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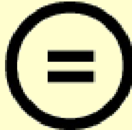

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A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Galectins from red-lip mullet, *Liza haematocheila* : Comparative expression profiling and functional aspects as pattern recognition receptor in host immune defense system.

Chaehyeon Lim

DEPARTMENT OF MARINE LIFE SCIENCES

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

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February 2020

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Galectins from red-lip mullet, *Liza haematocheila* : Comparative expression profiling and functional aspects as pattern recognition receptor in host immune defense system.

Chaehyeon Lim

(Supervised by Professor Jehee Lee)

A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science

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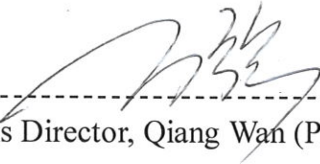

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Summary

Activation of immunity depends on the recognition of pathogens by innate immune cells through diverse pattern recognition receptors (PRRs) that initiate their signaling pathway and facilitate secretion of pro- and anti-inflammatory cytokines. Galectins, a family of carbohydrate binding protein have emerged as soluble mediators in infected cells and pattern recognition receptors responsible for evoking and controlling innate immunity. In previous studies, it was demonstrated that various galectin family members are concerned with the physiological turnover of particular innate immune cells; neutrophils, mast cells, macrophages, and dendritic cells.

Redlip mullets (*Liza haematocheila*) are belonged to the Mugilidae family which consists of more than 72 species. The statistics have shown that all species of mullets accounted for 8% of the total consumption and cultivation in Korea. The amount of total fish production of the coastal fishery has been decreased consistently. Recently, high mortality emerged in red lip mullet fishery in Korea, and it was identified that *Lactococcus garvieae*, which caused green liver syndrome in redlip mullet lead the mass mortality in mullets. Therefore, not only the identification of immune-related genes but study on their response to pathogens is essential for the prevention of diseases and development of the aquaculture industry.

In this study, two immune associated genes, galectin-1 and galectin related protein B like were identified from the established redlip mullet cDNA database and defined as *LhGalectin-1* and *LhGalectin related protein B like*. Moreover, mRNA expression patterns of both genes were comparatively analyzed in tissue distribution as well as in several timepoints of specific tissues induced by Lipopolysaccharide (LPS), polyinosinic: polycytidylic acid (poly I:C) and *Lactococcus garvieae* as immune stimulants. In addition, recombinant LhGal-1 and LhGal B like were examined their carbohydrate binding ability with lactose, galactose and

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glucose by ELISA method. Furthermore, microbial binding assay and agglutination assay was performed to figure out their functional characterization as a PRRs.

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요약문

초기면역시스템이 활성화되기 위해서는 다양한 패턴인식수용체들을 통한 선천면역세포들의 병원체 인식이 가장 먼저 이루어진다. 패턴인식수용체가 외부 병원체를 인식하면 세포 내부로의 신호전달이 개시되고 전염증성 또는 항염증성 사이토카인의 분비를 촉진시킨다. galectin 은 당결합단백질로써 세포 내부 또는 외부의 리간드에 결합하거나 외부에서 침입한 병원체 표면의 glycan 에 결합하여 패턴인식수용체로 작용함으로써 선천면역반응을 이끌어내고 그 반응들을 조절하는데 기여하는 것으로 알려져 있다. 이전 연구에서도 galectins 이 호중구, 비만세포, 대식세포와 같은 특정한 선천면역세포의 분화에 있어서 생리학적인 변화를 조절하는데 관련이 있다고 밝혀졌다.

