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Evaluation of *Lactococcus lactis* HNL12 combined with *Schizochytrium limacinum* algal meal in diets for humpback grouper (*Cromileptes altivelis*)

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ABSTRACT

The humpback grouper (*Cromileptes altivelis*) is a commercially valuable species of the family Epinephelidae; however, its marketization suffers from slow growth speed, low survival rate, and various pathogenic diseases. *Lactococcus lactis* and *Schizochytrium limacinum* are commonly used as immunostimulants due to their health benefits for the aquatic organisms. In the present study, we assessed the effects of dietary supplementation with *L. lactis* HNL12 combined with *S. limacinum* algal meal on the growth performances, innate immune response, and disease resistance of *C. altivelis* against *Vibrio harveyi*. The results showed that fish fed with a combination diet of *L. lactis* and *S. limacinum* exhibited significantly higher final weight, percent weight gain, and specific growth rate compared with groups fed with them alone. A bacterial challenge experiment indicated that the group fed with the *L. lactis* combined with *S. limacinum* diet achieved the highest relative percent of survival value (68.63%), suggesting that *L. lactis* and *S. limacinum* significantly improved the disease resistance against *V. harveyi* after a 4-week feeding trial. Moreover, the respiratory burst activity of macrophages of fish fed with a *L. lactis* combined with *S. limacinum* diet was significantly higher than that of fish fed the control diet after 1, 2, and 3 weeks of feeding. The serum superoxide dismutase of fish fed with a *L. lactis* combined with *S. limacinum* diet significantly increased compared to those fed the control diet after 1 and 2 weeks of feeding, while the serum alkaline phosphatase of fish fed with a *L. lactis* combined with *S. limacinum* diet after 2 and 4 weeks was significantly increased, compared to the control group. The serum lysozyme activities of fish fed with a *L. lactis* combined with *S. limacinum* diet significantly increased compared to the control group after 2 weeks of feeding. Furthermore, transcriptome sequencing of the *C. altivelis* head kidney was conducted to explore the immune-regulating effects of the *L. lactis* combined with *S. limacinum* diet on *C. altivelis*. A total of 86,919 unigenes, annotated by at least one of the reference databases (Nr, Swiss-Prot, GO, COG, and KEGG), were assembly yielded by *de novo* transcriptome. In addition, 157 putative differentially expressed genes (DEGs) were identified between the *L. lactis* combined with *S. limacinum* group and the control group. For pathway enrichment, the DEGs were categorized into nine KEGG pathways, which were mainly related to infective diseases, antigen processing and presentation, digestive system, and other immune system responses. The findings of this study suggest that the *L. lactis* combined with *S. limacinum* diet can induce positive effects on the growth, immunity, and disease resistance of *C. altivelis* against *V. harveyi*. This study expands our understanding of the synergistic combinations of probiotics and prebiotics in aquaculture.

1. Introduction

The humpback grouper (*Cromileptes altivelis*) is a member of Percifomes, Serranidae, Epinephelinae, *Cromileptes*, and is a favored commercially valuable species with high market value due to its unique shape, beautiful color, delicious flavor, and high nutritional value [1–3]. Recently, the population of wild *C. altivelis* is increasingly exploited, while at the same time, artificial *C. altivelis* cultures suffer from many problems. Slow growth speed, low survive rate, and various pathogenic diseases are the major limiting factors of *C. altivelis* farming [3–5]. Therefore, healthy and safe alternatives to improve the growth performance and immunity of *C. altivelis* are urgently required.

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Currently, both prebiotics and probiotics are the main candidates as environmentally safe feed supplements in aquaculture [6]. Numerous prebiotics have been reported to confer health benefits to aquatic organisms [7–9]. Microalgae are important prebiotics, since they contain many different polysaccharides with prebiotic potential [10,11]. Microalgae are widely consumed as feed supplement and are commercially available as microalgae algal meal for aquatic animal feed [10–13]. The microalgae algal meal is enriched in protein, vitamins, mineral trace elements, bioactive substances, and a variety of unsaturated fatty acids such as eicosapentaenoic acid (EPA) and twenty-two carbon hexaenoic acid (DHA) [14,15]. Numerous reports showed that the microalgae algal meal as a feed supplement induces positive effects on growth, feed utilization, protein digestibility, immune responses, and disease resistance in cultured fish species [11,15–18]. With regard to the microalgae used in this study, *Schizochytrium limacinum* is one of the main species of seawater microalgae and well known for its enriched high levels of lipids and polyunsaturated fatty acids (PUFAs), particularly, its high level of DHA (30–70%) [19,20]. The *S. limacinum* algal meal as a prominent sustainable source of n-3 and n-6 long-chain PUFAs has been applied to various aquatic animals and has been reported to affect the host by stimulating growth and enhancing immunity [19–24]. To date, *S. limacinum* has not been applied as feed supplement for *C. altivelis* aquaculture.

In addition to prebiotics, probiotics are well known to have important roles for enhancing the growth performance and disease resistance of fish and may be used as alternative to antibiotics due to the production of inhibitory compounds [25–27]. Probiotics are defined viable microorganisms, and sufficient amounts of these reach the intestine in an active state where they exert positive health effects [28]. Lactic acid bacteria (LAB) are most commonly used as probiotics in fish and specific LAB, such as, *Lactobacillus* spp. and *Lactococcus* spp., have been supported by an increasing number of *in vitro* and *in vivo* experiments to enhance both innate and systemic immunity [29–32]. In our previous work, the probiotic strain *Lactococcus lactis* HNL12, which was isolated from the gut of wild *C. altivelis*, was investigated as a dietary supplement and was found to be effective in improving the growth response, immunity, and disease resistance against *Vibrio harveyi* in *C. altivelis* [33]. In fish, most available studies focused on the effects of microalgae or probiotics as the dietary supplements individually; however, the available information regarding the application of synergistic combinations of microalgae and probiotics is limited [34,35]. Taking into account the previous data, this study evaluated the effects of dietary supplementation with *L. lactis* HNL12 combined with *S. limacinum* on growth performances, innate immune response, and disease resistance of *C. altivelis* against *V. harveyi*. This is the first study to examine the influences of synergistic combinations of prebiotics and probiotics to improve growth, immunity, and disease resistance of *C. altivelis*.

