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Sargassum horneri as a Prebiotic Dietary Supplement for Immunity Development in *Streptococcus parauberis* Infected Zebrafish Model

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Sargassum horneri (SH) is a brown macroalgal species commonly found along the coast of Japan, China, and Korea. SH possesses valuable bioactive compounds that can be developed as functional food ingredients as well as pharmaceutical agents for both humans and animals. In this study, SH was tested for its potential prebiotic effect. Several solvent-assisted extracts of SH were tested on the growth of three species of probiotics (LAB) *(Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus brevis*) and fish pathogen bacteria (*Streptococcus iniae, Streptococcus parauberis, Edwersiella tarda*) both *in vitro* and *in vivo*. According to the *in vitro* results, Celluclast extract (SHC) and crude polysaccharide extract (SHCPs) of SH showed outstanding growth enhancing activity in all LAB species and excellent antibacterial activity against pathogenic bacteria dose-dependently. Both SHC and SHCPs induced the production of secondary metabolites from LAB. The secondary metabolites successfully reduced pathogenic bacterial growth. Furthermore, *in vivo* experiments revealed that co-treatment with LAB and SHC/SHCPs diminished the mortality of *Streptococcus parauberis* infected zebrafish by modulating iNOS, COX-2 expressions. Similarly, SH act as an anti-inflammatory agent against *S. parauberis* infection by hindering NF-κB pathway activation. Conclusively, the results achieved from the study suggest that *S. horneri* has the potential to be used as a prebiotic dietary supplement and possesses a protective effect against *S. parauberis* infections in the aquaculture industry.

Keywords: *Sargassum horneri*, prebiotics, *Streptococcus parauberis*, secondary metabolites, Diet

1 INTRODUCTION

Aquaculture, the rapidly growing food sector plays a dynamic role in providing food, employment, and income in South Korea. Aquaculture products are considered a high-quality protein source for human consumption therefore, demand for such products increases continuously. Over the past few years, aquaculture food production and demand have been severely affected by outbreaks of infectious viral, bacterial, and parasitic diseases (Lee et al., 2016). Bacterial diseases have been the most important causative factor of major losses in aquaculture food productions, mainly due to the ability of bacteria to survive in environments independently from their host, as well as the

retention of bacteria in packed seafood products (Fernández-No et al., 2012; Woo and Park, 2013). Gram-negative bacteria such as *Aeromonas, Edwardsiella, Pseudomonas*, and *Vibrio*, as well as gram-positive bacteria such as *Lactococcus* and *Streptococcus*, have been identified as the common disease-causing bacteria in the aquaculture (Pridgeon and Klesius, 2012). *Streptococcus parauberis* is a non-motile, gram-positive, and alpha-hemolytic bacterium belonging to the family *Streptococcaceae* which was initially identified as a causative agent of Bovine mastitis. *S. parauberis* has been reported causing streptococcosis in most aquaculture fish species, and it was reported as a major causative agent in an outbreak of olive flounder *Paralichthys olivaceus*, which is a popular seafood source in Jeju Island, South Korea (Baeck et al., 2006).

Bacterial infections have been controlled using the crude application of antibiotics such as tetracycline, amoxicillin, and cefazolin, as well as chemicals such as formalin, hydrogen peroxide, and sodium chloride (Harikrishnan et al., 2010). Recently, the application of antibiotics has been reduced due to the detrimental effects on human health and the environment, as well as the development and transfer of resistance in other bacterial species (Cabello, 2006). In a study conducted on olive flounder, an antibiotic-resistant *S. parauberis* strain was isolated. Therefore, new alternative approaches are required to overcome these types of resistance in the pathogenic bacteria (Schmidt et al., 2000; Park et al., 2009). Probiotics have emerged as an alternative antibiotic source and play a significant role in antimicrobial applications in fisheries. It is well known that the gut microbiota is essential for supporting host health and well-being against pathogenic bacteria. The incorporation of probiotics with feed provides a number of advantages, such as increased feed value, inhibition of resistant pathogens, promotion of growth, and increased immunity (Wang et al., 2005; Yanbo and Zirong, 2006). In the last decade, lactic acid bacteria have gained recognition as probiotics and dietary supplements for the prevention and management of diseases. Probiotic activity is enhanced by prebiotics, non-active food constituents that have the ability to enhance the growth and production of secondary metabolites (Gibson et al., 2017). Prebiotics shift to the colon and is selectively fermented and become accessible to the probiotics. Seaweeds are underexploited plants with prebiotic activities. They provide a source of rich and valuable bioactive compounds, which have been proven to have prebiotic effects on several probiotic bacteria in both humans and animals (O'Sullivan et al., 2010).

According to previous reports, feeding fish with seaweed extracts improved the growth and reduced the mortality rate of pathogen-infected fish species (Eom et al., 2015; Lee et al., 2016). However, there is little information on the prebiotic role of brown seaweed *Sargassum horneri* (SH) on the effect of probiotics and its pathogen inhibition activity. Hence, this study was carried out to investigate the prebiotic potential of the products extracted from SH on lactic acid bacteria, pathogenic bacterial growth, and the mechanism of anti-inflammatory activity against *S. paruberis* infections in fish.

2 MATERIALS AND METHODOLOGY

2.1 Materials

S. horneri samples were collected along the coast of Jeju Island (33° 27' 30.6" N, 126° 56' 31.2" E) in South Korea in February 2020 and washed with fresh water to remove salt, epiphytes, sand, and other external contaminants, that had been attached to the surface of the sample. The samples were then dried using a Hybrid hot water Goodle dryer (Nagahawatta et al., 2021) and pulverized using a multifunctional grinder (Hanil electronics, model 12339938004). Analytical grade solvents were used to prepare crude samples (Dae-Jung Chemicals & Metals, Seoul, Korea). Carbohydrate-degrading enzymes Termamyl, Viscozyme, Ultraflo, Amyloglucosidase, and Celluclast) were purchased from Novo Nordisk (Bagsvaerd, Denmark).

2.2 Preparation of Distilled Water Extract (SHD), Ethanol Extract (SHE), Crude Polysaccharide Extract, and Five Types of Enzymatic Extract From *Sargassum horneri*

Dried seaweed powder was extracted using ethanol and distilled water, as previously described (Lee et al., 2016). Briefly, powdered SH was homogenized with solvents and evaporated using a rotary evaporator. Obtained samples were freeze-dried and used for further experiments. Five carbohydrases; Termamyl (Term), Viscozyme (Visco), Ultraflo (Ultra), Amyloglucosidase (AMG), and Celluclast were used for the enzymatic extraction of SH (Charoensiddhi et al., 2015). In short, 1 g of freeze-dried SH powder was dispersed in 100 mL distilled water and enzymes were separately introduced. pH was adjusted according to the optimal pH of each enzyme with the addition of 1 M HCl or 1 M NaOH. Enzymatic hydrolysis was performed for 24 h under optimum temperatures for each enzyme. Optimum pH and temperature for each enzyme are as follows: Viscozyme (pH 4.5, 50°C), Celluclast (pH 4.5, 50°C), Ultraflo (pH 7, 60°C), Amyloglucosidase (pH 4.5, 60°C) and Termamyl (pH 6, 60°C) (Shanura Fernando et al., 2018). Enzymes were heat-inactivated by heating in a boiling water bath for 10 min and hydrolysates were filtered, and pH was readjusted for 7. Crude polysaccharides were obtained from the Celluclast enzymatic extract of SH. Ethyl alcohol (1:3, v/v) was used to facilitate the precipitation of crude polysaccharides.

2.3 Culturing of LAB and Pathogenic Bacteria

The potential probiotic bacteria *Lactobacillus brevis* (KCCM 40399), *Lactobacillus plantarum* (KCCM 12116), *Lactobacillus pentosus* (KCCM 40997), and pathogenic bacteria such as *Streptococcus iniae* (KCTC 3657), *Streptococcus parauberis* (KCTC 3651), and *Edwersiella tarda* (KCTC 12267) were purchased from the Korean Culture Center of Microorganisms (KCCM) and KCTC (Korean Collection for Type Culture, Daejeon, Korea) respectively. LAB were cultured and incubated at 30°C in De Man, Rogosa, and Sharpe (MRS) broth and MRS agar medium

(Difco Laboratories, Detroit, MI, USA), whereas pathogenic bacteria were cultured in brain heart infusion medium (BHIF) broth, and BHIF agar medium (Difco Laboratories, Detroit, MI, USA) at 30°C until needed.

