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Effects of Fermented Kefir as a Functional Feed Additive in *Litopenaeus vannamei* Farming

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Abstract: *Litopenaeus vannamei*, known as whiteleg shrimp, is susceptible to infection by pathogenic microorganisms such as viruses and bacteria. Therefore, the prevention of infections in this shrimp is important to regulate the outbreaks of pathogenic microorganisms. In this study, we investigated the effects of kefir as a functional feed additive on innate immunity, survival against WSSV (White Spot Syndrome Virus) and productivity of *L. vannamei*. As a result, the treatment of kefir could upregulate six of seven genes crucial for innate immunity of *L. vannamei*. Also, the treatment of kefir directly improved the survival rate of *L. vannamei* against WSSV infection. Finally, in order to determine whether kefir can improve the productivity of shrimp, we carried out field tests in three aquaculture farms in South Korea. The weight of shrimp fed kefir was increased by 120% as well as the length, compared with that of the control group. These results demonstrate that kefir can be utilized as a functional feed additive to improve both innate immunity and productivity of *L. vannamei* in shrimp farming with no use of antibiotics.

Keywords: fermented kefir; *Litopenaeus vannamei*; innate immunity; survival rate against WSSV; productivity; functional feed additive

1. Introduction

Litopenaeus vannamei, known as whiteleg shrimp, is an important aquaculture species in the Asia–Pacific region because this region is the largest producer contributing to nearly 80% of the value and volume of the global shrimp market [1]. This shrimp can resist poor farming conditions such as enrichment of nitrogen sources, but it is susceptible to infection by viruses and bacteria, including *Vibrio* sp. [2,3]. These infections cause the sudden death of shrimp; the prevention of these infections is important to maintain the productivity of shrimp farming [2]. Antibiotics have been effective in preventing these infections, but there has been growing concern of serious problems such as the emergence of antibiotic-resistant microorganisms [4,5]. Therefore, the use of antibiotics in aquaculture is strictly regulated to minimize their negative effects on the environment and human health [4,5]. This has encouraged researchers to develop substances such as probiotics and identify medicinal herbs, as alternatives to antibiotics, to control infections in shrimp [1,6–11].

Kefir is milk fermented by kefir grains and contains beneficial microorganisms such as lactic acid bacteria, *Bacillus* spp., and yeast. These microorganisms have health-promoting and antimicrobial

activities [12–14]. Furthermore, kefir as a nutraceutical has several beneficial properties such as immunological, antimicrobial, antitumor, and hypo-cholesterolemic effects in animals and humans [15]. Also, Uluköy et al. found out that juvenile rainbow trout fed with kefir increased nonspecific immune response and improved disease resistance against lactococcosis and yersiniosis [16]. However, there are few studies on the effects of fermented kefir on *L. vannamei*. In this study, we investigated the effects of kefir as a functional feed additive on innate immunity and productivity of *L. vannamei* without the use of antibiotics.

2. Materials and Methods

2.1. Preparation of Lyophilized Kefir Cell Pellet

Kefir grain used in this study was collected in a private house, South Korea. Kefir grains were inoculated in 4% (*w*/*v*) whole fat milk medium and cultivated at 30 °C for 2 days without agitation. For main fermentation, we developed the medium based on de Man, Rogosa and Sharpe (MRS); yeast extract-peptone-dextrose (YPD); and nutrient broth (NB) media. To determine the optimum conditions for kefir fermentation, we modified the composition of glucose, whey protein, and dipotassium phosphate in the medium, and inoculation size of seed culture. The main fermentation of kefir was carried out in 300 L working volume of a 500 L fermentor at 30 °C for 1 d. For fermentation, sterilized air was supplied at 2 vvm in the fermentor, and the mixing rate was maintained at 200 rpm using an impeller. After fermentation, the total cells were harvested by continuous centrifugation with 8000 rpm and the cell pellet was mixed with 20% (*w*/*v*) sterilized skim milk solution. Thereafter, this mixture was lyophilized for 3 days. To determine the number of lactic acid bacteria, *Bacillus* spp., and yeast cells in the lyophilized sample, viable colonies were counted. Briefly, the sample was serially diluted to 0.85% with sterilized saline solution, and 100 µL of the diluted sample was spread on MRS agar for lactic acid bacteria, NB agar for *Bacillus* spp., and YPD agar for yeast culture. After incubation at 30 °C for 1 day, the colonies were counted.

2.2. Monitoring the Expression Level of Genes Related to Innate Immunity in L. vannamei after Treatment with Kefir

For this experiment, we prepared eight cages controlled by recirculating aquaculture system and transferred 30 *L. vannamei* into each cage. Thereafter, feed was supplied at 3% (*w*/*w*) of the total weight of shrimps in each cage three times per day. Furthermore, lyophilized kefir was supplied at 0.2% (*w*/*w*), 0.4% (*w*/*w*), and 0.8% (*w*/*w*) of the weight of feed supplied on 1 day. Lyophilized kefir was fed one time per day. The treatment was carried out for 30 days. The number of lactic acid bacteria, *Bacillus* spp., and yeast in lyophilized kefir was 5.6×10^9, 6.9×10^9, and 4.8×10^9 CFU/mL, respectively.

