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Original Research Article

Effects of different protein and lipid levels on the growth performance and intestinal microflora of loach (*Paramisgurnus dabryanus*)

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ABSTRACT

The aim of this study was to examine the effects of dietary protein and lipid levels on the growth performance and homeostasis of the intestinal flora in *Paramisgurnus dabryanus*. An 8-week 3 × 3 two-factorial experiment was conducted to investigate the interaction between dietary crude protein (CP: 30%, 35%, 40%) and ether extract (EE: 6%, 10%, 14%) on the growth rate and the intestinal microflora of *P. dabryanus*. A total of 2,160 fish (5.19 ± 0.01 g) were randomly allotted to 36 aquariums each with 60 fish. Fish were fed the experimental diet twice daily. Results revealed that weight gain rate (WGR), specific growth rate (SGR), protein efficiency ratio and net protein utilization significantly increased when increasing protein levels from 30% to 40% ($P < 0.05$). Both WGR and SGR enhanced first but reduced thereafter with maximum value at 10% lipid level as dietary lipid increased from 6% to 14% ($P < 0.05$). Significant interactions between protein and lipid were found with feed conversion rate, lipid efficiency ratio and net lipid utilization ($P < 0.05$). At the phylum level, Proteobacteria and Actinobacteria were the dominant bacteria; at the genus level, *Burkholderia-Caballeronia-Paraburkholderia* was the dominant bacteria. Fish fed the diet containing 10% lipid had a higher abundance of Proteobacteria and *unclassified_f_Eenterobacteriaceae* than those fed the 14% lipid diet, and a higher abundance of *Rhodobacter* than those fed the 6% lipid diet ($P < 0.05$). Analysis of the predicted functions showed that metabolism in the intestine of fish in the CP40EE10 group was more active than that in CP30EE14 group. Polynomial regression analysis found that a diet containing 40.87% protein and 9.88% lipid can be considered optimal for *P. dabryanus*.

1. Introduction

Protein is one of the most important nutrients for fish, as it not only provides amino acids for biological synthesis, but is also the main component of metabolically active substances (Yan et al., 2017; Ma et al., 2020, 2021). Though growth can be restricted by a diet insufficient in protein (Yue et al., 2019; Liu et al., 2021), excessive dietary protein will increase feed cost and ammonia nitrogen excretion and pose threats to the environment and fish health (Mir et al., 2020). Meanwhile, dietary lipid, as a non-protein energy source, can provide essential fatty acids, phospholipids, sterols and fat-soluble vitamins for maintaining normal function in various physiological, developmental and reproductive processes of fish (Ai et al., 2004; Chaitanawisuti et al., 2011; Ghanawi et al., 2011; Meng et al., 2019). Dietary lipid has a protein-retaining effect, which increases efficient utilization of dietary protein for maximum nitrogen retention and growth performance (Welengane et al., 2019; Liu et al., 2021; Rahimnejad et al., 2021). Therefore, carnivorous fish fed with sufficient lipids can obtain better growth performance (Meng et al., 2019; Ma et al., 2020). Nevertheless, excessive lipid levels in diets may reduce feed intake capacity along

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with fat deposition in fish, resulting in fatty fish (Sun et al., 2018). Therefore, it is very important to optimize dietary protein and lipid levels for cultured species to develop cost-effective feed and achieve good growth performance.

The relationship between dietary protein and lipid levels on growth performance and gut microbiota has received increasing attention. Intestinal flora is considered to be a large and complex ecosystem in the host, which plays an important role in intestinal physiology and biological function (Wu et al., 2012; Fan and Li, 2019). Maintaining the homeostasis of intestinal flora is essential for the growth and regulation of immune function of fish (Tran et al., 2018; Zhou et al., 2020). Since feed is one of the most important factors affecting the homeostasis of intestinal flora, the content of dietary protein and lipid will directly affect the homeostasis of intestinal flora (Hartviksen et al., 2014; Yao et al., 2019; Nikouli et al., 2021). At present, there are few studies about the effects of dietary protein and lipid ratios on the intestinal flora of aquatic animals. Diets with different protein levels could affect the composition and dynamic balance of intestinal flora, and low-protein diets could reduce the diversity of intestinal microbes in tilapia (*Oreochromis niloticus*) (Zhu et al., 2020; Yang et al., 2021a). Meanwhile, it was found that both low-fat and high-fat diets influenced the composition and diversity of intestinal flora in hybrid yellow catfish (*Tachysurus fulvidraco*♀ × *Pseudobagrus vachellii*♂) (Zhu et al., 2021). For instance, a high-fat diet disturbed intestinal microbiota homeostasis through the deterioration of the ecological network (Peng et al., 2019). In all cases mentioned, optimal growth performance is ascribed to improve gut health of farmed fish by changing the protein and lipid levels in the diet. Thus, there is an urgent need to pay more attention to the effects of dietary protein and lipid levels on the gut microbiota of aquatic animals.

Paramisgurnus dabryanus (a member of Cypriniformes, Cobitidae, *Paramisgurnus*) is one of the most popular reared aquatic species in China. It is widely cultivated because of its fast growth, strong disease resistance and high survival rate (Gao et al., 2017). Moreover, *P. dabryanus* is rich in high-quality protein, fatty acids, minerals, B vitamins and other nutrients, thus attracting the majority of consumers (Yan et al., 2017; Yang et al., 2021b). As an aquatic product with great economic benefits in China, there is an increasing nutritional demand for *P. dabryanus* (Chen and Chen, 2019). At present, few studies pay attention to the dietary protein and lipid requirements of *P. dabryanus*. There is also a knowledge gap regarding the effect of different dietary protein and lipid levels on the gut microbiota of this species. In previous studies, dietary protein requirements of *P. dabryanus* (3 to 6 g) were from 33% to 36% and lipid levels were from 5% to 7% to achieve optimal growth performance (Gao et al., 2017; Victor et al., 2019; Chen et al., 2020; Du et al., 2020). Thus, 3 protein levels of 30%, 35%, 40% and 3 lipid levels of 6%, 10%, 14% were designed to evaluate the interaction effects of different dietary protein and lipid levels on growth performance and intestinal microflora of *P. dabryanus*. The study will provide scientific guidance for research on the nutritional requirements of *P. dabryanus*.

2. Materials and methods

2.1. Animal ethics

All experimental procedures were carried out in accordance with the approval of the Animal Care and Use Committee of Jiangxi Agricultural University and the guidelines in the China Law for Animal Health Protection and Instructions (Ethics approval No. SCXK (YU2005-0001)).

2.2. Experimental design and diets

Fish meal and soybean meal were used as the main protein sources and fish oil, soybean oil and soy lecithin were used as lipid sources. A 2-factor and 3-level (3 × 3) interaction experiment was designed. Nine groups were formulated with 3 crude protein (CP: 30%, 35%, and 40%; dry matter basis) levels and 3 ether extract (EE: 6%, 10%, and 14%; dry matter basis) levels (Table 1). All ingredients were ground to 80 mesh particle size. The micro-components, such as vitamin and mineral premixes, were then mixed thoroughly by the progressive enlargement method. Finally, lipids and distilled water were added to the ingredients and mixed until homogenous in a mixer. Then, a granulator was used to form feed pellets of 2 mm diameter. The pellets were stored frozen (−20 °C) in sealed plastic bags until their use in the feeding trial.

