymes in serum and hepatopancreas.

	C	FB	FBA	FBW	FBP	FBR
Serum AST (U/L)	22.60 ± 0.20^{c}	28.57 ± 0.12^{a}	19.67 ± 0.07^{d}	18.77 ± 0.12^{d}	16.97 ± 0.26^{e}	24.47 ± 0.30^{b}
Serum ALT (U/L)	18.03 ± 0.03^{a}	7.77 ± 0.08^{c}	13.8 ± 0.01^{b}	13.80 ± 0.10^{b}	5.73 ± 0.13^{d}	14.07 ± 0.03^{b}
Serum TP (g/L)	20.43 ± 0.67^{c}	32.17 ± 0.18^{a}	27.53 ± 0.09^{b}	25.90 ± 0.06^{b}	21.40 ± 0.06^{c}	24.70 ± 0.06^{b}
Serum ALB II (g/L)	5.40 ± 0.12^{e}	9.33 ± 0.09^{a}	7.67 ± 0.12^{b}	7.13 ± 0.12^{c}	6.33 ± 0.07^{d}	7.53 ± 0.03^{bc}
Serum TC (mmol/L)	2.16 ± 0.08^{b}	3.07 ± 0.03^{a}	2.14 ± 0.06^{b}	1.80 ± 0.04^{b}	2.20 ± 0.10^{b}	$2.47 \pm 0.34a^{b}$
Serum TG (mmol/L)	1.56 ± 0.04^{d}	2.71 ± 0.07^{a}	2.01 ± 0.04^{c}	2.30 ± 0.08^{b}	2.09 ± 0.07^{bc}	1.61 ± 0.05^{d}
Hepatopancreas AST (U/gprot)	1523.33 ± 1.13^{d}	6534.83 ± 31.93^{a}	2712.97 ± 12.77^{c}	2815.80 ± 7.46^{c}	1615.47 ± 0.24^{d}	4467.07 ± 7.03^{b}
Hepatopancreas ALT (U/gprot)	92.10 ± 0.17^{e}	1620.43 ± 9.04^{a}	124.17 ± 0.07^{d}	616.30 ± 1.51^{b}	430.17 ± 0.27^{c}	164.77 ± 0.19^{d}
Hepatopancreas TP (g/gprot)	5.30 ± 0.06^{e}	8.30 ± 0.06^{a}	5.50 ± 0.06^{d}	6.40 ± 0.06^{b}	6.03 ± 0.03^{c}	5.47 ± 0.03^{d}
Hepatopancreas ALB II (g/gprot)	1.07 ± 0.07^{c}	$3.03 \pm 0.03a$	0.7 ± 0.01^{d}	2.23 ± 0.03^{b}	2.07 ± 0.09^{b}	0.47 ± 0.03^{d}
Hepatopancreas TC (U/gprot)	0.19 ± 0.01^{c}	0.35 ± 0.01^{b}	0.85 ± 0.03^{a}	0.26 ± 0.01^{c}	0.37 ± 0.02^{b}	0.27 ± 0.03^{c}
Hepatopancreas TG (U/gprot)	0.77 ± 0.03^{b}	0.49 ± 0.02^{d}	0.97 ± 0.05^{a}	0.22 ± 0.01^{e}	0.85 ± 0.03^{b}	0.65 ± 0.02^{c}

Note: AST, aspartate aminotransferase; ALT, Alanine aminotransferase; TP, total protein; ALB II, Albumin II; TC, total cholesterol; TG, triglycerides. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group. Different superscript letters represented significant difference ($P < 0.05$).

alcohol extract group and FB water extract group were higher than those of the FB group ($P < 0.05$).

There was no clear tendency in the activity of T-SOD or CuZn-SOD. On the other hand, the activity of glutathione peroxidase (GSH-Px) was significantly higher in FB group than other groups ($P < 0.05$), especially in hepatopancreas. The hepatopancreatic activity of GSH-px in FB water and FB alcohol extract groups were lower than that of the FB group ($P < 0.05$), suggesting that water and alcohol extraction could reduce the oxidative components from FB. In addition, the activities of intestinal lipase (LP), amylase (AMS) and trypsin (Tryp) were increased in the four FB extract groups (Table 6). The highest activities of LP, AMS, and Tryp were found in the FB water extract, FB alcohol extract and FB protein group, respectively.

