

DIET INFLUENCES THE ACCUMULATION OF SHORT-CHAIN FATTY ACIDS ASSOCIATED WITH THE GUT MICROBIOTA IN THE GRASS CARP (*CTENOPHARYNGODON IDELLUS*) HINDGUT

LI, Z. F.^{1,2} – XIE, J.^{1,2*} – YU, E. M.^{1,2} – WANG, G. J.^{1,2} – ZHANG, X. K.³ – YU, D. G.^{1,2} – ZHANG, K.^{1,2} – GONG, W. B.^{1,2}

¹Key Laboratory of Tropical and Subtropical Fishery Resource Application and Cultivation, Pearl River Fisheries Research Institute, Chinese Academy of Fishery Sciences, No.1, Xingyu Road, Liwan, Guangzhou, Guangdong 510380, China

²Guangdong Ecological Remediation of Aquaculture Pollution Research Center, Guangzhou, Guangdong 510380, China

³Research Center of Aquatic Organism Conservation and Water Ecosystem Restoration in Anhui Province, Anqing Normal University, Anqing, Anhui 246011, China

*Corresponding author

e-mail: xiejunhy01@126.com; phone: +86-20-8161-6178

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Abstract. Diet changes the composition and function of the gut microbiota (GM). Short-chain fatty acids (SCFAs) are mainly produced through carbohydrate fermentation by the GM. It remains unclear whether changing the diet influences the accumulation of SCFAs in the grass carp (*Ctenopharyngodon idellus*) gut. To address this question, we compared SCFA accumulation with GM changes in the hindgut contents of grass carp in groups fed hybrid giant napier (*Pennisetum sinense* Roxb, HG), formula feed (FG), and broad bean (*Vicia faba*, BG). Our results showed that diet significantly influenced the SCFA concentrations in the hindgut of grass carp. By eliminating the influences of dietary SCFA, acetate and total SCFA accumulation in the hindgut of grass carp turned out to be positively associated with dietary cellulose contents. Most of the enriched bacterial biomarkers in the HG group that consumed the most dietary cellulose were SCFA-producing bacteria. These results implied that diet could regulate the relative abundances of potential SCFA-producing bacteria and SCFA accumulation in the grass carp hindgut. These findings shed light onto GM functions related to SCFA production and associative functions between SCFAs and the GM of grass carp.

Keywords: aquaculture, carbohydrate fermentation, cellulose, high-throughput sequencing, microbial metabolism

Introduction

In mammals, short-chain fatty acids (SCFAs) such as acetate, butyrate, and propionate are essential host energy sources (Stevens and Hume, 1998; Spiljar et al., 2017; Leblanc et al., 2017), which act as signal-transduction molecules via G protein-coupled receptors and as epigenetic regulators of gene expression by inhibiting histone deacetylase (Kasubuchi et al., 2015; Cohen et al., 2017). SCFAs are involved in regulating glucose and lipid metabolism, stimulating the proliferation and differentiation of intestinal enterocytes, and reducing the intestinal pH (thereby promoting the absorption of minerals by increasing their solubility) (Barczynska et al., 2016; Postler and Ghosh, 2017). SCFAs are also key regulators of virtually every aspect of the intestinal immune system, and they play key roles in the prevention and treatment of metabolic syndromes, bowel disorders, and cancer (Kasubuchi et al., 2015; Postler and Ghosh, 2017; Fukuda et al., 2012; Flint et al., 2015; Safari et al., 2016). Although

comparatively less research has been conducted with fish than with mammals, high intestinal SCFA concentrations occur in some marine herbivorous fishes and SCFAs produced by the hindgut microbiota are used as a blood fuel for energy purposes or for lipid synthesis by the host fish (Clements et al., 1994; Clements and Choat, 1995, 1997; Seeto et al., 1996; Mountfort et al., 2002).

Naturally, SCFAs are mainly produced through carbohydrate fermentation by gut microbiota in the host's intestines (Flint et al., 2015; Clements and Choat, 1995; Mountfort et al., 2002; Hao et al., 2017b). However, with the collapse of wild fishery resources, aquaculture has become the main way to produce fish protein. In addition, formula feed has replaced natural food to become the primary, or even sole food for cultured fish.

Diet is believed to be the major factor that changes the composition and functions of fish gut microbiota (Ni et al., 2014a; Faith and Gordon, 2011; Claesson et al., 2012; Bolnick et al., 2015; Miyake et al., 2015; Hao et al., 2017a). In addition, the quantities and number of SCFA species are markedly influenced by carbohydrate substrates (Hao et al., 2017a; Mountfort et al., 1993; Kihara and Sakata, 1997, 2002). Kihara and Sakata (2002) reported that alpha starch is the best substrate for producing SCFAs in the tilapia (*Oreochromis niloticus*) gut by microbial fermentation. Mountfort et al. (1993) isolated a bacterium, *Eubacterium* sp. P-1 (DSM 6788), from the mullet gut contents, which anaerobically ferments pectin to produce 163 mol acetate, 30 mol ethanol, 88 mol methanol, and 48 mol formate per 100 mol pectin monomer, whereas it anaerobically ferments hexose in cellobiose or starch to produce 39 mol acetate, 128 mol ethanol, and 41 mol formate per 100 mol hexose. However, it remains unclear whether changing the diet from natural food to artificial feed reduces SCFA accumulation in the fish gut. Since acetate absorption is driven by an SCFA concentration gradient between the blood and intestines, and the rates of absorption increase with intestinal concentrations (Titus and Ahearn, 1991), the answer to the question will show which feed formula promotes SCFA accumulation in hindgut and enhances the growth and health of fish, considering there are many studies have shown that using SCFAs and their salts as feed supplements enhance fish production (Goosen et al., 2011; Ringø, 1992; Da et al., 2013).

