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Rice Husk Silica Enhances Innate Immune in Zebrafish (*Danio rerio*) and Improves Resistance to *Aeromonas hydrophila* and *Streptococcus iniae* Infection

Yong-Han Hong [1], Chung-Chih Tseng [2,3], Desy Setyoningrum [4,5], Zu-Po Yang [6], Maftuch [5] and Shao-Yang Hu [4,7,*]

1. Department of Nutrition, I-Shou University, Kaohsiung 824, Taiwan; yonghan@cloud.isu.edu.tw
2. Department of Dentistry, Zuoying Branch of Kaohsiung Armed Forces General Hospital, Kaohsiung 81357, Taiwan; caviton@g-mail.nsysu.edu.tw
3. Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 80424, Taiwan
4. Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan; desy.setyoningrum16@gmail.com
5. Department of Fisheries and Marine Science, University of Brawijaya, Malang 65145, Indonesia; maftuch@ub.ac.id
6. Institute of Photonic System, National Chiao Tung University, Tainan 71150, Taiwan; zupoyang@nctu.edu.tw
7. Research Center for Animal Biologics, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan

* Correspondence: syhu@mail.npust.edu.tw

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Abstract: Rice husk (RH) contains abundant silica such that RH silica (RHS) may be useful for possible industrial exploitation. Here, amorphous silica nanoparticles with multiple pore structures were acquired from RH by simple thermochemical processes. RHS antimicrobial activity and effects on zebrafish innate immunity against pathogen infections were evaluated. A toxicity assay showed that zebrafish exposed to an RHS dose lower than 200 μg/mL did not exhibit damage to zebrafish embryonic development or juvenile survival. RHS showed a wide spectrum of bacteriostatic activity against a variety of pathogens including antibiotic-resistant pathogens, implying its potential application as an antimicrobial agent in diverse industries. Fish exposed to 20 or 200 μg/mL RHS exhibited significantly increased mRNA expression of immune-related genes, including IL-1β, IL-6, IL-15, TNF-α, COX-2a, TLR-4a, lysozyme, and complement C3b. RHS-treated zebrafish exhibited a higher cumulative survival compared to that in control fish after infecting with *Aeromonas hydrophila* and *Streptococcus iniae*. The present results showed that a safe RHS dose enhanced innate immunity against infections without toxic effects in healthy fish, suggesting that RHS may be developed as an immunostimulant for improving health status in aquaculture.

Keywords: rice husk silica; innate immunity; disease resistance; zebrafish

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1. Introduction

Aquaculture is considered the fastest growing food production industry in the world that is responsible for fish production to support the impressive increase in food demand for human consumption. According to the Food and Agriculture Organization of the United States (FAO) 2018 report, global aquaculture production in 2016 reached 80 million tons of food fish, including 54.1 million tons of finfish, 17.1 million tons of mollusks, 7.9 million tons of crustaceans, and 938,500 tons

of other aquatic animals that in total create a production value of USD 231.6 billion [1]. With limited land for aquaculture, an intensive culture approach is usually practiced in worldwide aquaculture in pursuit of high production. However, the accumulation of organic waste and rapid deterioration of water quality often cause fish stress and disease outbreaks, resulting in a severe economic loss in aquaculture. Bacterial diseases most frequently occur in aquaculture, which is one of the major issues inhibiting the sustainable development of aquaculture. For example, *Aeromonas hydrophila* and *Streptococcus* spp. are typical pathogens that cause motile Aeromonas septicemia (MAS) and streptococcosis, respectively, in freshwater fish, resulting in hemorrhagic septicemia, congestion, erratic swimming, exophthalmos with clouding of the cornea, and mass mortality [2,3]. In response to disease, antibiotics and chemicals are usually extensively used in aquaculture for therapeutic purposes. However, heavy use of antibiotics in aquaculture has caused impacts, including the emergence of antibiotic-resistant fish pathogens in aquatic environments, alterations of the bacterial flora in microbial ecology, reduced therapeutic efficacy of antibiotics, increased risk of food safety by residual antibiotic contamination, and increased horizontal transfer of antibiotic resistance genes to pathogens that pose risks to animals and human health [4]. Thus, the development of alternatives to antibiotics for biocontrol in aquaculture is quite important and urgent. Alternative strategies to antibiotics, such as vaccines [5], antimicrobial peptides [6], probiotics [7,8], natural extracts, and their bioactive compounds [9,10], have been developed as antimicrobials or immunostimulators for disease control in aquaculture. Although some alternatives are workable for disease control in the laboratory, they are difficult to apply practically in aquaculture due to their high cost and lack of ease of use. Thus, developing effective, inexpensive, and accessible antimicrobials or immunostimulators in aquaculture is a necessity.

Rice husk (RH), which constitutes approximately 20%~22% of rice, is an agricultural byproduct that is abundantly available. According to the FAO, yearly global rice production has been estimated at 759.6 million tons for 2017, which implies more than 150 million tons of RH production [11]. Although the application of RH has already been utilized in diverse fields, such as use as a biofertilizer in agriculture, a material for animal husbandry, and an absorbent or pest control agent, it is still often considered a waste of the rice milling industry and is usually burned in open air or dumped on wasteland, resulting in land and environmental pollution. Interestingly, a high silica (SiO_2) content was found in RH, which contains approximately 17%~20% ash that consist of mainly silica content (>90%). The abundant presence of silica in RH has attracted attention for use as a material to be used in various possible industrial exploitations. A review indicated RH-derived silica nanomaterials for a variety of practical applications [12]. Due to the increasing demand for silica in diverse applications, studies on the extraction procedure for silica from RH have been widely pursued. For example, Vaibhav et al. [13] found that a nanosized silica particle with a diameter of 20~40 nm was obtained with a yield of 78% from RH by burning RH by heat treatment at 900 °C for 7 h and sodium hydroxide/sulfuric acid treatment. Liu et al. [14] reported that active silica particles with a diameter of 40~50 nm and an optimal pore volume were obtained by burning RH at 900 °C in air. Porous silica with a high specific surface area was acquired from RH ash by a low pH and high temperature (500 °C) procedure [15]. These reports elucidate that silica nanoparticles could be recovered and purified from RH by simple thermochemical processes, suggesting that RH-derived silica is a low-cost and easily-accessible material.

