

Article

Effects of Dietary Supplementation with Endogenous Probiotics *Bacillus subtilis* on Growth Performance, Immune Response and Intestinal Histomorphology of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

Jing Wang, Dan Fan, Ran Zhao, Tongyan Lu, Shaowu Li *  and Di Wang *

Key Laboratory of Aquatic Animal Diseases and Immune Technology of Heilongjiang Province, Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Harbin 150070, China; wangjing@hrfri.ac.cn (J.W.); zhaoran@hrfri.ac.cn (R.Z.); lutongyan@hrfri.ac.cn (T.L.)

* Correspondence: lishaowu@hrfri.ac.cn (S.L.); wangdi@hrfri.ac.cn (D.W.)

Abstract: The rainbow trout (*Oncorhynchus mykiss*) is an important commercial fish. Studies have shown that probiotics can promote the healthy growth of fish. In this study, we assessed the impact of an endogenous *Bacillus subtilis* strain (RT-BS07) on the growth, survival and immunological response of juvenile rainbow trout. Additionally, the morphology of rainbow trout's intestinal tissue was measured. Control (CT) and experimental groups (B) were fed with conventional and probiotic-supplemented diets (1.0×10^8 CFU per gram) for 42 days, respectively. Following feeding, a challenge experiment was carried out with *Aeromonas hydrophila* at 1.0×10^7 CFU/mL to determine the experimental fish's tolerance to the bacteria. The results showed that the weight gain rate (WGR) in group B was 118.2%, which was significantly higher than that in group CT (54.86%) after 42 days. The height of intestinal villi and the number of goblet cells increased in group B, thus increasing the resistance of rainbow trout to pathogenic bacteria. The alkaline phosphatase (AKP) activity (U/mg prot) in the liver, spleen and intestinal tissues of group B was significantly higher than that in the CT group. The expression of *interleukin-1 β* (IL-1 β) in the intestinal tissue of group B was 6-fold higher than that in group CT. Furthermore, the expression of *growth hormone-2* (GH-2) in the spleen was 3-fold higher than that in group CT. After 12 days of *A. hydrophila* injection, the survival rate was 40% and 70% in group CT and group B, respectively. Collectively, the present study demonstrated that the addition of endogenous *B. subtilis* strain (1.0×10^8 CFU per gram) to the feed can effectively promote the growth of rainbow trout and enhance immunity, which is beneficial to rainbow trout culture.

Keywords: *Oncorhynchus mykiss*; *Bacillus subtilis*; growth; immunity; intestinal histology

Key Contribution: *Bacillus subtilis* (RT-BS07) is an endogenous probiotic that has beneficial effects on rainbow trout. It can enhance the growth and immune response of rainbow trout as a feed additive.



Citation: Wang, J.; Fan, D.; Zhao, R.; Lu, T.; Li, S.; Wang, D. Effects of Dietary Supplementation with Endogenous Probiotics *Bacillus subtilis* on Growth Performance, Immune Response and Intestinal Histomorphology of Juvenile Rainbow Trout (*Oncorhynchus mykiss*). *Fishes* **2024**, *9*, 229. <https://doi.org/10.3390/fishes9060229>

Academic Editor: Kyeong-Jun Lee

Received: 23 May 2024

Revised: 8 June 2024

Accepted: 14 June 2024

Published: 15 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The rainbow trout (*Oncorhynchus mykiss*) is an economic fish that has been cultured worldwide for more than 100 years [1]. With the public concern for nutrition and food security, the high demand for rainbow trout has promoted the development of intensive cultures, yielding about 920,000 tons of global production per year [2]. However, expansion in the field of rainbow aquaculture from extensive to intensive culture will lead to increased occurrences of many epidemic diseases, as well as environmental problems and economic losses [3,4]. Antibiotics and chemotherapeutants are widely used for the prevention and control of diseases, accompanied by many problems such as damage to the immune system of the host, environmental pollution, drug residues, multiple-drug resistance, food safety, etc. [5,6]. Studies have shown that high environmental concentrations of sulfamethoxazole (SMX) and clarithromycin (CLA) cause damage to the zebrafish complement system [7].

Considered as environmentally and consumer-friendly alternatives that can be used to confront the critical situations caused by antibiotics and chemotherapy drugs, the use of probiotics has been widely accepted in sustainable aquaculture globally [8]. Dietary supplementation with probiotics could benefit the health of aquatic animals by improving immune responses, increasing digestive enzyme activities, promoting growth performance, modulating the intestinal flora diversity, improving the water quality and controlling various diseases [9,10]. Many genera of probiotics have been used in aquaculture for their different beneficial properties, such as *Bacillus*, *Lactococcus*, *Micrococcus*, *Lactobacillus*, *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Vibrio*, *Enterococcus*, etc. [11,12]. Among them, *Bacillus* species are widely used in aquafeeds due to their spore-forming ability, which gives them the capacity to survive in extreme environments [13,14]. In addition, *Bacillus* spp. could counteract a wide range of virulence factors by producing a variety of extracellular substances such as trypsin, lipase, amylase and antimicrobials [15,16]. The application of *Bacillus* spp. in rainbow trout aquaculture has been proved to be effective for enhancing their growth performance, immunity, disease resistance and many other aspects [13,17].

It is worth noting that most probiotics used in aquaculture are not derived from aquatic animals or the aquatic environment. For this reason, researchers have begun to pay more attention to host-associated probiotics and their application within aquaculture in recent years [18]. Zhang et al. reported on the safety issues when using probiotics obtained from other species in aquaculture, and suggested developing probiotic strains specific to each species rather than directly extending probiotic strains isolated from one species to others [19]. In our previous study, a *Bacillus subtilis* strain RT-BS07 was isolated from the gut of healthy juvenile rainbow trout. RT-BS07 could produce protease and cellulase, inhibit *Aeromonas* spp. and *Yersinia* spp., have the abilities to survive in extreme environment and adhere to the anterior intestinal mucosa in vitro. An in vivo challenge test also proved that RT-BS07 is non virulent to rainbow trout. Therefore, we selected RT-BS07 as a feed additive. Our hypothesis suggests that using RT-BS07 as a feed additive can promote rainbow trout growth, increase nutrient utilization and enhance immunity to pathogenic bacteria. This study provided a basis for future research on the use of *B. subtilis* (RT-BS07) in rainbow trout and other aquatic species.