Grass carp (*Ctenopharyngodon idella*) is one of the most important native Chinese freshwater herbivorous fish, representing the largest freshwater aquaculture product worldwide (Ni et al., 2014a; FAO, 2014; Wang et al., 2015). Recently, a high-density culture formulation of grass carp feed with broad bean (*Vicia faba*) or formula feed in ponds has started spreading in China. The changes of the gut microbiota in grass carp caused by diet were described previously (Ni et al., 2014a). However, given that broad bean and formula feeds contain high protein and low cellulose concentrations, it is unclear whether those changes alter SCFA accumulation in the hindgut of grass carp. Considering the usability of carbohydrate substrates in fermentation by the gut microbiota, the nutritional contents of feeds, and the co-evolution between the gut microbiota and host's feeding habits, we hypothesized that whereas feeding formula feeds would increase grass carp growth and SCFA fermentation in the hindgut, feeding natural grass would enrich the cellulose-degrading bacteria in the hindgut of grass carp. To test this hypothesis, we compared the compositions of gut microbiota with cellulose levels and SCFA accumulation in hindgut of grass carp fed hybrid giant napier (*Pennisetum sinense* Roxb), formula feed, and broad beans.

Materials and methods

Experimental design and sample collection

The experiment was conducted in the precise aquaculture base of the Pearl River Fisheries Research Institute of China (113°13'02" E, 23°04'07" N) over a period of 60 days from May 23, 2015 to July 24, 2015. Grass carps weighing approximately 500 g individually from the same parents was collected from an artificial pond at Foshan Tongwei Fisheries Co. (Guangdong, China). The experiment was divided into three groups. Each group, including 30 individuals, was fed with hybrid giant napier (H), formula feed (F), or broad bean (B), respectively. The daily feed amount was 3% of the total weight of grass carps in formula feed group and broad bean group, and 10% of the total weight of grass carps in hybrid giant napier group. The uneaten feed was removed after 1 h of feeding. Broad beans were marinated in fresh water 24 h before they were fed to grass carp. After 2 months of being fed hybrid giant napier, formula feed, or marinated broad bean at 09:00 and 16:00 each day in the artificial pond, three carps in each diet group were collected and euthanized by submersion in 60 mg/L tricaine methane sulfonate solution.

After two months, the grass carp were dissected under sterile conditions to obtain their hindgut contents for total microbial DNA extraction and SCFA concentration measurements, according to a previously described method (Ni et al., 2014a; Li et al., 2018). The groups were named based on the feed type. For example, FG indicates that the microbiota was collected from the hindgut of grass carp fed with formula feed.

Determination of nutritional contents in feeds

The crude fiber contents, crude ash contents, crude protein contents, and crude fat contents in fresh hybrid giant napier, marinated broad beans, and formula feed were determined according to standard methods (ISO 6541:1981, ISO 5984:2002, ISO 5983:1979, and ISO 6492:1999).

Determination of SCFA concentrations

SCFAs (acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate) in feeds and the hindgut contents of grass carp fed with different feeds were analyzed by gas chromatography using the method proposed by Schwiertz et al. (2012), with some modifications. Briefly, each sample of 300 mg (wet weight) was weighed (10^{-4} g precision) and placed in a 2-ml EP tube with 1.2 ml ddH₂O. The mixture was vortexed for 30 s, homogenized in a ball mill for 4 min at 45 Hz, and sonicated for 5 min in ice water. The mixture was centrifuged at 5,000 rpm for 20 min at 4 °C, and the supernatant (0.6 ml) was collected for metabolite extraction. Next, 0.15 ml H₂SO₄ (50%) and 50 µg SCFA/ml ether were added to each supernatant, after which the supernatant was vortexed for 30 s and centrifuged (10 min, 12000 rpm, 4 °C). The supernatant was passed through a nylon filter (0.45 µm) into a gas chromatography vial. SCFAs were analyzed using a GC2025 gas chromatograph (Shimadzu; Kyoto, Japan) with an automatic injector (AOC20i) at 240 °C, using a flame-ionization detector (Shimadzu Deutschland GmbH, Duisburg, Germany) at 240 °C, equipped with an HP-Innowax capillary column (Agilent, Santa Clara, USA; 30 m × 0.25 mm i.d. × 0.25 µm f.d.). The injection volume was 1 µl, the carrier gas was helium (flow rate, 1 ml/min), and the mode of injection was splitless. The oven temperature program was 50 °C for 3 min,

followed by an increase to 180 °C (held for 0 min) at 8 °C /min, and a further increase to 200 °C (held for 5 min) at 50 °C/min. Other parameters used were: gas helium flow, 30 ml/min; hydrogen flow, 40 ml/min; and airflow, 400 ml/min.

DNA extraction and sequencing

Total genomic DNA was extracted from the gut samples using a Gut Microbial DNA Extraction Kit (Omega, USA). DNA concentrations and purities were evaluated using 1% agarose gels. DNA samples were diluted to 1 ng/μl using sterile water before amplification was performed (Li et al., 2017).

The V4 region of the 16S rRNA gene was amplified using primers 515F and 806R, which contain sample-specific barcodes (Yan et al., 2016). PCR was performed in 30-μl reaction volumes with 15 μl of the Phusion[®] High-Fidelity PCR Master Mix (New England BioLabs, USA), 0.2 μM of the forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling conditions used were as follows: initial denaturation at 98 °C for 1 min; followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 60 s; and a final elongation step at 72 °C for 5 min (Li et al., 2018).

The PCR products were mixed with the same volume of 1× loading buffer (containing SYBR Green) and electrophoresed on 2% agarose gels for detection. Samples between 400 and 450 bp in size were chosen for further experiments. The PCR products were mixed at an equidensity ratio; then, the mixed PCR products were purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA).

Sequencing libraries were constructed using the NEB Next[®] Ultra[™] DNA Library Prep Kit (NEB, USA), according to the manufacturer's recommendations, and index codes were added. The library quality was assessed using a Qubit[®] 2.0 Fluorometer (Thermo Scientific, USA) and an Agilent Bioanalyzer 2100 system (Agilent Technologies, USA). Finally, the libraries were sequenced using the Illumina MiSeq platform, and 250-bp paired-end reads were generated.