Nanoparticles have been widely used as materials for various biological and medical applications [16]. However, a review reported that overexposure to nanoparticles can cause toxic harm and affect immune functions in many animal species, including fish [17]. Innate immunity consists of mainly diverse immune cells (monocytes/macrophages, dendritic cells, and phagocytic leukocytes) and proteins that are nonspecific and the first line of the host defense system, which depend on the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), such as the family of Toll-like receptors (TLRs) [18]. Hence, the innate immune system plays an essential role in the early recognition and subsequent production of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α to respond to inflammation.

Recently, reviews have detailed the actions of nanoparticles on innate immunity through TLR signaling pathways [19,20], potentially elucidating that exposure to nanoparticles causes excessive cytokine production and inflammation. Notably, the immunological effects of nanoparticles on the host are associated with the physicochemical properties (size, shape, and charge) and exposure dose of nanoparticles. Recently, reports showed that exposure to silver nanoparticles significantly increased innate immunity against white spot syndrome virus (WSSV) infection in shrimp (*Penaeus vannamei*) [21]. Angujam et al. [22] reported that dietary-supplemented β-1,3-glucan-binding protein-based zinc oxide nanoparticles enhance innate immunity against *A. hydrophila* infection in tilapia (*Oreochromis mossambicus*). Opposite results may result from the different physicochemical properties and treatment doses of nanoparticles. Although the toxicity or immunomodulatory effects of diverse nanoparticles have been widely studied, the effects of RH silica (RHS) nanoparticles on innate immunity in animals have not been investigated.

The present study attempted to study the effects of RHS on the immune modulation of fish and to develop RHS as an immunostimulator in aquaculture. The evaluation of the efficacy of various potential immunostimulators on diverse food fish species is an essential task in aquaculture. However, directly evaluating the functions of immunostimulators in aquaculture fish species usually requires high costs and a large culture space. Zebrafish provide an ideal animal model to validate the functions of immunostimulators for aquaculture research due to the advantages of ease of handling in breeding and experimentation, short generation time (approximately three months), and available genome sequences allowing evaluation of the effects of immunostimulators on the molecular level of immune responses, and results in zebrafish are applicable to important commercial fish [23,24]. The present study assessed the toxicity of RHS on the health status of zebrafish. The effects of RHS on the expression of innate immune-related genes were evaluated. The efficacy of RHS on disease resistance against *S. iniae* and *A. hydrophila* was also evaluated. These results could provide reference values for practical applications of RHS in aquaculture.

2. Materials and Methods

2.1. Fish and Bacterial Strains

Adult AB strain zebrafish (*Danio rerio*) with body lengths of 4.07 ± 0.23 cm and body weights of 0.82 ± 0.16 g were purchased from a domestic hatchery in Pingtung, Taiwan. Fish were reared acclimated in a 60 L aquarium at 28 °C for two weeks and fed with a commercial diet (MeM Prime, BERNAQUA, Olen, Belgium) twice daily. All fish were treated according to local animal welfare regulations. Zebrafish embryos were acquired from intercross mating of adult AB strain zebrafish. Zebrafish embryos cultured until they became one-month-old juveniles were used for an RHS toxicity assay. The clinical pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) was kindly provided by Dr. Huang et al. [25], and *Streptococcus iniae* was kindly provided by Dr. Gong et al. [26]. The foodborne pathogens *Escherichia coli* BCRC11634, *Staphylococcus aureus* BCRC12991, *Salmonella typhimurium* BCRC12947, and *Listeria monocytogenes* BCRC14932; clinical pathogen *Pseudomonas aeruginosa* BCRC12902; and plant pathogen *Burkholderia gladioli* BCRC13899 were purchased from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan. Other aquatic pathogens were obtained from Dr. Liu et al. [27–30]. The origin, culture conditions and maintenance of pathogens were as described previously [7].

2.2. Preparation of RHS

RH was purchased from a local rice miller and soaked in a 3% aqueous organic acid solution containing citrate and tartaric acid for 2 h to remove lignin and other crude fibers. Subsequently, the RHs were washed three times with distilled water and treated by smoldering at 300 °C for 2 h and then burning samples at 800 °C for 2 h to harvest RHS. The form and size of RHS were estimated by X-ray diffraction (Bruker D2 Phaser, Bruker, Billerica, Massachusetts, USA) and EDS-SEM (JeoL

JSM-7800F Prime, JeoL, Akishima, Tokyo, Japan). The harvested RHS was dissolved in a 1.25 M NaOH solution to obtain 2% (w/v) aqueous RHS. The silicon content in the aqueous RHS was determined to be 9800 ± 150 μg/mL by ICP-MS analysis (THERMO-ELEMENT XR, Thermo, Waltham, MA, USA). The aqueous RHS was serially diluted with deionized water and used as the tested sample in the following experiments.

