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Effect of different fishmeal levels in diets on growth performance, tissue morphology, intestinal microflora, and muscle volatile compounds of grass carp

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Introduction: Fishmeal is beneficial to the growth performance of grass carp. However, it remains unclear whether fishmeal can enhance the flavor of muscle, and contribute to the regulatory mechanisms involved in its interaction with the intestinal microbiome.

Methods: In this study, grass carp (70.01 ± 5.34 g) were fed with diets containing 0% (FL), 3% (FM), and 6% (FH) fishmeal for 60 days. The growth performance, intestinal and muscle histomorphology, intestinal microflora, and muscle volatile compounds (VOCs) were evaluated.

Results: The results showed that compared with the FL group, the final body weight (FBW), weight gain rate (WGR), specific growth rate (SGR), and intestinal villus height of grass carp in the FM and FH groups were significantly increased. Compared with the FL and FM groups, the muscle fiber density in the FH group was significantly increased, and the muscle fiber diameter was significantly reduced. Microbial data analysis found that the three treatment groups were well separated in the NMDS plot, and different levels of fishmeal had a significant impact on microbial community composition (Stress = 0.084). In the LefSe analysis (LDA value > 3.0), *Actinobacteria* in the FL group, *Petrimonas* in the FM group, *Bacteroides paurosaccharolyticus*, and *Erysipelatostridiaceae* in the FH group all showed significant responses. The intestinal microbiota composition was closer in the FM and FH groups compared with the FL group. GC-IMS analysis indicated that 53 VOCs were detected in the muscle. The FL group had fewer VOCs, and most of the VOCs had lower contents than those in the FH group. Spearman correlation analysis showed that aldehydes were significantly positively correlated with *Corynebacterium*, *Bacteroides*, *Cetobacterium*, *Erysipelatoclostridium*, *Aeromonas*, *Shewanella*, and *Vibrio*.

Discussion: Based on the evaluation of growth performance, intestinal morphology, and microflora, the diet containing 3% fishmeal is suitable. However, the muscle fiber characteristics and VOCs profile of grass carp fed with 6% fishmeal diet is superior to that of the 3% fishmeal group.

KEYWORDS

fish meal, grass carp, growth performance, intestinal microflora, volatile compounds

1 Introduction

The grass carp (*Ctenopharyngodon idellus*) belongs to the carp and minnow family (*Cyprinidae*) (Cudmore and Mandrak, 2004) and is the most productive freshwater fish species in aquaculture in China. It is reported that the annual production of grass carp exceeded 5.94 million tons in 2023 (China Fishery Statistical Yearbook, 2024). In its natural habitat, grass carp primarily feeds on riparian and aquatic plants (Zhang et al., 2022). Plant proteins such as soybean meal, rapeseed meal, and cottonseed meal are widely used in grass carp feed. However, the anti-nutritional factors in rapeseed meal and cottonseed meal have negative impacts on the health of grass carp, such as reducing growth performance and impairing intestinal function (Choi et al., 2020; Han et al., 2024). The addition of an appropriate level of fishmeal has been proven to enhance the growth performance of grass carp (Peng et al., 2020). Fishmeal is a valuable source of animal protein and is widely used in aquaculture feed due to its balanced amino acid profile, high crude protein content, and good digestibility. The inclusion of fishmeal in feed has been shown to promote grass carp growth by improving feed conversion efficiency, increasing palatability, and facilitating the digestion and absorption of nutrients (Hodar et al., 2020). Nonetheless, the impact of increased levels of fishmeal in the feed on muscle flavor in farmed grass carp remains unclear.

The interaction between the intestine and the endogenous microbiota contributes to the growth, maintaining intestinal homeostasis, enhancing immunity, and disease resistance in aquatic animals (Nayak, 2010). The intestinal microbiota of fish is involved in the synthesis of vitamins, digestive enzymes, and metabolic products (Rimoldi et al., 2018). The absence of probiotics such as *Lactobacillus* (Aghamohammad et al., 2022) and *Bacteroides* may lead to the host suffering from inflammatory bowel disease (Zhang et al., 2024). Dietary factors have been proven to affect the composition of the gut microbiota (Nayak, 2010; Wu et al., 2012). Ringø et al. observed that the dominance of *Gram-positive bacteria* belonging to *Brochothrix* and *Carnobacterium* species in the gastrointestinal tract of Atlantic cod (*Gadus morhua* L.) fed with fishmeal, while *Chryseobacterium* spp, *Psychrobacter glacincola* and *Gram-positive bacteria* belonging to *Carnobacterium* dominated the gastrointestinal tract when fed with soybean meal. In the context of grass carp farming, replacing fishmeal with rapeseed meal has been found to decreased the relative abundance of *Proteobacteria* while increased the relative abundance of *Firmicutes* and *Actinobacteria* in the fish intestine, potentially causing enteritis and inhibiting growth (Ringø et al., 2006). Replacing fishmeal with rapeseed meal reduces the relative abundance of *Proteobacteria* in the gut of grass carp, while increasing the relative abundance of *Firmicutes* and *Actinobacteria*, and may lead to enteritis and growth inhibition (Yao et al., 2019).

Volatile compounds (VOCs) play a key role in determining the aroma of fish products (Mu et al., 2017). Dietary components, such as fishmeal and meat meal, have been proven to affect the composition of VOCs in fish muscle, thereby influencing the overall flavor profile of the meat (Williams et al., 2003). For

instance, a low-fishmeal diet has been shown to reduce the content of certain categories of VOCs in the muscle tissue of large yellow croaker (*Larimichthys crocea*) (Mu et al., 2017). Gas chromatography-ion mobility spectrometry (GC-IMS) leverages the separation characteristics of GC, the advantages of fast response and high sensitivity of IMS, and is capable of detecting a large number of compounds with different chemical groups (Zhang et al., 2020). The application of GC-IMS enables more complex analysis of VOCs in fish products (Zhang et al., 2020). Moreover, GC-IMS requires a lower injection temperature, which can retain the original flavor information of the sample, making the analytical results more authentic and convincing (Lin et al., 2022). In this study, GC-IMS was employed to investigate the effects of fishmeal levels on the variations of VOCs in grass carp muscle and to establish distinct flavor profiles.

Typically, fishmeal is included at a 3% level in grass carp feeds. Building on this, we therefore investigated the effects of zero supplementation and excessive supplementation on muscle flavor and the intestinal microbiota of grass carp through application of GC-IMS to analyze VOCs profiles, and high-throughput sequencing (HTS) in grass carp. Ultimately, the central question is not merely how much fishmeal can be omitted or added, but how these low-, standard- and high-fishmeal treatments will translate into consumer acceptance. By deliberately designing this gradient, we seek to pinpoint the exact formulation that best elevates flesh texture, flavour and overall sensory appeal—turning grass carp from a routine staple into a product that shoppers actively reach for. Additionally, a Spearman correlation analysis was conducted to investigate the relationship between the predominant microbiome and VOCs.

2 Materials and methods

2.1 Experiment diets

The experiment utilized soybean meal, cottonseed meal, and rapeseed meal as primary protein sources, soybean oil and fish oil as primary lipid sources. The composition and formulation of the experimental diets are detailed in Table 1. The feed fishmeal-free (FL) is used as the basal diet. Experimental diets are prepared by adding 3% (medium level) fishmeal (referred to as FM) and 6% (high level) fishmeal (referred to as FH) to the basal diet. All diets ingredients were ground and filtered through a 40-mesh screen, and then pelletized using a twin screw extruder (TSE65, Modern Yanggong Machinery Technology Development Co., Ltd, Beijing, China) (2 mm × 2 mm). The pellets are subsequently dried in a dryer at 60°C and stored in sealed bags at room temperature until feeding.