이번 연구에서는 가숭어의 두 가지의 면역관련 유전자인 galectin-1 과 galectin related protein B like 를 규명하였고 조직내 전사체 발현 패턴을 분석하기 위해 면역 자극제인 LPS, poly I:C 그리고 가숭어로부터 분리한 *Lactococcus garvieae* 를 이용하여 challenge 실험을 진행하였다. 그리고 challenge 후 시간대별로 각 조직을 채취하고 조직으로부터 RNA 를 분리하고 cDNA 를 합성하여 real-time qPCR 을 통해 두 유전자의 각 조직별 mRNA 발현 양상뿐만 아니라 면역 자극 후 시간대별 발현 양상을 확인하였다. 두 유전자의 기능 및 활성을 시험해보기 위해 재조합 단백질을 생산하고 당에 대한 특이성이 있는 지 알아보기 위해 ELISA 방법을 이용하여 sugar binding assay 를 진행하였고, microbial binding assay 와 agglutination assay 를 통해 rLhGal-1 과 rLhGal B like 가 PRR 로써 기능을 규명하였다.

Galectin-1 과 galectin related protein B like 는 가숭어로부터 유래된 유전자이며 통계에 의하면 가숭어는 한국 어류 전체 소비량 및 생산량의 8 퍼센트를 차지하는 양식 어종이다. 하지만 최근에 한국의 가숭어 양식장에서 Green liver syndrome 이라고 하는 질병이 발병하여 대량 폐사가 잇따라 일어났으며, 원인체는 *Lactoccocus garvieae* 로 밝혀졌다. 따라서 이번 연구에서는 가숭어 유래 면역유전자에 대한 규명하였고 규명된 유전자가 병원체에 대해 어떠한 작용을

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하는 지에 대한 연구가 진행되었으며 이는 앞으로 가숭어의 질병 예방과 가숭어 양식 산업의 발전에 있어서 바탕이 되는 중요한 기초자료가 될 것이다.

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Galectins from red-lip mullet, *Liza haematocheila* : Comparative expression profiling and functional aspects as pattern recognition receptor in host immune defense system.

1. Introduction

Lectins

Lectins are a group of carbohydrate binding proteins known to play an important role in intercellular and extracellular functions including cell differentiation, opsonization, apoptosis, proliferation and phagocytosis. They are primarily involved in pathogen recognition which is the first step of the innate immune response to subsequently trigger pro-inflammatory responses against invading pathogens through protein-carbohydrate interactions (Innate & Recognition, 2013). According to the structure and functions of carbohydrate recognition domains (CRD), lectins have been sorted into C-type, F-type, P-type, ficolins, pentraxins, and galectins.

Galectins family

Galectins are a conserved family of carbohydrate binding proteins that include at least one CRD and widely distributed in mammals, birds, amphibians, fish, nematodes, sponges, and some fungi. In contrast with other lectins, galectins function in Ca^{2+}-independent manner and have two properties; one is a characteristic affinity for ß-galactosides, and the other is having a conserved carbohydrate recognition domain (CRD) sequence motif. Evolutionally conserved galectins have generally classified into proto, chimera and tandem-repeat types according to structural differences of the CRDs. Proto-type galectins include one CRD with non-covalently linked homodimers. Chimera-type galectins are comprised of a C-terminal CRD and N-terminal domain rich in proline and glycine. Tandem-repeat type galectins are embodied with two CRD and a functional linker

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peptide which are required for joining two domains (Vasta, 2012).

Galectins have potential roles in mediating innate and adaptive immune responses. In previous studies, major functions of galectins were reported in terms of mediators in the developmental process including cell differentiation, tissue organization and regulation of immune homeostasis by binding endogenous (self) glycans (Poirier, Hedlund, Qian, Leffler, & Carlsson, 2004). Moreover, it has become clear that galectin family can bind to exogenous (non-self) glycans on the surface of viruses, bacteria, parasites and fungi so-called pathogen-associated molecular patterns (PAMPs) as pattern recognition receptors PRRs in the innate immune system (Vasta, Quesenberry, Ahmed, & O'Leary, 1999).

