A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Immune-enhancing activities of Combination of the *Sargassum horneri* polysaccharides and *Lactobacillus plantarum* in macrophages and zebrafish model

Jin Hwang

DEPARTMENT OF MARINE LIFE SCIENCES

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

August, 2021

Immune-enhancing activities of Combination of the *Sargassum horneri* polysaccharides and *Lactobacillus plantarum* in macrophages and zebrafish model

Jin Hwang

(Supervised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of
MASTER OF SCIENCE

August, 2021

This thesis has been examined and approved by

Thesis director, BoMi Ryu (PhD), Research Professor of Marine Life Science, Jeju National University

Hye-Won Yang (PhD), Research Professor of Marine Life Science, Jeju National University

You-Jin Jeon (PhD), Professor of Marine Life Science, Jeju National University

Data: August, 2021

DEPARTMENT OF MARINE LIFE SCIENCES

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Immune-enhancing activities of Combination of the *Sargassum horneri* polysaccharides and *Lactobacillus plantarum* in macrophages and zebrafish model

Jin Hwang

Department of Marine Life sciences

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2021.08

CONTENTS

국문 초록 iii

LIST OF FIGURES vii

ABSTRACT 1

INTRODUCTION 2

MATERIALS AND METHODS 4

Reagents and chemicals 4

Preparation of crude polysaccharides from *S.hornei* (SHC.Ps) 4

Monosaccharide composition analysis of crude polysaccharides 5

FT-IR characterization of crude polysaccharides 5

Analysis of polysaccharides, polyphenols and sulfate contents of crude polysaccharides from *S. horneri* celluclast-enzyme extract 5

Acid hydrolysis for depolymerization of SHC.Ps 5

Characterization of molecular weight of SHC.Ps by Size-exclusion chromatography Multi Angle Light Scattering (SEC-MALS) 6

Mass spectrometric analysis of SHC.Ps 6

Preparation of probiotics 7

Cell culture 7

Cytotoxicity and NO production by the combination of SHC.Ps and LAB induced in RAW 264.7 macrophages 7

Phagocytosis assay 8

Fish maintenance and diet preparation 8

Flow cytometric analysis on immune-enhancing effects of the combination of SHC.Ps and LAB in zebrafish 9

Histopathology on immune-enhancing effects of the combination of SHC.Ps and LAB in zebrafish intestinal 9

Pro-inflammatory cytokines production against the combination of SHC.Ps and LAB in zebrafish 9

Western blot 10

Statistical analysis 10

RESULTS 11

Extraction of *S.horneri* and General and monosaccharides composition of SHC.Ps 11

FT-IR characterization of SHC.Ps.....13

Determination of absolute molecular weight and mass spectrometric analysis of SHC.Ps.....15

Effects of the combination of SHC.Ps and LAB on cell viability and NO production 16

Effects of the combination of SHC.Ps and LAB on for immune-enhancing activity using phagocytic analysis 17

Effect of the combination of SHC.Ps and LAB on immune-enhancing activity of pro-inflammatory cytokines production 23

Effect of the combination of SHC.Ps and LAB on immune-enhancing activity in though the JNK/p38 pathway 25

Effect of the combination of SHC.Ps and LAB on immune-enhancing activity in zebrafish kidney using FACS analysis 27

Effect of combination of SHC.Ps and LAB on immune-enhancing activity in zebrafish intestinal using the histological analysis 30

DISCUSSIONS 32

CONCLUSION 35

국문 초록

면역반응은 우리 몸을 보호하기 위한 자기방어 기작으로서 외부 인자로부터 유입된 다양한 감염원을 제거 또는 무력화하는 과정을 포함하고 항상성을 유지하기 위해 단핵구 (monocyte), 호중구 (neutrophile) 및 포식세포 (phagocyte) 등과 같은 다양한 면역 세포들 간의 상호작용에 의하여 그 체계가 유지된다. 체내 면역반응은 크게 선천적 면역반응과 후천성 면역반응 두가지로 구분되며, 각기 면역작용을 나타내는 담당 면역세포가 다른 특징을 가지고 있다. 선천적 면역반응을 매개하는 세포 중 대식세포 (macrophage)는 외부 항원을 포식하고, 면역 조절인자인 nitric oxide (NO)와 tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), IL-6 와 같은 염증성 사이토 카인 (pro-inflammatory cytokine)을 분비하여 자신의 포식작용을 증가시키거나 자연살해세포, 수지상세포 등의 선천면역의 활성을 유도하는 한편 외부로부터 유입된 항원을 면역 T 세포에 전달하여 세포의 활성화와 분화를 조절함으로써 적응 면역의 활성에도 기여하는 것으로 알려져 있다. 또한 NK cell은 세포 독성 과립을 방출하여 암세포를 직접적으로 살해할 수 있으며, interferon-γ (IFN-γ)와 같은 사이토 카인을 분비하여 대식세포 및 T 세포를 동시

에 활성화할 수 있다. 따라서 인체의 면역기능 저하는 결국 감염 및 질병의 발생 뿐만 아니라 종양의 발생 및 암환자의 회복을 지연시키는 요인이 되기도 한다. 이러한 관점에서 면역자극은 체내의 면역 방어체계 향상을 위한 중요한 전략으로 대두되고 있으며 이러한 요구에 부응하여 면역 잠재력을 향상시킬 수 있는 천연물 및 식이 소재 개발에 관심이 집중되고 있다. 또한 최근 연구에 의하면 천연물에서 추출한 다당류 및 단백질과 같은 고분자 물질들은 의약품에 비해 비교적 낮은 독성을 나타내는 장점을 가지고 있으며 생체내에서 대식세포, 림프구, 자연 살해 세포 등 면역세포의 활성화를 통해 면역조절 활성을 증진시킨다고 알려져 있다.

해양에 서식하는 다양한 해양생물들 중 해조류는 다량의 무기질을 함유하고 있을 뿐만 아니라, 다당류의 함량이 높아 다양한 생리활성을 가지는 천연자원으로 그 중요성이 점차 주목받고 있으며, 특히 갈조류의 경우 항암, 항염증 및 항산화 등 생리활성 물질이 풍부한 것으로 알려져 있다. 갈조류 중 모자반목 모자반과에 속하는 괭생이 모자반 (*Sargassum horneri*)은 우리나라 동해안과 남해안 및 일본의 전연안과 중국에도 보편적으로 분포하는 종으로 우리나라에서 현재까지는

해안에 밀려와 쌓여 악취를 풍기는 원인으로써 사료로 이용되는 점 이외에는 해상에서 인간에게 피해를 입히는 해조류로 인식되고 있었다. 그러나 일본의 경우 오래 전부터 식용으로 이용되고 있는 것으로 보고되고 있으며 해조류 추출물 및 이차대사물들이 가지는 건강증진효과로 인해 최근 들어 식단에서 주요식품의 한 급원으로 인식되기 시작하여 우리나라에서도 점차 괭생이 모자반의 건강기능성에 대한 연구가 활발해지고 있다. 괭생이 모자반에 대한 현재까지의 연구를 살펴보면, 괭생이 모자반 추출물이 골다공증 방지 기능이 있는 것으로 보고되어 있다. 또한 괭생이 모자반의 추출물이 항산화 및 항암효과를 가지고 헤르페스 바이러스 (Herpes simplex virus type 1)의 억제 효과가 있음이 보고되었다.

Zebrafish(*Denio rerio*)는 인간의 유전자 정보 및 장기 체계가 높은 상동성을 보이는 척추 동물로, 세대 기간이 2-3 개월로 짧고 발생이 매우 빨라 생후 첫 3 일 안에 중요한 기관이 모두 형성되는 장점이 있어 유전학, 생물학 등에서 널리 사용되고 있으며, 최근 선천성 면역계와 후천 면역계가 잘 발달되어 있기 때문에 면역연구에 유용한 동물 모델이다.

장내 정상 균총 (normal flora)은 숙주의 대사과정을 효율적으로 활용하며 안정적인 군집을 형성함과 동시에, 인간과 공생하면서 외부의 위험을 차단하는 기능도 함께 수행한다. 외부에서 유입된 병원균은 장내 상피 세포에 부착하기 위하여 우선 정상 균총과 경쟁하게 된다. 정상 균총은 특히 장내 점막면역계의 발달과 성숙에 필수 요소로서, 특정 미생물군은 특정 종류의 면역세포의 분화와 황성화를 유도하여 면역 관용 (immune tolerance)와 면역 자극 (immune stimulation) 간의 균형을 조절한다. 한편 장내 점막 면역계의 발달에는 고블렛 세포 (goblet cell) 이 관여하는 것으로 알려져 있다.

본 연구에서는 RWA264.7 대식세포에 괭생이 모자반의 효소 추출물을 처리하여 이들 이 세포생존, 사이토 카인 및 NO 생성에 미치는 영향을 비교하는 한편 mitogen-activated protein kinases (MAPKs) 신호전달경로 및 iNOS, COX-2 활성화 등에 미치는 영향을 관찰하였으며, 또한 제브라 피쉬 동물 모델에서 장내 점막 형성 및 상피세포의 면역에 관여하는 고블렛 세포을 관찰하였으며, 신장에서의 조혈모 세포에서의 면역세포들의 구성을 확인함으로서 이들의 면역증강활성 및

분자적 작용기전을 규명하고, 면역증강 소재 로서의 개발 가능성을 알아보고자 하였다.