2. Materials and methods

2.1. Experimental diets

Commercial feed was used as basal diet, the composition of which is listed in Table S1. The *S. limacinum* algal meal was supplemented to a basal diet at the 1% level. Briefly, 1 g of the *S. limacinum* algal meal was diluted with 20 ml phosphate-buffered saline solution (PBS; pH = 7.2) and thoroughly homogenized, then sprayed onto 100 g of the basal diet.

The probiotic strain *L. lactis* HNL12 was kept in our laboratory and administering 10^8 CFU/g *L. lactis* HNL12 was previously identified as effective to enhance growth, immunity, and disease resistance of *C. altivelis* [33]. The diet used in this study added 10^8 CFU/g *L. lactis* HNL12, prepared as previously described [33]. Briefly, the final dose of 10^8 CFU/g of *L. lactis* HNL12 was diluted with PBS, thoroughly homogenized, and then uniformly sprayed onto the basal diet. This diet was prepared fresh each week to ensure the quantity of *L. lactis* HNL12[Table S1].

2.2. Fish and experimental design

Healthy juvenile *C. altivelis* (average weight: 3.94 ± 0.43 g) were provided by the Hong Yuan fishery company of Sanya (Hainan province, China) where all experiments were conducted. Prior to the experiment, fish were fed with commercial feed for 2 weeks. A total of 120 fish were randomly distributed into four tanks (30 fish per tank) with recirculating aerated seawater, which would be further used in the growth performance analysis and the challenge test. Fish were fed four different diets: basal diet added PBS (control), basal diet added 10^8 CFU/g *L. lactis* HNL12 (*L. lactis*), basal diet added 1% *S. limacinum* (*S. limacinum*), and basal diet added 10^8 CFU/g *L. lactis* HNL12 combined with 1% *S. limacinum* (*L. lactis* combined with *S. limacinum*). The experimental diets were prepared and dried at 25 °C with the aid of an air conditioner, then stored at 4 °C until use. Fish were fed respective diets twice a day at 7:00 a.m. and 18:00 p.m. at a 3% feed rate of body weight. Uneaten food and waste were removed before each feeding and the feeding experiment lasted for 4 weeks. The experiments were repeated once. To evaluate the non-specific immune responses, young *C. altivelis* (average weight: 24.66 ± 0.53 g) were randomly grouped (25 fish/group) and administrated as mentioned above.

2.3. Fish growth performance

At the beginning of the feeding trial, all juvenile *C. altivelis* were weighted to calculate the initial weight. After 4 weeks of feeding trial, all fish were starved for 24 h and then weighted to calculate the final weight, percent weight gain (PWG), and specific growth rate (SGR) according to the following formulas:

Percent weight gain (%) (PWG) = 100 × [(Final weight - Initial weight)/Initial weight]; Specific growth rate (%) (SGR) = 100 × [Ln (Final weight) - Ln (Initial weight)]/Experimental days.

2.4. Sample preparation and non-specific immune responses analysis

2.4.1. Sampling

After 1, 2, 3, and 4 weeks of feeding, five young *C. altivelis* of each group (average weight: 24.66 ± 0.53 g) were randomly chosen for the measurement of non-specific immune parameters. Blood was withdrawn from each fish using 1 ml syringe and stored at 4 °C for overnight, and centrifuged at 800 × *g* for 15 min at 4 °C. Then the serum was collected to assess the non-specific immune parameters. The head kidney was excised and macrophages were harvested as previously described [33]. Cell viability was evaluated using the trypan blue exclusion test.

2.4.2. Respiratory burst activity

The respiratory burst activity produced by macrophages of the head kidney was measured according to the nitroblue tetrazolium (NBT, Sigma Aldrich) assay as previously described by Hong et al. [36]. The spectrophotometric result was measured at 620 nm and KOH/DMSO was used as blank.

2.4.3. Serum immune activities

The activities of superoxide dismutase (SOD), alkaline phosphatase (AKP), and lysozyme (LZM) in the serum were determined according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.5. Challenge test

The pathogenic *V. harveyi* strain QT520 has been responsible for mortalities of the golden pompano [37]. The challenge test was performed after 4 weeks of feeding trial. Twenty-five juvenile fish (average

weight: 3.94 ± 0.43 g) from each group were randomly selected and intraperitoneally injected with 100 μL of *V. harveyi* at a LD_{50} dose of 1×10^6 CFU/mL. Then, the mortality was monitored for 14 days after injection. The relative percent of survival (RPS) was calculated as RPS = [1 - (% mortality in treatment fish/% mortality in control fish)] × 100. The challenge test was repeated once.

2.6. Sample collection, total RNA extraction, and qualification

To investigate the immune response of fish fed with the diets added *L. lactis* combined with *S. limacinum* or not, transcriptomic analysis was performed. Based on the results of non-specific immune parameters, the fish (average weight: 22.32 ± 0.47 g) fed with the diets added *L. lactis* combined with *S. limacinum* or not for 14 days were used in transcriptomic analysis. Briefly, head kidneys were picked and pooled from six fish from the experimental and control group above, which were fed with the diets added *L. lactis* combined with *S. limacinum* or PBS for 14 days, and kept in liquid nitrogen for further RNA extraction. Extraction of total RNA was performed by a HP total RNA Kit (Omega Bio-tek, Doraville, GA, USA) following the manual. The quality and integrity of the extracted RNA were determined by agarose gel electrophoresis, and the concentration was measured with the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then, an equal amount of the extracted RNA per group was used for further analysis of gene expression.

