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Limosilactobacillus fermentum SWP-AFFS02 Improves the Growth and Survival Rate of White Shrimp via Regulating Immunity and Intestinal Microbiota

Bao-Hong Lee [1,†], Wei-Hsuan Hsu [2,3,4,†], You-Zuo Chen [2], Kung-Ting Hsu [4] and Tzu-Ming Pan [5,6,*]

1 Department of Horticulture, National Chiayi University, Chiayi 600355, Taiwan; bhlee@mail.ncyu.edu.tw
2 Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan 701401, Taiwan; whhsu@mail.ncku.edu.tw (W.-H.H.); xzp666842@gmail.com (Y.-Z.C.)
3 Center of Allergy and Mucosal Immunity Advancement at the National Cheng Kung University, Tainan 701401, Taiwan
4 Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 701401, Taiwan; az690857@gmail.com
5 Department of Research and Development Division, SunWay Biotech Co., Ltd., Taipei 114067, Taiwan
6 Department of Biochemical Science and Technology, College of Life Science, National Taiwan University, Taipei 10617, Taiwan
* Correspondence: tmpan@ntu.edu.tw
† These authors contribute equally to this work.

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Abstract: White shrimp *Litopenaeus vannamei* is an important species of farmed shrimp. Intestinal bacterial composition and immune activity play important roles in regulating the health condition of shrimp. Lactic acid bacteria *Limosilactobacillus fermentum* SWP-AFFS02 was isolated from the intestine of sea fish *Rachycentron canadum*, and the potential of its effect on growth, immunity, and intestinal microbiota of *L. vannamei* shrimp was investigated. Shrimps received feed with or without the addition of 8 log CFU/g *L. fermentum* SWP-AFFS02 thrice a day for 8 weeks. After 8-week treatment, weight gain, feed conversion rate, and survival rate of shrimp were greater in the *L. fermentum* SWP-AFFS02-feed group than in the control group. *L. fermentum* SWP-AFFS02 treatment increased the number of granular cells and semi-granular cells and decreased hyaline cell number when compared to the control group. *L. fermentum* SWP-AFFS02 promoted prophenoloxidase (PO) activity through increasing immune-associated gene expression in the hepatopancreas of shrimp. In addition, administration of feed containing *L. fermentum* SWP-AFFS02 regulated intestinal microbiota via decreasing the ratio of pathogenic bacteria, such as *Vibrionaceae* and *Enterobacteriaceae*, in the intestine of shrimp. This study demonstrated that administration of *L. fermentum* SWP-AFFS02 effectively prevented infection of *L. vannamei* shrimp by regulating intestinal microbiota and enhancing immunity in shrimp to increase the growth and improve their health status, which acted as a probiotic and provided beneficial effects on shrimp.

Keywords: gut microbiota; lactic acid bacteria; *Litopenaeus vannamei*; white shrimp; *Limosilactobacillus fermentum*; intestinal immunity

1. Introduction

Litopenaeus vannamei, commonly known as white shrimp, is native to Central and South America, which has strong tolerance to the environment, disease resistance, and osmotic pressure regulation in low-salinity waters. It is characterized by its feed conversion rate, fast growth rate, low residual food rate, and suitability for high breeding density, which have made it an internationally important species of farmed shrimp. Developing an intensive farming model to enable an increase in stocking density per unit area has caused problems, such as water contamination. Chemicals, growth of pathogenic bacteria,

and immunity decline lead to the occurrence of diseases in the aquaculture industry [1]. However, the use of antibiotics causes the pathogenic bacteria to develop drug resistance, which makes it more difficult to treat diseases during the aquaculture period.

There are many types of bacteria in the intestinal tract. These bacteria can successfully adsorb on the surface of intestinal mucosal cells and proliferate in the intestinal tract. Moreover, intestinal bacteria can be roughly divided into three categories: (i) normal flora that often exists in the intestine of the host, (ii) probiotics that are beneficial to the health of the host, and (iii) opportunistic or pathogenic bacteria. The interactions (bacterial interference, bacterial antagonism, barrier effect, and competitive exclusion) between probiotics and pathogenic bacteria play an important role in stabilizing the intestinal physiological functions of the host, including intestinal immune activity and intestinal bacterial composition [2].

Feeding grass shrimp (*Penaeus monodon*) with *Lactobacillus* spp. as a probiotic can increase the resistance to the pathogenic bacteria *Vibrio harveyi* [3], and feeding with the shrimp *Bacillus subtilis* BP11 can increase their survival rate, improve immune activity, and reduce *V. harveyi* infection [4,5]; adding *Lactobacillus plantarum* to the feed can improve the intestinal function and enzyme secretion capacity of *L. vannamei* to elevate the metabolic utilization of feed and increase the growth rate [6]. In addition, studies have shown that the use of prebiotics to maintain water quality during shrimp farming can effectively resist the growth of pathogenic bacteria and increase the growth rate and production of shrimp [7]. Sha et al. (2016) found that adding different probiotics (*L. pentosus* and *Enterococcus faecium*) or the bacteria-free supernatant of a *L. pentosus* culture to the feed can significantly increase *Actinobacteria* in the intestine and maintain physiological health in *L. vannamei* shrimp [8]. Therefore, *B. subtilis* and lactic acid bacteria (LAB) are gradually being used in aquaculture. According to a recent study, the benefits of probiotics isolated from aquaculture species (fish and shrimp) are greater than those obtained from non-aquaculture species [9]. Dash et al. (2014) showed that another kind of LAB (approximately 3–9 cfu/mL) can also be determined in pond water after adding *L. plantarum* to the pond water of *Macrobrachium rosenbergii* [10], indicating that probiotic treatment may improve the quality and microbiota of the water environment, which promote the growth of other types of probiotics. These results suggest that LAB are adaptable to the environment. We isolated the bacterium *Limosilactobacillus fermentum* SWP-AFFS02 from the intestine of the sea fish *Rachycentron canadum*. The aim of this study was to evaluate the potential of LAB feed containing *L. fermentum* SWP-AFFS02 to regulate the growth, immunity, and intestinal microbiota of *L. vannamei* shrimp. The results in this study are the first to prove that *L. fermentum* SWP-AFFS02 has the potential to act as a probiotic.