2.3. Experimental fish and feeding trial

P. dabryanus were purchased from the Loach Breeding Professional Cooperative of Fengcheng City, Jiangxi Province. The breeding experiment was carried out in the greenhouse of the aquaculture base of Jiangxi Agricultural University. At the beginning of the experiment, 2,160 *P. dabryanus* with neat specifications, strong physique, and similar body weight (5.19 ± 0.01 g) were randomly divided into 9 treatments with 4 replicates in each treatment, and 60 *P. dabryanus* were stocked in each bucket (80 cm × 66 cm × 64 cm) with freshwater hyacinth (*Eichhornia crassipes*) to rest and shade. During the trial, water was exchanged once a week with 1/2 water per change and aeration was continued throughout the experiment. The photoperiod was determined by the natural lighting, the water temperature was 25 to 28 °C and the dissolved oxygen was above 5 mg/L. According to 3% of the fish body weight, fish were manually fed with experimental diets (Table 1) at 08:00 and 17:00, respectively, to apparent satiation for 8 weeks.

2.4. Sample collection

At the termination of the feeding trial, loach number and tank weight were obtained after starvation for 24 h to estimate growth performance. Three fish per barrel were anesthetized with MS-222 (100 mg/L). Then, the surface of the loach body was swabbed with 75% ethanol. The intestinal contents of one fish from each tank were collected on ice, collected into sterile microcentrifuge tubes and put into liquid nitrogen for quick freezing. All samples were transferred to an ultra-low-temperature refrigerator at −80 °C for preservation.

All chemical composition analyses of diets were conducted according to the methods specified by the AOAC (1995). Moisture was analyzed by 105 °C normal temperature drying method (GB/T 6435-2014), crude protein (N × 6.25) by Kjeldahl method (GB/T 6432-1994), crude lipid by Soxhlet extraction method (GB/T 6433-2006) and ash analysis by combustion at 550 °C using a muffle furnace (GB/T6438-2007).

2.5. Performance measurement

Survival rate (%) = 100 × (final number of fish/initial number of fish);

Table 1
The composition and nutrient level of experimental feed (%, air-dry basis).

Item	Groups								
	CP30EE6	CP30EE10	CP30EE14	CP35EE6	CP35EE10	CP35EE14	CP40EE6	CP40EE10	CP40EE14
	EE/CP 0.20	EE/CP 0.33	EE/CP 0.47	EE/CP 0.17	EE/CP 0.29	EE/CP 0.40	EE/CP 0.15	EE/CP 0.25	EE/CP 0.35
Ingredients									
Fish meal	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
Full-fat expanded soybean	5.00	10.00	15.00	5.00	10.00	15.00	5.00	10.00	15.00
Soybean meal	22.50	19.00	15.50	22.50	19.00	15.50	22.50	19.00	15.50
Rapeseed meal	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Wheat flour	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Corn meal	20.00	20.00	20.00	20.00	17.60	12.90	15.00	10.10	5.40
Fish oil: Soybean oil (1:1)	2.00	5.40	8.60	2.00	5.40	8.60	2.00	5.40	8.60
Soy protein concentrate	0.00	0.00	0.00	7.50	7.50	7.50	15.00	15.00	15.00
Cellulose	11.0	6.10	1.40	3.50	1.00	1.00	1.00	1.00	1.00
Sodium carboxymethyl cellulose	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
$Ca(H_2PO_4)_2$	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Soy lecithin	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride (50%)	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Nutrient level[2]									
Moisture	9.51	9.36	8.80	9.77	9.41	8.62	9.38	9.12	8.92
CP	29.81	29.90	29.77	34.80	34.22	35.53	39.10	39.98	40.54
EE	3.56	8.21	12.07	3.63	8.38	12.67	4.85	9.22	14.24
GE[3], kJ/g	14.63	16.49	18.28	15.88	17.34	18.32	16.26	17.28	18.26
Ash	7.18	7.18	7.19	7.50	7.64	8.45	8.33	8.74	8.76

CP = crude protein; EE = ether extract; GE = gross energy.
[1] The premix provided the following per kilogram of diet: vitamin A 5,000 IU, vitamin B_1 25 mg, vitamin B_2 45 mg, vitamin B_6 20 mg, vitamin B_{12} 0.1 mg, vitamin K_3 10 mg, vitamin E 200 mg, vitamin C 200 mg, vitamin D_3 2,500 IU, inositol 200 mg, pantothenic acid 60 mg, niacin 200 mg, folic acid 10 mg, biotin 1.5 mg, $NaSeO_3 \cdot 5H_2O$ 0.3 mg, $CoCl_2 \cdot 6H_2O$ 0.4 mg, KI 0.8 mg, $CuSO_4 \cdot 5H_2O$ 10 mg, $MnSO_4 \cdot 4H_2O$ 20 mg, $ZnSO_4 \cdot H_2O$ 50 mg, $FeSO_4 \cdot 7H_2O$ 150 mg, $MgSO_4 \cdot 7H_2O$ 500 mg, NaCl 1,000 mg.
[2] Nutrient levels were measured values.
[3] Calculated using the mean values for carbohydrates (17.2 kJ/g), proteins (23.6 kJ/g), and lipids (39.5 kJ/g) according to NRC (2011).

Weight gain rate (WGR, %) = 100 × (FBW − IBW)/IBW;

Specific growth rate (SGR, %/d) = 100 × [Ln (FBW) − Ln (IBW)]/d;

Feed conversion rate (FCR) = FI/(FBW − IBW);

Protein efficiency rate (PER) = (FBW − IBW)/PI;

Net protein utilization (NPU) = (FBW × FBP − IBW × IBP)/PI;

Lipid efficiency rate (LER) = (FBW − IBW)/LI;

Net lipid utilization (NLU) = (FBW × FBL − IBW × IBL)/LI;

where IBW means the initial body weight (g), FBW means the final body weight (g); FI, PI or LI refers to feed, protein or lipid intake (g, air-dry basis); IBP or IBL stands for the initial body protein or lipids; FBP or FBL represents the final body protein or lipids; d denotes days of feeding.

2.6. DNA extraction, PCR amplification, and illumina sequencing

Microbial community genomic DNA was extracted from loach samples with the method of the E. Z.N.A. soil DNA Kit (Omega Biotek, Norcross, GA, U.S.). The DNA samples, having passed the quality test, were sent to Major Bio-Technology Co., Ltd., for amplification and sequencing. V3–V4 region of 16S rDNA was amplified by fusing primers with 338F and 806R barcodes and an Illumina MiSeq platform was used for high-throughput sequencing. According to barcode and primer sequencing, all raw data were screened by using Quantitative Insight into Microbial Ecology (QIIME) (Han et al., 2019; Wang et al., 2020). All of the sequencing data are available in the Sequence Read Archive (SRA) database at NCBI under accession number SRP363340.