2. Materials and Methods

2.1. Fish and Bacteria

A collection of 300 juvenile rainbow trout (35 ± 3 g) was obtained from the Bohai Cold Water Fish Experimental Station of Heilongjiang River Fisheries Research Institute. Randomized bacteriological tests ensured that the experimental fish were healthy and without pathogenic infections. The fish were temporarily cultured in a recirculating system for two weeks. During this period, rainbow trout were fed a basal diet (Alle, Qingdao, China) with a caliber of 2 mm at 7 am and 19 pm daily to achieve satiation. The experiment was conducted using a recirculating water system where 30–40% of the tank water was exchanged daily to keep all tanks clean. A 12 h:12 h light–dark cycle was adjusted. Also, 24 h of uninterrupted oxygen supply was provided with a water temperature of 15.0 ± 0.2 °C, pH of 7.3 ± 0.3 , dissolved oxygen of 8.5 ± 0.5 mg/L and ammonia nitrogen concentration of <0.1 mg/L. All of the animal procedures in this study were approved by the Committee of the Ethics on Animal Care and Experiments at Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences (20230320-001).

The probiotic *B. subtilis* (RT-BS07) used in this study was isolated from the gut of healthy rainbow trout and stored in our laboratory [20]. *Aeromonas hydrophila* 2016-76 was recovered from the spleen of diseased rainbow trout in a Chinese fish farm, and had been kept at the China Center for Type Culture Collection (CCTCC M2019532) [21,22].

2.2. Experimental Diets Preparation

RT-BS07 bacterial suspension with an effective live bacterial count of 1.0×10^9 CFU/mL was used in this experiment. Bacterial solutions were sprayed into commercial feeds (Aller,

Qingdao, China) based on the methods described in the studies of Abidin, Zaenal et al. [23] and Hooshyar, Yalda et al. [24] to achieve a final concentration of 1.0×10^8 CFU per gram. The control diet was sprayed with an equal amount of $1 \times$ PBS. Feeds were ventilated in a cool and dry place, dried and stored temporarily at 4°C . To determine the survivability of the sprayed bacteria, 1 g of feed was dissolved at various time intervals (1, 3, 7, and 14 days), and bacterial counts were performed on culture medium. It was shown that preparation of supplemental feed every three days ensured the viability of the sprayed bacteria.

2.3. Experimental Procedure

After two weeks of temporary culture, 180 fish with average weight of 35.052 ± 3.14 g were randomly divided into control (CT) and experimental (B) groups in triplicate (30 fish per tank). After that, fish were given two different diets for 42 days: one supplemented with RT-BS07 and the other with PBS as a control. The amount of feeding was adjusted weekly according to the body weight of experimental fish.

2.4. Growth Performance and Sample Collection

The initial body weight (IBW) of experimental fish was recorded after acclimation and the final body weight (FBW) was recorded after 42 days of the feeding trial. The total feed amount taken by fish was recorded at the end of the trial. Growth parameters and feed efficiency were calculated as follows:

$$\text{Body weight gain (BWG)} = \text{FBW} - \text{IBW} \quad (1)$$

$$\text{Weight gain rate WGR (\%)} = \text{BWG} / \text{IBW} \times 100 \quad (2)$$

$$\text{Feed conversion ratio (FCR)} = \text{feed intake (FI)} / \text{BWG} \quad (3)$$

$$\text{Specific growth rate (SGR) (\%)} = (\ln \text{FBW} - \ln \text{IBW}) / T \times 100 \quad (4)$$

$$\text{Hepatosomatic index (HSI) (\%)} = \text{liver weight (LW)} / \text{FBW} \times 100 \quad (5)$$

$$\text{Spleen somatic index (SSI) (\%)} = \text{spleen weight (SW)} / \text{FBW} \times 100 \quad (6)$$

$$\text{Intestines length index (ILI) (\%)} = \text{intestines length (IL)} / \text{body length (BL)} \times 100 \quad (7)$$

Prior to sampling, fish were anesthetized with 150 mg/L MS-222 (Sigma-Aldrich, Steinheim, Germany) after 24 h starvation. Samples of liver, spleen, muscle and intestine were collected for each replicate of 6 fish ($n = 18$, as described in 'Materials and Methods 2.3') from Group CT and Group B at each time point (including days 0, 14, 28 and 42). They were used for subsequent experiments.

2.5. Histology Observation

Separate intestinal specimens (approximately 1 cm^2) were cut and fixed in Bouin's solution for more than 24 h. Fixed intestinal samples were then dehydrated, embedded, sectioned, and stained for histological analysis by microscopy (Eclipse Ci-L).

2.6. Determination of Enzyme Activities

Samples of liver, spleen and intestine of six fish from group CT and B on day 42 were selected for enzyme activity assay, respectively. For each sample, 0.1 g of tissue was placed in a 1.5 mL centrifuge tube, followed by the addition of 1 mL of 100 mM phosphate-buffered saline (PBS pH 7.4) and homogenized at 4°C to break down the tissue cells. Finally, the homogenate was centrifuged at 12,000 rpm for 10 min, and the supernatant was removed and placed on ice for testing. The activities of three digestive enzymes in intestinal tissues were determined using three commercial assay kits including trypsin, α -amylase, and lipase (Grace Biotechnology, Suzhou, China). Catalase (CAT), superoxide dismutase (SOD), lysozyme (LZM), acid phosphatase (ACP) and alkaline phosphatase (AKP) activities in liver, spleen and intestine were determined using commercial assay kits (Grace Biotechnology,

Suzhou, China) [25,26]. Calculation of enzyme activity was performed according to the kit instructions.

2.7. Gene Expression Analysis

At each of the four time points (including days 0, 14, 28 and 42), RNA was collected from the liver, spleen, intestine and muscle of six fish from each of the CT and B groups. To examine gene expression levels of *growth hormone-2* (GH-2) and *interleukin-1 β* (IL-1 β). Total RNA was extracted using TIANGEN RNA simple Total RNA Kit (Tiangen, Beijing, China) [27]. Synthesize cDNA using ReverTra Ace[®] qPCR RT Master Mix (TOYOBO, Osaka, Japan) [28]. The specific primer pairs used for RT-qPCR are listed in Table 1 and β -actin gene was used as the reference gene for normalization. RT-qPCR was performed on an ABI 7500 Real-Time PCR System using the kit (TOYOBO, Osaka, Japan). The RT-qPCR reactions were carried out following the procedures described by Liu et al. [29]. The relative gene expression level was analyzed using the $2^{-\Delta\Delta CT}$ method [30].

