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Vitamin D impacts on the intestinal health, immune status and metabolism in turbot (*Scophthalmus maximus* L.)

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Abstract

Vitamin D (VD) plays a vital role in various physiological processes in addition to its classic functions on maintaining the balance of Ca and P metabolism. However, there still are gaps to understand in depth the issues on the precise requirement, metabolic processes and physiological functions of VD in fish. In this study, we investigated the effects of VD on the growth, intestinal health, host immunity and metabolism in turbot (*Scophthalmus maximus* L.), one important commercial carnivorous fish in aquaculture, through the supplementation of different doses of dietary VD_3 (0, 200, 400, 800 and 1600 μg VD_3/kg diet). According to our results, the optimal VD_3 level in the feed for turbot growth was estimated to be around 400 μg/kg, whereas VD_3 deficiency or overdose in diets induced the intestinal inflammation, lowered the diversity of gut microbiota and impaired the host resistance to bacterial infection in turbot. Moreover, the level of $1\alpha,25(OH)_2D_3$, the active metabolite of VD_3, reached a peak value in the turbot serum in the 400 μg group, although the concentrations of Ca and phosphate in the turbot were stable in all groups. Finally, the deficiency of dietary VD_3 disturbed the nutritional metabolism in turbot, especially the metabolism of lipids and glucose. In conclusion, this study evaluated the optimal dose of dietary VD_3 for turbot and provided the evidence that VD has a significant impact on intestinal health, host immunity and nutritional metabolism in fish, which deepened our understanding on the physiological functions and metabolism of VD_3 in fish.

Key words: Vitamin D_3: Metabolism: Microbiota: Inflammation: Infection

Vitamin D_3 (VD_3) belongs to the family of steroid hormones and plays a crucial role in maintaining the metabolic homoeostasis of Ca and P and promoting bone development in animals[1]. In mammals, VD_3 is mainly metabolised and degraded by three cytochrome P450 (CYP) enzymes, 25-hydroxylase, 1 α-hydroxylase and 24-hydroxylase, which are encoded by the genes of *cyp2r1, cyp27b1, cyp24a1*, respectively[2]. Moreover, VD_3 exerts its physiological functions through its active form $1\alpha,25(OH)_2D_3$ by binding to vitamin D receptor (VDR)[1,3].

The metabolic process of VD_3 is evolutionarily conserved[2]. Previous studies demonstrated that a complete endocrine VD_3 system, including three metabolic enzymes and VDR, first appeared in fish[4]. In most terrestrial higher animals, the photolysis of 7-dehydrocholesterol and subsequent conversion to VD_3 is induced in skin by thermal isomerisation exposure to UV light at 290–320 nm[4]. In contrast, many fish live in deep water where the sunlight cannot penetrate; therefore, it is believed that VD_3 found in the fish liver and adipose tissues mainly comes from the food chain[5]. In addition, unlike mammals having only one VDR, most fish have two subtypes of VDR (*vdra* and *vdrb*)[6,7].

It has been reported that VD_3 deficiency in fish is manifested as epidermis thinning, the necrosis of underlying muscle tissues and a significant decrease in blood Ca concentrations[8]. Moreover, VD_3 contributes to increase the activity and number of osteoblasts in fish[9,10]. According to the previous reports, the minimum VD_3 supplemental levels of tilapia (*Oreochromis niloticus O. aureus*), channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) were 360, 500 and 1600 μg/kg diet, respectively[11]. The requirement of dietary VD_3 for optimum growth of Wuchang bream (*Megalobrama amblycephala*) was around 5000 μg/kg[12], while 750 and 431 μg/kg of dietary VD_3 were required for the optimum growth of marine fish orange-spotted grouper (*Epinephelus coioides*) and Japanese sea bass (*Lateolabrax japonicus*), respectively[13,14]. In addition, it has been known that fish oil as an

Abbreviations: OTU, operational taxonomic unit; VD, vitamin D; VDR, vitamin D receptor; VD_3, vitamin D_3.

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important lipid source in aquatic animal feeds is rich in VD[15]. Therefore, it is important to supplement adequate VD in fish feeds when fish oil is replaced with other lipid sources that contain much lower VD_3. On the other hand, it seems that most fish possess a high tolerance to excessive VD_3[8,16]. However, the European panel on additives and products or substances used in animal feed (FEEDAP) has authorised the maximum content of dietary VD_3 which is 3000 μg/kg feed in fish[16].

In addition to the regulation of the homoeostasis of Ca and P, VD_3 also has a wide range of immunomodulatory functions[17], and VDR is extensively expressed in immune cells, such as monocytes, dendritic cells, B cells and T cells[18,19]. It is noteworthy that $1\alpha,25(OH)_2D_3$ can promote the expression of antimicrobial peptides in macrophages[20,21], thus improving the host resistance to pathogenic infection[20,22,23]. Furthermore, the studies have also shown that VD_3 affects the immunity and antibacterial activity in fish[24–27]. Furthermore, accumulating evidence from mammalian studies also shows that VD_3 regulates the metabolism of lipids and carbohydrates, and VD_3 deficiency leads to obesity, hyperglycaemia and related metabolic syndromes[17]. Unlike mammals, fish are less able to utilise dietary carbohydrates and have a low level of insulin secretion, which are considered to be congenital 'diabetics'[28]. So far, the effects of VD_3 on the glucose metabolism of fish have not been reported. However, the study on zebrafish has demonstrated that $1\alpha,25(OH)_2D_3$ promotes fatty acid oxidation in fish adipose tissues[29].

Interestingly, VD_3 has a significant impact on the gut microbiota. The study on *VDR* knockout animals has suggested that the lack of VD_3 signalling pathways leads to an imbalance of gut microbiota and induces various metabolic diseases[30,31]. Interestingly, the synthesis of VD_3 is significantly reduced in germ-free mice[32]. However, there are no reports about the interactions between VD_3 and gut microbiota in fish.

Turbot (*Scophthalmus maximus* L.) is an important commercial carnivorous fish[33]. In this study, we determined the precise requirement of turbots for VD_3 in the feed by the addition of different doses of dietary VD_3 and explored the potential physiological functions of VD_3 in fish.

Materials and methods

Animal ethics

The Experimental Animal Ethics Committee of Ocean University of China has approved all animal care and handling procedures in the present study.

Reagents

All ingredients in the experimental diets (except casein and VD_3) were supplied by Great Seven Biotechnology Co., Ltd.; casein and VD_3 were purchased from Sigma; VD_3 was added in the form of cholecalciferol; methanol used for HPLC was purchased from Merck KGaA; all reagents used for the measurement of body and diet composition were purchased from Sinopharm; Trizol was purchased from Takara; HiScript® III RT SuperMix for qPCR was purchased from Vazyme; SYBR green qPCR kit was purchased from Accurate Biology; 4 % paraformaldehyde solution was purchased from Biosharp; the reagents for the dehydration and staining of histological sections were purchased from Thermo Scientific; QIAamp DNA Stool Mini Kit was purchased from Qiagen and PBS was purchased from Solarbio.

Fish maintenance

Turbots (*Scophthalmus maximus* L.) weighing around 13 ± 0·08 g were purchased from a commercial fish farm in Shandong Province, China. The feeding trial was carried out in a flow-through system located in Longhui Aquatic Product CO. Ltd. The fish were acclimatised in the system for 2 weeks. After the acclimation period, the fish were weighed and fasted for 24 h before they were randomly allocated to fifteen tanks (500 l) with a lid, and sixty fish per tank. Each diet was randomly assigned to triplicate tanks. The fish were fed twice daily at 07.00 and 19.00 hours, and the feeding experiment was performed for 8 weeks, since significant differences in growth were attained already at 8 weeks. The water quality during the feeding experiment was monitored as follows: the temperature ranged from 16 to 18°C; salinity was from 27 to 29‰; the concentrations of ammonia-nitrogen and nitrite were less than 0·1 mg/l and the dissolved oxygen was approximately 7 mg/l. The husbandry and handling of the fish in the present study were performed strictly according to the Management Rule of Laboratory Animals (Chinese order no. 676 of the State Council, revised 1 March, 2017).

Diet formulation

The previous reports showed that the requirement of dietary VD_3 for the optimum growth of marine fish, such as orange-spotted grouper and Japanese sea bass, was 750 and 431 μg/kg, respectively[13,14]. Accordingly, five formulations of experimental diets with different VD_3 contents (0, 200, 400, 800 and 1600 μg/kg) were designed in our study. The composition of the experimental diets is shown in Table 1. Briefly, casein and gelatin were utilised as the dietary protein sources, and crystal amino acids were added to meet the basic nutritional requirements of turbots[34]. Fish oil, soyabean oil and soya lecithin were added as the primary lipid source. All the ingredients were ground into a fine powder through a 120-mesh sieve. After VD_3 was completely blended with other ingredients, fish oil and soyabean oil were further kneaded with the premixed ingredients. After the oil was fully mixed with all ingredients, some water (100 g/kg diet) was added to increase the diet viscosity. Finally, a manual granulator was used to pelletise the diets. The diets were dried in the shade and stored at –20°C until further use.

