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Effects of three feed attractants on the growth performance and meat quality of the largemouth bass (*Micropterus salmoides*)

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The trial was conducted to investigate the effects of limonene, allicin and betaine supplementation in low fish meal (FM) diet on growth performance, antioxidant capacity, meat quality and intestinal health in largemouth bass (*M. salmoides*). The biting-balls test and feeding trial were successively conducted. For the one, the results of the biting-ball test showed that with the increase of the concentration of the three attractants, the attracting effect firstly increased, then decreased, and the effect reached maximum at 0.2% concentration. ($P < 0.05$). Further, a 9-week feeding trial was conducted using five diets, including a basal diet with 30% and 40% fish meal without attractant, 30% fish meal supplemented with 0.2% limonene, 0.2% allicin or 0.2% betaine (the diets were named FM30, FM40, FM30 + L, FM30 + A, FM30 + B, respectively). The results demonstrated that adding limonene, allicin and betaine at concentration of 0.2% to the low fish meal feed could improve final body weight, weight gain rate, and specific growth rate of *M. salmoides* but only in 4 weeks ($P > 0.05$). Besides, dietary supplementation with attractants could significantly reduce the content of MDA in serum and liver, and increase the activity of GSH in liver ($P < 0.05$). Compared with FM30 group, the supplementation with limonene, allicin or betaine diet had higher pH, redness (a^*), yellowness (b^*) ($P > 0.05$), and lower refrigeration loss, cooking loss values ($P < 0.05$). Furthermore, supplementation with attractants groups had higher values for villus height, lamina propria, crypt depth, submucous layer, and serous layer ($P < 0.05$). Taken together, these results indicated that limonene, allicin and betaine had a time effect on the growth performance, and could improve antioxidant capacity, meat quality and intestinal health of *M. salmoide*.

KEYWORDS

feed attractants, largemouth bass, meat quality, physiological biochemistry, intestinal health

Introduction

Fish meal is the preferred protein source for manufacturing aquafeed due to its nutritional contents, such as protein, fatty acids, and amino acid profile, as well as its excellent digestibility and palatability (Niu et al., 2020). However, resource depletion and rising prices seriously limited the use of fish meal in aquaculture (Li X. et al., 2021). Earlier, a number of studies conducted on various fish species demonstrated that low-fishmeal (LFM) diets can lead to poor feed palatability, decrease food intake and reduce the growth performance. For instance, olive flounder (*Paralichthys olivaceus*) (Niu et al., 2019), rainbow trout (*Oncorhynchus mykiss*) (Lazzarotto et al., 2018), Nile Tilapia (*Oreochromis niloticus*) (Wattanakul et al., 2019), Japanese seabass (*Lateolabrax japonicu*) (Rahimnejad et al., 2019). While, the attractants such as L-amino acids, taurine, betaine, glycine, fish meal, earthworms, Chinese herbs, and herbal extracts (Lunger et al., 2007; Shamushaki et al., 2007; Pu et al., 2017; Rufchaei et al., 2019; Xu et al., 2020) supplementation in LFM diets were considered as one of the most effective and reliable ways to improve the feed palatability (Hirt-Chabbert et al., 2012; Dar et al., 2019). But, it was also found that such odorants supplementation in fish feeds could affect the foraging behaviors of some species (Schmachtenberg, 2015). Therefore, the formulation of fish feeds using plants with distinct smells merits investigation to discover beneficial effects on feeding attractant activity.

Limonene is an aromatic compound in essential oils, commonly used food additive obtained from oranges, grapefruits, and lemons (Cicero et al., 2015; Giarratana et al., 2016; Ravichandran et al., 2018). It has been reported that limonene has with a variety of beneficial impact including growth improvement (Kesbiç et al., 2019), nutrient absorption (Aanyu et al., 2018), antioxidant enzymatic activity (Djenane, 2015), and can also improve the specific immunity (de Souza et al., 2019; Han et al., 2019). Similarly, allicin is an important biologically active sulfur containing organic compound extracted from the bulbs of garlic (Huang et al., 2020). Currently, various studies have shown that allicin could improve the growth performance (Lee et al., 2014; Ajiboye et al., 2016), reduce oxidative stress (Abdel-Daim et al., 2015), strengthen immunity (Hamed et al., 2021) as well as improve meat quality (Kaswinarni, 2015) of fish. And it has been found that allicin could promote the daily feed intake of many fish such as *Litopenaeus vannamei* (Samadi et al., 2016), common carp (*Cyprinus carpio L*) (Mohammad, 2020), Nile Tilapia (*Oreochromis niloticus*) (Soltan and Amal Elfeky, 2016), benni fish (*Mesopotamichthys sharpeyi*) (Milad Maniat et al., 2014) and African catfish (*Clarias gariepinus*) (Gabriel et al., 2019). In addition, diet replenished with allicin improved the survival and growth of large yellow croaker (*Larimichthys crocea*) larvae probably by promoting the intestinal development, alleviating inflammation and enhancing appetite (Huang et al., 2020). Betaine, a stable and non-toxic natural substance, is mainly extracted from the processing of sugar beet (Zhao et al., 2018) and was observed to improve growth performance, health status, feed digestibility, as well as flesh quality and the immune status of fish (Hirt-Chabbert et al., 2012; Pinedo-Gil et al., 2017; Ismail et al., 2020; Sun et al., 2020). It has been proven that betaine could act as a feed attractant and appetizer through stimulating the olfactory bulb, leading to increase the feed intake, which minimize the feed wastage and water pollution (Danaceau and Lucero, 2000).

In China, largemouth bass (*Micropterus salmoides*) typically a freshwater carnivore fish traditionally been cultured due to high commercial values and over the past decade its production has expanded over 600,000 tons because of its suitability for aquaculture, marketability, and high nutritional value (China Fishery Statistics Yearbook 2020). So far, there are no comprehensive studies have been reported though using betaine, limonene and allicin as a natural attractant in largemouth bass fed low fishmeal diets. Thus, the current study aimed to evaluate the effects of three herbal extracts on feed intake, growth performance, antioxidant capability, meat quality and intestinal health for largemouth bass supplemented low fishmeal diets.