Table 1. Primers used for gene expression analysis [22,31].

Gene	Primer (5'-3')	Product Size (bp)
GH-2	F: TCGTCCTAACCCTGACTTC R: AAGCCTCTCTCTCCACACACA	104
IL-1 β	F: CGAGCTGGACATGGAGGAGC R: TCGTCCCAGTTGGTGACGAT	103
β -actin	F: GATGGGCCAGAAAGACAGCTA R: TCGTCCCAGTTGGTGACGAT	119

2.8. Challenge Test

Pathogenic *A. hydrophila* 2016-76 which was previously reported in our works was used for the challenge test. After the six-week feeding trial, 10 fish from each of the control and experimental groups were intraperitoneally injected with 0.1 mL of 2016-76 bacterial suspension at a concentration of 1.0×10^7 CFU/mL. Another 10 fish from each group were intraperitoneally injected with 0.1 mL of sterile PBS for control. Fish condition was monitored daily for 12 days of observation and cumulative survival rate was determined.

$$\text{Survival rate} = (\text{number of survivors}) / (\text{original total}) \times 100\%$$

2.9. Statistical Analysis

SPSS v.22 (IBM, Chicago, IL, USA) was utilized to verify the normality of the data. Data among two groups were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple diversity test by GraphPad Prism 8.0. Homogeneity of variance was checked using Levene's test. Data are presented as means $\pm$ standard deviation. p values of <0.05 were considered statistically significant.

3. Results

3.1. Growth Performance

Information on growth performance and feed utilization of juvenile rainbow trout is shown in Table 2. After 42 days, the survival rate was 100% in both of the groups. There were no clinical signs seen in the livers, spleens or intestines of the fish. It is worth noting that the fish in group B performed more positively in swimming and feeding when compared to the fish in group CT. The growth performance of the rainbow trout in the *B. subtilis*-supplemented group showed an advantage. There were extremely significant differences ($p < 0.01$) in the body weight, BWG, WGR, SGR and FCR between group CT and group B after 42 days of the feeding trial. The WGR of group B reached to 118.20%, which was significantly higher than that of group CT ($p < 0.01$). FCR was significantly lower in group B. HSI, SSI and ILI gradually increased during the feeding trial without a significant difference.

Table 2. Growth and feed utilization of rainbow trout.

Growth Index	Control Group (CT)			Experimental Group (B)		
	0 d	28 d	42 d	0 d	28 d	42 d
BWG (g)	-	8.67 ± 3.20	19.37 ± 5.39	-	21.55 ± 2.53 **	43.36 ± 5.16 **
WGR (%)	-	24.57 ± 1.94	54.86 ± 3.78	-	58.73 ± 1.77 **	118.20 ± 6.97 **
SGR (%)	-	0.78 ± 0.03	1.04 ± 0.14	-	1.65 ± 0.17 *	1.86 ± 0.19 **
FCR	-	0.91 ± 0.03	0.92 ± 0.05	-	0.84 ± 0.04 **	0.83 ± 0.06 *
HSI (%)	0.99 ± 0.13	1.11 ± 0.85	1.55 ± 0.85	1.04 ± 0.51	1.15 ± 0.69	1.56 ± 0.68
SSI (%)	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.09 ± 0.02
ILI (%)	30.10 ± 0.99	32.71 ± 1.12	36.54 ± 1.56	29.82 ± 1.03	33.41 ± 1.15	39.22 ± 1.56
Survival rate (%)	100.00	100.00	100.00	100.00	100.00	100.00

All values were obtained from 6 individual fish and presented as mean ± SD. * means the difference is significant ($p < 0.05$); ** means the difference is extremely significant ($p < 0.01$).

3.2. Histological Observation of Intestine

Photomicrographs of the rainbow trout intestines are shown in Figure 1. Intestinal in feed supplemented with *B. subtilis* RT-BS07 (Figure 1C,D) did not reveal any histopathological changes and was similar to the control (Figure 1A,B). Feed supplementation with *B. subtilis* resulted in an increase in the number of goblet cells in the rainbow trout intestine and a reduction in the intestinal villus width (VW) compared to the control group (Table 3). In addition, the intestinal villus height (VH) was significantly increased in group B (Table 3).

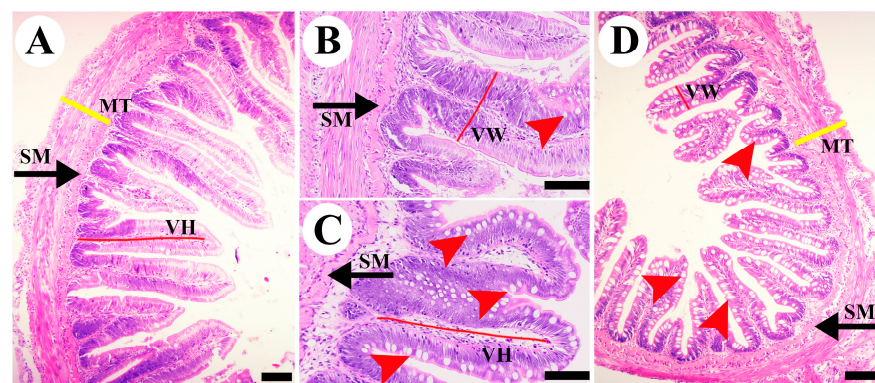


Figure 1. Photomicrographs of hematoxylin-eosin-stained cross sections of rainbow trout intestine under light microscope. (A,B) are control groups and (C,D) are *B. subtilis*-added groups. Abbreviation: villus height (VH), villus width (VW), sub-epithelial mucosa (SM), muscular thickness (MT). Red arrows point to goblet cells. The black scale is 100 μm.

Table 3. Intestinal morphology of rainbow trout fed different diets.

Items	Control Group (CT)	Experimental Group (B)
Villus height (μm)	378.5 ± 21.4	428.9 ± 14.3 *
Villus width (μm)	95.7 ± 14.3	76.6 ± 8.25
Goblet cell number (per 100 μm)	9.9 ± 0.4	19.3 ± 1.5 *

All values were obtained from 3 individual photomicrographs and presented as mean ± SD. * means the difference is significant ($p < 0.05$).

