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The Effects of Silkworm-Derived Polysaccharide (Silkrose) on Ectoparasitic Infestations in Yellowtail (*Seriola quinqueradiata*) and White Trevally (*Pseudocaranx dentex*)

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Abstract: The effect of silkworm-derived polysaccharide silkrose on fish ectoparasites was investigated. When juvenile yellowtail (*Seriola quinqueradiata*) fed diets containing silkrose were artificially infected with *Benedenia seriolae*, a fish ectoparasite, the numbers of parasitized *B. seriolae* were significantly lower compared to that in fish in the control group without silkrose treatment. Furthermore, when juvenile yellowtails were severely infected with *B. seriolae*, no mortality was observed in the silkrose-treated group, compared to more than 60% in the control group. In field studies carried out at a fish farm with yellowtail and white trevally (*Pseudocaranx dentex*), oral treatment with silkrose significantly reduced *B. seriolae* parasitism in yellowtail and *Caligus longipedis* and *Neobenedenia girellae* parasitism in white trevally. Silkrose treatment also reduced blood levels of cortisol, a stress hormone in both species. The changes in gene expression in the epidermis of yellowtail by silkrose treatment were also investigated, showing that the expression of various genes, including factors involved in immunity, stress response, and wound healing, was changed by the treatment. These findings indicate that silkworm-derived silkrose effectively prevents infection by external parasites in yellowtail and white trevally.

Keywords: polysaccharide; insect for feed; silkworm; innate immunity; ectoparasite; fish culture; immunostimulant

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1. Introduction

Ectoparasitic diseases are a serious problem in fish aquaculture. Parasitic infestation by sea lice such as *Lepeophtherius salmonis* and *Caligus elongatus* is a major problem in salmonid sea farming, and the damage caused by such infestation is very serious [1,2]. In Japan, ectoparasitic diseases are also a major problem in the mariculture of yellowtail, amberjack, red sea bream, and white trevally [3,4]. High infestations of ectoparasites in farmed fish can cause significant damage, because the fish are chronically stressed, resulting in reduced immune activity and increased risk of bacterial and viral infections [5,6]. The control of ectoparasites in fish has been carried out by bath treatment in hydrogen peroxide or oral administration of medications such as praziquantel [7–10]. However, these treatments are highly toxic to fish, and the environmental impact of these substances cannot be ignored [11–13]. Although various extermination techniques for ectoparasites have been developed, these methods are not effective solutions at present [14–16]. Under these conditions, it is necessary to develop ectoparasite prevention and removal techniques that do not have negative impacts on farmed fish and the environment of fish farms.

In recent years, insects have attracted great interest worldwide not only as a promising protein source for animal feed production [17–19], but also for their capability of producing

a variety of useful substances that can optimize animal health [20–22]. Insect meal, such as housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), and black soldier fly (*Hermetia illucens*), has been widely used in various commercial fish and shellfish diets to modulate the immune response and increase resistance to bacterial infection, with remarkable success [23–30].

We recently reported that the larvae of melon fly (*Bactrocera cucurbitae*), black soldier fly, the pupae of oak silk moth (*Antheraea yamamai*), and silkworm (*Bombyx mori*) were found to contain dipterose-BC [31], dipterose-BSF [32], silkrose-AY [33], and a novel bioactive polysaccharide named silkrose-BM [34], respectively. These insect-derived bioactive polysaccharides can effectively induce nitric oxide production and pro-inflammatory cytokine and interferon β expression in RAW264.7 mouse macrophages via the Toll-like receptor (TLR)/nuclear factor κB (NF-κB) pathway [31–34]. In particular, silkrose has been analyzed for disease resistance in fish and shrimp; the addition of this polysaccharide to feed has been effective against vibriosis in shrimp and edwardsiellosis in fish, and it has been used in investigations of disease resistance in fish at the molecular level [34–36].

Yellowtail (*Seriola quinqueradiata*) and white trevally (*Pseudocaranx dentex*) are important aquaculture species in Japan. These species are frequently affected by ectoparasites, and it is desirable to develop a safe and labor-saving method to prevent these diseases [3,4,37,38]. In this study, the effects of silkrose on ectoparasites of yellowtail and white trevally were examined through challenge tests in rearing and demonstration tests in aquaculture cage at sites inhabited by parasites, and a comprehensive investigation of the changes in gene expression induced by silkrose on the fish skin, which is the site of ectoparasitism.

2. Materials and Methods

2.1. Silkrose

The silkrose used in this study is a product that is widely available on the market. The silkrose was provided by Shintoa Corporation (Tokyo, Japan). This silkrose is produced from the pupae of silkworm (*Bombyx mori*) and has been confirmed to contain 5% pure silkrose-BM by NO production assay using RAW 264 cells [34].

2.2. Experiment I (Laboratory-Based Experiment) Feeding Trial of Yellowtail with Silkrose in Tanks

2.2.1. Parasitic Challenge with Low Counts of *Benedenia seriolae* in Juvenile Yellowtail

Yellowtails (*Seriola quinqueradiata*) under 1 year of age were obtained from commercial fisheries (Todo-suisan, Ehime, Japan) for use in this challenge test. At the initiation of the experiment, the bodyweight of these fish was 79.8 ± 5.40 g and the fork length was 18.2 ± 0.37 cm.

The fish were divided into two groups and stored in a 500 L tank. One group was fed a diet (Sustain, Skretting Japan Co., Ltd., Fukuoka, Japan) with 0.1% of silkrose, and the other was fed a diet without silkrose at 5% of body weight every day.

At 52 days after the initiation of feeding, the challenge test started. For this test, ten fish from each group were randomly selected from stock tanks and identified using internal tags (PIT tag BIO12A, Biomark, Boise, ID, USA). The body weight and fork length of these fish were measured, and they were divided into two 250 L aquariums for each group, with five fish in each aquarium. To remove all parasites, all fish were immersed in freshwater for 5 min before the start of the challenge test. After freshwater treatment, the fish were visually checked for the presence of parasites.

Laid eggs of *B. seriolae* for the challenge test were collected as follows. Ten yellowtails infected with *B. seriolae* were brought from the fish farm (Uwajima, Ehime, Japan) into the 500 L tank containing 100 yellowtails that were confirmed free of ectoparasites and kept for two months. After confirming the spread of Benedeniasis, five yellowtails were picked up and all the ectoparasites were collected from these fish for morphological observation of the parasites. As a result, all 253 external parasites collected were morphologically confirmed to be *B. seriolae* according to the criteria of Kinami et al. [37]. We used this as a collecting tank for the eggs of *B. Seriolae*. Eggs of *B. seriolae* were collected by putting 25 cm^2

polyethylene nets (mesh size 5 mm) into this collecting tank to keep yellowtail infected with this parasite. The eggs, which were entangled in the nets, were incubated for several days in the shading container with a continuous flow of seawater filtered through a 100 µm mesh. The incubated eggs were irradiated with natural light to promote hatching, and the hatched oncomiracidia of *B. seriolae* were collected and used for challenge test.