2.4 Effects of Different SH Extracts on the Growth of LAB and Pathogenic Bacteria

The extracted SH samples were screened with LAB using a colony counting assay (Lee et al., 2016). LAB was cultured in MRS broth containing 100 µL of extracted samples and incubated for 24h at 30°C. Each bacterial suspension was diluted and incubated on MRS agar under the same incubation conditions. The number of colony-forming units (CFU) was counted, and growth rates were calculated. In a previous study, conducted to identify the prebiotic role of *Ecklonia cava*, Celluclast extract of *E. cava* (ECC) showed the highest growth effect in LAB (Lee et al., 2016). Therefore, ECC was used as a positive control in the experiment.

The antibacterial effect of the SH enzymatic extracts (SH-Visco, SH-Celluclast, SH-AMG, SH-Ultra and SH-Term) on three species of pathogenic bacteria, as well as the growth enhancement effect of the enzymatic extracts on LAB, were assessed using colony counting assays as mentioned above. Freeze-dried samples of extracts were added to bacteria-inoculated broth media at concentrations of 0.01, 0.1, 1, and 10 mg/mL, and the relative growth rate was calculated as a percentage value. In the above experiment, Celluclast extract (SHC) and crude polysaccharide extract (SHCPs) of SH showed superior activity compared to other extracts. Therefore, SHC and SHCPs were selected for further experiments. The chemical composition of the SHC and SHCPs were measured and analyzed in our previous study (Sanjeewa et al., 2019).

2.5 Effects of SHC and SHCPs on LAB Growth

For the identification of the influence of SHC and SHCPs on LAB growth, a colony counting assay was performed as described in section 2.4, with concentrations of 0.5, 1, 5, and 10 mg/mL. The relative growth rate was calculated as a percentage value.

2.6 Recovery of Secondary Metabolites From *L. plantarum* and *L. pentosus* Incubated With SHC and SHCPS

L. plantarum and *L. pentosus* were cultured in MRS broth medium with or without SHC (Lee et al., 2016). In brief, 100 µL of SHC was added to the MRS broth and autoclaved. *L. plantarum* and *L. pentosus* (10^9 CFU/mL) were inoculated into the MRS broth with or without containing SHC and incubated for 24h at 30°C. Each culture medium was then centrifuged three times at 11200 g for 30 min and supernatants were filtered using 0.45 µm filters and freeze-dried. The weight of the supernatant containing secondary metabolites were measured. Supernatants containing metabolites were used in the following experiments.

2.7 Effect of Secondary Metabolites of *L. plantarum* and *L. pentosus* on Pathogen Bacterial Growth

S. iniae, S. parauberia, and *E. tarda* (10^3 CFUs) were cultured in BHIF broth medium containing 100 µL of secondary metabolites (0.5, 1, 5, and 10 mg/mL) obtained from *L. plantarum* and *L. pentosus* cultured with or without SHC and incubated for 24h. After 24h, the bacteria were diluted and incubated on BHIF agar plates for another 24h. The number of CFUs were counted, and growth rates were calculated as percentages based on the number of bacteria in the BHIF broth medium only.

2.8 Analysis of Secondary Metabolites From *L. plantarum* Incubated With SHC

An Agilent Head Space-Gas Chromatography-Tandem Mass Spectrometer system (Agilent Technologies, CA, US) was used to conduct GC/MS analysis to identify secondary metabolites produced by *L. plantarum* when cultured in MRS medium containing SHC (Lee et al., 2016). Data analysis was done with the help of Wiley version 8.0 and NIST version 5.0 mass spectral libraries. An HP-5ms capillary column coated with (5%-phenyl)-methylpolysiloxane was used, whereas helium was used as the carrier gas at 1.5 mL/min constant flow rate. A flow rate of 1.5 mL/min was maintained in the injector with an injection temperature of 280°C. The initial column temperature was maintained at 44°C and was raised to 170°C at a rate of 3°C/min after sample injection.

2.9 *In Vivo* Experiments

Animal experiments were conducted in accordance with the experimental animal guidelines provided by Jeju National University Animal center and were authorized by the Animal Care Use Committee (IACUC) of Jeju National University (protocol 2020-0049)

2.9.1 Maintenance of Zebrafish

Zebrafish were purchased from Seoul Aquarium, Korea. Fish were separated into tanks (260 x 155 x 160 mm and volume of 3.5 L) (15 fish per tank) under controlled conditions (29°C with 14/10h light and dark cycle) and acclimated for two weeks with temperature 28.5 ± 1°C, salinity 0 g/L and a flow rate of 700 mL/min. They were fed twice daily with tetrabits flakes (Tetrabits GmbH D-49324 Melle, Germany) with a feeding rate of 3% of body weight in feed per day.

2.9.2 Experimental Diet Preparation With *L. plantarum,* SHC, and SHCPs

The formulated commercial fish feed (Tetrabit Gmbh D49304, 47.5% crude protein, 6.5% crude fat, 2.0% crude fiber, 10.5% crude ash) was mixed with 1% pure culture of *L. plantarum* (w/w) and SH extracts (1%, 3%, and 5% (w/w)). Briefly, *L. plantarum* cells were collected by centrifuging the incubated MRS medium and freeze-dried to obtain *L. plantarum* powder. Freeze-dried bacterial samples and freeze-dried SH extract (SHC or SHCPs) were dissolved in autoclaved distilled water and mixed with

commercial feed. The extruded fish feed was freeze-dried and made into a powder. The fish were fed twice a day at 9.00 and 19.00 with the experimental diet for four weeks.

Fish were separated into 11 groups according to the experimental dietsas blank, positive control, 1% L. plantarum only, 1% SHC only, 3% SHC only, 5% SHC only, 1% SHCPs only, 1% L. plantarum + 1% SHC, 1% L. plantarum + 3% SHC, 1% L. plantarum + 5% SHC, and 1% L. plantarum +1% SHCPs. Fish, only fed with commercial fish feed was kept as a negative control to determine the normal mortality rate during the study period. A total of 330 zebrafish were used for the *in vivo* experiment.

2.9.3 Measurement of Body Weight of Zebrafish

The bodyweight of zebrafish was batch weighed every week from the start of the experimental diet (Ohaus Pioneer PX4201/E Precision scale, Seoul, South Korea). Growth performance was analyzed using fish weight gain following the method given in a previous study (Kesbiç and Yigit, 2019; Kesbiç et al., 2020).

2.9.4 Mortality Measurement of *S. parauberis* - Infected Fish

Fish were challenged with *S. parauberis* intraperitoneally with 10 µL PBS containing 1.1x 10^8 cells/µL in the fourth week of the experimental diet. The number of dead fish was recorded daily for 17 days.

2.9.5 Measurement of Inflammatory Responses in Pathogen Challenged Zebrafish

Sampling was done 48 h post-infection with *S. parauberis*. Four fish were selected from each tank randomly and anesthetized with 50 mg/L MS-222 (Tricaine Methanesulfonate) (Sigma Aldrich Inc. Louis USA). Muscles of *S. parauberis*-infected zebrafish were extracted, and protein lysates were prepared. Protein concentrations were measured using the BCA protein assay kit. Cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and NF-κB pathway protein expression levels were analyzed by Western blot technique (Fernando et al., 2017). Proteins were subjected to SDS-PAGE, and protein bands were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 5% skimmed milk and incubated with primary and secondary antibodies. Enhanced chemiluminescence reagents (iNtRON, Sungnam, Korea) were used to develop the blots, and a FUSION SOLO Vilber Lourment system was used to photograph the developed blots. ImageJ software was aided in quantifying band intensities.

2.9.6 Hematoxylin and Eosin (H&E) Staining of *S. parauberis* Infected Tissues

Tissue samples were obtained from pathogen-infected fish at 48h post-infection. Samples were subjected to H&E staining (Paul, 2017). In short, samples were fixed in 10% buffered formalin and dehydrated using alcohol to remove water from the tissue. The tissues were then cleared using xylene or chloroform and impregnated with paraffin wax. Sections were de-paraffinized using xylene and rehydrated using ethanol and distilled water. H&E staining was used to stain the slides. Stained slides were dehydrated, mounted with coverslips, and examined with a microscope under a magnification of 40 to 1000X (Olympus, Tokyo, Japan).

2.10 Statistical Analysis

Data were analyzed using the GraphPad Prism 7 statistical analysis package (version 5.01; GraphPad Software Inc. San Diego, CA, USA). All data have been analyzed a minimum of three times and expressed as mean ± standard error (SE). Significant values were evaluated using one-way ANOVA test and Dunnett's multiple range tests.