List of figures

Fig 1. (A) Polysaccharide, Polyphenol compositions and sulfate contents of SHC and SHC.Ps were estimated. (B) Monosaccharide content of SHCF2 was analyzed using an HPAE-PAD spectrum compared with a standard monosaccharide mixture..... 12

Fig 2. Infrared analysis spectroscopy (FT-IR) of commercial fucoidan. (A) Crude polysaccharide (B) obtained from *S. horneri via* Celluclast enzymatic extraction.....14

Fig 3. (A) Size exclusion chromatography of crude polysaccharide obtained from *S. horneri via* Celluclast enzymatic extraction was conducted using SEC-MALS instruments. (B) Mass spectrometry analysis of crude polysaccharide obtained from *S. horneri via* Celluclast enzymatic extraction with MALDI-TOS mass spectrometer after mild acid hydrolysis.....16

Fig 4. NO production (A) and Cytotoxicity (B) of the SHC.Ps present or absent of LAB on RAW 264.7 cells. The viability of cells without samples and LPS has taken as reference and the level of NO production is expressed as a percentage of that of the group treated with LPS alone. Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, #### $p < 0.0001$ compared to blank group (no sample treated group); * $p < 0.05$, *** $p < 0.001$ compared to control group (LPS 100 ng/ml) 18

Fig 5. Phagocytosis assay with Hoechst Dye-stained murine macrophage-like RAW264.7 cells. RAW 264.7 cells were treated with SHC.Ps presence or absence of LAB in different doses, non-treated group, and control (100 ng/ml of LPS). (A, B) FACS (A) and analysis pf phagocytosis of latex beads by RAW264.7 (B) treated with different agents for 2 h (C) The immunofluorescence of phagocytosis of the combination of SHC.Ps and LAB (Blue; RAW

264.7 Green; latex beads) Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, #### $p < 0.0001$ compared to a blank group (no sample treated group); * $p < 0.05$, *** $p < 0.001$ compared to control group (LPS 100 ng/ml) 20-22

Fig 4. The combination of SHC.Ps and LAB inhibit productions of TNF-α, IL-6, IL-1β in RAW 264.7 cells. Supernatants were collected, and levels in the culture supernatant were determined by ELISA according to the manufacturer's instructions. Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, #### $p < 0.0001$ compared to a blank group (no sample treated group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$ compared to control group (LPS 100 ng/ml) 24

Fig 7. The immune-enhacing effects of the SHC.Ps presence or absence of LAB though JNK/p38 protein activation (A(A) the protein expression (JNK/p38) on RAW 264.7 cells. (B) The JNK expression in RAW 264.7 cells. Relative amounts of COX-2 expressions were compared to that of the standard protein. (C) The p-38 expression in RAW 264.7 cells.The gel shown is a representative of the results from three separate experiments. Means with same letters are not significantly different at 0.05 sigma level. 26

Fig 8. FACS analysis of hematopoietic cells from the adult zebrafish's kidney. (A) Adult zebrafish were isolated and analyzed by FACS for total subfraction by light-scatter gating. Gated populations are as follows: erythrocytes (red), lymphocyte (Green), and myelomonocytes (Blue). Cell size is represented by forward scatter (FSC), and granularity is represented by side scatter (SSC). Mean percentage of cells in indicated for each gated subpopulation. Data are compared with that for the control group (the second bar) and are

represented as means ± SD of three independent experiments; ns, not significant, *** $p < 0.001$, **** $p < 0.0001$ compared to control group (LPS 100 ng/ml) 28

Fig 9. Representative images of H&E stain colon sections illustrate the grades of epithelial hyperplasia and of goblet cell is increasing. Increasing of goblet cells that display clear mucus droplets. In combination of SHC.Ps and LAB is increasing with Control group (LPS 1mg/ml). Blank; normal colon mucosa with intact epithelium Control; treated 1mg/Kg of LPS, goblet cells 31

Abstracts

Sargassum horneri (*S.horneri*) is brown algae that are invasive into the coast of South Korea, particularly Jeju island from the east coast of China which causes serious economic losses. Current researches on polysaccharides from *S.horneri* (SHC.Ps) have many bioactivities such as anti-inflammation, anti-allergic, immunomodulating activities. On the other hand, Probiotics, in particular, *Lactobacillus Plantarum* (LAB) have shown for immune-enhancing activities. Therefore, in this study, we evaluated the immune-enhancing activities potential of the combination of SHC.Ps and LAB in LPS- stimulated murine macrophages and zebrafish. we investigated the immune-enhancing activity of the combination of SHC.Ps and LAB, using murine macrophages and zebrafish model. In vitro experiment, the phagocytic activities against IgG-opsonized FITC particles were measured using a phagocytosis assay kit.

Also, we identified that the combination of SHC.Ps and LAB was increasing pro-inflammatory cytokines such as IL-6, tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), and immune regulator's nitric oxide (NO) which is stimulated its own phagocytosis or induces innate immunity such as natural killer cells and dendritic cells while immunizing antigens introduced from outside T cells.

The monocytes were known to occur when you are recovering from an acute infection were increased in kidney marrow. We confirmed the combination of SHC.Ps and LAB can be revealed the increase of lymphocytes and myelomonocytes by flow cytometric analysis of cells from adult whole kidney marrow in zebrafish models. Also, we observed that goblet cells involved in the formation of the intestinal mucosa, and immunity of epithelial cells was observed and were increasing by a combination of SHC.Ps and LAB similar to the control group. These results indicate that the combination of SHC.Ps and LAB exerted the enhanced immunostimulatory activity and contained more nutritional components, and it may be applied as an agent for immune-boosting therapy.

1. Introduction

Polysaccharides obtained from marine natural sources are known to affect a variety of biological responses, especially the immune response (Cho et al., 2015). Many polysaccharides have been shown to possess adjuvant potential on specific cellular and humoral immune responses against antigens (Sun, Wang, Xu, & Ni, 2009). It had been suggested that polysaccharide from *Sagarssum horneri* (SHC.Ps) possesses potent antitumor, immunomodulatory, and antiviral activities (Ahmadi, Zorofchian Moghadamtousi, Abubakar, & Zandi, 2015; D.-S. Kim et al., 2018; Shao, Liu, Chen, Fang, & Sun, 2015). SHC.Ps has been shown to have a strong potential to increase both cellular and humoral immune responses with its low toxicity. However, the underlying mechanisms of SHC.Ps in the regulation of immune response need to be investigated. Therefore, polysaccharides were extracted from *S.horneri* and analyzed the molecular structures and biological activities of SHC.Ps.

Probiotics are microbial organisms beneficial to health and have been used in a wide range of human and veterinary intestinal diseases. The mechanisms of action are obscure, but the beneficial effects on the host immune system have also been reported. Previous studies have investigated that the mechanism of action of probiotics includes the improvement of the immunoregulatory effects which is induced cytokine production following contact with macrophage (Cross, Ganner, Teilab, & Fray, 2004; Morita et al., 2002). *Lactobacillus* has been reported to be able to stimulate host immunity, enhance host resistance against infection and peritoneal macrophages.

Immunity plays as a primary line of self-defense mechanism that protects the host from numerous pathogenic infections (viruses and bacteria) in mammals (Netea et al., 2016). Activation of macrophages secretion among these immune responses is an important process considered to be a target immune cell for combating infection and inflammation by inducing several pro-inflammatory agents such as nitric oxide (NO), interferon (IFN), tumor necrosis factor α (TNF-α), interleukin-6 (IL-6). (Li et al., 2017). NO carries out a variety of tasks for the innate immune system such as the killing of virus-infected cells, tumor cells, and parasitic pathogens (Ivec et al., 2007). Therefore, we confirmed that the combination of SHC.Ps and LAB could affect the pro-inflammatory agents for immune-enhancing effects.

In the biological activity analysis, the combination of the SHC.Ps and LAB is a kind of probiotic that is known to have effects on immune function showed that enhance immunity *in vitro* and *in vivo*. Specifically, the immune-enhancing effects of the combination of SHC.Ps and LAB and its phagocytosis, production of inflammatory mediators, including nitric oxide (NO), and pro-inflammatory cytokines which include tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 on macrophages were investigated in RAW264.7 cells. *In vivo* studies, we assessed that immunology research, permitting the separation and identification of specific cell populations from kidney marrow and blood, and immunohistopathology assay in zebrafish. Therefore, the present study was aimed to investigate the immunomodulatory effects of the combination of the SHC.Ps and LAB in *in vitro* and in zebrafish model.

2. Material and methods

2.1. Reagent and chemical

The cell culture reagents were obtained from GIBCO/Invitrogen (Carlsbad, CA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbohydrate-degrading enzyme (celluclast) was donated by Novo Nordisk (Bagsvaerd, Denmark). RAW264.7 macrophages were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco′s Modified Eagles (DMEM), Fetal Bovien Serum (FBS), penicillin and streptomycin were purchased from GIBCO INC. USA. Primary antibodies of COX-2, and iNOS were purchased from Santa Cruz Biotechnology USA. Adult zebrafish were purchased from a commercial fish trader South Korea. ELISA kits (Mouse IL-1beta, Mouse IL-6, TNF-α, and PGE2) were purchased from eBioscience, Inc., USA. Phagocytosis assay kit was purchased from Cayman (Chemical, Ann Arbor, MI, USA). All other chemicals and reagents used in these experiments were of analytical grade.

2.2. Preparation of crude polysaccharides from *S.hornei* (SHC.Ps).

Crude polysachharides of S.horneri were prepared according to the method described by preivous study (Sanjeewa et al., 2018). Briefly, *S.hornei* was collected on the coast of Jeju Island, South Korea. 30 g of *S,horneri* freeze-dried powder was homogenized with 3,000 mL of distilled water and then mixed with 300 µl of Celluclast enzyme (pH adjusted to 4.5 by using HCl). Each reactant was adjusted to be within the optimum pH and temperature range of the respective enzyme and enzymatic reactions were performed for 24 h. Following extraction, the extract was boiled for 10 min at 100 ℃ to inactivate the enzymes. Then, samples were clarified by centrifugation (3000 rpm, for 20 min at 4 °C) to remove the residue. These extracts were adjusted to pH 7.0 hereafter and designated to as enzymatic extract. protein free enzymatic digest was mixed with 3 times of 95 % ethonol solution and then stored at 4℃ for 24 h to precipitate polysaccarides and then precipiates were collected by centrifugation at 10,000 × *g* for 20 min at 4 °C. Then, extraction the sample was kept in -20℃

and freeze dried for further experiments.

2.3. Characterization of crude polysaccharides from *S. horneri* celluclast-enzyme extract

2.3.1. Monosaccharide composition analysis of crude polysaccharides

To analyze monosaccharides composition of the crude polysaccharides, SHC.Ps were hydrolyzed with Hydrochloric aicd for 4h at 100 ℃. Then the monosaccharide contents of the samples were determined by LC (Bio-LC system, Dionex, USA) coupled with high-performance anion exchange chromatography (HPAE-PAD) using a CarboPacTM PA1 anion-exchange column (4.5 mm × 50 mm). The monosaccharides were detected under optimal isolation conditions (eluent: 18 mM NaOH / 200 mM NaOH; flow rate: 1 mL/min; injection volume: 20 μL) at the Carbohydrate Bioproduct Research Center in Seoul, South Korea.