The procedure for infection of yellowtail by *B. seriolae* was as follows: 135 individual oncomiracidia counted visually using a stereomicroscope were added to each tank and exposed to parasites for 4 h in the dark and without water supply. The flow-through system for each tank was switched to the closed-circulation system to maximize the infection rate. After infection, the seawater supply to the tanks was restarted and the infected fish were bred under natural light conditions. Feeding was continued during the experimental period in the same manner as before the infection. At 10 days after infection, when the infection was achieved [39], the body weight and fork length of fish were measured, and the parasitized *B. seriolae* were collected by immersing the fish in freshwater for 5 min according to the previous study [14] and then counted. After the challenge test, the body weight and fork length were measured, and the percentages of body weight gain and fork length gain were calculated as follows:

$$\text{Fork length gain (cm)} = \text{fork length at trial end-initial fork length},$$

$$\text{Body weight gain (g)} = \text{body weight at trial end-initial body weight},$$

$$\text{Percentage of fork length gain} = \text{fork length gain/initial fork length} \times 100,$$

$$\text{Percentage of body weight gain} = \text{body weight gain/initial body weight} \times 100$$

2.2.2. Challenge with High Counts of *Benedenia seriolae* in Yellowtail

For the next challenge, 30 fish were randomly selected from stock tanks of each group and identified using internal tags. Fish were divided into four 250 L aquariums: one control group and three silkworm diet groups. Each tank held six to ten fish. A polyethylene net with 5000 to 10,000 parasite eggs was set in each tank to promote parasite infection. After promotion of infection, the fish were kept for 24 days and observed for survival. During the experimental period, the infected fish were fed and bred under natural light conditions.

In all laboratory-based experiments, tanks used a flow-through system and the flow rate of the supplied seawater was 750 L/h. The seawater conditions during these experiments were as follows; the water temperature averaged at 24.4 °C, pH 8.1, the dissolved oxygen concentration was 6 to 7 ppm, and nitrate-nitrogen was not detected.

2.3. Experiment II (Field-Based Experiment) Feeding Trial of Yellowtail with Silkrose at Aquaculture Field Site

The effects of silkrose on ectoparasites of yellowtail were investigated at the aquaculture site of the Hamaei fish farms in Hisayoshi, Ainan, Ehime, Japan, from 7 July to 28 July 2019. The seawater temperature during this period ranged from 24.4 °C to 26.3 °C. Two fish cages (13 m long, 13 m wide, 10 m deep) containing 9000 one-year-old yellowtails (average body weight 2.3 kg) were prepared. The fish in one cage were fed extruded pellet (EP) feed containing 0.1% of silkrose as an experimental group, and the fish in the other cage were fed EP without silkrose as a control group. The base feed used in this experiment was EPs with a diameter of 5.6 or 6.5 mm (Max, Skretting Japan Co., Ltd., Fukuoka, Japan). For the experimental group, silkrose was added to the EP feed at a concentration of 0.1% (dry matter) at the time of pellet preparation on consignment by Skretting Co. Ltd. EP were produced on consignment by Skretting Co. Ltd.

Before the experiment, the ectoparasites on the experimental fish were removed by 0.065% Marinesour SP30 including 30% hydrogen peroxide treatment according to the manufacturer's instructions (Katayama Kagaku Kougyou Co., Ltd., Osaka, Japan).

On 28 July, 16 fish in each group were collected and anesthetized with seawater containing 0.05% 2-phenoxyethanol [40], and then sacrificed by excision of the medulla

oblongata [41,42]. Within 1 min after sacrifice, 1 mL blood sample was collected from the caudal vasculature using a syringe, then the body weight and fork length were measured. The fish bodies were then used for experiments on ectoparasite parasitism, and blood samples were used for measurement of cortisol levels.

All ectoparasites on all collected fish surfaces were detached from the skin by sprinkling tap water on the fish and collected using a 100 µm nylon mesh, then they were counted and fixed with 1% glutaraldehyde seawater. All collected ectoparasites were identified as *B. seriolae* based on their morphological characteristics [37].

2.4. Experiment III (Field-Based Experiment) Feeding Trial of White Trevally (Pseudocaranx dentex) at Aquaculture Field Site

The effects of silkrose on the ectoparasites of white trevally were investigated at the aquaculture site of Nishikawa Hotoku Fisheries Co., Ltd., in Uwajima, Ehime, Japan, from 10 August to 30 November. Two fish cages (13 m long, 13 m wide, 10 m deep) containing approximately 6000 3-year-old white trevally (average body weight 1.1 kg) were prepared on 20 July. The fish in one cage were fed moist pellet (MP) feed containing silkrose and 5 mm EP as an experimental group, and the fish in the other cage were fed the same feed without silkrose as a control group. The base feed used in this experiment was an EP feed with a diameter of 5 mm (Dainichi Co., Ltd., Ehime, Japan) and moist pellets for white trevally made by Dainichi Co., Ltd. In the feed for the experimental group, the amount of silkrose added to the MP was adjusted to 0.1% of the dry weight of the total feed at the time of pellet preparation by Dainichi Co. Ltd.

Prior to the start of the experiment, the ectoparasites of the experimental fish were removed by 0.065% Marinesour SP30 including 30% hydrogen peroxide treatment according to the manufacturer's instructions (Katayama Kagaku Kougyou Co., Ltd., Osaka, Japan).

The feeding trial of silkrose-containing feed started on 10 August. Until the start of the trial, the fish in both cages were fed the diet without silkrose. On 20 June, 2 September, 20 October, and 30 November, when the trial was completed, 100 fish in each cage were photographed using a stereo video camera (AM-100, AQ1 Systems Pty. Ltd., Tasmania, Australia), and the fork length and body weight of the trial fish were measured. The photographed measurements were corrected by actual measurements of fork length, body depth, and body weight. During the trial period, dead fish were pulled up and their numbers were recorded. The seawater temperature at the time of measurement was 22.8 °C on 20 June, 23.7 °C on 2 September, 22.6 °C on 20 October, and 19.6 °C on 30 November.

The observation of ectoparasites parasitizing white trevally was carried out on 13 September. September is the time when the seawater temperature is high and there are the most ectoparasites in the experimental sea area.

Ten fish in each group were collected for blood sampling and body measurements in the same manner as described above for yellowtail. Fish bodies were used for experiments on ectoparasite parasitism, and blood samples were used for measurement of cortisol levels.

Each fish body was individually transferred to a bucket filled with fresh water and then ectoparasites were carefully picked off with forceps and fixed with 1% glutaraldehyde seawater. The collected parasites were confirmed to be *Caligus longipedis* and *Neobenedenia girellae* based on the morphological characteristics [37,38]. The numbers of *C. longipedis* and *N. girellae* collected were counted for each individual fish.