3 RESULTS

3.1 SHC and SHCPs Showed the Highest Growth Rate in All Three LAB Species

A colony counting assay was performed to evaluate the effects of different solvent-assisted extracts of SH namely ethanol extract (SHE), distilled water extract (SHD), Celluclast extract (SHC), and crude polysaccharide extract of SH (SHCPs). According to the calculated CFU, SHC and SHCPs showed a comparative peak effect on the growth of all three types of LAB compared to the untreated controls (**Figure 1A**). Moreover, *L. plantarum* showed a significantly high growth rate in SHC (177.38 ± 8.46%) and SHCPs (192.37 ± 11.03%) groups compared to *L. pentosus* and *L. brevis*.

3.2 SHC Showed the Highest Effect on LAB Growth and Highest Inhibition of Pathogenic Bacterial Growth Among the SH Enzymatic Extracts

The five types of SH enzymatic extracts significantly induced LAB growth in a dose-dependent manner. Among the enzymatic extracts, Celluclast extract treatment (SHC) showed excellent and significant dose-dependent growth enhancement in all LAB species. The highest growth of *L. plantarum* was observed in Celluclast extract treated group at the concentration of 10 mg/mL (293.81± 9.32%). AMG treatment showed no significant differences in *L. plantarum* growth compared to untreated control in 0.1 and 1 mg/mL concentrations (**Figure 1B**). The growth of *L. pentosus* was significantly induced with treatment of Celluclast and Viscozyme enzymatic extracts under all concentrations (0.1, 1 and 10 mg/mL) but all the other enzymatic extracts showed no significant increase in growth at 0.1 mg/mL concentration (**Figure 1C**). In contrast to *L. plantarum* and *L. pentosus*, *L. brevis* showed significant increase in growth in all the enzymatic extracts treated growth medium (**Figure 1D**). These results indicate that the enzymatic extracts of SH have the potential to stimulate beneficial probiotic bacterial growth.

To assess the protective action of the above-mentioned enzymatic extracts against *S. parauberis*, *S. iniae*, and *E. tarda*, a colony counting assay was carried out. As shown in **Figure 2**, the enzymatic extracts of SH actively inhibited the growth of pathogenic bacteria dose dependently. Results indicated that Celluclast extract treatment led to a higher growth inhibition

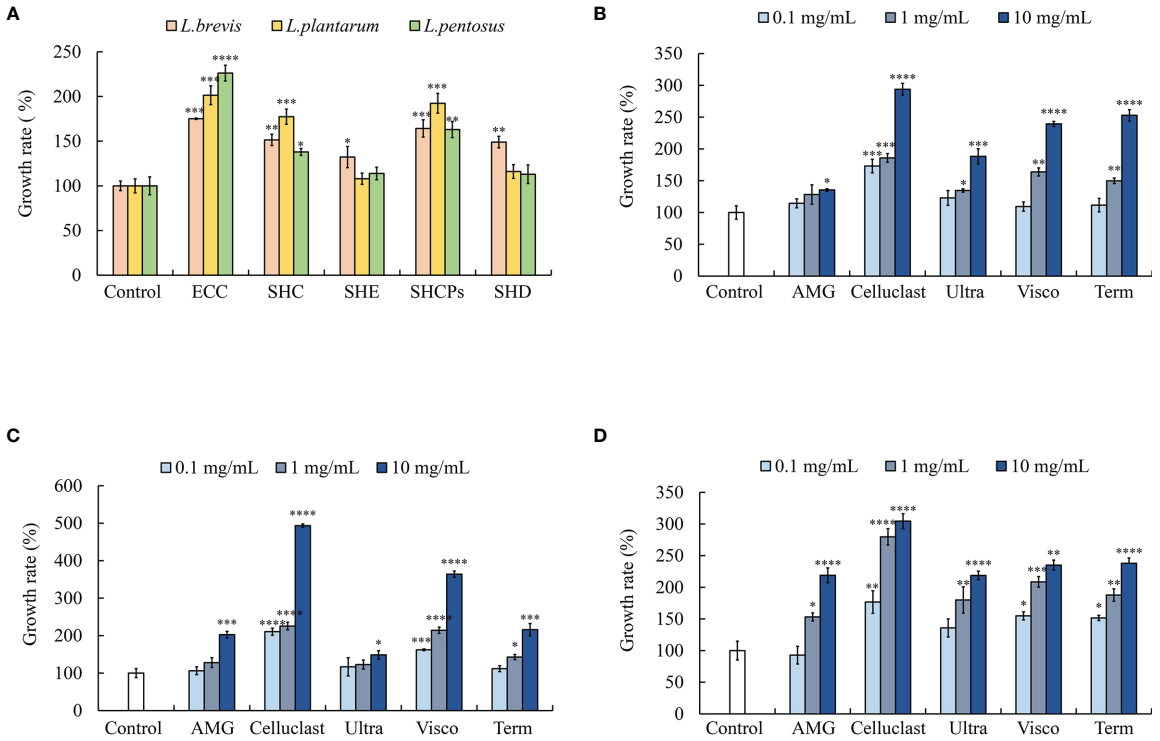

FIGURE 1 | Effect of SH extracts on lactic acid bacterial (LAB) growth. **(A)** Growth of LAB in different solvent extracts of SH. Growth of **(B)** *L. plantarum*, **(C)** *L. pentosus*, and **(D)** *L. brevis* in different enzymatic extracts of SH. Experiment was triplicated and statistical evaluation was performed by comparing experimental and control groups. Values are significantly different from the control group at *p<0.05, **p<0.01, ***p<0.005 and ****p<0.0001. (SHE, *S. horneri* ethanol extract; SHC, *S. horneri* Celluclast extract; SHCPs, *S. horneri* crude polysaccharide extract; SHD, *S. horneri* distilled water extract; ECC, *Ecklonia cava* Celluclast extract; AMG, *S. horneri* AMG enzymatic extract; Celluclast, *S. horneri* Celluclast extract; Ultra, *S. horneri* Ultraflo enzymatic extract; Visco, *S. horneri* Viscozyme enzymatic extract and Term, *S. horneri* Termamyl enzymatic extract).

on all pathogenic bacterial species compared to the non-treated groups, especially, the highest growth inhibition was observed in *S. iniae* compared to the untreated control group (14.34 ± 7.06%) (**Figure 2C**). These results indicate that SH possesses antibacterial effects against pathogenic bacteria.

3.3 SHCPs Was More Effective in Stimulating the Growth of LAB

The proximate composition analysis of SHC and SHCPs was reported in our earlier study (Sanjeewa et al., 2019). According to the results, the polysaccharide contents of SHC and SHCPs were 57.94%, and 65.01% respectively whereas the sulfate content of SHCPs was 12.5%. Therefore, to analyze the effect of polysaccharide content on the prebiotic activity of SH extracts, the growth rates of all three species of LAB corresponding to SHC and SHCPs were tested and shown in **Figure 3**. Both samples displayed potent growth-increasing ability in LAB, and SHCPs were proven to be more effective for the growth of all three LAB species. The treatment of SHCPs caused a significant increase in *L. brevis* growth when at 5 and 10 mg/mL concentrations, while a significant change in growth in SHC was only observed at 10 mg/mL (**Figure 3A**). *L. pentosus* showed significant growth enhancement in all concentrations of SHCPs and in 1,5 and 10 mg/mL concentrations in SHC (**Figure 3B**). **Figure 3C** shows that the treatment of SHCPs and SHC resulted in a gradual increase in growth of *L. plantarum* in a dose increasing manner. Therefore, the samples showed dose-dependent growth improvement in LAB species.

3.4 SHC and SHCPs Improved the Secondary Metabolite Production of LAB in MRS Medium

Secondary metabolites are considered auxiliary compounds produced by microbes that are not required for normal cell growth. Nonetheless, they have other beneficial biological activities, such as antibacterial effects. Therefore, the effects of SHC and SHCPs on secondary metabolite production from LAB were tested in this study. Co-culturing SHC and SHCPs with MRS medium was demonstrated to induce the production of secondary metabolites by *L. plantarum, L. pentosus, and L. brevis* (**Table 1**). However, *L. plantarum* cultured in SHC and SHCPs containing MRS media showed a significant increase in the production of secondary metabolites (718.70 ± 10.0 mg and 574.17 ± 5.0 mg respectively) compared to *L. pentosus and L. brevis* when given the same culture conditions. Moreover, it was observed that *L. plantarum* cultured in SHC treated medium produced higher amount of secondary metabolites than *L. plantarum* cultured in SHCPs containing medium.