Sampling

At the end of the feeding experiment, all the fish fasted for 24 h. Turbot from each group were anaesthetised with 20 mg/l tricaine and weighed. The liver from each fish was collected and weighed for the calculation of the hepatosomatic index. Serum, liver and gut samples from three fish per tank were collected and frozen in liquid N_2 immediately, followed by storage at −80°C for further analysis. Another three fish from each tank were euthanised with 20 mg/l tricaine and stored at −80°C for the measurement of body composition.

Table 1. Ingredients and proximate compositions of the basal diet (g/kg)

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Casein (vitamin free)	380	380	380	380	380
Dextrin	280	280	280	280	280
Gelatin	95	95	95	95	95
Fish oil	65	65	65	65	65
Amino acid mixture*	54	54	54	54	54
Soyabean oil	28	28	28	28	28
Soya lecithin	25	25	25	25	25
Monocalcium phosphate	25	25	25	25	25
Vitamin premix (vitamin D_3 free)†	15	15	15	15	15
Mineral premix‡	15	15	15	15	15
Attractants§	10	10	10	10	10
Choline chloride	5	5	5	5	5
Calcium propionate	1	1	1	1	1
Yttrium oxide	1	1	1	1	1
Ethoxy quinoline	1	1	1	1	1
Vitamin D_3 (μg/kg)	0	200	400	800	1600
Proximate composition (% DM)					
DM	93·58	93·09	93·74	93·52	93·58
Crude protein	52·21	52·53	52·15	52·60	52·36
Crude lipid	12·43	12·64	12·53	12·48	12·39
Vitamin D_3 (μg/kg)	3·53	190·35	380·46	789·23	1549·22

* Amino acid premix (g/100 g diet): arginine, 1·69; histidine, 0·55; isoleucine, 0·22, leucine, 0·14; lysine, 0·73; phenylalanine, 0·50; threonine, 0·61; valine, 0·13; alanine, 1·32; aspartic acid, 1·63; glycine, 1·62; serine, 0·42; cystine, 0·40; and tyrosine, 0·10.
† Vitamin premix (mg/kg diet): retinyl acetate, 32; *a*-tocopheryl acetate, 240; menadione sodium bisulphite, 10; ascorbic acid, 120; cyanocobalamin, 10; biotin, 60; choline dihydrogen citrate, 7000; folic acid, 20; inositol, 800; niacin, 200; D-calcium pantothenate, 60; pyridoxine HCl, 20; riboflavin, 45; thiamine HCl, 25; microcrystalline cellulose, 16 473.
‡ Mineral premix (mg/kg diet): $MgSO_4{\cdot}7H_2O$, 1200; $CuSO_4{\cdot}5H_2O$, 10; $FeSO_4{\cdot}7H_2O$, 80; $ZnSO_4{\cdot}H_2O$, 50; $MnSO_4{\cdot}H_2O$, 45; $CoCl_2$, 5; Na_2SeO_3, 20; calcium iodine, 60; zeolite powder, 8485.
§ Attractant (g/kg diet): betaine, 4; DMPT, 2; threonine, 2; glycine, 1; inosine-5′-diphosphate trisodium salt, 1.

The measurement of VD_3 in the diets

The contents of VD_3 in the diets were confirmed by HPLC as described previously[35]. Briefly, 0·2 g of the diet was mixed with 1 ml of ascorbate ethanol solution (5 g/l) and 200 μl of potassium hydroxide solution (500 g/l), and the mixture was saponified at 50°C for 4 h. After that, 2 ml of ethyl ether was added into the saponification solution and followed by the centrifugation for 10 min at 7000 ***g*** for three times. The ethyl ether phase after the centrifugation was collected and redissolved with 300 μl of methanol after nitrogen blowing. The HPLC conditions were as follows: mobile phase methanol, flow rate 1 ml/min, column temperature 25°C and detection wavelength 265 nm. As shown in Table 1, the actual contents of VD_3 in five diets were 3·53, 190·35, 380·46, 789·23 and 1549·22 μg/kg, respectively.

The measurement of 1α,25(OH)$_2$D$_3$ in serum

The concentrations of 1*α*,25(OH)$_2$D$_3$ in fish serum were measured with the 1,25-Dihydroxyvitamin D_3 (Calcitriol) ELISA Kit (Abbexa) according to the manufacturer's instructions.

The measurement of Ca and phosphate in serum

The concentrations of Ca and phosphate in fish serum were measured with the Calcium Assay Kit (Jiancheng Biotech Co.) and Phosphate Assay Kit (Jiancheng Biotech Co.) according to the manufacturer's instructions.

The composition analysis of fish body and diets

The body composition of turbots was confirmed by previously described methods[36]. Briefly, the samples were dried at 105° C to determine the moisture contents. Besides, the contents of crude protein and lipids were measured by Kjeldahl method of nitrogen determination (FOSS, Sweden) and soxhlet ether extraction (Buchi, Switzerland), respectively. The ash content of the samples was assessed by burning in a muffle furnace for 10 h.

Quantitative real-time PCR analysis

The liver and hindgut samples from three turbots per tank (nine fish per group) were collected as described above. Total mRNA was isolated using Trizol, and cDNA was synthesised from total RNA by PrimeScript RT reagent kit according to the manufacturer's instructions. The real-time PCR was performed in a thermo-cycler CFX96 instrument (BioRad), and the expression of target genes was normalised to *β-actin*. The sequences of all primers used in the present study are provided in Table 2.

Transcriptomic analysis

To further identify the potential effects of VD deficiency on turbot, two groups, that is, VD deficiency (0 μg/kg) and VD optimum (400 μg/kg), were compared in this experiment. Briefly, 1 μg RNA extracted from the livers of the turbots in 0 μg and 400 μg groups (three turbots per tank and total nine fish per group) was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB), and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina). After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

Feature Counts v1.5.0-p3 was used to count the read numbers mapped to each gene. Furthermore, fragments per kilobase per million mapped reads (FPKM) of each gene was calculated based on the length of the gene and read count mapped to this gene to calculate the gene expression level.

Differential expression analysis of two groups was performed using the DESeq2 R package (1.16.1). DESeq2 provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. Genes with an adjusted *P*-value < 0·05 found by DESeq2 were assigned as differentially expressed. Kyoto Encyclopedia of Genes and Genomes is a database resource for understanding high-level functions and utilities of the biological system. The cluster Profiler R package was used to test the statistical enrichment of differential expression genes in Kyoto Encyclopedia of Genes and Genomes pathways.

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Table 2. Primer sequences used for qRT-PCR

Gene	NCBI accession No.	Forward	Reverse
cyp2r1	XM_035629276.1	CGCCTTCCCTTGGATTGAGT	CCGCTCTTGTATGTTGGGGT
cyp27b1	XM_035632718.1	GAGTTCTATCGCTTCGGCCT	AGCACGGTCAACACCTCATC
cyp24a1	XM_035632779.1	TTCGTTGGCAGAGTTGGACA	ATCTGTCGGCGTTCCTCTTC
fgf23	XM_035603866.1	CCATCTGCCTCAAAGACGAA	GTCCGACTCAGGTAGACGCT
il-1β	XM_035640817.1	GAGAGCATCGTGGAAGAACA	GTTTCGGACCAGAACGAAGT
il-8	XM_035638412.1	GGCAGACCCCTTGAAGAATA	TGGTGAACCCTTCCCATTAT
tnfα	XM_035638223.1	GGGTGGATGTGGAAGGTGAT	GGCCTCTGTTTGGCTTGACT
il-6	XM_035621201.1	TTCTTTTATCCCAACCCCGC	TTCTGGTCCCGCTTCGTTTC
occludin	KU238182.1	CGTGCGTTGCCTCCACTCTC	CTCCCACTCCGCCCATCTGC
zo-1	KU238184.1	CCCAAGAGGAGAAGAAGTAA	TCAAAATGTGTCCGAATGTA
trpv6	XM_035643442.1	AGGACAATCTGCGGCTGG	TGGACGATGGGGGGAGTA
slc20a2	XM_035641206.1	CCTTGGCTTCGTCATTGC	ATACACCCTGCCGTTTGG
β-actin	XM_035614479.1	GCGTGACATCAAGGAGAAGC	TGGAAGGTGGACAGGGAAGC

The assessment of gut micromorphology

The hindguts of three turbots per tank (nine fish per group) were fixed with paraformaldehyde (BL539A, Biosharp) for 24 h and transferred to 75 % alcohol for the preservation. The fixed tissue around 1 cm long was cut, dehydrated routinely with a series of alcohols and embedded in paraffin. Tissue sections of approximately 7 microns were then cut, placed on slides and stained with haematoxylin and eosin. The slides were examined under a light microscope (Olympus, DP72) equipped with a camera (Nikon E600) and Cell Sens Standard Software (Olympus).

Analysis of gut microbiota

Total genome DNA of the whole gut from the turbots in 0, 400 and 800 μg groups (three turbots per tank and total nine fish per group) was extracted using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's protocols. 16S rDNA of distinct regions (16S V3-V4) was amplified using specific primers. All PCR were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs).

Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced by an Illumina NovaSeq platform.

Sequences analysis was performed by Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/)[37]. Sequences with ≥ 97 % similarity were assigned to the same operational taxonomic units (OTU). The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva Database (http://www.arb-silva.de/)[38] was used based on the Mother algorithm to annotate taxonomic information. OTU abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. Beta diversity analysis was used to evaluate differences of samples in species complexity.

Bacterial challenge

In order to further verify the difference of anti-infectious ability of turbot under different VD conditions (deficiency, optimum and excess), ten fish from 0, 400 and 800 μg groups were randomly selected for a bacterial challenge at the end of the feeding trial. *Edwardsiella tarda* was isolated from diseased turbot and determined by sequencing. For challenge experiments, bacterial cultures were centrifuged at 8000 ***g*** for 1 min, and the pellet was subsequently suspended in PBS to a final 2×10^7 CFU/ml before use.

After fasted for 24 h, the turbots were anaesthetised with tricaine (20 mg/l). The weight of each fish was recorded before intraperitoneal injection with 0·5 ml PBS containing 2×10^7 CFU/ml *E. tarda* using medical syringes. Fish mortality was monitored four times per day until 7th day, and the final weight of each fish was recorded. The average daily weight loss was calculated by (final weight – initial weight)/days.

Calculations and statistical analysis

The growth parameters were calculated as follows: weight growth rate (%) = 100 × (final individual weight – initial individual weight)/initial individual weight; specific growth rate (% per d) = 100 × (Ln final individual weight – Ln initial individual weight)/number of days; viscerosomatic index (%) = 100 × viscera weight of final individual fish/final individual weight (g); hepatosomatic index (%) = 100 × liver weight of final individual fish/final individual weight; survival rate (%) = 100 × (final number of fish)/(initial number of fish). The broken-line regression model and the quadratic regression model were used to estimate the optimum VD_3 requirement of turbot based on the weight growth rate.

Results were presented as means with their standard error of the mean unless otherwise stated. Raw data were analysed by the one-way ANOVA after normality and homogeneity of variance was confirmed. Multiple comparisons were conducted with Tukey's post-hoc test. $P < 0·05$ was considered as statistical significance. Statistical analysis was performed using the GraphPad Prism 9 (GraphPad Software Inc.).

Result

The growth and body composition of the turbot

To identify the optimum dose of VD_3 addition in the diet for turbot growth, the juvenile turbots with average initial weight 13 ± 0·08 g were fed with the diets containing five different levels of

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Table 3. The effects of different dietary vitamin D_3 contents on growth indices* (Mean values with their standard errors of the mean)

	Dietary vitamin D_3 contents (μg/kg)									
	0		200		400		800		1600	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Final weight (g)	48·61	1·35[a]	50·95	1·15[a]	57·60	1·70[b]	53·95	1·08[ab]	54·21	0·95[ab]
WGR (%)†	168·49	3·20[a]	182·28	3·16[b]	201·25	4·04[c]	200·46	3·45[c]	194·93	3·48 [bc]
SGR (%/d)‡	1·74	0·05[a]	1·82	0·04[a]	2·04	0·05[b]	1·94	0·03[ab]	1·93	0·04[ab]
VSI (%)§	5·00	0·14[a]	4·63	0·07[ab]	4·57	0·06[b]	4·57	0·09[b]	4·57	0·57[b]
HSI (%)‖	1·10	0·05	1·02	0·06	1·00	0·03	0·95	0·04	1·03	0·08
Survival rate (%)¶	100		100		100		100		100	

* *n* 9 fish. Values in the same row sharing the same superscript letter are not significantly different determined by the one-way ANOVA.
† WGR (%): weight growth rate (%) = 100 × (final individual weight (g) – initial individual weight (g))/initial individual weight.
‡ SGR (% per d): specific growth rate (% per d) = 100 × (Ln final individual weight (g) – Ln initial individual weight (g))/number of days.
§ VSI (%): viscerosomatic index (%) = 100 × viscera weight (g) of final individual fish/final individual weight (g).
‖ HIS (%): hepatosomatic index (%) = 100 × liver weight (g) of final individual fish/final individual weight (g).
¶ Survival rate (%) = 100 × (final number of fish)/(initial number of fish).

VD_3: 0, 200, 400, 800 and 1600 μg/kg, that is, 0, 5, 10, 20 and 40 μg/kg for 8 weeks. The feed efficiency calculated as [(final body weight − initial body weight)/feed intake] for each group was 0·74, 0·77, 0·78, 0·75 and 0·80, respectively, which was quite similar among all groups. As the results showed in Table 3, the fish fed with 400 μg/kg VD_3 in the diet displayed the highest values of final body weight, specific growth rate and weight growth rate. The broken-line model showed the optimal dietary requirement of VD_3 was 397·01 μg/kg under the conditions of this study (Fig. 1(a)). Further analysis based on a regression model also exhibited a significant correction between weight growth rate and dietary VD contents, and the optimal requirement of turbot for dietary VD_3 was estimated as 846·25 μg/kg according to the quadratic regression model (Fig. 1(b)). Besides, the viscerosomatic index of the fish was lowest at 400 μg diet, while the hepatosomatic index did not differ significantly among the groups. Furthermore, there was no significant difference in crude protein, water content and ash content between the treatment groups, except that the contents of crude fat in the fish fed 400 μg/kg dietary VD_3 were significantly lower than those in the 0 μg group (Table 4).

The histological structure of turbot hindgut

Moreover, we analysed the effects of VD_3 on the morphology of fish guts. According to our results, the hindgut anatomy of turbots fed with different doses of VD_3 displayed no difference (Fig. 2(a)). The villus height (Fig. 2(b)), enterocytes height (Fig. 2(c)) and lumen diameter (Fig. 2(d)) of the turbot hindguts in all groups exhibited no significant difference. Besides, the gene expression of *occludin* and *zo-1*, which are the markers of intestinal barrier, did not change significantly in all groups (Fig. 2(e) and (f)).

The composition and diversity of gut microbiota in the turbot

On the other hand, the compositions of gut microbiota in 0, 400 and 800 μg groups were analysed. A total number of 665 735 clean reads were generated, covering 5829 OTU (97 % similarity level) after the sequence quality control. Venn diagram showed that three groups shared 2448 OTU, and the number of unique

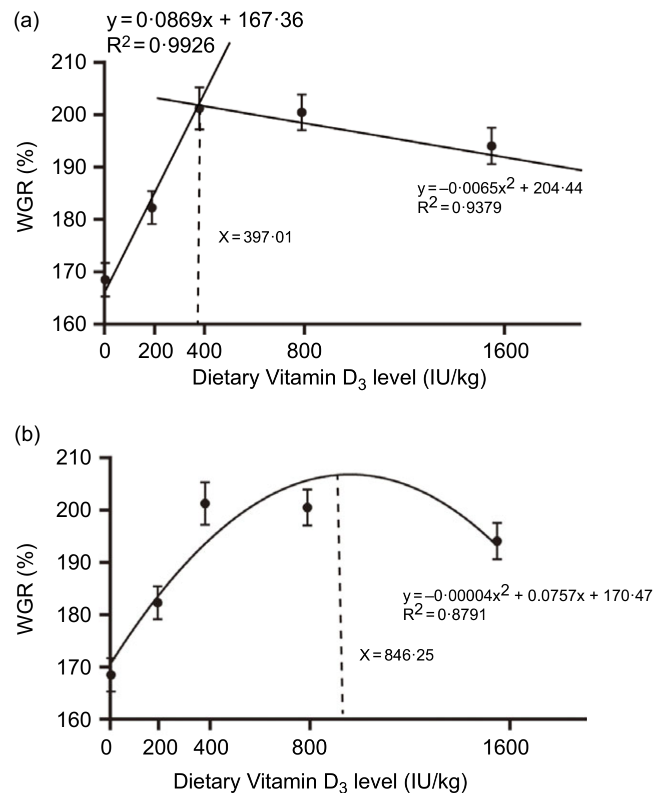

Fig. 1. Effects of dietary VD_3 levels on WGR of turbot. (a) Based on the broken-line model of WGR corresponding to dietary VD_3 contents (y = 0·0869x + 167·36, R^2 = 0·9926; y = − 0·0065x + 204·44, R^2 = 0·9379), the optimum level of dietary VD_3 for juvenile turbot was estimated to be 397·01 μg/kg. (b) Based on the quadratic regression model of WGR corresponding to dietary VD_3 contents (y = –0·00004x^2 + 0·0757x + 170·47, R^2 = 0·8791), the optimum level of dietary VD_3 for juvenile turbot was estimated to be 846·25 μg/kg. Error bars were indicated as means and standard deviations (*n* = 24 fish).