2.5. Measurement of Blood Cortisol Levels

The blood samples collected from fish were kept at 4 °C overnight. The serum was separated by centrifugation at 1500× *g* and stored at −40 °C until analysis. Serum cortisol levels were measured using an enzyme-linked immunoassay method with a Cortisol ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA).

2.6. Tissue Sampling and RNA Extraction for Differential Gene Expression Analysis of Yellowtail Epidermis

For sampling, yellowtails from the silkrose feeding trial at the aquaculture field site were used. Approximately, 50 mg of skin under the dorsal fin was collected from 10 fish each in the experimental and control groups. Collected tissues were placed in a sterile tube containing 1 mL of RNAlater (Invitrogen, Waltham, MA, USA) and stored at 4 °C for 24 h. These samples were then transferred to a freezer at −80 °C until further analysis.

For RNA extraction, 50 mg of RNAlater-stabilized skin tissue was homogenized in 1 mL ISOGEN II (Nippon Gene Co., Ltd., Toyama, Japan) and then subjected to total RNA extraction according to the manufacturer's instructions. The quantity of total RNA was analyzed by an Implen NanoPhotometer P330 (Implen, Munchen, Germany).

2.7. RNA-Seq Library Construction, Sequencing, and Bioinformatics Analysis

RNA-seq analysis was performed in two groups: the experimental group with silkrose treatment and the control group without silkrose treatment. Equal amounts of total RNA from five individual skin samples in each group were pooled into one sterile tube for RNA-seq library construction. The volume of this master pool was adjusted to be more than 20 µL and the total RNA concentration to be more than 50 ng/µL.

The concentration and purity of pooled total RNA were quantified using the Quanti-Fluor RNA System (Promega, Madison, WI, USA) and the Agilent 5200 Fragment Analyzer System with HS RNA Kit (Agilent Technologies, Santa Clara, CA, USA), respectively. The cDNA library was prepared using an MGIEasy RNA Directional Library Prep Set (MGI Tech Co., Ltd., Shenzhen, China) according to the manufacturer's instructions. After generating the clusters, library sequencing was performed on a DNBSEQ-G400RS platform to create paired-end reads with a length of 100 bp.

Raw sequence data were first processed to remove adaptors, low-quality reads (average QC value < 20), and paired reads with fewer than 40 bases [43,44]. Then, Hisat2 (v. 2.2.0) [45] was used to align the remaining reads to the reference genome of *Seriola lalandi dorsalis*. The output data from the mapping process were converted [46] and the transcription level for each gene was counted [47]. Differentially expressed genes (DEGs) between dietary treatments were identified by the TCC package (v. 1.26.0) in R software [48]. For DEG analysis, the adjusted *p*-value was set below 0.05 with log2(fold-change) $\geq$ 2.

Enrichment analysis for gene ontology (GO) and pathway was performed with a hypergeometric distribution test ($p < 0.05$) using clusterProfiler (v. 3.18.1) [49]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database for *Seriola lalandi dorsalis* was used for pathway enrichment analysis (https://www.kegg.jp/; accessed on 28 October 2021).

2.8. Confirmation of RNA-Seq Results Using Quantitative RT-PCR (qRT-PCR)

To examine the reliability of the RNA-seq results, four differentially expressed genes from RNA-seq results were selected for validation by quantitative RT-PCR (qRT-PCR) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Total RNA was extracted from the same sample sets used in RNA-seq analysis. First-strand cDNA was synthesized with 500 ng total RNA in each reaction system using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers designed based on transcript sequences are listed in Table S1. Melting-curve analysis was performed to ensure a single product for all tested genes. Yellowtail 18S ribosomal RNA was used as internal control during all qPCR runs. The amplification process was carried out in a quantitative RT-PCR detection system (Bio-Rad Laboratories), and consisted of initial enzyme activation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 55 °C for 5 s. The mRNA relative expression levels were calculated using the comparative threshold (CT) cycle method ($2^{-\Delta\Delta Ct}$) described by Livak and Schmittgen [50].

2.9. Statistical Analysis

Each item measured was expressed as mean ± standard error. Statistical analysis of survival rates in the challenge test was performed using the Kaplan–Meier survival estimator and a log-rank test at the 0.05 significance level. Statistical analysis of sequential changes of growth on cultured white trevally was done by two-way factorial analysis of variance (ANOVA) and Sidak multiple comparisons post-hoc test. For other statistically significant differences between the two groups, Mann–Whitney's U test was used. Differences of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of Silkrose on Ectoparasite Infection of Yellowtail Juveniles (Experimental I)

The effect of silkrose on *Benedenia seriolae* infection of yellowtail was investigated by a parasitic challenge test. Figure 1 indicates the number of parasitized *B. seriolae* on each tested yellowtail, and Figure 2 indicates the weight and length gain rates of tested fish in this challenge test.

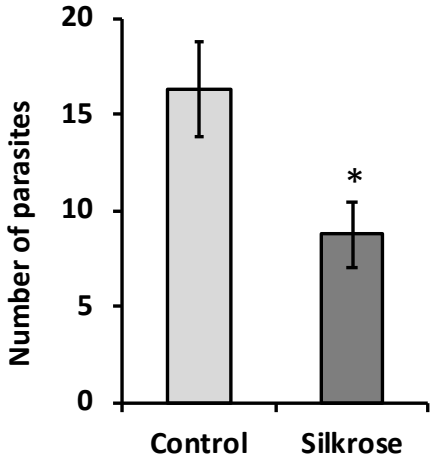

Figure 1. Effect of silkrose treatment on the number of parasites of *Benedenia seriolae* per individual yellowtail. Diet containing silkrose at a concentration of 0.1% was fed to yellowtail ($n = 10$) for 10 days. Results are presented as mean ± standard error. Asterisk indicates statistically significant differences versus control group (* $p < 0.05$).

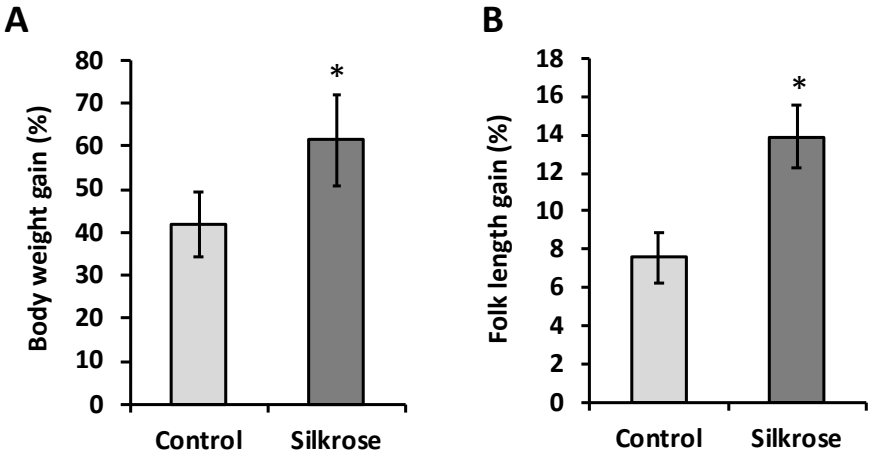

Figure 2. Effect of silkrose treatment on (**A**) body weight and (**B**) fork length gain rate of yellowtail during parasitic challenge experiment. Results are presented as mean ± standard error. Asterisk indicates statistically significant differences versus control group (* $p < 0.05$).

