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Short communication

Characterization of Galectin-2 from Nile tilapia (*Oreochromis niloticus*) involved in the immune response to bacterial infection

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ABSTRACT

galectin-2 plays important roles in innate and adaptive immunity. In this study, galectin-2 (*OnGal-2*) was identified from Nile tilapia (*Oreochromis niloticus*). Its tissue distribution and expression patterns following bacterial infection were also investigated. OnGal-2 is widely distributed in various tissues of healthy tilapia. After *Streptococcus agalactiae* challenge, OnGal-2 expressions were significantly up-regulated in all tested tissues. Meanwhile, the recombinant OnGal-2 (rOnGal-2) protein showed strong agglutinating activities against both Gram-negative bacteria and Gram-positive bacteria. Moreover, rOnGal-2 could promote phagocytosis of macrophages. Taken together, the present study indicated that OnGal-2 might play roles in the immune responses of Nile tilapia against bacterial pathogens.

1. Introduction

Galectin is a family of endogenous lectins with high affinity to polysaccharides containing β-galactoside residues [1]. Galectin proteins contain conserved carbohydrate recognition domains (CRD), which are necessary for splicing activity [2]. So far, 15 members of the Galectin family have been identified in many species. Structurally, Galectins can be divided into three categories: “prototype” (galectin-1, -2, -5, -7, -10, -11, -13, -14, -15), “chimera” (galectin-3) and “tandem repeat” (galectin-4, -6, -8, -9, -12) [3]. In extracellular, galectin acts as an adhesion molecule that mediates cross-linking between adjacent cells, cells and extracellular matrix proteins [4]. Besides, galectin can mediate the developmental processes of embryo implantation and myogenesis [5]. Galectin-2 (Gal-2) is a member of the galectin family, belonging to single-CRD prototype, playing vital roles in pathogen recognition, T cell apoptosis [6] and NK cell activation [7]. Although Gal-2 have been identified in crustacean to mammals [8], the information about fish Gal-2 is rare.

Nile tilapia (*Oreochromis niloticus*) is one of the most important economic culture of fish in southern China [9]. However, the bacterial pathogen *Streptococcus agalactiae* has caused enormous economic losses to the tilapia industry in recent years [10]. Therefore, there is an urgent need to understand the defense mechanism of tilapia. It has been proven that Gal-2 is involved in the production of cytokines and chemokines [11]. But the function of tilapia Gal-2 remain largely unknown. In this study, a Gal-2 homolog is isolated and characterized from Nile tilapia, *Oreochromis niloticus* (OnGal-2). The mRNA expression pattern of OnGal-2 of tilapia different tissues of healthy fish and post-infection fish were performed. In addition, a recombinant OnGal-2 protein was produced and its agglutination and promote phagocytosis were investigated. The data will help us to better understand the immune response of fish during bacterial infection.

2. Materials and methods

2.1. Fish preparation and bacterial challenge

Nile tilapia were acquired from a commercial fish farm in Zhangjiang, Guangdong, China. These fish had an average size of about 50 g without any disease and were kept in a 1000 L water tank at room temperature for 2 weeks [12]. To study the gene expression level of different tissue, samples of 10 tissues (heart, brain, spleen, liver, thymus, head kidney, intestine, muscle, gill, and skin) were obtained from the healthy tilapia, and quickly frozen in liquid nitrogen and

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Table 1
Primers used in this study.

Primers	Nucleotide Sequence (5′→3′)	Comment
OnGal-2-F	ATGAGTATGGAACTCGAACTGAAGA	ORF amplification
OnGal-2-R	TTAGCAGATCTTAAAGGATTTCAGTTT	ORF amplification
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	CAGGAAACAGCT ATGACC	Sequencing
qOnGal-2-F	GATGGTGCTGTGCTGGTGTG	RT-PCR
qOnGal-2-R	CCTCTGTAGGGGGTTGTGAA	RT-PCR
β-actin-F	CGAGAGGGAAATCGTGCGTGACA	RT-PCR Control
β-actin-R	AGGAAGGAAGGCTGGAAGAGGGC	RT-PCR Control
EOnGal-2-F	CGCGGATCCAGTATGGAACTCGAACTGAAGAATG	Protein expression
EOnGal-2-R	CCGCTCGAGGCAGATCTTAAAGGATTTCAGTTTG	Protein expression

*Bam*HI and *Xho*I sites are underlined.