After 30 days, hemolymph and hepatopancreas were isolated from all *L. vannamei*. To determine the total hemocyte count, the hemolymph sample was fixed in 4% formalin in fixation buffer (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, and 9 mM EDTA; pH 7.0). Subsequently, the total hemocytes were counted using a Neubauer chamber. To monitor the expression level of genes related to antimicrobial peptides and the immune defense system against major pathogens (Table 1), we investigated the expression level of each gene by RT-PCR previously reported [17]. Briefly, the hepatopancreas was homogenized with liquid nitrogen, and then the total RNA was extracted using the TRIzol® Plus RNA Purification System (Thermo Fisher Scientific, Carlsbad, CA, USA). cDNA was synthesized by reverse transcription with oligo dT primer, and 0.1 µg/µL cDNA, as a template, was amplified by PCR with primers, designed using those in GenBank (https://www.ncbi.nlm.nih.gov/genbank)as described below. After PCR, the intensity of each PCR product was analyzed using the Gel Documentation System (Bio-Rad, Hercules, CA, USA) after running on 1% agarose gel. The intensity of the amplified cDNA was analyzed using ImageJ software (National Institutes of Health, Betheda, MD, USA) for quantification.

Table 1. Information of target genes and primers for monitoring the expression level of genes related to antimicrobial peptides and the immune defense system against major pathogens.

Gene	Primer	Sequence	GenBank No.
βGBP	Forward Reverse	5′-CGTGAGGTTCCCCAGTATGG-3′ 5′-TTCGGTTTGGATGGCTAAAG-3′	AY249858
proPO	Forward Reverse	5′-GGAATTGTTTTACTACATGCATCAGC-3′ 5′-GGAACAAGTCATCCACGAGCTT-3′	AY723296
Crustin	Forward Reverse	5′-ATTCTGTGCGGCCTCTTTAC-3′ 5′-ATCGGTCGTTCTTCAGATGG-3′	AF430076
Penaeidin 3a	Forward Reverse	5′-AGCCTCACCTGCAGAGACCA-3′ 5′-AATCAGGATCRCAGKCTCTTCAC-3′	Y14926
Lysozyme	Forward Reverse	5′-TTCGGGAAGTGCGAATTCG-3′ 5′-AATGGAAACCCTTGGTGAC-3′	AY170126
SOD	Forward Reverse	5′-GAGAAGAAGTTGGCTGAGCT-3′ 5′-ATGTTGGGTCCAGAAGATGG-3′	AY486424
Hemocyanin	Forward Reverse	5′-AATGCAGCCTACTTCCGTCAG-3′ 5′-TTATCGGGGTACACGCCATG-3′	X82502
β-actin	Forward Reverse	5′-TGTGTGACGACGAAGTAGCC-3′ 5′-TGGTCGTGAAGGTGTAACCA-3′	AF300705

2.3. Monitoring the Effect of Kefir through WSSV (White Spot Syndrome Virus) Challenge Test

Two hundred and forty *L. vannamei* were used for this challenge test and it was confirmed that these shrimps were specifically pathogen free by National Institute of Fisheries Science, Rep. of Korea. We divided the total amount into four groups: negative, positive and two kefir-treated groups according to kefir concentration. Each group was subdivided into three subgroups. Each 15 shrimps were tested in a plastic aquarium (1 m × 1 m × 0.7 m). Average weight of shrimps for this experiment was 4.70 g. Before infection of WSSV, all shrimps were acclimated for 7 days in plastic aquarium.

To prepare the WSSV filtrate, we obtained dead shrimps by infection of WSSV and homogenized the infected organ of dead shrimp in TNE buffer (50 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, pH 7.5). Next, the homogenate was centrifuged at 6000 rpm and 4 °C for 15 min and the supernatant was centrifuged again at 12,000 rpm and 4 °C for 15 min for 30 min. The final supernatant was filtrated by 0.45 μm syringe filter. To determine LD50 as the dose used for this experiment, we injected 0.1 mL of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} diluted filtrate in the dorsal body part of one shrimp and checked that LD50 of filtrate was 10^{-4} diluted filtrate. The infection of WSSV was carried out by the injection of the 0.1 mL of 10^{-4} diluted filtrate into the dorsal body of all shrimps without a negative control group. In the negative control group, the buffer was injected instead of the filtrate. After injection, we monitored the number of dead shrimp day by day for 15 days. The feeding for this experiment was carried out like described above.