2.3.2. FT-IR characterization of crude polysaccharides

To investigate and compare with fucoidan (sigma aldrich) and SHC.Ps, IR spectrum of the separated crude polysacharides were recorded using a FT-IR spectrometer (Nicolet 6700, FT-IR spectrometer). SHC.Ps were homogenized with KBr powderd and then press into pellets. The FT-IR spectrum of each pellet measured in the frequenct range of 500-4000 cm-1.

2.3.3. Analysis of polysaccharides, polyphenols and sulfate contents of crude polysaccharides from S. horneri celluclast-enzyme extract

The total polysaccharides and phenolic contents of SHC.Ps were measured according to the procedures in AOAC Official Methods for Analysis [10]. The sulfate contents of SHC.Ps was determined by the BaCl2 gelatin method [11].

2.3.4. Acid hydrolysis for depolymerization of SHC.Ps

Mild acid hydrolysis of SHC.Ps was performed ws carried out using 0.01N hydrogen chloride

(HCl) for 16~18h overnight. Then neutralization of solution using 0.01M sodium hydroxide followed by solid-phase extract was enriched and remove salt content. After that depolymerized SHC.Ps was injected into MALDI-TOF mass spectrometry.

2.3.5. Characterization of molecular weight of SHC.Ps by Size-exclusion chromatography Multi Angle Light Scattering (SEC-MALS)

The molecular weight of the SHC.Ps was determined by size-exclusion chromatography-multiangle static light scattering (SEC-MALS). The SHC.Ps was separated by PL aquagel OH-MIXED-H column (Agilent technologies). The molecular weight was measured with a Dawn HELEOS II multiangle laser light scattering photometer (Wyatt, Santa Barbara, CA, USA) and a subsequent Optilab T-rEX differential refractometer. The mobile phase was 0.10 mol/l Na2HPO4 (pH 7) and the flow rate was 0.5 ml/min. The injection volume was 100 μl. The data were obtained and processed using Astra (v. 6.1) software (Wyatt, Santa Barbara, CA, USA).

2.3.6. Mass spectrometric analysis of SHC.Ps

MALDI-TOS MS analysis was performed on pretreated SHC.Ps in negative ion reflectron mode. The SHC.Ps reaction products were dissolved in water, and 1 μl of the solubilized products was spotted onto a stainless-steel target plate, followed by addition of 0.8 μl of 50 mg/ml 25-dihydroxybenzoic acid in 50% (w/w) acetoniltrile. The spot was rapidly dried in vaccum for homogenous crystallization. Matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) was performed on a Bruker ultrafleXetreme system Bruker Daltonics, Portland, OR). The mass spectra were externally calibrated using commercial fucoidan. A ladder of fucose polymers, spaced at 1 fucose unit (146.058 Da) apart, provided comprehensive coverage of the entire mass acquisition range, enabling accurate mass calibration of the MALDI-TOF/TOF instrument immediately before the sample analysis. Raw MS data were processed using the FlexAnalysis software (version 3.3; Bruker daltonics). MS peaks were filtered with a signal-to-noise ratio of 3.0 and manually inspected to detect common adducts. All peaks were then deconvoluted, and a list of all neutral

masses in the samples was generated with the abundance values reperesented by MS peak intensities.

2.4. Immunomodulatory activity of SHC.Ps in in vitro and in vivo

2.4.1. Preparation of probiotics

Lactobacillus plantarum (*L.plantarum*) was purchased from the Korean Collection for Type Cultures (KCTC, Seoul, Korea) and cultivated in a De Man, Rogosa and sharpe agar (MRS) medium at 37℃ for 48 h. *L.plantarum* cells were collected into pellets by centrifugation at 1000 g for 30 min. Pellets were washed in pH 7.4 of PBS (phosphate buffered saline) three times and re-suspended at a final concentration of 1×10^5 cells/ml.

2.4.2. Cell culture

The murine macrophage cell line RAW 264.7 were cultured in DMEM supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10 % FBS (fetal bovine serum). The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and cultured cells from passage 3-6 were used for the experiments.

2.4.3. Cytotoxicity and NO production by SHC.Ps induced in RAW 264.7 macrophages.

The cytotoxicity if the SHC.Ps to RAW 264.7 cells was evaluated by MTT assay (Bounous, Campagnoli, & Brown, 1992). Briefly, the cells (2 × 105 cells/ml) were seeds in a 48 well plate and incubated for 24 h. Then the cells were treated with various concentrations of samples (25, 50, 100 μg/ml) or LPS (100 ng/ml). Another 24 h, MTT reagent (2 mg/ml) was added to each well. After 2 h of incubation, the formazan crystals were dissolved in DMSO, and the amount of purple formazan was determined by measuring the absorbance at 540 nm. The optical density of the formazan generated in non-treated control cells was considered to represent 100% viability. The data are expressed as mean percentages of the viable cells versus the respective blank. To estimation the effect of SHC.Ps on NO production in Raw 264.7 cells, we carried out Griess assay described previous study (Leiro, Alvarez, Garcıa, &

Orallo, 2002). After pre-incubation of RAW 264.7 cells (2×10^5 cells/ml) without samples (blank), with samples (LPS 100 ng/ml, 25, 50, 100 μg/ml) at 37 ℃ for 24 h, the quantity of nitrite accumulated in the culture medium was measured as an indicator of NO production. In short, 50 μl of cell culture medium was mixed with 50 μl of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader (BioTek Instruments, Inc., Winooski, USA).

2.4.4. Phagocytosis assay.

A commercially available kit was used as per the manufacturer's protocol to determine the phagocytosis activity of macrophages. Briefly, 200 μl suspension (1×10^5) of cells were seeded. Then 24 h incubation, cells were treated with various concentration (0, 25, 50, 100 μg/ml) and LPS 100 ng/ml. After 24 h of treated samples on cells, add the latex bead-rabbit lgG-FITC complex directly to each well to a final dilution of 1:1000 or no beads(shaded) and incubated at 37 ℃ for 2 h. Cells were gently washed with assay buffer twice, followed by counterstaining with Hoechst 33342 for 10 min at 37 ℃. After two washes, cells were visualized at 20x magnification with a microscope for observing the phagocytosis. And performed a flow cytometer for flow cytometric analysis. The effect of SHC.Ps on macrophages was assessed by histograms generated by the computer program of Cell Quest and Mod-Fit (New York, USA.), and determined changes in the percentage of FITC fluorescence were gated and shown.

2.4.5. Fish maintenance and diet preparation.

Wild-type zebrafish (Danio rerio) were kept 10 fish were kept in one tank under the following condition (28.5 ± 1 ℃, fed two times a day with a 14/10 h light/dark cycle). The sample diets (SHC.Ps 5 %, *L.plantarum* 1 %, SHC.Ps 5 % + *L.plantarum* 1 %) were mixed and freeze-dried with commercial feed supplement. In the blank, control groups diets, an equivalent volume of sterile PBS was added to the commercial feed. Zebrafish were quantitatively fed according to their number in each treatment group. After 14-day sample treatment, fish were

intraperitoneal injected with LPS (1 mg/kg) and blanks were injected with PBS. After 48 h, post-injection fish were used for further experiments.

2.4.6. Flow cytometric analysis on immune-enhancing effects of SHC.Ps in zebrafish.

To determine differentially expressed immune cells of zebrafish in *in vivo*. Kidneys and blood were harvested from post-injected fish. Harvested kidneys and blood were minced in FACS buffer (PBS+2 % FBS) with heparin. The tissue was gently filtered through 40 μm filter mesh and cells were scanned using high-end performance cytometer (Becton–Dickinson, Germany) and scans were analyzed by the computer program of Cell Quest and Mod-Fit (New York, USA.) the exact numbers of the different immune cells (lymphocytes, monocytes) populations in the harvested tissues were calculated using the percentage of each cell type and the total cell numbers estimated for each tissue.

2.4.7. Histological analyses on immune-enhancing effects of SHC.Ps in zebrafish intestine.

Post-injection fish were sampled, and their organs fixed in 4 % formalin and after dehydration in alcohols with increasing concentrations, samples of the intestinal were embedded in paraffin and sliced (5 μm) using a microtome and stained with hematoxylin and eosin. Histological preparations were evaluated using a microscope (LIONHEART FX automated live-cell imager) and measuring the images (20× magnification) 5 times in different places using Gen5 3.08 software.

2.4.8. ELISA.

Macrophage cells were cultured (2×10^5 cells/ml) in 6-well plates and cells were treated with various concentrations of SHC.Ps for 24 h after cell seeding. For another 24 h, cell-free culture supernatants were collected and analyzed for PGE2, IL-1β, IFN-γ, and IL-6 using ELISA kits (R&D System Inc., Minneapolis, MN, USA). These experiments were conducted using commercially available ELISA kits following the manufacturer's instructions.

2.4.9. Western blot.

Pretreated and stimulated macrophage cells were harvested using a lysis buffer. After analyzing the lysed protein concentration, standardization was done by using a concentration gradient of BSA. Cytoplasmic proteins (20 μg) were separated on the 10 % polyacrylamide gel and protein bands were transferred onto nitrocellulose membranes and blocked with 5 % skim milk in TBST for 2 h. Blocked membranes were incubated overnight with relevant primary antibodies (both phosphorylated and totaled p-38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p65, p50 and β-actin) at 4℃. Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature. A FUSION SOLO Vilber Lourmat system imager with a chemiluminescent substrate was used for the visualization, and relative protein intensity was analyzed using Image J software.

2.4.10. Statistical analysis.

The data analysis was performed using GraphPad Prism 7 (GraphPad Prism Software, San Diego, CA, USA) and evaluated using two-way ANOVA and Dunnett's multiple range tests. All data were performed a minimum of three times and expressed as Mean ± SD (standard deviation). ns; not significant, Significant differences were represented at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.001$ compared to untreated control group (blank; untreated control group).