In the control group fed the diet without silkrose, the number of *B. seriolae* parasitized per fish was 16.3 ± 2.5. In the experimental group fed the diet with silkrose, the number was 8.8 ± 1.7, which is a significantly low level ($p < 0.05$). Silkrose treatment also affected fish growth. During the experimental period, both fork length and body weight were significantly increased by oral administration of silkrose. This challenge test was done in duplicate, and both tests showed similar patterns.

The rates of both body weight gain and fork length gain during experimental period were significantly higher in the experimental group than in the control group.

The survival rate of yellowtail in the case of severe infection with parasites is shown in Figure 3. In the case of severe parasite infection, not a single death was observed in all three tanks in the experimental group. In the control group, no mortalities were observed in one of the four tanks, but all fish in the other three tanks died during the experimental period. The final survival rate was 100% in the experimental group fed the diet with silkrose and 35.7% in the control group fed the diet without silkrose. There was a significant difference between the survival rates of the experimental and control groups ($p < 0.001$).

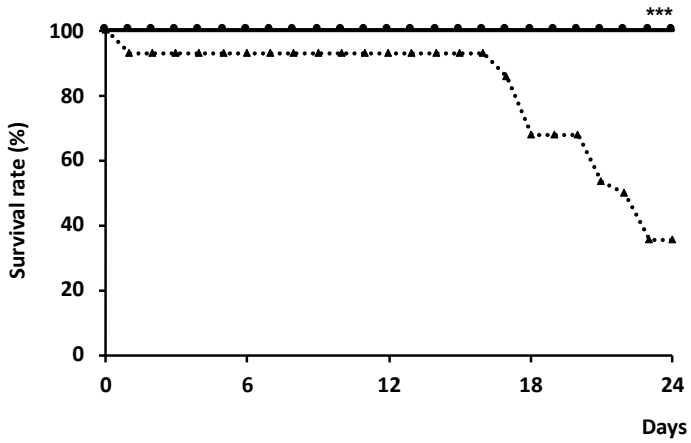

Figure 3. Survival curves for severely infected yellowtail with parasites. Solid and dotted lines indicate silkrose-treated and control group, respectively. Asterisks indicate statistically significant differences compared with control group by log rank test with Bonferroni correction (*** $p < 0.001$).

3.2. Effect of Silkrose on B. seriolae on Yellowtail at Aquaculture Field Site (Experimental II)

The effect of silkrose on infection of yellowtail by external parasites was investigated at the aquaculture field site. As mentioned above, all external parasites obtained were identified as *B. seriolae* based on their morphological characteristics [37]. The numbers of *B. seriolae* parasites per individual fish are shown in Figure 4. *B. seriolae* were detected in both groups of fish. The number of *B. seriolae* per fish was 20 ± 4.6 in the control group and a much lower 6.3 ± 1.3 in the silkrose-treated group. During the experimental period, there was no significant difference in growth between the two groups.

3.3. Effect of Silkrose on Parasites of White Trevally at Aquaculture Field Site (Experimental III)

The effect of silkrose on infection of white trevally by external parasites was investigated at the aquaculture field site. The number of parasites per individual fish after 12 weeks from the start of the experiment is shown in Figure 5. The parasites on the fish in this collection were *Caligus longipedis* [38] and *Neobenedenia girellae* [37].

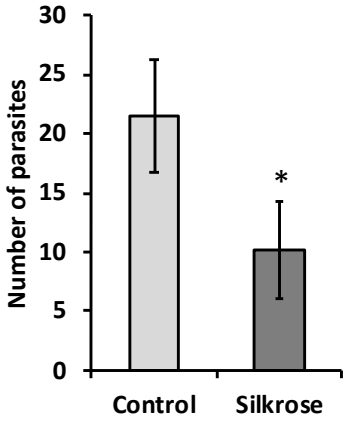

Figure 4. Effect of silkrose treatment on the number of *Benedenia seriolae* parasites per individual yellowtail at aquaculture field site. Results are presented as mean ± standard error. Asterisk indicates statistically significant differences compared with control group (* $p < 0.05$).

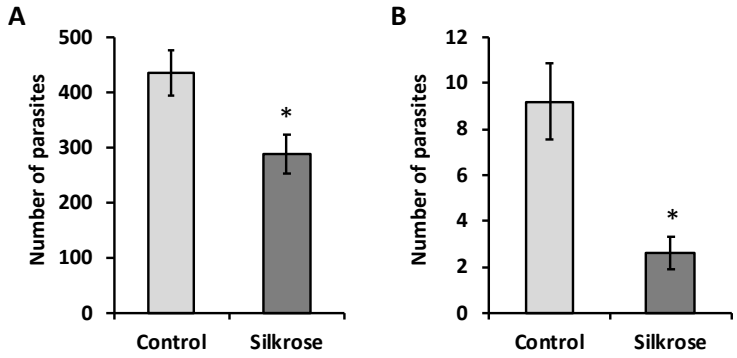

Figure 5. Effect of silkrose treatment on the number of parasites of (**A**) *Caligus longipedis* and (**B**) *Neobenedenia girellae* per individual white trevally at aquaculture field site. Results are presented as mean ± standard error. Asterisk indicates statistically significant differences compared with control group (* $p < 0.05$).

White trevally in the control group were highly parasitized by *C. longipedis*, averaging 435.1 ± 42.0 per individual. In the silkrose-treated group, the number of these parasites was significantly reduced, averaging 289.7 ± 35.3 per individual ($p < 0.05$). By comparison, the fish were parasitized by *N. girellae* at an average of 9.2 ± 1.7 per individual in the control group, and the number was significantly reduced to an average of 2.6 ± 0.7 per individual in the silkrose-treated group ($p < 0.05$).

Silkrose treatment affected white trevally growth (Figure 6). At the start of the experiment on 20 June, there were no differences in body weight and fork length between the groups; however, by 2 September the fish in the silkrose-treated group were larger than those in the control group, and by 20 November, the weight of the fish in the control group was 1394 ± 26.5 g and the fork length was 38.1 ± 22.7 cm, while in the silkrose-treated group, body weight was 1529 ± 31.9 g and fork length was 39.1 + 25.1 cm. During the experimental period, the difference in growth between the two groups was statistically significant for both body weight and fork length ($p < 0.0001$).

The number of dead fish during the feeding trial from 20 June to 30 November was 174 in the silkrose-treated group compared to 482 in the control group.