For this experiment, the temperature of all of aquariums had been kept at 28 °C and all aquariums were aerated by air blower. The range of parameters of water quality like dissolved oxygen, pH, total ammonium nitrogen, and nitrate was kept on according to Holmström (2003) [5]. Survival rate by kefir treatment was calculated by Kaplan–Meier survival analysis provided in Prism 8 (GraphPad Software, San Diego, CA, USA).

2.4. Monitoring Weight Gain in L. vannamei after Treatment with Kefir through Field Test

For the field test, three shrimp farms located in Taean-Gun, Chungcheongnam-do, South Korea, were selected. The shrimp were treated with lyophilized kefir one time per week from May to October. The amount of lyophilized kefir in a tank of 1 m depth was 10 kg/ha, and the number of lactic acid bacteria, *Bacillus* spp., and yeast in lyophilized kefir was 5.6×10^9, 6.9×10^9, and 4.8×10^9 CFU/mL,

respectively. To monitor the length and weight of the shrimp, we collected 100 shrimps before transfer to the main farm, at July, and at the end of farming. We measured the length and weight of the shrimps collected. The occurrence of significant differences in the average weight by treatment of lyophilized kefir was tested using Student's *t*-test.

2.5. Statistical Analysis

Statistical analyses were accomplished using SPSS 22.0 (IBM Corp., Armonk, NY, USA). The obtained data were analyzed by the paired *t*-test for evaluating the association and significance between variables. A p value < 0.05 indicated significance.

3. Results

3.1. Optimization of Culture Conditions for the Growth of Kefir

For the main fermentation of kefir, seed culture of kefir was carried out in whole fat milk medium for 2 days at 30 °C without agitation. To optimize the conditions of the main culture, we tested several parameters such as the content of glucose as a carbon source, whey protein as a nitrogen source, and dipotassium phosphate as a phosphate source, and the size of seed inoculated. The colony forming unit (CFU) of lactic acid bacteria, *Bacillus* spp., and yeast increased at 2% of glucose, 1% of whey protein and 0.02% and 0.1% of dipotassium phosphate, compared to other concentrations of nutrients (Figure 1A–C). In addition, we examined how the CFU of these microorganisms changed according to the size of inoculation of seed culture. Interestingly, the CFUs were the maximum with 2% inoculation of seed culture, rather than 10% (Figure 1D). Based on these results, we finally chose the optimal content of glucose, whey protein, and dipotassium phosphate, and size of inoculum (Table 2).

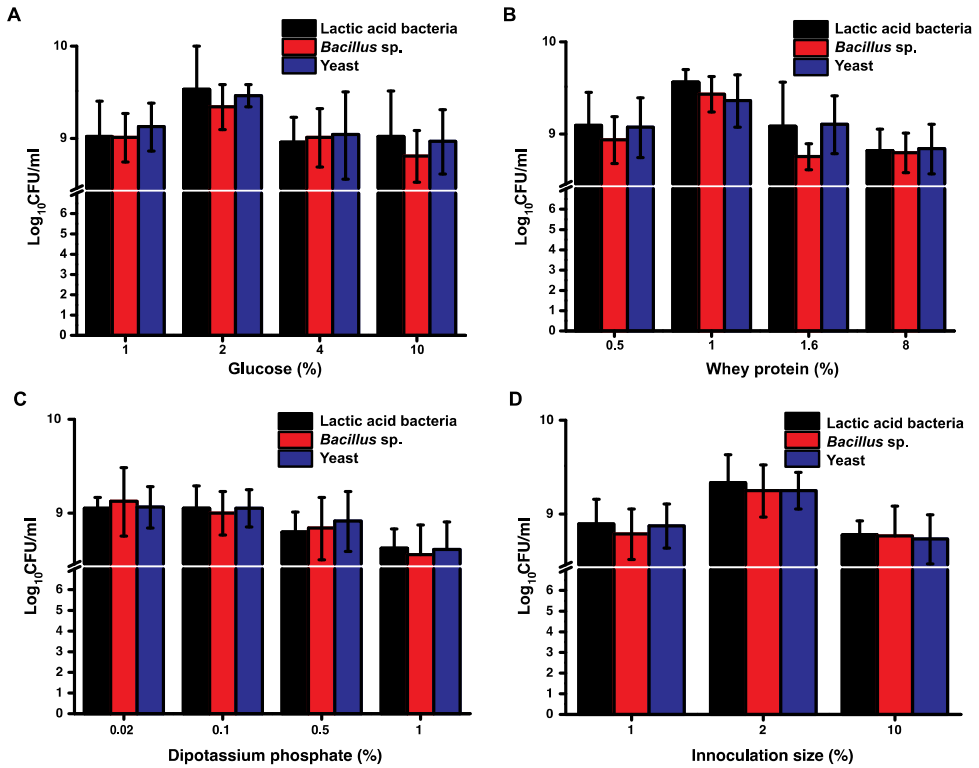

Figure 1. Proportion of lactic acid bacteria, *Bacillus* spp., and yeast in kefir according to culture conditions. (**A**) The proportion of lactic acid bacteria, *Bacillus* spp., and yeast in kefir by glucose content. (**B**) The proportion of lactic acid bacteria, *Bacillus* spp., and yeast in kefir by whey protein content. (**C**) The proportion of lactic acid bacteria, *Bacillus* spp., and yeast in kefir by dipotassium phosphate content. (**D**) The proportion of lactic acid bacteria, *Bacillus* spp., and yeast in kefir by inoculation size.