3.3. Digestive and Immune Related Enzymes Activities

As shown in Figure 2, the digestive enzyme activities in the rainbow trout intestinal tissues were determined in the control and *B. subtilis* supplemented groups on day 42. The trypsin activity in group B was 40.08 U/mgprot, which was significantly higher than that in group CT (Figure 2b, $p < 0.05$). The lipase activity of group B was 224.33 U/mgprot, which was three times higher than that of group CT, and the difference was highly significant

(Figure 2c, $p < 0.01$). However, there was no significant difference in α -amylase activities between the two groups.

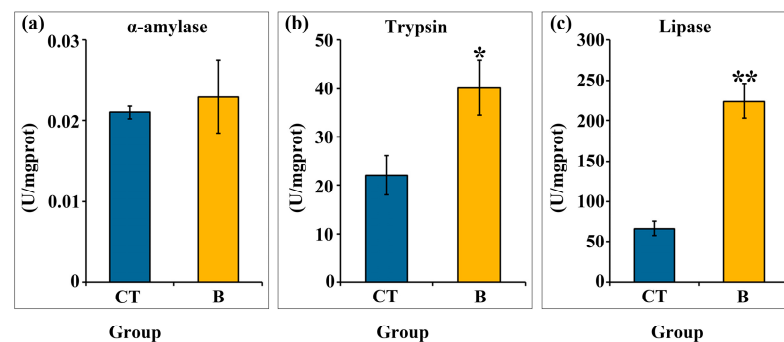


Figure 2. The intestine digestive enzyme activities of *O. mykiss* fed *B. subtilis* supplemented diets for 42 days. (a): α -amylase; (b): trypsin; (c): lipase. CT: control group; B: experimental group. All values were obtained from 6 individual fish ($n = 6$). * means the difference is significant ($p < 0.05$); ** means the difference is extremely significant ($p < 0.01$).

As shown in Figure 3, the immune and antioxidant enzyme activities in several tissues were determined to evaluate the effect of the dietary probiotic supplements on the rainbow trout after 42 days of feeding. The results revealed that *B. subtilis* could evoke higher AKP activities than the control group in all the tested tissues (Figure 3e). Although the SOD, LZM and ACP enzyme activities increased, there was no significant difference compared to the control group.

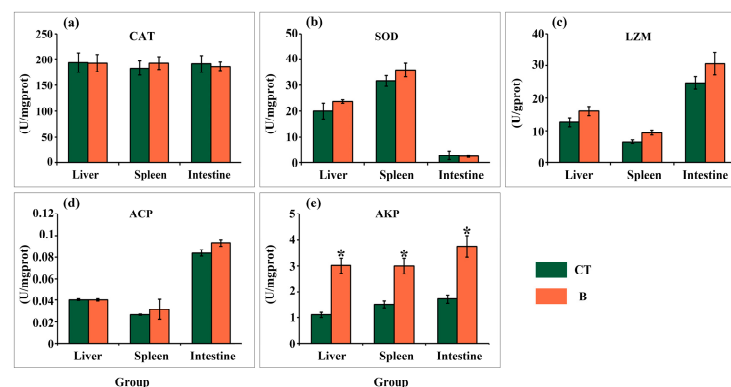


Figure 3. The antioxidant (a,b) and immune enzyme (c–e) activities of *O. mykiss* fed *B. subtilis* supplemented diets for 42 days. CT: control group; B: experimental group. All values were obtained from 6 individual fish ($n = 6$). * means the difference is significant ($p < 0.05$).

3.4. Gene Expression

Changes in the expression of growth and immune genes were detected in the liver, muscle, intestine and spleen of rainbow trout for 42 consecutive days (Figure 4). *IL-1 β* was significantly up-regulated in the intestine and muscle starting from day 14 (Figure 4b,d). In the intestine, its expression was six times higher compared to the control group, showing a highly significant difference ($p < 0.01$). The relative mRNA expression of *GH-2* was significantly up-regulated in the liver (Figure 4e) and spleen (Figure 4g) in group B from day 14 ($p < 0.05$). *GH-2* expression in the intestine (Figure 4h) was also significantly up-regulated at 42 days and was three times higher than in the control group ($p < 0.05$).