Materials and methods

The biting-balls test

A biting-ball test device was prepared as reported previously and the schematics was shown in Figure 1 (Yu et al., 2021). A total of 150 fishes were placed into 3 tanks evenly [(150 × 150 × 60 cm) (height × width × length)], supplied with dechlorinated water. The water depth was maintained at 40 cm during the experiment and the experiment was carried out twice a day at 8:30 and 17:00 for three days. Five different concentrations (0.0%, 0.1%, 0.2%, 0.6% and 1.0%) solution of limonene, allicin and betaine were prepared and stored at 4°C, then injected into a cotton ball and wrapped with gauze, respectively. The biting-ball was fixed with iron wire and submerged 5 cm under the water's surface to allow the fish to touch or bite it. In addition, a 10 cm diameter circle was drawn at the tank's bottom as an effective region based on the center of the biting ball. The mobile phone recorded the number of bites of each bait ball and entries into the effective region within 10 minutes in order to determine the proper concentration of limonene, allicin, and betaine.

Experimental design and diet preparation

Five experimental diets were formulated and the formulation, and proximate composition of the experimental

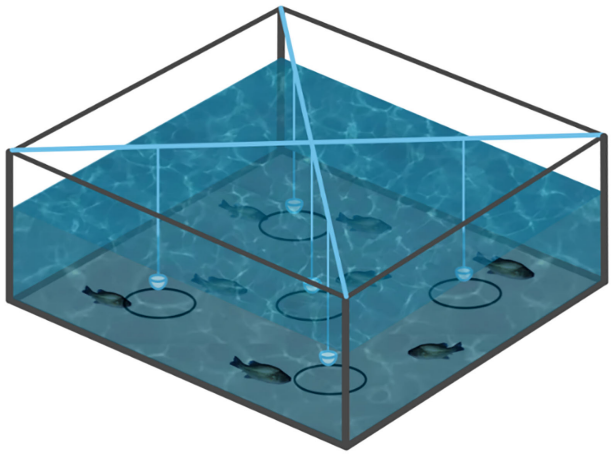

FIGURE 1
Schematic diagram of biting-balls test device.

diets are presented in Table 1. The basal diet was prepared with fish meal, soybean meal and peanut meal as the main protein source, and fish oil, and wheat flour as the main lipid and carbohydrate source respectively. According to the results in the biting-balls test, we selected the same concentration (0.2%) of allicin, betaine, limonene for further experiments. All the five test diets were designed as follows: (1) the normal fishmeal group (FM 40); (2) the low fishmeal group (FM 30); (3) the low fishmeal diet supplemented with 0.2% limonene (FM30 + L); (4) the low fishmeal supplemented with 0.2% allicin (FM30 + A); (5) the low fish meal supplemented with 0.2% betaine (FM30 + B) as presented Table 1. Crystalline amino acids (lysine, methionine) also were added to the diet to balance the dietary amino acid requirements in low fish meal diets. All dry ingredients were mixed thoroughly, and then oil and water were added. The mixture was extruded as an expanded particle diet (diameter of 1.5 mm) using a DS32-II type two-screw extruder (Guangzhou Vilavi Mechanical Equipment Co., Ltd.) after water addition, then air-dried and stored at -20°C until use.

Feeding trial and experimental conditions

M. salmoides were obtained from Guangdong Ho's Aquatic Products Co., Ltd. (Guangdong, China) and cultured in recirculating water system in Foshan University. Throughout the experiment, water temperature, pH, NH_{4+}, nitrite, nitrate and dissolved O_2 in water were maintained at 24-30°C, 6.5-7.5, < 1 mg/L, < 1 mg/L, < 20 ppm, and > 6 mg/L, respectively. After acclimation for 2 weeks, a total of 600 fish with similar body weight (mean initial weight 6.26 ± 0.01 g) were randomly assigned into 20 tanks. Each group contained four replicate tanks (30 fish/tank). All groups were fed two times per day at 8:30 and 17:00. The weight of the fish in each tank was recorded at fourth and sixth week.

Sample collection

After fasting for 24 h, fishes were anaesthetized with buffered MS-222, and the fishes in each tank were weighed to evaluate the growth performance parameters. Three whole fishes from each tank were sampled and stored at -20°C for subsequent proximate composition analysis. Blood was collected from the caudal vein of eleven fishes of each tank and blood samples were centrifuged (3000 r/min, 15 min) at 4°C, and the supernatant (serum) was stored at -80°C for further analysis. The livers and intestines of five fish per tank were collected and used for histopathological and enzyme activity analyses. Similarly, the dorsal muscles of six fish/tank were collected for flesh quality parameters analysis.

Enzyme assays

The collected livers were centrifuged for 10 min (2000 r/min, 4°C) before collecting the supernatant and then kept at -80°C. The supernatant of livers and serum were used to determine the superoxide dismutase (SOD) (determined by AST-1 method), malondialdehyde (MDA) (determined by thiobarbituric acid (TBA) test method), catalase (CAT) (determined by ammonium molybdenum acid method), glutathione (GSH) (determined by microplate method) and total protein (TP) (determined by coomassie blue staining) using the kits purchased from Nanjing Jiancheng Bioengineering Institute, China. All the analyses were performed according to the instructions of the manufacturer.

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TABLE 1 Composition and nutrient levels of experimental diets for *M. salmoides* (dry-weight basis).

Ingredients (%)	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Fish meal	30	40	30	30	30
Soybean meal	22	22	22	22	22
Peanut meal	21	10	21	21	21
Wheat flour	8.6	11	8.4	8.4	8.4
Vital wheat gluten	6	6	6	6	6
Beer yeast	3	3	3	3	3
Soybean lecithin	1	1	1	1	1
Fish oil	3	3	3	3	3
Choline chloride	0.5	0.5	0.5	0.5	0.5
Calcium dihydrogen phosphate	1.5	1.5	1.5	1.5	1.5
Compound premix[a]	3	3	3	3	3
Crystalline lysine	0.29	0	0.29	0.29	0.29
Crystalline methionine	0.11	0	0.11	0.11	0.11
Limonene[b]	0	0	0.2	0	0
Allicin[c]	0	0	0	0.2	0
Betaine[d]	0	0	0	0	0.2
Totel	100	100	100	100	100
Proximate composition					
Crude protein	44.73	47.88	46.08	45.92	46.54
Crude lipid	13.93	14.02	14.16	13.96	14.39
Crude ash	11.65	12.52	11.72	11.82	11.98