Redlip mullet

Redlip mullets (*Liza haematochelia*) are belonged to Mugilidae family, which consists of more than 72 species from 17 fish genera, with a worldwide distribution (Minos, 2014). Redlip mullet is the only species of being cultured among them so that they are regarded as valuable aquaculture species in South Korea, China, Japan and in the North West Pacific Ocean. The statistics published by the Korean Ministry of Maritime Affairs and Fisheries have shown that all species of mullets accounted for 8% of the total consumption and cultivation in Korea as well as the amount of total fish production of the coastal fishery have been decreased consistently. Recently, there was high mortality in red lip mullet fishery in Korea, and it was identified that *Lactococcus garvieae*, a gram-positive bacteria which caused green liver syndrome in red lip mullet. (Han, Lee, Kim, & Jung, 2015). Notwithstanding the outbreak of disease from red-lip mullet, the study of the host immune defense mechanism is deficient. Therefore, not only the identification of immune-related genes but the study on their response to pathogens is essential for the prevention of diseases and the development of the aquaculture industry.

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In this study, two immune associated genes, galectin-1 and galectin related protein B like were identified from the established redlip mullet cDNA database and defined as *LhGalectin-1* and *LhGalectin related protein B like*. Moreover, mRNA expression patterns of both genes were comparatively analyzed in tissue distribution as well as in several timepoints of specific tissues induced by Lipopolysaccharide (LPS), polyinosinic: polycytidylic acid (poly I:C) and *Lactococcus garvieae* as immune stimulants. In addition, recombinant LhGal-1 and LhGal B like were examined their carbohydrate binding ability with lactose, galactose and glucose by Enzyme linked immuno sorbent assay (ELISA) method. Furthermore, microbial binding assay and agglutination assay was performed to figure out their functional characterization as a PRRs.

Figure 1. Redlip mullet (*Liza haematocheila*)

2. Material and method

2.1. Fish rearing, immune challenge, and tissue isolation

Healthy red lip mullets were obtained from the Sangdeok fishery in Hadong, South Korea with average body weight and length of 100 g, 40 cm respectively. Selected fish were sustained at 20 °C in 300 L tanks for seven days preliminary to experimentation.

For immune stimulation experiment, unchallenged mullets with average body weight

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100 g were divided into three different immune stimulants groups and one control group; Lipopolysaccharide (LPS; 1.25 μg/g, from *Escherichia coli* 055:B5; Sigma, St. Louis, MO, USA), polyinosinic:polycytidylic acid [poly(I:C) 1.5 μg/g], *Lactococcus garvieae* (1×10^3 colony-forming units [CFU]/μL) were prepared with 1× phosphate-buffered saline (PBS). 100 μL of each stimulant was injected intraperitoneally into individual fish and 100 μL of PBS injection was carried out for the control group. After the challenge, blood, spleen and head-kidney were collected and stored in the same method as tissue distribution.

For the tissue-specific mRNA expression analysis, five healthy mullets were anatomized; kidney, spleen, gill, intestine, stomach, heart, blood, liver, muscle, skin, brain and heart. Blood were drawn from the caudal vein of fish by sterile syringes coated with heparin sodium salt (USB, USA) and blood cells were collected thereby centrifugation at 3000 × g, 4°C for 10 minutes. Eleven different tissues were carefully isolated and immediately become solid with liquid nitrogen and they were stored in -80°C freezer until used for RNA extraction.

2.2. Total RNA extraction and cDNA synthesis

Prior to total RNA extraction, the collected tissues were pooled (n = 5) for tissue distribution and immune challenge, respectively. Total RNA was isolated by RNAiso plus kit (TaKaRa, Japan) and subsequently clean-up with RNeasy spin column (Qiagen, USA). After RNA extraction, quality and concentration value of purified RNA were measured with μDrop Plate (Thermo Scientific) at 260 nm and followed by running 1.5 % agarose gel electrophoresis. Thereafter, cDNA synthesis was carried out with the PrimeScript™ II 1st strand cDNA synthesis kit (TaKaRa, Japan) using the final volume of 20 μL reaction mixture which contained 2.5 μg of RNA. The synthesized cDNA samples were diluted 40-fold and stored at -80°C.