2. Materials and Methods

2.1. Culture of L. fermentum SWP-AFFS02

LAB *L. fermentum* SWP-AFFS02 was isolated from the intestine of the sea fish *R. canadum* via MRS selection medium with $CaCO_3$. After the Gram stain and catalase test, the strain was identified by 16S ribosomal DNA sequencing. LAB were cultured in MRS broth under anaerobic equipment (Oxoid, Basingstoke, Hampshire, England) at 37 °C, and the numbers of the LAB were detected on MRS agar plates (Difco Laboratories, Detroit, MI, USA). The commercial feed (young eel feed No. F; Tung Li Feed Industrial Co., LTD, PingTung, Taiwan) was inoculated with precultured LAB in MRS for 30 min (feed: MRS = 2:1; *w*/*w*). Finally, the LAB-dietary feed was freeze-dried by a freeze drier system (Rebers, Taoyuan, Taiwan) and stored at −20 °C.

2.2. The Number of LAB in Feed

According to the method of Wirunpan et al. (2016) [11], the feed with added LAB (1 g) was crushed, mixed with 9 mL of PBS, and diluted to appropriate concentration by serial dilution, then 0.1 mL of the suspension was inoculated on MRS agar. After cultivation for 24 h at 37 °C, counting colony numbers was carried out.

2.3. Husbandry Conditions and Feeding Trial

Shrimps used for this study were collected from an aquafarm at Kaohsiung, Taiwan. A total of 180 *L. vannamei* shrimps (initial body weight: 8.22 ± 0.38 g and 8.27 ± 0.53 g in blank and *L. fermentum* SWP-AFFS02 feed, respectively) were evenly distributed into 3 groups (60 shrimps/group), and they were stocked in a fiber reinforced plastics (FRP) bucket with a flow-through sea water system and continuous aeration. The indoor cement pool was maintained under natural light/dark regime. During the experimental period, the monitored water quality parameters (mean ± S.D.) were as follows: water temperature was 28.3 ± 2.4 °C, pH was 8.4 ± 0.5, and the salinity was 33.6 ± 0.2 ppt. All shrimp groups were fed feed with or without LAB to apparent satiation thrice a day (at 6:00, 17:00, and 22:00) for 8 weeks. The remaining feed residues after feeding were removed by siphoning and dried by using a freeze drier system to subtract from total feed intake. Body weight was also recorded once every two weeks.

$$\text{Weight gain} = ((\text{final weight} - \text{initial weight})/\text{initial weight}) \times 100\% \quad (1)$$

$$\text{Feed conversion rate (FCR)} = \text{feed supplied}/(\text{final weight} - \text{initial weight}) \quad (2)$$

$$\text{Survival (\%)} = (\text{number of individuals at the end of the evaluation period}/\text{initial number of individuals stocked}) \times 100\% \quad (3)$$

2.4. Detection of Total Bacteria and *Vibrio* spp. Counts in Seawater Surrounding

The 1 mL of aquaculture breeding water was diluted to the appropriate ratio by a 10-fold serial dilution method, and the 100 µL dilution solutions were smeared on 3% tryptone soy agar (TSA) plate medium (BD, Franklin Lakes, NJ, USA) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Sigma–Aldrich, St. Louis, MO, USA) for total bacteria and *Vibrio* spp. cultivation, respectively. After cultivation for 24 h at 30 °C, the bacterial numbers between 25–250 colonies were counted [12].

2.5. Assay for Immune Activity (Hymolymph Parameters)

2.5.1. Preparation of Hemolymph Solution

The 0.8 mL of hemolymph was withdrawn from the ventral sinus in the first abdominal segment using a 26-gauge hypodermic needle. Each syringe was pre-filled with 0.2 mL of anticoagulant (10 mM Tris–HCl, 250 mM sucrose, 100 mM sodium citrate, pH 7.6). More anticoagulant was added to make an equal volume ratio of hemolymph to anticoagulant. The hemolymph samples of 180 *L. vannamei* shrimps (60 shrimps/group) from each treatment were analyzed individually [13].