Sequence data analysis

Paired-end reads from the original DNA fragments were merged using FLASH software. Paired-end reads were assigned to each sample based on the sample-specific barcodes. Sequences analysis was performed using the UPARSE software package with the UPARSE-OTU and UPARSE-OTUref algorithms (Edgar, 2013). QIIME 1.9.0 software (Caporaso et al., 2010) and the vegan package (Dixon, 2003) in the R platform (R Core Team, 2014) were used to analyze alpha (within samples) and beta (among samples) diversity. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). We selected representative sequences for each OTU and used the RDP classifier to annotate the taxonomic information for each representative sequence (Wang et al., 2007). To compute alpha diversities, we rarified the OTU table and calculated three metrics: Chao1 estimated species abundance, observed species estimated as the number of unique OTUs found in each sample, and the Shannon index. Rarefaction curves were generated based on these three metrics.

Correspondence analysis (CA), non-parametric multivariate analysis of variance (MANOVA) (Anderson, 2001), and non-parametric significance testing of differences were conducted using the vegan package in the R platform. A heatmap profile was drawn using HemI 1.0 (Deng et al., 2014).

All sequencing data were deposited in the NCBI Sequence Read Archive (SRA) under accession number SRP080980. The SRA records can be accessed at <http://www.ncbi.nlm.nih.gov/sra/SRP080980>.

SCFA data analysis

Statistical analysis was performed using R software, version 3.0.1. Data are displayed as the mean \pm standard error (S.E.). The Kruskal–Wallis test was applied to analyze global differences among three groups. Tukey’s method was used as a post-hoc test for multiple comparisons of means.

Results

Nutrient content in the three feeds and growth of grass carp

As expected, fresh hybrid giant napier contained the highest content of crude fiber ($23.96 \pm 1.68\%$, dry matter), followed by formula feed ($9.70 \pm 0.46\%$, dry matter) and marinated broad beans ($1.98 \pm 0.89\%$, dry matter). Fresh hybrid giant napier contained the lowest content of crude protein ($12.36 \pm 1.92\%$, dry matter), followed by formula feed ($28.78 \pm 0.00\%$, dry matter) and marinated broad beans ($31.78 \pm 1.86\%$, dry matter) (Table 1).

Table 1. Nutrient contents in three feeds used to feed grass carp (% dry matter). The superscripted lowercase letters indicate significant differences observed between different feeds. The same superscripted letters reflect cases where no significant difference was found, whereas different superscripted letters represent cases where significant differences were found ($p < 0.05$)

Nutrient component	Hybrid giant napier (%)		Broad bean (%)		Formula feed (%)	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
Crude fiber content	23.96 ^a	1.68	1.98 ^c	0.89	9.70 ^b	0.46
Crude ash content	13.33 ^a	1.20	3.76 ^c	0.00	7.53 ^b	0.07
Crude protein content	12.36 ^a	1.92	31.78 ^c	1.86	28.78 ^b	0.00
Crude fat content	3.23 ^b	0.13	< 1.11* ^a	NA	5.42 ^c	0.26

*The content of crude fat in the marinated broad beans was below the limit of detection

At the start of the experiment, the average weights of grass carp in groups fed hybrid giant napier, formula feed, and marinated broad bean were 501.5 ± 20.17 g, 501.33 ± 15.00 g, and 498.83 ± 21.44 g, respectively. No significant differences were observed. However, at the end of the two-month experiment, the average weights were 680.17 ± 15.17 g, 918.00 ± 46.67 g, and 602.50 ± 10.50 g, respectively. The weight increases were 178.67 ± 13.67 g, 416.67 ± 53.33 g, and 103.67 ± 15.11 g, which represented significant differences, in each case (Appendix 1).

Concentrations of SCFAs in feeds and gut contents of grass carp fed different feeds

The concentrations of acetate, propionate, and i-valerate in broad beans were the highest among the three feeds, whereas the absolute concentrations of all SCFAs in the

hindguts of grass carp fed with formula feed were the highest (1.95 ± 1.06 mmol/kg, 0.54 ± 0.16 mmol/kg, and 0.13 ± 0.01 mmol/kg for acetate, propionate, and butyrate, respectively), followed by those from grass carp fed marinated broad beans and fresh hybrid giant napier (Table 2). Although the absolute concentrations were different, the three most dominant SCFAs in the guts of all of grass carp fed with different feeds were acetate, propionate, and butyrate. The ratios of their concentrations (approximately 14.3: 2.5: 1) were similar in all grass carp contents (Table 2). These results showed that the SCFA concentrations in the feeds and the metabolic activity of the hindgut microbiota markedly influenced SCFA concentrations in the hindgut of grass carp.

Table 2. Absolute concentrations of short-chain fatty acids (SCFAs) in the three feeds and in the hindguts of grass carp fed with the three feeds. HG, hindgut contents of grass carp fed with fresh hybrid giant napier (*Pennisetum sinense* Roxb); BG, hindgut contents of grass carp fed with marinated broad beans (*Vicia faba*); FG, hindgut contents of grass carp fed with formula feed; H, fresh hybrid giant napier; B, marinated broad beans; F, formula feed. The superscripted lowercase letters indicate significant differences observed between different feeds. The same superscripted letter shows instances where no significant difference was found, and different superscripted letters indicate cases where significant differences ($p < 0.05$) were found. Data are displayed as the mean \pm standard error (S.E.)