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2.3. Quantitative real-time PCR

For analyzing expression pattern of mRNA in each tissue and timepoints after challenge, qPCR (TaKaRa Thermal Cycler Dice: TP850 Real Time System) was performed using synthesized cDNA as a template and gene-specific primers: LhGal-1 (Forward (F): 5′- GTCGCTAAACCTGACGCTTCCAAC -3′ / Reverse (R): 5′- ATTGTGTGCAACTCCTACCAGGGA -3′) and LhGal B like (F: 5′- TGCACCTGGAGAACCCTTCAAGAT -3′ / R: 5′- TCCCTCACTGCCTTACGAGTCTTC -3′). Mullet elongation factor 1α (EF1α) gene (Accession No: MH017208) (F: 5′- CCCTGGTCAGATCAGTGCTGGTTAT -3′ and R: 5′- AGCGTCGCCAGACTTTAGGGATTT -3′) was served as the internal control for qPCR. The total reaction in 10 μL was contained 3 μL of diluted cDNA, 5 μL of 2× SYBR® Premix Ex Taq™, 0.4 μL of each primer (10 pmol/ μL) and 1.2 μL of nuclease-free water. To amplify and detect a fluorescent signal the thermal cycler was used as following conditions: initial denaturation at 94 ℃ for 10 sec; 35 cycles of 94 ℃ for 5 sec, 58 ℃ for 30 sec, and 72 ℃ for 1 min; and a final cycle of 95 ℃ for 30 sec, 72 ℃ for 30 sec, and 95 ℃ for 15 sec. Each sample of cDNA was tested in triplicate to increase accuracy.

2.4. Identification and *in silico* analysis of *LhGal-1* and *LhGal B like* sequence.

Mullet cDNA database was constructed using Illumina and PacBio full-length transcriptome sequencing technology. The sequence of LhGal-1 and LhGal B like were identified by searching on the Basic Local Alignment Search Tool (BLAST) (Altschul, Gish, Miller, Myers, & Lipman, 1990) at the National Center for Biotechnology Information (NCBI) and compared with other known orthologous genes sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frames (ORFs) and their

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translated amino acid sequence were determined by an online software, ORF finder from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Then, NCBI Conserved Domain Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and ExPASy-PROSITE (https://prosite.expasy.org/) were used to analyze the extrapolated protein domain sequence of LhGal-1 and LhGal B like. Besides, SignalP-5.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to ascertain the location and potential of signal peptide in LhGal-1 and LhGal B like sequences. Based on several sequence information, the cloning and real-time qPCR primers were designed by Integrated DNA Technologies (IDT) (https://sg.idtdna.com/pages). Multiple sequence alignment was performed by CLC Main Workbench 8.0.1 to determine the conserved domain and residues. Also, the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software was used for phylogenetic analysis of Neighbor-joining method with 5000 bootstrap replicates to figure out speciation and evolutionary relationships between different kinds of species based upon similarities and difference within their molecular genetic characteristics.

2.5. Cloning and purification of rLhGal-1 and rLhGal B like

For cloning the ORF of LhGal-1 and LhGal B like, PCR was performed to amplify the target sequences with designed primers appending restriction enzyme sites. After purifying the PCR products, pMAL-c5X and inserts were digested with the same restriction enzymes; *Eco*RI and *Eco*RV and ligated the digested expression vector and target genes using DNA Ligation Kit (Mighty Mix) from Takara. To realize the mass production of recombinant plasmid DNA, the transformation was performed into *E.coli* DH5α. Then, the recombinant plasmid was extracted by AccuPrep® Plasmid Mini Extraction Kit and samples were sent to Macrogen, Korea to confirm their sequences. The confirmed LhGal-1 and LhGal B like were transformed into competent *E.coli* ER2523 cells and overexpressed by using

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isopropy-b-thiogalactopyranoside (IPTG) induction. Briefly, transformed *E.coli* ER2523 cells were incubated in 500 mL of LB broth containing 500 µL of ampicillin (100 µg/mL) and 0.2 % glucose at 37 °C. When the optical density at 600 nm (OD_{600}) reached 0.6, IPTG (final concentration of 1 mM) was added to induce the expression of rLhGal-1 and rLhGal B like fusion protein and then cultured mediums were further incubated at 25 °C for 8 hours. Then, the induced cells were harvested by centrifugation (3500 rpm for 15 min at 4 °C) and subsequently washed with column buffer (20 mM Tris-HCL, pH 7.4 and 200 mM NaCl) 2 times and stored at -20 °C. Column chromatography was used for protein purification followed by pMAL™ Protein Fusion & Purification System manual (New England BioLabs Inc.). Finally, the concentration of purified protein was measured by the Bradford method and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the size and purity of the target fusion protein. The eluted protein was stored at -80 °C.