2.2 Experimental fish and feeding trial

The experiment was carried out in a fish farm in Xidongting district (Changde, Hunan, China). Grass carps were obtained from

TABLE 1 Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients (%)	Treatments		
	FL	FM	FH
Fish meal	0.00	3.00	6.00
Soybean meal	24.00	24.00	24.00
Cottonseed meal	14.00	14.00	14.00
Rapeseed meal	35.00	29.00	25.00
DL-Met (98.5%)	0.05	0.05	0.05
Wheat flour	17.9	20.90	21.9
Ca (H ₂ PO ₄) ₂	2.00	2.00	2.00
Fish oil	2.50	2.50	2.50
Soybean oil	1.25	1.25	1.25
Vitamin premix ^a	1.00	1.00	1.00
Mineral premix ^a	2.00	2.00	2.00
Choline chloride ^b	0.25	0.25	0.25
Ethoxyquin ^b	0.05	0.05	0.05
Total	100	100	100
Nutrient contents			
Crude protein ^c	37.58	37.72	37.89
Crude lipid ^c	4.92	5.12	5.04
Water content ^d ,%	10	10	10
Crude fiber ^d ,%	7.7	7.6	7.5
Ash ^d ,%	9.2	9.1	9.0
Nitrogen-free extract ^d ,%	34.9	35.4	35.9
Digestible energy ^d , MJ/kg	12.8	13.0	13.0

^aVitamin premix (g/kg): retinyl acetate (500,000 IU g⁻¹), 0.386; cholecalciferol (500,000 IU g⁻¹), 0.40; D, L- α -tocopherol acetate (50%), 23.23; menadione (22.9%), 0.83; cyanocobalamin (1%), 0.94; D-biotin (2%), 0.75; folic acid (95%), 0.42; thiamine nitrate (98%), 0.09; ascorhyl acetate (95%), 9.77; niacin (99%), 4.04; meso-inositol (98%), 19.39; calcium-D-pantothenate (98%), 3.85; riboflavin (80%), 0.73; pyridoxine hydrochloride (98%), 0.62. All ingredients were diluted with maize starch to 1 kg; Mineral premix (g/kg): MnSO₄·H₂O (31.8% Mn), 2.6590; MgSO₄·H₂O (15.0% Mg), 200.0000; FeSO₄·H₂O (30.0% Fe), 12.2500; ZnSO₄·H₂O (34.5% Zn), 8.2460; CuSO₄·5H₂O (25.0% Cu), 0.9560; KI (76.9% I), 0.0650; Na₂SeO₃ (44.7% Se), 0.0168. All ingredients were diluted with maize starch to 1 kg.

^bCholine chloride with a purity of 50%; Ethoxyquin with a purity of 30%.

^cNutrient contents were actual values.

^dNutrient levels were theoretical values.

a commercial farm (Changde, Hunan, China). The grass carp are acclimated in cages for two weeks. The grass carps were subjected to a 24-hour fasting period before the formal experiment. A total of 270 healthy grass carps (average weight 70.01 ± 5.34 g) were selected and randomly divided into nine floating cages (2.0 × 1.5 × 1.5 m). The experiment was divided into three treatment groups, each with three replicates (n = 30). After the experiment begins, the fish are fed three times a day (at 7:00, 12:00, and 17:00). During the 60-day experimental period, the mortality and feeding conditions of the grass carp are recorded daily. Culture unit: 20-hectare earthen pond, average depth 2 m. Water volume: ~400,000 m³. Water

exchange: 10% of pond volume per day via continuous inflow/outflow. Throughout the experiment, water quality parameters are monitored: water temperature ranges from 24.5 to 30.3°C, pH values range from 7.3 to 7.8, and dissolved oxygen levels range from 6.5 to 7.5 mg/L.

2.3 Sample collection

At the end of the experiment, grass carp were fasted for 8 h. Then, three fish were randomly selected from each cage. Their body weight was measured, and samples of the posterior intestine contents were collected. These samples are rapidly frozen in liquid nitrogen and then stored in a -80°C freezer. The samples from three fish in the same net cage were pooled into one sample for further analysis of the intestinal microbial community. The remained grass carp are then fasted for another 16h, and the total number and body weight in each cage were recorded. Additionally, three fish from each cage were randomly selected and anesthetized, and then the body length, body weight, visceral, and liver weight were measured. The section of posterior intestine (1 cm×1 cm) and the left dorsal muscle samples (1 cm×1 cm×1 cm) were collected for histomorphology analysis. The right dorsal muscle samples were collected. The samples from the three fish in the same cage are mixed into one sample, rapidly frozen in liquid nitrogen, and stored at -80°C, and stored in a -80°C freezer for the subsequent measurement of VOCs.

The growth performance of the grass carp was determined as follows:

$$\text{Weight gain rate (WGR, \%)} = 100 * ((\text{final body weight}) - (\text{initial body weight})) / (\text{initial body weight})$$

$$\text{Special gain rate (SGR)} = 100 * (\text{Ln (final body weight)} - \text{Ln (initial body weight)}) / \text{time (days)}$$

$$\text{Feed conversion rate (FCR)} = \text{feed intake} / ((\text{final body weight}) - (\text{initial body weight}))$$

$$\text{Hepatosomatic index (HSI, \%)} = 100 * (\text{liver weight} / \text{body weight})$$

$$\text{Viscerosomatic index (VSI, \%)} = 100 * (\text{visceral weight} / \text{body weight})$$

$$\text{Condition factor (CF, \%)} = 100 * (\text{body weight} / \text{total length}^3)$$

2.4 Histomorphology analysis

Intestinal and muscle sections are prepared according to the method of T. Tang (Tang et al., 2021). Sections are photographed using a fluorescence microscopy (Olympus, Tokyo, Japan). The complete intestinal villus height and villus width are measured by ImageJ software. The number of intact myofibers in various regions

TABLE 2 Effect of dietary fishmeal on growth performance of grass carp.

Parameters	Treatments		
	FL	FM	FH
IBW (g)	70.09 ± 0.07	70.00 ± 0.32	69.95 ± 0.18
FBW (g)	209.15 ± 7.48 ^a	236.72 ± 10.47 ^b	238.18 ± 11.25 ^b
WGR (%)	198.39 ± 10.94 ^a	238.22 ± 15.87 ^b	240.52 ± 16.96 ^b
SGR (%)	1.73 ± 0.06 ^a	1.93 ± 0.08 ^b	1.94 ± 0.08 ^b
FCR	3.22 ± 0.13	2.85 ± 0.34	2.59 ± 0.32
HSI (%)	1.94 ± 0.16	2.27 ± 0.57	1.83 ± 0.17
VSI (%)	12.20 ± 0.22	13.23 ± 1.09	12.15 ± 0.25
CF (%)	2.10 ± 0.18	2.10 ± 0.12	2.06 ± 0.13

IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate; FCR, feed conversion ratio; HSI, hepatosomatic index; VSI, viscerosomatic index; CF, condition factor. FL: grass carp fed diet without fishmeal; FM: grass carp fed diet with 3% fishmeal; FH: grass carp fed diet with 6% fishmeal. Data are expressed as means ± SD. Value with different superscripts are significantly different ($P < 0.05$, $n \geq 3$).

of the transected muscle is measured, and the fiber density was calculated as the number of fibers per square millimeter (fibers/mm (Zhang et al., 2022)), and the fiber diameter ($\geq 10 \mu\text{m}$) is calculated based on the area = $\pi \times r^2$.

2.5 Intestinal microflora analysis

Total DNA is extracted from intestinal contents using the sodium dodecyl sulfate method according to the manufacturer's instructions. Next-generation HTS is employed to analyze the composition of the gut microbiota. The V4 hypervariable region of the bacterial 16S rRNA gene is amplified using specific primers with barcodes, 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), followed by sequencing on the NovaSeq 6000 platform. Samples are annotated using the SILVA database and the Mothur method. After dereplication of valid tags, singletons are removed based on abundance, and then clustering at 97% similarity. Weighted UniFrac distance is used to calculate the sample distance matrix to investigate similarities among different samples. The UPGMA clustering tree is generated using the QIIME software (version 1.9.1). A species abundance clustering heatmap (top 30) is created based on species annotation and abundance information at the genus level using R software (version 3.1.0). Non-Metric Multi-Dimensional Scaling (NMDS) analysis is conducted using the vegan package in R software. LEfSe (LDA Effect Size) analysis is performed using LEfSe software (version 1.0) to identify species with significant differences among groups (Wen et al., 2021).

Sequencing and routine data analysis were conducted by Novogen Co., Ltd. (Novogen, Beijing, China).

2.6 Muscle gas chromatography-ion mobility spectrometry analysis

VOCs in muscle samples are analyzed using an integrated system composed of an Agilent 490 Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) and IMS instrument (FlavorSpec[®], Gesellschaft für Analytische Sensorsysteme mbH, Dortmund, Germany). The detection method follows the protocol outlined by Zhang Q et al (Granato et al., 2018; Zhang et al., 2020). N-Ketones C4-C9 (Sigma-Aldrich, St. Louis, MO, USA) are used as external references to calculate the retention indices (RI) of aromatic compounds. Aromatic compounds in muscle samples are identified by comparing their RI values with standards in the GC-IMS library. Quantification of each VOC in muscle samples is performed using established calibration curves, with results expressed as the relative content of each VOC relative to the total aroma content ($n=3$) (Song et al., 2021). For downstream analysis, individual VOCs were grouped into chemical classes (aldehydes, alcohols, ketones, etc.). The abundance of each class was calculated as the sum of the peak areas of all compounds belonging to that class.