	Sample size	Acetate (mmol/kg)	Propionate (mmol/kg)	Butyrate (mmol/kg)	Iso-butyrate (mmol/kg)	Valerate (mmol/kg)	Iso-valerate (mmol/kg)
HG	3	0.31 ± 0.03	0.02 ± 0.00^a	0.01 ± 0.00^a	0.01 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a
BG	3	1.07 ± 0.51	0.14 ± 0.03^a	0.04 ± 0.02^a	0.01 ± 0.00^a	0.00 ± 0.00^a	0.01 ± 0.00^a
FG	3	1.95 ± 1.06	0.54 ± 0.16^b	0.13 ± 0.01^b	0.03 ± 0.00^b	0.02 ± 0.00^b	0.03 ± 0.00^b
H	3	0.14 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
B	3	1.82 ± 1.15	0.26 ± 0.34	0.05 ± 0.06	0.02 ± 0.01	0.01 ± 0.00	0.04 ± 0.01
F	3	1.05 ± 0.04	0.20 ± 0.01	0.08 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00

To measure the SCFA increment in the hindgut region of grass carp fed with different feeds, the ratios of SCFA concentrations in the hindgut of each group to those in the feed were calculated. In terms of the total SCFA concentration (i.e., the sum of the concentrations of acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate), the ratio for HG was the highest (1.98), followed by those for FG (1.95) and BG (0.58). The crude fiber contents in different feeds positively associated with SCFA ($R^2 = 0.890$) and acetate ($R^2 = 0.989$) increments in grass carp, but negatively associated with propionate ($R^2 = 0.895$) and butyrate ($R^2 = 0.837$) increases (Fig. 1). These findings implied that incremental changes of SCFAs, especially acetate in the hindguts of grass carp, were associated with the fiber contents of feeds.

Gut microbiota composition of grass carp fed different feeds

In this study, 451,160 high-quality spliced sequences from 9 samples were obtained. To eliminate the impediment of sequencing depths for each sample in determining the reliability, each sample was re-sampled at 29,918 sequences to assess their microbiotas. After re-sampling, 628 OTUs from 284 genera were detected using the criterion of 97% sequence similarity. An average of 190 OTUs (ranging from 93

to 310) were detected in each sample (*Appendix 2*). The alpha diversity (Chao1 and Shannon indexes) of the gut microbiota was highest in the BG group, followed by the HG group, and lowest in the FG group (*Appendix 2*). The rarefaction curves of the OTUs showed that the sequence datasets could be used to represent the diversities and structures of each microbiota (*Appendix 3*).

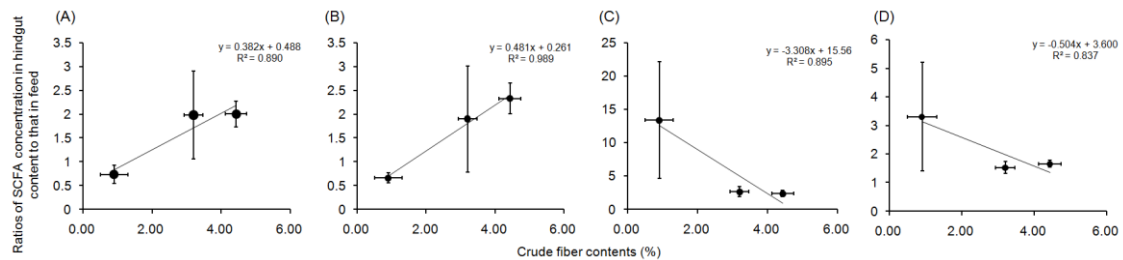


Figure 1. Ratios of SCFA concentrations in the hindguts of grass carp to those in different feeds. (A) Ratios of all SCFA concentrations (i.e. total concentrations of acetate, propionate, butyrate, iso-butyrate, valerate, and iso-valerate). (B) Ratios for acetate. (C) Ratios for propionate. (D) Ratios for butyrate

Except for a minority of sequences (ranging from 0.03% to 0.17%) that could not be classified into any phylum, 19 phyla consisting of 1 Archaea phylum and 18 Bacteria phyla were found. Fusobacteria (40.92%), Proteobacteria (26.40%), Firmicutes (25.04%), Bacteroidetes (5.61%), and Cyanobacteria (1.46%) were the dominant phyla in the gut microbiota of the grass carp (*Fig. 2A*). In this study, differences in the phylum compositions of the gut microbiota among the grass carp fed different diets were observed. The relative abundance of Bacteroidetes in the hindgut microbiota of the BG group was significantly higher than that found with the other diets, whereas the relative abundance of Fusobacteria was significantly lower (*Fig. 2B*). The relative abundance of Firmicutes in the hindgut microbiota of the FG group was significantly lower than that in the other groups (*Fig. 2B*). Moreover, the relative abundance of Proteobacteria in the BG group was significantly higher than that of the HG group (*Fig. 2B*). These results indicated that diet could fundamentally change the gut microbiota composition at the phylum level.

CA based on the OTUs and genus compositions of the samples showed a clear separation of the community composition among three treatment groups and revealed that diet significantly changed the hindgut microbiota of grass carp (*Fig. 3A and B*). MANOVA also revealed distinct differences among the gut microbiota of grass carp fed with different feeds ($P < 0.05$).

Fifty-three dominant genera (relative abundances $> 0.1\%$ in at least one sample) was detected. They clustered into three different groups according to diet (*Fig. 4A*). The relative abundances of *Cetobacterium* in the FG and HG were obviously higher than those in the BG group. The relative abundances of *Lactobacillus*, *Pediococcus*, *Bacillus*, an unidentified Streptophyta genus, and an unidentified Staphylococcaceae genus in the HG group were obviously higher than those in the FG and BG groups. The relative abundances of *u114* of Fusobacteriaceae and an unidentified Betaproteobacteria genus in the FG group were obviously higher than those in the BG and HG groups, whereas the relative abundances of *Bacteroides*, *Tolomonas*, an unidentified Lachnospiraceae genus, and an unidentified Enterobacteriaceae genus

were obviously higher in the BG group than those in the HG and FG groups (Fig. 4A). LEfSe results showed that the increased genus biomarkers in the HG group mostly concentrated in *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Enterococcus*, and *Leptotrichia*; the increased genus biomarkers in the BG group mostly concentrated in *Rhodobacter* and *Uliginosibacterium*; and the increased genus biomarker in the FG group was mostly represented by *Fusobacteria* (Fig. 4B).