2.7. Bio-informational analysis

The raw data were demultiplexed, quality-filtered by Fastp (version 0.19.6)[1] and merged by FLASH (version 1.2.11).[2] Based on valid data, the operational taxonomic units (OTUs) were clustered by Upares (version 7.0.1090)[3] with 97% identity as the division standard (Wang et al., 2020). The taxonomy of each OTU representative sequence was analyzed by RDP Classifier (version 2.11)[4] against the Silva Database (version 138),[5] using a confidence threshold of 0.7. Alpha diversity statistics (the Simpson diversity index, the Shannon diversity index, the Ace estimator, the Chao1 richness estimator and the coverage) for each sample were calculated by Mothur (version 1.30.2).[6] Beta diversity (principal coordinates analysis (PCoA)) was evaluated by similarity analysis (ANOSIM) based on weighted UniFrac distance of OTUs. Bacterial taxonomic differences between 9 groups at the genus or other taxonomic level were analyzed using LEfSe.

2.8. Data analysis

All values were expressed as mean ± SE and were analyzed using SPSS 25.0. Two-way ANOVA was used to analyze the effects of dietary protein and lipid levels and their interaction with growth performance and feed utilization of *P. dabryanus*. $P < 0.05$ indicated a significant difference. When there was a significant interaction between protein and lipids, a one-way ANOVA was performed for all data, and Tukey's test was used for multiple comparisons to

[1] https://github.com/OpenGene/fastp.
[2] https://ccb.jhu.edu/software/FLASH/index.shtml.
[3] http://www.drive5.com/uparse/.
[4] https://sourceforge.net/projects/rdp-classifier/.
[5] https://www.arb-silva.de/.
[6] https://www.mothur.org/wiki/Download_mothur.

analyze the significance among treatment groups. The above criteria (v, w, x …) ($P < 0.05$), and the effect of a single influencing factor were not considered. When there was no significant interaction between protein and lipids, the protein or lipid level was fixed, then the effect of the other factor was analyzed: when the lipid level was fixed, the effect of protein level was analyzed, the above standard (a, b, c …) indicated a significant difference between treatment groups ($P < 0.05$); when the protein level was fixed, the effect of lipid level was analyzed, the above standard (A, B, C …) indicated a significant difference between treatment groups ($P < 0.05$).

3. Results

3.1. Performance

Growth of *P. dabryanus* was responsive to dietary protein and lipid levels ($P < 0.05$) but not to their interaction (Table 2). Fish fed a diet with 40% protein showed significantly higher FBW, WGR, SGR, PER and NPU values than those fed with diets containing 30% or 35% protein, regardless of dietary lipid levels ($P < 0.05$). Fish fed a diet with 10% lipid exhibited significantly higher FBW, WGR, SGR, PER and NPU values than those fed with diets containing 6% or 14% lipid, regardless of dietary protein levels. While dietary protein and lipid had an opposite effect on FCR, LER and NLU, both factors had significant interaction ($P < 0.05$). FCR was the lowest in the CP40EE10 group, whereas LER and NLU were the highest in the CP40EE6 group. With the increase in protein level, FCR, LER and NLU all increased significantly ($P < 0.05$), reaching the highest value at 40% protein level. With the increase in lipid level, fish fed a diet with 10% lipid exhibited significantly lower FCR value than those fed with a diet containing 14% lipid, and LER and NLU values significantly decreased.

The relationship between EE/CP ratios and WGR ($y = -955.22x^2 + 461.64x + 77.386$, $R^2 = 0.3224$) was observed and the optimal EE/CP ratio calculated from the regression equation was 0.24. According to the linear regression analysis of protein level and EE/CP ratios ($y = -0.008x + 0.5686$, $R^2 = 0.9962$), the optimal protein level was 40.87% and the optimal lipid level was 9.88%.

3.2. 16S rDNA gene sequencing and alpha diversity

In total, 1,522,844 good quality V3–V4 regions of 16S rDNA sequences were detected from 9 groups. Each sample was resampled to the minimum number of sample sequences (31,772 reads per sample) and clustered, resulting in 2,464 OTUs of 97% identity, ranging from 1,544 to 2,447 in each group. The coverage index of all samples reached up to 99%, indicating that the depth of sequencing was sufficient (Supplementary Table S1). The alpha diversity of intestinal flora was calculated by diversity index (Shannon and Simpson) and richness index (Ace and Chao1). As can be seen from Fig. 1, dietary protein and lipid had a significant interaction effect on Shannon and Simpson indexes ($P < 0.05$). When the protein level was 30%, the Shannon index of the high-lipid group (CP30EE14) was significantly higher than that of other 2 groups (CP30EE6 and CP30EE10) ($P < 0.05$). When the protein level was 40%, the Shannon index of CP40EE10 group was significantly higher than that of the low-lipid group (CP40EE6) ($P < 0.05$), but not significantly different from that of the high-lipid group (CP40EE14) ($P > 0.05$). When the protein level was 30%, the Simpson index in CP30EE14 was significantly lower than that in the low-lipid group (CP30EE6) ($P < 0.05$). There was no significant interaction effect of dietary protein and lipid on Ace and Chao1 index ($P > 0.05$) (Supplementary Table S1). The Ace index was mainly affected by the protein level. The Ace index of 30% protein was significantly higher than that of 35% protein ($P < 0.05$). Dietary protein and lipid had no significant effect on the Chao1 index ($P > 0.05$). Based on the above indices, when the dietary protein level was lower than 40%, increasing the lipid level could increase the diversity of intestinal flora; however, when the dietary protein level was 40%, the diversity of intestinal flora with 10% lipid was basically similar to that of 14% lipid.

Table 2
Effects of dietary protein and lipid levels on growth performance of *Paramisgurnus dabryanus* (mean ± SE, $n = 4$).