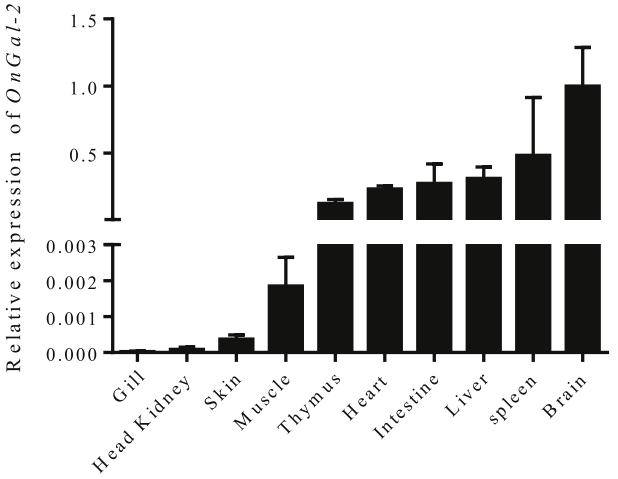

Fig. 1. Tissue distribution of OnGal-2 mRNA in healthy Nile tilapia. The ratio refers to the gene expression in different tissues relative to that in gill, and target gene expression is normalized to β-actin. Data are presented as means ± standard deviation.

stored at −80 °C. All experiments were conducted according to the principles and procedures of Guangdong Province laboratory animal management regulations.

S.agalactiae ZQ0910, used in the experiment was isolated from Nile tilapia and kept in our laboratory. The *S. agalactiae* was dissolved in phosphate-buffered saline (PBS) with a final concentration of 1×10^7 cells/mL [13]. The challenge group was injected with 0.1 mL of *S. agalactiae*, in triplicates. Tissue samples (gill, thymus, head kidney, spleen) were collected at seven time points (0 h, 4 h, 12 h, 24 h, 48 h, 72 h, 96 h) with 0 h was performing as control. All the primers used in this study were designed by primer 6.0 (Primer design software) and showed in Table 1.

2.2. Cloning of OnGal-2

Total RNA was extracted from the control fish according to the manufacturer's protocol of EasyPure RNA Kit (TransGen, Beijing, China). Using the EasyScript One-Step cDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) to synthesize the first strand of cDNA from the above total RNA. The ORF of OnGal-2 was cloned based on laboratory Nile tilapia transcriptome data (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA244908) and the prediction of NCBI Basic Local Alignment Search Tool.