2.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

A colorimetric assay using resazurin as substrate was used to determine MIC and MBC of the RHS according to the protocol described previously [9]. Briefly, a 10 μL of different concentrations of RHS was added respectively into a 96-well microplate, whereby each well comprised 90 mixture solution (10 μL of the tested pathogen (10^7 CFU/mL), 67 μL of tryptic soybean broth (TSB) medium, 13 μL of 0.025% (wt/v) resazurin solution). After incubating at 28 °C for 12 h, the lowest RHS concentration with a dark green color in the test well was defined as MIC. The entire sample with a dark green color in each well was spread on TSB agar plate and incubated at 28 °C for 24 h. The lowest RHS concentration in the sample without colony formation on the TSB plate was defined as MBC. A total of 10 μL of various concentrations of kanamycin presented in the well was used as control. The experiment was conducted in triplicate.

2.4. Toxicity Assessment of RHS to Zebrafish Embryos and Juveniles

The protocol for toxicity assay was modified in accordance with a previous report [31]. Zebrafish embryos at 5 hpost-fertilization (hpf) were collected and used for assessing toxicity of RHS. Fifty embryos were placed in 9 cm petri dish and immersed with 30 mL of aerated deionized-distilled water (ddH_2O) containing RHS at a final concentration of 2, 20, 200, or 2000 μg/mL, and then incubated at 28 °C for 91 h. The survival rate, hatching rate, and malformation at 3 days post fertilization (dpf) and 4 dpf were used as parameters to evaluate toxicity of RHS. To assess the toxicity of RHS to juvenile zebrafish, thirty one-month-old juvenile zebrafish were cultured in 3 L acrylic cylinder fish tanks containing 1 L of aerated ddH_2O and various concentrations (2, 20, 200, or 2000 μg/mL) of RHS. The survival rate was recorded daily for 7 days. Zebrafish embryos and juveniles immersed in aerated ddH_2O were used as the control group. The aerated ddH_2O containing different concentration of RHS concentration was renewed daily for maintenance of the RHS concentration. The experiment was done in triplicate and three times for each condition.

2.5. Analysis of Innate Immune-Related Genes by Real-Time PCR

Six zebrafish treated with different concentrations of RHS were sampled for extraction of total RNA using TriPure isolation reagent (Roche, Mannheim, Germany). The cDNA synthesis was carried out by an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The expression levels of innate immune-related genes were determined by real-time PCR (Applied Biosystems StepOnePlus, Foster City, CA, USA) using SYBR Green PCR reagents. The PCR condition for amplifying each gene and specific primers are described in previous reports [9,32] and listed in Table 1. The relative expression level of each gene normalized to EF-1α expression was expressed as the mean ± standard error (S.E.).

Table 1. Primer sequences used in this study.

Gene Name	Primer Sequence (5′→3′)	PCR Product Size (bp)	Accession Number
Interleukin-1β (IL-1β)	TGGACTTCGCAGCACAAAATG CACTTCACGCTCTTGGATGA	147	AY340959
Interleukin-6 (IL-6)	TCAACTTCTCCAGCGTGATG TCTTTCCCTCTTTTCCTCCTG	73	JN698962
Interleukin-15 (IL-15)	ATGTCATTGGAACTCAGAGGTTT CTGTTCTGGATGTCCTGCTTGA	100	BC162843
Tumor necrosis factor-α (TNF-α)	AAGGAGAGTTGCCTTTACCG ATTGCCCTGGGTCTTATGC	152	BC165066
Toll-like receptor-4a (TLR-4a)	TTTCAGATGCCACATCAGA TCCACAAGAACAAGCCTTTG	150	EU551724
Cyclooxygenase-2a (COX-2a)	GATCTCCCAAATGCCAAGCA GGGCGAAGAAAGCAAACATG	100	NM_153657
Complement component C3b	CGTCTCCGTACACCATCCATT GGCGTCTCATCAGGATTTGTTAC	100	NM_131243
Lysozyme	CGTGGATGTCCTCGTGTGAAG CCAATGGAGAATCCCTCAAA	100	NM_139180
Elongation factor-1α (EF-1α)	AACAGCTGATCGTTGGAGTCAA TTGATGTATGCGCTGACTTCCT	100	AY422992

2.6. Treatment of Adult Zebrafish with RHS and Challenge Experiment

Adult zebrafish were randomly distributed into three groups. Fifteen fish in each group were cultured in a 5 L acrylic cylinder aquarium containing 3 L of aerated ddH$_2$O without RHS (control group) or with RHS at a final concentration of 20 or 200 μg/mL. Each group was conducted in triplicate and cultured at 28 °C for 7 days. The aerated ddH$_2$O containing different concentration of RHS concentration was renewed daily for maintenance of the RHS concentration. The protocol of the challenge test was modified from a previous report [32,33]. Briefly, *A. hydrophila* and *S. iniae* were cultured respectively in TSB at 28 °C for 24 h, and then the culture solution was centrifuged at 6100× *g* for 15 min at 4 °C to collect the bacterial cells. The bacterial pellets were suspended in sterile water to a concentration of 1×10^{10} CFU/mL and adjust the cell concentration by sterile water. The 7-day median lethal dose (LD$_{50}$) was determined by injecting intraperitoneally with serial doses of *A. hydrophila* and *S. iniae* into 10 fish (10^5, 10^6, 10^7, and 10^8 CFU/fish), respectively. At the end of the RHS-treatment trial, the effect of RHS on the cumulative survival was evaluated by injecting each fish with 10 μL of dilute *A. hydrophila* or *S. iniae* solution (10^6 CFU/fish for *A. hydrophila* and 10^5 CFU/fish for *S. iniae*; equivalent to the 7-day LD$_{50}$). Fish cultured in aerated ddH$_2$O and injected with saline were used as control group. The cumulative survival in each group was recorded for 7 days post infection.