2.7. cDNA library preparation and mRNA sequencing

To construct a cDNA library, the mRNA was enriched with oligo-dT-attached magnetic beads and fragmented into small pieces. First-strand cDNA was synthesized using mRNA fragments as templates with random hexamers. Second-strand cDNA was synthesized with DNA polymerase I, dNTPs, buffer, and RNase H. Subsequently, double-stranded cDNA was purified, end-repaired, added a poly (A) tail, and ligated the Illumina's paired-end adapters. A suitable template size range was selected using agarose gel electrophoresis and the template was amplified by PCR. The resulting paired-end cDNA library was sequenced on an Illumina HiSeq 2000 platform by Beijing Novogene Bioinformatics.

2.8. Data filtering, de novo assembly, and annotation

Clean reads were obtained by removing adapter sequences and ambiguous or low-quality nucleotides from raw reads, and assembled by Trinity software to receive the transcripts according to the guidelines suggested by manufactures. These transcripts were analyzed using Corset software to obtain unigenes. To understand the functional information, unigenes were annotated against the following public databases: NCBI non-redundant (Nr) protein, Swiss-Prot, Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and EuKaryotic Orthologous Groups (KOG). The Blast2GO program was performed to annotate unigenes to three main GO categories (biological processes, molecular functions, and cellular components) based on the Nr annotations.

2.9. Differentially expressed genes (DEGs) analysis

Clean reads from each sample were mapped back onto the assembled transcriptome using RSEM software. In order to obtain gene expression levels, the number of aligned clean reads in different unigenes/treatment conditions was normalized by the reads per kilobase of exon model per million mapped reads (RPKM) method. The differentially expressed genes (DEGs) between two samples were identified through the method of DEGseq package in R. Stringent criteria with a q value ≤ 0.005 and $|\log_2\text{FoldChange}| \geq 1$. The GO and KEGG enrichment analyses were performed by hypergeometric tests and the results were used to identify over-represented functional genes and pathways. A threshold of the corrected P-value < 0.05 was used to determine the significantly enriched putative DEGs.

Table 1
Primers used in this study.

Primer name	Primer sequences (5′-3′)
IgM-F	AAAGGACGACTGGAGGAGCG
IgM-R	TCTACAGGCGTTGTGGTATGGA
MHCIα-F	TCGACCAAATGAAGACGACACC
MHCIα-R	ACAAGACAACCAGCACCACACC
MHCIIα-F	GGCCAGCCTGTCTTTCAGTCCA
MHCIIα-R	CAGCAGTCTGCCAGCCGATGTT
TFRC-F	GCCTTTTCTGGCATTCCCTC
TFRC-R	GGACCAGCCTGAGCACCAT
MAP3K5F	GCCTCAGCACATCTCCACCC
MAP3K5-R	GGAAGCCAAACAGCACGACC
NR4A1-F	CAGAAAGAGGACGCCAGTGACA
NR4A1-R	GCGAGACGCAAGATGAAGAGTT
TAX1BP1–F	TGGGACAGCAATGTGGTGTGTATC
TAX1BP1-R	GGCGGTTTGTCCATCGTTCA
HERC4-F	CCTCTCCTACGACCAGCAGTACC
HERC4-R	GCTCAGGGCCTTTGTCAGCAT
TNFα-F	GTGAGAAGGCTCTTGAAGGGACA
TNFα-R	TGCATCTGAATAGCAGGGAGGT
TLR2-F	GCTGGGTGGAAAACTTCTTGG
TLR2-R	AGGAAGGAAGTCCCGCTTGT
β-actin-F	CGCTGACAGGATGCAGAAGG
β-actin-R	TGAAGTTGTTGGGCGTTTGG

2.10. Validation of RNA-seq data by quantitative real time PCR (qPCR)

Quantitative real time PCR (qPCR) analysis of 10 selected DEGs was performed to validate the obtained RNA-seq data. RNA was extracted from head kidney samples of both experimental and control groups as described above. cDNA synthesis was performed as previously reported [38]. qPCR was performed using the SYBR ExScript qRT-PCR Kit (Promega, Madison, USA) on the QuantStudio™ 6 Flex Real-Time PCR System (ABI, Singapore). Gene specific primers used for qPCR were designed and are listed in Table 1 *β-actin* was used as internal control to assess the relative mRNA transcript levels as described in our previous study [33]. The assay was repeated three times.

2.11. Statistical analysis

Data were analyzed by SPSS 17.0 software (SPSS. Inc., Chicago, IL, USA). Significant differences were detected by One-way ANOVA analysis. The results were considered statistically significant at P-values < 0.05.

3. Results

3.1. Growth performance

The data on the growth performance of *C. altivelis* subjected to experimental diets are shown in Table 2. After 4 weeks of feeding, statistical analysis showed that the fish fed with the diets that contained *L. lactis* alone, *S. limacinum* alone, or *L. lactis* combined with *S. limacinum* had significantly increased (P < 0.05) final length, final weight, PWG, and SGR (Table 2), compared with the control group. In addition, the final weight, PWG, and SGR of fish fed with *L. lactis* combined with *S. limacinum* were significantly higher than that of fish fed with *L. lactis* or *S. limacinum* alone (P < 0.05).

3.2. Non-specific immune responses assays

The results of the respiratory burst activity from head kidney macrophages are presented in Fig. 1A. The respiratory burst activity of

Table 2
Weight gain, percent weight gain (%) (PWG), specific growth rate (%) (SGR) of *Cromileptes altivelis* fed with diets containing *Lactococcus lactis*, *Schizochytrium limacinum*, or *L. lactis* combined with *S. limacinum* for 4 weeks.