[a]Compound premix: (kg^{-1} of diet): vitamin A, 250,000 IU; riboflavin, 750 mg; pyridoxine HCL, 400 mg; cyanocobalamin, 1 mg; thiamin, 250 mg; menadione, 250 mg; folic acid,125 mg; biotin, 10 mg;α-tocopherol, 2.5 g; myo-inositol, 8000 mg; calcium pantothenate,1250 mg; nicotinic acid, 2000 mg; choline chloride, 8000 mg; vitamin D3,45,000 IU; vitamin C, 7000 mg; $ZnSO4{\cdot}7H_2O$, 0.04 g; CaCO3, 37.9 g; KCl, 5.3 g; KI, 0.04 g; NaCl, 2.6 g; $CuSO4{\cdot}5H_2O$, 0.02 g; $CoSO4{\cdot}7H_2O$, 0.02 g; $FeSO4{\cdot}7H_2O$, 0.9 g; $MnSO4{\cdot}H_2O$, 0.03 g; $MgSO4{\cdot}7H_2O$, 3.5 g; $Ca(HPO_4)_2{\cdot}2H_2O$, 9.8 g.
[b]Purchased from Xi'an Victory Biochemical Technology Co., Ltd (Victorybio).
[cd]Purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

Muscle quality measurement

The muscle quality related parameters including the pH, lightness (L^*), redness (a^*), yellowness (b^*) and water holding capacity (included thawing loss, refrigeration loss, centrifugal loss, cooking loss, drop loss and pressure loss) of dorsal muscle were measured as earlier been reported by Caimi et al. (Caimi et al., 2021). The L^*, a^* and b^* of muscle were analyses using colorimeter (SCQ-1A Tenovo International Co., Limited) while, muscle pH was measured with a direct pH meter (accurate to 0.01, pH star, Mets, Germany).

Intestinal morphology analysis

The whole intestines were fixed in 4% paraformaldehyde, dehydrated in a graded alcohol series, cleared in xylol, embedded in paraffin, sectioned at 5 μm thickness, and hematoxylin and eosin (H&E) staining were performed. Lastly, the stained sections were observed under the microscope camera NLCD 500 (Nanjing China). Image J software (W. Rasband, NIH, USA) was used to measure the villi height (VH), villi width (VW), muscle thickness (ML), lamina propria (LP), crypt depth (CD), submucous layer (SML) and serous layer (SL).

Calculation and statistical method

Growth performance of *M. salmoides* was calculated as follows:

Final body weight (FBW) = the weight of fish in the tank/the number of fish in the tank;

Weight gain rate (WGR, %) = 100 × (final body weight-initial body weight)/initial body weight;

Daily feed intake (DFI, g/fish) = (amount of feed consumed by all fish in a tank/(days of the experiment × (IBW +FBW)/2) ×100%);

Specific growth rate (SGR, %/d) = 100 × (Ln final body weight-Ln initial body weight)/days of the experiment;

Feed conversion ratio (FCR) = feed intake/body weight gain;

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

Condition factor (CF, g/cm^3) = 100 × body wet weight (g)/body length (cm)^3;

Hepatosomatic index (HSI, %) = 100 × (liver weight/whole body weight);

Viscerosomatic index (VSI, %) = 100 × (viscera weight/whole body weight);

Intestinal index (ISI, %) = 100 × (intestine weight/whole body weight);

Intestinal length index (ILI, %) = 100 × (intestine length/body length).

All the data were statistically analyzed by using SPSS 26.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA). One-way ANOVA followed by Duncan's multiple range tests was used and all the results were presented as means ± S.E.M (standard error of the mean). Whereas, the values of $P \leq 0.05$ were considered as level of significance.

Results

The biting-balls test

It has been observed that at 8:00, only the effect of 0.2% allicin and betaine as food attractants was significantly higher than that of the 0.0% group ($P < 0.05$). While at 17:00, all the three food attractants (limonene, allicin, and betaine) with 0.2% concentration have a substantially higher effect than that of the 0.0% group ($P < 0.05$). Furthermore, limonene, allicin, and betaine as a food attractant with different concentrations 0.0%, 0.1%, 0.2%, 0.6%, and 1.0% are given in Table 2.

Growth performance

Similarly, the growth performance, feed utilization and biometric indices were also evaluated and are presented in Table 3. At 4th week the group fed with FM40 diet presented significantly higher FBW, WGR, and SGR than the group fed with FM30 diet ($P < 0.05$), meanwhile, no difference was observed for FBW, WGR, or SGR among all the attractant groups ($P > 0.05$). Although, both the FM30 and FM40 groups at 6th week exhibited an insignificant ($P > 0.05$) differences for DFI and FCR whereas, at 6th week the DFI in FM30 + A group was significantly higher than that of the FM30 group ($P < 0.05$). Additionally, no significant difference was observed for FBW, WGR, and SGR among the experimental groups ($P > 0.05$) at 9th week.

Furthermore, the CF, ISI and ILI was not changed among the experimental groups after 9 weeks ($P > 0.05$). Besides, the FM30 + L diet group had significantly higher levels of HSI than the FM30 diet group ($P < 0.05$) after 9 weeks, but there was no significant difference in HSI between the FM40 and the supplementation with limonene, allicin or betaine groups ($P > 0.05$).

Whole-body and muscle chemical composition

All the dietary treatments had an insignificant ($P > 0.05$) effect on the contents of the crude protein, crude lipid, and moisture levels of the whole body and muscle mass. However, the contents of the crude ash in FM30, FM30 + L, FM30 + A and FM30 + B groups were lower than that in FM40 group to varying degrees, and the FM30 + B group was significantly lower than that in FM40 group. The results of the whole body and muscle composition analysis are depicted in Tables 4, 5.

Liver antioxidant capability

A significantly higher contents of the MDA were detected in FM30 diet group ($P < 0.05$) as compared to the FM30 + L and FM30 + B groups, while no change in MDA contents were observed between the FM40 group and the supplementation with limonene, allicin or betaine groups ($P > 0.05$) as shown in Figure 2A. Similarly, the GSH contents in FM30 + A and FM30 + B diet groups were significantly higher than that of the FM30 diet group ($P < 0.05$), but insignificant difference was perceived between the FM40 group and

TABLE 2 The effects of different concentrations of limonene, allicin and betaine on attracting *of M. salmoides* at 8:00 and 17:00.