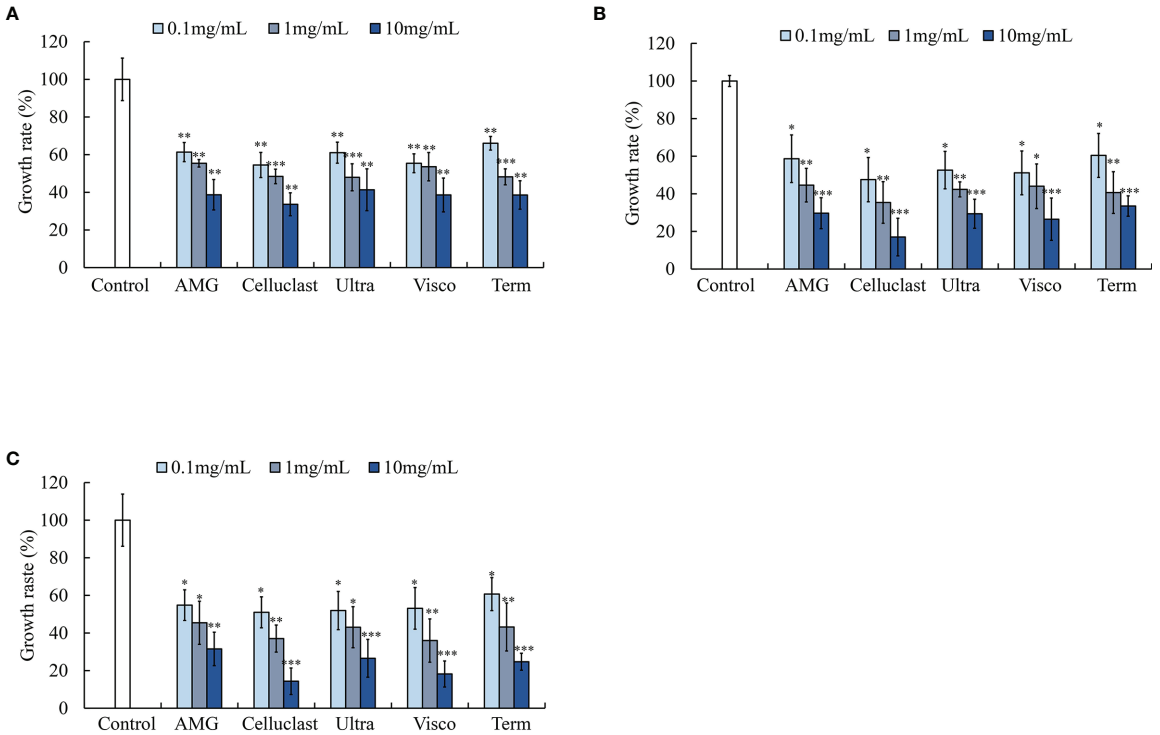

FIGURE 2 | Effect of various enzymatic extracts of SH on pathogenic bacterial growth **(A)** *S. parauberis*, **(B)** *E. tarda* and **(C)** *S. iniae* growth. Experiment was triplicated and statistical evaluation was done to compare the experimental and control groups. Values are significantly different from the control group at *p<0.05, **p<0.01, and ***p<0.005 (AMG: *S. horneri* AMG enzymatic extract; Celluclast: *S. horneri* Celluclast extract; Ultra: *S. horneri* Ultraflo enzymatic extract; Visco: *S. horneri* Viscozyme enzymatic extract and Term: *S. horneri* Termamyl enzymatic extract). The samples were treated to the bacterial culture at different concentrations as 0.1,1 and 10 mg/mL.

3.5 Secondary Metabolites of *L. plantarum* and *L. pentosus* Reduced the Growth of Pathogenic Bacteria

To identify the potential functions of secondary metabolites produced by *L. plantarum* and *L. pentosus*, we tested their antibacterial activity (**Table 2**). Secondary metabolites produced by both *L. plantarum* and *L. pentosus* cultured with or without SHC containing media inhibited the growth of pathogenic bacteria in a significant manner. Secondary metabolites obtained from the LAB cultured in an SHC-containing medium noticeably inhibited pathogen growth (*S. parauberis, S. iniae*, and *E. tarda*) compared to the control groups. In this experiment, SHCPs was not used due to the lower secondary metabolite production by both *L. plantarum* and *L. pentosus* in SHCPs containing medium compared to SHC containing medium. Similarly, *L. brevis* was not used due to the slower growth rate, as well as the lower production of secondary metabolites.

Obtained results showed that SH is a potential prebiotic for beneficial bacteria, with SHC and SHCPs showing superior activity over other enzymatic extracts. Hence, further experiments were planned with SHC and SHCPs, while *L. plantarum* was used as the model probiotic bacteria in this study due to its popularity as a starter probiotic as well as its abundance in the gastrointestinal tract in both humans and fish (Arasu et al., 2016). *S. parauberis* was used as the pathogenic bacteria *in vivo* experiments due to its high infectivity and its popularity as a common disease-causing bacterium in aquaculture.

3.6 Feeding of Prebiotics Improved the Growth Performance and Reduced the Mortality of *S. parauberis* Infected Zebrafish Significantly

The experimental diet was fed to zebrafish for 30 days and the weight gain was recorded each week. The effects of diet on growth performance are shown in **Figure 4A**. The results showed that co-treatment of *L. plantarum* with SHC and SHCPs had a direct effect on zebrafish growth. After 30 days of feeding the diet, the final biomass of the fish had improved in all groups compared to the group fed with commercial feed only. However, there was no significant difference between the experimental groups.

For the assessment of the role of SH as a prebiotic, zebrafish were injected intraperitoneally with 1.18 x 10^8 cells/μL of *S. parauberis* after four weeks of experimental diets, and the number of dead fish was counted. The results showed that infected fish displayed symptoms especially, darkened body coloration, cachexia (**Figure 4B**), and abnormal swimming patterns. During this period, the number of dead fish in the infected groups increased significantly compared to that in the non-infected group. However, supplementation with *L. plantarum* and SH extracts

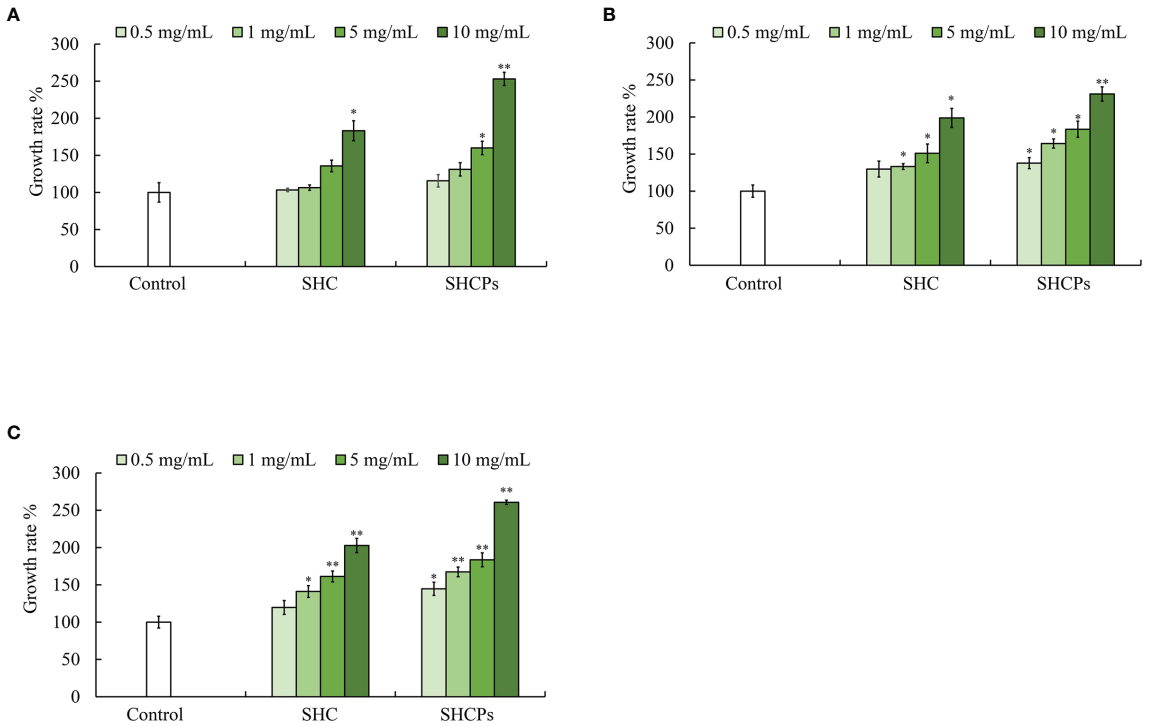

FIGURE 3 | Effect of SHC and SHCPs on probiotic bacterial growth Growth of **(A)** *L. brevis*, **(B)** *L. pentosus* and **(C)** *L. plantarum*. Experiment was triplicated and statistical evaluation was performed to compare the experimental and control groups. Values are significantly different from control group at *p<0.05, **p<0.01 (SHCPs: *S. horneri* crude polysaccharide extract; SHC: *S. horneri* Celluclast extract). The samples were treated to bacterial culture with different concentrations as 0.5,1,5 and 10 mg/mL.