3.7. Carbohydrate metabolism

Both whole FB and FB extracts had significant effects on carbohydrate metabolism (Table 7). The acetyl-CoA content and activity of glucose-6-phosphatase (G-6-P), hexokinase (HK), citrate synthase, pyruvate kinase (PK), and succinate dehydrogenase (SDH) were the lowest

Table 6
Oxidative stress of serum and hepatopancreas and intestinal enzymes.

	C	FB	FBA	FBW	FBP	FBR
Serum T-SOD(U/mL)	3.65 ± 0.04^{a}	2.49 ± 0.02^{e}	2.76 ± 0.01^{d}	2.97 ± 0.01^{c}	1.33 ± 0.02^{f}	3.26 ± 0.02^{b}
Serum CuZn-SOD(U/mL)	2.19 ± 0.01^{a}	1.36 ± 0.01^{e}	1.65 ± 0.01^{d}	1.75 ± 0.01^{c}	1.29 ± 0.01^{f}	1.88 ± 0.01^{b}
Serum MDA (mmol/mL)	5.46 ± 0.12^{e}	17.78 ± 0.40^{d}	27.87 ± 0.17^{b}	20.83 ± 0.76^{c}	32.59 ± 0.19^{a}	26.34 ± 0.17^{b}
Serum H_2O_2 (mmol/L)	32.18 ± 0.22^{e}	96.04 ± 1.01^{a}	38.49 ± 0.22^{d}	62.334 ± 0.30^{c}	85.84 ± 0.89^{b}	32.77 ± 0.22^{e}
Serum GSH-Px (U/mL)	329.25 ± 7.82^{b}	291.13 ± 2.30^{a}	288.50 ± 1.64^{b}	168.89 ± 2.56^{c}	161.99 ± 2.81^{c}	289.82 ± 1.14^{b}
Serum POD (U/mL)	115.26 ± 0.93b	140.63 ± 2.72^{a}	77.85 ± 0.70^{c}	80.07 ± 0.54^{c}	75.44 ± 0.17^{c}	40.40 ± 1.39^{d}
Hepatopancreas T-SOD (U/mgprot)	16.27 ± 0.22^{a}	9.97 ± 0.09^{e}	14.56 ± 0.22^{c}	12.25 ± 0.12^{d}	14.84 ± 0.11^{c}	15.34 ± 0.06^{b}
Hepatopancreas CuZn-SOD (U/mgprot)	18.67 ± 0.24^{a}	11.73 ± 0.11^{c}	18.14 ± 0.17^{a}	15.88 ± 0.13^{b}	15.94 ± 0.10^{b}	18.13 ± 0.13^{a}
Hepatopancreas MDA (g/gprot)	1.04 ± 0.04^{d}	0.83 ± 0.01^{e}	1.20 ± 0.03^{c}	1.65 ± 0.03^{a}	1.38 ± 0.04^{b}	1.33 ± 0.03^{b}
Hepatopancreas H_2O_2 (g/gprot)	7.11 ± 0.19^{c}	12.39 ± 0.61^{a}	3.94 ± 0.12^{d}	8.25 ± 0.15^{b}	8.53 ± 0.04^{b}	6.54 ± 0.10^{c}
Intestinal LP (U/L)	533.70 ± 12.75^{c}	819.63 ± 6.09^{b}	812.50 ± 12.93^{b}	1006.85 ± 11.97^{a}	791.35 ± 12.08^{b}	803.85 ± 20.89^{b}
Intestinal Tryp (U/L)	1060.78 ± 56.86^{d}	1924.20 ± 49.03^{b}	1453.35 ± 48.28^{c}	1425.08 ± 22.27^{c}	2155.25 ± 23.86^{a}	1870.88 ± 42.45^{b}
Intestinal AMS (U/L)	228.28 ± 6.72^{c}	301.48 ± 6.06^{b}	410.47 ± 4.86^{a}	292.83 ± 3.59^{b}	427.78 ± 4.42^{a}	286.38 ± 2.73^{b}

Note: T-SOD, total superoxide dismutase; MDA, malondialdehyde; H_2O_2, Hydrogen peroxide; GSH-Px, glutathione peroxidase; LP, lipase; AMS, amylase; Tryp, trypsin. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group. Different superscript letters represented significant difference ($P < 0.05$).