	L. lactis	*S. limacinum*	*L. lactis* combined with *S. limacinum*	Control
Initial length (cm)	8.35 ± 0.65[a]	8.39 ± 0.45[a]	8.40 ± 0.49[a]	8.42 ± 0.65[a]
Final length (cm)	10.56 ± 0.22[b]	9.75 ± 0.63[b]	10.28 ± 0.33[b]	9.37 ± 0.42[a]
Initial weight (g)	3.89 ± 0.33[a]	4.00 ± 0.44[a]	3.97 ± 0.39[a]	3.95 ± 0.40[a]
Final weight (g)	13.78 ± 1.05[b]	14.68 ± 0.78[b]	15.69 ± 0.45[c]	10.27 ± 0.94[a]
PWG (%)	254.24 ± 21.82[b]	267.16 ± 19.51[b]	294.82 ± 11.30[c]	160.00 ± 13.50[a]
SGR (%)	4.52 ± 0.41[b]	4.64 ± 0.19[b]	4.90 ± 0.10[c]	3.41 ± 0.31[a]

Note: Data (mean ± standard deviation) at the same sampling time with different letters significantly differ ($P < 0.05$) among treatments.

fish fed with diet of *L. lactis* combined with *S. limacinum* was significant higher after 1, 2, and 3 weeks of feeding compared with the control group ($P < 0.05$). Moreover, significantly higher respiratory burst activity was observed in the *L. lactis* combined with *S. limacinum* group after 3 weeks, compared with the groups fed with *L. lactis* or *S. limacinum* alone ($P < 0.05$).

As shown in Fig. 1B, the SOD activities in the serum of fish fed three experimental diets were significantly higher than that of fish fed the control diet after 1 week ($P < 0.05$). At week 2, a significantly increase ($P < 0.05$) of SOD activity was observed in fish fed with *S. limacinum* or *L. lactis* combined with *S. limacinum* diet, compared with the *L. lactis* or control diet.

Compared with the control group, the AKP activity was increased significantly ($P < 0.05$) in fish fed with *S. limacinum* diet among 4 weeks of feeding (Fig. 1C). At the end of week 2 and week 4, all three experimental diets exhibited significant higher AKP activity ($P < 0.05$) than the control group. In addition, after 2 weeks of feeding, fish fed with the *L. lactis* combined with *S. limacinum* diet caused a significant ($P < 0.05$) increase in this parameter compared with fish fed with *L. lactis* or *S. limacinum* alone.

The LZM activities in *L. lactis* group and *L. lactis* combined with *S. limacinum* group were significantly higher than those of the control group or *S. limacinum* group after 2 weeks of feeding ($P < 0.05$) (Fig. 1D). In contrast, fish fed with *S. limacinum* showed no statistically significant difference in LZM activity ($P > 0.05$) among 4 weeks of feeding, compared with the control group.

3.3. Disease resistance

After 4 weeks of feeding trial, fish were challenged by *V. harveyi* and their mortality was recorded daily. At 14 days after the challenge, all groups of fish reached a steady state and no further mortality was monitored afterwards. These results showed that the protection against *V. harveyi* infection of fish fed with the experimental diets was significantly higher than in the control group ($P < 0.05$ or $P < 0.01$) (Fig. 2). The mean survival rates of fish fed with diets containing *L. lactis* alone, *S. limacinum* alone, *L. lactis* combined with *S. limacinum*, or control (PBS) were 72%, 74%, 80%, and 36%, respectively. Therefore, fish fed with the diet containing *L. lactis* combined with *S. limacinum* exhibited the highest RPS value (68.63%), while the RPS of fish fed diets added with either *L. lactis* or *S. limacinum* addition alone were 56.08% and 59.41%, respectively.