(SHC and SHCPs) led to a decline in mortality rates effectively (**Figures 4C, D**). The mortality rate of the group of infected fish fed with the control diet (commercial feed) only reached 66.67%, whereas the mortality rates of other treated groups were lower than 30%. The lowest mortality rate of pathogen-infected fish was observed in the groups co-treated with SH and *L. plantarum.*

3.7 Supplementation of SHC Reduced the Expressions Levels of iNOS and COX-2 Proteins in *S. parauberis* Infected Zebrafish

The anti-inflammatory effect of the SH diet on inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein levels, in *S. parauberis*-infected fish, were assessed *via* Western blot analysis (**Figures 5A, B**). The protein expression results for iNOS and COX-2 are shown in **Figures 5C, D**. The results indicated that the expression of iNOS and COX-2 was significantly increased in pathogen-infected zebrafish. Meanwhile, the expression of proteins was downregulated following the supplementation of experimental diet. Significant reduction of iNOS and COX-2 expressions in the SHCPs treated group was observed compared to the control group and 5% SHC treated group showed the highest inhibition of iNOS expression in infected fish (**Figure 5C**). The overall highest inhibition of iNOS and COX-2 expression was observed in 1% *L. plantarum* + 5% SHC treated group (0.1591 ± 0.0014, 0.1385 ± 0.0003 respectively) (**Figure 5D**). Overall, the results show that SHC amended the mortality of *S. parauberis*-infected zebrafish *via* obstructing the inflammatory mediator expression.

TABLE 1 | Effects of SHC and SHCPS on secondary metabolite production by LAB.

		Control	SHC	SHCPs
Secondary metabolites (mg)	*L. plantarum*	402.00 ± 8.0	718.70 ± 10.0****	574.17 ± 5.0**
	L. pentosus	351.80 ± 14.0	586.07 ± 10.0****	516.07 ± 8.0***
	L. brevis	303.50 ± 10.0	422.05 ± 11.0****	464.42 ± 7.0****

**Statistical evaluation was performed to compare the experimental and control groups. Values are significantly different from control group at **p<0.01, ***p<0.005 and ****p<0.0001.*
SHC: Celluclast enzymatic extract of S. horneri.
SHCPs: Crude polysaccharide extract of S. horneri.

TABLE 2 | Effects of secondary metabolites produced by LAB on pathogenic bacterial growth (%).

		Control	SHC-	SHC+
Secondary metabolites of *L. plantarum*	*S. parauberis*	100.00	64.11 ± 6.66*	54.85 ± 4.52***
	S. iniae	100.00	70.09 ± 5.23*	61.55 ± 4.49***
	E. tarda	100.00	53.14 ± 4.24**	48.31 ± 4.53****
Secondary metabolites of *L. pentosus*	*S. parauberis*	100.00	53.09 ± 6.12*	45.48 ± 4.32****
	S. iniae	100.00	63.05 ± 6.03*	50.53 ± 4.49***
	E. tarda	100.00	50.07 ± 6.54*	41.61 ± 3.56****

**Statistical evaluation was performed to compare the experimental and control groups. Values are significantly different from control group at *p < 0.05, **p < 0.01, ***p < 0.005 and ****p < 0.0001.*

SHC⁻, secondary metabolites obtained from LAB cultured in culture medium without SH Celluclast extract.

SHC⁺, secondary metabolites obtained from LAB cultured in culture medium containing SH Celluclast extract.

3.8 SHC Significantly Inhibited the Activation of NF-κB Signaling Pathway

To explore whether the anti-inflammatory activity of SH extracts in *S. parauberis*-infected zebrafish is mediated by NF-κB pathway-related protein expression, the levels of NF-κB protein expression were determined by Western blot. Thus, we evaluated the expression of p65 and IKB-α in zebrafish infected with *S. parauberis* and treated with SH extracts. As shown in **Figures 5E, F**, the results revealed that compared to the non-infected group, expression levels of p65 and IKBα in zebrafish were increased due to *S. parauberis* infection. Moreover, supplementation with the experimental diet containing SH extracts and *L. plantarum* actively

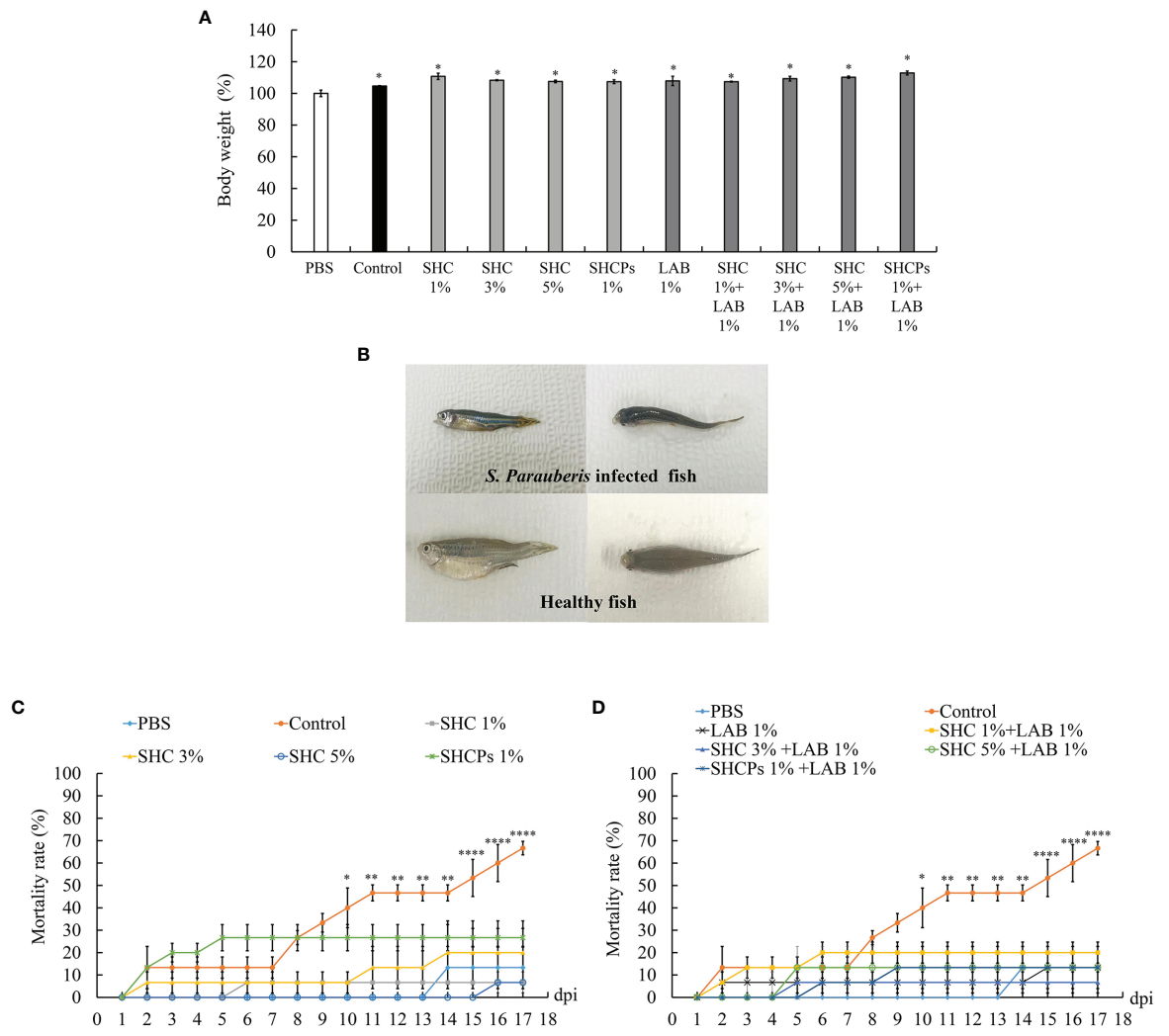

FIGURE 4 | Effect of experimental diet on S. parauberis infected zebrafish. **(A)** growth performance of zebrafish, **(B)** morphology, **(C)** accumulated mortality (diet containing SH extracts only), and **(D)** accumulated mortality (diet containing LAB and SH extracts). Statistical evaluation was performed to compare the experimental and control groups. Values are significantly different from PBS group at *p<0.05, **p<0.01, and ****p<0.0001.

inhibited the phosphorylation of the above-mentioned proteins. As indicated in **Figure 5F**, the highest inhibition of p-p65 and p-IKBα was observed in fish fed with 1% *L. plantarum* + 5% SHC group (0.1763 ± 0.0003 and 0.1691 ± 0.0050 respectively). These results suggest that SH possesses anti-inflammatory activity *via* inhibiting the activation of NF-κB signaling pathway.