OTU in 0, 400 and 800 μg diets was 416, 1166 and 393, respectively (Fig. 3(a)). Interestingly, the fish fed 400 μg/kg VD_3 in the diets exhibited the highest diversity of gut microbiota (Fig. 3(b)). In addition, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were identified as the predominant bacterial phyla in the guts of turbots from all groups. The relative abundance of *Cyanobacteria*,

Table 4. The effects of different dietary vitamin D_3 contents on the body composition of turbots (dry weight)* (Mean values with their standard errors of the mean)

	Dietary vitamin D_3 contents (μg/kg)									
	0		200		400		800		1600	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Moisture (%)	77·87	0·47	77·82	0·31	77·62	0·51	77·50	0·43	77·34	0·14
Crude lipid (%)	13·7	$0{\cdot}72^{a}$	12·57	$0{\cdot}90^{ab}$	12·4	$0{\cdot}29^{b}$	13·87	$0{\cdot}86^{ab}$	13·76	$0{\cdot}29^{ab}$
Crude protein (%)	71·31	$0{\cdot}25^{ab}$	71·09	$1{\cdot}45^{ab}$	69·45	$0{\cdot}07^{a}$	73·71	$0{\cdot}40^{b}$	73·76	$0{\cdot}30^{a}$
Ash (%)	16·94	0·07	17·07	0·22	16·71	0·00	17·33	0·02	17·10	0·19

* *n* 3 of replicates of nine fish. Values in the same row sharing the same superscript letter are not significantly different determined by the one-way ANOVA.

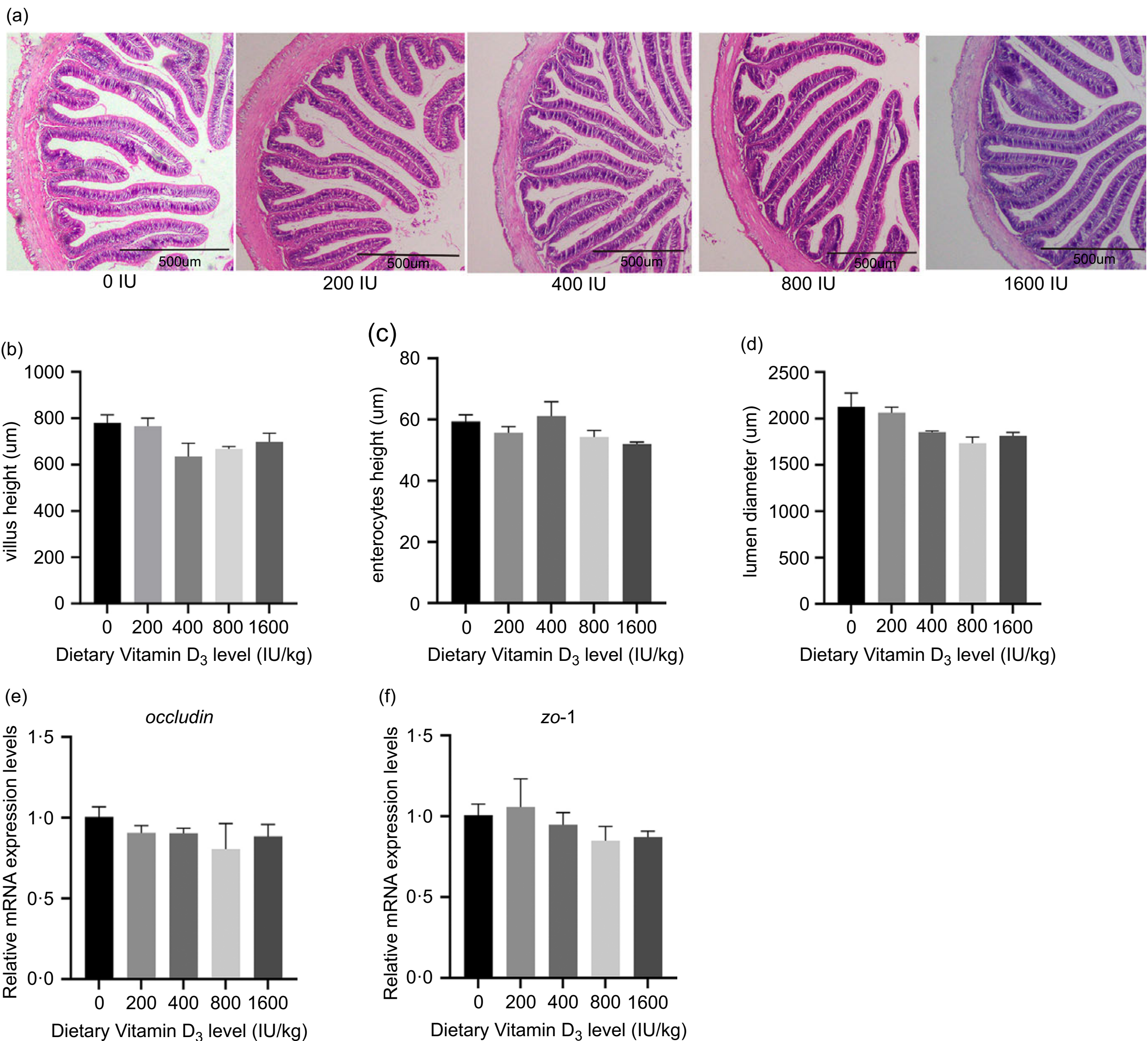

Fig. 2. Effects of dietary VD_3 contents on the hindgut histology of turbot. (a) The hindguts of turbot in different VD_3 groups were collected and sectioned. After the fixation by haematoxylin and eosin (H&E), the hindgut morphology in different groups was observed. The images were representative of at least three independent experiments. (b)–(d) The micromorphology, including villus height (b), enterocyte height (c) and lumen diameters (d) of the turbot guts in three groups was evaluated. (e), (f) The gene expression of *occludin* and *zo-1* in the hindgut of the turbots fed with different VD_3 doses was analysed by RT-PCR (*n* 9 fish). Error bars indicate means with their standard error of the mean. *zo-1*, zona occludens-1.

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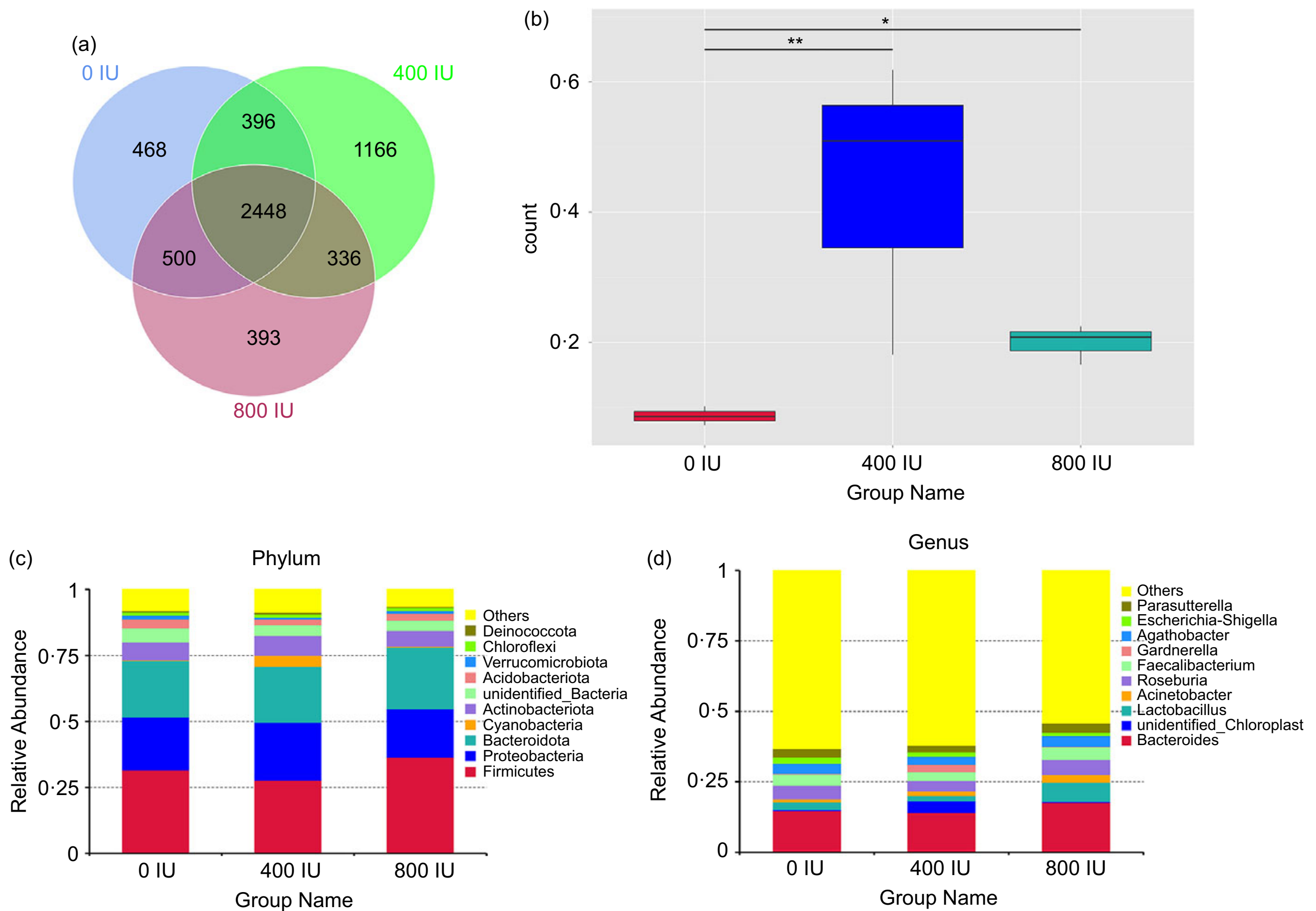

Fig. 3. Effects of dietary VD_3 contents on the composition and diversity of gut microbiota of turbot. (a) Every circle in Venn diagram represents one group. The value from the overlapping part of different circles represents the shared OTU between groups, and the value from the non-overlapping part of one circle represents the unique OTU of that group. (b) The beta diversity index of intestinal microbiota from three groups (0, 400, 800 μg) was calculated. Error bars indicate means with their standard error of the mean; $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. (c), (d) The taxonomy classification of reads at the phylum (c) or genus (d) taxonomic level. Only top ten most abundant (based on the relative abundance) bacterial phyla or genera were shown. Other phyla or genera were all assigned as 'Others', *n* 9 fish.