2.7. Statistical Analysis

Statistical analysis in relative gene expression levels between each group were performed using one-way ANOVA and Tukey's multiple comparison tests. The data between each group was considered significant difference when a probability value of less than 0.05 ($p < 0.05$). Statistical analysis of cumulative survival in the challenge test was performed by the Kaplan–Meier method using SAS software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Toxicity Assay of RHS in Zebrafish

A white powder of RHS could be acquired by a simple two-stage (300 and 800 °C) thermochemical process. X-ray diffraction and EDS-SEM analysis showed that the RHS consisted of primarily amorphous hydrated silica (SiO_2) and nanoparticles with pore sizes less than 100 nm (Figure 1). ICP-MS analysis showed that the purity of SiO_2 in the RHS was more than 98% but less than 99.0%. Before potential application of RHS as an immunomodulator in aquaculture, the toxicity of RHS to

zebrafish embryos and juvenile fish was assessed using survival rate, hatching rate, and malformation rate as criteria. As shown in Table 2, a significant decrease in the survival rate and hatching rate at 3 and 4 dpf was observed in zebrafish in the presence of 2000 μg/mL RHS. Moreover, embryos exposed to 2000 μg/mL RHS exhibited a significantly increased malformation rate, and the defects manifested as mainly pericardial edema, spinal curvature, yolk sac edema, and irregular muscle shape phenotypes. Embryos exposed to RHS concentrations lower than 200 μg/mL showed normal development, and the survival rate, hatching rate, and malformation rate compared with those of the control group exhibited no significant difference. Similarly, Table 3 shows an obvious increase in mortality in juvenile zebrafish exposed to 2000 μg/mL RHS for seven days, suggesting that the concentration is toxic to zebrafish. The survival rates between the control group and the groups treated with RHS below 200 μg/mL were not significantly different. These results suggested that a concentration of RHS less than 200 μg/mL is the optimal dosage for treating zebrafish.

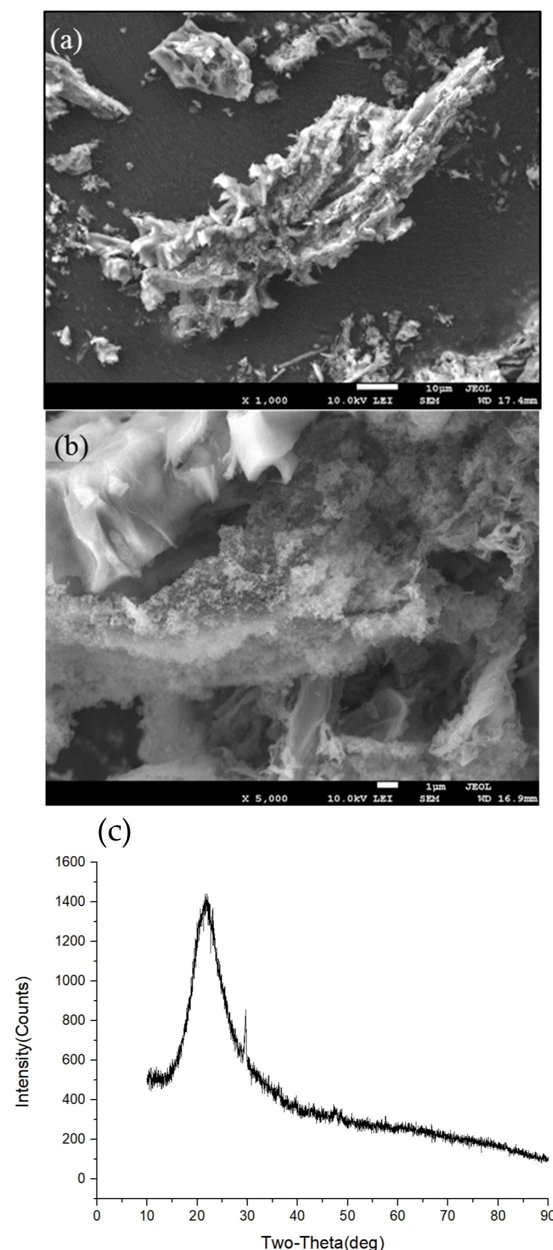

Figure 1. SEM images of RHS at (**a**) low (1000×) and (**b**) high magnifications (5000×) and (**c**) X-ray reflectivity pattern of RHS.

Table 2. Survival rate, hatching rate, and malformation rate of zebrafish embryos exposed to different concentrations of RHS solution at 3 and 4 dpf.

Concentration of RHS Solution (µg/mL)	3 dpf			4 dpf		
	Survival Rate (%)	Hatching Rate (%)	Malformation Rate (%)	Survival Rate (%)	Hatching Rate (%)	Malformation Rate (%)
Control	89 ± 4% (n = 134/150) [a]	100 ± 0% (n = 134/134) [a]	2 ± 3% (n = 2/134) [a]	89 ± 4% (n = 134/150) [a]	100 ± 0% (n = 134/134) [a]	2 ± 3% (n = 2/134) [a]
2	84 ± 3% (n = 126/150) [a]	98 ± 3% (n = 123/126) [a]	1 ± 2% (n = 1/123) [a]	83 ± 4% (n = 125/150) [a]	100 ± 0% (n = 123/123) [a]	2 ± 2% (n = 2/123) [a]
20	89 ± 5% (n = 133/150) [a]	100 ± 0% (n = 134/134) [a]	0 ± 0% (n = 0/134) [a]	88 ± 5% (n = 132/150) [a]	100 ± 0% (n = 132/132) [a]	0 ± 0% (n = 0/132) [a]
200	84 ± 3% (n = 126/150) [a]	98 ± 4% (n = 123/126) [a]	1 ± 1% (n = 2/123) [a]	84 ± 3% (n = 126/150) [a]	100 ± 0% (n = 123/123) [a]	1 ± 1% (n = 2/123) [a]
2000	30 ± 13% (n = 50/150) [b]	39 ± 8% (n = 20/50) [b]	66 ± 4% (n = 33/50) [b]	19 ± 5% (n = 28/150) [b]	43 ± 1% (n = 12/28) [b]	69 ± 10% (n = 19/28) [b]

Data in the same column with different superscripts are significantly different ($p < 0.05$) among the treatments. Data are presented as the mean ± S.E. from triplicate experiments.