2.7 Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) 23 software (IBM, Armonk, USA). Data were presented as mean ± standard deviation (SD), the analysis was conducted using one-way ANOVA, with a significance threshold level set at $P < 0.05$. Laboratory Analytical Viewer (LAV, G.A.S., Dortmund, Germany) software and GC-IMS Library Search were used to analyze the aroma profiles "Fingerprint" gallery plots and principal component analysis (PCA) of muscle samples are analyzed using laboratory analytical viewer (LAV, G.A.S., Dortmund, Germany) software and GC-IMS Library Search. In R software, Spearman correlation is used to calculate the correlation between the top 30 bacterial genera in gut microbiota abundance and VOCs with statistical differences.

3 Results and discussion

3.1 Growth performance

The growth performance results of grass carp are shown in Table 2. Compared with the FL group, the FBW, WGR, and SGR of the FM and FH groups are significantly increased ($P < 0.05$). This is similar to the results obtained by Peng, K.-S. et al. regarding the impact of fish meal substitution for soybean meal on the growth performance of grass carp (Peng et al., 2020). Fishmeal is a widely used feed ingredient which contains a higher percentage of protein,

energy, minerals, and vitamins (Cho and Kim, 2011). Fishmeal applied with other animal and vegetable proteins in the diet had synergistic effects which could balance the amino acid composition, promote fast growth of fish, and reduce feeding costs (Cho and Kim, 2011). Therefore, completely or partially replacing plant protein with fishmeal can positively impact the growth performance of grass carp.

This experiment adopts a stepwise feed formulation: fishmeal increases from 0% to 6% while rapeseed meal decreases from 35% to 25%. Rapeseed meal contains several known antinutritional factors—such as glucosinolates, tannins, and phytates—that suppress fish growth and disturb the gut microbiota. Hence, part of the observed improvements in growth performance and gut microbiota cannot be ruled out as arising from the reduction of rapeseed meal rather than from the increase in fishmeal, and the overall feed effect may concurrently benefit from the lower rapeseed meal level.

Ethoxyquin was included at 0.05% of the diet as a necessary antioxidant to ensure feed stability during storage. Ethoxyquin was added only as an antioxidant. However, published data indicate that even this level can transiently suppress respiratory burst and lysozyme activity in tilapia (Yamashita et al., 2009) and accumulate as dimer residues in salmonid tissues (Ørnsrud et al., 2011). Such mild immunomodulation could have dampened diet-related inflammatory responses, although growth performance remained unaffected (Wang et al., 2015). At current ingredient prices, the 6% formulation increases feed cost by approximately 8%–10% per tonne compared with the 3% formulation. Therefore, based on the effect–cost trade-off, 3% fishmeal represents the economically optimal recommendation; a 6% fishmeal formulation is considered only if the market offers a premium for “flavour” that exceeds 10%. Subsequent studies quantify the actual revenue gain from this flavour improvement through consumer sensory tests and economic modelling to confirm the strategy’s feasibility.

3.2 Tissue morphology

The morphological results of grass carp intestines and muscles are shown in Figures 1A and 1B. The results in Figure 1C indicate that, compared with the FL group, the intestinal villus height of the FM and FH groups is significantly increased ($p < 0.05$). The intestine is the primary site for the digestion and absorption of nutrients, and the height of intestinal villi is positively correlated with nutrient absorption (Zhu et al., 2012). Therefore, the addition of 3% and 6% fishmeal may enhance the intestinal digestive and absorptive capacity of grass carp, thereby promoting their growth, by increasing the height of intestinal villi to expand the intestinal absorptive surface area. The results in Figures 1E and 1F show that, compared to the FL and FM group, the fiber diameter of the FH group significantly decreased while the fiber density significantly increased ($p < 0.05$). Changes in muscle fiber size are achieved through the balance between protein synthesis and degradation (Yu et al., 2017). Nutritional status is mirrored in the muscle fibers’ characteristics, including their size, shape, and classification

(Rodriguez-Torres et al., 2020). An increase in fiber density and a decrease in fiber diameter can improve meat tenderness (Kopač and Ručigaj, 2024). Therefore, a fishmeal level of 6% may elevate the quality of grass carp by improving the morphology of muscle fibers.

3.3 Intestinal microflora

The sequencing result of the intestinal microbiome is shown in Figure 2. The results of the UPGMA phylogenetic tree analysis indicate that *Firmicutes*, *Actinobacteria*, *Bacteroidota*, and *Fusobacteriota* were the predominant bacteria at the phylum and species level in all groups (Figures 2A, B). This is consistent with the findings of Wu, S. and Zhang, X. et al (Wu et al., 2012; Zhang et al., 2014). Furthermore, the composition of bacterial phyla in the FM and FH groups was closer to each other than to the FL group. Intestinal *Firmicutes* possess genes responsible for fermenting dietary fiber and interact with the intestinal mucosa to help maintain host homeostasis (Sun et al., 2023). *Actinobacteria*, especially *Bifidobacteria*, are crucial for gut homeostasis and widely used as probiotic with beneficial effects in various pathological conditions (Binda et al., 2018). *Bacteroidetes* play an important role in gut health by producing butyrate, which is the end product of colonic fermentation and is considered to have antineoplastic properties (Kim and Milner, 2007). *Fusobacteria* activate the host’s inflammatory response to prevent pathogens from promoting tumor growth (Kelly et al., 2018). Dysbiosis, characterized by an imbalance in the taxonomic composition, often manifests as an increase in *Firmicutes* relative to *Bacteroidetes* in metabolic disorders (Shin et al., 2015). Studies have shown that an increase in *Firmicutes* and *Actinobacteria*, and a decrease in *Bacteroidetes* and *Fusobacteria*, are similar to the microbial patterns of diseased fish (Tran et al., 2018). Compared with the FM and FH groups, we observed this disease-like microbial pattern in the FL group. The gut microbiota may indirectly affect intestinal inflammatory pathways and the synthesis and catabolism of nutrients (Przewłócka et al., 2020). There are complex interactions among different groups within the gut microbial community, including competition and symbiosis (Przewłócka et al., 2020). Changes in fishmeal levels may disrupt the balance of these interactions, leading to changes in the relative abundance of certain groups. In the absence of fish meal, certain microbial populations may become more prominent. For example, *Photobacterium* was highly represented in gilthead seabream (*Sparus aurata*) fed with a fishmeal-free diet (Kelly et al., 2018). Some studies have also found that when fishmeal is used to replace cottonseed protein, the proportion of the relative abundance of the phylum *Proteobacteria*, which encompasses both pathogenic and beneficial symbionts in the gut of juvenile golden pompano (*Trachinotus ovatus*) gradually decreased with the increase in fish meal levels (Thomas et al., 2011). *Proteobacteria* is a metabolically diverse phylum that includes not only opportunistic pathogens but also beneficial commensals such as *Vibrio alginolyticus*, which can act as a probiotic and reduce mortalities caused by *Aeromonas* and *Vibrio* spp (Spring et al., 1995). Similarly, with the increasement of