2. Results

3.1. Extraction of *S.horneri* and General and monosaccharides composition of SHC.Ps

We have previously reported, digestion of S. horneri with Celluclast enzyme for 24 h (pH 4.5 and 50 °C) is the best enzymatic digestion method to separate bioactive crude polysaccharides from S. horneri. Following the same method, we extracted CP from S. horneri. S. horneri crude extracts (SHC) contained 25.53 ± 3.87 % polysaccharides and 3.53 ± 0.23 % polyphenol and 10.31 ± 1.38 % of sulfate. S.horneri crude polysaccharides (SHC.Ps) contained 36.73 ± 1.42 % of polysaccharides, 2.01 ± 0.12 % of polyphenol, and 17.83 ± 2.75 % of sulfate. In this study we noted that, SHC.Ps contains a high percentage of polysaccharides (Fig. 1A). According to the results, SHCF2 had 66.08 % fucose, 23.03 % of galactose, and 0.29 % of rhamnose.

(A)

Proximate contents (%)			
Sample	Polysaccharide	Polyphenol	Sulfate
SHC	25.53 ± 3.87	3.53 ± 0.23	10.31±1.38
SHC.Ps	**36.73 ± 1.42**	2.01 ± 0.12	**17.83±2.75**

(B)

No.	Monosugar	Monosaccharide compositions (%)
1	Fucose	**66.08**
2	Rhamnose	0.29
3	Galactose	23.03

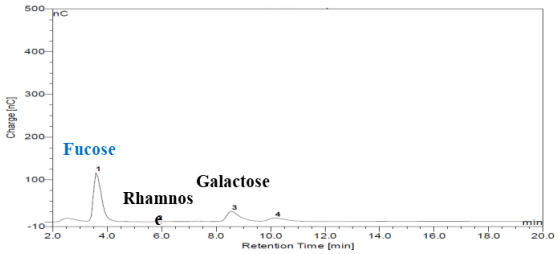


Fig 1. (A) Polysaccharide, Polyphenol compositions and sulfate contents of SHC and SHC.Ps were estimated. (B) Monosaccharide content of SHCF2 was analyzed using an HPAE-PAD spectrum compared with a standard monosaccharide mixture.

3.2. FT-IR characterization of SHC.Ps

The Fourier-transform (FT-IR) spectrum of SHCF (Fig. 2), contained specific absorbance at 1035, 1240, 2950, and 3000 cm−1 which are marker peaks of natural fucoidan. Other than that, FT-IR spectrums of crude and purified polysaccharide with commercial fucoidan, we observed that major absorbance at 3000 cm−1 only present in crude polysaccharide and commercial fucoidans. The FT-IR results strongly support that SHCF2 has more similarities to the commercial fucoidan.

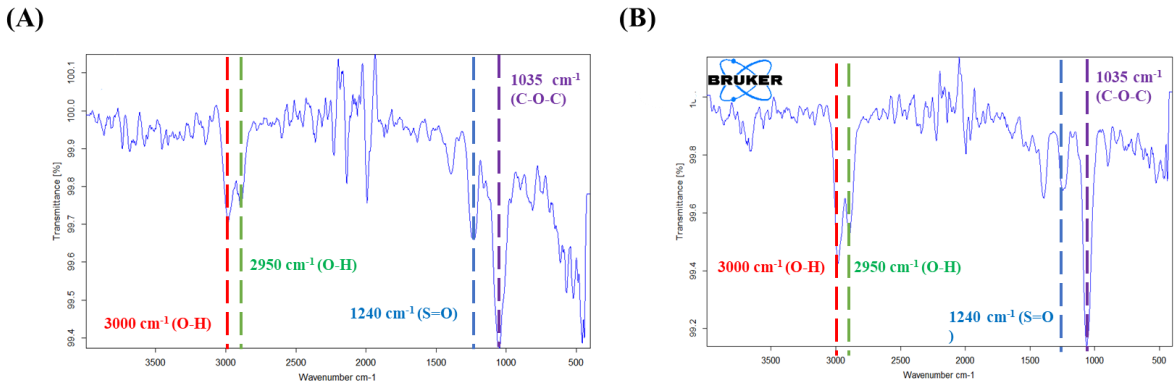


Fig 2. Infrared analysis spectroscopy (FT-IR) of commercial fucoidan. (A) Crude polysaccharide (B) obtained from *S. horneri via* Celluclast enzymatic extraction.

3.3. Determination of absolute molecular weight and mass spectrometric analysis of SHC.Ps

SEC-MALS analysis of the SHC.Ps revealed a very high molecular weight average of 813.3 (±6.4) kDa, and molecular number average of 673.8 (±8.3) kDa. The molar mass was broadly distributed between 250 kDa and 2.5 MDa.

Figure 3A shows the MALDI-TOF mass spectrum of the acidic-hydrolized samples. There are one major oligomeric series and four minor oligomeric series. The oligomeric series were observed with signal separation of 146 Da, corresponding to the fucose residue. The following series of peaks were identified: series red circles with m/z 243.03, 389.09, 535.16, 681.22, 827.29, 973.35, 1119.41, 1265.48 and 1411.55; series check red circles with m/z 405.09, 551.16; series purple circles with m/z 567.15, 713.21, 859.28, 1005.34, 1151.40 and 1297.48; series yellow circles with m/z 521/12. 667.21, 813.28, 959.34, 1105.41, 1251.47 and 1397.54 and series green circles with m/z 507.04, 653.14, 799.20, 945.29, 1091.39, 1237.45 and 1383.52. All major signals correspond to a deprotonated parent ion ($[M-H]^-$). Each series is in the form of continuous fucose in common, showing difference in the parent body. Series red circle is the major form in which fucose is continuously linked to sulfate fucose. Other series are sulfate fucose linked galactose and xylose continuously linked to fucose.

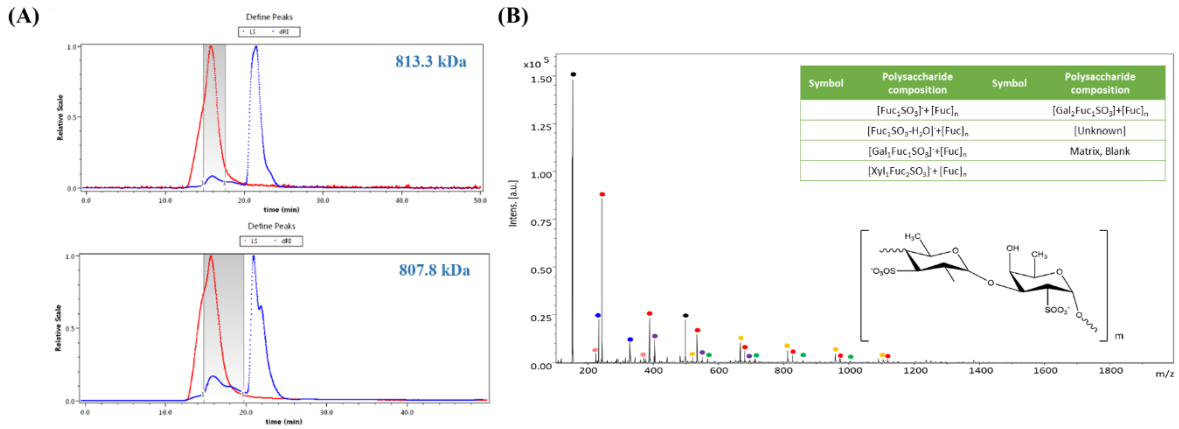


Fig 3. (A) Size exclusion chromatography of crude polysaccharide obtained from *S. horneri via* Celluclast enzymatic extraction was conducted using SEC-MALS instruments. (B) Mass spectrometry analysis of crude polysaccharide obtained from *S. horneri via* Celluclast enzymatic extraction with MALDI-TOS mass spectrometer after mild acid hydrolysis.

3.4. Effects of SHC.Ps and LAB on cell viability and NO production

The macrophages are well known for playing a vital role in the innate and adaptive immune response. The NO production and cell viability of a combination of SHC.Ps and LAB on RAW264.7 cells were evaluated by the Griess assay and MTT colorimetric assay. As shown in Fig 1, after being treated with increasing SHC.Ps present or absent of LAB concentration (25,50,100 μg/ml), the cell viability indicated that the samples exhibited a non-toxic effect on Raw 264.7 cells. Also, the effect of the combination of SHC.Ps and LAB on NO production, compared with the blank (non-treated) group, the NO production was significantly increasing in SHC.Ps (25, 50, 100 μg/ml) and combination (SHC.Ps presence of LAB) group. Compared to the control group (LPS 100 ng/ml), the NO production rate of the highest combination group increased similarly to the LPS treated group. The results indicated that the combination of SHC.Ps and LAB could mediate the up regulation of intracelluar NO production.

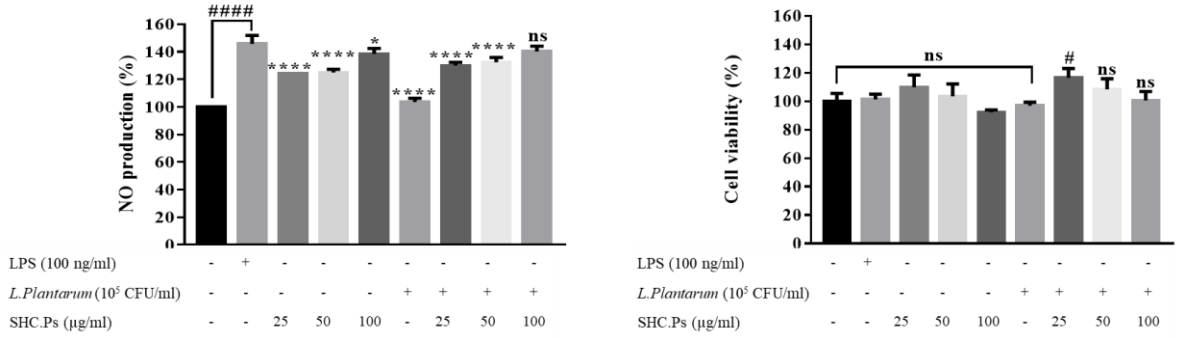
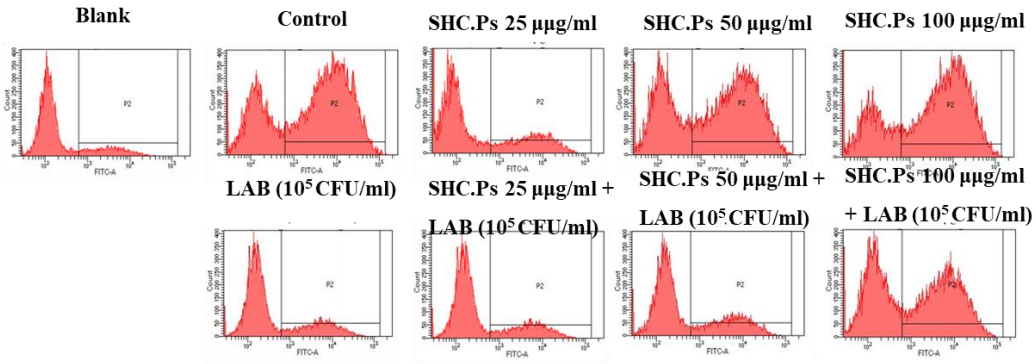