Table 3. Survival rate of juvenile zebrafish exposed to different concentrations of RHS solution for seven days.

Concentration of RHS Solution (µg/mL)	Survival Rate (%)						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	100 ± 0% (n = 90/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]
0.2	100 ± 0% (n = 90/90) [a]	100 ± 0% (n = 90/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]
2	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]	97 ± 6% (n = 87/90) [a]
20	100 ± 0% (n = 87/90) [a]	97 ± 6% (n = 87/30) [a]	97 ± 6% (n = 87/30) [a]	93 ± 6% (n = 84/90) [a]	90 ± 0% (n = 81/90) [a]	90 ± 0% (n = 81/90) [a]	90 ± 0% (n = 81/90) [a]
200	93 ± 6% (n = 84/90) [a]	93 ± 6% (n = 84/90) [a]	93 ± 6% (n = 84/90) [a]	90 ± 6% (n = 81/90) [a]	90 ± 10% (n = 81/90) [a]	90 ± 10% (n = 81/90) [a]	90 ± 10% (n = 81/90) [a]
2000	70 ± 10% (n = 63/90) [b]	60 ± 17% (n = 54/90) [b]	47 ± 15% (n = 42/90) [b]	43 ± 12% (n = 39/90) [b]	40 ± 10% (n = 36/90) [b]	40 ± 10% (n = 36/90) [b]	40 ± 10% (n = 36/90) [b]

Data in the same column with different superscripts are significantly different ($p < 0.05$) among the treatments. Data are presented as the mean ± S.E. from triplicate experiments.

3.2. Antimicrobial Activity of RHS against Diverse Pathogens

The bacteriostatic and bactericidal activities against diverse pathogens were evaluated by determining the MIC and MBC using a colorimetric assay. As shown in Table 4, RHS exhibited effective bacteriostatic activity against the aquatic pathogens *A. hydrophila*, *S. iniae*, *V. parahaemolyticus*, *V. alginolyticus, V. vulnificus,* and *D. hansenii*; the foodborne pathogens *E. coli*, *S. aureus*, *S. typhimurium,* and *L. monocytogenes*; the clinical pathogens methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*; and the plant pathogen *B. gladioli*. The RHS yielded MICs between 100 and 240 μg/mL for all tested pathogens. However, although RHS has bacteriostatic potency against diverse pathogens, RHS does not have bactericidal potency. RHS yielded high MBCs for most tested pathogens, at more than 2000 μg/mL, a concentration at which it is harmful and toxic to zebrafish hosts. Compared with the antimicrobial potency of the antibiotic kanamycin, the antimicrobial potency of RHS was far less. However, RHS exhibited bacteriostatic activity against the antibiotic-resistant pathogens MRSA, *V. alginolyticus,* and *V. parahaemolyticus*, suggesting its potential as an alternative to antibiotics for treating antibiotic-resistant pathogen infections.

Table 4. Antimicrobial potency of RHS against various pathogens.

Pathogens and Sources	RHS (μg/mL)		Kanamycin (μg/mL)	
	MIC	MBC	MIC	MBC
Aeromonas hydrophila	120	1000	0.5	2
Streptococcus iniae	140	2000	3	6
Vibrio parahaemolyticus [a]	140	2000	NE [c]	NE [c]
Vibrio alginolyticus [a]	200	NE [c]	NE [c]	NE [c]
Vibrio vulnificus	180	20,000	3	6
Debaryomyces hansenii	100	1000	NE [c]	NE [c]
Escherichia coli BCRC11634 [b]	180	20,000	1	2
Staphylococcus aureus BCRC12991 [b]	160	NE [c]	0.5	2
Salmonella typhimurium BCRC12947 [b]	180	NE [c]	1	2
Listeria monocytogenes BCRC14932 [b]	200	NE [c]	1	3
Methicillin-resistant S. aureus (MRSA) [a]	240	NE [c]	NE [c]	NE [c]
Pseudomonas aeruginosa BCRC12902 [b]	160	NE [c]	2	4
Burkholderia gladioli BCRC13899 [b]	180	NE [c]	3	4

[a] Antibiotic-resistant pathogens. [b] Pathogens were obtained from BCRC. [c] NE indicated no effect.

3.3. RHS Immersion Treatment Enhanced Innate Immunity in Zebrafish

The immunomodulatory function of RHS was evaluated by determining the expression of genes involved in the innate immune response. The expression levels of interleukin (IL)-1β, TLR-4, cyclooxygenase (COX)-2a, complement component C3b, and lysozyme from the whole body of zebrafish in the presence of 200 μg/mL RHS were significantly higher than those from the control group, and there were no significant differences between zebrafish in the presence of 2 or 20 μg/mL RHS and the control group. The expression levels of IL-6 and IL-15 in zebrafish that were immersion treated with 20 and 200 μg/mL RHS were significantly higher than those in the control group. The mRNA expression of tumor necrosis factor (TNF)-α in zebrafish treated with 2, 20, and 200 μg/mL RHS was significantly induced compared to that in the control group (Figure 2). Significant increases in the cytokine genes IL-1β, IL-6, and IL-15; TLR-4a; COX-2a; TNF-α; C3b and lysozyme in the zebrafish treated with RHS suggested that fish exposure to RHS could modulate immune immunity.