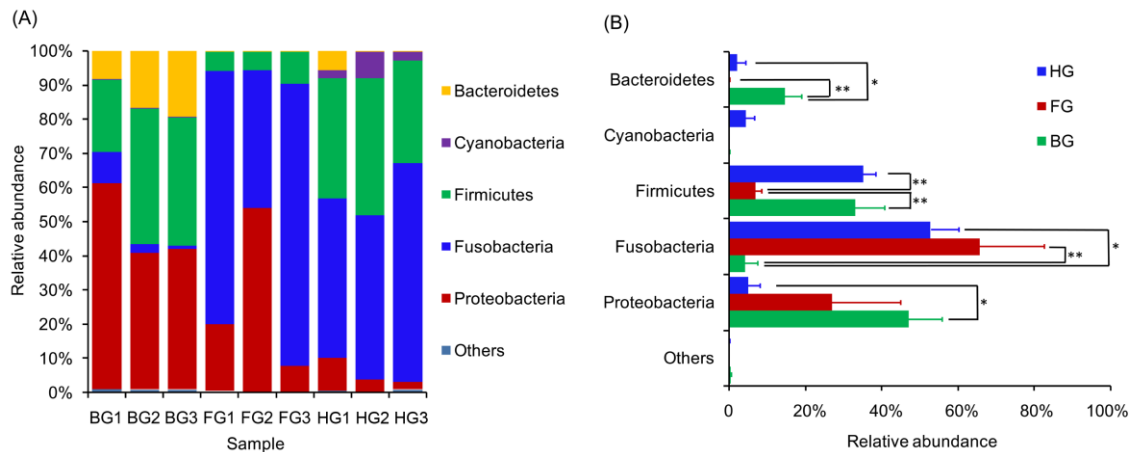


Figure 2. Phylum compositions of the gut microbiota in grass carp fed with different feeds. (A) The relative abundance of each bacterial phylum in each hindgut microbiota of grass carp fed with different feeds. (B) Significant differences in phylum abundances were found between the gut microbiota of grass carp fed with different feeds. FG, HG, and BG indicate the hindgut microbiota of grass carp fed with formula feed, fresh hybrid giant napier, and marinated broad beans, respectively. * $p < 0.05$, ** $p < 0.01$

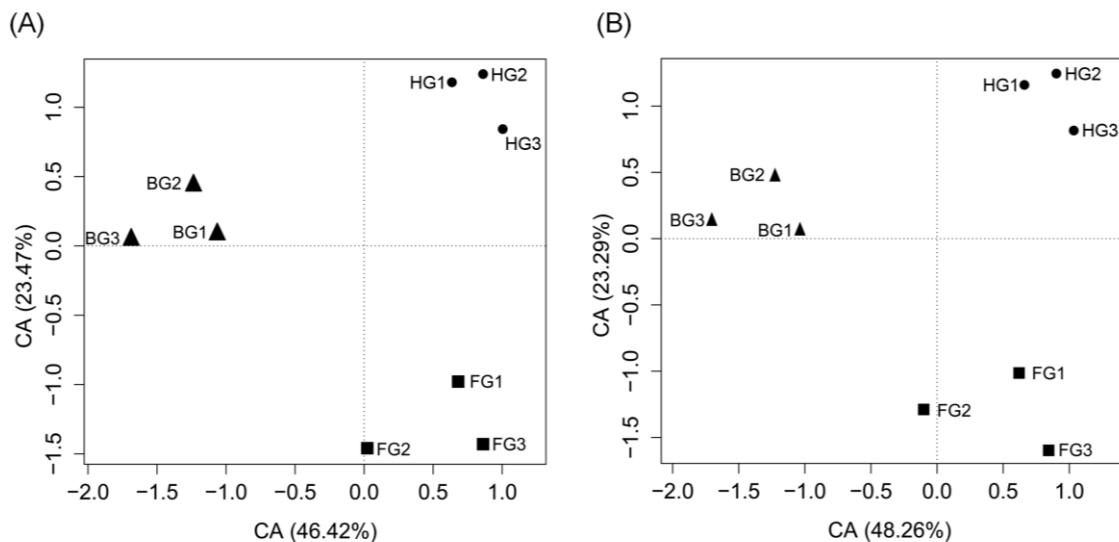


Figure 3. Correspondence analysis (CA) profiles of the gut microbiota genotypes in grass carp fed with different feeds. (A) and (B) show the CA profiles after analysis, based on the OTU and genus compositions, respectively. FG, HG, and BG indicate the hindgut microbiota of grass carp fed with formula feed, fresh hybrid giant napier, and marinated broad beans, respectively