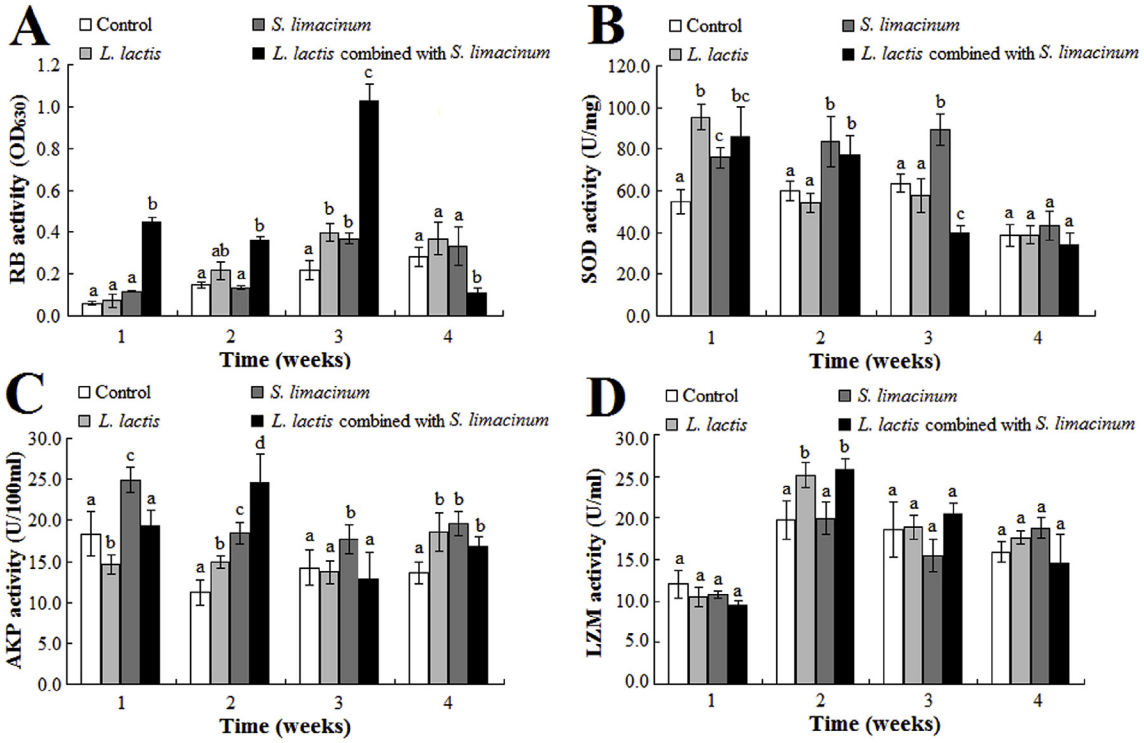

Fig. 1. The effect of different experimental diets on the respiratory burst (RB) activity (A), superoxide dismutase (SOD) activity (B), alkaline phosphatase (AKP) activity (C), lysozyme (LZM) activity (D) in the serum of *Chromileptes altivelis*. Each bar represents the mean value from five determinations with the standard deviation (SD). Significant difference ($P < 0.05$) between the experimental group and control group at the same sampling time was denoted by different letters.

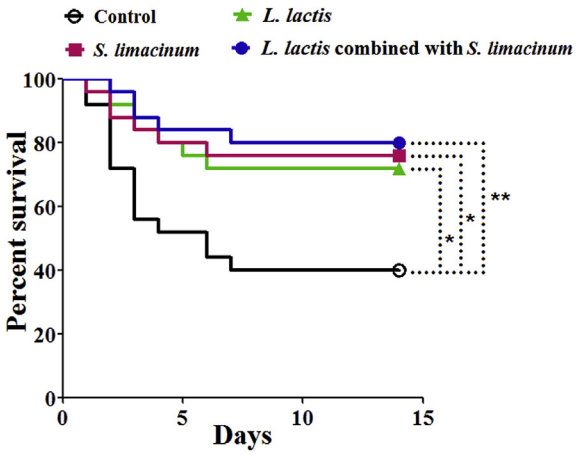

Fig. 2. Survival percentages of different administrating fish. *Chromileptes altivelis* fed with diets containing *Lactococcus lactis*, *Schizochytrium limacinum*, *L. lactis* combined with *S. limacinum*, or control (PBS) for 4 weeks and then were challenged with *Vibrio harveyi*. Survival percentages were monitored daily. "*" represents significance between the survivals of the different administrating fish and control fish, which was determined with Chisqare test. *P < 0.05, **P < 0.01.

3.4. Sequencing and de novo transcriptome assembly

The cDNA library was successfully established from pooled head kidney RNA of fish fed with the diet of *L. lactis* combined with *S. limacinum* or PBS for 14 days. In total, 264,573 transcripts (min length: 201 bp, max length: 16,847 bp, N50:1337) were assembled using Trinity. Furthermore, 158,468 sequences (min length: 201 bp, max length: 16,847 bp, N50:1689) were obtained after clustering and redundancy filtering. These were defined as unigenes.

3.5. Functional annotation of unigenes

After being blasted against public databases (Nr, Swiss-Prot, GO, KEGG, and KOG), 54.85% (86,919) unigenes were annotated by at least one of the above databases. The numbers of annotated unigenes at Nr, Swiss-Prot, GO, KEGG, and KOG databases were 77191 (89.61%), 63837 (73.44%), 59652 (68.63%), 41138 (47.33%), and 28629 (32.94%) respectively (Table 3).

3.5.1. GO functional annotation

The above 59652 annotated unigenes at GO database classified into three categories is shown in Fig. 3. For biological process, the most represented GO terms were cellular process, metabolic process, signal-organism process, biological regulation, and regulation of biological process. For cellular component, cell was the most common category, followed by cell part, organelle, macromolecular complex, and membrane. For molecular functions, the five most abundant categories were binding, catalytic activity, transporter activity, nucleic acid binding transcription factor activity, and molecular transducer activity.

Table 3
Statistics of annotation results for *Cromileptes altivelis* head kidney transcriptome unigenes.

	Number of unigenes	Percentage
Annotated in Nr	77191	89.61%
Annotated in Swiss-Prot	63837	73.44%
Annotated in GO	59652	68.63%
Annotated in KEGG	41138	47.33%
Annotated in KOG	28629	32.94%
Total annotated unigenes	86919	100%

3.5.2. KEGG functional annotation

In our study, a total of 41138 unigenes were grouped into 231 known KEGG pathways. The classification statistics showed that these 231 pathways were subjected to five types: cellular processes (14740, 35.83%), environmental information processing (8654, 21.04%), genetic information processing (6077, 14.77%), metabolism (8700, 21.15%), and organismal systems (8295, 20.16%) (Fig. 4). Among these unigenes, a total 3212 unigenes (7.81%) were grouped into the cluster of immune system.

3.6. Identification and analysis of DEGs

After the clean reads were mapped, a total of 57, 248, 327 (40, 324, 787 mapped) reads were obtained from the experimental group, while the number of reads in the control group was 58, 783, 614 (43, 182, 656 mapped). Then, DEGs between the experimental group and the control group were assessed. In total, 157 putative DEGs were identified, which contained 85 up-regulated genes and 72 down-regulated genes (Fig. S1). Moreover, GO and KEGG enrichment analyses of the putative DEGs were performed. The results of GO enrichment analyses showed that no GO terms were significantly enriched. However, DEGs were mainly enriched in nine specific KEGG enrichment pathways, including PI3K-Akt signaling pathway, graft-versus-host disease, allograft rejection, ECM-receptor interaction, autoimmune thyroid disease, antigen processing and presentation, phagosome, apoptosis, and protein digestion and absorption (Table 4, Fig. S2).