Table 2. The optimized medium condition for kefir fermentation.

Component	Composition (%, *w*/*v*)
Glucose	2
Whey protein	1
Dipotassium phosphate	0.02
Yeast extract	2
Ammonium sulfate	0.1
$MgSO_4$	0.01
$MnSO_4$	0.05
Inoculation size	2

3.2. Effect of Kefir on the Innate Immunity of *L. vannamei*

To monitor the effect of kefir on the innate immunity of *L. vannamei*, we treated lyophilized kefir with different doses. After 30 days of treatment, the total hemocytes in the hemolymph of *L. vannamei* were counted. Next, we examined the expression level of immune-related genes such as beta-glucan binding protein (βGBP), prophenoloxidase (proPO), crustin, peaneidin 3a, lysozyme, superoxide dismutase (SOD), and hemacyanin in the hepatopancreas of *L. vannamei*. The treatments of kefir did not change the number of total hemocytes (Table 3). However, the expression of immune-related genes was substantially upregulated after treatment of kefir (Figure 2). The expression of βGBP was upregulated fourfold in the group treated with 0.8% lyophilized kefir compared with that in the control group (Figure 2). Moreover, the expression of proPO, lysozyme, and SOD was substantially upregulated by lyophilized kefir treatment in a dose-dependent manner (Figure 2). The treatment of lyophilized kefir strongly induced the expression of genes encoding crustin and penaedin-3a by threefold compared with the control (Figure 2). These results indicate that kefir can contribute to the enhancement of the immune system in *L. vannamei* by upregulating the expression of immune-related genes and not cellular immunity by increasing hemocytes.

Table 3. The number of total hemocytes in *L. vannamei* by treatment of fermented kefir.

Kefir Treated (%)	Number of Total Hemocytes ($\times 10^7$/mL)
0	7.53 ± 0.30
0.20	7.46 ± 0.11
0.40	7.40 ± 0.2
0.80	7.6 ± 0.2

3.3. Effect of Kefir in *L. vannamei* against WSSV Infection

After infection of WSSV filtrate, the number of dead shrimps immediately increased and the survival rate at the end of this experiment was calculated to be 33.33% in the positive group (Figure 3). In the case of the 0.2% kefir-treated group, the death rate was retarded early, compared to the positive group. However, the survival rate was the same at the end of the experiment (Figure 3). Meanwhile, in the case of the 0.8% kefir-treated group, the survival rate was dramatically improved and calculated to be 56.8% at the end of this experiment. This result means that the enhancement of innate immunity of *L. vannamei* by treatment of kefir can increase the survival rate against WSSV infection.

3.4. Effect of Kefir on Productivity of *L. vannamei*

In the development of a functional feed additive for shrimp farming, the improvement of shrimp productivity is an important aspect. Therefore, to examine whether kefir can improve the productivity of *L. vannamei*, we treated lyophilized kefir in three farms in South Korea after transfer to the main pond for 6 months; we measured the weight of shrimp before transfer to the main pond, and at 3 and 6 months after transfer. The shrimps in the group treated with lyophilized kefir were longer than those in the control group (Figure 4A–D). Furthermore, the average weight of *L. vannamei* in the group

treated with lyophilized kefir increased by up to 120% compared with that of *L. vannamei* in the control group (Figure 4E–G). The increase in both length and weight of shrimp in the group treated with lyophilized kefir was observed in all three farms. These results indicate that the supplementation of kefir can improve the utilization of feed to increase the length and weight of *L. vannamei*.