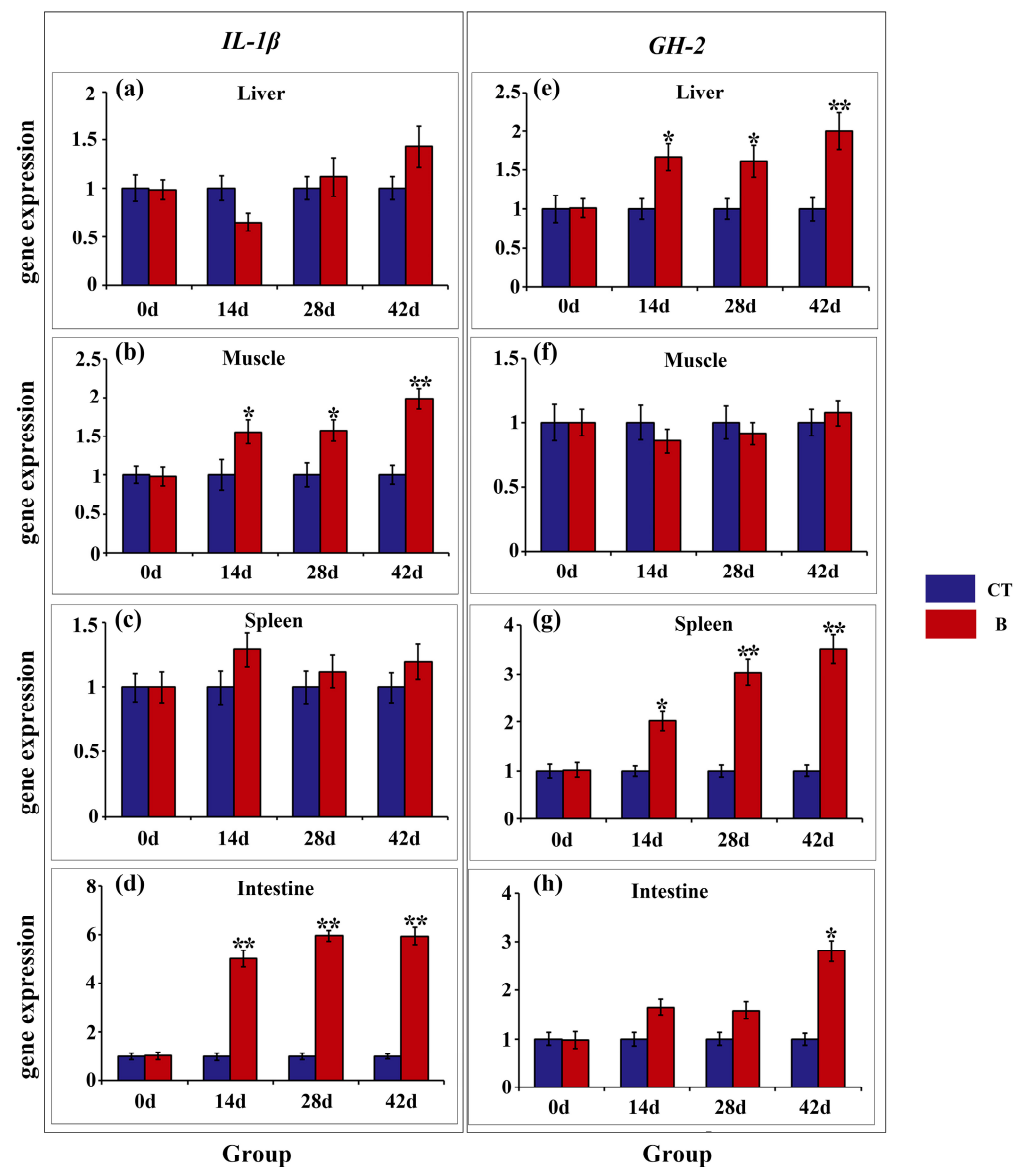


Figure 4. Expression levels of growth and immune-related genes in four tissues (liver, muscle, spleen, intestine) of *O. mykiss*. *IL-1β*: interleukin-1 β (a–d); *GH-2*: growth hormone-2 (e–h). CT: control group; B: experimental group. All values were obtained from 6 individual fish ($n = 6$). * means the difference is significant ($p < 0.05$). ** means the difference is extremely significant ($p < 0.01$).

3.5. Challenge Test

After 12 days of a challenge test with *A. hydrophila* by intraperitoneally injection, the survival rates in group CT and group B were 40% and 70%, respectively, indicating that *B. subtilis* RT-BS07 had some positive effect against *A. hydrophila* infection in the rainbow trout. Our clinical observations showed that fish challenged with *A. hydrophila* exhibited a loss of appetite, slow swimming and enteritis to varying degrees, appearing more obviously in the fish from group CT.

4. Discussion

Probiotics have been widely applied in aquaculture and recognized as a safe and environmentally friendly strategy to improve their growth and immune functions, in addition to enhancing the disease resistance of aquatic animals [32–34]. Among numerous probiotics, *Bacillus* species appear to be well presented and used due to their confirmed probiotic effects [17]. In rainbow trout aquaculture, *Bacillus* species and *Pediococcus acidilactici*

have been also documented to be effective for immunity and disease resistance [35,36]. However, the use of host-associated probiotics and their application have not been well reported on in rainbow trout. In the present study, the probiotic effects of a host-associated *B. subtilis* on rainbow trout were evaluated and the results indicated that *B. subtilis* RT-BS07 could promote the growth significantly and also benefit the feed utilization, immune function, disease resistance through dietary administration. Because it can withstand harsh conditions, generates cellulases and proteases and inhibits *A. hydrophila*, RT-BS07 is adequate for its function in the rainbow trout gut.

RT-BS07 strain, isolated from healthy rainbow trout intestine, showed a remarkable growth-promoting effect on juvenile rainbow trout in this study, while commercialized strains from other sources had no significant effect on the growth parameters of rainbow trout [37]. However, host gut-derived *B. subtilis* could significantly promoted the weight gain rate (WGR), specific growth rate (SGR) of hybrid groupers [33]. Some reports also indicated that bacterial sporulating isolates with diverse carbohydrate activities from the gut of *Dicentrarchus labrax* also showed an excellent positive effect on growth and feed utilization efficiency of their juveniles [38]. Therefore, it is speculated that the beneficial effects of probiotics on the host are influenced by the source of the strain, and native probiotics may be more suitable.

Intestinal morphology affects the absorption and metabolism of nutrients, thus an intact intestinal barrier facilitates normal growth of the animal [39]. The results of this study showed that feeding with RT-BS07 did not cause damage or inflammation to rainbow trout intestinal tissue. In addition, rainbow trout fed RT-BS07 had an increase in the height and density of intestinal villi and a significant increase in the number of goblet cells compared to the control group. Intestinal villi prevent the colonization of harmful bacteria and facilitate the absorption of nutrients [40]. Greater intestinal villi height and density translate into more surface area, which improves intestinal function and nutrition absorption [41,42]. Goblet cells accelerate the absorption of nutrients by producing and releasing mucus, while forming the first line of defense to protect the intestinal lining [43]. Therefore, feeding rainbow with RT-BS07 trout may increase their number of goblet cells, which in turn may boost mucus secretion in the intestines and improve intestinal defensive systems.

Gram-positive bacteria do secrete a wide range of external enzymes, and the presence of probiotics may stimulate the production of endogenous enzymes in some way to affect digestion and improve body growth performance by producing enzymes [44]. RT-BS07 exhibits the same probiotic properties, it can stimulate an increase in the enzyme activity of trypsin, lipase and α -amylase in rainbow trout intestines when added to the feed. Meanwhile, the expression of the growth hormone-2 (*GH-2*) gene in the intestine, liver and spleen were significantly increased, which also verified the growth-promoting effect of RT-BS07. On the other hand, in the process of artificial domestication and breeding, the feed components of rainbow trout change greatly [45]. Studies have shown that the composition of fish gut microbial communities changes in response to the host's diet [46]. RT-BS07 being isolated from the intestines of healthy rainbow trout may also be an important reason for its good adaptability to rainbow trout juvenile and its good promotion of digestion and growth.