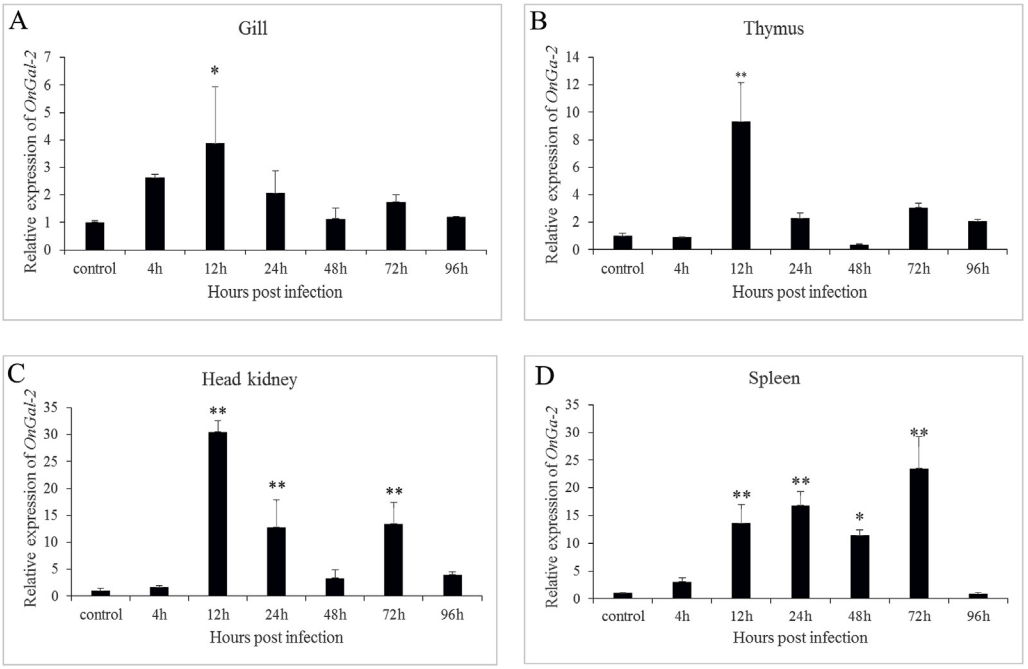

Fig. 2. Expression analysis of *OnGal-2* in head kidney, thymus, gill, and spleen after *S. agalactiae* challenge was performed by relative quantitative RT- PCR. Data are shown as mean ± SDs. Significant difference was indicated by asterisks as *0.01 < P < 0.05 and **P < 0.01.

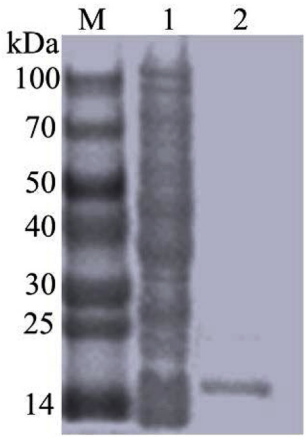

Fig. 3. SDS-PAGE and Western blot of rOnGal2. (A) Lane M: markers (14–100 kDa); lane 1, bacteria liquid before IPTG induction; lane 2, purified rOnGAL2. (B) Lane M, markers (15–180 kDa); lane 1, Western blot analysis of rOnGAL2. The gel used in this study is 10%.

2.3. Bioinformatics analysis of OnGal-2

The correct positive clone results were spliced with above steps, the DNAMAN Version 6 software was used for splicing to obtain the coding sequence of the OnGal-2. The BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to conduct the nucleotide alignment and amino acid sequences. The ORF Funder (http://www.ncbi.nim.nih.gov/govf/govf.html) and ExPASy Proteomics Server (http://ca.expasy.org) were used to predict the amino acid sequence, molecular weight, open reading frame and hydrophilic parameters of the sequence. Using Signal P 4.0 Server (http://www.cbs.dtu.dk/services/signalp) to predict the signal peptide sequence. Using Clustalw2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to analyze the multiple alignment of the reported OnGal-2 amino acid sequences. Using MEGA-6 to build phylogenetic trees [14]. Using PORTER (http://www.distill.ucd.ie/porter) to predict the secondary structure. Using SWISSM-MODEL (http://www.fundp.ac.be/sciences/biologie/Urbm/bioinfo/esypred) to predict the tertiary structure.

2.4. Quantitative real-time PCR of OnGal-2 mRNA

RNA from 10 different tissues (heart, brain, spleen, liver, thymus, head kidney, intestine, muscle, gill, and skin) was extracted and then reverse-transcribed into cDNA as described in 2.2. The β-action (housekeeping gene) was taken as the internal reference gene. Quantitative RT-PCR (qRT-PCR) was operated on Roche LC384 Lightcycler™ (Roche, Switzerland) with a PCR reaction volume of 10 μL including 5 μL of FastStart Essential DNA Green Master (Roche, Switzerland), 0.5 μL of each primer, and 3.5 μL of nuclease free water. The program was performed as follows: 1 cycle of 10 min at 95 °C, 40 cycles of 10 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C. The relative expression level of OnGal-2 mRNA were calculated using $2^{-\Delta\Delta Ct}$ method [15].