3.9 Pathological Features of Zebrafish Infected With *S. parauberis*

The infection with *S. parauberis* leads to serious injuries in zebrafish, especially in the gastrointestinal (GI) tract. Histological sections of tissues from the GI tract demonstrated the presence of bacteria in the intestinal lumen of infected fish. We also

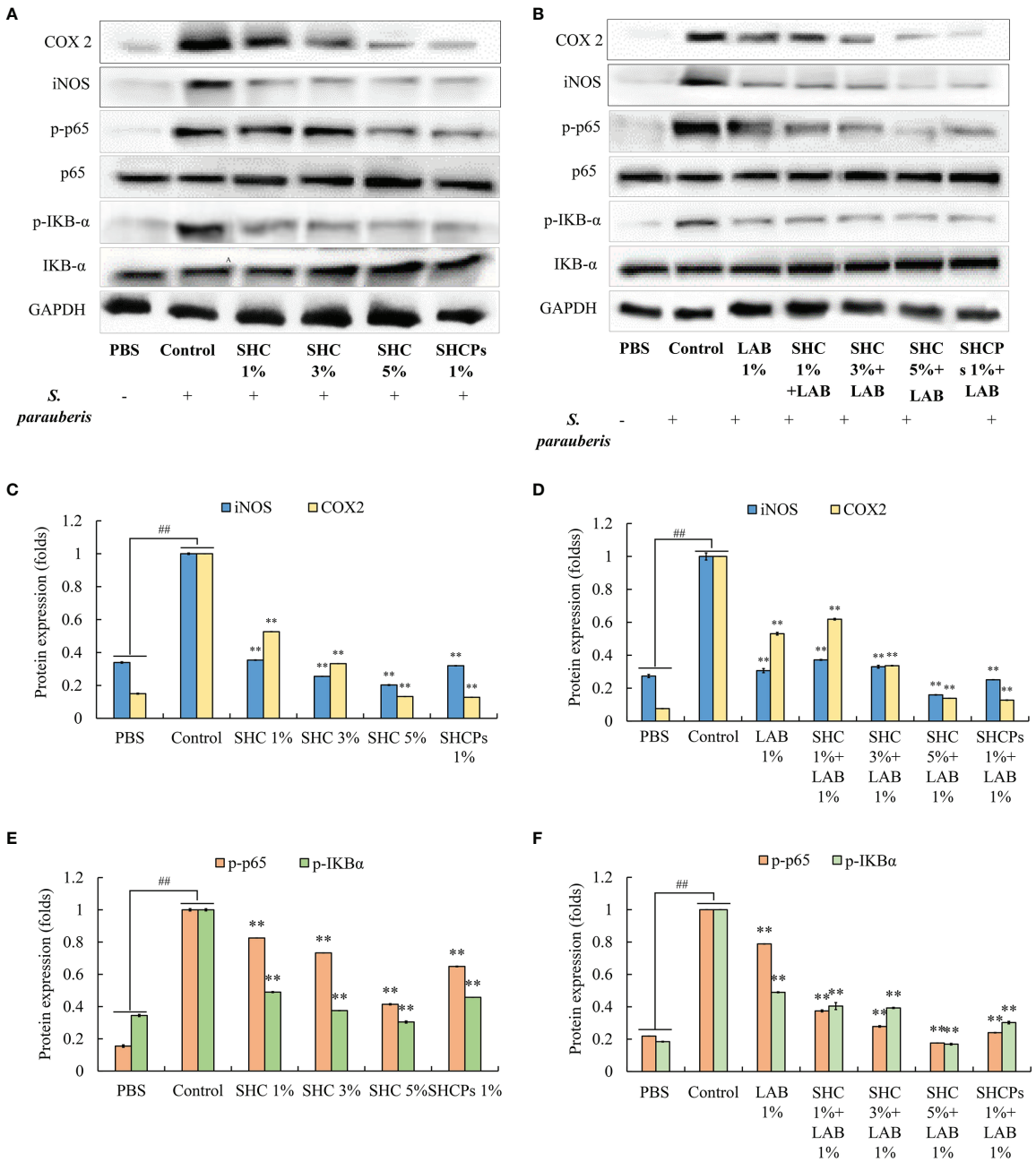

FIGURE 5 | Effect of experimental diet on expression levels of inflammatory mediators and NF-κB pathway related proteins. **(A, B)** Protein expression inhibition by SH extracts, **(C, D)** iNOS and COX-2 expression **(E, F)** NF-κB pathway protein expression. Statistical evaluation was done to compare the experimental and control groups. Values are significantly different from control group at $^{**}p<0.01$ or $^{\#\#}p < 0.01$ compared to non-treated group (PBS group).

observed an increase in the number of goblet cells involved in the intestinal mucosal formation and immunity of epithelial cells in the experimental diet-supplemented fish compared to infected fish fed with the control diet. According to the H&E staining results, fish in the 5% SHC+1% *L. plantarum* supplemented group had the highest increase in goblet cell density. Similarly, we observed extensive necrosis in epithelial cells in the intestine caused by pathogenic bacterial infection, which was lessened with the supplementation of SHC (**Figure 6**).

3.10 Incubation of *L. plantarum* With SHC Produced Several Secondary Metabolites Compounds That May Affect Antibacterial Activity

According to the results obtained from *in vivo* experiments, SHC showed higher activity in inhibiting *S. parauberis* infection when co-treated with *L. plantarum*. Therefore, for the identification and analysis of secondary metabolites produced by *L. plantarum* in an SHC-containing growth medium, GC/MS analysis was performed. For the purpose of providing a negative control, GC/MS analysis was also performed with MRS broth without SHC treatment. Results of the analysis of secondary metabolites in SHC-treated broth are shown in **Figure 7**. According to the results, the MRS broth containing *L. plantarum* contained compounds with similar peaks to those of in SHC-treated broth, such as L-Lactic acid, N-methoxy-N-methyl acetamide, diethyl phthalate, and propanoic acid.

Interestingly, tetradecanamide, 2,8,9-Trioxa-5-aza-1-silabicyclo [3.3.3] undecane, 5, 10-Diethoxy-2, 3, 7, 8-tetrahydro- 1H,6H-dipyrrolo[1,2-a:1',2'-d] pyrazine,1H-Indole-3- carboxaldehyde, and 5- fluoro-1-phenyl were only observed in MRS medium supplemented with SHC. Such compounds were identified by comparing their spectra to those in the mass spectra library. Compounds identified from *L. plantarum* secondary metabolites were aromatic and heterocyclic, which are suspected to possess the antibacterial effects on pathogenic bacteria used in this study.