Item	SR, %	FBW, g	WGR, %	SGR, %/d	FCR	PER	NPU	LER	NLU
Individual treatment means[1]									
CP30EE6 (GE 14.63)	95.42 ± 1.72	10.18 ± 0.11	95.74 ± 2.19	1.20 ± 0.02	3.44 ± 0.13^{x}	0.98 ± 0.04	0.12 ± 0.01	8.21 ± 0.32^{x}	0.53 ± 0.02^{x}
CP30EE10 (GE 16.49)	93.75 ± 1.85	10.21 ± 0.17	96.00 ± 3.35	1.20 ± 0.03	3.50 ± 0.16^{x}	0.96 ± 0.05	0.14 ± 0.01	3.50 ± 0.17^{z}	0.42 ± 0.01^{yz}
CP30EE14 (GE 18.28)	98.33 ± 2.36	9.28 ± 0.30	79.19 ± 5.73	1.04 ± 0.06	3.74 ± 0.18^{x}	0.90 ± 0.05	0.11 ± 0.01	2.23 ± 0.11^{z}	0.33 ± 0.01^{z}
CP35EE6 (GE 15.88)	94.58 ± 2.49	11.48 ± 0.20	121.59 ± 3.82	1.42 ± 0.03	2.78 ± 0.15^{y}	1.04 ± 0.05	0.14 ± 0.01	10.01 ± 0.51^{w}	0.64 ± 0.03^{vw}
CP35EE10 (GE 17.34)	96.67 ± 1.18	12.00 ± 0.23	129.93 ± 4.44	1.49 ± 0.03	2.42 ± 0.14^{y}	1.22 ± 0.07	0.18 ± 0.01	4.98 ± 0.27^{y}	0.43 ± 0.02^{y}
CP35EE14 (GE 18.32)	97.78 ± 0.39	11.36 ± 0.21	119.72 ± 4.10	1.40 ± 0.03	2.55 ± 0.06^{y}	0.96 ± 0.15	0.12 ± 0.02	2.68 ± 0.43^{z}	0.36 ± 0.04^{yz}
CP40EE6 (GE 16.26)	96.67 ± 0.68	12.66 ± 0.23	144.32 ± 4.52	1.59 ± 0.03	2.00 ± 0.01^{z}	1.28 ± 0.01	0.21 ± 0.00	10.32 ± 0.06^{w}	0.68 ± 0.00^{v}
CP40EE10 (GE 17.28)	97.08 ± 1.72	13.81 ± 0.04	165.09 ± 0.84	1.74 ± 0.01	1.88 ± 0.05^{z}	1.33 ± 0.03	0.20 ± 0.00	5.77 ± 0.14^{y}	0.55 ± 0.01^{wx}
CP40EE14 (GE 18.26)	94.42 ± 1.60	12.38 ± 0.19	138.99 ± 3.63	1.56 ± 0.03	2.56 ± 0.13^{y}	0.97 ± 0.05	0.15 ± 0.01	2.77 ± 0.14^{z}	0.33 ± 0.01^{z}
Means of main effects[2]									
Protein									
30%	95.83 ± 1.01	9.89 ± 0.17^{c}	90.31 ± 3.17^{c}	1.14 ± 0.03^{c}	3.56 ± 0.09^{a}	0.95 ± 0.02^{b}	0.12 ± 0.00^{c}	4.65 ± 0.78^{b}	0.43 ± 0.03^{c}
35%	96.34 ± 0.93	11.61 ± 0.14^{b}	123.75 ± 2.54^{b}	1.44 ± 0.02^{b}	2.58 ± 0.08^{b}	1.07 ± 0.06^{ab}	0.15 ± 0.01^{b}	5.89 ± 0.95^{a}	0.48 ± 0.04^{b}
40%	96.06 ± 0.82	12.95 ± 0.21^{a}	149.47 ± 3.83^{a}	1.63 ± 0.03^{a}	2.15 ± 0.10^{c}	1.19 ± 0.05^{a}	0.18 ± 0.01^{a}	6.28 ± 0.94^{a}	0.52 ± 0.04^{a}
Lipid									
6%	95.56 ± 0.97	11.44 ± 0.32^{B}	120.55 ± 6.28^{B}	1.40 ± 0.05^{B}	2.74 ± 0.19^{B}	1.10 ± 0.04^{A}	0.15 ± 0.01^{A}	9.51 ± 0.34^{A}	0.62 ± 0.02^{A}
10%	95.83 ± 0.95	12.01 ± 0.45^{A}	130.34 ± 8.67^{A}	1.48 ± 0.08^{A}	2.60 ± 0.21^{B}	1.17 ± 0.05^{A}	0.17 ± 0.01^{A}	4.75 ± 0.30^{B}	0.47 ± 0.02^{B}
14%	96.84 ± 0.80	11.01 ± 0.41^{C}	112.63 ± 7.89^{C}	1.33 ± 0.07^{C}	2.95 ± 0.18^{A}	0.94 ± 0.05^{B}	0.13 ± 0.01^{B}	2.56 ± 0.16^{C}	0.34 ± 0.01^{C}
Two-way ANOVA (*P*-values)									
Protein	0.992	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
Lipid	0.569	<0.001	<0.001	<0.001	0.007	0.001	<0.001	<0.001	<0.001
Interaction	0.154	0.054	0.061	0.092	0.032	0.139	0.058	0.026	0.001

FBW = final body weight; SR = survival rate; WGR = weight gain rate; SGR = specific growth rate; FCR = feed conversion rate; PER = protein efficiency ratio; NPU = net protein utilization; LER = lipid efficiency ratio; NLU = net lipid protein utilization; CP = crude protein; EE = ether extract; GE = gross energy.

[1] Treatment means represent the average values for 4 tanks per treatment; treatment means followed by a different superscript letter (v, w, x, y, z) in the same column are significantly different ($P < 0.05$).

[2] Main effect means followed by a different superscript letter (dietary protein = lowercase, a, b, c; dietary lipid = uppercase, A, B, C) in the same column are significantly different ($P < 0.05$).

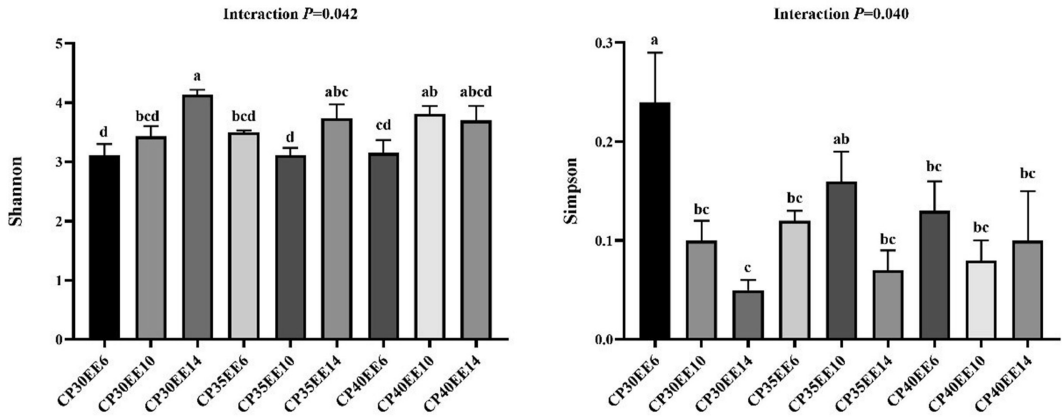

Fig. 1. Difference in alpha diversity of intestinal flora of the 9 groups of samples. Shannon and Simpson are diversity indexes.

3.3. Composition of intestinal microflora in P. dabryanus

According to the results of compositional analysis, a total of 34 phyla and 804 genera were identified. After all species with a sample abundance of less than 1% were merged, all samples were found to be concentrated in 12 phyla and 46 genera (Supplementary Fig. S1 and Fig. S2). At the phylum level, Proteobacteria and Actinobacteria were defined as the dominant phyla with relative abundance ≥10%. Proteobacteria dominated in each group, accounting for 57.04% to 81.45%. Actinobacteria accounted for 7.78% to 18.74% in each group, belonging to the second most dominant phylum. The abundance of Fusobacteria in CP40EE10 was higher than that in other groups (Supplementary Table S2). Next, we analyzed the composition of intestinal flora at the genus level of *P. dabryanus*. According to the definition of dominant species with relative abundance ≥10%, *Burkholderia-Caballeronia-Paraburkholderia* was the dominant genus in all groups. In CP40EE10 group, the abundance of *Mycobacterium* (6.98% vs 2.69%) and *Cetobacterium* (3.33% vs 2.35%) was higher than that in CP30EE14 group (Supplementary Table S3).