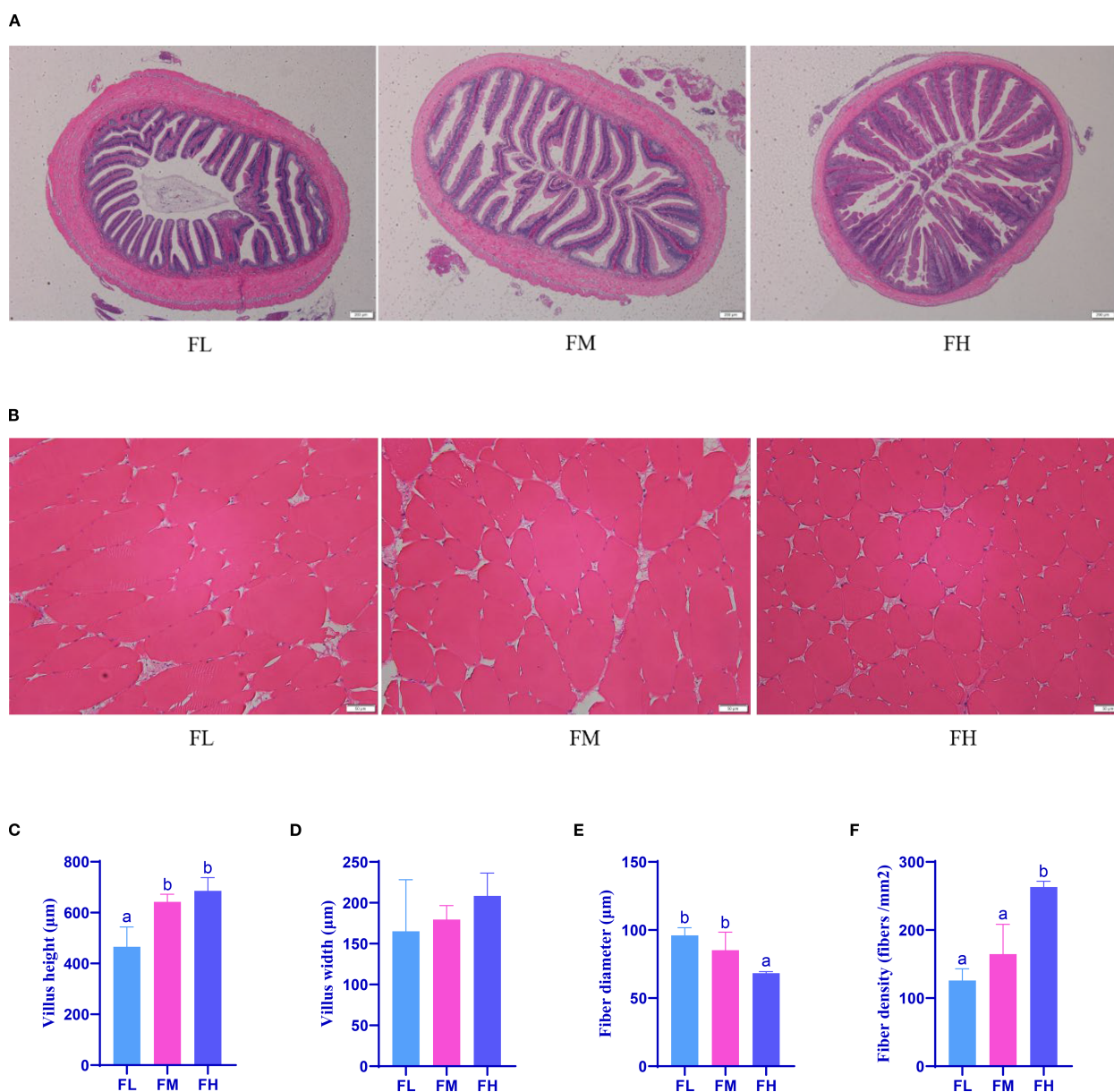


FIGURE 1

Effect of dietary fishmeal on tissue morphology of grass carp. Note: (A) Images of hematoxylin and eosin-stained sections of the posterior intestines of grass carp. (B) Images of hematoxylin and eosin-stained sections of the dorsal muscle of grass carp. (C) Effect of dietary fishmeal on grass carp villus height. (D) Effect of dietary fishmeal on grass carp villus width. (E) Effect of dietary fishmeal on grass carp dorsal muscle fiber diameter. (F) Effect of dietary fishmeal on grass carp dorsal muscle fiber density. FL: grass carp fed diet without fishmeal; FM: grass carp fed diet with 3% fishmeal; FH: grass carp fed diet with 6% fishmeal. Value with different superscripts are significantly different ($P < 0.05$, $n \geq 3$).

fishmeal levels, probiotic bacteria (*Lactococcus*) increase while pathogenic bacteria (*Enterococcus*) decrease in the common carp (*Cyprinus carpio*) intestine (Shin et al., 2015). Therefore, increasing fishmeal levels can balance the gut microbiota and maintain intestinal health.

The clustering heatmap based on the top 30 bacterial genera with relative abundance is shown in Figure 2C. ANOSIM analysis of dietary fishmeal on intestinal microflora of grass carp: FL vs. FM and FM vs. FH were showed in Figure 2D. The FL group is dominated by *Staphylococcus*, *Paenibacillus*, *Lactobacillus*, *Kocuria*, and *Corynebacterium*. In contrast, *Butyrivibrio*,

Acinetobacter, *Rothia*, *Dermaococcus*, *Ralstonia*, *Pseudomonas*, *Alteromonas*, *Brevinema*, *Geobacter*, and *Pseudoalteromonas* are enriched in the FM group. The dominant genera in the FH group include *Dielma*, *Romboutsia*, *Clostridium sensu stricto* 1, *Erysipelatoclostridium*, *Aeromonas*, *Shewanella*, *Anaerostignum*, and *Vibrio*. Recent studies have identified various pathogenic microorganisms (*Staphylococcus* (Surana and Kasper, 2014), *Paenibacillus* (Li et al., 2022), *Acinetobacter* (Joly-Guillou, 2005), *Corynebacterium* (Bernard and Funke, 2015), and *Brevinema* (Paster, 2015)) and beneficial microorganisms (such as *Dermaococcus* (Rangseekaew et al., 2022)) in the intestines of

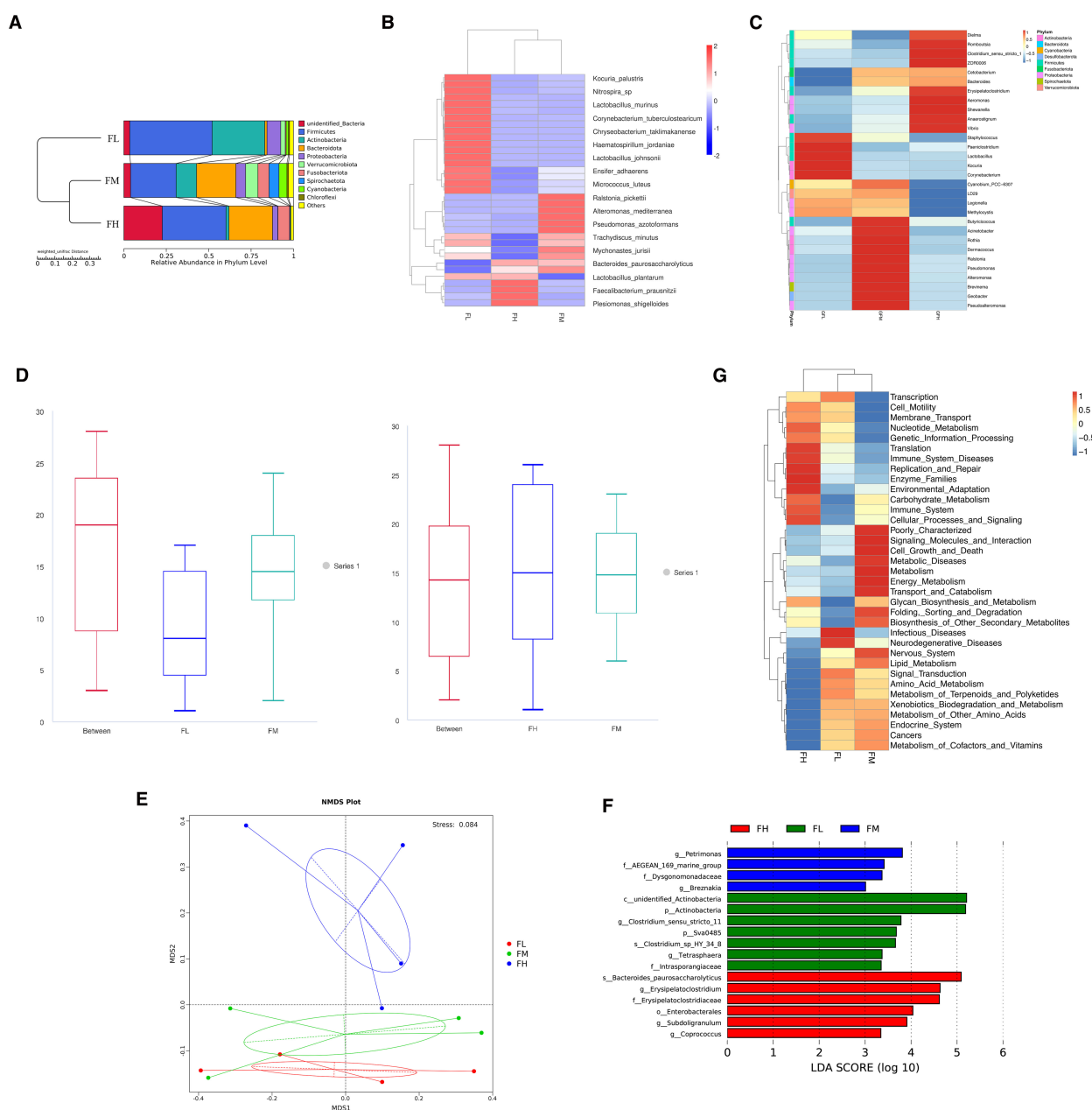


FIGURE 2

Effect of dietary fishmeal on intestinal microflora of grass carp. Note: **(A)** UPGMA clustering tree depicting all intestinal microbiome samples. Only the top 10 most abundant (based on relative abundance) bacterial phyla are displayed in the figures. **(B)** Heatmap of relative abundance (top 10 species) at the species level. **(C)** heatmap of species abundance clustering. Only the top 30 most abundant bacterial genera (based on relative abundance) are shown in the figures. **(D)** ANOSIM analysis of dietary fishmeal on intestinal microflora of grass carp: FL vs. FM (left) and FM vs. FH (right). **(E)** NMDS analysis of dietary fishmeal on intestinal microflora of grass carp. **(F)** LefSe analysis of dietary fishmeal on intestinal microflora of grass carp. **(G)** PICRUSt (level2) analysis of dietary fishmeal on intestinal microflora of grass carp (Top 35). FL: grass carp fed diet without fishmeal; FM: grass carp fed diet with 3% fishmeal; FH: grass carp fed diet with 6% fishmeal.