Fig 4. NO production (A) and Cytotoxicity (B) of the SHC.Ps present or absent of LAB on RAW 264.7 cells. The viability of cells without samples and LPS has taken as reference and the level of NO production is expressed as a percentage of that of the group treated with LPS alone. Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, #### $p < 0.0001$ compared to blank group (no sample treated group); * $p < 0.05$, *** $p < 0.001$ compared to control group (LPS 100 ng/ml)

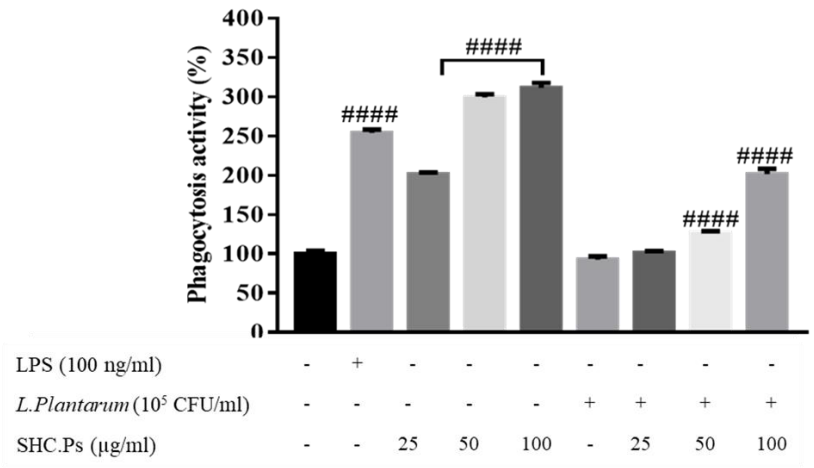
3.5. Effects of SHC.Ps on for immune-enhancing activity using phagocytic analysis.

To explore, whether the combination of SHC.Ps and LAB could directly activate macrophages in vitro, we primarily examined macrophages phagocytosis using the Cayman's Phagocytosis Assay Kit (IgG FITC). As shown in Fig 5, the cells uptake intensity of the combination of SHC.Ps and LAB-treated groups was found enhanced in a dosage-dependent manner. The results indicated that the combination of SHC.Ps and LAB can significantly enhance the pinocytic and phagocytic function of macrophages, thus can scavenge foreign invasion and enhance the host immune function.

(A)



(B)



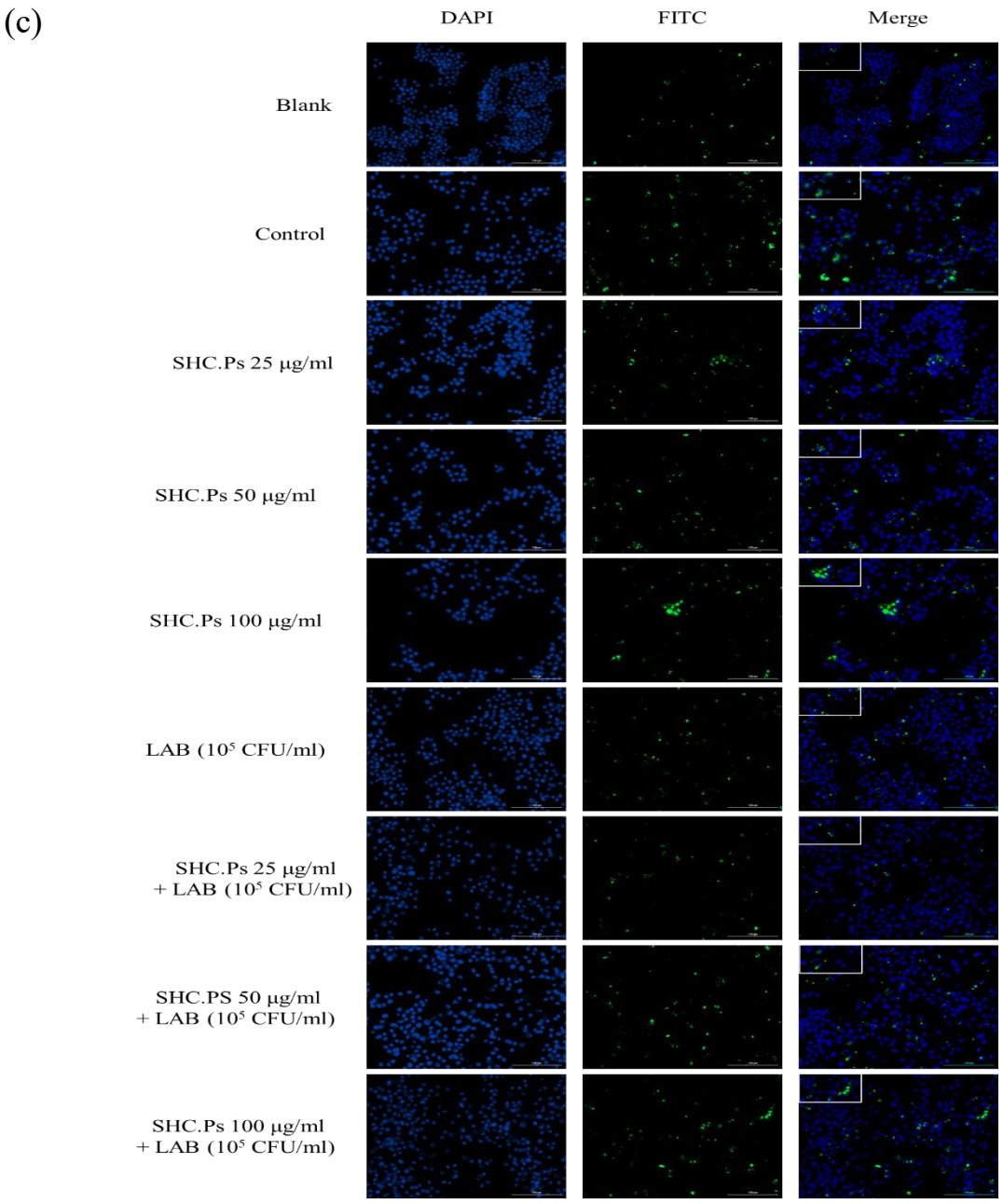


Fig 5. Phagocytosis assay Hoechst Dye-stained murine macrophage-like RAW264.7 cells. RAW 264.7 cells were treated with SHC.Ps presence or absence of LAB in different doses, non-treated group, and control (100 ng/ml of LPS). (A, B) FACS (A) and analysis pf phagocytosis of latex beads by RAW264.7 (B) treated with different agents for 2 h (C) The immunofluorescence of phagocytosis of the combination of SHC.Ps and LAB (Blue; RAW 264.7 Green; latex beads) Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant,

$p < 0.0001$ compared to blank group (no sample treated group); * $p < 0.05$, *** $p < 0.001$ compared to control group (LPS 100 ng/ml)

3.6. Effect of the combination of SHC.Ps and LAB on immune-enhancing activity of pro-inflammatory cytokines production

It is a well-established fact that the pro-inflammatory cytokines, including TNF-α, IL-6, IL-1β has an important role in the immune stimulated in the body. To examine the potential immune boosting effects of the combination of SHC.Ps and LAB on RAW 264.7 cells, the levels of TNF-α is decreased, and not significantly increased in the SHC.Ps group. However, IL-6, IL-1β was significantly increased in SHC.Ps present or absent groups similarly with control group. In immunity, TNF-α and IL-6 is negative feedback each other, it might be affected in level of TNF-α. It secretes pro-inflammatory cytokines such as IL-6 to increase its own phagocytosis or induces innate immunity such as natural killer cells and dendritic cells, while immunizing antigens introduced from outside T cells. It is known to contribute to the activity of adaptive immunity by transmitting to the cells to regulate the activation and differentiation of cells. In addition, NK cells can directly kill cancer cells by releasing cytotoxic granules, and they can simultaneously activate macrophages and T cells by secreting cytokines such as interferon-γ (IFN-γ). Therefore, the combination of SHC.Ps and LAB could have immune-enhancing activity.