2.5. Challenge experiment

To investigate the expression pattern of OnGal-2 after Nile tilapia stimulated by *S. agalactiae*, the total RNA of 4 tissues (gill, thymus, head kidney, spleen) at different points in time, which were mentioned in section 2.1, were extracted, the cDNA were synthesized, and the qRT-PCR were operated as section 2.4. Statistical significance was presented to assess the difference expression levels of OnGal-2 between pre- and post-infected tissues [13].

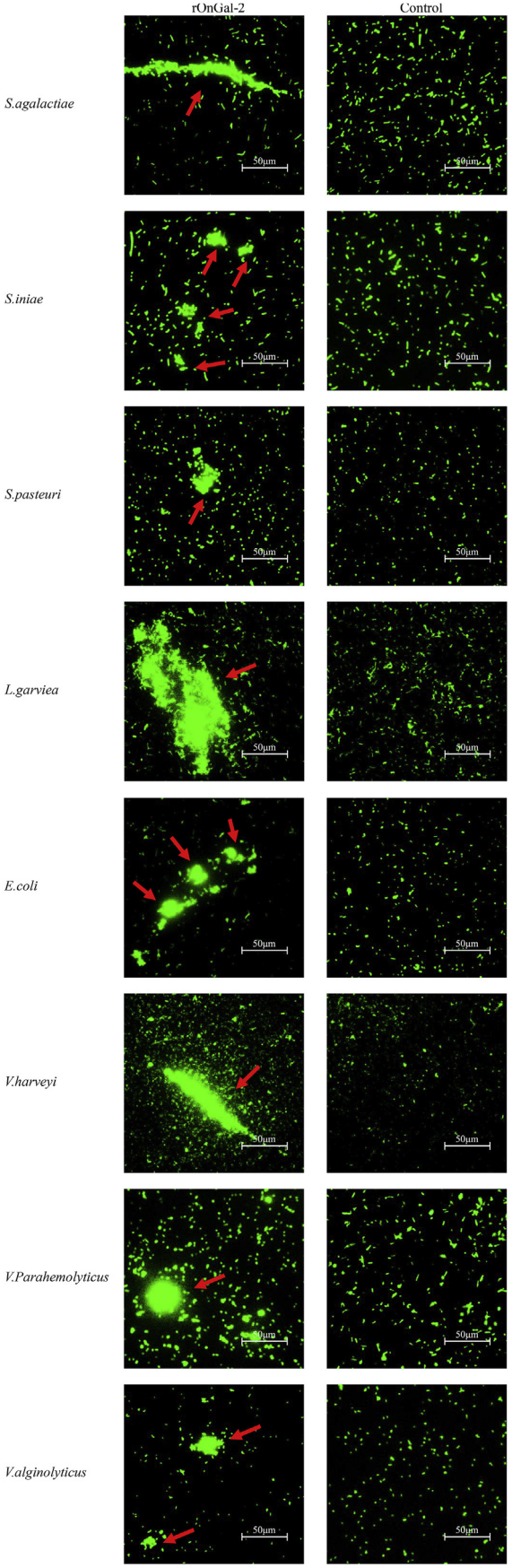

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Fig. 4. Agglutinating activity of rOnGal-2 against FITC-labeled *Vibrio harvey*, *Vibrio alginolyticus*, *Vibrio parahemolyticus*, *Escherichia coli*, *Streptococcus agalactiae*, *Streptococcus iniae*, *Staphylococcus pasteuri*, and *Lactococcus garviea*. PBS was incubated with bacteria as a negative control. The agglutination was presented with red arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. Expression and purification of rOnGal-2

A pair of primers (EOnGal-2-F and EOnGal-2-R) with restriction sites (*Bam*HI and *Xho*I) were designed (Table 1), and tilapia spleen cDNA was used as a template for OnGal-2 PCR amplification. PCR product was purified and ligated into the pMD18-T vector. After 30 min at 37 °C, the plasmid was transformed into *E. coli* DH5α competent cells. The cells were shaken for 1 h in a constant temperature shaker at 37 °C, and then plated the cells onto an LB plate containing Amp + and cultured for 8 h. Single colonies were picked and colonies were identified using the universal primer M13/RV. The positive clones were sent to Shanghai Sangon Biotech for sequencing.