3.7. Validation of 10 DEGs by qPCR

To validate the RNA-seq data, the transcript levels of 10 DEGs were determined by qPCR. These 10 selected DEGs were mainly immune and metabolism related genes, including immunoglobulin M (IgM), major histocompatibility complex (MHC) class Iα and class IIα, transferring receptor (TFRC), mitogen-activated protein kinase kinase kinase 5 (MAP3K5), nuclear receptor subfamily 4 group A member 1 (NR4A1), tax1-binding protein 1 (TAX1BP1), E3 ubiquitin ligase HERC4 (HERC4), tumor necrosis factor alpha (TNFα), and toll-like receptor 2 (TLR2). The qPCR results of all selected DEGs presented similar expression patterns compared with their corresponding RNA-seq results (Fig. 5). The calculated correlation of the expression levels of 10 putative DEGs acquired by RNA-seq and qPCR was 0.77027, indicating that the *de novo* transcriptome assembly was reliable.

4. Discussion

Dietary supplementation of different feed additives e.g. probiotics and prebiotics have been found to be beneficial to improve the immune status, feed efficiency, and growth performance of cultured fish species [27,39–42]. Despite the potential benefits of both probiotics and prebiotics to health performance as reported for various aquatic animals, the combined use of probiotics and prebiotics in fish has been less investigated. In this study, we evaluated the effects on the growth performance, innate immune parameters, and resistance of *C. altivelis* against *V. harveyi* after fish fed with *L. lactis* HNL12 combined with *S. limacinum* diet. The present study clearly indicates that the diet containing *L. lactis* and *S. limacinum* at the same time achieved a better health benefit in the *C. altivelis* than using them alone.

Probiotics are generally used to promote growth and immunity due to their ability to improve the nutrient digestibility of the host by enhancing the nutrient absorption, vitamins synthesis, and digestive enzyme activities [43,44]. Previous studies demonstrated that many probiotics such as *Lactobacillus sakei* [30], *Bacillus subtilis* [45], and *L. lactis* [32,46,47] could beneficially enhance the growth performance and pathogen inhibition in fish. To microalgae, their chemical composition (include different polysaccharides) and enriched nutrition may lead to an improvement in growth performance in the host [7,48,49]. Li

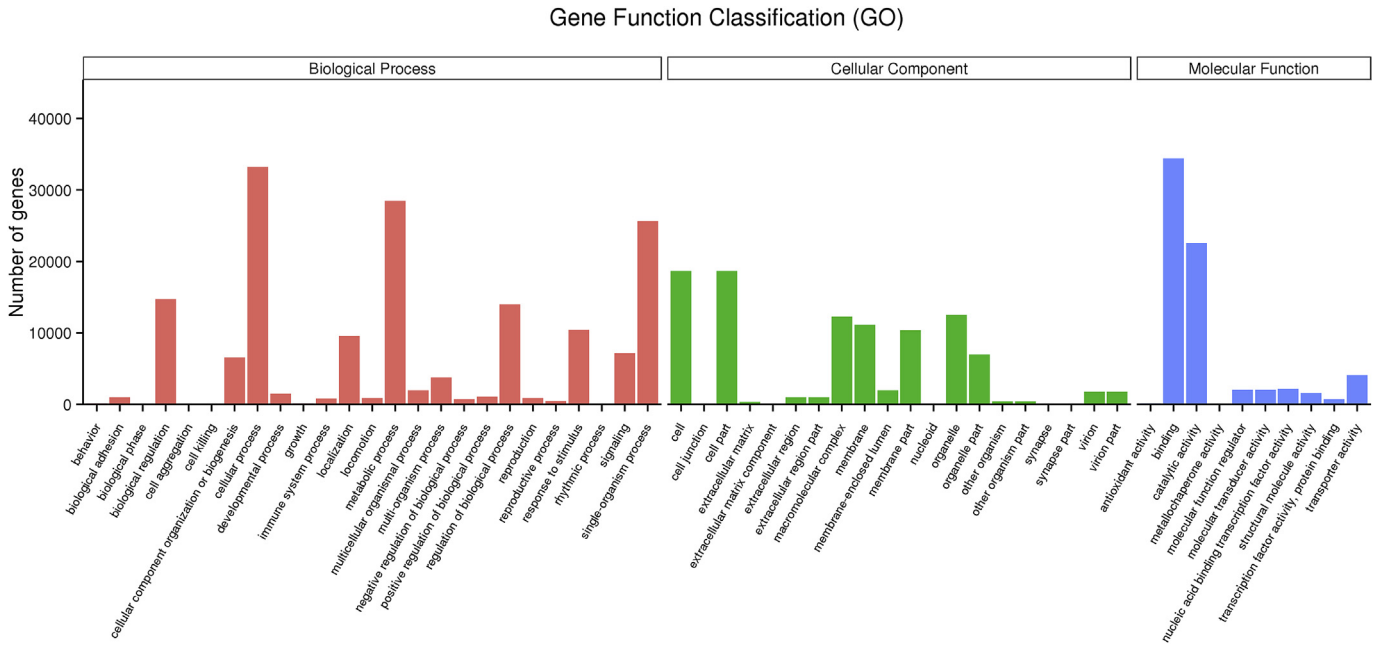

Fig. 3. Gene ontology functional classification of the assembled unigenes. 59652 unigenes were classified into three GO categories.