3.4. Beta diversity of intestinal flora in P. dabryanus

Then we investigated whether the different dietary protein and lipid levels were associated with gut microbiome community composition. PCoA plots were used to show the microbial community compositions for loach fed with different diets based on the weighted UniFrac distance. The PCoA result showed that when both species presence and species abundance were considered, the 9 groups could be separated (ANOSIM: $R = 0.2447$, $P = 0.002$). The gut microbiota composition changed significantly with the increase in dietary lipid levels (ANOSIM: $R = 0.1997$, $P = 0.003$) (Fig. 2). There was a significant difference in intestinal flora composition between the low-lipid level (6%) and the high-lipid level (14%) groups. The samples of 6% and 14% lipid levels were clustered, while the samples of 10% lipid level were scattered. In contrast, there was no significant difference in β diversity among the different protein level groups (ANOSIM: $R = 0.0123$, $P = 0.572$) (Supplementary Fig. S3).

3.5. Difference in microflora composition in P. dabryanus

Kruskal–Wallis H test was used to test the significant differences between the 9 groups, and the species with significant differences were screened out. At the phylum level, there was no significant difference between groups ($P > 0.05$). At the genus level (top 10 different species in abundance), the relative abundance of *Pseudomonas* and *unclassified_f_Enterobacteriaceae* was significantly different among the 9 groups ($P < 0.05$) (Supplementary Fig. S4).

Dietary protein had no significant effect on species composition abundance at both phylum and genus levels ($P > 0.05$) (Supplementary Fig. S5 and Fig. S6). Dietary lipid level had a significant effect on the relative abundance of Proteobacteria and Bacteroidetes at the phylum level. The abundance of Proteobacteria at a lipid level of 10% was significantly higher than that of 14% ($P < 0.05$), but not obviously different from the 6% lipid level ($P > 0.05$). The abundance of Bacteroidota at a lipid level of 14% was significantly lower than that of 6% ($P < 0.05$) (Fig. 3A). In addition, dietary lipid level had a significant effect on the relative abundance of *Rhodoblastus*, *unclassified_f_Eenterobacteriaceae* and *Burkholderia-Caballeronia-Paraburkholderia* at the genus level ($P < 0.05$). At a lipid level of 10%, the abundance of *Burkholderia-Caballeronia-Paraburkholderia* was not significantly different from that of other lipid levels, whereas the abundances of *Rhodoblastus* and *unclassified_f_Eenterobacteriaceae* were significantly higher than that of 6% and 14%, respectively (Fig. 3B).

To find biomarkers more accurately between the 9 groups, LEfSe multilevel species difference discriminant analysis was used to identify the key roles with significant effects between the groups, with LDA threshold of 4.0 (Fig. 4). *Norank_f_Caldilineaceae* (LDA = 4.15, $P = 0.043$) and *unclassified_f_Chloroflexaceae* (LDA = 4.14, $P = 0.044$) were predominant in the CP30EE6 group, while *unclassified_f_Enterobacteriaceae* (LDA = 4.76, $P = 0.006$) was predominant in the CP30EE10 group. *norank_f_Rhizobiales_Incertae_Sedis* (LDA = 4.062, $P = 0.002$) and *Actinomycetospora* (LDA = 4.107, $P = 0.028$) were predominant in the CP30EE14 group. Gammaproteobacteria (LDA = 5.324, $P = 0.010$), *Pseudomonas* (LDA = 4.899, $P = 0.009$) and *Beijerinckiaceae* (LDA = 4.598, $P = 0.004$) were biomarkers of CP35EE10, CP40EE6 and CP40EE14, respectively.

3.6. Functional prediction

Functional prediction using the KEGG database was annotated based on 16S rDNA sequencing data. PICRUSt2 was used to predict the function of intestinal flora in the 9 groups, and it was found that they were mainly related to metabolism, environmental information processing, cellular processes, genetic information processing, diseases and biological systems. According to the functional annotation and abundance information of all samples in the KEGG database, the top 20 third-level functions and their abundance information in each sample were selected to draw heat maps. KEGG

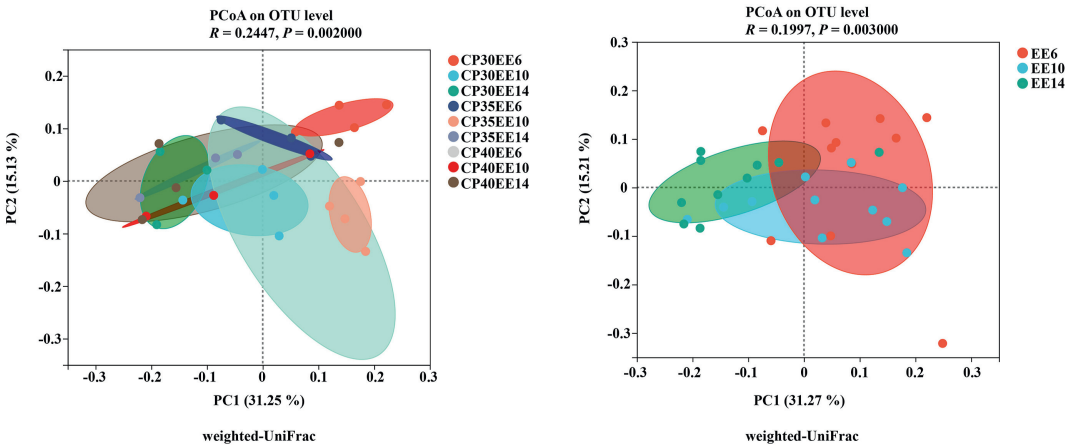

Fig. 2. Principal coordinates analysis (PCoA) based on weighted-UniFrac of the intestinal flora community. CP = crude protein; EE = ether extract; CP30EE6: 30% crude protein, 6% ether extract; CP30EE10: 30% crude protein, 10% ether extract; CP30EE14: 30% crude protein, 14% ether extract; etc.

pathways at level 3 were mainly enriched in metabolic pathways, biosynthesis of secondary metabolism, microbial metabolism in diverse environments, ABC transporters, etc. Clusters of orthologous groups (COG) functional classification statistics showed that the relative abundance of "amino acid transport and metabolism", "energy production and conversion" and "carbohydrate transport and metabolism" was higher (Fig. 5). From the KEGG functional abundance statistics table (Supplementary Table S4), it was found that the abundance of metabolism-related pathways in the CP40EE10 group was higher than that in the CP30EE14 group.