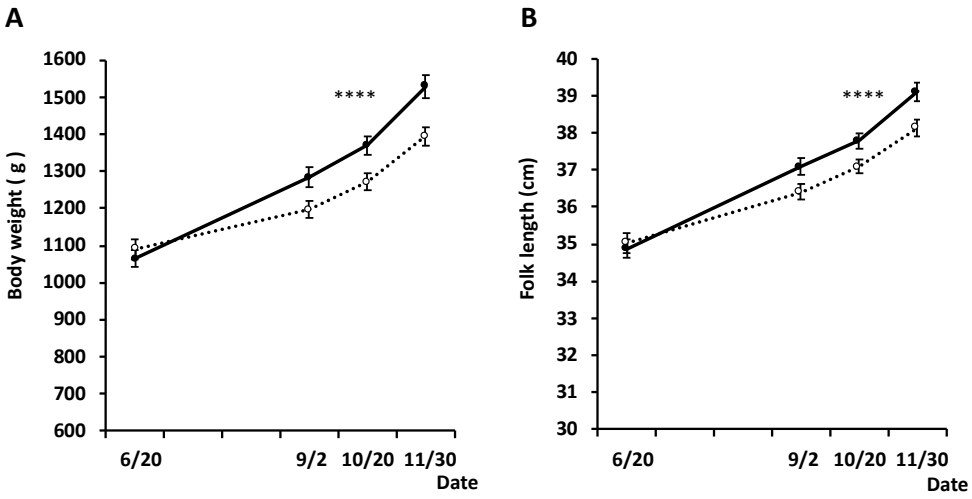

Figure 6. Effect of silkrose treatment on (**A**) sequential body weight and (**B**) fork length of white trevally at aquaculture field site. Solid line represents the results of silkrose-treated group, and dotted line represents results of control group. Results are presented as mean ± standard error. Asterisks indicate statistically significant differences compared with control group (**** $p < 0.0001$).

3.4. Effect of Silkrose Treatment on Blood Cortisol Levels in Yellowtail and White Trevally

The blood cortisol levels of silkrose-treated yellowtail and white trevally in the field test are shown in Figure 7. The cortisol level in yellowtail was 7.9 ± 0.85 ng/mL in the control group and significantly lower in the silkrose-treated group, at 4.33 ± 0.89 ng/mL ($p < 0.05$). The cortisol level in white trevally was 53.3 ± 7.06 ng/mL in the control group and significantly lower in the silkrose-treated group, at 33.8 ± 3.34 ng/mL ($p < 0.05$).

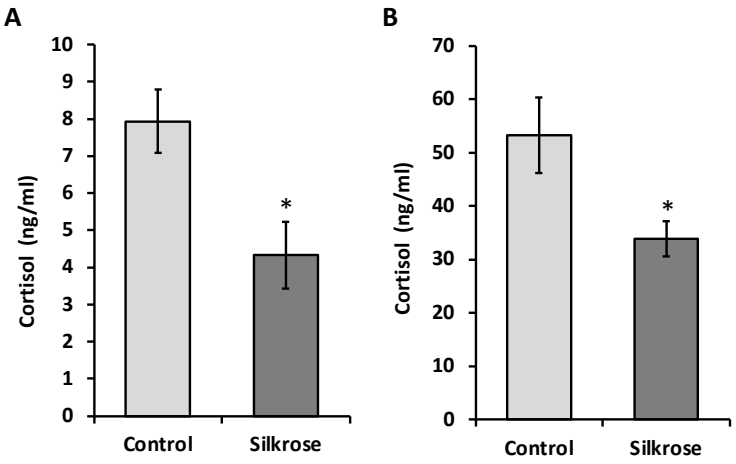

Figure 7. Effect of silkrose treatment on blood cortisol levels in (**A**) cultured yellowtail and (**B**) white trevally. Results are presented as mean ± standard error. Asterisk indicates statistically significant differences compared with control group (* $p < 0.05$).

3.5. Sequencing and Transcriptomic Analysis

RNA-seq using the DNASEQ-G400 yielded 16,240,375 ± 853,005.17 raw reads (Table S2). After quality trimming and adapter clipping, we obtained 15,415,264 ± 830,664.13 clean reads, accounting for 94.65 to 95.23% of raw reads. The results of quality control analysis showed values of Q20 and Q30 of more than 95.1 and 85.6%, respectively (Table S2). These reads were then mapped back to the reference sequence of *Seriola lalandi dorsalis* using Hisat2 (v. 2.2.0) [45] for each sample, and the mapped rate for each sample was over 61.12%, with a total of 8,750,174 ± 186,948 aligned clean reads (Table S3). After assembling and calculating the transcripts using RPKM and TPM normalization, a total of 43,172 transcripts

were detected in assembled transcripts, of which 34,785 and 34,595 were identified as being expressed in the control and experimental group, respectively, accounting for 80.56 and 80.12% of expressed transcripts.

DEGs were identified using a threshold of log2(fold-change) ≥ 2 with adjusted *p*-value below 0.05. There were 134 genes differentially expressed in the skin of yellowtail fed a diet including silkrose, 96 upregulated and 38 downregulated genes. The resulting DEGs were plotted into an MA plot using TCC package to visualize the distribution of upregulated and downregulated genes in the skin of yellowtail, as shown in Figure S1.

To determine the molecular/pathway mechanism underlying the dietary effect of silkrose on the skin of yellowtail, a gene functional enrichment analysis was conducted. DEGs were used to perform GO term and KEGG pathway enrichment analysis using the R package clusterProfiler. A total of 17 GO terms, including 8 biological processes, 5 cellular components, and 4 molecular functions, with adjusted *p*-value < 0.05, were considered significantly enriched in the skin of yellowtail. Among them are cell division, microtubule-based movement, response to hypoxia, and mitotic cytokinesis (Figure 8). In addition, nine KEGG pathways with adjusted *p*-value < 0.05 were observed, including cell cycle, oocyte meiosis, cardiac muscle contraction, PPAR signaling pathway, p53 signaling pathway, drug metabolism, and pyrimidine metabolism.