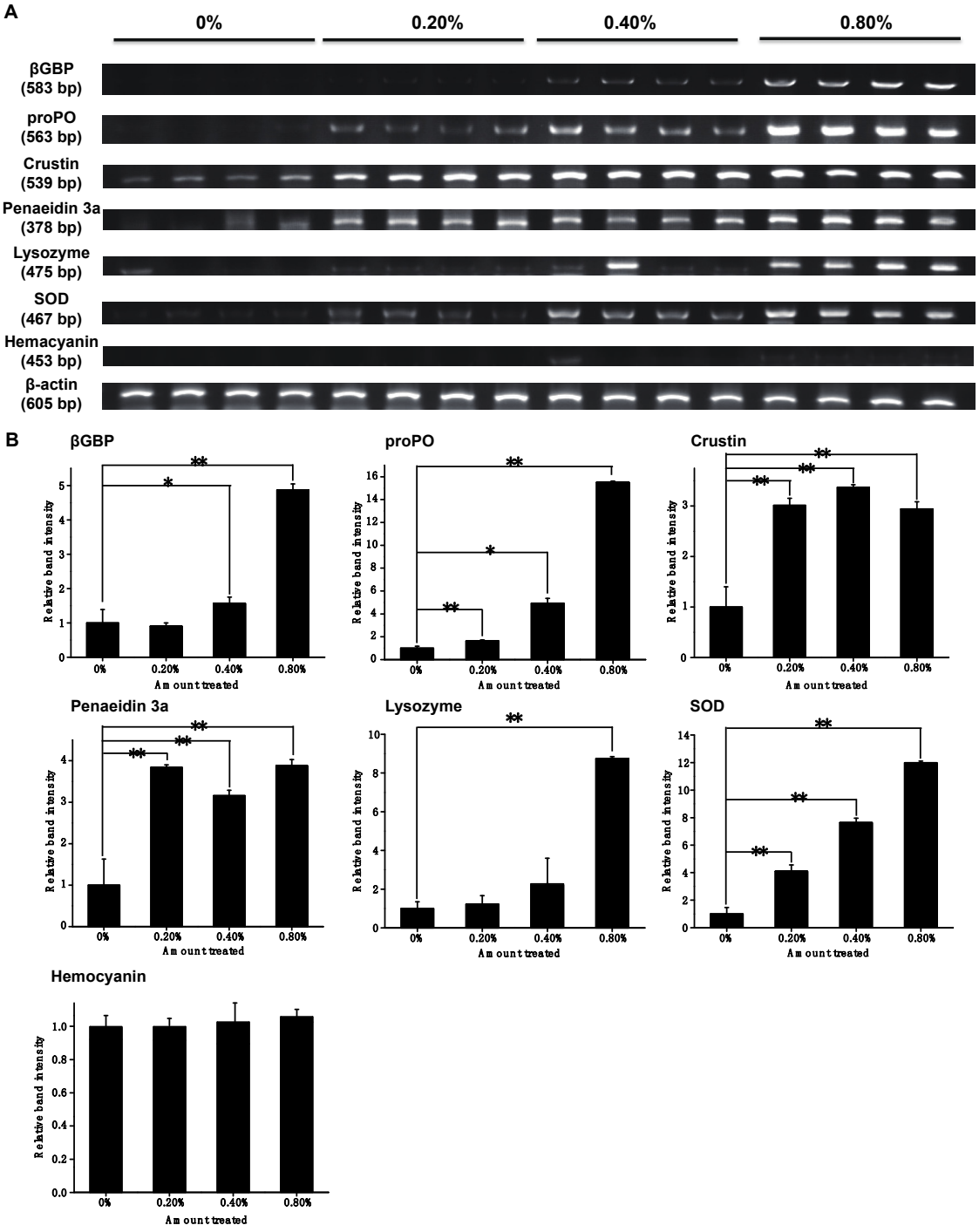

Figure 2. Transcription level of genes related to innate immunity in *L. vannamei* after treatment with kefir. (**A**) The amplified cDNA genes related to innate immunity in *L. vannamei* after treatment with kefir. (**B**) Quantification of the amplified cDNA genes related to innate immunity in *L. vannamei* using ImageJ. For quantification of the amplified cDNA genes, the intensity of the amplified cDNA in four wells was averaged and normalized by dividing with amplified cDNA intensity of the non-treated group. * $p < 0.05$ and ** $p < 0.01$, accessed using the paired t test.

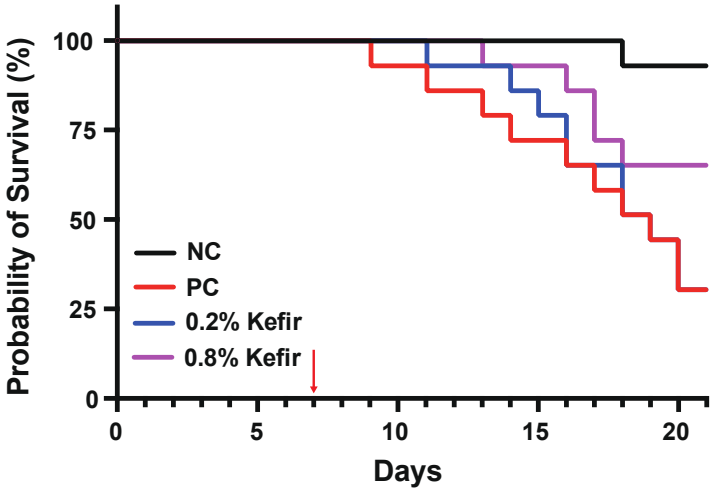

Figure 3. Survival rate of *L. vannamei* against WSSV infection according to treatment of kefir. NC and PC indicate negative and positive control group, respectively. Red arrow indicates the day when WSSV filtrate was injected except for the negative control group.