Acidobacteriota and *Deinococcota* was significantly improved in the gut of the turbots in the 400 μg group compared with that in the other two groups. Moreover, the lower relative abundances of *Firmicutes* in the 400 μg group were found compared with that in the 0 μg group, although no statistical difference was observed (Fig. 3(c)). At the genus level, the 800 μg diet increased the relative abundance of *Bacteroides*, *Acinetobacter* and *Lactobacillus*. Meanwhile, the 400 μg diet reduced relative abundances of the *Roseburia* and *Faecalibacterium* but improved relative abundances of *Gardnerella* than the 0 μg diet (Fig. 3(d)).

Intestinal inflammation and anti-infectious ability of the turbot

In the following experiments, we measured the gene expression of several inflammatory cytokines, including *il-1β*, *il-8 il-6* and *tnf-α* in the liver and hindgut of turbots in the five groups. As shown in Fig. 4(a), the gene expression of all pro-inflammatory cytokines was down-regulated in the liver of turbot in the 400 μg group compared with other groups. The similar results were obtained in the hindgut of the turbots, except that the gene expression of *il-6* in the 800 μg group was lower than that in the 400 μg group (Fig. 4(b)).

According to our results in bacterial challenge, the survival rate of the fish in the 400 μg group was around 80 % at 6th day after infection, compared with 30 % in the 0 μg group and 20 % in the 800 μg group (Fig. 4(c)). Corresponding to the mortality, the daily weight loss of turbots in the 0 μg and 800 μg groups was also significantly higher than that in the 400 μg group (Fig. 4(d)).

Vitamin D metabolism in the turbot

First, the gene expression of VD metabolic enzymes, including *cyp2r1*, *cyp27b1* and *cyp24a1* in different tissues of juvenile turbots, was analysed. According to our results, the genes of *cyp2r1*, *cyp27b1* and *cyp24a1* were mainly expressed in the liver, and a lower abundance of *cyp27b1* transcript in other tissues was also detected (Fig. 5(a)). We further measured the contents of $1\alpha,25(OH)_2D_3$ in the turbot sera of all groups, and the results demonstrated that the sera from the turbot in the 400 μg group contained the maximum level of $1\alpha,25(OH)_2D_3$ (Fig. 5(b)). Consistently, the gene expression of *cyp2r1* and *cyp27b1*

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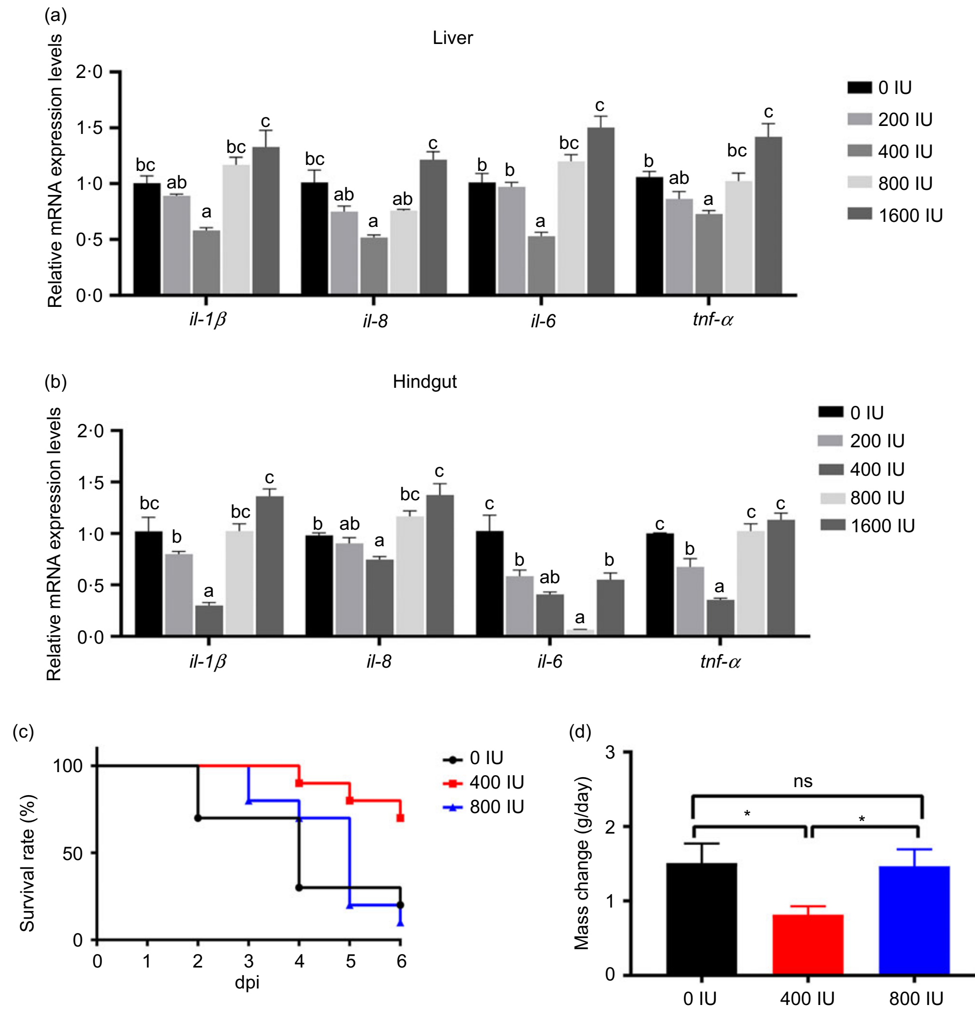

Fig. 4. Effects of dietary VD_3 contents on immunity and anti-infection ability of turbot. (a), (b) The gene expression of inflammatory cytokines in the liver and hindgut of turbots from different VD_3 groups. The gene expression of inflammatory cytokines in the liver (a) and hindgut (b) of turbot in different VD_3 groups was analysed by RT-PCR (*n* 9 fish). Error bars indicate means with their standard error of the mean. The different letters above the bars indicate significant differences. *il-1β*, interleukin-1beta; *il-8*, interleukin-8; *il-6*, interleukin-6; *tnf-α*, tumour necrosis factor-alpha. (c), (d) The mortality and weight loss in the infected turbots fed with different VD_3 doses. Ten turbots were randomly selected in 0, 400 and 800 μg VD_3 groups at the end of feeding experiment, and each fish was intraperitoneally injected *E. tarda* (1×10^7 bacteria per fish). (C) The survival rates of the turbots were recorded every 24 h in 6 d (*n* 10). (D) The daily mass change of each turbot before death was calculated. Error bars indicate means with their standard error of the mean ± SEM. *$P < 0·05$.

reached the highest level in the livers of the turbots in the 400 μg group (Fig. 5(c) and (d)). In contrast, the gene expression of *cyp24a1* was lower in the 200 μg and 400 μg groups, compared with that in 0, 800 and 1600 μg groups (Fig. 5(e)). In addition, the gene expression of fibroblast growth factor 23 (*fgf23*), a factor for negative feedback during VD_3 metabolism, was elevated with the increase of VD_3 concentration in the diets (Fig. 5(f)).

The concentrations of Ca and phosphate in the turbot

Considering the well-known effects of VD_3 on the regulation of Ca and P homoeostasis in animals, the concentrations of Ca and phosphate in the turbot sera were analysed. It appeared that the concentrations of Ca (Fig. 6(a)) and phosphate (Fig. 6(b)) were at a similar level in turbot sera from all groups, regardless of the VD_3 dose in the diets. However, the gene expression of Ca transporter *trpv6* and P transporter *slc20a2* in the hindgut of turbots from different groups showed an opposite trend to $1\alpha,25(OH)_2D_3$ content in turbot sera (Fig. 6(c) and (d)).