In this study, the SOD activity increased in the intestine with probiotic supplementation. The amount of AKP in intestine was significantly increased, which indicated that the ability to digest invading organisms and to digest and absorb nutrients has been strengthened. Just like Dai et al. reported that *B. subtilis* could effectively mitigate the decrease in AKP enzyme activity caused by cadmium exposure in *Procambarus clarkia* [47]. Meanwhile, Liao et al. reported that host gut-derived *B. subtilis* could significantly boost serum lysozyme (LZM), superoxide dismutase (SOD) and acid phosphatase (ACP) levels. Probiotics supplementation has been shown to activate the innate immune response in the fish host [48]. The dietary supplementation with *B. subtilis* enhanced the immune response of rainbow trout in this study, as shown by increased enzyme activity of LZM and the expression of *IL-1 β* in the intestine. Similar observations were described in *Nile tilapia* and

shrimp fed with *Bacillus* [5,32,49]. Moreover, the expression of *IL-1 β* was up-regulated in the intestine starting from day 14. This observation is consistent with the probiotic property of inducing immune cells to produce cytokines, such as *IL-1 β* [50]. This study supports the probiotic role of *B. subtilis* [34–36,49] by showing that dietary endogenous *B. subtilis* can effectively improve the resistance of *O. mykiss* to the pathogenic bacteria *A. hydrophila*.

5. Conclusions

In conclusion, supplementation with endogenous *B. subtilis* probiotics (1.0×10^8 CFU per gram) promoted growth performance and enhanced non-specific immunity in juvenile rainbow trout. Meanwhile, RT-BS07 can improve rainbow trout's intestinal structure, increase absorption capacity and block harmful bacteria colonization by sticking to the digestive tract, boosting rainbow trout's resistance to *A. hydrophila*. Future studies can focus on developing a feeding strategy for *B. subtilis* RT-BS07, which would help in accelerating the pace of commercial applications.

Author Contributions: J.W.: Investigation, Conceptualization, Methodology, Data curation, Writing—Original draft preparation, Writing—review and editing. D.F.: Software, Validation, Investigation, Data curation. R.Z.: Data curation; Methodology; Visualization. T.L.: Data curation; Methodology; Visualization. S.L.: Supervision, Resources, Project administration. D.W.: Investigation, Conceptualization, Methodology, Data curation, Writing—review and editing, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Central Public Interest Scientific Institution Basal Research Fund, CAFS (NO. 2023TD45), the Key Research and Development Program of Heilongjiang Province (NO. JD22A017) and the Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO. HSY202411Q).

Institutional Review Board Statement: All of the animal procedures in this study were approved by the Committee of the Ethics on Animal Care and Experiments at Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Approval code: 20230320-001).

Informed Consent Statement: Not applicable.

Data Availability Statement: All datasets generated for this study are included in the article.

Acknowledgments: The authors would like to express our gratitude and appreciation to those who have taken the time to critically review this manuscript, as well as those who has given lots of help during this research at Heilongjiang River Fisheries Research Institute, CAFS.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Bobe, J.; Marandel, L.; Panserat, S.; Boudinot, P.; Berthelot, C.; Quillet, E.; Volff, J.-N.; Genêt, C.; Jaillon, O.; Crollius, H.R.; et al. The rainbow trout genome, an important landmark for aquaculture and genome evolution. In *Genomics in Aquaculture*; Academic Press: Cambridge, MA, USA, 2016; pp. 21–43.
2. Food and Agriculture Organization of the United Nations. *The State of World Fisheries and Aquaculture: Towards Blue Transformation*; FAO: Rome, Italy, 2022.
3. Xie, J.J.; Liu, Q.Q.; Liao, S.; Fang, H.H.; Yin, P.; Xie, S.W.; Tian, L.X.; Liu, Y.J.; Niu, J. Effects of dietary mixed probiotics on growth, non-specific immunity, intestinal morphology and microbiota of juvenile pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* **2019**, *90*, 456–465. [[CrossRef](#)] [[PubMed](#)]
4. Costello, M. The global economic cost of sea lice to the salmonid farming industry. *J. Fish Dis.* **2009**, *32*, 115. [[CrossRef](#)] [[PubMed](#)]
5. Kewcharoen, W.; Srisapoome, P. Probiotic effects of *Bacillus* spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). *Fish Shellfish Immunol.* **2019**, *94*, 175–189. [[CrossRef](#)] [[PubMed](#)]
6. Bueno, I.; Travis, D.; Gonzalez-Rocha, G.; Alvarez, J.; Lima, C.; Benitez, C.G.; Phelps, N.B.D.; Wass, B.; Johnson, T.J.; Zhang, Q.; et al. Antibiotic Resistance Genes in Freshwater Trout Farms in a Watershed in Chile. *J. Environ. Qual.* **2019**, *48*, 1462–1471. [[CrossRef](#)] [[PubMed](#)]
7. Pereiro, P.; Rey-Campos, M.; Figueras, A.; Novoa, B. An environmentally relevant concentration of antibiotics impairs the immune system of zebrafish (*Danio rerio*) and increases susceptibility to virus infection. *Front. Immunol.* **2023**, *13*, 1100092. [[CrossRef](#)] [[PubMed](#)]