2.5.2. Total Hemocyte Count (THC) and Differential Hemocyte Count (DHC)

A volume of 50 µL anticoagulated hemolymph was fixed with an equal volume of neutral buffered formalin (10%) for 30 min to measure the THC. Fixed hemolymph was smeared on a slide and stained with Giemsa solution (10%) for 10 min. The DHC was then characterized according to Tsing et al. (1989) [14], and 250–300 cells from each smear were counted. The remaining anticoagulated hemolymph was centrifuged at 300× *g* for 10 min at 4 °C to separate the hemocytes from plasma. Hemocytes were suspended and adjusted to 5 × 10^6 cells/mL in ice-cold cacodylate buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$, pH 7.0). A volume of 2.5 mL (5 × 10^6 cells/mL) hemocyte suspension was respectively separated for superoxide anion and phenoloxidase activity [13].

2.5.3. Measurement of Superoxide Anion Respiratory Burst

According to recent study [15], after adding 100 µL (5 × 10^6 cells/mL) of diluted hemolymph to the wells of a 96-well microplate, the plate was centrifuged at 800× *g* at 4 °C for 20 min. The supernatant was discarded, and the pellets were rinsed with MCHBSS buffer (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, he mocytes were mixed with 100 µL of DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) and zymosan (1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in the 96-well plate. After a 30-min incubation in

the dark, cells were washed twice with MCHBSS buffer. The supernatant was discarded 30 min post-incubation and the pellets were rinsed once using MCHBSS buffer. Finally, the fluorescent intensity was measured using a spectrofluorometer with an excitation wavelength of 480 nm and an emission wavelength of 523 nm.

2.5.4. Assay for Phenoloxidase (PO) Activity

The PO activity was assayed, as described by Sung et al. (1994) [16]. using L-3,4-dihydroxyphenyl-alanine (L-dopa) (Sigma-Aldrich) as a substrate. To obtain lysates from hemolymph (200 µL; 5×10^6 cells/mL), frozen hemocytes were thawed and frozen several times. Hemocyte lysate supernatants (HLS) and plasma were assayed for PO activity using a spectrophotometer (Hitachi U-2000) to measure the $OD_{490\ nm}$. The concentration of total plasma protein was determined by the Bradford method [17] using bovine serum albumin (Bio-Rad Protein assay Kit II) as a standard. One unit of PO activity was defined as an increase in absorbance of 0.001 min/mg protein [18].

2.5.5. Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA from hepatopancreas was obtained using the Trizol reagent (Gibco BRL Life Technologies, Inc., Gaithersburg, MD, USA), according to the manufacturer's instructions. Primers were synthesized by MD-Bio, Inc. (Taipei, Taiwan). The gene expression level was determined by relative quantification using RT-qPCR (CFX Cycler System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers: HSP70 (F): CAACGATTCTCAGCGTCAGG and HSP70 (R): ACCTTCTTGTCGAGGCCGTA; beta-actin (F): GCCCTGTTCCAGCCCTCATT and beta-actin (R): ACGGATGTCCACGTCGCACT [19]. PGx (F): GGCACCAGGAGAACACTAC and GPx (R): CGACTTTGCCGAACATAAC; LGBP (F): CCATGTCCGGCGGTGGAA and LGBP (R): GTCATCGCCCTTCCAGTTG; PPAF (F): GAGAAGGAGCTGAACCTGTAC and PPAF (R): AGCGCCTGAGTTGTAGTTAG [20]. PCR conditions were according to the literature [20].

2.6. Microbiota Sequencing

Each intestine of shrimp was collected and immediately soaked in liquid nitrogen and stored at −80 °C for subsequent use. Total genomic DNA from samples was extracted using QIAamp PowerFecal DNA Kit (Qiagen) for 16S rRNA gene sequencing. V3-V4 regions (515F-806R) were amplified, and PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The sequencing library was prepared and sequenced on an Illumina MiSeq platform.

2.7. Statistical Analysis

Experimental results were analyzed in five repeats and expressed as the mean ± standard error of mean. The results were examined by using one-way analysis of variance (ANOVA) and Duncan's multiple range tests, and the significance of differences between sample means was calculated. A p value ≤ 0.05 was considered significant.

3. Results

3.1. Variation of LAB Feed-Contained L. fermentum SWP-AFFS02 after Stroage

L. fermentum SWP-AFFS02 was collected after cultivation for 48 h (approximately 10^9 CFU/mL) in MRS medium at 37 °C. The standard feed (sterilized) was mixed with a culture solution or deionized water (1/1: w/v) under laminar airflow and stored at 4 °C or 25 °C for 7 days after freeze-drying and vacuum-packaging. Finally, the LAB numbers in the *L. fermentum* SWP-AFFS02-rich feed were measured and calculated on MRS agar under anaerobic conditions. As shown in Table 1, the bacterial counts were 4.47×10^8 CFU/g on day 0. However, a decrease in bacterial levels was observed with increasing storage days (during days 0–7) in both 25 °C and 4 °C treatments. However, maintaining a low temperature could effectively retain the number of viable bacteria.

Table 1. The variation of *L. fermentum* SWP-AFFS02 level in LAB feed after storage for 7 days at 25 °C or 4 °C.