Table 7
The activity of enzymes related to carbohydrate metabolism in hepatopancreas.

	C	FB	FBA	FBW	FBP	FBR
Hepatopancreas G-6-P (U/prot)	388.47 ± 32.28^{c}	711.03 ± 11.16^{b}	792.92 ± 24.68^{b}	731.45 ± 18.82^{b}	804.82 ± 48.22^{b}	982.37 ± 18.02^{a}
Hepatopancreas Acyl-CoA (U/gprot)	84.54 ± 6.09^{b}	191.18 ± 5.59^{a}	197.46 ± 3.40^{a}	187.14 ± 5.83^{a}	197.51 ± 3.95^{a}	204.11 ± 5.66^{a}
Hepatopancreas HK (mU/prot)	560.28 ± 15.62^{d}	616.97 ± 16.72^{cd}	1000.22 ± 13.23^{a}	630.63 ± 7.96^{c}	1044.00 ± 15.60^{a}	784.90 ± 16.19^{b}
Hepatopancreas CS (U/prot)	13.54 ± 0.83^{d}	$21.48 \pm 0.^{c}$	34.05 ± 0.37^{a}	28.59 ± 0.71^{b}	28.46 ± 0.31^{b}	31.46 ± 0.77^{a}
Hepatopancreas PK (mU/gprot)	286.62 ± 5.69^{c}	530.28 ± 6.61^{a}	446.02 ± 8.86^{b}	577.73 ± 10.53^{a}	448.90 ± 6.85^{b}	585.63 ± 40.68^{a}
Hepatopancreas SDH U/gprot)	61.08 ± 3.98^{c}	143.85 ± 3.32^{a}	142.98 ± 3.29^{a}	129.55 ± 2.32^{ab}	134.26 ± 2.01^{a}	103.15 ± 14.60^{b}

Note: G-6-P, glucose-6-phosphatase; Acyl-CoA, acetyl-CoA; HK, hexokinase; CS, citrate synthase; PK, pyruvate kinase; SDH, succinate dehydrogenase. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group. Different superscript letters represented significant difference ($P < 0.05$).

in the control group. The FB water extract and the FB residues groups tended to have enhanced carbohydrate metabolism compared to other groups ($P < 0.05$).

3.8. The interactions between intestinal microbiota and physiological enzymes

RDA/CAA analysis and a heat map of correlations (relative abundance > 0.1) were constructed for different groups, among microbial bacteria (relative abundance > 0.1% at genus levels) and physiological enzymes (Fig. 3). RDA analysis showed that the microbial bacteria of the control, FB, FB alcohol extract, FB water extract and FB residues groups were positively correlated with the TG, TC, POD, LP and ALB, AST, Tryp, while were negatively correlated with the ALT, GSH-px and CuZn-SOD (Fig. 3a). CCA analysis showed the microbial bacteria of FB water extract, FB protein and FB residues groups were positively correlated with the TC, ALB, AST and Tryp, while were negatively correlated with the ALT, GSH-px, CuZn-SOD and T-SOD (Fig. 3b). According to the heat map of the correlations (Fig. 3c), AST and POD were positively correlated with Romboutsia and Cetobacterium, respectively; AST, TC and POD were negatively correlated with Bacteroides; TC and Tryp were positively correlated with Akkermansia.