Items	0	0.1%	0.2%	0.6%	1%
8:00					
Limonene	17.67 ± 1.58	19.44 ± 0.62	24.22 ± 3.12	21.67 ± 6.77	14.67 ± 4.51
Allicin	11.22 ± 2.31^{ab}	19.44 ± 2.78^{bc}	23.56 ± 4.33^{c}	6.00 ± 3.18^{a}	9.78 ± 3.36^{ab}
Betaine	12.33 ± 1.33^{a}	20.44 ± 1.25^{b}	25.67 ± 2.07^{b}	14.00 ± 2.80^{a}	8.89 ± 1.11^{a}
17:00					
Limonene	14.56 ± 0.78^{a}	19.00 ± 2.71^{ab}	25.22 ± 1.50^{b}	22.00 ± 2.34^{b}	14.67 ± 2.85^{a}
Allicin	10.89 ± 4.05^{a}	20.33 ± 0.33^{ab}	27.78 ± 2.31^{b}	11.00 ± 5.50^{a}	10.78 ± 5.31^{a}
Betaine	16.78 ± 2.35^{a}	27.22 ± 2.74^{ab}	34.78 ± 1.83^{b}	26.78 ± 4.44^{ab}	21.11 ± 4.29^{a}

Values marked with different letters are significantly different (P < 0.05) between treatments.

TABLE 3 Effects of limonene, allicin and betaine on growth performance of *M. salmoides* for 4, 6 and 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
4 weeks					
IBW (g)	6.26 ± 0.00	6.25 ± 0.00	6.25 ± 0.01	6.26 ± 0.01	6.26 ± 0.01
FBW (g)	18.99 ± 0.51^{a}	22.58 ± 0.70 ^{b}	20.94 ± 0.20^{ab}	19.89 ± 0.54^{a}	20.96 ± 1.14^{ab}
WGR (%)	203.41 ± 8.20^{a}	261.02 ± 11.09^{b}	234.81 ± 3.15^{ab}	217.98 ± 9.15^{a}	230.34 ± 6.65^{ab}
SGR (%/d)	3.96 ± 0.09^{a}	4.55 ± 0.11^{b}	4.31 ± 0.04^{ab}	4.12 ± 0.10^{ab}	4.25 ± 0.24^{ab}
DFI (%/d)	3.62 ± 0.17^{ab}	3.38 ± 0.05^{ab}	3.60 ± 0.08^{ab}	3.76 ± 0.02^{b}	3.49 ± 0.04^{ab}
FCR	1.01 ± 0.06^{b}	0.84 ± 0.02^{a}	0.93 ± 0.01^{ab}	1.01 ± 0.03^{b}	0.91 ± 0.03^{ab}
6 weeks					
FBW (g)	29.28 ± 0.54	32.19 ± 0.40	30.23 ± 0.26	29.40 ± 1.14	30.41 ± 1.14
WGR (%)	367.92 ± 8.62	414.74 ± 3.51	383.36 ± 4.14	370.14 ± 18.50	385.72 ± 17.88
SGR (%/d)	3.67 ± 0.04	3.90 ± 0.02	3.75 ± 0.02	3.68 ± 0.09	3.76 ± 0.09
DFI (%/d)	2.79 ± 0.03^{ab}	2.74 ± 0.06^{a}	2.92 ± 0.06^{bc}	2.96 ± 0.06^{c}	2.78 ± 0.05^{ab}
FCR	0.91 ± 0.01^{ab}	0.85 ± 0.01^{a}	0.93 ± 0.02^{b}	0.95 ± 0.03^{b}	0.88 ± 0.02^{ab}
9 weeks					
FBW (g)	48.05 ± 1.53	49.05 ± 0.59	47.92 ± 1.87	46.78 ± 0.62	48.85 ± 1.24
WGR (%)	667.90 ± 24.42	684.23 ± 8.87	666.38 ± 30.73	648.14 ± 10.3	680.29 ± 19.49
SGR (%/d)	3.23 ± 0.05	3.27 ± 0.02	3.23 ± 0.06	3.19 ± 0.02	3.26 ± 0.04
DFI (%/d)	2.30 ± 0.03^{ab}	2.22 ± 0.01^{a}	2.34 ± 0.05^{ab}	2.41 ± 0.05^{b}	2.27 ± 0.03^{a}
FCR	0.91 ± 0.01^{ab}	0.86 ± 0.01^{a}	0.91 ± 0.03^{ab}	0.94 ± 0.02^{b}	0.88 ± 0.01^{ab}
SR (%)	99.17 ± 0.83	100.00 ± 0.00	100.00 ± 0.00	98.33 ± 0.96	98.33 ± 0.96
CF (g/cm^{3})	1.25 ± 0.03	1.29 ± 0.03	1.29 ± 0.02	1.28 ± 0.02	1.28 ± 0.02
HSI (%)	2.58 ± 0.19^{a}	3.14 ± 0.15^{b}	3.22 ± 0.17^{b}	2.85 ± 0.12^{ab}	2.86 ± 0.16^{ab}
VSI (%)	7.51 ± 0.18^{abc}	7.73 ± 0.21^{bc}	7.90 ± 0.21^{c}	7.3 ± 0.14^{ab}	7.16 ± 0.14^{a}
ISI (%)	0.79 ± 0.03	0.71 ± 0.02	0.73 ± 0.05	0.71 ± 0.03	0.78 ± 0.02

Values marked with different letters are significantly different (P < 0.05) between treatments.

the supplementation with limonene, allicin or betaine group (P > 0.05) (Figure 2B). In addition, among all the groups (P > 0.05) the activity of SOD was not differ (Figure 2C). Moreover, the CAT activity of the FM30 + L, FM30 + A and the FM30 + B group were significantly lower than that of the FM30 and the FM40 groups (P > 0.05) as presented in Figure 2D.

Serum antioxidant capacity

The contents of MDA in FM30 + A group was significantly lower than that of the FM30 group (P < 0.05), while was not differ between the FM40 group and supplementation with limonene, allicin or betaine group (P > 0.05) (Figure 3A). Similarly, the GSH contents in FM30 + A and FM30 + B groups were markedly lower than the FM40 group (P < 0.05) (Figure 3B). Moreover, the FM30 + A group had lower SOD activity compared to other groups (P < 0.05) (Figure 3C), however, no difference has been observed for CAT activity among all the groups (P > 0.05) (Figure 3D).

Meat quality

As shown in Table 6, the pH, L^*, thawing loss, centrifugal loss, drop loss and pressure loss were not differ among all the experimental groups (P > 0.05), while, the a^* and b^* of FM40 group was significantly higher than that of the FM30 group (P <

TABLE 4 Effects of limonene, allicin and betaine on whole-body composition (dry-weight basis) of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Crude protein (%)	59.52 ± 0.42	62.81 ± 1.46	62.23 ± 1.76	60.93 ± 0.71	61.20 ± 0.19
Crude lipid (%)	23.18 ± 0.37	22.82 ± 0.29	23.87 ± 0.33	23.49 ± 0.33	23.47 ± 0.47
Crude ash (%)	13.52 ± 0.19^{b}	13.42 ± 0.10^{ab}	13.29 ± 0.13^{ab}	13.24 ± 0.09^{ab}	13.00 ± 0.19^{a}
Moisture (%)	2.70 ± 0.08	2.28 ± 0.10	2.54 ± 0.14	2.58 ± 0.14	2.45 ± 0.11

Values marked with different letters are significantly different (P < 0.05) between treatments.