2.6. Sugar binding assay

The sugar-binding ability of LhGal-1 and LhGal B like was examined by Enzyme linked immuno sorbent assay (ELISA) method under three different carbohydrates; α-lactose, galactose and glucose. (47287-U, Sigma Aldrich, USA,) In brief, each lactose, galactose and glucose were dissolved in carbonate-bicarbonate buffer (50 mmol L^{-1}, pH 9.6) at the final concentration of 100 mM and coated on 96-well micro titer plate at 25 ℃ overnight. After washing the plate three times with TBS-T and the plate was blocked by 5 % skim milk in TBS-T. The plate was washed 5 times and then 50 µL of serially diluted rLhGal-1, rLhGal B like and MBP were treated. After incubated at 37 ℃, 10 rpm for one hour, each well was washed again 3 times and added 100 µL of mouse anti-MBP antibody diluted in 1:5000 ratio as the primary antibody. The plate was incubated at 37 ℃ for 2

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hours and washed again with TBS-T. One hundred microliters of goat anti-mouse IgG (FC) HRP conjugate diluted 1:5000 were treated and the plate was incubated at the same condition with primary antibody. Finally, the plate was washed 5 times and reacted with TMB solution at room temperature for 5 to 10 minutes in dark. For terminating the reaction, 50 µL of stop solution (1 M H_2SO_4) was added and the absorbance was measured by Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA) at 450 nm wavelength.

2.7. Microbial binding assay

Enzyme linked immuno sorbent assay (ELISA) method was used with total seven kinds of bacteria; three of Gram-positive (G+) species (*L. garvieae, S. iniae, S. parauberis*) and four of Gram-negative (G-) species (*E. coli, E. tarda, V. anguillarum, V. harveyi*) so that determine microbial binding affinity of LhGal-1 and LhGal B like. Briefly, seven bacteria were cultured in medium (LB broth: *E. coli, V. anguillarum* and BHIS with 1.5% NaCl: *L. garvieae, S. iniae, S. parauberis, E. tarda, V. harveyi*) overnight and harvested by centrifugation at 13,000 × g for 10 min 4 °C. Harvested cells were washed with PBS twice and resuspended in PBS to $O.D_{600}$ = 1 and diluted with carbonate-bicarbonate buffer (50 mmol L^{-1}, pH 9.6) to 1 x 10^8/mL. One hundred microliters of diluted bacteria cells with coating buffer (1 x 10^7/well) were coated in 96-well plate at 25 °C overnight. After washing the plate three times with TBS-T, each well was blocked with 250 µL of 2% skim milk/TBS-T at 37 °C for one hour. Then, 100 µL of rLhGal-1 and rLhGal B like and MBP (final concentration 10 µg/mL) were treated in wells then the plate was incubated at 37 °C for 2 hours and elution buffer was added for the control group. Since washing the plate three times with TBS-T, one hundred microliters of mouse anti-MBP antibody diluted in 1:5000 ratio added into individual wells and let them react at 37 °C for 2 hours. The plate

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was washed again and 50 μL of secondary antibody, goat anti-mouse IgG (FC) HRP conjugate diluted 1:5000 was treated and incubated at 37 °C for 2 hours. After washing with TBS-T for five times, 50 μL of TMB solution was used for detection and let it react for five to ten minutes at room temperature in dark. To stop the reaction, an equal volume of stop solution (1M H_2SO_4) was added into each well and the absorbance of the plate was measured by Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, USA) at 450 nm.