4. Discussion

Faba bean is reported to improve fish textural quality in many studies (Yu et al., 2017; Yang et al., 2015), but also causes growth retardation. The present study demonstrated that FB water extract and FB alcohol extract improved the growth performance of grass carp, and FB water extract still retained the activity to improve muscle texture. The present feeding trial using isonitrogenous and isoenergic diets overcomes the limitation of our previous studies (Ma et al., 2020a, 2020b), in which the four extracts were simply added to the same commercial diet achieving the same total weight. Although the formulation strategy used in this study resulted in another problem that the inclusion levels of each conventional ingredient differ depending on the diet, it is important to note that the results of the present and previous (Ma et al., 2020a, 2020b) experiments are generally consistent in terms of the improvement of muscle texture and growth parameters. Thus, it should be reasonable to conclude that FB water and alcohol extracts, not other conventional ingredients commonly used in aquafeed, are likely responsible for the observed changes in muscle texture and growth parameters. Indeed, water and alcohol extraction are effective means to isolate functional compounds from plants. It was reported that adding 0.14% alcohol extract of lotus leaf to feed promoted the growth performance of grass carp (Zhu et al., 2019). Hydro-alcohol extract of wood betony (*Stachys lavandulifolia* Vahl) at 2~8% was found to enhance the growth of rainbow trout (Sarvi Moghanlou et al., 2018). The ginger (*Zingiber officinale*) alcohol extract at a 0.2% level effectively improved the growth and health status of common carp (Mohammadi et al., 2020). So, the FB water extract and alcohol extract can be used as aquafeed additives in future. On the other hand, the lower growth performance of FB, FB protein and FB residues groups is possibly related to the anti-nutritional factors (tannin, saponin, etc.). Specific components involved in growth performance need further clarification.

Intestinal microbiota plays indispensable roles for fish physiology, immunity and growth (Xiong et al., 2019). Studies have shown that intestinal microbiota not only affects fish intestinal digestion and regulates immune system (Wang et al., 2018), but also can be used as a biomarker of fish health and metabolic capacity (Clarke et al., 2014). High alpha diversity (mean species diversity at the habitat level) is frequently detected in healthy fish (Li et al., 2016). In this study, the alpha diversity indexes (reads and Chao1 indexes) and OTUs number of FB water and alcohol extract groups were higher than those of other groups, indicating that FB water and alcohol extracts significantly enhanced microbial diversity. Indeed, at the Phylum and Genus levels,