different species. In our study, the three pathogenic microorganisms *Staphylococcus*, *Paenibacillus*, and *Corynebacterium* accumulate in the FL group. Additionally, the butyrate-producing genera *Butyrivibrio* and *Rothia* are enriched in the FM group. *Pseudomonas* (lipase) (Gong et al., 2021), *Alteromonas* (Ivanova et al., 2005), and *Pseudoalteromonas* (amylase and chitinase) (Parrilli et al., 2021) known for their vigorous metabolic capabilities, are more abundant in the FM group.

Clostridium_sensu_stricto_1 (Gong et al., 2021) and *Romboutsia* (Gerritsen et al., 2019) are important for amino acid utilization in animal protein diets, displaying a range of metabolic capabilities, including carbohydrate utilization, fermentation of single amino acids, anaerobic respiration, and production of metabolic end products. *Clostridium* can induce regulatory T cells in the colonic lamina propria of mice, thereby providing protective effects in mouse models of colitis and allergies (Li et al., 2022). *Clostridium*

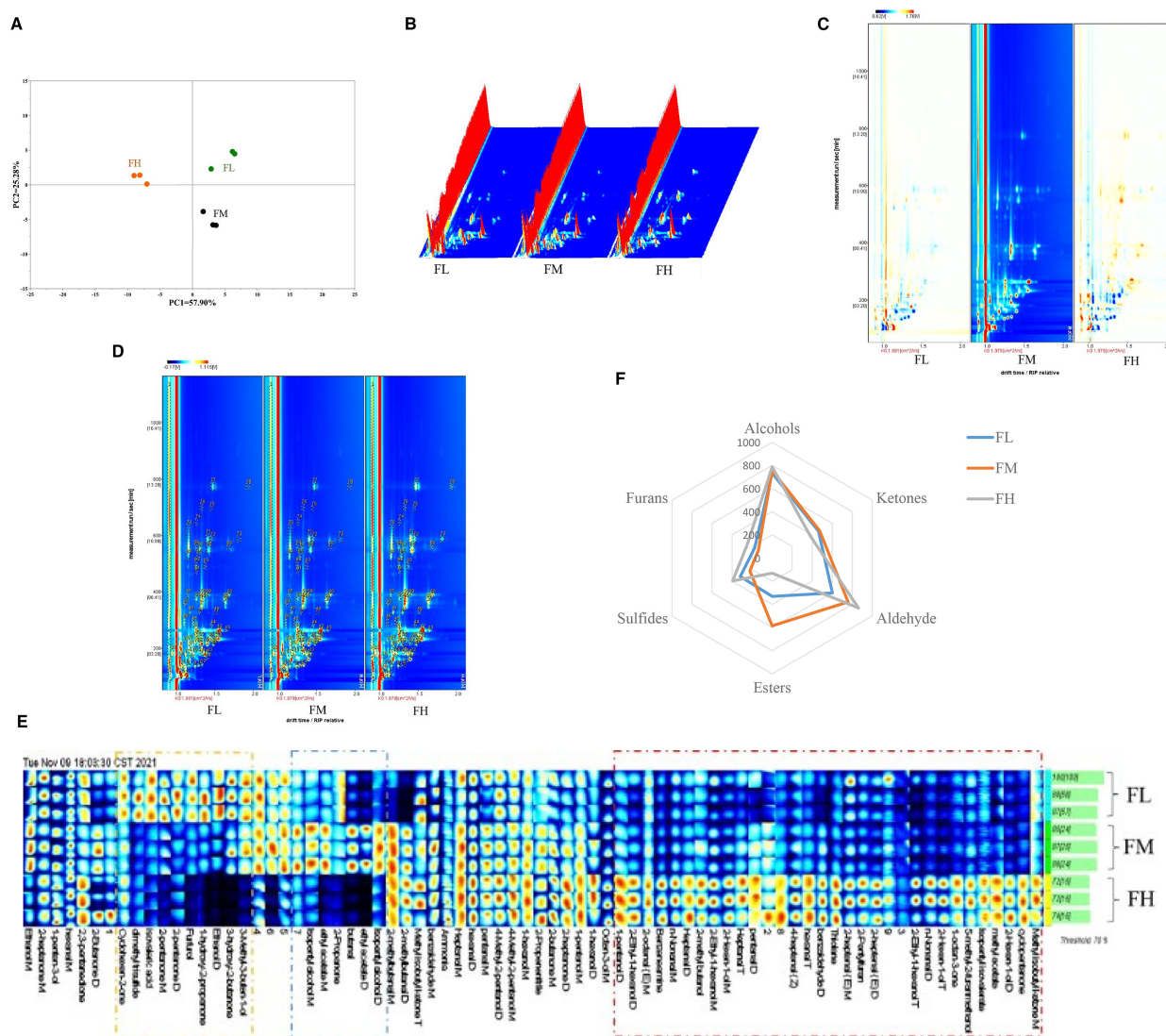


FIGURE 3

Effect of dietary fishmeal on muscle volatile compounds of grass carp. Note: (A) Principal Component Analysis (PCA) of GC-IMS. (B) 3D topographic plots of VOCs in muscle. (C) 2D topographic plots of volatile compounds in muscle; the x-axis and y-axis represent ion-relative drift time and retention time, respectively. The background color of the figure is blue, with a red vertical line at the abscissa 1.0 indicating the reactive ion peak. The entire spectrum represents the total volatile compounds in the samples; signal intensity is shown in different colors, where darker colors indicate higher intensity (white for lower intensity and red for higher intensity). (D) Topographic plots of GC-IMS spectra with identification of individual volatile compounds labeled 1-78 (as listed in Table 3); using FM as the reference, white corresponds to the same concentration of volatiles, with colors closer to red indicating higher concentration (blue corresponds to lower concentration). (E) Gallery plot of grass carp samples with different treatments; each row represents a sample and each column represents a signal peak. (F) Radar map of VOCs with statistically significant differences. VOC categories (aldehydes, alcohols, ketones, etc.) correspond to the summed peak areas of all compounds within each class. FL: grass carp fed diet without fishmeal; FM: grass carp fed diet with 3% fishmeal; FH: grass carp fed diet with 6% fishmeal. Value with different superscripts are significantly different ($n=3$).

and *Romboutsia* can enhance the digestion of cellulose (Wu et al., 2012), and they are enriched in the FH group.

Through non-metric multidimensional scaling (NMDS) analysis, as shown in Figure 2E, we visualized the structure of microbial communities fed with different levels of fish meal. The results indicate that the three treatment groups are well separated in the NMDS plot, with a Stress value of 0.084. This demonstrates that different levels of fish meal significantly affect the composition of microbial communities and that our two-dimensional ordination

plot effectively represents the original distances between samples, ensuring high reliability of the results (Zha et al., 2018).

In the LefSe analysis (Figure 2F), different levels of fish meal exert significant effects on microbial community structure. The LDA value, used to assess the impact of microbial groups that show significant differences between groups, indicates that an LDA value greater than 3 is generally considered to represent biomarkers with high credibility (Ma et al., 2020). In the FL group, *Actinobacteria*, *Clostridium_sensu_stricto_11*, *Sva0485*, *Clostridium_sp_HY_34_8*,

TABLE 3 Identification of muscle volatile compounds based on GC-IMS.