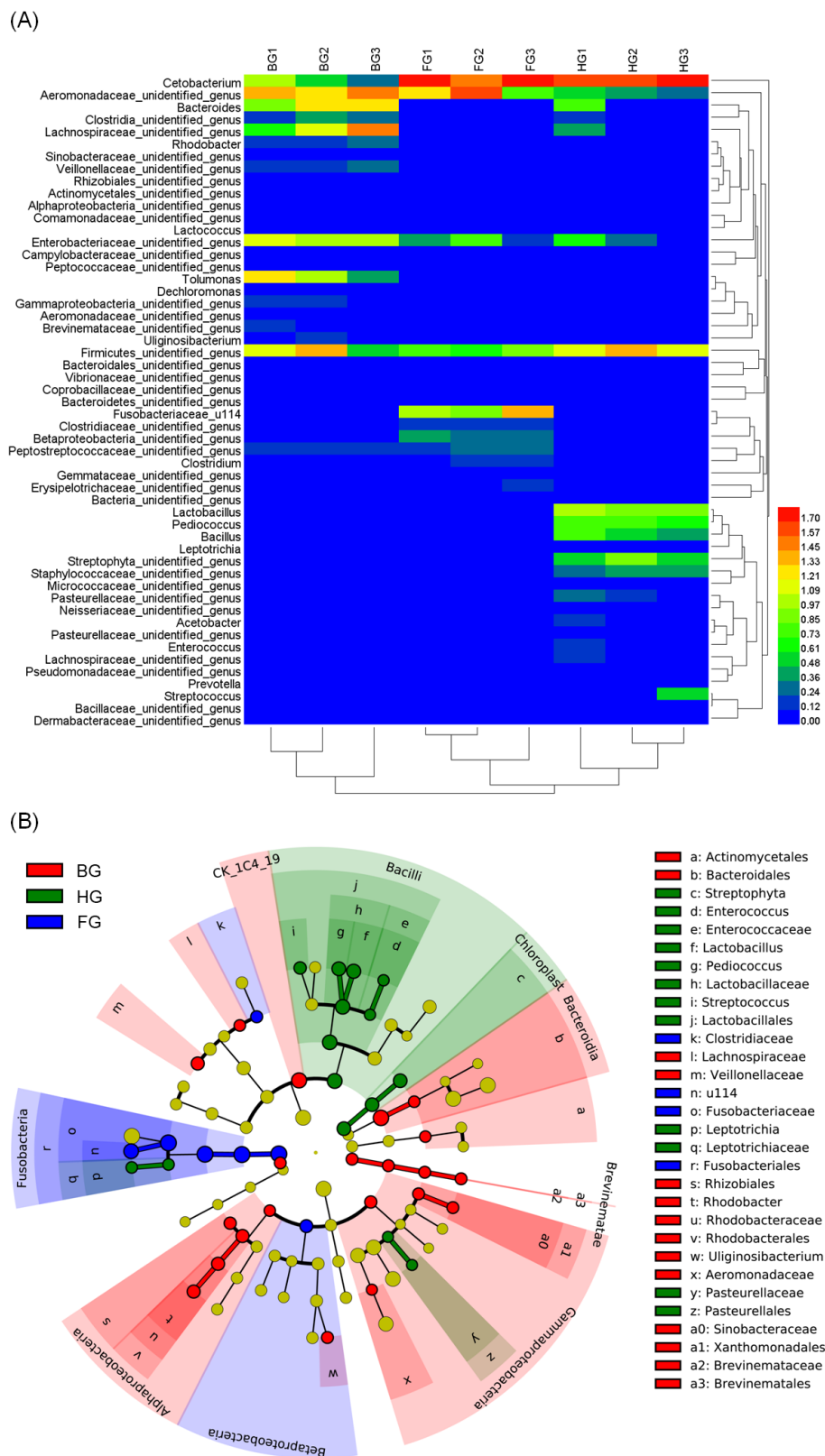


Figure 4. Heatmap (A) and LEfSe (B) profiles showing the relative abundances of significantly different gut microbiota OTUs in grass carp fed with different feeds. FG, HG, and BG indicate the hindgut microbiota of grass carp fed with formula feed, fresh hybrid giant napier, and marinated broad beans, respectively

Discussion

Effects of diet on hindgut SCFA accumulation and the growth of grass carp

Our results showed that changing the diet from natural food to formula feed significantly influenced the accumulation of SCFAs in the hindgut of grass carp (*Table 2*). Two dietary factors influenced SCFA accumulation in the hindgut of grass carp. Firstly, the diet directly supplied SCFAs to the hindgut of grass carp. Although SCFAs are thought to be mainly produced through carbohydrate fermentation by microorganisms, all three feeds had detectable SCFAs in this study (*Table 2*). Secondly, the amounts and species of SCFAs are significantly influenced by carbohydrate substrates (Hao et al., 2017a; Mountfort et al., 1993; Kihara and Sakata, 1997; Probert et al., 2004; Geraylou et al., 2014). Starch is seemingly more easily utilized to produce SCFAs through fermentation by gut microbiota in many fish (Kihara and Sakata, 1997; Van, 1994; Leenhouders et al., 2007). This could explain the increased growth in grass carp fed formula feed compared with those fed with hybrid giant napier or broad beans, as formula feed contains more starch and higher luminal concentrations of SCFAs (*Table 2*).

Proportional model and concentrations of SCFA metabolites in the grass carp hindgut

In mammals and fishes, the SCFA metabolites produced during gut fermentation were mostly acetate, propionate, and butyrate (Clements et al., 1994; Hao et al., 2017b; Bergman, 1990; Smith et al., 1996; Cook and Sellin, 1998), and these metabolites were produced at different ratios. Acetate accumulation was most abundant (~60%), followed by propionate (~25%) and then, to a much lesser degree, by butyrate (~15%) in human intestines (Fukuda et al., 2012). Although the ratios of acetate, propionate, and butyrate fluctuate among different species, the trends of these ratios (acetate > propionate ≥ butyrate) were identical between mammals and fishes (Clements et al., 1994; Hao et al., 2017b; Bergman, 1990; Smith et al., 1996), with few exceptions in marine fishes (Kandel et al., 1994). Mountfort et al. (2002) reported the ratio of their concentrations in three species of marine fish as ranging from approximately 6.4: 3.2: 1 to 28.8: 9.8: 1, on average. In this study, the ratio of these concentrations was approximately 14.3: 2.5: 1 (*Table 2*). Therefore, changes in the acetate concentration primarily influenced the concentration of total SCFAs.

We found that the SCFA concentration in the grass carp hindgut ranged from 0.35 to 2.70 mmol/kg, which is similar to findings reported by Hao et al. (2017a), but were lower than those reported by Paris et al. (Paris et al., 1977). Clements et al. (2014) compared the SCFA concentrations in the hindgut contents of 18 fishes. The SCFA concentrations ranged from 1.70 to 38.00 mM. In addition, the SCFA concentrations in hindgut contents of freshwater herbivore fish were commonly lower than in other fishes (Clements et al., 2014).