The metabolism of lipids and carbohydrates in the turbot

To further identify the potential effects of VD deficiency on turbot, the transcriptomic sequencing of turbot livers from two groups, that is, VD deficiency (0 μg/kg) and VD optimum (400 μg/kg) groups, was performed. As the results showed, a total of 1175 genes were differentially expressed (adjusted by *P*-value < 0·05) between two groups. Among these genes, the transcripts of 454 genes were up-regulated, while the transcripts of 721 genes were down-regulated in the 400 μg group compared with those in the 0 μg group (Fig. 7(a)). The enrichment results of Kyoto Encyclopedia of Genes and Genomes metabolic pathways showed that a series of pathways in nutritional metabolism were significantly influenced, such as fatty acid biosynthesis, PPAR signalling pathway, protein export and amino

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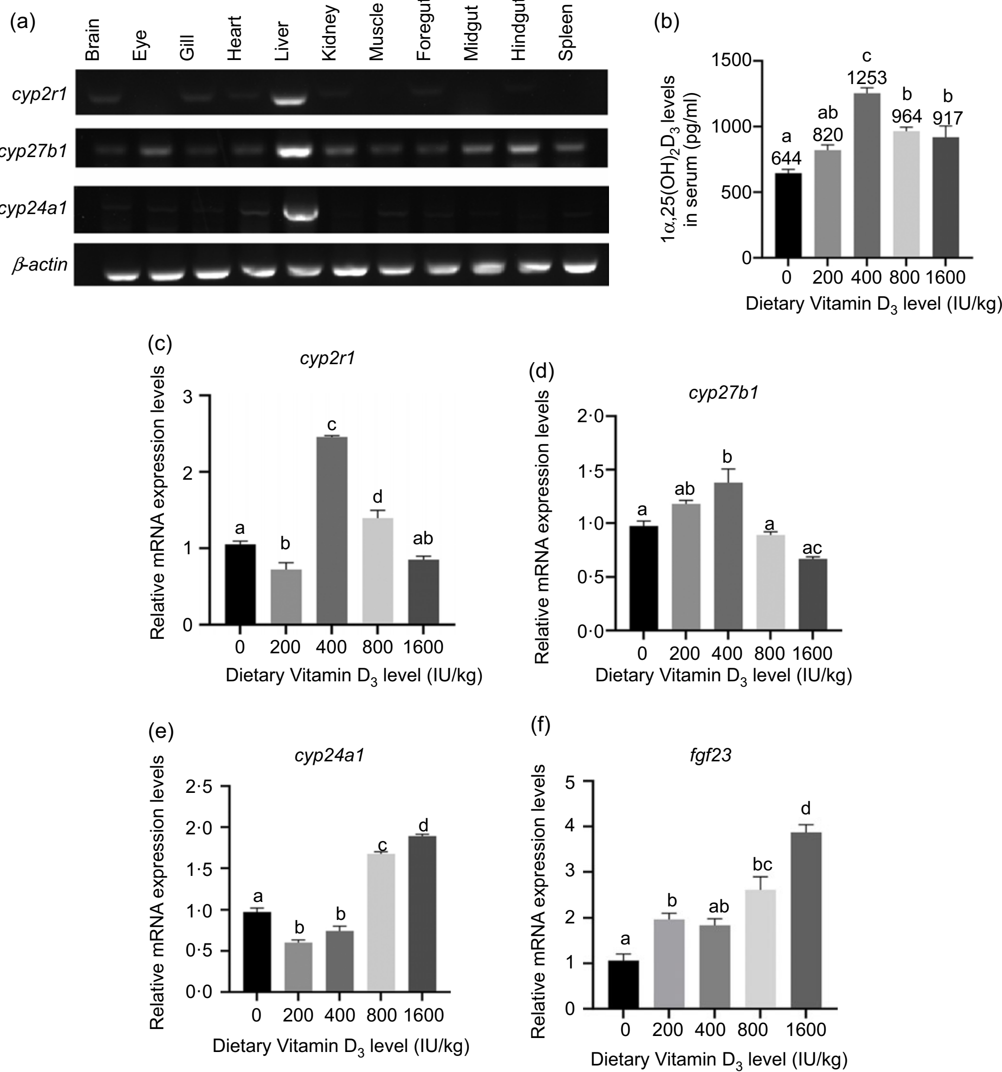

Fig. 5. VD metabolism of turbot fed with different dietary VD_3. (a) The gene expression of *cyp2r1, cyp27b1, cyp24a1* in different tissues of juvenile turbots was measured by PCR. The image was representative of at least three independent experiments. (b) $1\alpha,25(OH)_2D_3$ concentrations in the serum of turbots fed with different VD_3 were determined by ELISA (*n* 9 fish). (c)–(f) The gene expression of *cyp2r1* (C), *cyp27b1* (D), *cyp24a1* (E), and *fgf23* (F) in the livers of the turbots fed with different VD_3 diets was analysed by RT-PCR (*n* 9 fish). Error bars indicate means with their standard error of the mean. The different letters above the bars indicate significant differences. *cyp2r1*, cytochrome P450, family 2, subfamily R, polypeptide 1; *cyp27b1*, cytochrome P450, family 27, subfamily B, polypeptide 1; *cyp24a1*, cytochrome P450, family 24, subfamily A, polypeptide 1; *fgf23*, fibroblast growth factor 23.

sugar metabolism (Fig. 7(b)). The heatmap of differentially expressed genes also revealed that many key genes in lipid metabolism (Fig. 7(c)) and glucose metabolism (Fig. 7(d)) changed significantly in VD-deficient group.

Discussion

In this study, we confirmed that VD_3 has extensive effects on different physiological processes in fish. We identified that dietary VD_3 influenced the growth, intestinal health and pathogen resistance in turbot, although the homoeostasis of Ca and phosphate appeared not affected. Moreover, we found that the nutritional metabolism was disturbed in the turbots with VD_3 deficiency in the diets. We have demonstrated for the first time that VD_3 influences the composition and diversity of gut flora in fish. To our knowledge, this is also the first study to investigate the effects of dietary VD_3 on the metabolism of VD_3 itself in fish.

The significance of VD_3 in fish has been reviewed by Lock and co-workers[8]. Actually, Barnett *et al.* proved for the first time the importance of VD_3 addition in fish feeds[39]. In this study we attempted to investigate the effects of VD on turbot from three levels of dietary VD_3: deficiency, optimum and excess. According to our results, the turbot fed with 400 IU/kg dietary VD_3 displayed the optimal growth performance (Fig. 1(a)), inflammatory status (Fig. 4(a) and (b)) and the highest concentration of the active VD_3 metabolite in serum (Fig. 5(b)) in all five groups, hence the group fed with 400 IU/kg VD_3 was considered as the optimum one. In the analysis of intestinal microbiota and anti-infectious ability of turbot, the fish fed with 0, 400 and 800

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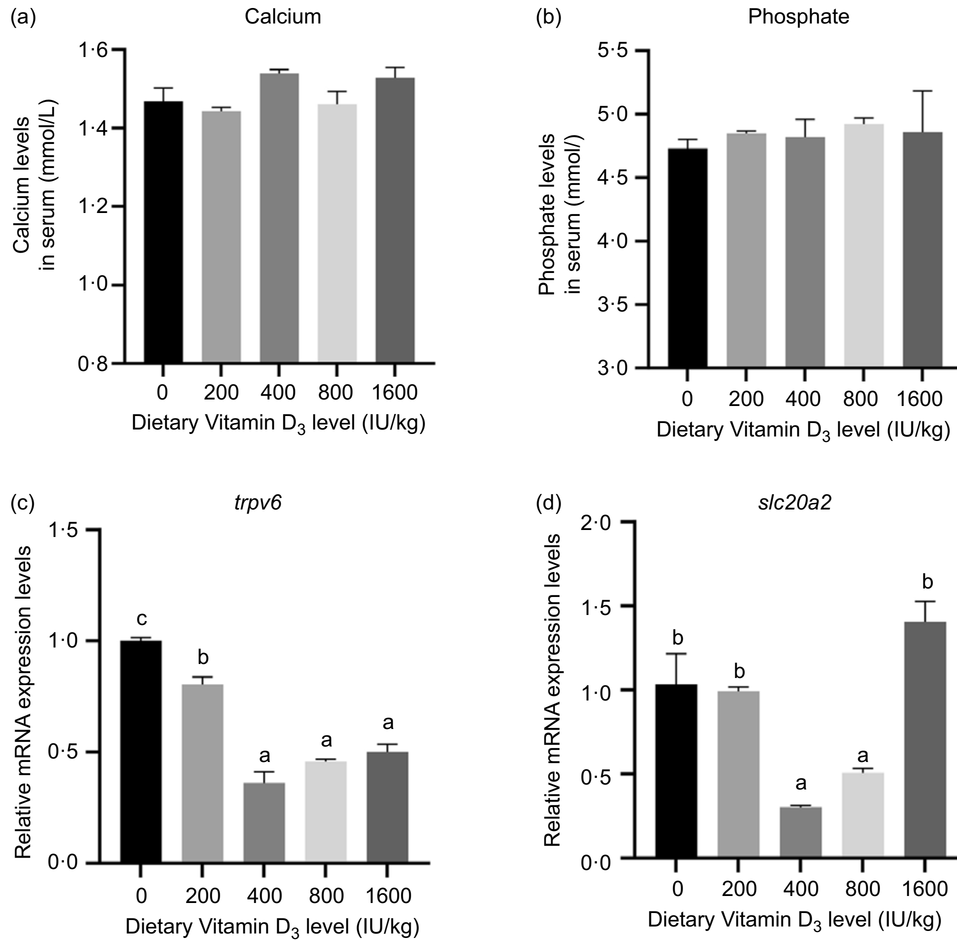

Fig. 6. Ca and phosphate metabolism of turbot in different VD_3 groups. (a), (b) The concentrations of Ca (a) and phosphate (b) in serum of the turbots with different dietary VD_3 levels were analysed (*n* 9 fish). (c), (d) The gene expression of *trpv6* and *slc20a2* in the hindgut of the turbots fed with different VD_3 doses was analysed by RT-PCR (*n* 9 fish). Error bars indicate means with their standard error of the mean. The different letters above the bars indicate significant differences. *trpv6*, transient receptor potential cation channel, subfamily V, member 6; *slc20a2*, solute carrier family 20 member 2.