Count	Compound	CAS#	MW	RI	Rt (sec)	Dt (a.u.)	Integral volume		
							FL	FM	FH
1	ethanol M	C64175	46.1	485.7	99.39	1.05	2166 ± 428 ^c	2777 ± 46 ^b	1399 ± 91 ^a
2	ethanol D	C64175	46.1	482	98.39	1.13	1609 ± 206 ^c	644 ± 26 ^b	83 ± 22 ^a
3	ammonia	C7664417	17	499.6	103.24	0.91	42625 ± 1389 ^a	44561 ± 690 ^a	57250 ± 3429 ^b
4	2-propanone	C67641	58.1	510.5	106.34	1.12	1587 ± 102 ^b	1451 ± 50 ^b	421 ± 26 ^a
5	2-propenenitrile	C107131	53.1	510.8	106.44	1.09	593 ± 82 ^a	833 ± 25 ^b	825 ± 76 ^b
6	methyl acetate	C79209	74.1	522.8	109.99	1.04	*	*	1037 ± 73
7	2-butanone M	C78933	72.1	604.1	137.29	1.07	823 ± 120 ^a	923 ± 76 ^{ab}	1133 ± 92 ^b
8	2-butanone D	C78933	72.1	600.7	136.03	1.24	390 ± 181	794 ± 122	469 ± 319
9	1	unidentified	0	602.7	136.77	1.16	287 ± 49	298 ± 37	323 ± 161
10	butanal	C123728	72.1	608.1	138.79	1.29	284 ± 34 ^b	754 ± 57 ^c	90 ± 36 ^a
11	ethyl acetate M	C141786	88.1	619.9	143.31	1.1	331 ± 13 ^b	585 ± 71 ^c	130 ± 27 ^a
12	ethyl acetate D	C141786	88.1	619.2	143.06	1.33	167 ± 15	724 ± 231	*
13	2-methylbutanal M	C96173	86.1	663.7	161.51	1.18	210 ± 89 ^a	668 ± 27 ^c	526 ± 32 ^b
14	2-methylbutanal D	C96173	86.1	663.2	161.3	1.4	79 ± 57 ^a	731 ± 29 ^b	877 ± 36 ^c
16	1-hydroxy-2-propanone	C116096	74.1	684.4	170.88	1.04	1015 ± 141	526 ± 25	*
17	2-pentanone M	C107879	86.1	692.8	175.67	1.12	449 ± 73 ^c	272 ± 8 ^b	127 ± 5 ^a
18	2-pentanone D	C107879	86.1	696.6	178.21	1.37	1220 ± 157	509 ± 95	*
19	2,3-pentanedione	C600146	100.1	698.8	179.67	1.22	377 ± 18 ^b	268 ± 38 ^a	474 ± 39 ^c
20	1-penten-3-ol	C616251	86.1	700.1	180.6	0.94	975 ± 4 ^a	1126 ± 9 ^b	1405 ± 46 ^c
21	2	unidentified	0	701.4	181.46	1.33	*	211 ± 12	223 ± 42
22	pentanal M	C110623	86.1	704.4	183.57	1.18	415 ± 170	596 ± 21	552 ± 54
23	pentanal D	C110623	86.1	705.3	184.16	1.42	97 ± 70	174 ± 13	189 ± 20
24	3	unidentified	0	708.2	186.21	1.30	*	125 ± 6	182 ± 15
25	4	unidentified	0	746.2	215.09	1.16	598 ± 53 ^b	502 ± 54 ^{ab}	489 ± 10 ^a
26	5	unidentified	0	741.9	211.6	1.28	441 ± 37 ^{ab}	500 ± 117 ^b	299 ± 26 ^a
27	3-hydroxy-2-butanone	C513860	88.1	743.5	212.96	1.33	1778 ± 35	1237 ± 90	*
28	methyl isobutyl ketone M	C108101	100.2	744.7	213.87	1.18	110 ± 4 ^a	106 ± 4 ^a	149 ± 12 ^b
29	methyl isobutyl ketone T	C108101	100.2	743.7	213.11	1.48	91 ± 18	83 ± 25	85 ± 23
30	6	unidentified	0	748.4	216.89	1.40	827 ± 81 ^b	944 ± 157 ^c	134 ± 22 ^a
31	isopentyl alcohol M	C123513	88.1	751.6	219.54	1.24	248 ± 32 ^a	440 ± 51 ^b	294 ± 31 ^a
32	isopentyl alcohol D	C123513	88.1	752.3	220.13	1.49	*	152 ± 4	*
33	cyclopentanone	C120923	84.1	757.2	224.25	1.11	*	*	86 ± 17
34	2-methyl butanol	C137326	88.1	763.6	229.84	1.47	159 ± 13 ^b	96 ± 17 ^a	201 ± 10 ^c
35	thiolane	C110010	88.2	765.4	231.37	1.32	267 ± 30 ^a	275 ± 59 ^a	635 ± 17 ^b
36	3-methyl-3-buten-1-ol	C763326	86.1	767.5	233.22	1.43	737 ± 22 ^c	658 ± 44 ^b	158 ± 7 ^a

(Continued)

TABLE 3 Continued

Count	Compound	CAS#	MW	RI	Rt (sec)	Dt (a.u.)	Integral volume		
							FL	FM	FH
37	4-methyl-2-pentanol M	C108112	102.2	768.7	234.28	1.26	459 ± 17 ^a	505 ± 18 ^a	606 ± 27 ^b
38	4-methyl-2-pentanol D	C108112	102.2	767.7	233.37	1.55	1290 ± 19 ^a	1287 ± 53 ^a	1458 ± 120 ^b
39	1-pentanol M	C71410	88.1	782.8	247.18	1.25	333 ± 28 ^a	430 ± 27 ^b	405 ± 32 ^b
40	1-pentanol D	C71410	88.1	782.6	247	1.51	235 ± 33 ^a	273 ± 7 ^a	378 ± 14 ^b
41	hexanal M	C66251	100.2	802.8	265.74	1.25	1957 ± 33 ^b	1995 ± 60 ^{ab}	1714 ± 122 ^a
42	hexanal D	C66251	100.2	800.8	263.86	1.56	4418 ± 640 ^a	4895 ± 257 ^a	6280 ± 134 ^b
43	hexanal T	C66251	100.2	799.4	262.52	1.65	107 ± 26 ^a	124 ± 6 ^a	247 ± 13 ^b
44	7	unidentified	0	818	280.74	1.46	74 ± 5 ^a	104 ± 15 ^b	66 ± 3 ^a
45	isovaleric acid	C503742	102.1	822.7	285.49	1.21	195 ± 23	98 ± 6	*
46	furfural	C98011	96.1	825.6	288.52	1.10	396 ± 44	232 ± 14	*
47	2-hexen-1-ol M	C2305217	100.2	859.4	325.91	1.18	137 ± 26 ^a	174 ± 32 ^a	344 ± 20 ^b
48	2-hexen-1-ol D	C2305217	100.2	859.2	325.58	1.30	67 ± 4 ^a	70 ± 3 ^a	209 ± 12 ^b
49	2-hexen-1-ol T	C2305217	100.2	859.7	326.23	1.51	*	*	112 ± 9
50	cyclohexen-2-one	C930687	96.1	884.4	356.6	1.11	63 ± 10 ^b	48 ± 5 ^a	68 ± 3 ^b
51	1-hexanol M	C111273	102.2	889	362.61	1.32	1087 ± 144	1188 ± 170	1032 ± 177
52	1-hexanol D	C111273	102.2	887.3	360.34	1.64	479 ± 49 ^a	596 ± 31 ^{ab}	805 ± 183 ^b
53	1-hexanol T	C111273	102.2	887.5	360.66	1.99	*	*	*
54	8	unidentified	0	896.9	373.49	1.35	256 ± 16 ^a	295 ± 20 ^a	522 ± 24 ^b
55	2-heptanone M	C110430	114.2	899.2	376.93	1.26	303 ± 5 ^b	240 ± 27 ^a	280 ± 10 ^b
56	2-heptanone D	C110430	114.2	897.7	374.69	1.63	434 ± 45 ^a	553 ± 53 ^b	604 ± 19 ^b
57	4-heptenal (Z)	C6728310	112.2	904.1	384.10	1.15	123 ± 30 ^a	150 ± 16 ^a	201 ± 3 ^b
58	heptanal M	C111717	114.2	908.1	390.15	1.33	626 ± 133	676 ± 32	610 ± 103
59	heptanal D	C111717	114.2	908.3	390.4	1.40	167 ± 5 ^a	181 ± 13 ^a	263 ± 33 ^b
60	heptanal T	C111717	114.2	908.4	390.55	1.68	357 ± 138 ^a	493 ± 50 ^a	745 ± 111 ^b
61	5-methyl-2-furanmethanol	C3857258	112.1	955.6	469.22	1.26	50 ± 7 ^a	44 ± 10 ^a	87 ± 10 ^b
62	2-heptenal (E) M	C18829555	112.2	968	492.29	1.25	272 ± 55 ^{ab}	195 ± 57 ^a	333 ± 48 ^b
63	2-heptenal (E) D	C18829555	112.2	967.8	491.96	1.38	126 ± 19 ^a	119 ± 25 ^a	257 ± 17 ^b
64	benzaldehyde M	C100527	106.1	983.9	523.73	1.15	450 ± 66 ^a	708 ± 105 ^b	730 ± 69 ^b
65	benzaldehyde D	C100527	106.1	984.1	524.14	1.28	118 ± 50 ^a	236 ± 25 ^b	360 ± 15 ^c
66	benzeneamine	C62533	93.1	993.5	543.49	1.43	865 ± 162 ^a	944 ± 62 ^a	2225 ± 57 ^b
67	dimethyl trisulfide	C3658808	126.3	996.7	550.22	1.31	370 ± 93 ^b	170 ± 26 ^a	154 ± 9 ^a
68	1-octen-3-one	C4312996	126.2	1001.8	559.03	1.68	*	*	118 ± 11
69	2-pentylfuran	C3777693	138.2	1002	559.32	1.25	175 ± 15 ^a	137 ± 31 ^a	246 ± 24 ^b
70	octen-3-ol M	C3391864	128.2	1008.8	571.38	1.16	2764 ± 226 ^a	2606 ± 291 ^a	3678 ± 62 ^b
71	2-ethyl-1-hexanol M	C104767	130.2	1019.7	590.97	1.4	832 ± 354 ^a	1020 ± 125 ^a	1542 ± 132 ^b
72	2-ethyl-1-hexanol D	C104767	130.2	1018.2	588.27	1.46	182 ± 83 ^a	226 ± 30 ^a	469 ± 39 ^b