Days	Storage at 25 °C	Storage at 4 °C
	Concentration (CFU/g)	
0	$4.47 \times 10^8 \pm 0.31 \times 10^7$	$4.47 \times 10^8 \pm 0.31 \times 10^7$
1	$4.01 \times 10^8 \pm 0.44 \times 10^7$	$4.56 \times 10^8 \pm 0.25 \times 10^7$
2	$3.77 \times 10^8 \pm 0.48 \times 10^7$	$4.11 \times 10^8 \pm 0.22 \times 10^7$
3	$3.52 \times 10^8 \pm 0.78 \times 10^7$	$3.96 \times 10^8 \pm 0.38 \times 10^7$
4	$3.21 \times 10^8 \pm 1.04 \times 10^7$	$3.78 \times 10^8 \pm 0.41 \times 10^7$
5	$2.84 \times 10^8 \pm 1.11 \times 10^7$	$3.55 \times 10^8 \pm 0.37 \times 10^7$
6	$6.45 \times 10^7 \pm 0.19 \times 10^7$	$3.32 \times 10^8 \pm 0.63 \times 10^7$
7	$4.08 \times 10^7 \pm 0.28 \times 10^7$	$3.18 \times 10^8 \pm 0.79 \times 10^7$

3.2. Regulations of Growth, Environmental Microbe, and Immunity in L. vannamei Shrimp by LAB Feed-Contained L. fermentum SWP-AFFS02

3.2.1. Growth, FCR, and Survival Rate of Shrimp

The initial body weights of *L. vannamei* shrimp were 8.22 and 8.27 g in the control and LAB feed groups, respectively. The weight gain (WG), feed conversion rate (FCR), and survival of *L. vannamei* shrimp in the two groups were evaluated after 8 weeks. We found that the final weight of *L. vannamei* shrimp in the LAB group was higher than that in the control group. WG, FCR, and survival rate were greater in the LAB feed group than in the control group (Table 2).

Table 2. The effects of LAB feed on the growth, FCR, and survival rate of *L. vannamei* shrimp.

Groups	Initial Weight (g)	Final Weight (g)	WG (%)	FCR	Survival Rate (%)
Control feed	8.22 ± 0.38	15.72 ± 2.75^{b}	45.95 ± 11.57^{b}	7.22 ± 5.05^{a}	68.8 ± 10.1^{b}
LAB feed	8.27 ± 0.53	22.09 ± 2.50^{a}	61.98 ± 6.09^{a}	4.38 ± 1.04^{b}	82.2 ± 6.9^{a}

WG: weight gain; FCR: Feed conversion rate. Significant difference was shown by different letters $^{(a, b)}$ ($p < 0.05$).

3.2.2. Environmental Microbial Population (Total Bacteria Level and Vibrio spp.)

The results indicated that the total bacterial levels in environmental seawater were higher in the LAB feed group than in the control group between weeks 2 and 8 (Table 3). *Vibrio* spp. resulted in growth interference and yield reduction in brine shrimp (*Artemia franciscana*) [21]. Therefore, the level of *Vibrio* spp. in the environment was monitored from week 0 to week 8. We found that the number of *Vibrio* spp. increased with increasing aquaculture period in environmental seawater in both the control and LAB feed groups, but no significant difference was found between these two groups (Table 4).

Table 3. The total bacterial numbers in environmental seawater.

Weeks	Total Bacterial Numbers (CFU/mL)	
	Control Feed	LAB Feed
0	$2.10 \times 10^4 \pm 0.24 \times 10^{3\,a}$	$1.49 \times 10^4 \pm 0.12 \times 10^{3\,b}$
2	$1.33 \times 10^4 \pm 0.18 \times 10^{3\,b}$	$1.94 \times 10^4 \pm 0.15 \times 10^{3\,a}$
4	$0.89 \times 10^3 \pm 0.71 \times 10^{2\,b}$	$1.65 \times 10^4 \pm 0.17 \times 10^{3\,a}$
6	$1.42 \times 10^4 \pm 0.27 \times 10^{3\,a}$	$1.57 \times 10^4 \pm 0.08 \times 10^{3\,a}$
8	$1.16 \times 10^3 \pm 0.62 \times 10^{2\,b}$	$1.61 \times 10^4 \pm 0.11 \times 10^{3\,a}$

Significant difference was shown by different letters $^{(a, b)}$ ($p < 0.05$) between control and LAB feed groups.

Table 4. The *Vibrio* spp. numbers in environmental seawater.

Weeks	*Vibrio* spp. Numbers (CFU/mL)	
	Control Feed	LAB Feed
0	$0.70 \times 10^3 \pm 0.08 \times 10^2$	$0.79 \times 10^3 \pm 0.12 \times 10^2$
2	$1.03 \times 10^3 \pm 0.03 \times 10^2$	$1.14 \times 10^3 \pm 0.05 \times 10^2$
4	$0.89 \times 10^3 \pm 0.06 \times 10^2$	$1.08 \times 10^3 \pm 0.11 \times 10^2$
6	$1.82 \times 10^3 \pm 0.11 \times 10^2$	$1.77 \times 10^3 \pm 0.13 \times 10^2$
8	$1.76 \times 10^3 \pm 0.15 \times 10^2$	$1.91 \times 10^3 \pm 0.09 \times 10^2$

3.2.3. Investigation for Immunity Index

Total hemocyte count (THC) is a marker to indicate shrimp immunity, and the THC ratio approximately reaching 2.0–4.0 $\times$ 10^7 cells/mL was reported [22]. Another study demonstrated that THC levels were affected by internal factors (including sex, shelling cycle, age, food intake, season, and body temperature), as well as external environmental factors (including pollutants, temperature, pH, salinity, and dissolved oxygen) in crustaceans [23]. Moreover, the THC level was markedly reduced in shrimp, thereby leading to infections caused by pathogenic bacteria or viruses [24]. We found that the LAB feed markedly elevated THC levels in shrimp when compared to the control group (Figure 1A).