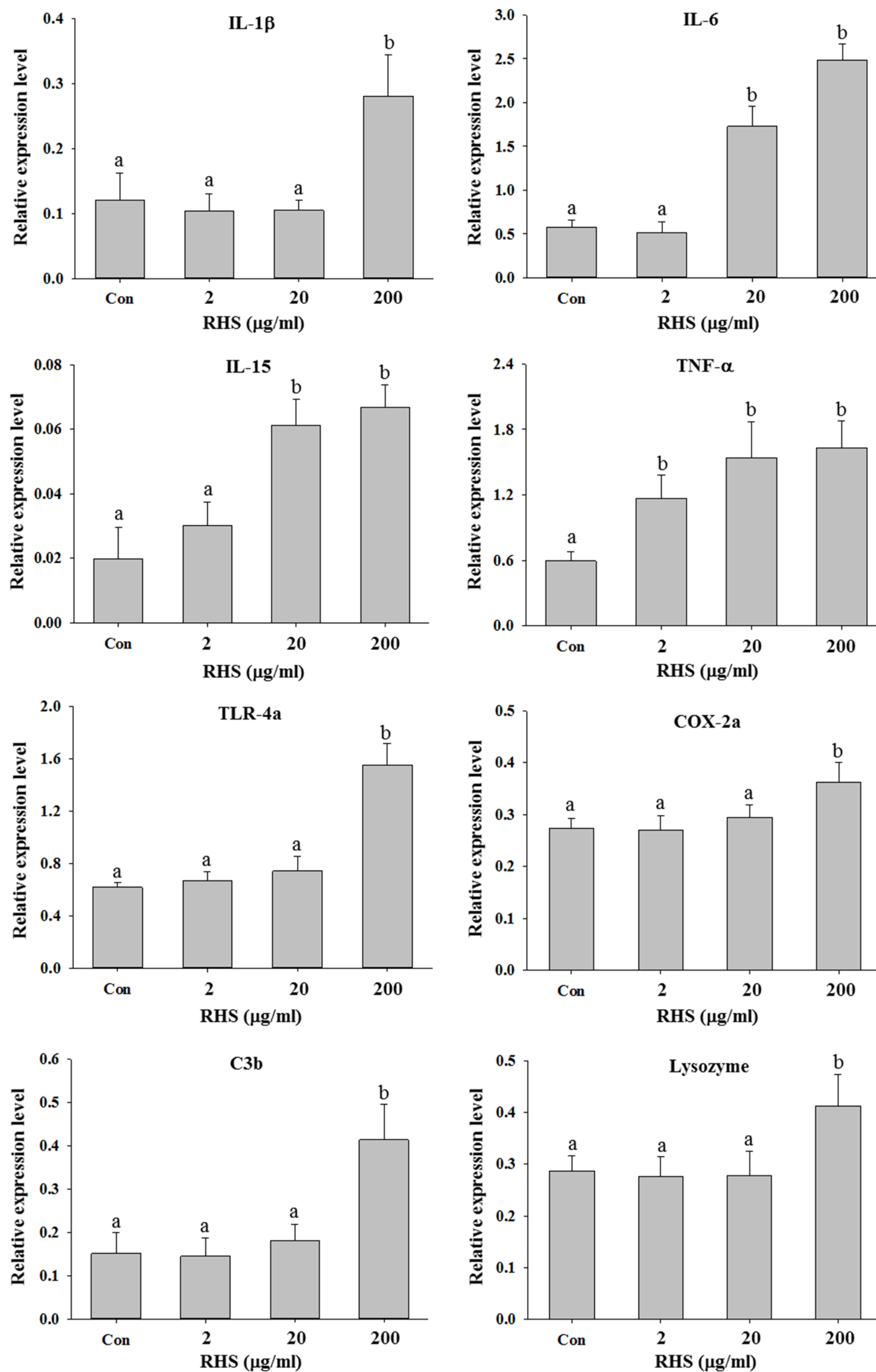

Figure 2. The relative expression levels of immune-related genes in zebrafish treated with RHS for seven days. The different superscripts between each bar indicate significant difference ($p < 0.05$, one-way ANOVA).

3.4. Treatment with RHS Enhanced Disease Resistance in Zebrafish

Enhancement of innate immunity in zebrafish after RHS immersion treatment inspire us to evaluate the efficacy of RHS on disease resistance. Because most innate immune-related genes were

induced in zebrafish exposed to 20 and 200 μg/mL RHS, those concentrations were used to evaluate cumulative survival after being infected with *A. hydrophila* and *S. iniae*. As shown in Figure 3, the cumulative survival of fish injected with saline during the seven days post injection was 100%. However, the cumulative survival of zebrafish injected with *A. hydrophila* and *S. iniae* obviously declined during the first three and four days post infection. Subsequently, a stable survival at 28.8% ± 3.84% and 17.7% ± 7.69%, respectively, was maintained at seven days post-infection. The survival of zebrafish exposed to 20 μg/mL RHS at seven days post infection for *A. hydrophila* and *S. iniae* infection was 33.3% ± 6.67% and 26.7% ± 6.67%, respectively. Although the cumulative survival of zebrafish in the presence of 20 μg/mL RHS was higher than that of zebrafish without RHS treatment after *A. hydrophila* and *S. iniae* challenge, there were no significant differences between no RHS- and 20 μg/mL RHS-treated zebrafish. Notably, the fish immersed with 200 μg/mL RHS exhibited a dramatically increased cumulative survival compared to that of zebrafish without RHS treatment. The survival rates at seven days post infection were 51.1% ± 3.85% and 48.9% ± 3.85% for *A. hydrophila* and *S. iniae*, respectively. There is no significantly different between the cumulative survival of fish in the presence of 20 and 200 μg/mL RHS. The results suggested that zebrafish exposed to 200 μg/mL RHS had enhanced diseases resistance to *A. hydrophila* and *S. iniae* infection (Figure 3).