The correct recombinant plasmid and pET-28 expression plasmid were extracted and sequenced, and *Bam*HI and *Xho*I were used for double digestion. The digested product was recovered and ligated with T4 ligase for 18 h and transformed into *E. coli* BL21 competent cells. The Kana resistance plate was used to screen the binding colonies. Single colonies were picked for PCR identification, and the positive clone recombinant plasmids were sent to Shanghai Sangon Biotech for sequencing.

The positive strain of pET-28 empty plasmid and the expression plasmid were inoculated in fresh Kana containing LB liquid medium at a ratio of 1:100, and cultured under shaking at 37 °C conditions until the OD_{600} reached 0.4–0.6, then add IPTG to a final concentration of 1 mmoI/L. The bacterial solution was collected after 5 h of induction, and then washed three times with PBS. Lysozyme was added to a final concentration of 1 mg/mL, and placed on ice for 30 min. The supernatant was purified using a His-tag protein purification kit (Beyotime, China), desalted and concentrated using an Amicon Ultra Centrifugal Filter (Amicon, USA). The purified protein was analyzed by reducing SDS-PAGE.

2.7. Agglutination assay

The ability of the recombinant protein to agglutinate different bacteria was determined by the method of Mu [16]. Briefly, four Gram-negative bacteria (*V.harveyi*, *V. alginolyticus, V. Parahemolyticus*, and *E. coli* and four Gram-positive bacteria (*S.agalactiae*, *S. iniae*, *L. garviea*, and *S. pasteuri*) were labeled using FITC(Solaribo, China) [17], incubated at 37 °C for 30 min, centrifuged three times to completely remove the FITC, and then incubate rOnGal-2 with the bacteria at room temperature for 1 h. The bacteria were applied to a grass slide and the results were observed with a fluorescent microscope.

2.8. Phagocytosis-promoting activity analysis

Macrophages were prepared according to the method of Huang et al. [13]. Briefly, the head kidney of tilapia was cut and filtered through a 40 μm stainless nylon mesh (Greiner Bio-OneGmbH, Germany) and the cell suspension was suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 25 U/mL heparin (Gibco). The cell suspension was carefully added to a 51%/34% percoll (GE Healthcare) density gradient, centrifuged at 400 g for 40 min, and the cell layer of the interface was carefully aspirated and then washed with PBS at 500 g for 10 min [13]. Cell viability was measured using a trypan Blue staining kit (Sangon Biotech).

Here, *S. agalactiae*, a Gram-positive bacteria, which was considered as the most serious pathogen in tilapia, and used with another Gram-positive bacteria, *V. harveyi*, to detect the phagocytosis-promoting activity of rOnGal-2. In brief, 200 μL of FITC-labeled bacterial were incubated with 200 μL of macrophages and 20 μL of rOnGal-2 (1 mg/mL) in a 1.5 mL EP tube in the dark, used PBS instead of rOnGal-2 as a control, shaken every 5 min [17]. Then the cell were centrifuged at 500 g for 10 min to remove the un-phagocytic bacteria in the upper layer and wash three times with PBS to ensure complete removal. Here, the macrophages plus bacteria plus rOnGal-2 group was used as an experiment, while macrophages plus bacteria plus PBS group was used as a control. The results were measured using flow cytometer. The fluorescence data for this experiment is limited to a Gate to ensure the accuracy of the analysis, and all data was repeated in triplicate.

2.9. Statistical analysis

All data in this study were displayed as means ± standard deviation (SDs). Statistical analysis was performed by the LSD (least significant difference) test using SPSS 17.0 software. Differences were considered significant at $p < 0.05$ (*) and highly significant at $p < 0.01$ (**).

3. Results

3.1. Cloning and sequence analysis of OnGal-2

Data not shown.