(Continued)

TABLE 3 Continued

Count	Compound	CAS#	MW	RI	Rt (sec)	Dt (a.u.)	Integral volume		
							FL	FM	FH
73	2-ethyl-1-hexanol T	C104767	130.2	1018.5	588.71	1.81	134 ± 95 ^a	170 ± 27 ^a	403 ± 85 ^b
74	9	unidentified	0	1049.8	648.85	1.26	83 ± 25	72 ± 7	83 ± 9
75	isopentyl isovalerate	C659701	172.3	1073.1	697.37	1.45	*	*	55 ± 9
76	2-octenal (E) M	C2548870	126.2	1073.8	699.03	1.33	147 ± 14	130 ± 21	160 ± 15
77	n-nonanal M	C124196	142.2	1107.4	775.66	1.47	874 ± 158 ^a	829 ± 41 ^a	1396 ± 153 ^b
78	n-nonanal D	C124196	142.2	1107	774.64	1.94	*	*	180 ± 50

MW, formula weight; RI, gas chromatography retention index; Rt, gas chromatography retention time; Dt, drift-time. M, D, and T after the substance represented an aggregation state of monomer and dimer, respectively. FL, grass carp fed diet without fishmeal; FM, grass carp fed diet with 3% fishmeal; FH, grass carp fed diet with 6% fishmeal. Data are expressed as means ± SD. Value with different superscripts are significantly different ($P < 0.05$, $n \geq 3$).

Tetrasphaera, and *Intrasporangiaceae*; in the FM group, *Petrimonas*, *AEGEAN_169_marine_group*, *Dysgonomonadaceae*, and *Breznakia*; and in the FH group, *Bacteroides paurosaccharolyticus*, *Erysipelatostridiaceae*, *Enterobacterales*, *Subdoligranulum*, and *Coproccoccus* all exhibit significant responses, with LDA values exceeding the threshold. This highlights the clear response of these microbial groups to different levels of fish meal feeding.

PICRUSt2-level-2 functional profiling revealed that the 3% fish-meal group was significantly enriched in pathways related to metabolism, energy metabolism, lipid metabolism, and cell growth and death (Figure 2G). These enriched functions provide mechanistic insight into the improved growth performance and enhanced muscle flavour observed with fish-meal supplementation, thereby furnishing more direct evidence for the hypothesis that gut-microbiota modulation mediates flavour development.

3.4 Muscle volatile compounds

In Figure 3A, the PCA model is selected as the separation model. Samples with similar aroma characteristics overlap or are closely positioned in their respective figures (Song et al., 2020). The cumulative variance contribution rate of PC1 (57.9%) and PC2 (25.28%) is 83.18%, indicating that these two PCs essentially cover the sample information and can characterize the odor composition of grass carp muscle at different fish meal levels (Pan et al., 2022). A distinct separation is observed between the samples of the FH group and the other samples along PC1 (57.9%), indicating that the odor changes in muscle samples with a high level of fish meal are more pronounced.

The muscle VOCs of FL, FM, and FH are analyzed using GC-IMS. VOCs are significant determinants of meat flavor (Liu et al., 2009), and they serve as an important basis for consumers to evaluate meat quality (Chen et al., 2022). Intensity variations in different muscles are represented as a 3D topographical model in Figure 3B. The 2D topographical visualization of the VOCs is depicted in Figure 3C. Most signals are observed to be located in

the retention time range of 100–400 s and a drift time of 1–1.5 s. The results in Table 3 show that 53 VOCs are detected in the samples, including 10 aldehydes, 12 alcohols, 9 ketones, 2 nitrogen compounds, 3 sulfides, and 3 esters. In addition, there are nine unknown compounds. Aldehydes, alcohols, ketones, acids, and hydrocarbons are the primary VOCs in fish and fish products (Liu et al., 2009). When compared to the FM group as the background, the FL and FH groups display varying degrees of blue and red spots. The FL group has more blue spots, indicating that more VOCs and their content are lower than in the FM group; the FH group has many red spots, indicating that more VOCs and their content are higher than in the FM group (Figure 3D; Table 3).

The comparison of VOC fingerprints among the three groups is shown in Figure 3E. The VOCs with significant differences are roughly categorized into aldehydes, alcohols, ketones, esters, sulfur compounds, and furans, and a radar chart is created as shown in Figure 3F. Compared with the FL and FM groups, a large number of aldehydes are significantly increased in the FH group, including 2-methylbutanal D, hexanal D, T, 4-heptenal (Z), heptanal, 2-heptenal (E) D, benzaldehyde D, and n-nonanal M ($P < 0.05$); the content of butanal and 2-methylbutanal M is highest in the FM group ($P < 0.05$). Aldehydes are highly volatile and have a robust fatty aroma (Lorenzo et al., 2013). They are crucial to the flavor of fish due to their diversity and low odor thresholds (Pan et al., 2022). It has been reported that butanal, hexanal, heptanal, and benzaldehyde are the key aroma compounds in *Hypophthalmichthys molitrix*, *Aristichthys nobilis*, *Lateolabrax japonicus*, and *Parabramis pekinensis* (Chen et al., 2022). Most linear aldehydes are produced through autooxidation reactions initiated by enzymes or microorganisms present in fish (Fu et al., 2015; Zhang et al., 2020). Genomic and transcriptomic data have confirmed that the cecal microbial community affects the VOCs of duck meat, especially pentanal, hexanal, and heptanal, by modulating metabolic pathways related to fatty acid synthesis and degradation (Xu et al., 2024). Hexanal has been recognized as a unique aroma-active component in fresh tuna (Pan et al., 2022) and raw red seabream (Chen et al., 2022), and it exerts a major influence on flavor perception in silver carp samples (Liu et al., 2009).