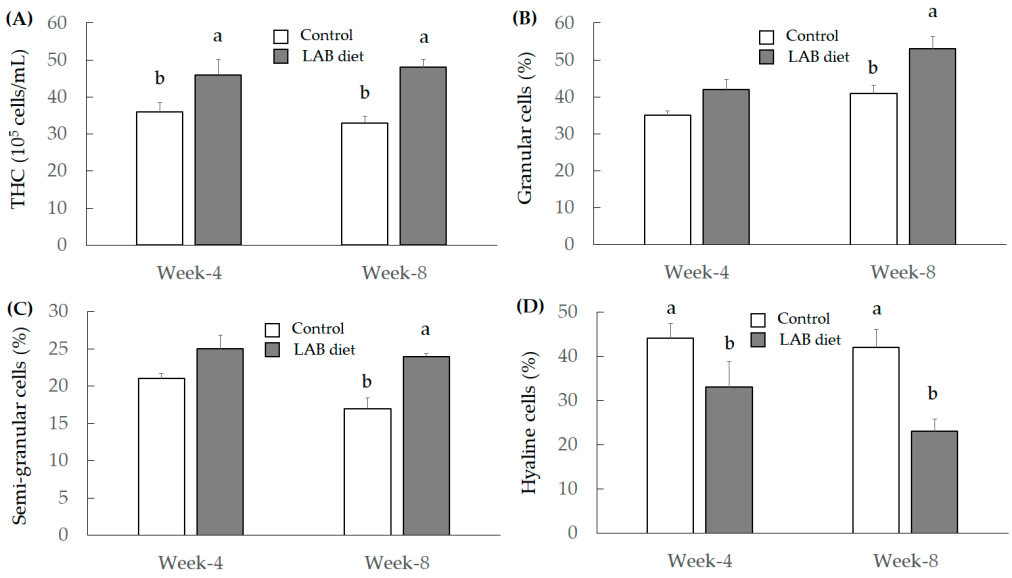

Figure 1. The variations of THC (**A**), granular cells (**B**), semi-granular cells (**C**), and hyaline cells (**D**) in shrimp feed with control or LAB feed. Significant difference is shown by different letters (a, b) ($p < 0.05$) between control and LAB feed groups.

Differential hemocyte count (DHC) was divided into three types of immunocytes, including granular cells, semi-granular cells, and hyaline cells, and these cells constituted THC. In addition, DHC plays different roles in regulating immune function. Granular cells and semi-granular cells exert cytotoxicity and activate the prophenoloxidase system (PO system), but hyaline cells do not activate the PO system. Phagocytosis and respiratory burst (production of superoxide anions) are the major immune abilities of hyaline cells [25]. We found that the LAB feed markedly increased the number of granular cells (Figure 1B) and semi-granular cells (Figure 1C) after 8 weeks of administration. However, the number of hyaline cells treated with the LAB feed was reduced both in week 4 and week 8, compared to the control group (Figure 1D).

Respiratory burst was monitored to identify the body defense ability mediated by superoxide anion production; respiratory burst activation has been found to improve the shrimp defense system [26]. The results indicated that LAB feed administration did not promote respiratory burst (Figure 2A), which may be because the LAB feed could

not promote hyaline cells. The activation of the PO system led to quinonoid production mediated by tyrosine oxidation, and quinonoid compounds exerted cytotoxicity [27]. As shown in Figure 2B, the LAB feed markedly promoted the PO activity of *L. vannamei* shrimp in both week 4 and week 8. These results demonstrate that *L. fermentum* SWP-AFFS02 could be developed as a probiotic to improve immunity and growth in *L. vannamei* shrimp, thereby elevating FCR efficacy.

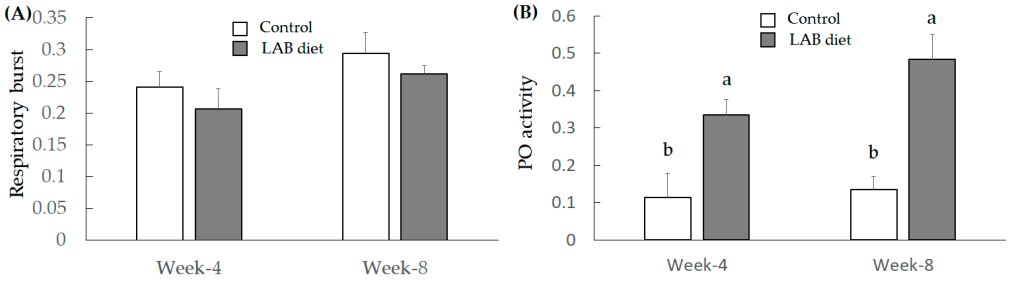

Figure 2. The regulation of LAB feed on respiratory burst (index of hyaline cell) (**A**) and PO activity (granular cells) (**B**). Significant difference is shown by different letters (a, b) ($p < 0.05$) between control and LAB feed groups.