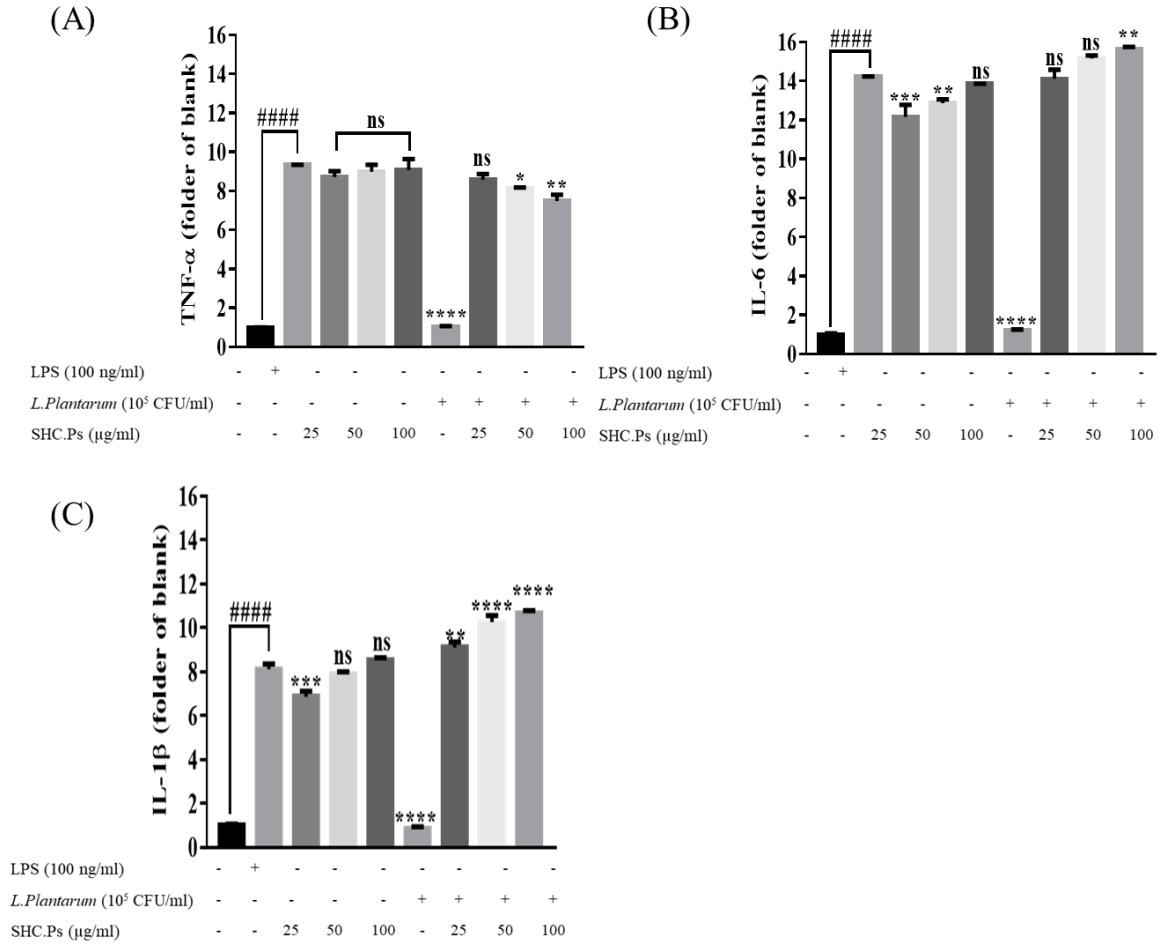


Fig 6. The combination of SHC.Ps and LAB inhibit productions of TNF-α, IL-6, IL-1β in RAW 264.7 cells. Supernatants were collected, and levels in the culture supernatant were determined by ELISA according to the manufacturer's instructions. Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, #### $p < 0.0001$ compared to a blank group (no sample treated group); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ **** $p < 0.0001$ compared to control group (LPS 100 ng/ml)

3.7. Effect of the combination of SHC.Ps and LAB on immune-enhancing activity in though the JNK/p 38 pathway

The Cells (1×10^5 cells/ml) were treated with 25–100 μg/ml treatment for 24 h. Each extract protein level was evaluated using BCA protein assay kit and was standardized (Bio-Rad, USA). Sodium dodecyl sulfate-polyacrylamide gels (10%) were used for the electrophoresis and then, transferred onto nitrocellulose membranes. The membranes were blocked with skim milk and were incubated overnight with relevant primary antibodies in 5% skim milk. Following the HRP-conjugated secondary antibodies were added to the membranes and were incubated. Then, signals were developed using a chemiluminescent substrate (Cyanagen Srl, Bologna, Italy), and membranes were photographed using a FUSION SOLO Vilber Lourmat system. The band intensities were quantified using the ImageJ program Using western blot analysis, we investigated the effect of the combination of SHC.Ps and LAB on JNK/p 38 activities on RAW 264.7 cells. As shown in Fig 7, the results demonstrated that the control (LPS 100 ng/ml) was increasing in JNK, p-38 compared to non-treated group. Also, the combination of SHC.Ps and LAB group was increased the expression of protein level. Combination of ths SHC.Ps and LAB downregulated the phosphorylation of p38 and JNK which related to cell proliferation. From these results, we suggest that Combination of the SHC.Ps and LAB has immune-enhancing effect via p38/JNK signaling.

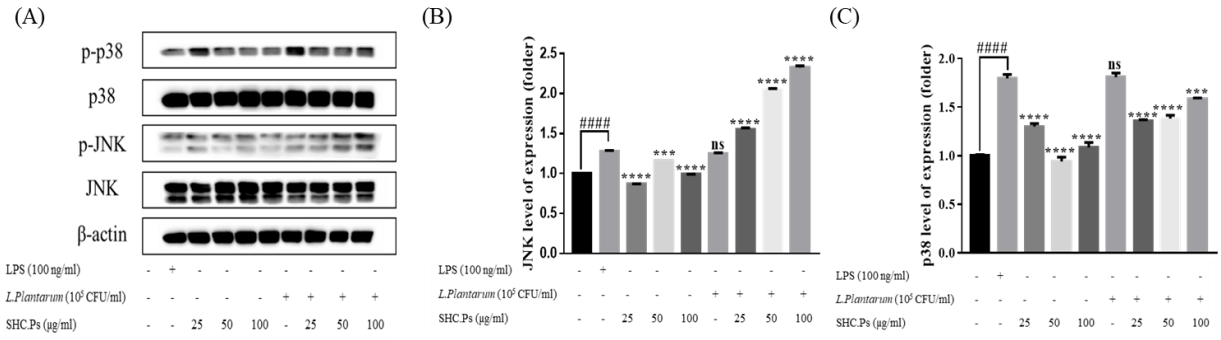


Fig 7. The immune-enhancing effects of the SHC.Ps presence or absence of LAB though JNK /p38 protein activation. (A) the protein expression (JNK/p38) on RAW 264.7 cells. (B) The JNK expression in RAW 264.7 cells. Relative amounts of JNK expressions were compared to that of the standard protein. (C) The p-38 expression in RAW 264.7 cells. The gel shown is a representative of the results from three separate experiments. Means with same letters are not significantly different at 0.05 sigma level.

3.8. Effect of SHC.Ps on immune-enhancing activity in zebrafish kidney using FACS analysis.

The FACS analysis of hematopoietic cells from the adult zebrafish's kidney. Adult zebrafish were isolated and analyzed by FACS for total subfraction by light-scatter gating. Gated populations are as follows: erythrocytes (red), lymphocyte (Green), and myelomonocytes (Blue). Cell size is represented by forward scatter (FSC), and granularity is represented by side scatter (SSC). In control group, both lymphocyte and myelomonocytes was increasing, and sample treated groups (SHC.Ps, LAB, SHC.Ps+LAB) also were increased. The combination of SHC.Ps and LAB group was highest increased among the group. In previous study, mature lymphocytes preferentially home to spleen, kidney, and gut, and we speculate that they may interact in these organs following an immune stimulus. kidney marrow cDNA libraries contain transcript of mature myeloid marker, also participating in immune reaction in secondary lymphoid organs in mammals are found in kidney, spleen, and gut of zebrafish, making these the major secondary lymphoid organs. Therefore, we evaluated the hematopoietic cells from kidney, core secondary lymphoid organ, lymphocyte and myelomonocytes was increasing in the sample treated groups, in particular. the combination of SHC.Ps and LAB groups was increased significantly than non-treated group.

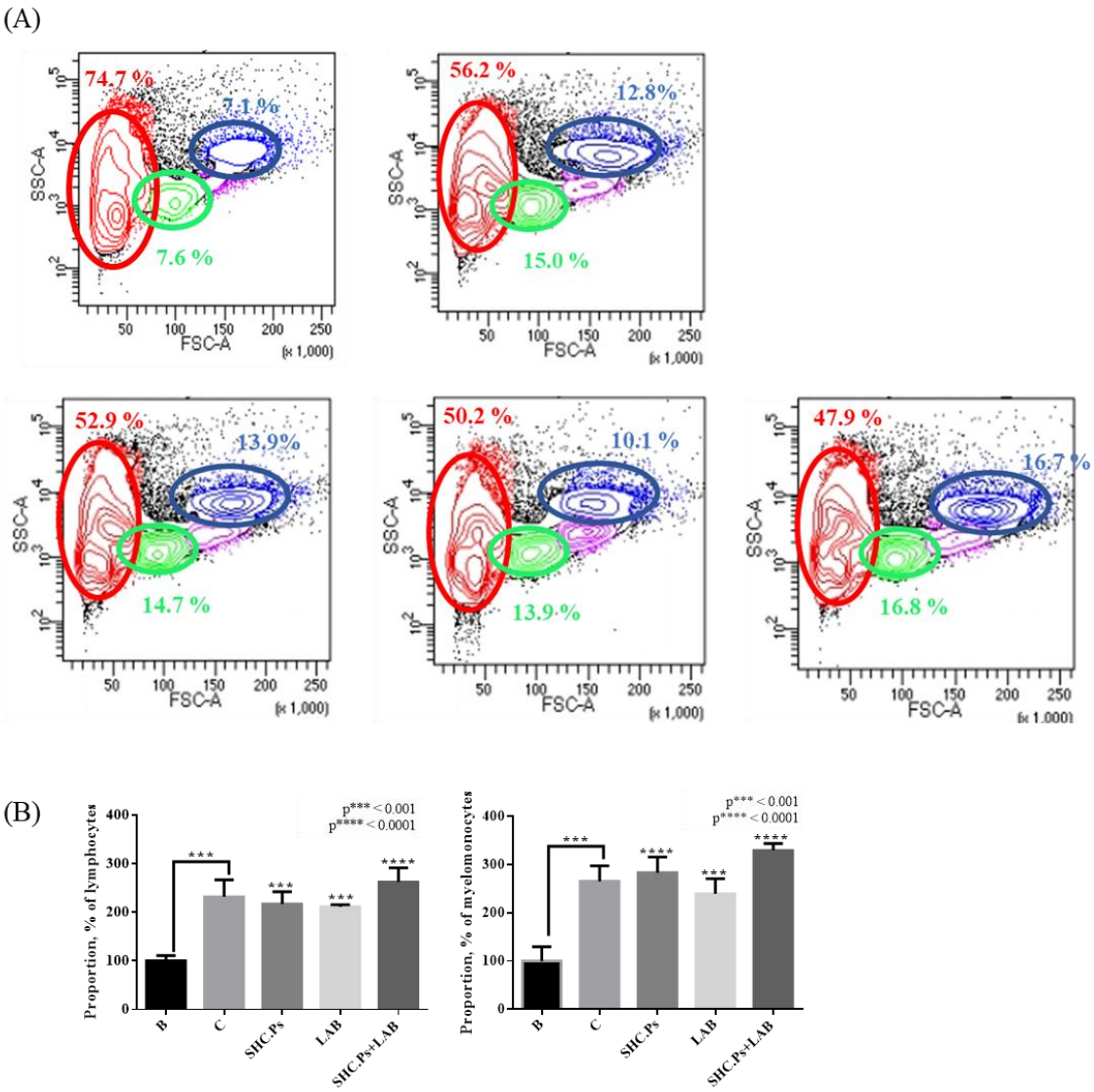


Fig 8. FACS analysis of hematopoietic cells from the adult zebrafish's kidney. (A) Adult zebrafish were isolated and analyzed by FACS for total subfraction by light-scatter gating. Gated populations are as follows: erythrocytes (red), lymphocyte (Green), and myelomonocytes (Blue). Cell size is represented by forward scatter (FSC), and granularity is represented by side scatter (SSC). Mean percentage of cells in indicated for each gated

subpopulation. Data are compared with that for the control group (the second bar) and are represented as means ± SD of three independent experiments; ns, not significant, *** $p < 0.001$, **** $p < 0.0001$ compared to control group (LPS 100 ng/ml)