Effect of dietary crude fiber contents on the SCFA increment of the grass carp hindgut microbiota

Gut microbiota ferment dietary cellulose to SCFAs as energy sources and signal-transduction molecules in humans and mammals (Spiljar et al., 2017; Postler and Ghosh, 2017; Bindels et al., 2012; Hullar and Fu, 2014). Data from previous studies

also indicated that the gut microbiota in herbivorous fish are capable of cellulose fermentation (Hao et al., 2017a, b; Ni et al., 2014a). Hao et al. (2017a) studied the influence of diets on the SCFA concentrations in the grass carp hindgut. Although they found that diets influenced SCFA concentrations in the hindgut, the influence of dietary SCFA concentrations was not demonstrated. The association between SCFA concentrations and the dietary cellulose contents was also not analyzed. However, the intestinal SCFA concentrations were clearly influenced by the dietary contents, as also shown in this study. Here, we used the ratios of intestinal SCFA concentrations to dietary SCFA concentrations to (i) exclude the influence of dietary SCFA concentrations on intestinal SCFA concentrations and to (ii) determine the SCFA increment. We found that the SCFA increment associated positively with the dietary cellulose contents in the three diets. The acetate increment, which represented the largest proportion of SCFAs, was highly positively associated with the dietary cellulose contents, but the increments of propionate and butyrate associated negatively with the dietary cellulose contents (*Fig. 1*). These results implied that acetate was probably produced in the hindgut of grass carp through cellulose fermentation, and the produced rates were faster than hindgut-absorption rates in the HG and FG, as the ratios were more than 1. However, most propionate and butyrate were probably not produced through cellulose fermentation, and their production and absorption in the hindgut were probably influenced by other undetected factors.

Effect of dietary cellulose contents on the structure of grass carp hindgut microbiota

In our study, we verified that the diet significantly changed the hindgut microbiota of grass carp, as reported previously (Ni et al., 2014b; Hao et al., 2017a). Changes in the hindgut microbiota of grass carp fed different diets also emerged at the phylum, genus, and OTU levels (*Figs. 2–4*). Similar to previous reports in grass carp and other fish (Hao et al., 2017b; Ni et al., 2014b; Miyake et al., 2015; Zhang et al., 2017), we found that Fusobacteria, Proteobacteria, Firmicutes, Bacteroidetes, and Cyanobacteria were the dominant phyla in the gut microbiota of the grass carp. However, their relative abundances acutely fluctuated between individuals fed different feeds or living in different habitats. Proteobacteria species dominate the intestines of many fish species, with differences found in the resident microbes (Zhang et al., 2017; Xia et al., 2014; Piazzon et al., 2017). Overgrowth of Proteobacteria was commonly associated with gut microbial dysbiosis and over-accumulation of energy in mammals. For instance, in Western-style diets, which are low in fiber, decreased beneficial Bacteroidetes and increased Proteobacteria levels are found, compared with those in a high-fiber diet (Geraylou et al., 2013). Dulski et al. (2018) speculated that Proteobacteria inhabiting the gut microbiota at an early stage of life are a necessary component of the pikeperch (*Sander lucioperca*) microbiome that may support proper nutrition of the fish. Fusobacteria species also dominated the intestines of grass carp in this study, as observed in other studies (Xia et al., 2014). However, the relative abundance of Fusobacteria species showed large fluctuations among different individuals under different conditions (Xia et al., 2014; Piazzon et al., 2017). Fusobacteria has been found to produce butyric acid as a major product of fermentation (Anand et al., 2016), and some species have been associated with diseases in mammals (Bennett and Eley, 1993).

CA of the 16S rRNA gene-sequencing results also revealed that gut bacterial communities from grass carp fed different diets formed different clusters (*Fig. 3A and B*). The *Pediococcus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, and *Leptotrichia*

genus biomarkers increased in the HG group (Fig. 4B). They were mostly represented by the Bacilli class, which contains many SCFA-producing bacteria, such as *Pediococcus* and *Lactobacillus* (Leblanc et al., 2017; Johansson et al., 1998; Li et al., 2016b, 2009; Dar et al., 2015).

Fermentation of cellulose by the gut microbiota is a major source of SCFA production (Leblanc et al., 2017; Kasubuchi et al., 2015; Flint et al., 2015). The grass carp is basically herbivorous (Ni et al., 2014a; Wu et al., 2012), and its cellulose-degrading capability is important for improving the utilization rate of plant polysaccharides and the production of SCFAs (Li et al., 2016a). In this study, most bacteria showing changes in abundance were related to SCFA production. For instance, the relative abundances of *Cetobacterium* in the HG and FG groups were obviously higher than that in the BG group. Tsuchiya et al. (2008) reported that the *Cetobacterium* abundance positively associated with acetic and propionic acid production through fermentation. The ratios of the SCFA concentrations in the HG and FG hindgut contents of grass carp to those in the feed were also obviously higher.