4. Discussion

4.1. Effect of protein and lipid levels on growth performance

In the present study, the optimal dietary protein and lipid levels for growth of *P. dabryanus* with an initial body weight of 5.19 g were 40.87% and 9.88%, respectively. Dietary protein and lipid levels had significant effects on growth performance, but with no significant interaction. Dietary protein can promote fish growth and has been considered a key factor in determining fish growth performance and feed conversion (Muhammadar et al., 2021). The results showed a significant improvement in *P. dabryanus* growth and reduction of feed conversion rate by increasing dietary protein levels, which were consistent with the results obtained in many species (Kumar et al., 2018; Méndez-Martínez et al., 2018; Wang et al., 2018). The optimal protein level in the present study was found to be 40.87%, which is similar to that of topmouth culter (*Cuter alburnus*) (Zhang et al., 2016). However, it is lower than that of *Misgurnus anguillicaudatus* (initial weight 0.40 g) with 50% protein level (Yan et al., 2017), and higher than that of 1.5 g *Misgurnus anguillicaudatus* with 30% protein level (Kim et al., 2013). These differences could be related to different dietary formulation, environmental conditions, fish types and the growth stage. In this study, the FCR of fish fed with the 40% protein level diet was significantly lower than that of fish fed with other protein level diets, which may be due to the fact that the content of amino acids (especially essential amino acids) of the 40% protein level diet was higher than that of other protein level diets. Similar findings have also been reported in yellow croaker (*Larimichthys polyactis*) (Ma et al., 2020). The increase in dietary protein levels had a significant effect on PER, NPU, LER and NLU values, which meant that fish fed the high-protein diets (40% dietary protein) utilized dietary protein and lipid more efficiently than fish fed the low-protein diet (30% and 35%). A similar trend has also been found by different authors for other fish species including *Siniperca scherzeri* (Sankian et al., 2017), *Channa striatus* (Aliyu-Paiko et al., 2010), *Macrobrachium Americanum* (Méndez-Martínez et al., 2018) and *Culter alburnus* (Zhang et al., 2016).

With the increase in dietary lipid level from 6% to 14%, the growth performance of *P. dabryanus* was initially improved, but then decreased significantly with further increases. At any given protein level, the highest FBW, WGR and SGR were obtained in the group fed diets containing 10% lipid, and growth was reduced by low or high lipid. FCR of the 10% lipid level was significantly lower than that of the 14% lipid level, but not significantly different from the 6% lipid level. Similarly, PER and NPU of the 10% lipid level were significantly higher than those of the 14% lipid level and with the increase in lipid level, LER and NLU both decreased significantly. This could be evidence of the protein sparing effect of lipid inclusion in *P. dabryanus*. Similarly, this effect was also found in some other species (Zhang et al., 2016; Xun et al., 2021). Moreover, there was apparent interaction of dietary protein and lipid on FCR, LER and NLU in the present study. The lowest FCR in the CP40EE10 group indicated that the formulation of this diet was efficiently utilized by *P. dabryanus*. The nutrient levels required by loach in this study are different from previous research results (Gao et al., 2017; Victor et al., 2019; Chen et al., 2020; Du et al., 2020), which may be due to the differences in loach species, growth stage, environmental conditions and dietary formulations.

4.2. Effect of protein and lipid levels on α and β diversity of intestinal microflora

The higher value of Shannon and the lower value of Simpson mean larger species diversity in the samples, whereas the higher value of Ace and Chao1 indicate larger community richness (Fang et al., 2021). In this study, dietary protein and lipid had significant interactions with diversity indices, but the protein level only had a significant effect on the Ace index, while the lipid level significantly increased the diversity index and had no obvious effect on the richness index. In fish nutrition, dietary lipids provide energy, essential fatty acids, phospholipids, and fat-soluble vitamins for fish growth, and feeding fat may affect the composition of animal gut microbiota (Zhu et al., 2021). In zebrafish, overfeeding dietary fat caused dysbiosis altering the diversity and composition of the intestinal microbiota (Navarro-Barrón et al., 2019). When fish possesses a highly diverse gut microbiome, they can make use of

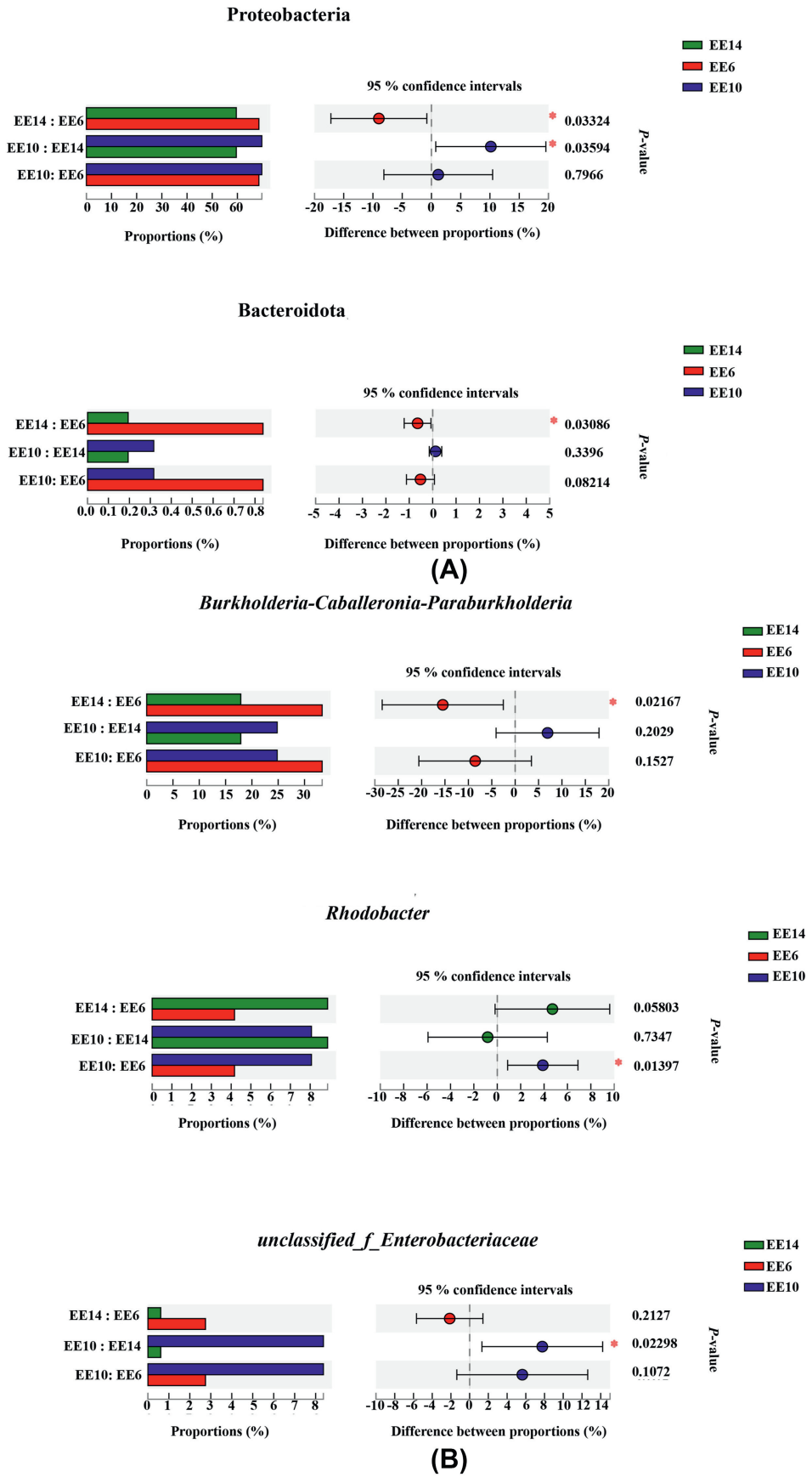

Fig. 3. Kruskal–Wallis H test difference diagram at the phylum level (A) and genus level (B) between different lipid levels groups. EE = ether extract.