3.2. Real-time PCR analysis of OnGal-2 mRNA expression in different tissues

Quantitative RT-PCR was used to detect the expression level of *OnGal-2* in healthy tilapia tissues. The data presented herein indicate that OnGal-2 mRNA is present in the all inspected tissues, with the highest level of expression in brain, followed by spleen, liver, intestine, heart, thymus, muscle, skin, head kidney, and gill (Fig. 1).

3.3. Real-time PCR analysis of the response of OnGal-2 mRNA expression to bacterial infection

Expression patterns of *OnGal-2* in different tissues of tilapia after *S. agalactiae* challenge was performed by qRT-PCR. At 4 h post infection, the gill began to show a clear upward trend of *OnGal-2*, which reached its highest level at 12 h, then began to decline and reached the lowest level at 48 h, which was similar to the initial level, and rose slightly in the last 72 h (Fig. 2A).

The expression levels of *OnGal-2* in thymus and head kidneys showed the same time-dependent trend, peaking at 12 h and 72 h, respectively, the former being much higher than the latter and reaching the lowest level at 48 h (Fig. 2B and C). Spleen at 12 h post infection showed significant up-regulation of *OnGal-2*, reached the first peak at 24 h, and the second peak was displayed at 72 h with a higher level (Fig. 2D).

3.4. Recombinant OnGal-2 expression and purification

The recombinant plasmid pET-28a-OnGal-2 was transformed into *E. coli* strain BL21 (DE3). The soluble recombinant protein was successfully expressed. As showed in Fig. 3, a band of approximately 17 kDa was detected, which was consistent with the predicted result.

3.5. Agglutinating activity of rOnGal-2

FITC-labeled Gram-positive (*S.agalactiae*, *S. iniae*, *S. pasteuri*, and *L. garviea*) and Gram-negative (*E. coli*, *V. harveyi*, *V. Parahemolyticus*, and *V. alginolyticus*) bacteria were used to test the agglutination ability of