TABLE 5 Effects of limonene, allicin and betaine on muscle composition (dry-weight basis) of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
Crude protein (%)	89.54 ± 0.11	89.54 ± 0.20	90.37 ± 0.51	90.39 ± 0.32	90.43 ± 0.48
Crude lipid (%)	11.57 ± 0.48	11.46 ± 0.55	10.80 ± 0.33	10.72 ± 0.72	11.01 ± 0.24
Crude ash (%)	5.88 ± 0.25	6.08 ± 0.10	5.87 ± 0.05	6.05 ± 0.06	6.00 ± 0.11
Moisture (%)	2.18 ± 0.21	1.97 ± 0.11	2.05 ± 0.04	2.03 ± 0.12	1.94 ± 0.20

Values marked with different letters are significantly different (P < 0.05) between treatment.

0.05), but no difference was there between the FM40 group and the supplementation with limonene, allicin or betaine groups ($P > 0.05$). The refrigeration loss of the FM30 + A and FM30 + B diets were significantly lower than that of the FM30 and FM40 groups ($P < 0.05$). The cooking loss of the FM30 + L group was also lower than that of the FM30 group ($P < 0.05$).

Intestinal morphology

The intestinal morphology showed that the villi of FM30 group were injured and broken, and the thickness of the small intestinal wall was heterogeneous as depicted in Figure 4A. The FM30 + B group had significantly higher number of villi compared with the FM40 group ($P < 0.05$) (Figure 4B; Table 7) But the width and muscular layer of fishes'villus were not differ in all groups ($P > 0.05$). The lamina propria of FM30 + A and FM30 + B groups were significantly higher compared to the FM30 group ($P < 0.05$) (Table 7). Meanwhile, FM40, FM30 + L, FM30 + A and FM30 +B groups had significantly deeper crypt depth compared with FM30 group ($P < 0.05$) (Figures 4C–E). The submucous layer value of FM30 + L and FM30 + A group were significantly higher than FM30 group ($P < 0.05$). while, the serous layer of FM30 + L group was significantly thicker than

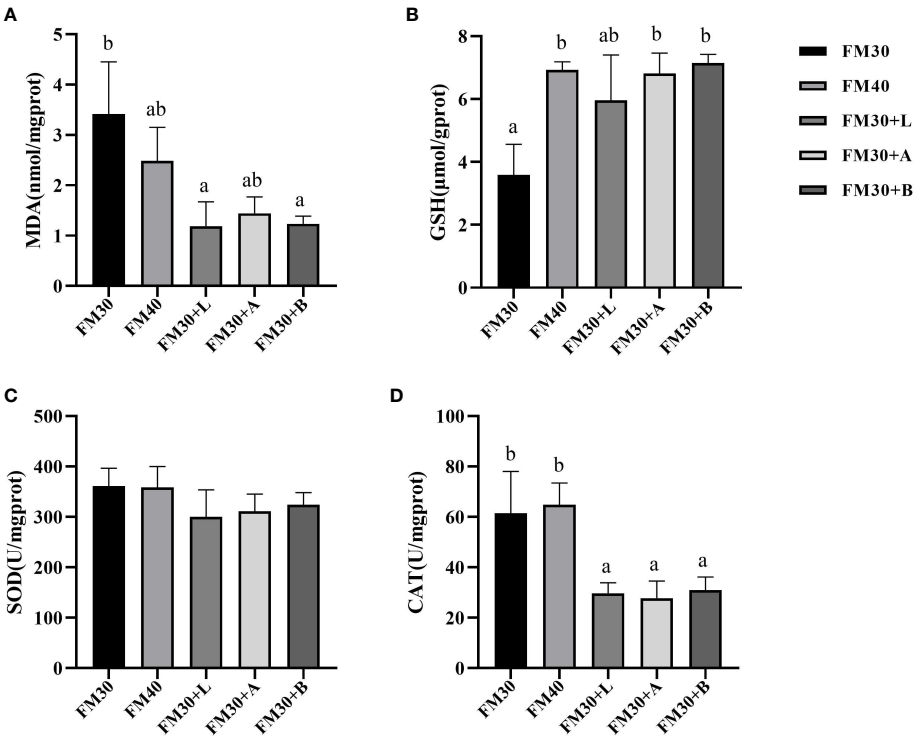

FIGURE 2
Effects of limonene, allicin and betaine on liver antioxidant capability of *M. salmoides* for 9 weeks. **(A)** Malondialdehyde (MDA); **(B)** glutathione (GSH); **(C)** superoxide dismutase (SOD); **(D)** catalase (CAT). Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05) between treatments.