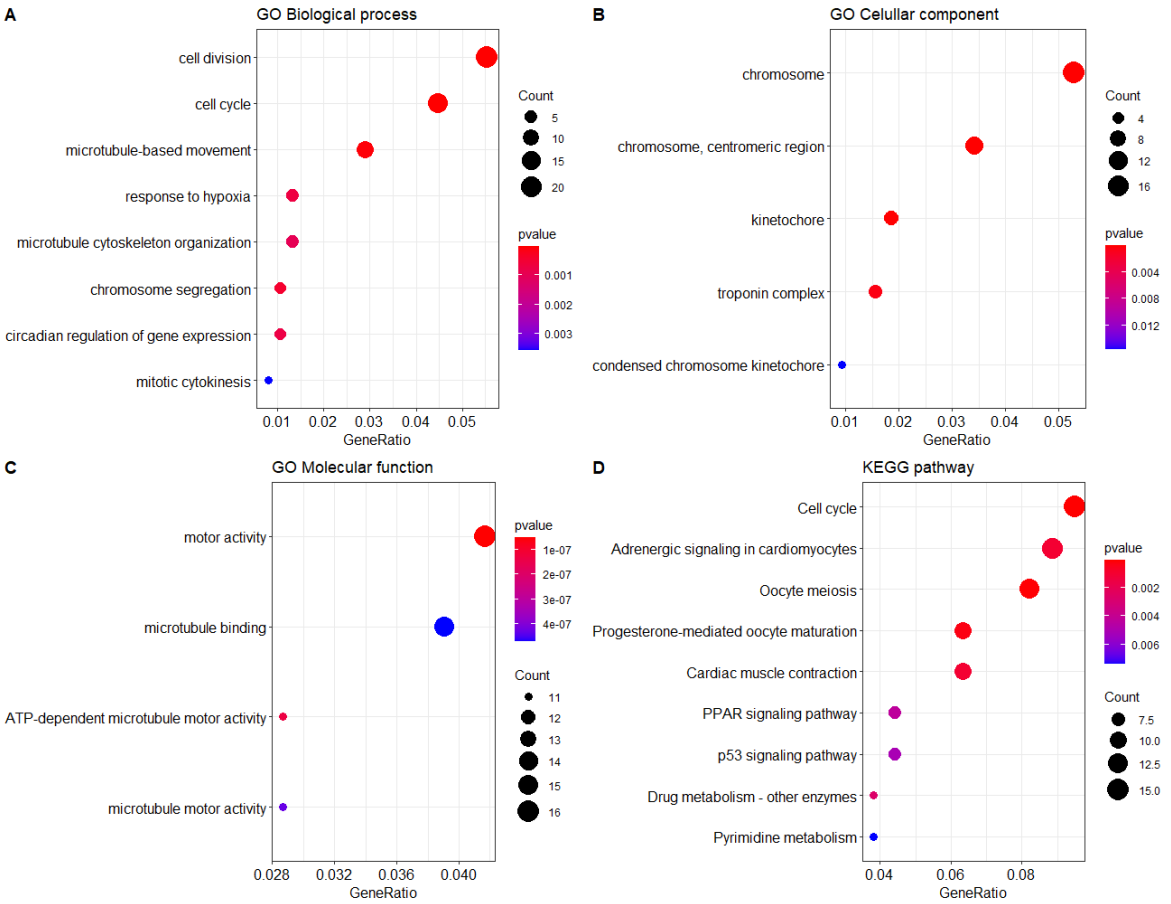

Figure 8. Representative analysis of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in skin of yellowtail using DEGs based on comparison between control and silkrose-treated groups. (**A**) Enriched biological process terms from DEGs. (**B**) Cellular component terms enriched in DEGs. (**C**) Enriched molecular function terms from DEGs. (**D**) Molecular pathways enriched by silkrose-containing diet. GO and KEGG enrichment analysis was conducted using clusterProfiler package (v. 3.18.1) [49] in R software.

Due to the limited annotation of yellowtail genome, resulting in a low mapping rate, we used a combination of KEGG pathway, GO annotation, and manual literature searches to identify immune-related genes that were differentially expressed, as shown in Table 1.

Table 1. List of upregulated immune-related genes (log2 fold-change > 2) and their expression ratio in skin of yellowtail after being fed silkrose-containing diet.

Gene ID	Gene Name	Gene Symbol	Fold Change (log2)
			Skin S-N/C-N
XM_023417751.1	collagenase 3-like, transcript variant X2	LOC111663530	5.668
XM_023417750.1	collagenase 3-like, transcript variant X1	LOC111663530	5.666
XM_023412500.1	galactose-specific lectin nattectin-like	LOC111659519	5.160
XM_023400417.1	interleukin-1 receptor type 2-like	LOC111650527	4.283
XM_023400815.1	nuclear receptor subfamily 4 group A member 3, transcript variant X2	nr4a3	3.844
XM_023400817.1	nuclear receptor subfamily 4 group A member 3, transcript variant X4	nr4a3	3.721
XM_023400816.1	nuclear receptor subfamily 4 group A member 3, transcript variant X3	nr4a3	3.654
XM_023400814.1	nuclear receptor subfamily 4 group A member 3, transcript variant X1	nr4a3	3.647
XM_023421640.1	FOS like 1, AP-1 transcription factor subunit	fosl1	3.563
XM_023417752.1	collagenase 3-like	LOC111663531	3.395
XM_023403589.1	heat shock 70 kDa protein 1-like	LOC111653120	3.144
XM_023405101.1	tumor necrosis factor receptor superfamily member 9-like	LOC111654192	2.705
XM_023395815.1	heat shock 70 kDa protein 1	LOC111646336	2.661
XM_023427402.1	matrix metallopeptidase 9	mmp9	2.432
XM_023431279.1	suppressor of cytokine signaling 3-like	LOC111673508	2.395
XM_023414302.1	C-C motif chemokine 20-like	LOC111660877	2.366
XM_023401030.1	class I histocompatibility antigen, F10 alpha chain-like	LOC111651075	2.240
XM_023419525.1	heat shock protein family A (Hsp70) member 5	hspa5	2.019
XM_023415374.1	complement C1q-like protein 4	LOC111661717	4.688
XM_023403629.1	claudin-17-like	LOC111653151	3.745
XM_023431223.1	mucin-5AC-like	LOC111673465	3.745
XM_023400530.1	low affinity immunoglobulin gamma Fc region receptor II-like	LOC111650619	3.504
XM_023412429.1	lysozyme C-like	LOC111659460	3.089
XM_023401049.1	tumor necrosis factor receptor superfamily member 5-like	LOC111651097	2.919
XM_023415322.1	complement C1q tumor necrosis factor-related protein 3-like	LOC111661670	2.852
XM_023422497.1	mucin-2-like	LOC111666964	2.714
XM_023416021.1	claudin-4-like	LOC111662227	2.714