The lipopolysaccharide and beta-1,3-glucan binding protein (LGBP) and the cascade via PO-activating factor (PPAF) both activate the PO system, thereby promoting immune function in shrimp [28]. In addition, heat-shock protein 70 (HSP70) and glutathione peroxidase (GPx) are defense agents associated with antioxidant activity against stress [29]. The LAB feed containing *L. fermentum* SWP-AAS02 significantly increased LGBP and PPAF expression in the hepatopancreas of *L. vannamei* shrimp (Figure 3A,B), but the levels of HSP70 and GPx were not elevated by LAB feed administration (Figure 3C,D). These results indicate that *L. fermentum* SWP-AFFS02 markedly elevated immune activity in *L. vannamei* shrimp.

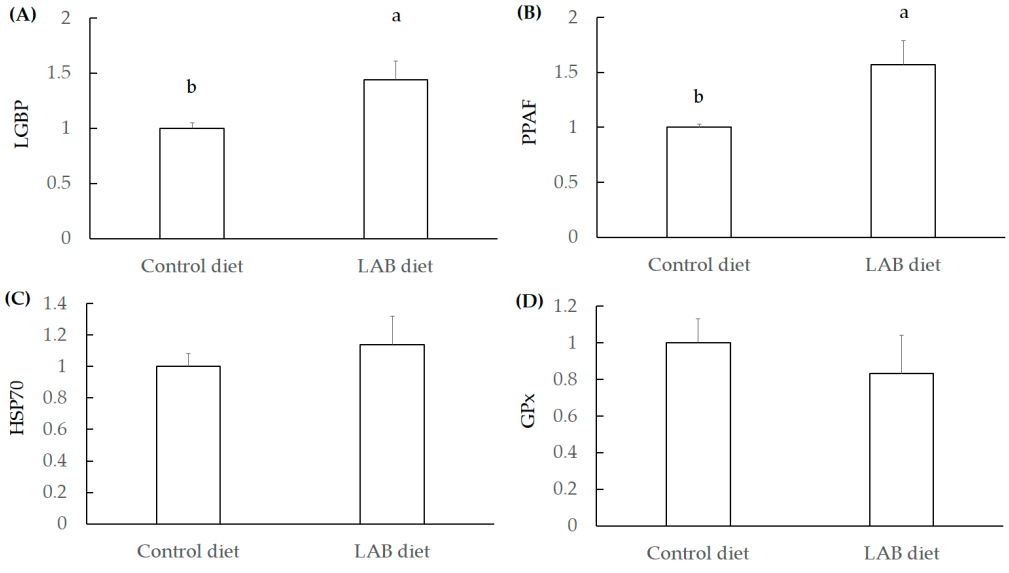

Figure 3. Expression levels of LGBP (**A**), PPAF (**B**), HSP70 (**C**), and GPx (**D**) in the hepatopancreas of *L. vannamei* shrimp fed with LAB feed containing *L. fermentum* SWP-AFFS02 as determined by RT qPCR. Significant difference is shown by different letters (a, b) ($p < 0.05$) between control and LAB feed groups.

3.3. Protection of Intestinal Microbiota in L. vannamei Shrimp by LAB Feed Containing L. fermentum SWP-AFFS02

There are 391 different types of bacteria within the intestinal stool of *L. vannamei* shrimp fed a control feed or LAB feed. However, the operational taxonomic unit (OTU)

numbers were 126 and 201 in the intestinal stool of *L. vannamei* shrimp fed a control feed or a LAB feed, respectively (Figure 4A). These results suggested that the LAB feed containing *L. fermentum* SWP-AFFS02 may potentially regulate microbial beta diversity in the intestine, compared to the control group, as shown by PCA. Furthermore, the microbial beta-diversity of seawater was different from that of the stools of *L. vannamei* shrimp fed with control and LAB feed (Figure 4B).

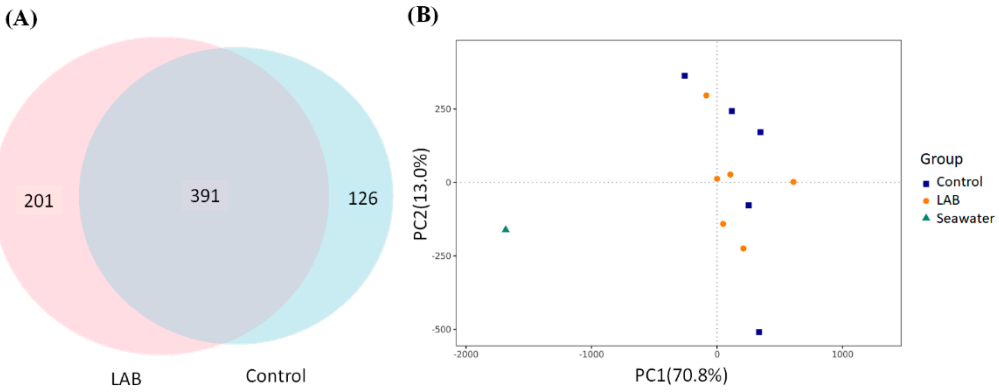

Figure 4. Alpha and Beta diversity of intestinal microbiota in *L. vannamei* shrimp. (**A**) Venn diagram. The overlapping part indicates the number of OTUs shared between control and LAB treatment groups, and the non-overlapping part indicates the number of unique OTUs between two groups. (**B**) Principal component analysis (PCA) for species. Each point in the figure represents one sample, and the same grouped samples are represented by the same color.