IU/kg VD_3 were selected to be representative of VD_3 deficiency, optimum and excess, respectively. To further investigate the effects of VD deficiency on turbot, two groups, i.e., VD deficiency (0 IU/kg) and VD optimum (400 IU/kg) were compared in transcriptomic analysis.

Based on the prediction by the broken-line model, the optimal requirement of dietary VD_3 in the feed for the growth of juvenile turbot is around 400 μg/kg (Fig. 1(a)), which is close to marine fish orange-spotted grouper[13] and Japanese sea bass[14]. It has been known that the predicted requirement of a nutrient for the maximal growth of animals could be greatly different depending on the selected mathematical model. Usually, the broken-line model predicts the nutrient requirement that is lower than that predicted by curvilinear curve-fitting procedures[40]. As our results showed, the optimal dietary VD_3 requirement predicted by quadratic regression model for the growth of juvenile turbot is 846·25 μg/kg (Fig. 1(b)). However, it seems the broken-line model fits better the data in our study considering the R^2 value is less than 0·90 in the quadratic regression model.

In fact, dietary VD_3 requirement of cultured fish greatly varies in different experiments. For example, the dietary VD_3 requirement of freshwater species Wuchang bream (initial weight was 17·71 ± 0·22 g) was estimated to be around 5000 μg/kg using the second-order polynomial regression model[12]. As predicted by the broken-line model based on the weight gain, the dietary VD_3 requirement of juvenile black carp (*Mylopharyngodon piceus*, initial weight 4·73 ± 0·13 g) and tilapia (initial weight around 0·80 g) was 534·2 IU/kg[41] and 374·8 IU/kg[42], respectively. Surprisingly, no significant difference in the growth and body composition was detected when the 21-d-old fry of freshwater species Rora (*Labeo rohita*, initial weight around 0·1 g), were fed with VD_3 deficient or 1650 IU/kg VD_3 supplemented feeds[43]. In addition, the study on the marine fish orange-spotted grouper (initial weight 81·5 ± 0·1 g) showed that the optimum addition level of dietary VD_3 was 750·19 IU/kg estimated by the broken-line model based on WGR[13]. The vast disparities among the different experiments may be caused by the various species and developmental stage of cultured fish. Considering the previous report that the fish fed with diets low in fish meal and high in plant protein seemed to require more dietary VD_3 to reach the optimal growth[44,45], the different composition of the feeds should be taken into account when the optimal requirement of dietary VD_3 for cultured fish was evaluated.

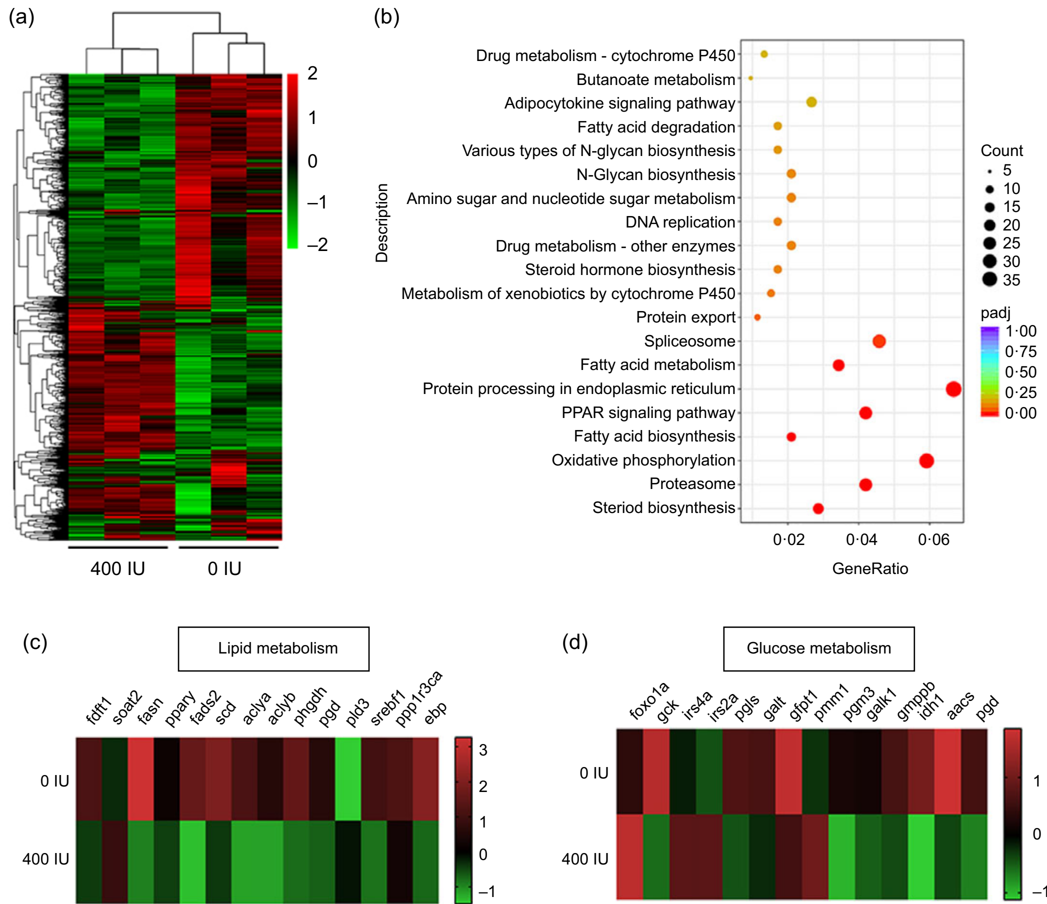

Fig. 7. Effects of dietary VD_3 deficiency on the turbot liver. Transcriptomic analysis of the liver from turbot fed with 0 μg and 400 μg VD_3 diet was conducted. (a) Cluster analysis of different gene changes between 0 and 400 μg vitamin D_3 treatment. (b) The top twenty statistics of KEGG pathway enrichment for differentially expressed genes (DEG). GeneRatio is the ratio of number of differentially expressed genes enriched in a certain pathway to total number of DEG. (c), (d) The heatmap of differentially expressed genes in the metabolism of lipids (C) and glucose (D). *n* 9 fish. The list genes include farnesyl-diphosphate farnesyltransferase 1 *(fdft1)*, sterol O-acyltransferase 2 *(soat2)*, fatty acid synthase *(fasn)*, peroxisome proliferator-activated receptor gamma *(ppary)*, fatty acid desaturase 2 *(fads2)*, stearoyl-CoA desaturase *(scd)*, ATP citrate lyase a *(aclya)*, ATP citrate lyase b *(aclyb)*, phosphoglycerate dehydrogenase *(phgdh)*, phospholipase D family member 3 *(pld3)*, sterol regulatory element binding transcription factor 1 *(srebf1)*, protein phosphatase 1*(ppp1r3ca)*, EBP cholestenol delta-isomerase *(ebp)*, forkhead box O1 a *(foxo1a)*, glucokinase *(gck)*, insulin receptor substrate 4a *(irs4a)*, insulin receptor substrate 2a *(irs2a)*; 6-phosphogluconolactonase *(pgls)*, galactose-1-phosphate uridylyltransferase *(galt)*, glutamine–fructose-6-phosphate transaminase 1 *(gfpt1)*, phosphomannomutase 1 *(pmm1)*, phosphoglucomutase 3 *(pgm3)*, galactokinase 1 *(galk1)*, GDP-mannose pyrophosphorylase B *(gmppb)*, isocitrate dehydrogenase (NADP(+)) 1 *(idh1)*, acetoacetyl-CoA synthetase *(aacs)*, phosphogluconate dehydro *(pgd)*.