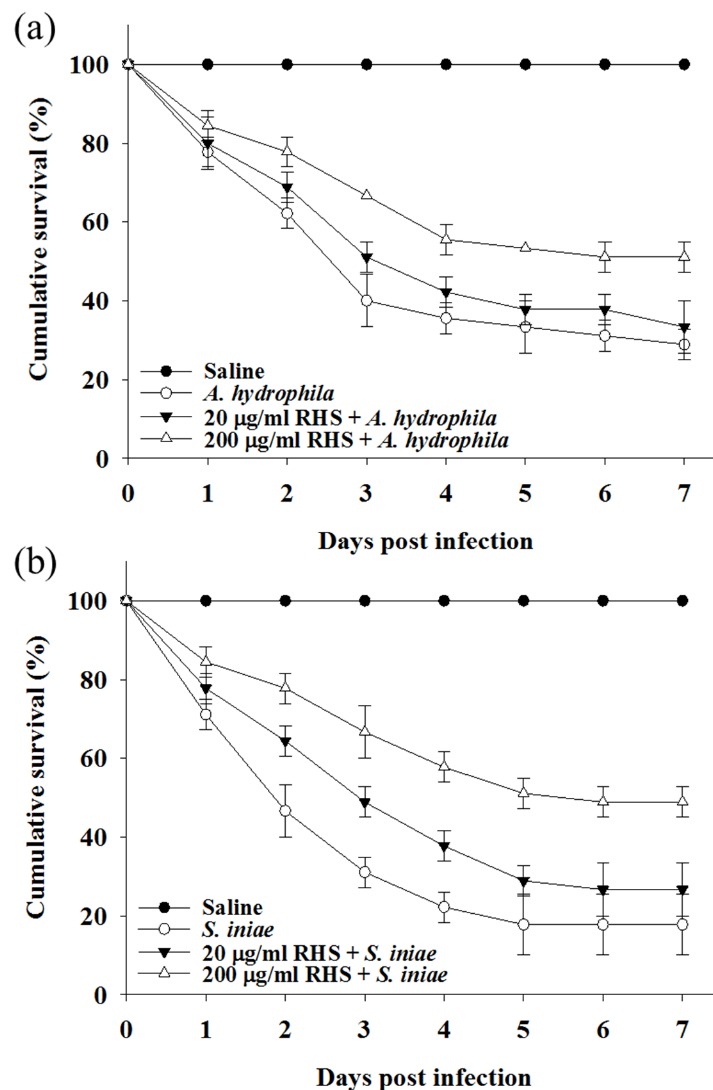

Figure 3. Cumulative survival of zebrafish infected with (**a**) *A. hydrophila* or (**b**) *S. iniae* after treatment with 20 or 200 μg/mL RHS for seven days. The cumulative survival rates of zebrafish in the 200 μg/mL RHS-treated groups were significantly higher ($p < 0.05$) than those in the control group.

4. Discussion

In the present study, the antibacterial effect of RHS in the aquaculture field was first explored. Silica (or silicon dioxide, SiO_2) is often used as a food additive in the food industry and is numbered E551. Based on the degree of purity, silica is divided into fumed silica and hydrated silica. Fumed silica is produced synthetically through a thermal process yielding an amorphous structure. Hydrated silica including precipitated silica and silica gel are produced synthetically by a wet process generating a water-absorbed surface. In pyrogenic (fumed) silica, the content of SiO_2 is not less than 99% on the basis of ignition loss, whereas in hydrated silica the content of SiO_2 ranges from 94% to 99%. The present study showed that the content of SiO_2 in RHS ranged from 98% to 99%, suggesting that the RHS is food grade between hydrated silica and fumed silica.

This study is the first exploration of applying silica from RHs in the field of aquaculture. As expected, RHS exhibited antimicrobial potency for aquaculture; however, the safety of RHS should be addressed in additional detail. In our unpublished data related this study, daily supplementation of RHS at a concentration of 200 μg/mL for eight months did not harm the growth and physiological functions of C57BL/6 mice. In addition to the discussion of silica nanoparticles, we also focused on the level of silicon. Here, the RHS we proposed belongs to a concentrated solution of silica and is assumed to provide aquatic animals with water-solubilized silica for intake. Based on our enumeration analysis data, zebrafish that were living in water containing 98 ± 15 ppm silicon (200μg/mL RHS) exhibited an increased potential to defend against bacteria. In our unpublished results, the contents of lead and arsenic trioxide (As_2O_3) in RHS were both less than 2 ppm, which are under restricted levels, thus indicating aqueous RHS as a comparable safety reagent.

Nanotechnologies conferring metal or metal oxide nanoparticles create many prospective applications in the fields of biomedicine, drug delivery, catalysis, and agriculture [34–36]. However, elimination or minimization of the toxicity of metal and metal oxide nanoparticles is an essential issue before applying these materials to living organisms. The present study evaluated the toxicity of RHS to zebrafish before applying RHS as a bacteriostatic agent or immunostimulant in aquaculture. A report showed that the survival and hatching rates of zebrafish embryos exposed to 100 μg/mL of two different-sized silica nanoparticles (25 and 115 nm) were not affected [37]. Similarly, the present study showed that zebrafish embryos and juvenile zebrafish exposed to less than 200 μg/mL RHS were not affected for survival rate, hatching rate, or malformation rate and exhibited a healthy physiological state, suggesting that 200 μg/mL RHS is a safe dose for zebrafish.