4 DISCUSSION

Due to its high polysaccharide content, seaweed can be used as a prebiotic ingredient for both human and animal health applications (O'Sullivan et al., 2010). The notable effect of prebiotics is by modulating the intestinal microbiota population, which results in inhibition of pathogenic bacteria by limiting their proliferation. This study demonstrated the potential of SH to enhance beneficial probiotic bacterial growth and secondary

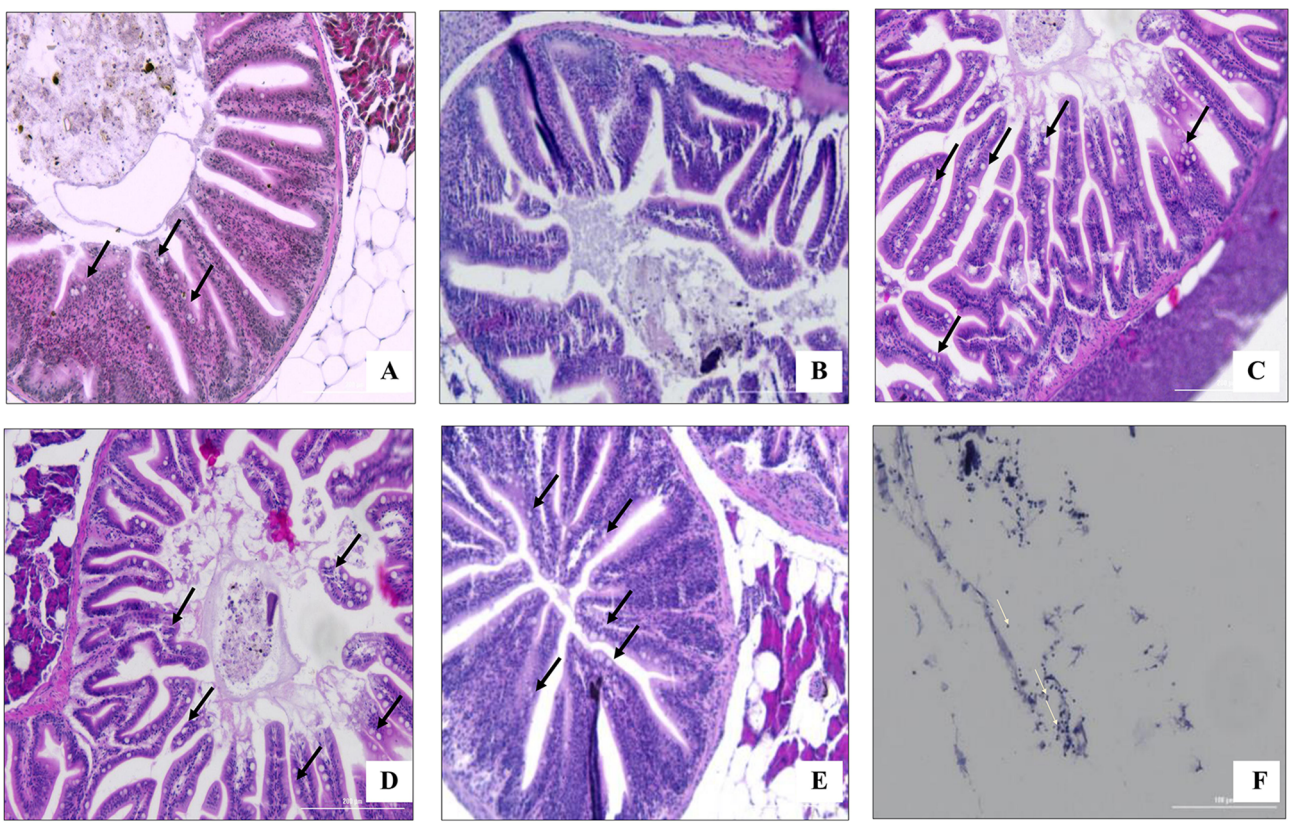

FIGURE 6 | Histological analysis of the goblet cells (black arrow) in the intestine of zebrafish challenged with *S. parauberis*. **(A)** non-infected group with control diet **(B)** infected group fed with control diet, **(C)** infected group fed with 5% SHC supplemented diet, **(D)** infected group fed with 1% *L. plantarum* supplemented diet, **(E)** infected group fed with 1% *L. plantarum* + 5% SHC supplemented diet and **(F)** bacterial cells in the intestinal lumen (white arrow). (SHC, S. horneri Celluclast extract).

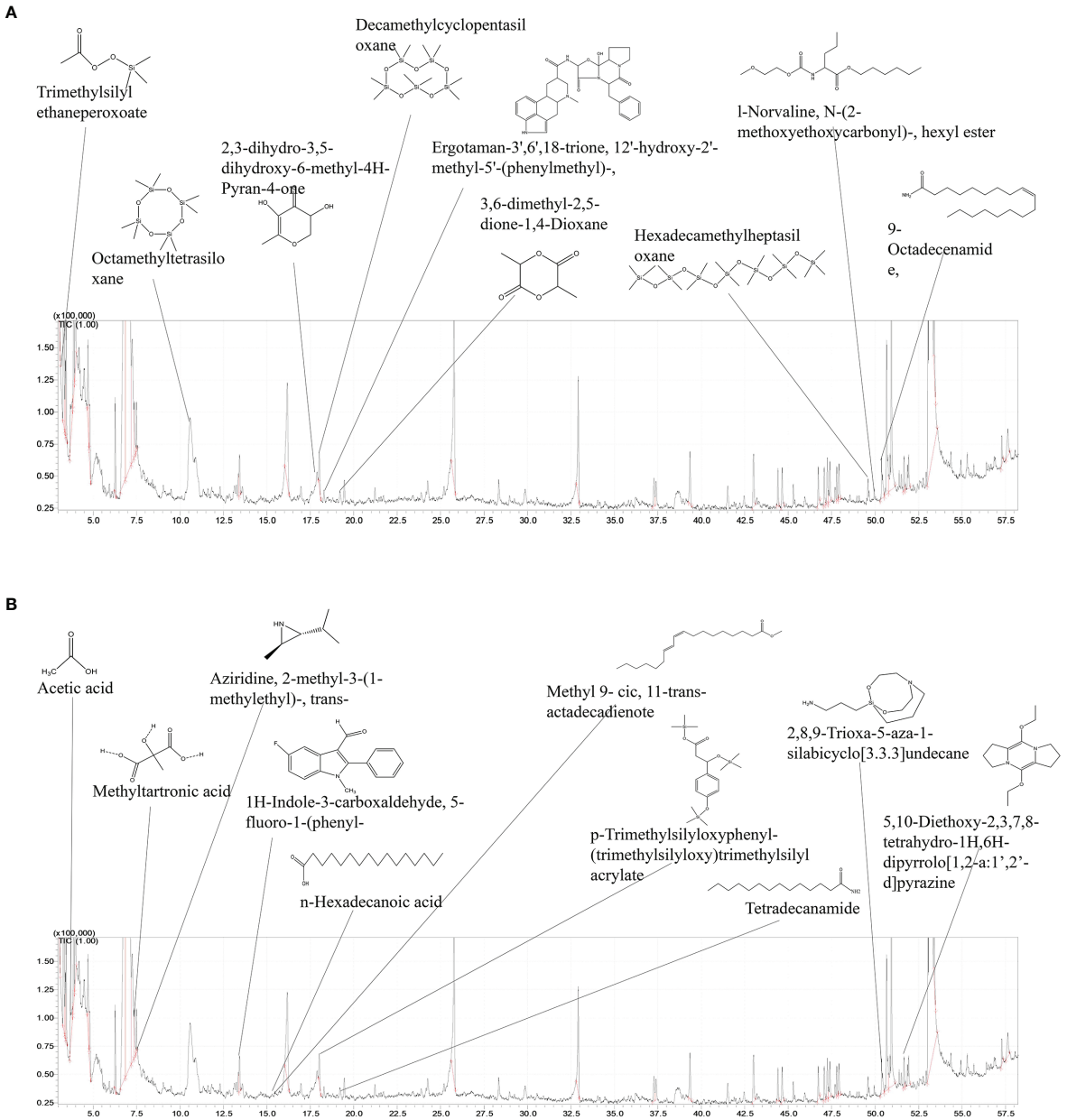

FIGURE 7 | GC/MS analysis of secondary metabolites obtained from *L. plantarum* incubated with SHC. **(A)** GC/MS spectrum of secondary metabolites produced in only MRS containing medium only **(B)** GC/MS spectrum of secondary metabolites.

metabolite production, thereby resulting in the reduction of pathogenic bacterial infections in fish. Likewise, supplementation with SH was also shown to result in increased body weight in zebrafish. Probiotics such as lactic acid bacteria (LAB) are beneficial microorganisms having numerous health benefits. LAB are a group of gram-positive, non-spore-forming anaerobic or facultative aerobic cocci or rods (Hayek and Ibrahim, 2013). They metabolize growth medium components and produce bacterial secondary metabolites that are important for the health and nutrition of other organisms (Ustyugova et al., 2012). LAB are widely used as probiotics in poultry and swine management practices (Patterson and Burkholder, 2003) and have also been recently used as an additive in fish feed, resulting in improved growth and management of diseases in the aquaculture (Ringo and Gatesoupe, 1998).

The use of seaweed as prebiotics is an important alternative approach for controlling diseases in fish through a variety of mechanisms. LAB breakdown components found in seaweed and produce bioactive compounds having antibacterial activity. Further, microbial fermentation of seaweed results in new bioactive complexes with antioxidant properties and other activities, as occurs, for example, through fermentation of *Ekclonia cava*

by *Candida utilis* (Wijesinghe and Jeon, 2012). Another study showed that fermentation of *Hizikia fusiforme* by *L. brevis* enhanced the antioxidant and anti-inflammatory activities of its water extract (Song et al., 2011). Also, fermentation of *Sargassum siliquanstrum* with LAB was proven to enhance its anti-inflammatory property (Lee et al., 2016). Such antioxidant properties and anti-inflammatory properties reduce the oxidative stress and inflammation caused by external factors such as pathogens in fish.