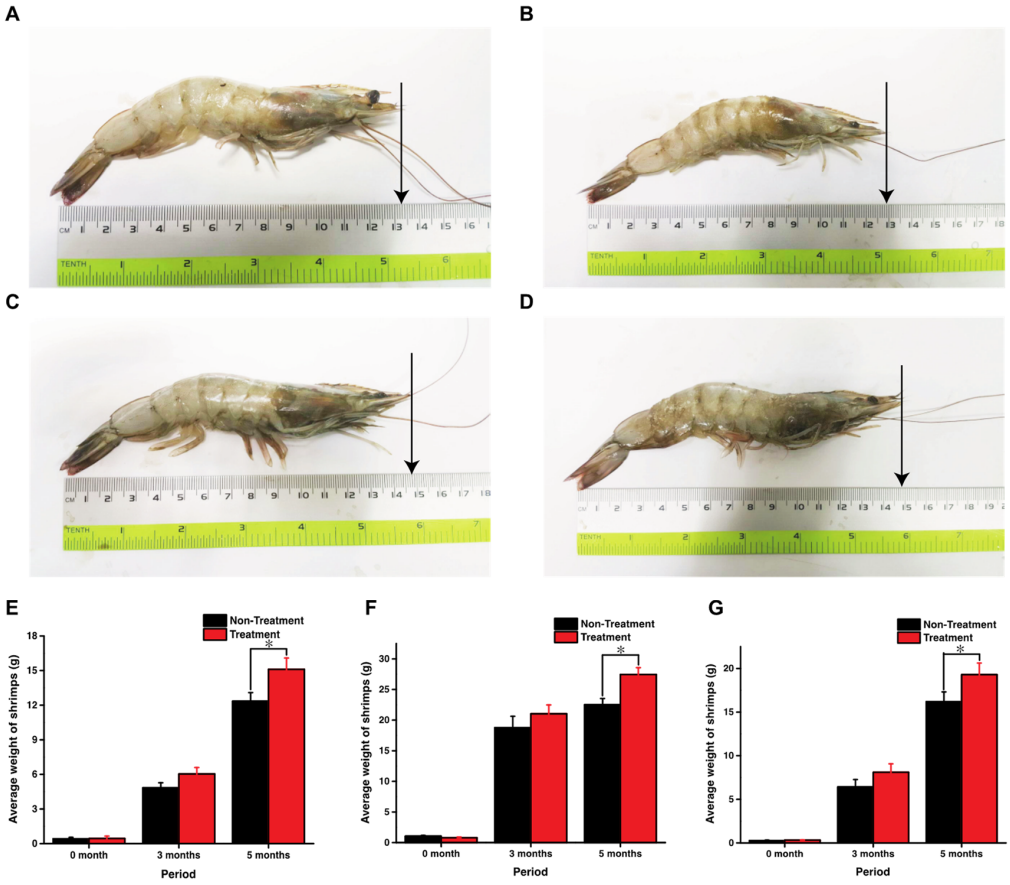

Figure 4. The growth of *L. vannamei* after treatment with kefir. (**A**,**B**) Representative images of the length of *L. vannamei* in the non-treated group. (**C**,**D**) Representative images of the length of *L. vannamei* in the kefir-treated group. Black arrows indicate the end of head of *L. vannamei*. (**E**) The average weight of *L. vannamei* after treatment with kefir in shrimp farm #01. (**F**) The average weight of *L. vannamei* after treatment with kefir in shrimp farm #02. (**G**) The average weight of *L. vannamei* after treatment with kefir in shrimp farm #03. The average weight of *L. vannamei* was calculated by the average weight of 100 *L. vannamei* individuals. * $p < 0.05$, accessed using the paired *t* test.

4. Discussion

The production of this shrimp is approximately 50 ton/ha/crop, which exceeds the production of Penaeus monodon, another popular shrimp species [18]. It has been reported that *L. vannamei* is highly tolerant to a wide range of salinity levels and requires a relatively low protein feed [18]. Furthermore, this shrimp can grow with high stocking densities and at low temperatures of up to 15 °C [19]. Even with these advantages, *L. vannamei* is prone to be infected by viruses such as Taura syndrome virus (TSV), white spot syndrome virus (WSSV), and yellow head virus (YHV), and pathogenic bacteria such as Vibrio parahaemolyticus [19]. Moreover, high-density farming of this shrimp that produces yields of 20,000 to 100,000 kg/ha/year results in the outbreak of infection by viruses and pathogenic bacteria because of eutrophication by unconsumed feeds and lots of shrimp feces [4]. Treatment with antibiotics is one of the approaches used to prevent these infections, but their use is strictly restricted because antibiotics can cause the development of antibiotic resistance among pathogens [4,5]. Thus, there is a need for alternatives to antibiotics in shrimp farming.