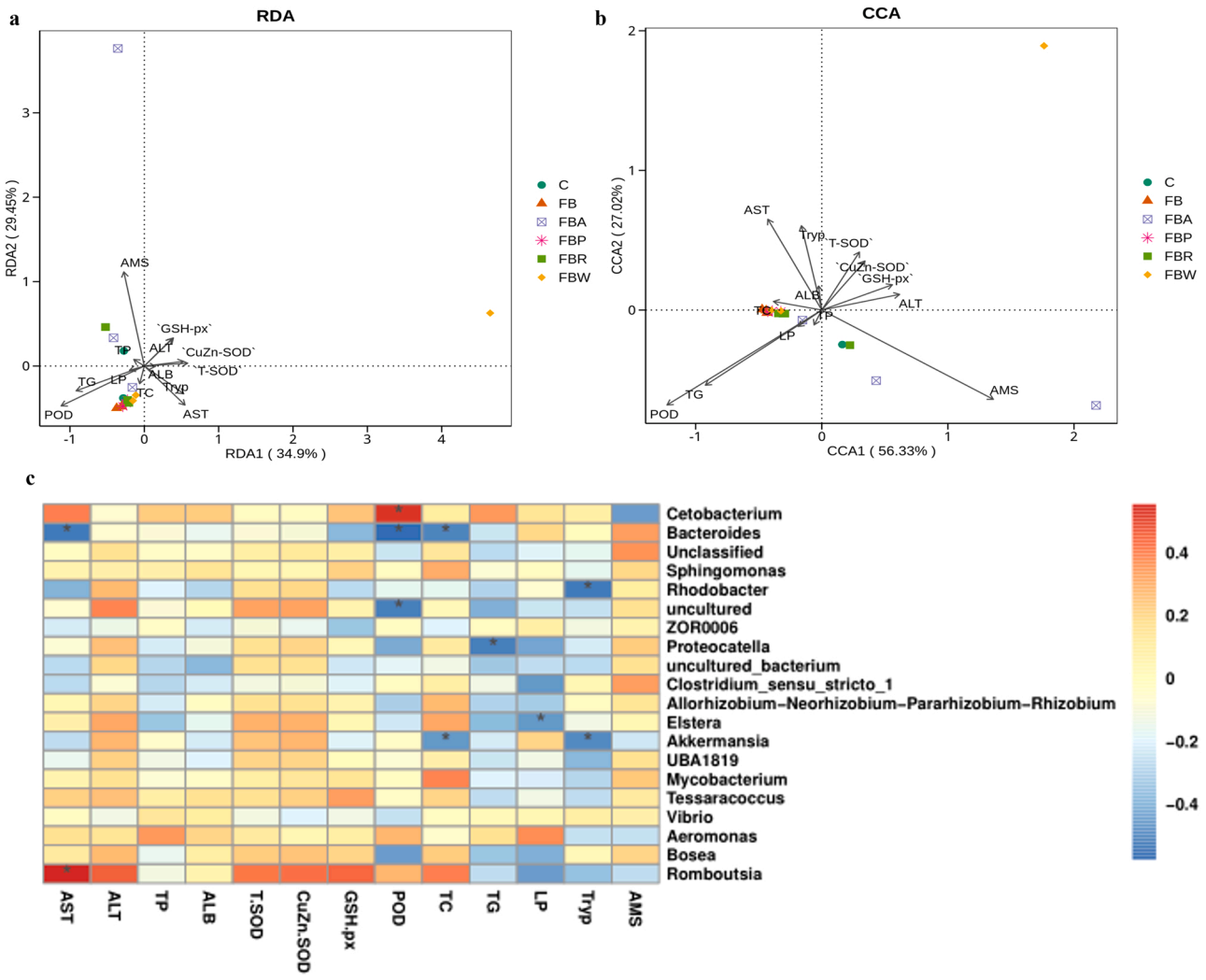

Fig. 3. The correlation analysis between intestinal microbiota (Genus levels) and physiological indexes. **a**, RDA, Redundancy Analysis; **b**, CCA, Canonical Correlation Analysis; **c**, Heat map. C, the control; FB, whole faba bean group; FBA, FB alcohol extract group; FBW, FB water extract group; FBP, FB protein group; FBR, FB residues group.

FB water extract group had more species of Acidobacteria, and FB alcohol extract group had more species of Bacteroides. Both Acidobacteria and Bacteroides are the dominant bacteria in the intestine that are helpful for fish health (Shi et al., 2020; Duan et al., 2021). So, it was suggested that FB alcohol and water extracts increased intestinal microbial diversity and enhanced immune function, thus promoting health and growth of grass carp. However, this possibility should be carefully tested because the diets used in this study contain different amount of conventional ingredients (rapeseed meal, cottonseed meal, etc.) to be isonitrogenous and isoenergetic. Compared to the changes in aquaculture parameters that were generally in line with our previous studies (Ma et al., 2020a, 2020b), it is more difficult to attribute the microbial changes in grass carp intestine, which was firstly observed in this study, solely to FB extracts, although many studies have used similar approach to investigate the effect of diets on intestinal microbiota (Hu et al., 2020; Li et al., 2020; Wei et al., 2020).

Our previous study found that FB induced intestinal inflammation by affecting pathways related to flagellar assembly and lipopolysaccharide biosynthesis in intestinal microbiota (Li et al., 2018). Here, we found that both FB water and alcohol extracts in aquafeed improved the composition of intestinal microbiota and decreased the abundance of potentially pathogenic Fusobacteriaceae. This change may have contributed to the enhanced growth performance, but high abundance of Fusobacteriaceae was also found in the carp of fast growth (Xie et al., 2021). So, the relationship between Fusobacteriaceae and the growth deserves further exploration taking different fish species into account. The present study also found that Rhodobacteraceae was unique in the FB alcohol extract group. The higher abundance of Rhodobacteraceae was observed in the intestine of shrimp with normal growth compared with the growth-retarded (Xiong et al., 2017), and Rhodobacteraceae members were capable of synthesizing vitamin B12, which was essential for shrimp growth (Sañudo-Wilhelmy et al., 2014). Thus, Rhodobacteraceae possibly plays a role in the health and growth in aquatic animals. Interestingly, in this study, we also found that the FB water extract group had the highest abundance of Sphingomonadacea. Sphingomonadacea was known to degrade aromatic compounds, some of which had negative effects on fishes (Asaf et al., 2020; Hao et al., 2020). It was speculated that FB water extract possibly promoted the growth of grass carp by increasing the abundance of intestinal Sphingomonadacea.