The ability of both lactobacilli and *Pediococcus* to produce SCFAs is well documented (Leblanc et al., 2017; Zhang et al., 2017). For instance, Geraylou et al. (2013) found that arabinoxylan-derived oligosaccharides could stimulate the growth of lactic acid bacteria and *Clostridium* sp. in the sturgeon gut and enhance the SCFA levels in the hindgut of sturgeon. Within 24 h, supplementation of *Lactobacillus salivarius* JCM 1230 and *Lactobacillus agilis* JCM 1048 in a simulated chicken cecum significantly increased propionate and butyrate formation (Meimandipour et al., 2010). Moreover, *L. acidophilus* CRL 1014 was also reported to increase the SCFA concentrations in a simulated human microbial ecosystem (Sivieri et al., 2013). Recently, *Lactobacillus rhamnosus* GG was included in a study with a mix of probiotic strains and prebiotics, where it could metabolize these prebiotics, leading to SCFA production (Leblanc et al., 2017). In our study, the relative abundances of *Lactobacillus* in the HG group were obviously higher compared to the other groups (Fig. 4A), as was the SCFA-production capability (Fig. 1A). Bacterial strains of *Pediococcus* have been widely reported as SCFA-producing bacteria (Dineshkumar, and Renu, 2008; Taheri et al., 2015). For instance, Iehata et al. (2010) reported *Pediococcus* sp. Ab1 treatment could improve the gut environment of the abalone, *Haliotis gigantea*. In our study, the relative abundances of *Pediococcus* in the HG group were obviously higher those in the other groups (Fig. 4A). Bacterial strains of *Bacillus* and *Bacteroides* were also reported to cause cell lysis and to be capable of SCFA and branched-chain short fatty acid biosynthesis (Kaneda, 1963; Mayhew et al., 1975; Dreher et al., 1976; Tsuchido et al., 1985; Pogribna et al., 2010; Ray et al., 2012; Hong et al., 2017). For instance, Li et al. (2016a) isolated some cellulolytic *Bacillus* strains from the gut contents of grass carp. Ray et al. (2012) reported that many *Bacillus* sp. could produce cellulase. Bacilli is the primary producers of butyrate (Flint et al., 2015; Kaoutari et al., 2013; Levy et al., 2016). Our results showed that the elevated genera in the HG group were mostly represented by Bacilli (Fig. 4B). These results implied that cellulolytic bacteria would be enriched with high-cellulose substrates in the hindgut of grass carp. In addition, SCFAs were also indirectly produced from other intermediates that were produced by polysaccharide fermentation. For instance, lactate, which is produced by many members of the human gut microbiome, can serve as a substrate for the bacterial production of propionate (e.g., *Coprococcus catus*) and butyrate (e.g., *Eubacterium hallii* and *Anaerostipes caccae*) (Duncan et al., 2004; Reichardt et al., 2014). These findings imply

that bacteria related to the SCFA concentration may not directly produce SCFAs, although they can increase the SCFA concentrations in the gut.

Conclusions

We conducted the first quantitative analysis of the association between SCFA accumulation and the dietary cellulose contents in this study. We found that the HG, which contained the highest dietary cellulose, exhibited the highest SCFA increase, even though the absolute concentration of SCFAs was higher in the FG, which contained more easily fermentable polysaccharides. Moreover, SCFA-producing bacteria, such as *Lactobacillus*, *Pediococcus*, and *Bacillus*, were significantly enhanced in the hindgut microbiota of the HG group. These results implied that the dietary cellulose content influenced the production of SCFAs in the grass carp hindgut by regulating the relative abundances of gut bacteria related to SCFA production. In addition, our results implied that adding appropriate proportion of cellulose or grass meal to grass carp feed could help to regulate the intestinal microbiota, and thereby increasing the content of SCFAs and increasing the growth and health of grass carp. However, the regulating mechanism of intestinal microbiota of grass carp to the productions of SCFAs needed to further study. These results would provide important reference for the design of grass carp feed formulation, and expand our understanding of the relationship between intestinal microbiota and grass carp metabolism.

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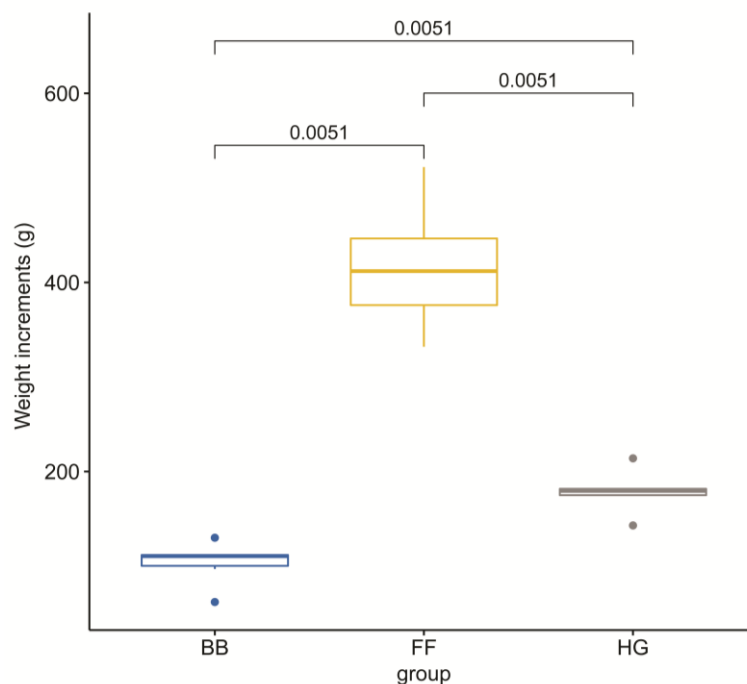
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APPENDIX

Appendix 1. Weight increments of grass carp feed two months of hybrid giant napier (HG), formula feed (FF), or marinated broad beans (BB), respectively



Appendix 2. Sequencing information of samples for microbiota analysis. FG, HG, and BG indicate the hindgut microbiota of the grass carp fed formula feed, hybrid giant napier, and marinated broad beans, respectively

Sample ID	Raw sequence	Clean sequence	Effective sequence	Average length (nt)	Q30	OUT number	Chao1 index	Shannon index
BG1	55,159	55,115	52,782	253	97.13	277	418.973	3.533
BG2	54,108	54,075	50,859	253	97.13	291	350.381	3.57
BG3	63,421	63,375	60,402	253	97	310	415	3.208
HG1	62,086	60,662	57,635	253	96.98	209	246.561	3.366
HG2	60,888	58,019	55,007	253	96.71	126	165	2.704
HG3	59,094	57,268	55,248	252	96.88	198	265.097	2.408
FG1	34,651	34,636	32,466	252	97.01	99	118.333	1.873
FG2	43,641	43,617	39,371	253	97.04	93	118.87	2.071
FG3	51,364	51,322	47,390	252	97.12	103	146.938	2.025

Appendix 3. OTU rarefaction curves of the samples. FG, HG, and BG indicate the hindgut microbiota of the grass carp fed formula feed, hybrid giant napier, and marinated broad beans, respectively