8. Rohani, M.F.; Islam, S.M.M.; Hossain, M.K.; Ferdous, Z.; Siddik, M.A.B.; Nuruzzaman, M.; Padeniya, U.; Brown, C.; Shahjahan, M. Probiotics, prebiotics and synbiotics improved the functionality of aquafeed: Upgrading growth, reproduction, immunity and disease resistance in fish. *Fish Shellfish Immunol.* **2022**, *120*, 569–589. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Ma, K.; Chen, S.; Wu, Y.; Ma, Y.; Qiao, H.; Fan, J.; Wu, H. Dietary supplementation with microalgae enhances the zebrafish growth performance by modulating immune status and gut microbiota. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 773–788. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Sun, Y.; Xiang, Y.; He, M.; Zhang, X.; Wang, S.; Guo, W.; Liu, C.; Cao, Z.; Zhou, Y. Evaluation of *Lactococcus lactis* HNL12 combined with *Schizochytrium limacinum* algal meal in diets for humpback grouper (*Cromileptes altivelis*). *Fish Shellfish Immunol.* **2019**, *94*, 880–888. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Wang, A.; Ran, C.; Wang, Y.; Zhang, Z.; Ding, Q.; Yang, Y.; Olsen, R.E.; Ringø, E.; Bindelle, J.; Zhou, Z. Use of probiotics in aquaculture of China—a review of the past decade. *Fish Shellfish Immunol.* **2019**, *86*, 734–755. [\[CrossRef\]](#) [\[PubMed\]](#)
12. El-Saadony, M.T.; Alagawany, M.; Patra, A.K.; Kar, I.; Tiwari, R.; Dawood, M.A.O.; Dhama, K.; Abdel-Latif, H.M.R. The functionality of probiotics in aquaculture: An overview. *Fish Shellfish Immunol.* **2021**, *117*, 36–52. [\[CrossRef\]](#)
13. Docando, F.; Nuñez-Ortiz, N.; Serra, C.R.; Arense, P.; Enes, P.; Oliva-Teles, A.; Díaz-Rosales, P.; Tafalla, C. Mucosal and systemic immune effects of *Bacillus subtilis* in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* **2022**, *124*, 142–155. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Setlow, P.; Eichenberger, P.; Driks, A. Spore Resistance Properties. In *The Bacterial Spore: From Molecules to Systems*; John Wiley & Sons: Hoboken, NJ, USA, 2014; pp. 201–215.
15. Yilmaz, M.; Soran, H.; Beyatli, Y. Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiol. Res.* **2006**, *161*, 127–131. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Wang, Y.-B. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture* **2007**, *269*, 259–264. [\[CrossRef\]](#)
17. Abbass, A.; Sharifuzzaman, S.M.; Austin, B. Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* **2009**, *33*, 31–37. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Van Doan, H.; Hoseinifar, S.H.; Ringø, E.; Ángeles Esteban, M.; Dadar, M.; Dawood, M.A.O.; Faggio, C. Host-associated probiotics: A key factor in sustainable aquaculture. *Rev. Fish. Sci. Aquac.* **2019**, *28*, 16–42. [\[CrossRef\]](#)
19. Zhang, Z.; Zhang, H.L.; Yang, D.H.; Hao, Q.; Yang, H.W.; Meng, D.L.; Meindert de Vos, W.; Guan, L.L.; Liu, S.B.; Teame, T.; et al. *Lactobacillus rhamnosus* GG triggers intestinal epithelium injury in zebrafish revealing host dependent beneficial effects. *iMeta* **2024**, *3*, e181. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Fan, D.; Deng, F.R.; Li, S.W.; Lu, T.Y.; Liu, H.B.; Wang, D. Isolation, identification and biological characterization of a *Bacillus subtilis* strain from rainbow trout (*Oncorhynchus mykiss*). *Period. Ocean. Univ. China* **2022**, *52*, 41–49.
21. Cao, Y.S.; Li, S.W.; Han, S.C.; Wang, D.; Zhao, J.Z.; Xu, L.M.; Liu, H.B.; Lu, T.Y. Characterization and application of a novel *Aeromonas* bacteriophage as treatment for pathogenic *Aeromonas hydrophila* infection in rainbow trout. *Aquaculture* **2020**, *523*, 735193. [\[CrossRef\]](#)
22. Cao, Y.S.; Li, S.W.; Wang, D.; Zhao, J.Z.; Xu, L.M.; Liu, H.B.; Lu, T.Y.; Mou, Z.B. Genomic characterization of a novel virulent phage infecting the *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*). *Virus Res.* **2019**, *273*, 197764. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Abidin, Z.; Huang, H.T.; Hu, Y.F.; Chang, J.J.; Huang, C.Y.; Wu, Y.S.; Nan, F.H. Effect of dietary supplementation with *Moringa oleifera* leaf extract and *Lactobacillus acidophilus* on growth performance, intestinal microbiota, immune response, and disease resistance in whiteleg shrimp (*Penaeus vannamei*). *Fish Shellfish Immunol.* **2022**, *127*, 876–890. [\[CrossRef\]](#)
24. Hooshyar, Y.; Abedian Kenari, A.; Paknejad, H.; Gandomi, H. Effects of *Lactobacillus rhamnosus* ATCC 7469 on Different Parameters Related to Health Status of Rainbow Trout (*Oncorhynchus mykiss*) and the Protection Against *Yersinia ruckeri*. *Probiotics Antimicrob. Proteins* **2020**, *12*, 1370–1384. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Tian, J.; Yang, Y.; Xu, W.; Du, X.; Ye, Y.; Zhu, B.; Huang, Y.; Zhao, Y.; Li, Y. Effects of β -1,3-glucan on growth, immune responses, and intestinal microflora of the river prawn (*Macrobrachium nipponense*) and its resistance against *Vibrio parahaemolyticus*. *Fish Shellfish Immunol.* **2023**, *142*, 109142. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Jia, R.; Hou, Y.; Feng, W.; Nomingere, M.; Li, B.; Zhu, J. Multi-Omics Analysis to Understand the Effects of Dietary Proanthocyanidins on Antioxidant Capacity, Muscle Nutrients, Lipid Metabolism, and Intestinal Microbiota in *Cyprinus carpio*. *Antioxidants* **2023**, *12*, 2095. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Wang, Q.; Pan, Y.; Huang, J.; Li, Y.; Wu, S.; Zhao, L.; Sun, T.; Kang, Y.; Liu, Z. Dietary supplementation of Chinese herbal medicines enhances the immune response and resistance of rainbow trout (*Oncorhynchus mykiss*) to infectious hematopoietic necrosis virus. *Front. Vet. Sci.* **2024**, *11*, 1341920. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Saengrungs, J.; Bunnay, A.; Du, X.; Huang, L.; An, R.; Liang, X.; Srisapome, P. Effects of ribonucleotide supplementation in modulating the growth of probiotic *Bacillus subtilis* and the synergistic benefits for improving the health performance of Asian seabass (*Lates calcarifer*). *Fish Shellfish Immunol.* **2023**, *140*, 108983. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Liu, S.; Wang, D.; Cao, Y.S.; Lu, T.Y.; Liu, H.B.; Li, S.W. Effects of propolis on the immune enhancement of the formalin-inactivated *Aeromonas salmonicida* vaccine. *Aquac. Res.* **2020**, *51*, 4759–4770. [\[CrossRef\]](#)
30. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. [\[CrossRef\]](#)