Intestinal microbiota is also closely related to fish immunity and stress responses, which in continuous direct contact with the gut mucosa, induces immune response through lymphoid tissue (Perez et al., 2010). Specifically, immunity and stress responses are affected by gut-microbiota-brain axis (Butt and Volkoff, 2019). In addition, intestinal microbiota affects the activity of intestinal enzymes (Wang et al.,

2018). Romboutsia covers a broad range of metabolic capabilities with respect to carbohydrate utilization and fermentation of amino acids (Gerritsen et al., 2019; Ramírez et al., 2018). Cetobacterium improves glucose homeostasis and increase insulin expression in fish (Wang et al., 2021). This study also found that aspartate aminotransaminase (AST) and peroxisome (POD) were positively correlated with Romboutsia and Cetobacterrium. So, Romboutsia and Cetobacterrium could be related to immunity and stress responses of grass carp.

In fish, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) are key enzymes for amino acid metabolism, and their levels are important indexes to evaluate hepatopancreatic conditions. High activities of these enzymes in the hepatopancreas generally imply that fish are under stressful conditions (Wu et al., 2017). In this study, the FB group showed higher activities of AST and ALT in hepatopancreas, implying that FB induced hepatopancreas damage and stressful conditions, further causing the health and growth of grass carp. Furthermore, immune response is closely related to reactive oxygen species (ROS) that regulate metabolism and chemical composition in various tissues of fish (Biller andTakahashi, 2018). It was reported that prolactin peptide-induced production of ROS suppressed specific immune responses in rainbow trout (Villalba et al., 2020). It was also found that the increased ROS levels had negative effects on immune function in meagre (*Argyrosomus regius*) juveniles (Mansour et al., 2017). Our previous study found that FB water extract alleviated the immunity response compared with the whole FB (Ma et al., 2020a, 2020b). Consistent with these findings, in this study, the ROS (H_2O_2) level was the highest in the FB group, followed by FB protein group, FB water extract group and FB alcohol extract group; the results of ALBII, and TP levels, which indicates the immune capacity, showed the opposite tendency (i.e., high in FB water and alcohol extract groups and the lowest in the FB group). So, the difference in ROS production levels is likely to contribute to the results of our feeding trial using isonitrogenous and isoenergetic diets, in which FB water and alcohol extracts showed low ROS levels and improved aquaculture characteristics. However, despite the decrease in H_2O_2 level, FB water and alcohol extracts significantly increased serum MDA, the peroxidation products of lipid, compared to the FB group.

The metabolism of major nutrients (lipid, carbohydrate, amino acid) is closely connected with fish growth and health condition (Ballantyne, 2001; Guo et al., 2019; Hemre et al., 2002), and generally high activity of digestive enzymes is associated with fast growth of fish (Zhou et al., 2013). FB water extract reduced fat accumulation (decreased contents of TC and TG) and promoted lipid metabolism (increased activity of intestinal lipase) in this study, which could account for the enhanced growth of grass carp (Jiang et al., 2018). FB alcohol extract also up-regulated carbohydrate metabolism (increased activity of HK, CS and SDH), which would have led to increasing the body energy supply and further promoting the growth in fish (Shi et al., 2018). It is also known that enhanced carbohydrate metabolism, or other energy metabolism, generally promotes fish growth (Hemre et al., 2002). In this study, FB water extract enhanced intestinal carbohydrate metabolism, and FB water and alcohol extracts increased intestinal amino acids metabolism in grass carp. It is speculated that the FB water and alcohol extracts have enhanced fish growth possibly by the enhancement of these mechanism systems.

In conclusion, the diet containing FB water extract not only improved the textural quality, but also promoted growth performance, abundance of intestinal microbiota and nutrients metabolism, and reduced immune damages in grass carp. FB alcohol extract diet promoted the growth performance, but it caused lipid accumulation and did not improve muscle texture. FB protein diet inhibited the growth and negatively affected the intestinal microbiota. Overall, this study demonstrated that FB water and alcohol extracts are promising functional feed additives that improve muscle quality and growth performance in fish. In our future studies, optimal supplementation levels of FB water and alcohol extract in grass carp diet will be determined. Their applications in other fishes also deserve further exploration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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