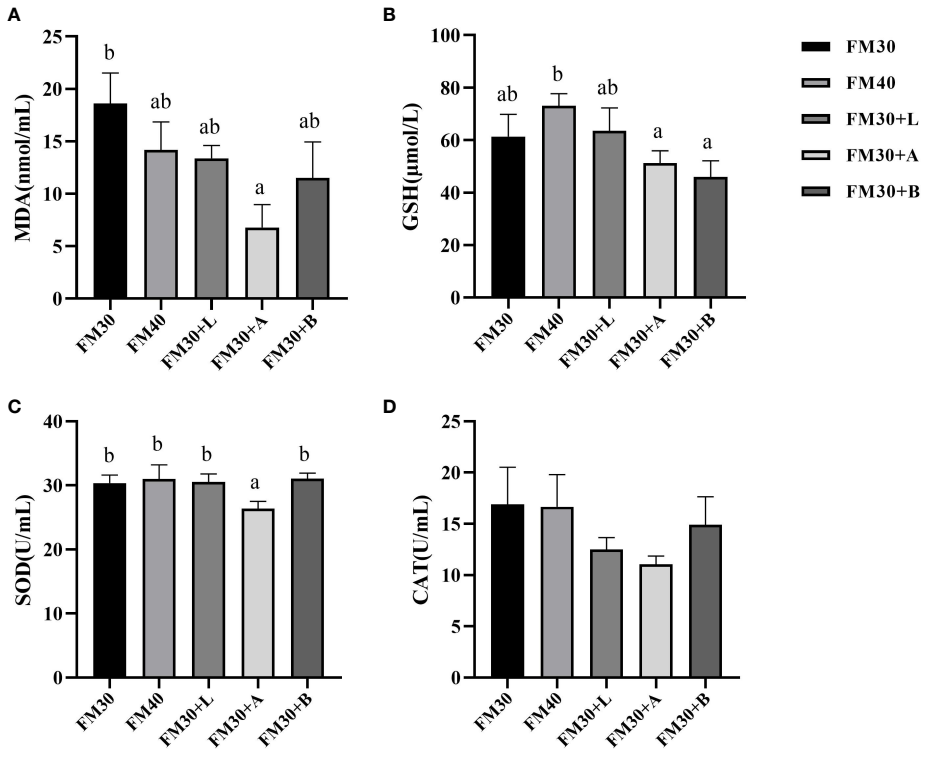

FIGURE 3
Effects of limonene, allicin and betaine on serum antioxidant capability of *M. salmoides* for 9 weeks. **(A)** Malondialdehyde (MDA); **(B)** glutathione (GSH); **(C)** superoxide dismutase (SOD); **(D)** catalase (CAT). Values (mean ± standard error of the mean, SEM) in bars that have the same letter are not significantly different (P > 0.05) between treatments.

FM30, FM40 and FM30 + B groups ($P < 0.05$). The villus height, villus width, muscular layer, lamina propria, crypt depth, and submucous layer values of fishes was not varied in limonene-, allicin- or betaine-supplementation groups ($P > 0.05$).

Discussion

In aquaculture, the commercial bait is composed of food-based basic materials and attractants, among which the attractants play a decisive role in the entire bait due to their characteristic flavour (Yu et al., 2021). Simultaneously, fish predominantly rely on their olfaction for a variety of fundamental behaviors such as foraging (Volz et al., 2020), and food attractants that could stimulate the olfactory receptors (Wang et al., 2021). Limonene is a translucent liquid with pleasant lemon-like odor (Ibáñez et al., 2020), allicin is the compound responsible for garlic's pungent odor (Borlinghaus et al., 2014), and betaine is a flavor enhancer used to reduce bitterness and imparting optimal sweetness and umami to food (Tu et al., 2020). Based on these theories, we designed this biting-balls test, it showed that these three compounds limonene, allicin and betaine do have positive impact on food consumption. And it is consistent with the results of our biting-balls test, indicating that limonene, allicin and betaine could stimulate the smell or taste receptors of *M. salmoides* and had a strong attraction effect (Reyes-Camacho et al., 2021). In addition, the attraction effects of different food attractants varied, which could be attributed to differences in the number of olfactory receptor genes responsible for detecting different odor molecules (Liu et al., 2021), resulting in different sensations or recognition capacities of olfactory and taste receptors to attractants in *M. salmoides*. In this study, when the concentrations of limonene, allicin and betaine were higher than 0.2%, the attraction effects on *M. salmoides* was gradually weakened, even lower than that of the control group. It might be because high concentrations of limonene, allicin and betaine were beyond the tolerance range of *M. salmoides*.

Interestingly, a gradual decrease in specific growth rate and daily feed intake was observed from 4th to 9th weeks, and the variations in growth performance progressively became inconspicuous among all the experimental groups. We speculate that *M. salmoides* might adapt to the taste and texture of the different diets with the extension of the feeding time, similar phenomenon was also reflected in previous researches (Tian et al., 2016; Lazado et al., 2019; Le et al., 2020; Martchenko et al., 2021). In this study, the crude

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TABLE 6 Effects of limonene, allicin and betaine on meat quality of *M. salmoides* for 9 weeks.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
H	6.16 ± 0.27	6.40 ± 0.03	6.42 ± 0.04	6.38 ± 0.02	6.33 ± 0.06
L^*	52.76 ± 2.07	55.37 ± 0.89	54.06 ± 1.75	52.08 ± 1.87	55.22 ± 1.34
a^*	1.98 ± 0.32^{a}	3.30 ± 0.49^{b}	2.25 ± 0.24^{ab}	3.15 ± 0.43^{ab}	2.40 ± 0.31^{ab}
b^*	4.64 ± 0.56^{a}	6.88 ± 0.61^{b}	5.33 ± 0.66^{ab}	5.97 ± 0.53^{ab}	6.36 ± 0.47^{ab}
Thawing loss (%)	2.01 ± 0.28	2.54 ± 0.37	2.17 ± 0.17	1.80 ± 0.16	2.10 ± 0.18
Refrigeration loss (%)	1.64 ± 0.13^{b}	1.75 ± 0.17^{b}	1.49 ± 0.13^{ab}	1.17 ± 0.15^{a}	1.16 ± 0.09^{a}
Centrifugal loss (%)	9.93 ± 0.80	8.83 ± 0.59	9.23 ± 0.71	9.43 ± 0.49	9.27 ± 0.69
Cooking loss (%)	22.04 ± 1.37^{b}	20.64 ± 0.36^{ab}	18.90 ± 0.5^{a}	20.19 ± 0.69^{ab}	21.00 ± 0.79^{ab}
Drop loss (%)	3.69 ± 0.33	3.01 ± 0.24	3.58 ± 0.29	3.04 ± 0.18	3.13 ± 0.21
Pressure loss (%)	5.26 ± 0.39	5.00 ± 0.26	4.61 ± 0.21	4.61 ± 0.15	5.07 ± 0.52

Values marked with different letters are significantly different ($P < 0.05$) between treatment.

protein, crude lipid and moisture of whole body and muscle did not differ among all the dietary treatments. The content of crude protein and moisture in whole fish and muscle of *M. salmoides* in the FM30 + L, FM30 + A and FM30 + B groups were closer to those of the FM40 group. Various factors have contributed to the nutritional composition of fish, such as genetic factors (Cai et al., 2021), water environment (Mohanty et al., 2019; Byrd et al., 2020), and season (Duarte et al., 2022), while the most important factor is the feed nutrition (Khalili Tilami and Sampels, 2017). At present, there have been many studies shown that supplementation of attractants, such as betaine (Yeşilayer and Kaymak, 2020), squid hydrolysate and squid meal (Novriadi et al., 2017), red seaweed eucheuma denticulatum (*Eucheuma denticulatum*) (Ragaza et al., 2015), taurine (Nguyen et al., 2020) to a low-fishmeal diet had no significant effect on fish chemical composition. However, the contents of the crude ash in FM30, FM30 + L, FM30 + A and FM30 + B groups were lower than that in FM40 group to varying degrees, and the FM30 + B group was significantly lower than that in FM40 group. Similar results were also shown in the study of Nile tilapia (*Oreochromis niloticus*) (Ahmad and Abdel-Tawwab, 2011) and juvenile tinfoil barb (*Barbonymus schwanenfeldii, Bleeker 1853*) (Nafees et al., 2022). In addition, previous study have shown that the crude ash content of fish decreases with the prolongation of starvation