It has been reported that kefir consists of lactic acid bacteria, *Bacillus* spp., and yeast that play a role as probiotics [20], and thus, it has various probiotic properties [21]. Furthermore, the effect of probiotics as an alternative to antibiotics has been studied in shrimp farming [1,6,7]. Therefore, in this study, we explored the effect of kefir as a functional feed additive in shrimp farming to improve the immune system and productivity of *L. vannamei* with no use of antibiotics. We treated lyophilized kefir and determined the number of hemocyte and expression level of genes related to the innate immune system of *L. vannamei*. In *L. vannamei*, hemocytes play pivotal roles in pattern-recognition system, phagocytosis, proPO-activating system, encapsulation, nodule formation, antimicrobial peptide release, and lysozyme activity [2]. In this study, lyophilized kefir did not directly affect the count of hemocytes, but it substantially upregulated the expression of the βGBP, proPO, crustin, peaneidin 3a, lysozyme, and SOD genes. βGBP is a pattern-recognition protein and proPO participates in the proPO cascade [22]. Furthermore, crustin and peaneidin 3a are antimicrobial peptides [23]. Lysozyme and SOD are known to participate in the post-phagocytosis process to remove toxic materials during phagocytosis [2]. Also, in WSSV challenge experiment, we observed that the survival rate of *L. vannamei*-treated 0.8% kefir was obviously improved, compared to positive control and the 0.2% kefir-treated group. So, it is shown that the enhancement of innate immunity of *L. vannamei* directly can improve the survival of these shrimp against WSSV infection.

In terms of economic status, the use of functional feed additive might increase the cost of management; therefore, an improvement in shrimp productivity is one of the important factors when choosing a functional feed additive [7]. In this study, we carried out a field test in three farms in South Korea. During farming, we monitored the weight of shrimps before the transfer of shrimp to the main pond and after 3 months of feeding and harvest of shrimp. After 3 months of feeding, the average weight of shrimp was higher than that of the control group shrimp; at harvest, the average weight of shrimp treated with lyophilized kefir increased by 120% compared with that of the control group. This suggests that multiple bacteria in kefir can promote the growth of *L. vannamei*. Based on these results, we conclude that kefir might be a promising feed additive for both enhancement of the immune defense system and improvement of productivity of *L. vannamei* without treatment of antibiotics.

Author Contributions: H.-C.K., H.L., and J.-W.S. designed the research and conducted all experiments. W.C. and C.-W.C. mainly carried out field test and monitored the expression level of genes and weight of shrimps. D.-B.S. and B.-C.J. carried out kefir fermentation, lyophilized kefir preparation, and viable cell counting in the sample. H.L. wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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