3.9. Effect of SHC.Ps on immune-enhancing activity in zebrafish intestinal using the histological analysis.

An activated immune response further increases mucus secretion from goblet cells in intestinal. These results signify combination group of SHC.Ps and LAB have goblet cell, mucus secretion. The control group was increased in goblet cells compared to the blank group, and the sample group (Only SHC.Ps and LAB) also confirmed an increase in goblet cells. And the combination group was similar increased in control group. Thus, the results showed that the SHC.PS affected intestinal immunity, and that the increase in the immune cell goblet cells confirmed the possibility of the resources combination of SHC.Ps and LAB as immune boosters.

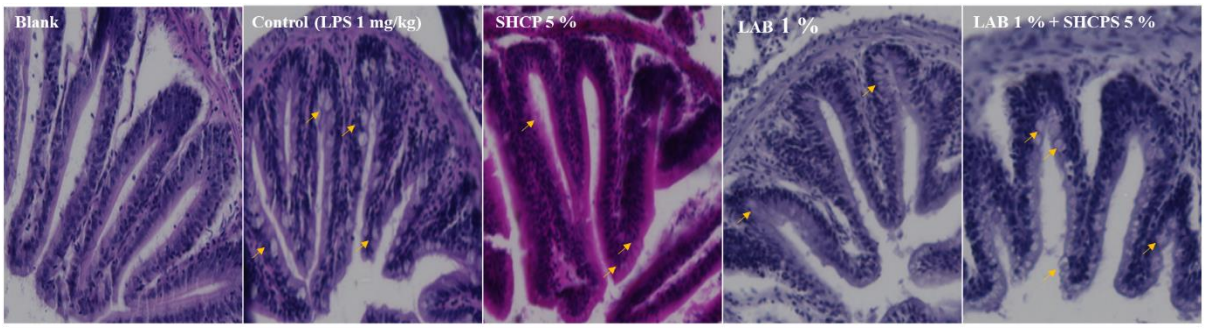


Fig 9. To observe the intestinal histopathological recovery, 10 fish were randomly chosen and fixing in 4 % formalin, and dehydrated using a series of graded ethanol, vitrified by dimethyl benzene, then these fish were embedded in paraffin wax, sectioned at 5 um thickness, and then stained with hematoxylin and eosin (H&E) staining colon sections for illustrate the grades of epithelial hyperplasia and of goblet cell is increasing. Increasing of goblet cells that display clear mucus droplets. The sample treated groups were increasing goblet cells. In particular, combination of SHC.Ps and LAB is increasing with Control group (LPS 1 mg/kg). Blank; normal colon mucosa with intact epithelium Control; treated 1 mg/kg of LPS, goblet cells.

4. Discussion

In the present study, crude polysaccharides from Sargassum horneri was extracted, and characterized by FT-IR, HPLC, SEC-MALS ans mass spectromeer. SHC.Ps is contained major oligomeric series of $[fuc_1SO_3]+[fuc]_n$ residues with Mw of 810.55 kDa. Moreover, minor series of SHC.Ps was demonstrated Galactose+sulfated fucose, xylose+sulfated fucose. All oligomeric series of SHC.Ps include fucose which is known that high immunomodulatory activity by improving immune cells.

Natural sources have utilized widely the inspiration for medicinal ingredients, and to this day, the advantage of natural compounds such as chemical diversity, and evolutionary pressure still has a tremendous influence in the pharmaceutical and nutraceutical industries (Harvey, 2007). As reported polysaccharides isolated from brown algae have various bioactivities (Jiao, Yu, Zhang, & Ewart, 2011) and several studies indicate that the immune-stimulating activity of polysaccharides occurred by stimulating NO production in macrophages through the activation of p 38 MAPK and NFκB pathways (Wang, Pan, Mehmood, Cheng, & Chen, 2018; Zhang et al., 2013). On the other hand, *L.plantaram* (LAB), a kind of lactic acid bacteria, is widely used in the food and dairy industry and has an immunomodulatory function (Saadat, Khosroushahi, & Gargari, 2019). Therefore, it is important to study the immuno-enhancing effect of polysaccharides from natural products. Moreover, there is no study about the combination of the immune-enhancing potential of polysaccharides separated from *S.horneri* via enzymatic digestion, and LAB has not yet been reported. This is the first study to demonstrate that the immunostimulatory activities of the combination of SHC.Ps and LAB, including its macrophage-activating and immune function-enhancing properties in murine macrophages and zebrafish model.

Macrophages occurred as a constant and regularly distributed cell population in almost all the tissues and regulate both innate and adaptive immunity (Navegantes et al., 2017). They are derived from peripheral blood monocytes and are involved in the chances of other immune cells via phagocytic activity and destruction of bacteria and other harmful organisms (Kubica et al., 2008). Activated macrophages generate great amounts of reactive nitric oxide (NO) which contribute to the killing of bacteria or virus-infected cells and tumor cells (Bogdan, 2015). Also, it regulates the production of cytokines and T lymphocytes in macrophages (Saito

& Nakano, 1996). Activated macrophages are associated with cytokines such as interferon-γ, IL-1β, IL-6, IL-10, and TNF-α to recruit and activate other cells for the initiation of the adaptive immune responses (Giacomini et al., 2001). To identify the most activated macrophages, we evaluated NO release and cytokines such as IL-1β, IL-6, and TNF-α. Among the investigated samples (Fig. 4), the combination of SHC.Ps and LAB induced the greatest NO production similar to the control (LPS 100 ng/ml) group (Fig. 4). also, the combination of SHC.Ps and LAB has been shown to significantly induce IL-6, IL-10, and TNF-α production in RAW 264.7 cells (Fig. 6)

Phagocytic activity by macrophages is the first immune response and also, a vital symbol of non-specific immune responses against invasion (Sharma et al., 2014). The phagocytic activity was monitored by detecting internalized IgG-opsonized FITC particles in macrophages and higher fluorescence emission intensity indicates increased phagocytosis activity using FACS. The combination of SHC.Ps and LAB enhanced the phagocytic activity of RAW 264.7 cells (Fig. 2) The present study suggests that the combination of SHC.Ps and LAB could enhance non-specific immune function in macrophages (Fig. 5).

Previous studies demonstrated that ERK and p38 MAPK contribute to plays a key role in macrophage activation and phagocytosis (Shiratsuchi & Basson, 2005). Activated ERK and p38 MAPK have linked to actin polymerization which is necessary for bacteria internalization by the phagocytic activity of macrophages (Xiao et al., 2002). In addition, it is reported that MAPK activation induces IL-10 production in murine-activated macrophages (Chanteux, Guisset, Pilette, & Sibille, 2007), and MAPK and of NO and TNF-α that regulate the innate immune response (Campbell et al., 2004). Also activated ERK, JNK, NF-κB singling mediates effects of enhancing phagocytic activity (Bi et al., 2017). The results of this *in vitro* experiment demonstrated that a combination of SHC.Ps and LAB may increase macrophage phagocytic activity through activation of the JNK/p38 pathway (Fig. 7). Moreover, activating the cytokines that regulate immune response (Fig. 8).

The zebrafish model has been developed as a model to study vertebrate's developments because of the ability to see development in *in vivo(Henry, Loynes, Whyte, & Renshaw, 2013)*. The developmental biology community has innovated a series of tools for controlling and visualizing physiological processes in *in vivo*, which has led to the zebrafish model being

widely utilized throughout the world (Renshaw, Trede, & mechanisms, 2012). Zebrafish models are not only cost-effective and have fast-growing, but also have important similarities to mammalian immune systems in terms of immunity (Rombout, Yang, Kiron, & Immunology, 2014). At the molecular level, zebrafish immunity has retained components, such as TLRs, complement proteins, pro-inflammatory cytokines, and acute-phase response proteins. Particularly, larval zebrafish allow insight into myeloid cell function, independently of adaptive responses, which develop later (Davis et al., 2002). In this situation, establishing the zebrafish as a vertebrate genetic model provides an attractive alternative and complementary tool to the use of mice and further out understanding of the study of genetic modifiers of the immune response to infections (Casanova, Abel, & Quintana-Murci, 2011).

In addition, a previous study speculated that mature lymphocytes preferentially home to the spleen, kidney, and gut, and also, they may interact in these organs following an immune stimulus (Trede, Langenau, Traver, Look, & Zon, 2004). Kidney marrow cDNA libraries contain a transcript of the mature myeloid marker, also participating in immune reaction in secondary lymphoid organs in mammals are found in kidney, spleen, and gut of zebrafish, making these the major secondary lymphoid organs (Trede et al., 2004). In this study, we confirmed the composition of immune cells in hematopoietic cells in the kidneys (Fig. 8) and the results was shown that the cell count of the both lymphocytes, and myelomonocytes were increasing in the combined group (SHC.Ps and LAB). Fish mainly depend on innate immune responses to environmental insults, and these appear to be more potent than in higher vertebrates (Whyte & immunology, 2007). Also, the number of goblet cells that play important roles in host defense against physical and chemical injury caused by endogenous and exogenous irritants, or microbial attachment, and invasion is an important parameter for evaluating innate immunity in the fish gut (J. J. Kim & Khan, 2013). The present study observed goblet cells involved in intestinal mucosal formation and immunity of epithelial cells in zebrafish animal models in a combination group (SHC.Ps and LAB treated). The results showed that the combination group was increasing the goblet cells parallel to the control group (LPS 1mg/ml treated). (Fig.9) These results indicated that the combination of SHC.Ps and LAB increasing the enhancing of the innate immune response in zebrafish model.