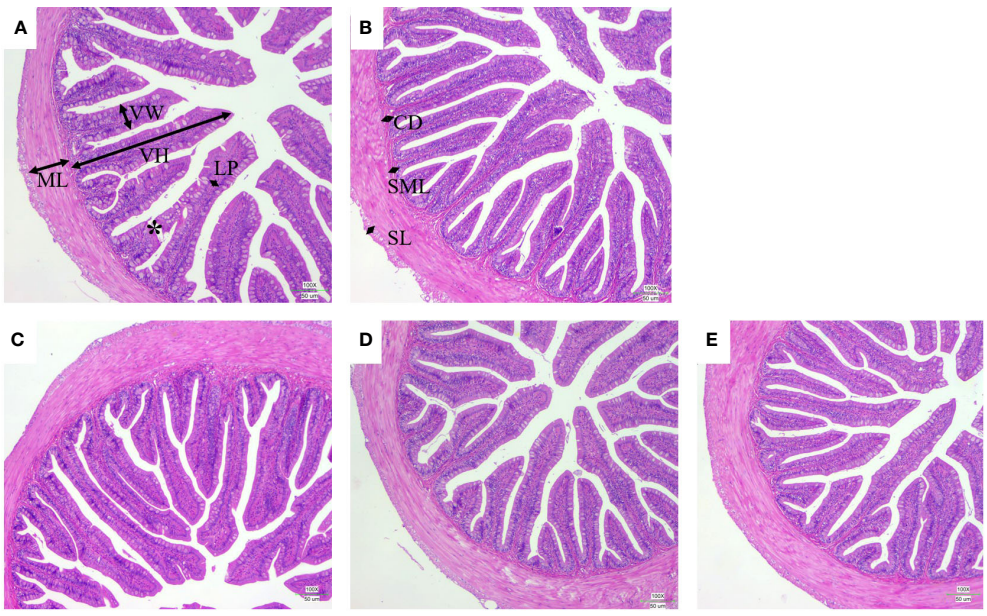

FIGURE 4
Effects of limonene, allicin and betaine on intestinal morphology of M. salmoides for 9 weeks. CD, crypt depth; LP, lamina propria; ML, muscular layer; SL, serous layer; SML, submucous layer; VH, villus height; VW, villus width. Scale bars = 50 μm. **(A)** FM30; **(B)** FM40; **(C)** FM30 + L; **(D)** FM30 + A; **(E)** FM30 + B.

TABLE 7 Histomorphometry of the intestine of *M. salmoides* fed different experimental diets.

Items	FM30	FM40	FM30 + L	FM30 + A	FM30 + B
VH (μm)	38.21 ± 3.01^{ab}	34.76 ± 2.88^{a}	43.09 ± 3.53^{ab}	38.17 ± 2.95^{ab}	44.64 ± 2.91^{b}
VW (μm)	9.15 ± 0.85	9.44 ± 1.03	12.43 ± 1.85	11.21 ± 1.24	12.59 ± 1.10
ML (μm)	14.70 ± 1.81	16.53 ± 3.10	18.26 ± 2.50	20.62 ± 1.64	20.09 ± 0.94
LP (μm)	2.99 ± 0.10^{a}	3.19 ± 0.19^{ab}	3.61 ± 0.26^{abc}	4.15 ± 0.47^{bc}	4.41 ± 0.40^{c}
CD (μm)	2.09 ± 0.24^{a}	3.43 ± 0.42^{b}	3.74 ± 0.44^{b}	3.85 ± 0.42^{b}	3.97 ± 0.58^{b}
SML (μm)	2.10 ± 0.18^{a}	2.66 ± 0.20^{ab}	3.21 ± 0.15^{b}	2.94 ± 0.24^{b}	2.61 ± 0.16^{ab}
SL (μm)	2.24 ± 0.23^{a}	2.43 ± 0.33^{a}	3.94 ± 0.47^{b}	3.16 ± 0.20^{ab}	2.86 ± 0.31^{a}

Values marked with different letters are significantly different ($P < 0.05$) between treatments.

time, (Abdel-Tawwab et al., 2006), thus, we speculate that largemouth bass fed a low fish meal diet were starved more quickly and fasted for longer, resulting in differences in the crude ash content of the whole fish. Thus, it has been perceived that growth performance, whole-body and muscle chemical composition as well as health parameters were not negatively affected by limonene, allicin, and betaine, even almost similar to FM40 group.

The SOD and CAT are typical antioxidant enzymes found in fish serum or liver that can prevent organisms from being harmed by reactive oxygen species (ROS), which can cause a variety of disorders by attacking macromolecules (Balaban et al., 2005). GSH is the most prominent non-enzymatic antioxidant in fish, as well as a free radical scavenger and detoxifier (Chen et al., 2015). MDA, a byproduct of lipid peroxidation that can interact with the free amino groups in protein causing cell damage (Xiao et al., 2022), and the contents of MDA in the fish liver can reflect the severity of the free radical attack on the liver or body cells (Całyniuk et al., 2016). Previous studies have demonstrated that limonene, allicin and betaine could improve the antioxidant capacity of fish (Abdel-Tawwab et al., 2021; Dong et al., 2021; Hamed et al., 2021; Ajiboye et al., 2016; Lopes et al., 2019; Lopes et al., 2020; Mohseni et al., 2021). In this study, the GSH and MDA contents variations in the liver as well as the MDA contents in the serum demonstrated the antioxidant effect of the aforesaid three attractants. On the contrary, the CAT activity in the liver and the GSH content as well as the SOD activity in the serum decreased in different group. This was because limonene, allicin and betaine could significantly reduce the oxidative stress damage, resulting in low concentration of catalytic substrates-free radicals, and SOD being unable to perform disproportionation reaction, whereas the CAT activity dropped as SOD activity declined. Furthermore, there were no significant differences between SOD in the liver and CAT in the serum, which might be because various antioxidant enzymes compete to respond with different degrees of antioxidative stress (Wang et al., 2019). Thus, it has been demonstrated that limonene, allicin, and betaine in a low fish meal diet may considerably minimize the degree of oxidative damage to body cells and our results are in line with the previous studies conducted on fruit fly (*Drosophila melanogaster*) (Nagpal and Abraham, 2017), Nile tilapia (*Oreochromis niloticus*) (Hamed et al., 2021), male rats (*Rattus norvegicus*) (Li et al., 2021b), broilers (Chen et al., 2021) and rats (Shan et al., 2021). These results indicated that limonene, allicin, and betaine might enhance *M. salmoides*' antioxidant capability by enhancing antioxidant enzymes and decreasing MDA levels.