On the other hand, it seemed that most fish exhibited high tolerance to excessive VD_3 in the diet, since many fish did not display significant impairment on their growth[16,46]. Similarly, our result showed that the growth performance and the intestinal anatomy of turbot were not significantly influenced when the higher doses of VD_3 up to 1600 μg/kg were added. However, when the fish were fed with the diets containing the higher doses of VD_3 than 400 μg/kg, the inflammation was induced in the gut and liver (Fig. 4). More importantly, the pathogen resistance of the fish in the 800 μg group was significantly lower than that in the 400 μg group, which confirmed that the immune status of the fish in the 800 μg group was impaired. A recent report has also demonstrated that the lower or higher than adequate dose of dietary VD_3 exhibits adverse effects on antioxidant capacities and innates immunity in black carp[41]. According to the perspective of 'precise nutrition', we claim that although most fish seem tolerant to much higher doses of VD_3 in the diets based on their growth performance, the immune status and other physiological functions might be impaired when the higher doses of VD_3 were fed to the fish. It is noteworthy that FEEDAP Panel has authorized the maximum content of dietary VD_3 is 3000 IU/kg feed in fish[16]. Nonetheless, some previous studies have demonstrated high doses of VD_3 are beneficial to the innate immunity of the fish[9,27,47]. The discrepancy could be caused by the difference in the species and the methods how to prepare VD_3-containing diets.

Previous studies have proved the functions of VD_3 on anti-inflammation and host immune regulation. For example, the VD/VDR pathway was involved in protecting the intestinal barrier during colon inflammation and relieved the symptoms of dextran sulphate sodium-induced colitis in mice[48]. The study on Atlantic salmon demonstrated that the co-incubation of VD_3 with macrophages reduced the adhesion of *Aeromonas salmonicida subsp. salmonicida* to macrophages[26]. Meanwhile, dietary VD_3 increased the activity of lysozyme in serum and

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the expression of hepcidin in the liver of juvenile black carp[41,46]. The study on European perch (*Dicentrarchus labrax* L.) also showed that VD_3 increased the phagocytosis of leucocytes in the head kidney, while inhibited the expression of *il-1β* in the head kidney and intestinal tract[49]. Furthermore, the addition of dietary VD_3 inhibited the up-regulation of pro-inflammatory cytokines induced by bacterial infection in yellow catfish[25] and Jian carp (*Cyprinus carpio var. jian*)[47]. Consistently, our results demonstrated that the gene expression of the pro-inflammatory cytokines, including il-*1β*, *il-8*, *il-6* and *tnf-α*, was significantly lower in the liver and gut of the turbots in the 400 μg group (Fig. 4(a) and (b)), indicating that VD_3 deficiency or overdose could induce inflammation in liver/gut axis in fish. In addition, our results also demonstrated that VD_3 significantly improved the anti-infection ability of turbots (Fig. 4(c)), and our recent published report has depicted the molecular mechanisms how VD_3 enhances the pathogen resistance in turbot[50]. It is well known that VD has a large impact on innate immunity[51]. For example, VD_3 significantly enhanced the expression of antimicrobial peptides in human macrophages[21] and in the fish cells[49,52]. Interestingly, the VD_3/VDR-type I interferon axis seems involved in the immunomodulatory functions of VD_3 in yellow catfish[27].

Our result from transcriptomic analysis in the turbot livers clearly demonstrated the metabolism of fatty acids and carbohydrates were interfered in the VD_3-deficient group (Fig. 7). In accord with this result, the crude fat contents in turbots fed VD_3-deficient diet significantly increased compared with those in the fish fed 400 μg/kg dietary VD_3 (Table 4), and the decrement on body weight was lowest in the 400 μg group during the bacterial infection **(**Fig. 4(d)**)**. In fact, the studies in higher animals have shown that VD_3 deficiency is closely related to obesity, hyperglycaemia and related metabolic syndromes[53]. Consistently, the transcriptomic analysis showed that $1\alpha,25(OH)_2D_3$ significantly affected the lipid metabolism pathway in the early embryo of zebrafish[54]. Furthermore, significant fat accumulation was also observed in *cyp2r1* knockout zebrafish, and the promotion of fatty acid oxidation by VD_3 in fish was also confirmed[29]. Importantly, it has been well known that gut microbiota plays a vital role in maintaining the homoeostasis of the host intestinal environment, and its imbalance often leads to various metabolic diseases[55]. The studies in mammals have provided the evidence that VDR is a key genetic factor for shaping the host microbiome[31,56,57]. Our results showed that the fish fed VD-deficient diet exhibited the lowest diversity of gut microbiome, and the addition of VD_3 in the diets shifted the composition of gut microbiome in turbots, increasing the abundance of the beneficial bacteria, including *lactobacillus* (Fig. 3). A recent report claimed that VDR affected the metabolism of carbohydrates, proteins/amino acids, lipids and exogenous organisms by regulating microbial metabolites[58]. Our experiments on zebrafish also demonstrated that the regulation of lipid and glucose metabolism by dietary VD_3 was dependent on intestinal flora (unpublished results). It would be intriguing to further clarify how VD/VDR signalling pathway impacts the nutritional metabolism via the regulation of microbial metabolites in fish.

Regarding to VD metabolism in fish, several points are different from that in mammals. Firstly, $1\alpha,25(OH)_2D_3$ is mainly synthesised in mammalian kidney, while $1\alpha,25(OH)_2D_3$ synthase (encoded by *cyp27b1*) is also expressed in fish liver, suggesting that the liver could be the primary source of $1\alpha,25(OH)_2D_3$ in fish[59,60]. Moreover, $1\alpha,25(OH)_2D_3$ is the primary circulating form of VD_3 metabolite in fish, instead of $25(OH)D_3$ in mammals[61]. Consistent with the previous reports, the gene expression of *cyp27b1* in turbots was mainly detected in liver, and a much lower abundance of *cyp27b1* transcript was also identified in kidney and gut (Fig. 5(a)). In addition, we discovered that the content of $1\alpha,25(OH)_2D_3$ in turbot serum reached the highest level when 400 μg/kg VD_3 was added in the diets, and the absence or overdoses of VD_3 in the diets lowered the serum contents of $1\alpha,25(OH)_2D_3$ in turbots (Fig. 5(b)).

In fact, VD metabolism is strictly regulated in higher animals, and fgf23 has been identified as a negative regulator of VD metabolism in higher animals[62]. When the content of $1\alpha,25(OH)_2D_3$ is too high *in vivo*, fgf23 inhibits the expression of cyp27b1 and reduces the synthesis of $1\alpha,25(OH)_2D_3$ to prevent poisoning[63]. Based on our results that the expression of *cyp2r1* and *cyp27b1* decreased and the expression of *cyp24a1* increased in liver when the fish were fed with the higher doses of VD_3 than 400 μg/kg in the diets, we inferred that it might be caused by the increase in *fgf23* expression when the fish were fed with dietary overdoses of VD_3, leading to the reduced $1\alpha,25(OH)_2D_3$ productions. In addition to fgf23, the evidence has showed that gut microbiota also influences VD_3 metabolism, since germ-free mice have lower serum levels of $25(OH)D_3$ than those in conventional mice[32]. Hence, further studies are worth to be conducted to clarify how the gut microbiota affect VD_3 metabolism in fish.

As early as the 1920s, VD_3 was identified to prevent rickets[64]. So far, the regulation of Ca and P homoeostasis by VD_3 in land animals and in fish has been extensively studied. Our results demonstrated that the concentrations of Ca and phosphate in turbot sera were stable, regardless of VD_3 doses in the diets (Fig. 6(a) and (b)). It is noteworthy that $1\alpha,25(OH)_2D_3$ concentration was still around 640 pg/ml in the serum of the turbot fed with 0 μg/kg dietary VD_3 for 2 months (Fig. 5b)). Different from mammals, it is believed that fish cannot synthesise VD_3 *in vivo*; they acquire VD_3 via food chain[65]. According to our analysis, there still was a very low amount of VD_3 (3·53 μg/kg) in the VD_3 absent diet, which could come from the fish oil in the diet and contribute to the source of $1\alpha,25(OH)_2D_3$ in the turbot serum from 0 μg group. A previous study has also shown that the bone development of European sea bass (*Dicentrarchus labrax*) seems not to be affected by the low dietary VD_3[66]. Thus, the basal content of $1\alpha,25(OH)_2D_3$ in the serum of fish in the 0 μg group could involve in the regulation of Ca and phosphate homoeostasis in fish. Moreover, the expression of Ca and P transporters was increased when the contents of $1\alpha,25(OH)_2D_3$ were lowered in turbot sera, which could also be beneficial to the maintenance of Ca and phosphate homoeostasis in fish.

Conclusion

Our study assessed the optimal VD_3 requirement in the feed for turbot and demonstrated the effects of dietary VD_3 on intestinal health, anti-infection ability and metabolism in fish. The results in

the present study deepened our understanding on the physiological functions and metabolism of VD_3 in fish and provided a reference to the evaluation of precise requirement for dietary VD_3 in aquatic animals.

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R. S. designed and performed the experiments, analysed the data and wrote the manuscript; J. L., Y. L., X. L., J. Z. and W. X. performed the experiments; K. M. supervised the project; Q. A. supervised the project and wrote the manuscript; M. W. supervised the project, designed the experiments, analysed the data and wrote the manuscript.

The authors declare no competing financial interests.

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