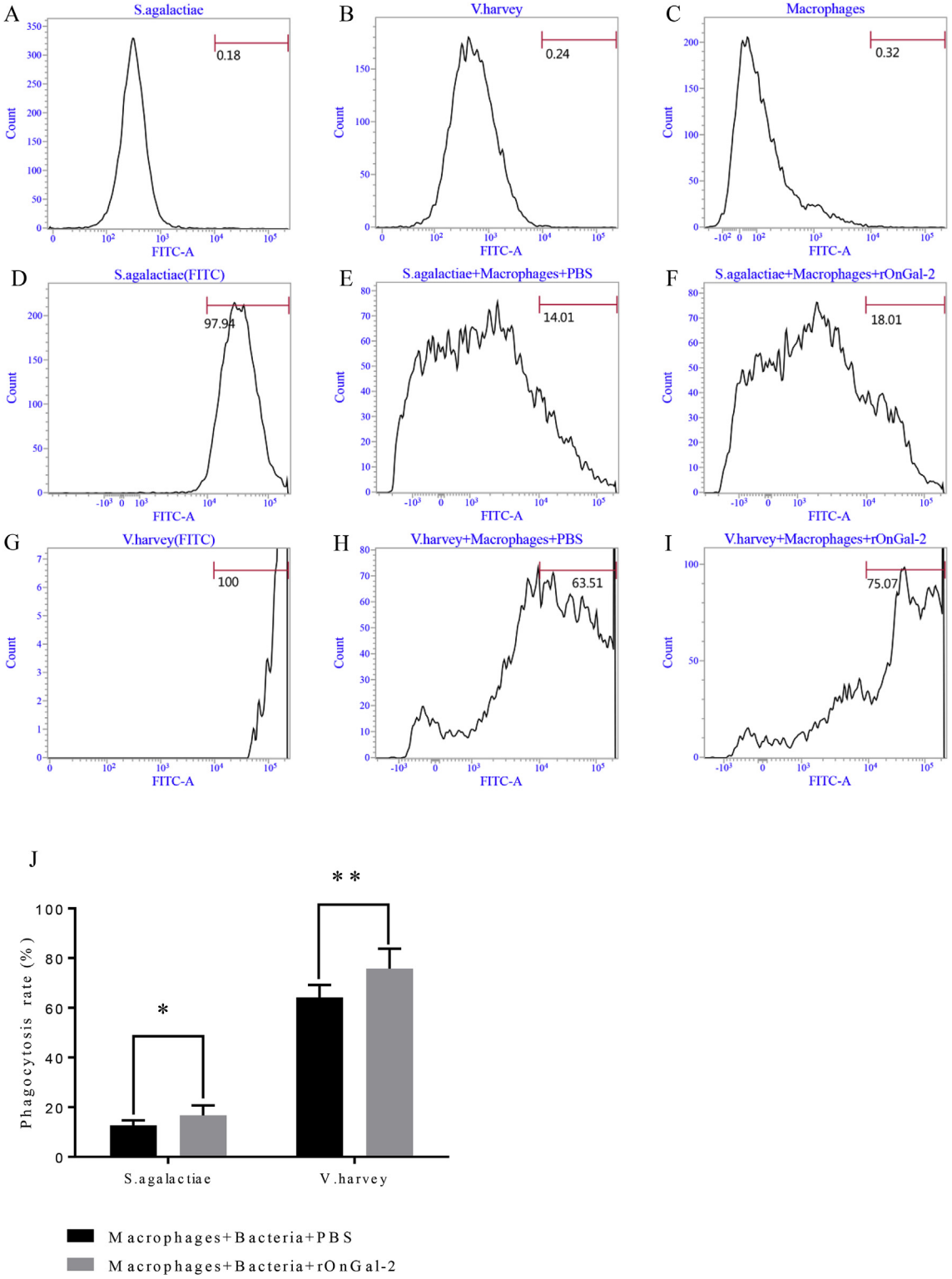

Fig. 5. Effects of (r)OnGal-2 on phagocytosis of Nile tilapia macrophages. Flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *V. harvey* which were treated as described in Section 2. Data show analyses of 10,000 events. (A, B, and C) The histogram of the *S. agalactiae*, *V. harvey*, and macrophages alone. The marker represented phagocytosis part. (D and G)The histogram of label *S. agalactiae* and *V. harvey*. (E, F, H, and I) The histogram of flow cytometric analyses of the macrophages phagocytosing *S. agalactiae* and *V. harvey* pre-incubated with PBS or (r)OnGal-2. The phagocytosis rates were shown near the marker. The results shown here were from one experiment out of three independent experiments. **(J)** Histogram showing phagocytosis rates. The average standard deviation was obtained from three experiments. The symbol * shows a significant difference from control ($p < 0.05$).

rOnGal-2. As showed in Fig. 4, rOnGal-2 could agglutinate all bacteria, while the PBS showed no agglutination activity for any bacteria.

3.6. Enhancement of phagocytosis by rOnGal-2

The ability of rOnGal-2 to promote the phagocytosis of macrophages by bacteria was determined by flow cytometry. As shown in this study, there were almost no fluorescent signals for the bacteria and phagocytic cells before labeling (Fig. 5A–C)th, and the bacteria could be completely labeled with FITC (Fig. 5D and G), so the intensity of the fluorescent signal can be used to represent the phagocytic efficiency. The phagocytosis results of the experimental group and the control group were shown in histogram (Fig. 5E, F, H, and I). Statistical analysis of the data indicate that the rOnGal-2-treated group had a stronger fluorescent signal compare to PBS-treated group (Fig. 5J, hinting that rOnGal-2 could effectively promote phagocytosis.

4. Discussions

Galectin, a member of β-galactoside binding protein family, play key roles in modulating innate and adaptive immune responses. In this study, a galectin (OnGal-2) was successfully identified and characterized from Nile tilapia. The *OnGal-2* transcript levels of various tissues could be induced by bacterial challenge. Further studies of rOnGal-2 have shown significant agglutination effects and effectively enhancement phagocytosis of bacteria by macrophages, suggesting that OnGal-2 might participate in immune response during bacterial infection.