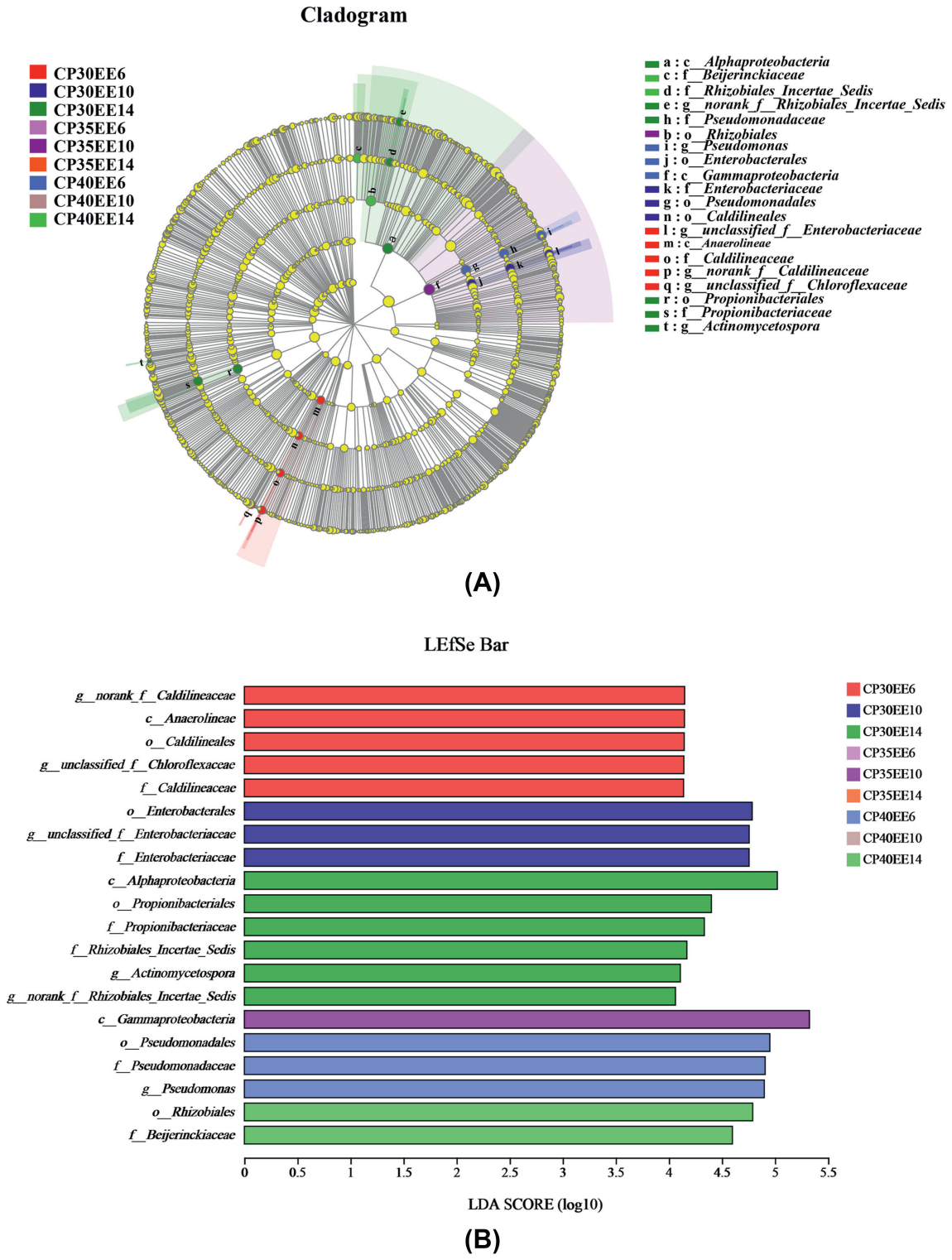

Fig. 4. LEfSe map of species differences between the 9 groups at the genus level. LEfSe cladogram (A), highly significant bacteria with LDA score >4 (B). CP30EE6: 30% crude protein, 6% ether extract; CP30EE10: 30% crude protein, 10% ether extract; CP30EE14: 30% crude protein, 14% ether extract; etc.

nutrients from food more efficiently because different microbiota can synergistically decompose and utilize nutrients (Fang et al., 2021). There was no significant difference in α diversity between CP30EE14 and CP40EE10, but the growth performance was diametrically opposite, which may be related to the species composition and abundance of the microflora.

β Diversity analysis showed that there were significant differences in community structure among the 9 groups, but only lipid level had a significant impact on it. Among the 9 groups, the aggregation of the CP30EE14 group was higher than that of the CP40EE10 group. The samples of 6% and 14% lipid levels were clustered, while the samples of the 10% lipid level were scattered.