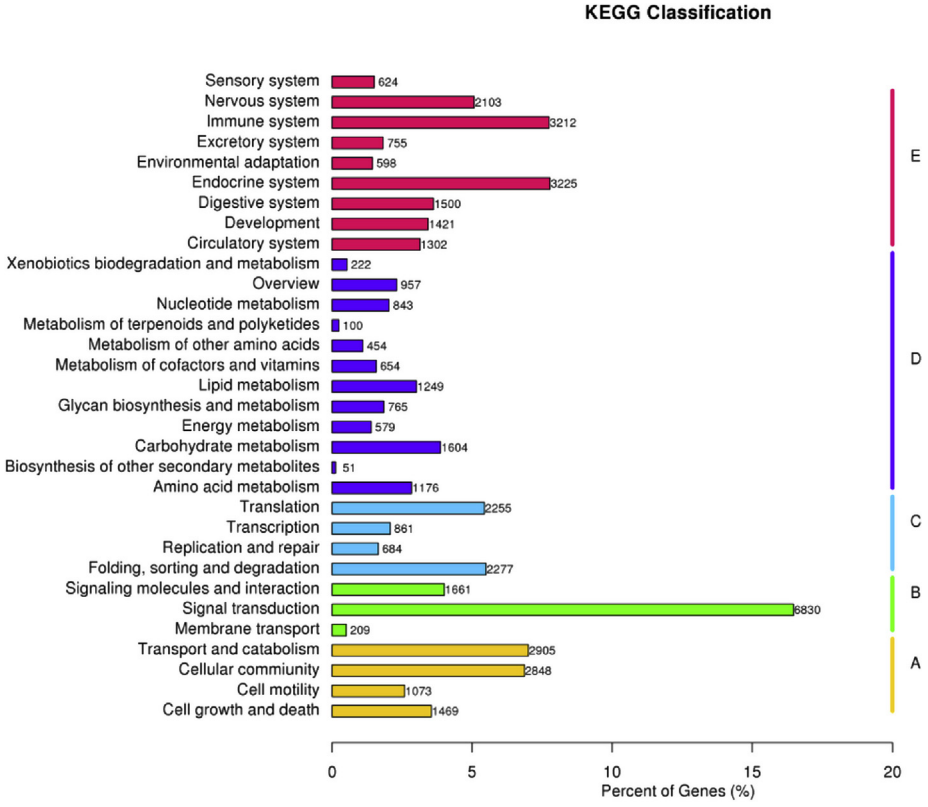

Fig. 4. Statistics of KEGG pathway classifications. The ordinate represents the name of the KEGG pathway; the horizontal represents the number and ratio of genes mapped to a specific pathway. A, Cellular processes; B, Environmental information processing; C, Genetic information processing; D, Metabolism; E, Organismal systems.

et al. reported that a diet containing 1.0–1.5% *Schizochytrium* meal significantly increased the weight gain in *Ictalurus punctatus* [24]. Similarly, many studies reported improvements of growth and feed utilization in aquatic animals treated with microalgae meal [50–52]. In this study, the diet of *L. lactis* combined with *S. limacinum* significantly improved the final weight, PWG, and SGR in *C. altivelis* after 4 weeks of feeding compared with either *L. lactis* or *S. limacinum* supplementation alone. These results agree with a previous report of *Lutjanus peru*, which showed that fish fed the diet supplemented with *Navicula* and *L. sakei* displayed significantly higher final weight compared with diets that provided *Navicula* or *L. sakei* alone or control diet [34]. Likewise, Rafiee et al. reported that a mixed diet of *Skeletonema costatum* and *B. subtilis* accelerated the growth of three larval stages (zoa, mysis, and post-larvae) of *Penaeus monodon* [53].

The immune defense in bony fish is mainly based on their innate immunity. The respiratory burst activity of phagocytes degrades internalized pathogens during phagocytosis and plays an important role in the innate immune system [54]. The level of respiratory burst activity

Table 4
KEGG classification of significant pathways of *Cromileptes altivelis* fed with *Lactococcus lactis* combined with *Schizochytrium limacinum*.

Pathway name	DEG number	Corrected *P*-value
PI3K-Akt signaling pathway	14	0.003
Graft-versus-host disease	4	0.004
Allograft rejection	4	0.004
ECM-receptor interaction	7	0.004
Autoimmune thyroid disease	4	0.004
Antigen processing and presentation	5	0.014
Phagosome	7	0.021
Apoptosis	7	0.021
Protein digestion and absorption	4	0.034

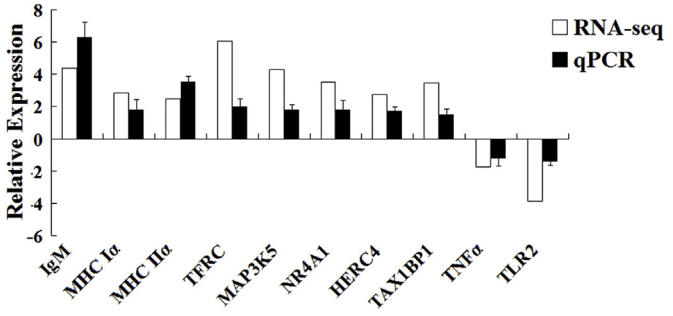

Fig. 5. Effect of the combined dietary of *Lactococcus lactis* combined with *Schizochytrium limacinum* on the expression of selected genes and qPCR validation of putative DEGs. *Cromileptes altivelis* were fed with the *L. lactis* combined with *S. limacinum* diet, and the expression of immunoglobulin M (IgM), major histocompatibility complex (MHC) class Iα and class IIα, transferring receptor (TFRC), mitogen-activated protein kinase kinase kinase 5 (MAP3K5), nuclear receptor subfamily 4 group A member 1 (NR4A1), tax1-binding protein 1 (TAX1BP1), E3 ubiquitin ligase HERC4 (HERC4), tumor necrosis factor alpha (TNFα), and toll-like receptor 2 (TLR2) in head kidney were determined by qPCR. Fold changes of selected genes were given either according to RNA-seq or qPCR results. The fold change in gene expression was normalized to *β-actin* gene and relative to the control group samples. qPCR data were reported as mean ± standard deviation.