Meat quality is an important feature for producers and consumers. In addition to sensory attributes (color, juiciness, and flavor) (Oliveira et al., 2017), the meat quality is reflected in its physicochemical parameters, such as WHC, pH, and nutrient composition (Maltin et al., 2007). The pH is one of the most important factors affecting many meat quality attributes, such as meat color, tenderness, the WHC and other characteristics of muscle (Cao et al., 2012). The fish meat tenderness decreased as the pH decline. Furthermore, fish color is one of the major criteria for determining freshness, with a significant influence on customer purchasing decisions (Truong et al., 2014). In this study, the pH of supplementation groups was all higher than that of the FM30 group, indicating that limonene, allicin and betaine could effectively maintain the relatively high pH in a short time, and improved the tenderness. Moreover, the a^* and b^* of fish fed the FM40 diet were significantly higher than that of fish fed the FM30 diet, whereas the a^* and b^* did not differ between the FM40 group and the supplementation groups, indicating that the low fish meal diet could affect the body color. However, limonene, allicin and betaine supplementation in low fish meal diet could restore a^* and b^* indices to the level of the normal fish meal diet, which were consistent with the earlier studies conducted on pigs (Lan et al., 2017) and chicks (Attia et al., 2009). Thus, we speculated that these are because of the antioxidant and antibacterial activity of the limonene and allicin (Bacanli et al., 2015; Costa et al., 2019; Dwivedi et al., 2019; Li D. et al., 2021). Limonene and allicin reduced the oxidation, degeneration and acidification rate of muscle, while increasing the pH. Betaine might improve the muscle pH by altering the anaerobic glycolysis and antioxidant capacity of muscle (Chen et al., 2020).

Water-holding capacity (WHC) is of great significance to the physical form, flavor and color of muscle, which can be

evaluated by thawing, refrigeration, centrifugal, cooking, drop and pressure loss. Our results showed that the refrigeration loss of fish fed FM30 + A and FM30 + B diets were significantly lower than that of fish fed FM30 and FM40 diets, and the cooking loss of fish fed FM30 + L diet was lower than that of fish fed FM30 diet, indicating that limonene, allicin and betaine could improve the flesh WHC. Earlier it has been illustrated that muscle WHC was positively correlated with the MDA content (Datta et al., 2015). Furthermore, studies have also been documented that the WHC of muscle is closely related to pH and decline in pH decreases results in lower electrostatic strength of muscle protein, which dipping the interaction between charges and the gap between myoprotein fiber and actin fiber. Water permeates from myofibrils to sarcoplasma and further into the extracellular space of muscle, resulting in increased water loss and lower WHC (Huff-Lonergan and Lonergan, 2005). In the study, the MDA content in the liver or serum in the supplementation groups were significantly lower than that of the FM30 group, and the pH was higher, indicating that limonene, allicin and betaine might improve the WHC by reducing the oxidative damage and increasing pH. Furthermore, the WHC had the highest cooking loss due to denaturation of muscle protein causing myofibril contraction, exposing more hydrophobic groups, and increasing water fluidity and eventually the water loss (Wang K. et al., 2020).

Intestinal health and integrity are directly connected to the precise fish physiological processes since it is a vital organ for nutrition absorption and utilization (Wang J. et al., 2020). In fish, intestinal villi are an important site for the secretion of digestive enzymes and nutrient absorption, therefore, the villus with regular shape and complete structure are the basic conditions to ensure fish intestinal health (Torrecillas et al., 2019; Yuan et al., 2019; Li W. et al., 2021). Crypt depth influence the process of migration, development and differentiation of tiny intestinal cells, hence influencing the process of digestion and absorption. Besides, the force of intestinal peristalsis generated from the contraction of smooth muscle, as the thickness of muscular layer increases, more will be the intestinal peristalsis which could improve the digestibility and absorption of the intestine. Previous studies have reported that allicin can improve digestion (Yan and Kim, 2013), intestinal microbiota and increase the beneficial microbiota of animals (Zhang et al., 2020; Guillamon et al., 2021). Furthermore, studies have also shown that betaine could improve intestinal barrier function (Shakeri et al., 2019) (Alhotan et al., 2021). In this study, compared with the FM30 group, lamina propria, muscular layer, serous layer, submucous layer, villus height, villus width and crypt depth of the supplementation groups were significantly higher, indicating that limonene, allicin and betaine could promote the development of intestinal villi and improve the structure of *M. salmoides* digestive tract by increasing the villi height, villi width, crypt depth and muscle thickness. However, further research is needed on whether limonene, allicin, and betaine improve intestinal structure by altering the intestinal microbiota of *M. salmoides*.

Conclusion

In conclusion, the results showed that the optimum attractant concentration of limonene, allicin and betaine was 0.2%. Adding limonene, allicin and betaine at concentration of 0.2% to the low fish meal feed could improve growth performance (increased final body weight, weight gain rate, and specific growth rate) of *M. salmoides* but only in 4 weeks. In addition, in 9th week, supplementation of limonene, allicin and betaine with concentration of 0.2% in low fishmeal feed improved antioxidant capacity in liver and serum (reduced the MDA contents.), meat quality (increased pH, a^*, and b^* values and decreased refrigeration loss, cooking loss values in the muscle) and intestinal morphology (increased villus height, lamina propria, crypt depth, submucous layer, and serous layer) of *M. salmoides*. Therefore, limonene, allicin and betaine may be recommended as promising attractants in the compound feed of *M. salmoides* and can alleviate the current shortage of fishmeal to a certain degree.

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 authors.

Ethics statement

All the experimental procedures including the animal experimentation were approved by the animal research committees of Foshan University Animal Ethics Committee (approval number: 2020056).

Author contributions

YuhuaY: conceptualization, data curation, writing - original draft. MC and XB: conducting a research and investigation process, specifically performing the experiments, or data/evidence collection. YingyY: conceptualization, supervision, methodology, writing - review & editing. WS: formulation or evolution of overarching research goals and aims. YL and YingY: writing - review & editing. HY: writing - review & editing. All authors contributed to the article and approved the submitted version.

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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.

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