5. Conclusion

In our study demonstrated for the first time that combinations of the polysaccharides from *S.horneri* and *L.plantarum* have strong active components and had immune enhancement effects by enhancing the phagocytic activity and stimulating NO production in macrophages. The combination of SHC.Ps and LAB was activated immune systems through the P38/JNK protein activation. In the zebrafish model, activated immune response further increased mucus secretion from goblet cells in the intestinal. These findings provided the basis for the development of the combination of SHC.Ps and LAB have the potential to be an immune-boosting therapeutic agent.

References

Ahmadi, A., Zorofchian Moghadamtousi, S., Abubakar, S., & Zandi, K. (2015). Antiviral potential of algae polysaccharides isolated from marine sources: a review. *BioMed research international,* 2015.

Bi, D., Zhou, R., Cai, N., Lai, Q., Han, Q., Peng, Y., . . . Bao, W. (2017). Alginate enhances Toll-like receptor 4-mediated phagocytosis by murine RAW264. 7 macrophages. *International Journal of Biological Macromolecules,* 105, 1446-1454.

Bogdan, C. (2015). Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in immunology,* 36(3), 161-178.

Bounous, D. I., Campagnoli, R. P., & Brown, J. (1992). Comparison of MTT colorimetric assay and tritiated thymidine uptake for lymphocyte proliferation assays using chicken splenocytes. *Avian Diseases*, 1022-1027.

Campbell, J., Ciesielski, C. J., Hunt, A. E., Horwood, N. J., Beech, J. T., Hayes, L. A., . . . Foxwell, B. M. (2004). A novel mechanism for TNF-α regulation by p38 MAPK: involvement of NF-κB with implications for therapy in rheumatoid arthritis. *The Journal of Immunology,* 173(11), 6928-6937.

Casanova, J.-L., Abel, L., & Quintana-Murci, L. (2011). Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. *Annual review of immunology,* 29, 447-491.

Chanteux, H., Guisset, A. C., Pilette, C., & Sibille, Y. (2007). LPS induces IL-10 production by human alveolar macrophages via MAPKinases-and Sp1-dependent mechanisms. *Respiratory research,* 8(1), 1-10.

Cho, C.-W., Han, C.-j., Rhee, Y. K., Lee, Y.-C., Shin, K.-S., Shin, J.-S., . . . Hong, H.-D. (2015). Cheonggukjang polysaccharides enhance immune activities and prevent cyclophosphamide-induced immunosuppression. *International Journal of Biological Macromolecules,* 72, 519-525.

Cross, M. L., Ganner, A., Teilab, D., & Fray, L. M. (2004). Patterns of cytokine induction by gram-positive and gram-negative probiotic bacteria. *FEMS Immunology & Medical Microbiology,* 42(2), 173-180.

Davis, J. M., Clay, H., Lewis, J. L., Ghori, N., Herbomel, P., & Ramakrishnan, L. J. I. (2002). Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. 17(6), 693-702.

Giacomini, E., Iona, E., Ferroni, L., Miettinen, M., Fattorini, L., Orefici, G., . . . Coccia, E. M. (2001). Infection of human macrophages and dendritic cells with Mycobacterium tuberculosis induces a differential cytokine gene expression that modulates T cell response. *The Journal of Immunology,* 166(12), 7033-7041.

Harvey, A. L. (2007). Natural products as a screening resource. *Current opinion in chemical biology,* 11(5), 480-484.

Henry, K. M., Loynes, C. A., Whyte, M. K., & Renshaw, S. A. J. J. o. l. b. (2013). Zebrafish as a model for the study of neutrophil biology. 94(4), 633-642.

Ivec, M., Botić, T., Koren, S., Jakobsen, M., Weingartl, H., & Cencič, A. (2007). Interactions of macrophages with probiotic bacteria lead to increased antiviral response against vesicular stomatitis virus. *Antiviral research,* 75(3), 266-274.

Jiao, G., Yu, G., Zhang, J., & Ewart, H. S. (2011). Chemical structures and bioactivities of sulfated polysaccharides from marine algae. *Marine drugs,* 9(2), 196-223.

Kim, D.-S., Sung, N.-Y., Park, S.-Y., Kim, G., Eom, J., Yoo, J.-G., . . . Kim, K.-A. (2018). Immunomodulating activity of Sargassum horneri extracts in RAW264. 7 macrophages. *Journal of Nutrition and Health,* 51(6), 507-514.

Kim, J. J., & Khan, W. I. J. p. (2013). Goblet cells and mucins: role in innate defense in enteric infections. 2(1), 55-70.

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., . . . Shaw, L. (2008). A potential new pathway for Staphylococcus aureus dissemination: the silent survival of S. aureus phagocytosed by human monocyte-derived macrophages. *PloS one,* 3(1), e1409.

Leiro, J., Alvarez, E., Garcıa, D., & Orallo, F. (2002). Resveratrol modulates rat macrophage functions. *International immunopharmacology,* 2(6), 767-774.

Li, M., Yan, Y. X., Yu, Q. T., Deng, Y., Wu, D. T., Wang, Y., . . . Zhao, J. (2017). Comparison of immunomodulatory effects of fresh garlic and black garlic polysaccharides on RAW 264.7

macrophages. *Journal of food science,* 82(3), 765-771.
Morita, H., He, F., Fuse, T., Ouwehand, A. C., Hashimoto, H., Hosoda, M., . . . Kurisaki, J.-I. (2002). Cytokine production by the murine macrophage cell line J774. 1 after exposure to lactobacilli. *Bioscience, biotechnology, and biochemistry,* 66(9), 1963-1966.
Navegantes, K. C., de Souza Gomes, R., Pereira, P. A. T., Czaikoski, P. G., Azevedo, C. H. M., & Monteiro, M. C. (2017). Immune modulation of some autoimmune diseases: the critical role of macrophages and neutrophils in the innate and adaptive immunity. *Journal of translational medicine,* 15(1), 1-21.
Netea, M. G., Joosten, L. A., Latz, E., Mills, K. H., Natoli, G., Stunnenberg, H. G., . . . Xavier, R. J. (2016). Trained immunity: a program of innate immune memory in health and disease. *Science,* 352(6284).
Renshaw, S. A., Trede, N. S. J. D. m., & mechanisms. (2012). A model 450 million years in the making: zebrafish and vertebrate immunity. 5(1), 38-47.
Rombout, J. H., Yang, G., Kiron, V. J. F., & Immunology, S. (2014). Adaptive immune responses at mucosal surfaces of teleost fish. 40(2), 634-643.
Saadat, Y. R., Khosroushahi, A. Y., & Gargari, B. P. (2019). A comprehensive review of anticancer, immunomodulatory and health beneficial effects of the lactic acid bacteria exopolysaccharides. *Carbohydrate polymers,* 217, 79-89.
Saito, S., & Nakano, M. (1996). Nitric oxide production by peritoneal macrophages of Mycobacterium bovis BCG-infected or non-infected mice: regulatory roles of T lymphocytes and cytokines. *Journal of leukocyte biology,* 59(6), 908-915.
Sanjeewa, K. A., Fernando, I., Kim, S.-Y., Kim, H.-S., Ahn, G., Jee, Y., & Jeon, Y.-J. (2018). In vitro and in vivo anti-inflammatory activities of high molecular weight sulfated polysaccharide; containing fucose separated from Sargassum horneri. *International Journal of Biological Macromolecules,* 107, 803-807.
Shao, P., Liu, J., Chen, X., Fang, Z., & Sun, P. (2015). Structural features and antitumor activity of a purified polysaccharide extracted from Sargassum horneri. *International Journal of Biological Macromolecules,* 73, 124-130.
Sharma, L., Wu, W., Dholakiya, S. L., Gorasiya, S., Wu, J., Sitapara, R., . . . Reddy, S. (2014). *Assessment of phagocytic activity of cultured macrophages using fluorescence microscopy and flow cytometry*. In *Cytokine Bioassays* (pp. 137-145): Springer
Shiratsuchi, H., & Basson, M. D. (2005). Activation of p38 MAPKα by extracellular pressure mediates the stimulation of macrophage phagocytosis by pressure. *American Journal of Physiology-Cell Physiology,* 288(5), C1083-C1093.
Sun, H.-X., Wang, H., Xu, H.-s., & Ni, Y. (2009). Novel polysaccharide adjuvant from the roots of Actinidia eriantha with dual Th1 and Th2 potentiating activity. *Vaccine,* 27(30), 3984-3991.
Trede, N. S., Langenau, D. M., Traver, D., Look, A. T., & Zon, L. I. (2004). The use of zebrafish to understand immunity. *Immunity,* 20(4), 367-379.
Wang, D.-D., Pan, W.-J., Mehmood, S., Cheng, X.-D., & Chen, Y. (2018). Polysaccharide isolated from Sarcodon aspratus induces RAW264. 7 activity via TLR4-mediated NF-κB and MAPK signaling pathways. *International Journal of Biological Macromolecules,* 120, 1039-1047.
Whyte, S. K. J. F., & immunology, s. (2007). The innate immune response of finfish–a review of current knowledge. 23(6), 1127-1151.
Xiao, Y. Q., Malcolm, K., Worthen, G. S., Gardai, S., Schiemann, W. P., Fadok, V. A., . . . Henson, P. M. (2002). Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-β. *Journal of Biological Chemistry,* 277(17), 14884-14893.
Zhang, S., Nie, S., Huang, D., Huang, J., Wang, Y., & Xie, M. (2013). Polysaccharide from Ganoderma atrum evokes antitumor activity via toll-like receptor 4-mediated NF-κB and mitogen-activated protein kinase signaling pathways. *Journal of agricultural and food chemistry,* 61(15), 3676-3682.