The current study found that OnGal-2 existed in all tested tissues with the highest expression in brain, followed by spleen, liver, intestine, heart, thymus, and lower expression in muscle, skin, head kidney, and gill. The highest expression in brain was also observed in rat Gal-2, suggesting that galectin-2 may be involved in synaptogenesis and neurite fasciculation [18,19]. Given that *S. agalactiae* can penetrate into the brain microvascular endothelial cell (BMEC) of tilapia [20] and induce complement-mediated lysis of the body to kill infected cells [21], implying that gal-2 could be involved in response against *S. agalactiae* invasion [12]. Additionally, relative high expressions of OnGal-2 were detected in immune related tissues, including spleen, liver and intestine. Similar distributions of gal-2 were also observed in *Hyriopsis cumingii* [22]. As we known, spleen and intestine are the major peripheral lymphoid organs in fish [23,24]. The widespread of OnGal-2 in these tissues indicated that OnGal-2 might possessed multiple functions during immune response.

It has been well-recorded that galectins act as a pattern recognition receptor during various bacterial pathogens infection [25]. In this study, the temporal expression patterns of *OnGal-2* upon different tissues (gill, thymus, head kidney and spleen) were investigated by qRT-PCR. Gill is the first barrier in fish mucosal immune system and is highly prone to infections [26], and the thymus is considered as the central immune organ [27]. Moreover, head kidney and spleen are the two main immune organs in teleost and main target organs attacked by bacterial infection [28]. The result showed that OnGal-2 was up-regulated in a time-dependent manner with distinct manifestations in the gill, thymus, head kidney and spleen after *S. agalactiae* infection, similar findings were observed in the study of *Labeo rohita* [29], *Hyriopsis cumingii* [22], and *Litopenaeus vannamei* [30]. In the gill, OnGal-2 increased quickly within 4 h p i., suggesting that tilapia OnGal-2 could participate in mucosal immunity and play an important role in the antibacterial response. In addition, the expression of OnGal-2 in thymus, head kidney, and spleen are up-regulated more dramatic at 12 h after being challenged, showing that OnGal-2 was able to make fast response following infection in immune related tissues [31]. The largest transcriptional response was observed in head kidney (30 folds, $p < 0.01$), which is consistent with its role as an important endocrine and hematopoietic-lymphoid organs in teleost [32]. Interestingly, OnGal-2 was significantly up-regulated again at 72 h post-injection in head kidney and spleen, which was consistent with the research of yellow catfish galectin [33]. Concerning our results, it can be explained as the melano-macrophage accumulations of the parenchyma are able to retain the antigen for longer time [29]. Taken together, these results exhibited that OnGal-2 might be involved in the immune response of tilapia.

To further explore the functions of OnGal-2 during pathogen invasion, recombination protein of OnGal-2 (rOnGal-2) was prepared and applied for further analysis. The results shown that rOnGal-2 can aggregate a variety of bacteria including Gram-positive and Gram-negative bacteria, similar result was found in other reported fish galectins [22,34]. The agglutinating activity of rOnGal-2 against bacteria will prevent these pathogens from entering cell and remaining in extracellular surface or matrix [35], which may facilitate phagocytosis of bacteria by macrophages. In addition, rOnGal-2 could significantly enhance phagocytosis of macrophages, similar result was observed in the study of Gal-2 in *S. constricta* [17]. The dual-functions in pattern recognition and opsonization of fish lectin protein were also reported in the study of Yin et al. [36]. Moreover, given that macrophage play crucial roles in the process of recognizing, ingesting, and eliminating microbial pathogens [37], these data imply that OnGal-2 might resist bacterial invasion through bacterial agglutinating activity and promoting macrophages phagocytosis.

5. Conclusion

In this study, a Gal-2 homolog was successfully identified and characterized from Nile tilapia (OnGal-2), which was widely distributed in immune related tissues of healthy tilapia and could be induced following bacterial challenge. In addition, the recombinant protein could significantly aggregate both Gram-positive and Gram-negative bacteria and promote phagocytosis activity of macrophages. These data suggest that OnGal-2 plays a vital role in immune response of tilapia.

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