References

1. Van Hai, N.; Fotedar, R. A Review of Probiotics in Shrimp Aquaculture. *J. Appl. Aquac.* **2010**, 251–266. [CrossRef]
2. Lin, Y.C.; Chen, J.C.; Siti, S.N.; Wan, W.Z.; Awangku, A.S.; Cheng, S.Y.; Hsu, C.H. Modulation of Innate Immunity and Gene Expressions in White Shrimp Litopenaeus Vannamei Following Long-Term Starvation and Re-Feeding. *Results Immunol.* **2012**, *2*, 148–156. [CrossRef]
3. Mohney, L.L.; Lightner, D.V.; Bell, T.A. An Epizootic of Vibriosis in Ecuadorian Pond-Reared Penaeus Vannamei Boone (Crustacea: Decapoda). *J. World Aquac. Soc.* **1994**, *25*, 116–125. [CrossRef]
4. Bermdez-Almada, M.C.; Espinosa-Plascenci, A. The Use of Antibiotics in Shrimp Farming. In *Health and Environment in Aquaculture*; IntechOpen Limited: London, UK, 2012. [CrossRef]
5. Holmström, K.; Gräslund, S.; Wahlström, A.; Poungshompoo, S.; Bengtsson, B.E.; Kautsky, N. Antibiotic Use in Shrimp Farming and Implications for Environmental Impacts and Human Health. *Int. J. Food Sci. Technol.* **2003**, *38*, 255–266. [CrossRef]
6. Farzanfar, A. The Use of Probiotics in Shrimp Aquaculture. *FEMS Immunol. Med. Microbiol.* **2006**, 149–158. [CrossRef] [PubMed]
7. Ninawe, A.S.; Selvin, J. Probiotics in Shrimp Aquaculture: Avenues and Challenges. *Crit. Rev. Microbiol.* **2009**, *35*, 43–66. [CrossRef] [PubMed]
8. Kumar, V.; Roy, S.; Meena, D.K.; Sarkar, U.K. Application of Probiotics in Shrimp Aquaculture: Importance, Mechanisms of Action, and Methods of Administration. *Rev. Fish. Sci. Aquac.* **2016**, 342–368. [CrossRef]
9. Sivasankar, P.; Santhiya, A.V.; Kanaga, V. A Review on Plants and Herbal Extracts against Viral Diseases in Aquaculture. *J. Med. Plants Stud.* **2015**, *3*, 75–79.
10. Immanuel, G.; Vincybai, V.C.; Sivaram, V.; Palavesam, A.; Marian, M.P. Effect of Butanolic Extracts from Terrestrial Herbs and Seaweeds on the Survival, Growth and Pathogen (Vibrio Parahaemolyticus) Load on Shrimp Penaeus Indicus Juveniles. *Aquaculture* **2004**, *236*, 53–65. [CrossRef]
11. Yogeeswaran, A.; Velmurugan, S.; Punitha, S.M.J.; Babu, M.M.; Selvaraj, T.; Kumaran, T.; Citarasu, T. Protection of Penaeus Monodon against White Spot Syndrome Virus by Inactivated Vaccine with Herbal Immunostimulants. *Fish Shellfish Immunol.* **2012**, *32*, 1058–1067. [CrossRef]
12. Bourrie, B.C.T.; Willing, B.P.; Cotter, P.D. The Microbiota and Health Promoting Characteristics of the Fermented Beverage Kefir. *Front. Microbiol.* **2016**, *7*, 647. [CrossRef] [PubMed]
13. Leite, A.M.D.O.; Miguel, M.A.L.; Peixoto, R.S.; Rosado, A.S.; Silva, J.T.; Paschoalin, V.M.F. Microbiological, Technological and Therapeutic Properties of Kefir: A Natural Probiotic Beverage. *Braz. J. Microbiol.* **2013**, 341–349. [CrossRef] [PubMed]
14. Laureys, D.; De Vuyst, L. Microbial Species Diversity, Community Dynamics, and Metabolite Kinetics of Water Kefir Fermentation. *Appl. Environ. Microbiol.* **2014**, *80*, 2564–2572. [CrossRef] [PubMed]
15. Liu, J.R.; Chen, M.J.; Lin, C.W. Antimutagenic and Antioxidant Properties of Milk-Kefir and Soymilk-Kefir. *J. Agric. Food Chem.* **2005**, *53*, 2467–2474. [CrossRef]
16. Uluköy, G.; Metin, S.; Kubilay, A.; Güney, Ş.; Yıldırım, P.; Güzel-Seydim, Z.; Kok-Tas, T.; Gümüş, E. The Effect of Kefir as a Dietary Supplement on Nonspecific Immune Response and Disease Resistance in Juvenile Rainbow Trout, Oncorhynchus Mykiss (Walbaum 1792). *J. World Aquac. Soc.* **2017**, *48*, 248–256. [CrossRef]
17. Wang, Y.C.; Chang, P.S.; Chen, H.Y. Tissue Expressions of Nine Genes Important to Immune Defence of the Pacific White Shrimp Litopenaeus Vannamei. *Fish Shellfish Immunol.* **2007**, *23*, 1161–1177. [CrossRef]
18. Thakur, K.; Patanasatienkul, T.; Laurin, E.; Vanderstichel, R.; Corsin, F.; Hammell, L. Production Characteristics of Intensive Whiteleg Shrimp (Litopenaeus Vannamei) Farming in Four Vietnam Provinces. *Aquac. Res.* **2018**, *49*, 2625–2632. [CrossRef]
19. Li, E.; Wang, X.; Chen, K.; Xu, C.; Qin, J.G.; Chen, L. Physiological Change and Nutritional Requirement of Pacific White Shrimp Litopenaeus Vannamei at Low Salinity. *Rev. Aquac.* **2017**, 57–75. [CrossRef]
20. Shi, X.; Chen, H.; Li, Y.; Huang, J.; He, Y. Effects of Kefir Grains on Fermentation and Bioactivity of Goat Milk. *Acta Univ. Cibiniensis Ser. E Food Technol.* **2018**, *22*, 43–50. [CrossRef]
21. Guzel-Seydim, Z.B.; Kok-Tas, T.; Greene, A.K.; Seydim, A.C. Review: Functional Properties of Kefir. *Crit. Rev. Food Sci. Nutr.* **2011**, 261–268. [CrossRef]

22. Amparyup, P.; Charoensapsri, W.; Tassanakajon, A. Prophenoloxidase System and Its Role in Shrimp Immune Responses against Major Pathogens. *Fish Shellfish Immunol.* **2013**, *34*, 990–1001. [CrossRef]
23. Tassanakajon, A.; Somboonwiwat, K. Antimicrobial Peptides from the Black Tiger Shrimp Penaeus Monodon—A Review. In *Dissease in Asian Aquaculture VII*; Asian Fisheries Society: Kuala Lumpur, Malaysia, 2011; pp. 229–240.