31. Biao, G. *The Effect of Acute Temperature Stress on Blood Components and Key Metabolism Mechanisms in Rainbow Trout (Oncorhynchus mykiss)*; Ocean University of China: Qingdao, China, 2014.
32. Van Doan, H.; Wangkahart, E.; Thaimuangphol, W.; Panase, P.; Sutthi, N. Effects of *Bacillus* spp. mixture on growth, immune responses, expression of immune-related genes, and resistance of Nile Tilapia against *Streptococcus agalactiae* infection. In *Probiotics and Antimicrobial Proteins*; Springer: Berlin/Heidelberg, Germany, 2021; pp. 1–16.
33. Liao, J.; Cai, Y.; Wang, X.; Shang, C.; Zhang, Q.; Shi, H.; Wang, S.; Zhang, D.; Zhou, Y. Effects of a potential host gut-derived probiotic, *Bacillus subtilis* 6-3-1, on the growth, non-specific immune response and disease resistance of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂). *Probiotics Antimicrob. Proteins* **2021**, *13*, 1119–1137. [[CrossRef](#)] [[PubMed](#)]
34. Du, R.; Zhang, H.; Chen, J.; Zhu, J.; He, J.; Luo, L.; Lin, S.; Chen, Y. Effects of dietary *Bacillus subtilis* DSM 32315 supplementation on the growth, immunity and intestinal morphology, microbiota and inflammatory response of juvenile largemouth bass *Micropterus salmoides*. *Aquac. Nutr.* **2021**, *27*, 2119–2131. [[CrossRef](#)]
35. Raida, M.K.; Larsen, J.L.; Nielsen, M.E.; Buchmann, K. Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *J. Fish Dis.* **2003**, *26*, 495–498. [[CrossRef](#)]
36. Villumsen, K.R.; Ohtani, M.; Forberg, T.; Aasum, E.; Tinsley, J.; Bojesen, A.M. Synbiotic feed supplementation significantly improves lipid utilization and shows discrete effects on disease resistance in rainbow trout (*Oncorhynchus mykiss*). *Sci. Rep.* **2020**, *10*, 16993. [[CrossRef](#)] [[PubMed](#)]
37. Hisar, O.; Yilmaz, S.; Yigit, M. Effects of different probiotic bacteria on growth, body composition, immune response and hematological parameters of rainbow trout (*Oncorhynchus mykiss*) under sublethal water temperature. *Mar. Sci. Technol. Bull.* **2015**, *4*, 21–28.
38. Cláudia, R.S.; Eduarda, M.A.; Inês, G.; Rafaela, S.; Merrifield, D.L.; Tavares, F.; Oliva-Teles, A.; Enes, P. Selection of carbohydrate-active probiotics from the gut of carnivorous fish fed plant-based diets. *Sci. Rep.* **2019**, *9*, 6384.
39. Xia, X.; Liang, N.; Ma, X.; Qin, L.; Chang, Z.; Zhang, X. Effect of dietary supplementation with *Leuconostoc mesenteroides* DH on the antimicrobial capacity and overall health of juvenile loach (*Misgurnus anguillicaudatus*). *Aquaculture* **2024**, *579*, 740208. [[CrossRef](#)]
40. Caspary, W.F. Physiology and pathophysiology of intestinal absorption. *Am. J. Clin. Nutr.* **1992**, *55*, 299S–308S. [[CrossRef](#)] [[PubMed](#)]
41. Standen, B.T.; Peggs, D.L.; Rawling, M.D.; Foey, A.; Davies, S.J.; Santos, G.A.; Merrifield, D.L. Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*. *Fish Shellfish Immunol.* **2016**, *49*, 427–435. [[CrossRef](#)] [[PubMed](#)]
42. Ferguson, R.M.W.; Merrifield, D.L.; Harper, G.M.; Rawling, M.D.; Mustafa, S.; Picchietti, S.; Balcázar, J.L.; Davies, S.J. The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia (*Oreochromis niloticus*). *J. Appl. Microbiol.* **2010**, *109*, 851–862. [[CrossRef](#)] [[PubMed](#)]
43. Kühlwein, H.; Merrifield, D.L.; Rawling, M.D.; Foey, A.D.; Davies, S.J. Effects of dietary β -(1,3)(1,6)-D-glucan supplementation on growth performance, intestinal morphology and haemato-immunological profile of mirror carp (*Cyprinus carpio* L.). *J. Anim. Physiol. Anim. Nutr.* **2013**, *98*, 279–289. [[CrossRef](#)]
44. Kwaku, A.; Huang, Q.; Tan, B.; Zhang, S.; Chi, S.; Yang, Q.; Liu, H.; Dong, X. Dietary supplementation of probiotic *Bacillus coagulans* ATCC 7050, improves the growth performance, intestinal morphology, microflora, immune response, and disease confrontation of Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish. Immunol.* **2019**, *87*, 796–808.
45. Vazquez-Duhalt, R.; Callet, T.; Médale, F.; Larroquet, L.; Surget, A.; Aguirre, P.; Kerneis, T.; Labbé, L.; Quillet, E.; Geurden, I.; et al. Successful selection of rainbow trout (*Oncorhynchus mykiss*) on their ability to grow with a diet completely devoid of fishmeal and fish oil, and correlated changes in nutritional traits. *PLoS ONE* **2017**, *12*, e0186705.
46. Zhang, Y.; Wen, B.; Meng, L.J.; Gao, J.Z.; Chen, Z.Z. Dynamic changes of gut microbiota of discus fish (*Symphysodon haraldi*) at different feeding stages. *Aquaculture* **2021**, *531*, 735912. [[CrossRef](#)]
47. Dai, C.; Lin, A.; Mo, A.; Yuan, Y.; Yuan, J.; Gu, Z.; Wang, J. Effect of dietary *Bacillus subtilis* supplement on Cd toxicokinetics and Cd-induced immune and antioxidant impairment of *Procambarus clarkii*. *Environ. Sci. Pollut. Res.* **2023**, *30*, 43914–43926. [[CrossRef](#)] [[PubMed](#)]
48. Dang, Y.; Sun, Y.; Zhou, Y.; Men, X.; Wang, B.; Li, B.; Ren, Y. Effects of probiotics on growth, the toll-like receptor mediated immune response and susceptibility to *Aeromonas salmonicida* infection in rainbow trout *Oncorhynchus mykiss*. *Aquaculture* **2022**, *561*, 738668. [[CrossRef](#)]
49. Panase, A.; Thirabunyanon, M.; Promya, J.; Chitmanat, C. Influences of *Bacillus subtilis* and fructooligosaccharide on growth performances, immune responses, and disease resistance of Nile tilapia, *Oreochromis niloticus*. *Front. Vet. Sci.* **2023**, *9*, 1094681. [[CrossRef](#)]
50. Yun, S.W.; Kim, J.K.; Lee, K.E.; Oh, Y.J.; Choi, H.J.; Han, M.J.; Kim, D.H. A probiotic *Lactobacillus gasseri* alleviates *Escherichia coli*-induced cognitive impairment and depression in mice by regulating IL-1 β expression and gut microbiota. *Nutrients* **2020**, *12*, 3441. [[CrossRef](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.