Table 1. *Cont.*

Gene ID	Gene Name	Gene Symbol	Fold Change (log2)
			Skin S-N/C-N
XM_023418843.1	C-C chemokine receptor type 7-like	LOC111664317	2.629
XM_023429071.1	galectin-2-like	LOC111671845	2.629
XM_023404531.1	cytokine inducible SH2 containing protein	cish	2.609
XM_023401015.1	C-type mannose receptor 2-like	LOC111651055	2.547
XM_023428424.1	interleukin 4 induced 1	il4i1	2.547
XM_023394796.1	interferon-inducible GTPase 5-like	LOC111645553	2.547
XM_023395884.1	interleukin 20 receptor subunit beta, transcript variant X1	il20rb	2.530
XM_023398726.1	RELT, TNF receptor	relt	2.524
XM_023399368.1	immunoglobulin lambda-1 light chain-like	LOC111649636	2.366
XM_023414416.1	interleukin-1 beta-like	LOC111660964	2.366
XM_023407830.1	adherens junctions associated protein 1	ajap1	2.366
XM_023401390.1	interferon regulatory factor 2 binding protein like	irf2bpl	2.214
XM_023419704.1	NFKB inhibitor delta	nfkbid	2.160
XM_023430276.1	heat shock protein HSP 90-alpha	LOC111672729	2.141
XM_023399529.1	claudin-23-like, transcript variant X2	LOC111649834	2.115
XM_023399530.1	claudin-23-like, transcript variant X3	LOC111649834	2.115
XM_023399527.1	claudin-23-like, transcript variant X1	LOC111649834	2.115
XM_023399111.1	gap junction Cx32.2 protein-like	LOC111649336	2.095
XM_023398423.1	cathepsin L1-like	LOC111648648	2.044
XM_023418047.1	interleukin-22 receptor subunit alpha-2-like, transcript variant X1	LOC111663746	2.004

3.6. Validation of RNA-Seq Results by Quantitative RT-PCR

To verify our RNA-seq results, the relative expression of four genes representing DEGs from the comparison between control and experimental groups was investigated using qRT-PCR. The qRT-PCR results showed that these genes were significantly upregulated in the skin of yellowtail fed with a silkrose-containing diet (Figure S2). A similar pattern of relative fold change between RNA-seq and qRT-PCR results was observed, as shown in Figure S2A. These results further confirm the reliability and accuracy of RNA-seq for gene expression analysis.

4. Discussion

Two types of challenge tests of yellowtail with *Benedenia seriolae* were performed with different objectives. One used a low count of parasites, 135 per tank of five individuals, to see the effect of silkrose on the parasitism of *B. seriolae* on yellowtail. The other used a high count of parasites, 5000 to 10,000 per tank, to observe the effect of silkrose on the mortality of yellowtail due to parasitism. Silkrose treatment significantly reduced the number of *B. seriolae* parasites in yellowtail in the test with a low count of parasites and prevented mortality due to excessive *B. seriolae* parasitism in the test with a high count of parasites. During the experiment with a low count of parasites, all yellowtails were identified by tags and changes in the weight and length of each one were observed to determine the effect of silkrose treatment on their growth. Both weight and length gain

were significantly increased in the silkrose-treated group compared to the control group. This is thought to be due to the lower number of parasites in the treated group and thus less stress caused by parasites, rather than a direct effect of the silkrose. The stress and growth are discussed below.

In the experiment with more parasites, there were no deaths in the silkrose-treated group. The main reason for the absence of mortality in this group may be the reduction in the number of *B. seriolae* by silkrose treatment.

Damage caused by ectoparasitism in aquaculture is thought to be due to morbidity from other infections from the parasite site rather than a direct cause of parasite infestation [5]. In the parasitic challenge test with high counts of *B. seriolae* in yellowtail, the mortality of the fish observed in the control group without silkrose treatment might be due to the same reason. Our previous studies have shown that silkrose activates the immune system of fish at the molecular level, leading to the acquisition of disease resistance [33–36]. Therefore, it is possible that the absence of mortality due to parasite infection after silkrose treatment was related not only to the reduced parasite numbers but also to the immune activation of the affected fish.

Silkrose treatment was also found to be effective against parasite infection under actual aquaculture conditions. In an experiment using yellowtail at an aquaculture field site, treatment with silkrose significantly reduced the number of *B. seriolae* infections compared to the control group. The number of parasites did not affect the growth of yellowtail, because the average number of parasites was fewer than 20 per individual. The number of dead fish in the cage of the silkrose-treated group was significantly different from that of the untreated control group, but this was probably due to the immunostimulation of yellowtail by silkworm, as shown previously, rather than the influence of parasites.

Silkrose treatment significantly reduced the number of parasites in white trevally at the aquaculture field site. Two species of parasites, *Caligus longipedis* and *Neobenedenia gillele*, were found in the cultured white trevally. Silkrose treatment was effective in reducing infection by both species.

In the present experiment, the parasitism of *C. longipedis* in white trevally was serious. In the control group cage, there were more than 400 *C. longipedis* parasites per fish, and there were many dead fish; 9.64% of the cultured fish died during the experimental period. The cause of death of these fish might be thought to be secondary effects of bacterial or viral infection from the damaged skin caused by the parasite rather than the direct effects of parasitism [5]. Further studies on this regard are still needed to confirm the cause of death.

Although the number of *C. longipedis* parasites was significantly reduced in the silkrose-treated group, there were nearly 300 per fish. However, the mortality rate of the silkrose-treated group was 3.48% during the experimental period, which was significantly lower than that of the control group, and the weight and fork length of the fish were also significantly increased compared to the control group, indicating that the silkrose treatment was effective for cultured white trevally.

In vertebrates, because serum levels of cortisol increase in response to stressful stimulation, it is used as an indicator of stress [51]. Since the serum level of cortisol is sensitive to fluctuations [52], the collection of blood samples must be rigorous. The data on cortisol levels of yellowtail and white trevally obtained in this study showed relatively low variability in measurements from individual fish, with standard errors ranging from 9.8 to 24.0% of the mean, suggesting that there were no technical problems with the blood collection and the values obtained indicate the stress levels of the experimental fish.

Comparing the blood cortisol levels in the control groups of yellowtail and white trevally, it can be seen that the values for white trevally are markedly higher. In general, levels of cortisol in fish blood under resting or unstressed conditions are less than 30–40 ng/mL [51]. These values are expected to differ greatly depending on the season and the rearing and growth conditions. In general, however, serum cortisol levels in yellowtail are close to those reported for other fish species, and the values for white trevally are

considered to be relatively high. This high serum cortisol level in white trevally may be caused by excessive parasitic infection.

Serum cortisol levels in white trevally were significantly decreased by the silkrose treatment. This decrease could be caused by a significant decrease in the number of parasites. However, it is also possible that the silkrose treatment itself causes a decrease in serum cortisol levels, as the treatment induced a significant decrease in serum cortisol levels even in yellowtail that had not been infected with many parasites. In agreement with the current result, naturally derived polysaccharides, such as those *from Astralagus propinquus* [53], *Ulva intestinalis* [54], *Gracilariopsis persica* [54], as well as β-glucan [55,56], have also been found to be associated with the reduction of serum cortisol level in various teleost species. These previous studies, however, did not establish the underlying mechanism of this cortisol-lowering effect. It is widely accepted that the secretion of cortisol in teleost is regulated by steroidogenic cells distributed in the head-kidney region [57]. Further investigation, such as omics profiling of head-kidney, is needed to get a better insight into how silkrose can lower the cortisol level in teleost. As mentioned above, a decrease in serum cortisol levels indicates a decrease in stress in the fish, and the growth promotion by silkrose treatment was possibly a result of the decreased stress.