Metal and metal oxide nanoparticles with intrinsic antimicrobial activity have received great attention as emerging antimicrobial agents for overcoming the current bacterial resistance to antibiotics. Reviews indicated that the type of metal or metal-derived materials and particle size are two critical parameters that affect the antimicrobial effectiveness of metal nanoparticles [38,39]. Nanoscaled silica has been reported to exhibit obvious antibacterial activity and inhibit pathogen adherence to medical devices [40]. In the present study, RHS showed a nanosized particle with multiple pores after certain thermochemical processes, potentially suggesting that RHS exhibited antibacterial activity. As shown in Table 2, although the bactericidal activity of RHS was limited, the RHS revealed an effective bacteriostatic activity against aquaculture, foodborne, clinical, plant, and antibiotic-resistant pathogens. The results suggested that RHS has broad-spectrum bacteriostatic activity and potential application as an antimicrobial agent in diverse industries.

Recently, the engineering of nanoparticles for application in the modulation of the fish immune system has become an emerging field. For instance, Na-Phatthalung et al. [41] reported that gilthead sea bream (*Sparus aurata*) exposure to gold nanoparticles could modulate antioxidant and innate immune gene expression in gills, which are well-known mucosal organs exhibiting abundant immune cells that serve as the indispensable first line of defense; a report revealed that shrimp (*Penaeus vannamei*) exposure to silver nanoparticles (0.011 nmol of metallic silver/shrimp) could activate innate immunity against WSSV infection [21]. Similarly, in the present study, the survival rate of zebrafish challenged with *A. hydrophila* and S. *iniae* significantly increased in the 200 μg/mL RHS-treated group compared with that

in the control group, suggesting that RHS could enhance immune activity against pathogenic infection. The present study investigated the immunomodulatory function of RHS on fish by determining the expression of genes related to innate immune responses in zebrafish. The innate immune system is the first line of defense that relies on the recognition of PAMPs through PRRs, such as the family of TLRs. TLR-4 is the major receptor for recognizing lipopolysaccharide (LPS), which is a major component of the gram-negative bacterial outer membrane. Increasing evidence suggests that TLR-4 acts as a crucial sensor reacting to metal nanoparticles to activate the innate immune response and proinflammatory cytokine production. For instance, a report showed that sea urchins exposed to titanium dioxide nanoparticles (TiO_2NPs) induced the expression of TLR-4-like proteins and participated in the TLR/p38 MAPK signaling pathway to stimulate the phagocytic activity of immune cells [42]; Bhanja et al. [43] reported that Ag nanoparticles injected into chicken embryos induced TLR-4 gene expression and improved immunocompetence, suggesting that Ag nanoparticles can act as potential agents for the enhancement of innate immunity in chickens. Although in vitro and in vivo evidence of silica nanoparticle-induced TLR-4 expression is still lacking, the present study showed that zebrafish exposed to RHS significantly increased TLR-4a expression, suggesting that TLR-4a is potentially involved in silica nanoparticle-induced innate immune responses. The TLR-4 signaling pathway plays an important role in triggering the innate immune response. Its activation activates NF-κB to induce the expression of proinflammatory cytokines and COX-2. The present study showed significant increases in gene expression levels for the proinflammatory cytokines IL-1β, IL-6, IL-15, and TNF-α and COX-2a in zebrafish in the presence of the tested RHS concentrations. COX-2 induces an immune response via the synthesis of prostaglandins that perform various important functions during all stages of inflammation [44]. Proinflammatory cytokines secreted from macrophages and monocytes play major roles in regulating the host innate immunity response to infection. The RHS-induced IL-1β, IL-6, IL-15, and TNF-α cytokine and COX-2a expression in zebrafish suggests that RHS could enhance immunity against bacterial infection. Studies have reported that exposure to silica nanoparticles induced cytokine (IL-1β, IL-6, and TNF-α) expression in cell lines, mice, and zebrafish, also supporting the results of the present study, although the subjects of these studies focus on the toxicity of silica nanoparticles [45–47]. Lysozyme is an indispensable enzyme that functions as a humoral component of the innate immune defense system in resisting pathogen infections by hydrolyzing the peptidoglycan of bacterial cell walls. When invading pathogens are engulfed by phagocytic cells via phagocytosis, and immune cells such as neutrophils and macrophages kill pathogenic bacteria by releasing lysozyme. In addition to antimicrobial activity, a recent report demonstrated that lysozyme-mediated bacterial lysis can modulate the immune response resistance to pathogen infection in the host by activating PRRs and the complement system [48]. Complement component C3 activation is essential for the function of the complement system, which is composed of a large number of distinct plasma proteins that react with one another to opsonize pathogens and induce a series of inflammatory responses that help fight infection [49]. In the present study, a significant increase in immune-related gene expression in response to RHS suggested that RHS could enhance immunomodulation in fish.

5. Conclusions

The application of metal nanoparticles in organisms is similar to a double-edged sword. Reviews have indicated that overdose exposure to metal nanoparticles will cause toxic harm in organisms. However, they potentially provide an immunomodulatory benefit to treated hosts at an appropriate dose. The present study demonstrated that zebrafish exposed to a safe dose (200 μg/mL) of RHS exhibited bacteriostatic activity and improved innate immunity against *A. hydrophila* and *S. iniae* infections. To the best of our knowledge, this research is the first study to report the immunostimulatory function of RHS and its potential application in aquaculture, and the results suggest that RHS has the potential to be developed as an immunostimulant for improving immunity against pathogen infections in aquaculture.

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