Co-culturing of LAB with seaweed extracts resulted in increased growth of LAB.

In particular, the growth of *L. plantarum* was enhanced significantly when treated with SHC and SHCPs compared to non-treated groups. In addition, compared to SHC, SHCPs led to a higher increase in lactic acid bacterial growth. This is likely due to the increased polysaccharide content in the extract (Sanjeewa et al., 2017), as an earlier study reported that a high polysaccharide content was found to result in a good prebiotic activity (O'Sullivan et al., 2010). A study conducted on several Indian seaweeds, has also proven that the high dietary fiber content and polysaccharide content in *E. compressa* results in higher growth of probiotics (Ajanth Praveen et al., 2019). Thus, prebiotics are able to boost the growth of beneficial probiotic bacteria and thereby promote the production of secondary metabolites that are beneficial to host health. The results from previous studies agreed with the results obtained from the present study. Apart from its prebiotic activity, seaweeds are also proven to have antibacterial activity against various infectious bacteria. In an early study, several cultivated seaweeds were screened for their antibacterial activity and verified the possible use of seaweeds as an antimicrobial source (Bansemir et al., 2006). Similarly, we observed that SH enzymatic extracts, especially SHC acted as a protective agent against fish pathogenic bacteria such as *E. tarda, S. parauberis* and *S. iniae*, independently from its prebiotic action on LAB. This was confirmed by culturing pathogenic bacteria in SH enzymatic extracts-containing culture medium and observing their growth compared to the non-treated control groups. Moreover, both SHC and SHCPs significantly improved the production of secondary metabolites by all three species of LAB compared to LAB cultured in MRS culture medium only. Furthermore, secondary metabolites produced from both *L. plantarum* and *L. pentosus* were proven to possess antibacterial action against the common fish pathogens used in this study. Based on GC/MS results, we identified that culturing of *L. plantarum* in *S. horneri*-containing medium produced tetradecanamide, 2,8,9-Trioxa-5-aza-1-silabicyclo [3,3,3] undecane, 5,10-Diethoxy-2,3,7,8-tetrahydro-1G, 6H-dipyrrolo[1,2-a:1,2-d] pyrazine, as secondary metabolites, all of which are suspected of having potential antibacterial activity against all three pathogenic bacterial species. This may be the reason for the higher antibacterial activity of secondary metabolites from *L. plantarum* against *S. parauberis.* However, further investigations are required to characterize its antibacterial activity. A previous, similar study reported that *L.plantarum* produced 3-ethyl-6-pentofuranosyl imidazo (4,5-c) pyrazole-1(6H)-carbaldehyde which has antibacterial properties when grown in MRS medium containing *Ecklonia cava* extract (Lee et al., 2016). Moreover, it has been reported that secondary metabolites, such as oleic acid, octanoic acid, butanamide, and decanoic acid derivatives produced by *L. plantarum* have antifungal activities (Poornachandra Rao et al., 2019).

Probiotics and prebiotics like immune-stimulants, act as alternatives to antibiotics and chemotherapeutics which have negative effects such as resistance development and mutations in pathogens (Lopez et al., 2003). Because of such benefits, probiotics and prebiotics have received substantial consideration for incorporation into fish feed. A study conducted on groupers (*Epinephelus coioides)* showed that feeding on *L. plantarum*, enhanced their growth and disease resistance in a significant manner (Son et al., 2009). In early studies, SH was proven to increase the growth performance of aquaculture fish such as black sea bream and turbot (Shi et al., 2019; Wang et al., 2019). Correspondingly, our study revealed that feeding on prebiotics resulted in comparatively higher weight gain in zebrafish compared to the non-treated group. The increase in body weight may be due to the supplementation of the experimental diet, which helps in augmenting the digestive activity *via* vitamin and enzyme synthesis and improving the microbial flora in the gut (Son et al., 2009; Ngamkala et al., 2010).

The pathogenic bacteria used in this study are responsible for the disease streptococcosis, which is related to acute and chronic mortality in many aquaculture fish species. The results of this study suggested that the co-application of *L. plantarum* and SH improved the survival rate of zebrafish infected with *S. parauberis*. Infected fish showed darkened skin, inflammation, and cachexia, symptoms which were relieved following the co-treatment with *L. plantarum* and SH. Similar findings were obtained from studies conducted on the prebiotic potential of *E. cava* in *E. tarda* -infected zebrafish (Lee et al., 2016) and olive flounder (Lee et al., 2016; Vidhya Hindu et al., 2018), indicating that the incorporation of seaweed in fish feed results in improved growth and decreased mortality of infected fish. Histological studies showed that co-treatment with *L. plantarum* and SH improved goblet cell density in infected fish. Goblet cells are specialized epithelial cells that play an important role in innate defense in the gut, especially by exerting a protective mechanism against exogenous conditions, including protection from pathogenic bacteria (Kim and Ho, 2010). Similar results were obtained in previous studies conducted on the effects of *U. pinnatifida* on intestinal health of gibel carp and the effect of chitosan silver nanoparticles on immunomodulation in zebrafish (Udayangani et al., 2017; Cui et al., 2020).

The first line of the defense system in fish is the innate immune system (Harikrishnan et al., 2010). Probiotic activation in the gut induces the activation of the innate immune system and induction of pro-inflammatory cytokines when invaded by foreign pathogens. Bacterial infections result in the production of inflammatory responses and production of NO, a free radical with numerous pathological functions. Increased generation of NO leads to the activation of iNOS and COX (Wang et al., 2007). The inducible form of COX, called COX-2, is produced extensively by macrophages at the site of inflammation (Wang et al., 1995). Previous work showed the expression of iNOS and COX-2 to be up-regulated in *S. parauberis*-infected zebrafish

in the non-treated group compared to the *L. plantarum* and SH treated groups. According to our study, co-application of *L. plantarum* significantly downregulated the expression of iNOS and COX-2. The reduction in inflammatory mediators was reported to decrease the effects of pathogenic bacteria in fish (Vojtech et al., 2009). Numerous studies have demonstrated the ability of SH to reduce iNOS and COX-2 expression during inflammation in *in vitro* and in *in vivo* models (Acheson, 1969; Sanjeewa et al., 2018). Due to the reduction of iNOS and COX-2, pain, swelling, and stiffness are decreased. The anti-inflammatory activity of SH was further tested by analyzing its effects on signal transduction pathways in *S. parauberis*-infected zebrafish.

It has been reported that inflammatory responses are due to the activation of complex signaling pathways, such as NF-κB. p65 and IKB-α are fundamental proteins in the NF-κB pathway, which are triggered by pathogen infection and eventually phosphorylated into the nucleus to bind with DNA, resulting in the regulation of mRNA expression of genes associated with inflammation including iNOS and COX-2 (Kim, 2014). The study showed that infection with *S. parauberis* resulted in an increase in phosphorylation of the above-mentioned proteins. However, treatment with SH extracts significantly suppressed the phosphorylation of NF-κB proteins, resulting in downregulation of NF-κB signaling cascade. These results collectively suggest that SH has anti-inflammatory activity against pathogenic bacteria-induced inflammation.

CONCLUSION

In agreement with the results obtained from our study, *S. horneri* (SH) efficiently improved the growth of probiotic bacteria such as *L.plantarum, L. pentosus*, and *L. brevis*, whereas it inhibited the growth of pathogenic bacteria such as *S. iniae, St. parauberis*, and *E. tarda.* Similarly, SH reduced the mortality of *S. parauberis*-infected zebrafish. These findings are also supported by the downregulation of several inflammatory mediators in the NF-κB pathway and the antibacterial activity against the pathogenic bacteria. Hence, these results strongly suggest that SH extracts possess potential prebiotic activity, which can be utilized in disease management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Use Committee (IACUC) of Jeju National University (protocol 2020-0049).

AUTHOR CONTRIBUTIONS

NL: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing- Original draft preparation; Y-SK: Resources, Data Curation, Supervision; DN: Software, Resources; TJ: Resources; HJi: Resources; H-WY: Resources; HJa: Resources; Y-JJ: Conceptualization, Supervision, Writing- Review and Editing, Project administration, Funding acquisition. All authors contributed to the article and approved the submitted version.

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