provides an effective index for the evaluation of the defense ability against host pathogens [55]. Serum parameters are typically used to monitor the physiological and pathological changes of fish [56]. In *Oncorhynchus mykiss* fed with *L. lactis*- and *L. mesenteroides*-containing diets has elevated the respiratory burst activity of head kidney leucocytes compared with the control group [57]. Diaz-Rosales et al. reported that *Porphyridium cruentum* administration for 4 weeks significantly increased the respiratory burst activity of *Solea senegalensis* [58]. Cerezuela et al. also found a significantly higher head kidney leucocyte respiratory burst in *Sparus aurata* L. fed with *Phaeodactylum tricornutum* for 2 weeks [10]. In *Lutjanus peru* fed with *Navicula* + *L. sakei*, the SOD activity was also significantly improved, compared with the control group [34]. Similar trends of AKP and LZM activities were found in previous studies on *Salmo trutta* fed with *L. lactis* [59], *L. peru* fed with *Navicula* + *L. sakei* or *L. sakei* alone [34]. In line with these studies, in the present study, the respiratory burst activity, SOD, AKP, and LZM activities of the fish fed with the diet containing *L. lactis* combined with *S. limacinum* were significantly higher than those of the control group after 2 weeks of feeding, suggesting that feeding with diets that contain both *L. lactis* and *S. limacinum* might induce a strong innate immunity in fish.

The bacterial challenge experiment was used as an indicator to evaluate the effectiveness of different diets with regard to protection against pathogens [60]. Kim et al. showed that *L. lactis* BFE20 protected *Paralichthys olivaceus* against infection caused by *Streptococcus iniae* [31]. Nguyen et al. demonstrated that *L. lactis* WFLU12 improved the survival rate of *P. olivaceus* infected with *Streptococcus parauberis* [32]. In addition, a recent study showed that dietary supplementation with *S. limacinum* significantly enhanced the disease resistance to *Aeromonas hydrophila* in *Cyprinus carpio* var. Jian [61]. In accordance with these studies, the results of the present study showed that the disease resistance to *V. harveyi* has been significantly improved in *C. altivelis* fed with diets containing *L. lactis* alone, *S. limacinum* alone, or a combination of *L. lactis* and *S. limacinum*, with the corresponding RPS of 56.08%, 59.41%, and 68.63%, respectively. Given these results, this study indicated that *L. lactis* and *S. limacinum* might exert synergistic effects on the growth, innate immunity, and pathogen defenses of *C. altivelis*.

To understand the reasons for these effective synergistic effects induced by *L. lactis* and *S. limacinum*, this study investigated the immune-regulating activities at the transcriptome level. A total of 157 putative DEGs were found between the *L. lactis* combined with *S. limacinum* group and the control group. Nine KEGG pathways were significantly enriched, such as, the PI3K-Akt signaling pathway, antigen processing and presentation, and protein digestion and absorption, which were mainly related to immune responses and digestive system. It has been reported that prebiotics can shift the microbial community structure to being dominated by beneficial bacteria, such as *Lactobacillus* spp. and *Lactobacilli* spp [13,62]. Previous studies have demonstrated that the microalgae *Spirulina platensis* can promote the growth of beneficial bacteria, such as *L. casei*, *Streptococcus thermophilus*, and *Lactobacillus acidophilus* [63,64]. In addition, it has been documented that microalgae may positively impact the host metabolism [34,65]. A previous study has shown that *S. limacinum* can increase the concentration of glucose and decrease the concentration of cholesterol in the blood of lambs, which might enhance the energy and lipid metabolisms of the host [65]. In accordance with those results, the current study showed the up-regulation of the glucose and lipid metabolism related gene NR4A1. This may indicate that the combined use of *L. lactis* and *S. limacinum* could activate specific metabolism pathways and positively modify the metabolism of *C. altivelis*. Therefore, the beneficial effects on growth performance of *C. altivelis* caused by the combined diet could be partially explained by the mutual promotion between both types of feed additives and consequently improve the growth of the host.

Previous studies have shown that *L. lactis* can induce the up-regulation of MHC in fish and murine [33,66]. When feeding with the microalgae *T. chuii*, Cerezuela et al. also found that MHC IIα, IgM, and transferrin were significantly up-regulated in *S. aurata* L [10]. Similarly, this study detected the up-regulation of immune related genes (IgM, MHC Iα, MHC IIα, and TFRC) in the transcriptome, which was further verified by qPCR. Furthermore, the down-regulation of TLR2 and TNFα was also observed. In line with these results, Li et al. reported that dietary supplementation with V_E combined with *S. limacinum* significantly down-regulated the expression of TLRs and NF-κB in *Fenneropenaeus chinensis* [67]. Previous studies conducted in mice also demonstrated that the crude polysaccharide extracts of microalgae showed significant anti-inflammatory effects [68,69]. In this study, the down-regulation might indicate an anti-inflammatory profile, which is likely because the diet of *L. lactis* combined with *S. limacinum* inhibits specific pathways (such as, NF-κB, MAPK, and PI3K-Akt signaling pathways) by acting on receptors, thus reducing the expression of pro-inflammatory cytokines (TLR2 and TNFα). As a result, *L. lactis* combined with *S. limacinum* might exert a good performance on the immunity, and protection against pathogens in *C. altivelis*.

In conclusion, the current study showed that the *L. lactis* HNL12 combined with *S. limacinum* promotes growth, enhances immunity, and confers protection in *C. altivelis* against infection by *V. harveyi*. These results suggest that the marine microalgae *S. limacinum* and the probiotic *L. lactis* have various health promoting properties and could be used as potential immunostimulants for farmed fish.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2019.09.059.

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