In the current study, we utilized a whole transcriptome analysis tool, the RNA-seq technique, to reveal the changes in all gene expression levels in the skin of yellowtail after they were fed dietary silkrose. There were not many changes in the expression of genes related to immunity. However, since more than 40% of the genes in this analysis were not mapped in the genome database (Table S3), it is possible that immune-related genes are included in these unmapped genes. Despite that, several genes related to the immune system were still observed as being potentially upregulated in the skin due to dietary treatment with silkrose, including inflammatory reaction, complement cascade, bactericidal effects, and response to stress (Table 1). Interestingly, similar patterns were also observed in our previous study of silkrose-BM and -AY in the liver and intestine of Japanese medaka (*Orzyias latipes*) [35,36]. These findings further indicate that oral administration of an immunostimulant, in this case silkrose, could affect transcriptomic changes in multiple tissues in teleosts, thus providing a better defense mechanism against various pathogenic infections.

Generally, the immunostimulatory activities of polysaccharides are strongly related with their structural properties being similar to the molecular pattern found in foreign matters that are generally absent from the host [58]. The addition of these unique structural features, which are called pathogen-associated molecular patterns (PAMPs), is then recognized by the host–pathogen recognition receptor (PRRs) to induce the activation of the innate immune system [59,60]. Our previous studies on mammalian RAW264.7 cells suggest that silkrose utilizes Toll-like receptors, a family of PRR, to modulate the activation of innate immunity [33,34]. Similar observations were also found in the intestine and liver of Japanese medaka (*Oryzias latipes*) fed with silkrose [35,36]. Taken together, silkrose may act as PAMP and utilize host PRRs to stimulate innate immune responses by mimicking the early phase of bacterial infection, thus leading to better preparation of host immune system against pathogen invasion [34–36]. In the current study, we did not conduct transcriptomic profiling in the intestine of yellowtail. However, the similarity between transcriptomic profiling patterns of yellowtails skin and the previous RNA-seqs resulting from the intestine and liver of medaka further indicates that silkrose may also be bound to the PRRs in the intestine and liver of yellowtail, thus inducing immune responses in multiple tissues in the host. Further analysis on the transcriptomic profiling of yellowtail's intestine fed with silkrose is needed to confirm these phenomena.

There are only limited studies that discuss the possible role of the fish immune system and its relationship with endoparasitic disease. However, recent studies have revealed that ectoparasitic infection could directly induce a dramatic immune response in the skin of aquatic vertebrates [61–65]. This phenomenon is often followed by a prominent decrease in the number of parasites in the teleost's skin [66]. In agreement with the previous studies,

our current result also show that fish treated with silkrose had a significant immune response in the skin and simultaneously showed a decrease in the number of *B. seriolae* compared to fish in the control group.

We predict that the change of immune response in our study is due to the upregulation of pro-inflammatory cytokines such as IL-1β, IL-1r2, TNFrSF5, IL4il, IL20rβ, RELT, and IL-22r (Table 1), resulting in the recruitment of inflammatory cells to the infected areas of the skin [67]. The presence of inflammatory cells such as neutrophils, eosinophils, basophils, and macrophage in the lesions could then alter various immune responses, including complement cascade (C1q and C1qTNF3) and bactericidal factor (lysozyme-C) [67–69], which we found upregulated in this study. Based on these results, we suggest that the improved yellowtail survival rate shown in Figure 3 could be an effect of silkrose as an immune modulator for the host, thus preventing secondary infection in the lesion site, rather than the direct effect of parasites. However, we also found a significant decrease in the number of parasites on fish fed with a silkrose-containing diet (Figures 1, 4 and 5). A report by Johnson [70] suggested that increased numbers of *Lepeophtherius salmonis* eggs in coho salmon (*Oncorhynchus kisutch*) was correlated with reduced immune system function caused by sexual maturation of the fish. Furthermore, other studies in parasitic copepods belonging to the genus *Lernaea* indicate that reinfection of parasite species from these genera to goldfish (*Carassius auratus*), big head carp (*Aristicthys nobilis*), and kissing gourami (*Helostoma temmmincki*) resulted in significantly reduced infectivity and a lower parasite egg hatching rate, thus suggesting the possibility that host-acquired immunity had developed [71–73]. Taking all the data together, the stimulation effect of silkrose in the host immune response might play vital role in the decreased parasite numbers in teleosts.

Besides immune-related genes, elevated expression of stress-related genes such as heat shock proteins (hsp70, hspa5, and hsp90α) was observed in the skin of yellowtail treated with dietary inclusion of silkrose (Table 1). This is comparable to our previous study on the livers of Japanese medaka fed with silkrose-BM [36]. It is widely believed that heat shock proteins are intimately connected to cortisol levels in teleosts, thus providing a possible clue regarding the decreased blood cortisol levels in fish fed with silkrose in this study [74–76]. Further study in this regard will be necessary to elucidate the detailed mechanism of silkrose as a stress-related factor. Furthermore, genes related to tissue remodeling/wound healing such as collagenase 3/matrix metallopeptidase 13 (mmp13) and mmp9 were also found to be upregulated in the skin of yellowtail fed with dietary silkrose [77]. These findings further suggest that fish fed with silkrose might have faster tissue/wound healing compared to controls, thus preventing the occurrence of secondary infection.

5. Conclusions

In this study, we reveal that silkworm-derived silkrose is effective in preventing infection by external parasites in fish. As mentioned above, ectoparasites cause a lot of damage to salmonids and other fish [1,2]. Silkrose is thought to be effective in preventing infection by these ectoparasitic diseases. Although a transcriptional analysis of yellowtail's skin was conducted in this study, further studies in other tissues, such as intestine, liver, and head kidney, are still needed to achieve wider understanding of the mechanism of silkrose in yellowtail. In the future, it is also necessary to investigate the effectiveness of this method for bacterial and viral diseases using cultured fish species.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fishes7010014/s1, Figure S1: RNA-sequencing analysis of skin of yellowtail (*Seriola quinqueradiata*), Figure S2: Confirmation of RNA-seq results by quantitative RT-PCR (qRT-PCR), Table S1: Primers used in quantitative RT-PCR (qRT-PCR) analysis (References [78,79] are cited in Table S1), Table S2: RNA-sequencing summary results of raw reads, clean reads, and values of Q20 and Q30, Table S3: RNA-sequencing summary results of mapping to reference genome.

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Institutional Review Board Statement: Animal experiments were carried out following the guidelines of Ehime University. The study protocol was accepted by the Institutional Animal Care and Use Committee (IACUC) of Ehime University (permit number: 08K2-1). In this study, 2-phenoxyethanol was used as an anesthetic agent at concentrations of 0.05% [40]. Euthanasia was conducted following procedures described by Underwood & Anthony (2020) [41] and Reilly (2001) [42]. All efforts were made to minimize suffering.

Data Availability Statement: The data presented in this study are available on request from the corresponding author, upon reasonable request.

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