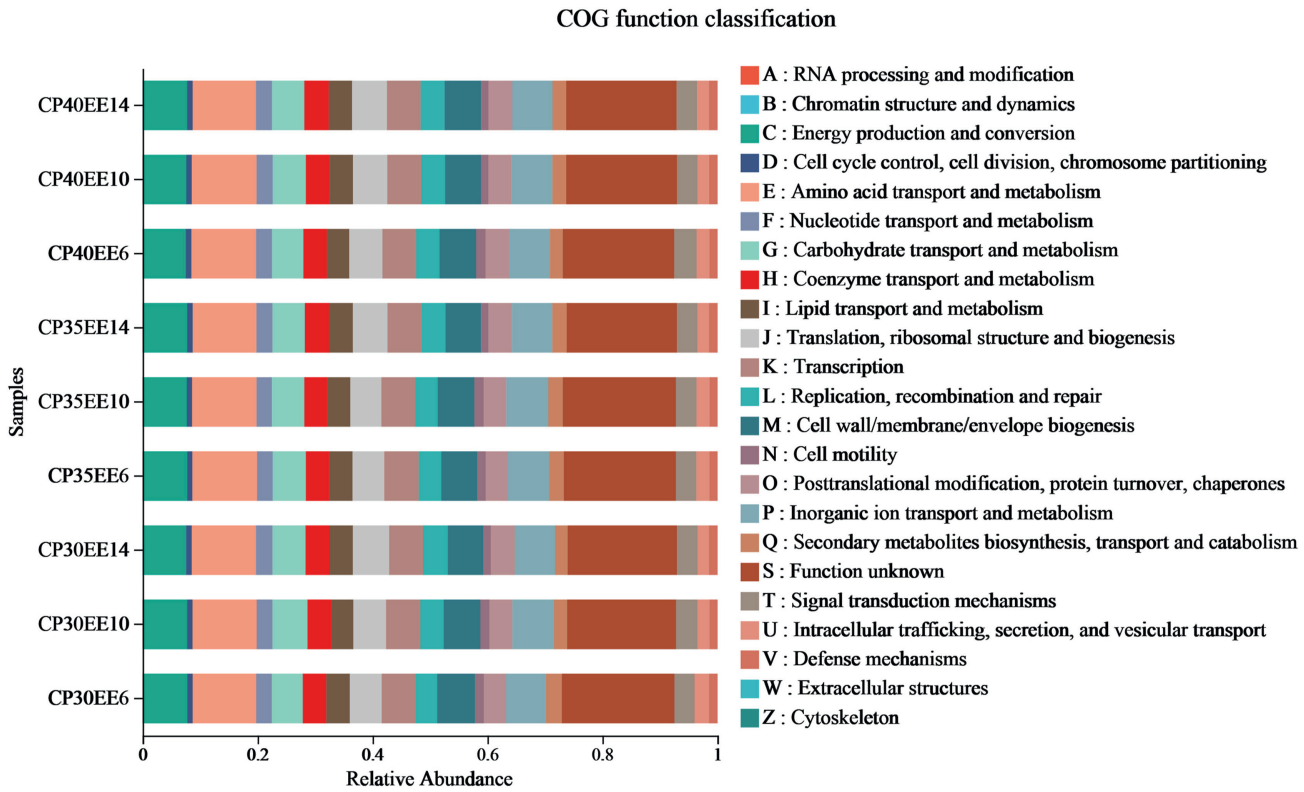

Fig. 5. The COG function classification of intestinal flora. CP30EE6: 30% crude protein, 6% ether extract; CP30EE10: 30% crude protein, 10% ether extract; CP30EE14: 30% crude protein, 14% ether extract; etc. COG = clusters of orthologous groups.

This indicated that the gut microbiota of *P. dabryanus* fed with 6% or 14% lipid level diets had higher specificity than those fed with the 10% lipid level diet, which was consistent with the results of the LEfSe analysis, suggesting that the gut microbiota of *P. dabryanus* varied with different dietary lipid levels (Huyben et al., 2020).

4.3. Effect of protein and lipid levels on community of intestinal microflora

The intestine is an important part of the digestion and absorption process of nutrients in fish, housing the highest number and types of bacteria. The normal microflora can secrete various enzymes to promote the digestion and absorption of nutrients, and also regulate immune function to maintain body health (Tran et al., 2018; Peng et al., 2019; Yang et al., 2021a). Studies have shown that the main intestinal flora of loach include Proteobacteria and Actinobacteria (Lin et al., 2021; Zhu et al., 2020; Yang et al., 2021a); comparable findings were also obtained in this experiment. In our experiments, the relative abundances of Fusobacterium, Firmicutes and Bacteroidetes did not reach more than 10%, which may be due to different species, diets, sampling methods and analysis methods (Nayak, 2010; Clements et al., 2014; Deng and Swanson, 2015).

Since protein has no significant effect on species at the phylum level, analysis of the intestinal flora composition of the 3 lipid level groups showed that the abundance of Proteobacteria at a lipid level of 10% was significantly higher than that at a lipid level of 14%. Proteobacteria, a group of gram-negative bacteria, play an important role in the degradation and fermentation of polysaccharides, proteins and other organic matter, and are the dominant flora in the intestinal microbial composition of many fish (Gao et al., 2017). The change in the abundance of Proteobacteria in the intestine is an important sign of an imbalance of intestinal flora (Shin et al., 2015). *Unclassified_f_Eenterobacteriaceae* and *Rhodoblastus* both belong to Proteobacteria, with their relative abundances at a lipid level of 10% being significantly higher than those of 14% and 6% lipid levels, respectively, indicating that the 10% lipid level was favorable for the growth of Proteobacteria. It is speculated that a high-lipid diet (14%) reduced the abundance of this dominant bacteria, thereby affecting gut health. It may explain why the diversity of fish fed the 14% lipid diet was higher, but the growth performance was lower than that of fish fed the 10% lipid diet. *Mycobacterium* and *Cetobacterium* belong to Actinobacteria and Fusobacterium, respectively. They were more abundant in the CP40EE10 group than the CP30EE14 group. Actinobacteria are a major source of many enzymes, vitamin-producing bacteria and active substances such as antibiotics. Increasing the abundance of this bacteria is beneficial to the decomposition and digestion of nutrients (Fan and Li, 2019). In addition, *Cetobacterium*, which can promote protein synthesis or glycolipid metabolism and can synthesize fats, carbohydrates, and proteins for the host's use (Lin et al., 2019), were only found in the CP30EE14, CP40EE10 and CP40EE14 groups. Furthermore, the abundance of *Cetobacterium* in the CP40EE10 group was higher than the CP30EE14 group, suggesting that *Cetobacterium* played a vital role in the growth of fish. The proportion of Bacteroidetes and Firmicutes is associated with obesity, which promotes the efficient absorption of energy from food (Peng et al., 2019; Zhu et al., 2021). In this study, although there was no significant difference between Firmicutes and Bacteroidetes among the 9 groups, the abundance of Firmicutes in most taxa was higher than that of Bacteroidetes. It may be related to energy metabolism. At a lipid level of 14%, the relative abundance of Bacteroidetes was significantly lower the 6% lipid level, but no significant difference was found with the 10% lipid level. Firmicutes was relatively more abundant than Bacteroidetes, which may indicate that a high lipid level (14%) has the

potential to increase fat deposition in fish (Yang et al., 2021a). These results revealed the reason for the poor growth performance of fish fed with the 14% lipid level diet, which may be related to the decrease in the abundance of bacteria that produce digestive enzymes, nutrients and energy metabolism, and may also be related to fat deposition in body tissues caused by a high lipid level diet.

4.4. Effect of protein and lipid levels on potential function of intestinal flora

In order to further understand the effects of dietary protein and lipid levels on intestinal microflora of *P. dabryanus*, the potential function of intestinal flora in 9 groups was predicted.

It was found that functional genes related to metabolism and biosynthesis were enriched in each group, amino acid transport and metabolism dominated, followed to a lesser extent by energy production and conversion, indicating that *P. dabryanus* can effectively utilize dietary protein and lipid for energy supply during the growth process. Although there were significant differences in species abundance of microorganisms among the 9 groups of samples, each sample had the same function pathway. This may be caused by the similarity of gene functions among different bacterial communities. The abundance of metabolism-related pathways in the CP40EE10 group was higher than that in CP30EE14 group (Table S4), indicating that the metabolism in the intestine of the CP40EE10 group was more active than that in the CP30EE14 group. However, these results are only based on functional predictions from existing reference genomes, and more research is needed in the future.

5. Conclusion

This study explored the relationship between growth performance and gut microbiota, and also revealed complex relationships between diet, intestinal microflora and host metabolism. Results demonstrated that *P. dabryanus* obtained the best growth performance when dietary protein was 40% and lipid was 10%. The intestinal microbiota community was mainly influenced by dietary lipid level. The intestinal metabolic capacity of *P. dabryanus* in the CP40EE10 group was higher than that in the CP30EE14 group. The optimal protein and lipid levels of *P. dabryanus* were 40.87% protein and 9.88% using quadratic regression analysis based on WGR.

Author contributions

Zirui Wang and **Qiubai Zhou** participated in the design of the study. **Shuyao Li** and **Yongan Li** performed the experiments. **Youjie Li** helped with loach culture and preparation of experimental materials. **Shuyao Li**, **Yongan Li**, **Youjie Li**, **Zhiwen Yuan**, **Guanghua Huang** collected samples. **Shuyao Li** analyzed data and drafted the manuscript. **Zirui Wang**, **Qiubai Zhou** and **Jinhua Zhang** participated in the coordination of the study, and revised the manuscript.

Declaration of competing interest

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

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2023.02.007.

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