We analyzed the most abundant microbes (top 35) at the genus and family levels in the stool of *L. vannamei* shrimp fed a control feed or a LAB feed. The results indicated that *Pseudomonas*, *Bacillus*, *Idiomarina*, NS5_marine_group, *Aliidiomarina*, and *Alcanivorax* at genus level (Figure 5A) and *Flavobacteriaceae*, *Pseudomonadaceae*, *Bacillaceae*, *Idiomarinaceae*, *Rhodobacteraceae*, *Legionellaceae*, *Halieaceae*, *Alcanivoracaceae*, *Paenibacillaceae*, *Halomonadaceae*, and *Shingomonadaceae* at family level (Figure 5B) in seawater were higher than in the stool of *L. vannamei* shrimp fed with control or LAB feed; however, other bacteria in seawater were markedly lower than those in the stool of *L. vannamei* shrimp, suggesting that some mechanisms may regulate special microbes remaining in the shrimp intestine, compared to seawater bacteria staying in shrimp. In addition, the levels of *Paraprevotella*, *Diallister*, *Oscillibacter*, *Lachnoclostridium*, and *Sutterella* at the genus level were elevated by LAB feed-containing *L. fermentum* SWP-AFFS02, compared to the control group; however, the levels of *Acinetobacter*, *Ruminococcaceae*_UCG_002, and *Escherichia_Shigella* were reduced by LAB feed-containing *L. fermentum* SWP-AFFS02, compared to the control group. Moreover, we found that the levels of *Vibrionaceae*, *Corynebacteriaceae*, *Marinifilaceae*, *Tannerellaceae*, and *Enterobacteriaceae* were suppressed by the LAB feed. These results indicate that the LAB feed potentially regulates the intestinal microbiota to improve host health in *L. vannamei* shrimp.

Moreover, short-chain fatty acids, including acetic acid, propionic acid, and butyric acid in the stools of *L. vannamei* shrimp fed the LAB feed were measured, as shown in Table 5. The results showed that acetic acid and butyric acid were elevated in the stools of *L. vannamei* shrimp fed the LAB feed, compared to the control group.

Table 5. The level of intestinal short chain fatty acid in *L. vannamei* shrimp fed with LAB feed-contained *L. fermentum* SWP-AFFS02.

Short-chain Fatty Acids	Acetic Acid	Propionic Acid	Butyric Acid
	Concentration (μM)		
Control feed	17.1 ± 1.4^{b}	11.2 ± 1.3	37.6 ± 5.3^{b}
LAB feed	29.8 ± 0.8^{a}	13.6 ± 1.8	63.3 ± 8.7^{a}

Significant difference is shown by different letters $^{(a, b)}$ ($p < 0.05$) between control and LAB feed groups.

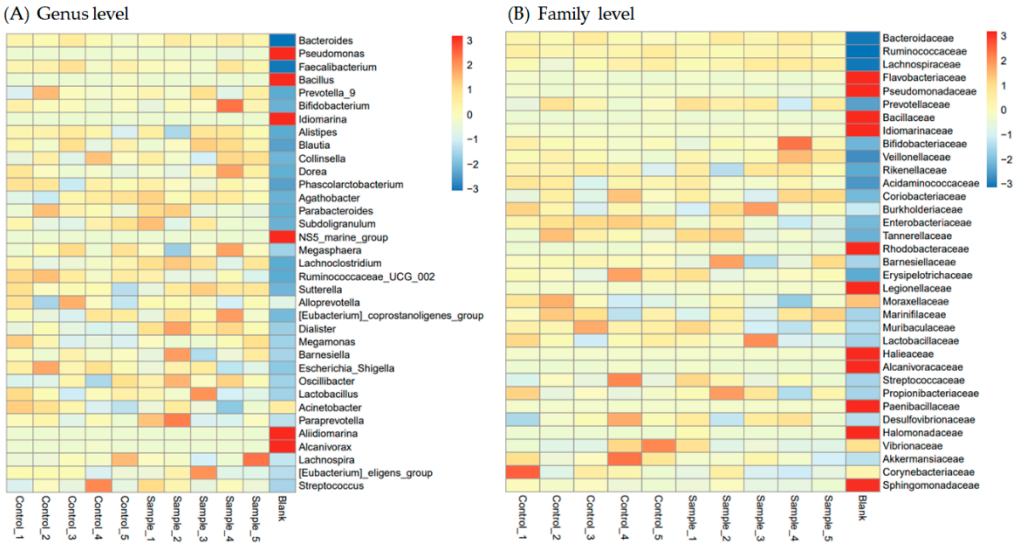

Figure 5. Heatmap of intestinal microbiota in *L. vannamei* shrimp. According to the species annotation and abundance information of all samples in the (**A**) genus level and (**B**) family level, we selected the top 35 in abundance.