2.8. Agglutination assay of *LhGal-1* and *LhGal B like* on bacteria

In order to examine the agglutination ability of rLhGal-1 and rLhGal B like, the agglutination assay was carried out with *E. coli*. Briefly, the bacteria were cultured in LB broth at 37 °C overnight and harvested by centrifugation at 13,000 × g for 10 min 4 °C. Harvested cells were washed with TBS (20 mM Tris, 137 mM NaCl, pH 7.8) twice and added 50 μL into the individual well of 96-well plate (5×10^6 CFU/ mL). Ten micrograms of rLhGal-1 and rLhGal B like was treated and at 25 °C for one hour. Ten millimole EDTA and MBP were treated for the control group. Bacterial cells in distinguishable conditions were observed by a light microscope to determine bacterial agglutination.

3. Result and discussion

3.1. Bioinformatic analysis

LhGal-1 and LhGal B like genes are characterized by sequencing and several bioinformatic analysis from constructed redlip mullet transcriptome database. The length of LhGal-1 ORF (open reading frame) have been presented 408 bp encoded 135 amino acid with a predicted molecular weight of 15.31 kDa and 4.89 p*I* (theoretical isoelectric point). In addition, the LhGal B like was comprised of 438 bp coding sequence encoded

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putative 145 amino acid residues with a predicted molecular weight of 16.07 kDa and 6.74 p*I*. A lack of signal peptide in their whole aa sequence was proved by signal 4.1 server, which suggests that these molecules might be secreted via the non-classical secretory pathway (Liu, Boulianne, Lu, Kües, & Aebi, 2015).

The deduced amino acid sequences of LhGal-1 and LhGal B like was possessed of a single GLECT carbohydrate recognition domain. It is supported that both genes are considered as the prototype of galectin. Besides, each domain contained eight conserved residues of sugar-binding pockets for β-galactoside (^{45}H, ^{47}N, ^{48}P, ^{49}R, ^{60}V, ^{62}N, ^{69}W, ^{72}E, ^{74}R / ^{59}Q, ^{61}K, ^{63}S, ^{70}Q, ^{72}N, ^{79}W, ^{82}S, ^{84}N) respectively according to InterPro. Although LhGal-1 has four cysteine residues and LhGal B like has two, the disulfide bond was not identified by ScanProsite. The predicted 3D models of LhGal-1 and LhGal B like were described as their common structure which is folded as a β sandwich composing two anti-parallel β-sheets. Furthermore, the five strands of β sheets are slightly bent and formed on the concave side and convex side. All the main residues for carbohydrate-binding take position on the concave side of β sheets. It is suggested that this groove allows holding long enough linear tetra-saccharide as a sugar-binding pocket (Poirier et al., 2004).

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1 **ATG** ATG AAA GAT CTG ATC ATA AAG AAC ATG TCC TTT AAG GTC GGA CAA ACA ATG ACC ATT 60
1 M M K D L I I K N M S F K V G Q T M T I 20

61 GTT GGA GTC GCT AAA CCT GAC GCT TCC AAC TTT GCA TTC AAC ATC GGC CCC AGT GAG GAG 120
21 V G V A K P D A S N F A F N I G P S E E 40

121 GAG ATT ACA ATG CAC ATC AAT CCT CGT TTT AAT GCC CAT GGA GAC GAG AAT ACG ATT GTG 180
41 E I T M H I N P R F N A H G D E N T I V 40

181 TGC AAC TCC TAC CAG GGA GGC AGC TGG TGT GAG GAG CAG CGG GAG TGC TGC TTC CCT TTC 240
61 C N S Y Q G G S W C E E Q R E C C F P F 80

241 CAG CAG GGG GAG GAG TTC AAG ATT GTC ATC GAA TTC ACC CCG TCA GAG TTC GTG GTG ACT 300
81 Q Q G E E F K I V I E F T P S E F V V T 100

301 TTA TCG GAC GGC TCT GCC ATC CAC TTC CCC AAC CGC ATG GGC GCT GAG AAA TAC CAG TTC 360
101 L S D G S A I H F P N R M G A E K Y Q F 120

361 ATC AGC TTC GAC GGG GAC GCT CGC ATC AGA AGC TTT GAG ATC AAG **TAA**
121 I S F D G D A R I R S F E I K

Figure 2. The nucleotide and deduced amino acid sequences of LhGal-1 from Redlip mullet. The start codon (ATG) and stop codon (TAA) are indicated by bold. The GLECT carbohydrate recognition domain is indicated in a gray box. The residues of sugar binding pocket are marked by the red letters. The residues of dimerization Interface are indicated by the green letters.