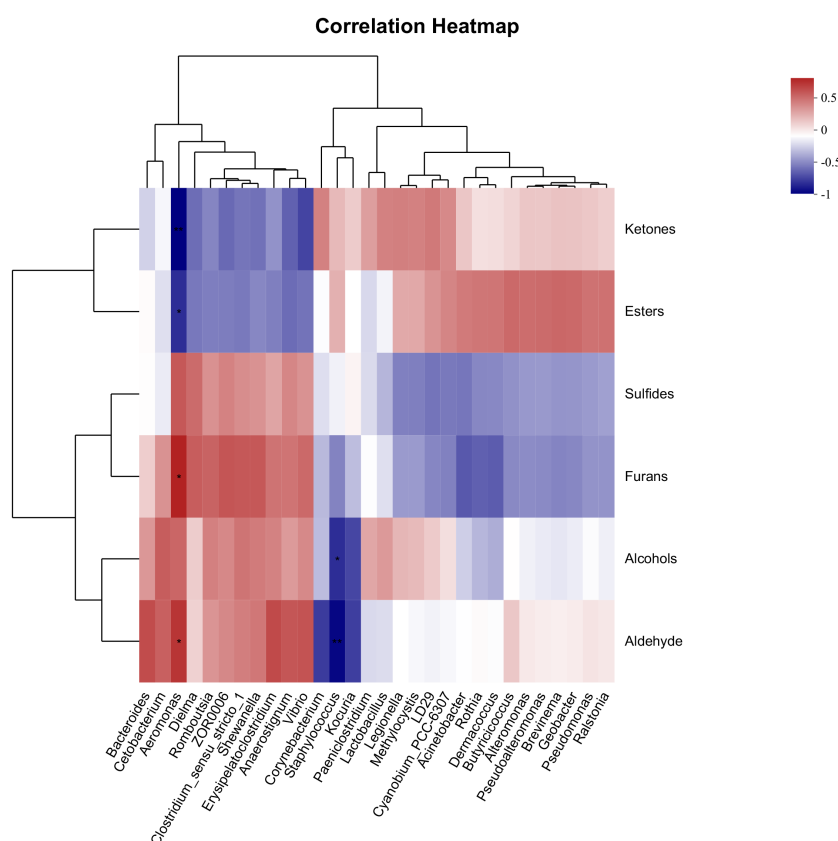


FIGURE 4

The correlation of top 30 genera and muscle volatile compounds in grass carp. Note: Firstly, classify the detected VOCs with statistically significant differences and calculate the total signal intensity for each VOC class. Then, based on the top 30 most abundant bacterial genera, perform a correlation analysis between the VOCs and the microbial community. Significant difference ($p < 0.05$ or $p < 0.01$) is indicated by * or **.

Previous research has indicated that (Z)-4-heptenal is present in extracts from turbot, characterized by a fishy or potato-like odor (Sérot et al., 2001). These aldehydes, which significantly contribute to the aroma of fish meat, impart a pleasant nutty, grassy, and fruity fragrance to the fish.

Compared to the FL and FM groups, the content of many alcohols is significantly increased in the FH group, including 1-penten-3-ol, 4-methyl-2-pentanol, 1-pentanol, 2-hexen-1-ol, 5-methyl-2-furanmethanol, octen-3-ol, and 2-ethyl-1-hexanol ($P < 0.05$). Alcohols are primarily derived from the oxidative decomposition of lipids or the reduction synthesis of carbonyl groups. They have higher thresholds compared to aldehydes and are closely related to the characteristic fatty flavor of meat (Zhang et al., 2020; Pan et al., 2022). Alcohols generally impart fruity, floral, and grassy notes to fish (Liu et al., 2009). Unsaturated alcohols typically have much lower thresholds than saturated alcohols and can significantly influence the flavor of food (Lorenzo et al., 2013). In our study, the content of ethanol is highest in the FL group, and the content of isopentyl alcohol M is highest in the FM group ($P < 0.05$). The elevated content of ethanol in the FL group suggests that it may not be conducive to preservation and can lead to the

generation of unpleasant odors (Pan et al., 2022). High concentrations of 1-hexanol and 1-pentanol have been detected in mature cod roe, and they may play a critical role in the product's aroma (Purrinos et al., 2011). The compound 1-octen-3-ol, originating from the hydroperoxide degradation product of linoleic acid, has been identified as one of the most important aroma-active compounds in fish samples (Salum et al., 2017; Pan et al., 2022). This may be one of the reasons why fish flavor compounds are affected.

Compared with the FL group, the content of many ketone compounds is significantly increased in the FH group, including 2-butanone M, 2,3-pentanedione, methyl isobutyl ketone M, and 2-heptanone D ($P < 0.05$); compared with the FH group, the content of 2-propanone and 2-pentanone M is significantly increased in the FL and FM groups ($P < 0.05$). Ketones may originate from the thermal oxidation of unsaturated fatty acids, the degradation of amino acids, or microbial oxidation (Zhang et al., 2019). Ketones have a unique buttery and fruity flavor (Pan et al., 2022). 2-Heptanone, primarily produced by the oxidation of linoleic acid, has been identified as a compound that can modulate the flavor of meat and meat products (Han et al., 2020). Studies have shown that 2-heptanone helps

enhance the aromatic characteristics of silver carp (Liu et al., 2009). In addition, among the esters, the content of ethyl acetate M is highest in the FM group, and the content of thiolane is highest in the FH group ($P < 0.05$). Esters are well-known for their significant contribution to fruity flavors (Peng et al., 2020) and play a crucial role in the unique aroma of fish (Liu et al., 2009). Ethyl acetate, with its slight fruity aroma, is considered a VOC that can enhance the aroma of fish (Mansur et al., 2003). The content of the sulfur compound dimethyl trisulfide is highest in the FL group ($P < 0.05$). Sulfur compounds, with their low thresholds, play an important role in the overall flavor of food, and thiols are generated from the Strecker degradation of methionine. Furans are one of the important compounds that produce meaty flavors (Pan et al., 2024). The content of the furan compound 2-pentylfuran is highest in the FH group ($P < 0.05$), and it makes a positive contribution to the flavor of grass carp. The diet-induced shifts in key volatile compounds are known to enhance the fresh, sweet, and umami notes while reducing 'fishy' odours. Consequently, fillets from fish fed the higher-fish-meal diets are expected to command better market prices and broader consumer acceptance, providing a clear economic incentive for producers.

3.5 Correlation analysis of microflora and muscular VOCs

Studies have proven that microbial communities are associated with muscle metabolic characteristics (Feng et al., 2022). Changes in the gut microbiota can regulate muscle fiber diameter (Lei et al., 2022). Short-chain fatty acids are produced during the fermentation process of gut microbiota, which promote the formation of oxidative muscle fibers (Sérot et al., 2001). Studies have shown that fecal microbiota transplantation is beneficial for the muscle growth and development of germ-free piglets (Qi et al., 2021). The microbial communities in the animal gut are diverse and play an important role in meat quality (Chen et al., 2022). VOCs with significant differences are categorized into aldehydes, alcohols, ketones, esters, sulfur compounds, and furans. Spearman correlation analysis is performed with the top 30 bacterial genera of the gut microbiota. The results are shown in Figure 4. Ketones and esters show no significant correlation with various bacterial genera ($P > 0.05$); sulfur compounds are extremely significantly correlated with *Dielma* ($P < 0.01$); furans are significantly positively correlated with *Aeromonas* ($P < 0.05$); alcohols are significantly positively correlated with *Clostridium-sense-stricto-1* and *Geobacter* ($P < 0.05$). Some gut microbes can catalyze the production of acids, thereby affecting the formation of flavor compounds in fish. For example, supplementing ducks with lactic acid bacteria has been shown to significantly increase the content of many VOCs in duck meat, including 1-octen-3-ol (Qi et al., 2021). *Clostridium-sense-stricto-1* can produce butyric acid through the butyrate kinase pathway (Chai et al., 2019). Aldehydes show significant positive correlations with multiple bacterial genera, including a significant positive correlation with *Bacteroides* ($P < 0.05$) and extremely significant positive correlations with *Erysipelatoclostridium*, *Aeromonas*, *Shewanella*, *Cetobacterium*, and *Vibrio* ($P < 0.01$). As a halophilic bacterium, *Vibrio* can produce a

variety of flavor compounds, thereby providing different flavors to the final product (Liu et al., 2021). Therefore, we speculate that the gut microbiota of grass carp shows a significant correlation with muscle VOCs.

4 Conclusion

Integrating growth performance, intestinal morphology, microbiota and muscle quality, the 3% fish-meal diet already fulfils production targets at the lowest cost. Raising fish-meal to 6% yields only marginal VOC increments (<15% for individual aldehydes and alcohols) without improving growth, and its 8–10% higher feed cost is economically justified only if a market flavour premium exceeds this margin. Although gut microbiota and VOCs correlate, causality remains unproven; thus, sensory tests and economic modelling are needed to verify consumer willingness-to-pay for any flavour advantage.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Hunan Normal University Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WG: Writing – original draft. WEL: Formal Analysis, Methodology, Writing – original draft. XZ: Investigation, Writing – original draft. GS: Methodology, Writing – original draft. FT: Methodology, Writing – original draft. ZW: Conceptualization, Project administration, Resources, Supervision, Visualization, Writing – review & editing. WLU: Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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