4. Discussion

A study has found that a *Bacillus subtilis*-fermented soybean meal could improve intestinal microbiota and disease prevention in shrimp [30]. Xie et al. (2017) indicated that piglets fed feed fermented by *Lactobacillus*, *Bacillus*, and *Saccharomyces cerevisiae* potentially improved growth performance [31]. In a similar study in fish, *Lactobacillus* and *Pediococcus* were abundant bacteria in the intestines of Atlantic salmon fed the fermented feed, and the expression of mucin 2 (muc2) and aquaporin 8ab (aqp8ab) significantly increased in the fermented feed group, compared to the control group [32].

Vibrio spp. are widely distributed in marine environments and are among the most abundant flora in shrimp digestive systems, and vibriosis is the most serious disease in worldwide aquaculture that causes shrimp death [33,34]. Huang et al. (2016) demonstrated that pathogenic *Photobacterium* and *Vibrio* were the predominant bacteria at the post-larval stage (80%) and juvenile stage (89.1% ~ 94.2%), resulting in shrimp infection [35]. Similar to this study, *Vibrio* spp. were predominantly found in the intestines of shrimp, but these pathogenic bacteria were reduced in shrimp fed a synbiotic feed [34], thereby reducing disease arising from *Vibrio* infection by promoting immunity [36,37], as well as by avoiding hepatopancreatic damage [38]. A previous study suggested that activation of the antioxidant system avoided these negative effects caused by *Vibrio* infection in shrimp [39]. The immune and antioxidant systems are two primary physiological mechanisms that protect aquatic animal health against *Vibrio* spp. infection [40]. Non-specific immunity is the major defense system for stress resistance and protection against pathogen infection in crustaceans, and the PO system is also one of the primary response pathways in shrimp [41]. PPAF and LGBP are the key regulators of the immune response in shrimp [28]. In our study, we found that the LAB feed containing *L. fermentum* SWP-AFFS02 potentially suppressed intestinal *Vibrionaceae* in shrimp (Figure 5B) and promoted immune-associated gene expression in the hepatopancreas of *L. vannamei* shrimp, including LGBP and PPAF (Figure 3A,B). *Acinetobacter* spp. is a pathogenic bacterium of *L. vannamei* shrimp [42]. *Corynebacterium* is a gram-positive (non-spore forming) and aerobic bacterium that opportunistically reduces immunity and defense systems in hosts associated with systemic infection [43]. *Escherichia_Shigella* (*Enterobacteriaceae* family) is a pathogenic bacterium that causes gut leakiness and infection [44]. We found that administration of LAB feed that containing *L. fermentum* SWP-AFFS02 markedly inhibited these pathogenic intestinal bacteria in *L. vannamei* shrimp.

The animal intestine plays an important role in nutrient absorption and metabolism and acts as the first line of defense against pathogen infection and environmental stress [45]. Intestinal bacteria and bacterial metabolites directly influence the physiology of the host, including absorption and metabolism, intestinal barrier integrity, and immune function [46]. We believe that the LAB feed containing *L. fermentum* SWP-AFFS02 could limit pathogenic bacteria in the intestine of *L. vannamei* shrimp, thereby improving growth, FCR, and survival (Table 2).

In a recent study, feeding *L. vannamei* with feed supplemented with bacteria (*Bacillus subtilis* and *L. rhamnosus*) enhanced the antioxidative status by increasing the activity of antioxidant enzymes in shrimp [47]. Thus, shrimp feed with bio-friendly agents. such as *Lactobacillus* spp., could be used as effective alternatives to antibiotics for treating bacterial infections, especially *V. harveyi* infection in shrimp aquaculture [48]. Pooljun et al. (2020) confirmed that, after feeding juvenile shrimp (*Penaeus vannamei*) with *L. acidophilus* and *Saccharomyces cerevisiae* mixture (1:1, at 10^8 and 10^9 CFU/kg feed), the adverse effects of acute hepatopancreas necrosis disease induced by *V. parahaemolyticus* infection were ameliorated by improving hemocyte parameters, including the THC, granular hemocyte percentage, and phenoloxidase activity, as well as by highly upregulating hemocyte genes (*crustin* and *penaeidin*), which lead to high shrimp survival rate [49].

5. Conclusions

This study aimed to evaluate the effects of feeding *L. vannamei* shrimp with a feed rich in the LAB *L. fermentum* SWP-AFFS02. The effect of *L. fermentum* SWP-AFFS02 on the immunity and microbiota, as well as the changes in size of white shrimp, were investigated. Our study makes a significant contribution to the literature due to *L. vannamei* being an economically important species of shrimp, which are often plagued by pathogenic bacteria that cause their death. We noticed a marked inhibition of pathogenic bacteria in the shrimp intestine, which could in turn promote growth and improve immunity in *L. vannamei*. This study provided useful insights for the use of *L. fermentum* SWP-AFFS02 in shrimp. Enhancing the health status of cultured shrimp by using beneficial microbes as a probiotic is a good approach to control pathogens.

Author Contributions: B.-H.L. and W.-H.H. performed the design for the framework of the study. B.-H.L. determined most of the experimental assays. Y.-Z.C. analyzed the data. T.-M.P. and W.-H.H. revised the manuscript. T.-M.P., B.-H.L., W.-H.H., and K.-T.H. participated in the study design and finished the draft. The corresponding author T.-M.P. was responsible for financial resources and funds for the project, supervision of the research activities, and the manuscript submission. The corresponding author T.-M.P. led the research group. All authors have read and agreed to the published version of the manuscript.

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