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1	**ATG**	GAA	GAA	ACG	GAC	AAG	AAA	GAA	AAT	GGG	GAG	TAT	ACC	GGA	GAA	ATA	AAA	GGT	GGG	ATG	60
1	M	E	E	T	D	K	K	E	N	G	E	Y	T	G	E	I	K	G	G	M	20
61	CGG	CCT	TCA	ATG	AAA	CTA	GTT	GTT	ATG	GGT	ATT	GTT	AAC	AAA	AAG	CCC	AAA	AGC	ATT	GAG	120
21	R	P	S	M	K	L	V	V	M	G	I	V	N	K	K	P	K	S	I	E	40
121	GTG	ACT	CTG	TCC	AGT	AAG	CCT	CAG	GAG	GAG	GAC	ACG	GAG	GGC	GAT	GTG	GGC	CTG	CAG	CTA	180
41	V	T	L	S	S	K	P	Q	E	E	D	T	E	G	D	V	G	L	Q	L	60
181	AAG	GTC	AGC	TTC	ATG	GAC	AAG	GCC	GTC	CAA	CGC	AAC	GCC	CGC	CTG	GCC	GGA	AAG	TGG	GGT	240
61	K	V	S	F	M	D	K	A	V	Q	R	N	A	R	L	A	G	K	W	G	80
241	CCG	TCA	GAG	AAT	ACG	CTC	TCC	TTT	TTT	CCT	TTT	GCA	CCT	GGA	GAA	CCC	TTC	AAG	ATG	GAG	300
81	P	S	E	N	T	L	S	F	F	P	F	A	P	G	E	P	F	K	M	E	100
301	ATC	GTT	TGC	GAG	CAC	CAG	CAG	TTT	CGC	ATC	CTG	GTG	GAC	GGG	CAG	CCT	CTG	TGC	GGC	TTC	360
101	I	V	C	E	H	Q	Q	F	R	I	L	V	D	G	Q	P	L	C	G	F	120
361	TCC	CAC	CGC	CTC	TCC	CCG	CTC	GCC	TCC	CTC	ACT	GCC	TTA	CGA	GTC	TTC	GGC	GAC	CTG	CAG	420
121	S	H	R	L	S	P	L	A	S	L	T	A	L	R	V	F	G	D	L	Q	140
421	CTC	ACC	AAG	GTG	GCC	**TAA**															
141	L	T	K	V	A	*															

Figure 3. The nucleotide and deduced amino acid sequences of LhGal-B like from Redlip mullet. The start codon (ATG) and stop codon (TAA) are indicated by bold. The GLECT carbohydrate recognition domain is indicated in a gray box. The residues of sugar binding pocket are marked by red letters. The residues of the putative alternate dimerization interface are marked by green letters. The residues of dimerization Interface are marked by blue letters.

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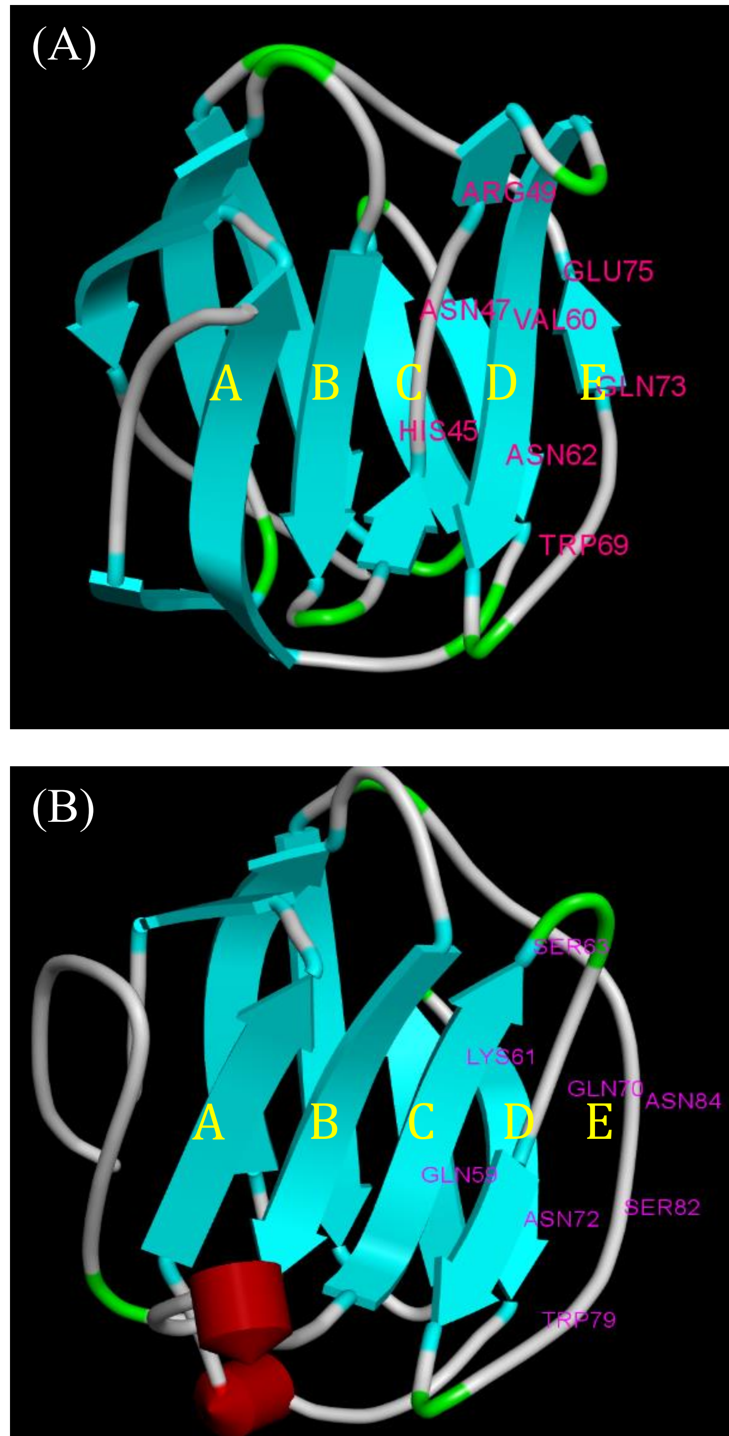

Figure 4. (A) The predicted molecular model of redlip mullet LhGal-1 3D structure. (B) The predicted molecular model of redlip mullet LhGal B like 3D structure. The yellow A, B, C, D, E indicate carbohydrate binding site and the main residue is named with pink color.

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Multiple sequence alignment was performed to infer sequence homology and conservation of protein domains throughout vertebrate evolution. LhGalectin-1 has conserved CRD motifs (H-NPR and -W-E-R) which is crucial and typical residues of galectin family, related with carbohydrate binding affinity. On the other hand, LhGal B like has no predominant CRD motif but there are conserved residues for sugar binding site instead. Moreover, the main residues for carbohydrate binding of LhGal-1 has highly conserved with other galectins from fishes, mammals, birds, reptiles and amphibians. However, LhGal B like has significantly different patterns compared with other species in which important residues for sugar binding pockets as well as more closely related to the fish group than the members of mammals and amphibians. The result of analyzing 3D structure and multiple sequence alignment of LhGal B like was shown that ^{62}N, ^{69}W--^{72}E-^{74}R was substituted for ^{72}N, ^{79}W--^{82}S-^{84}N and asparagine^{72} was highly conserved throughout the species but tryptophan^{79}, serine^{82} and asparagine^{84} was attributively conserved in the fish group.

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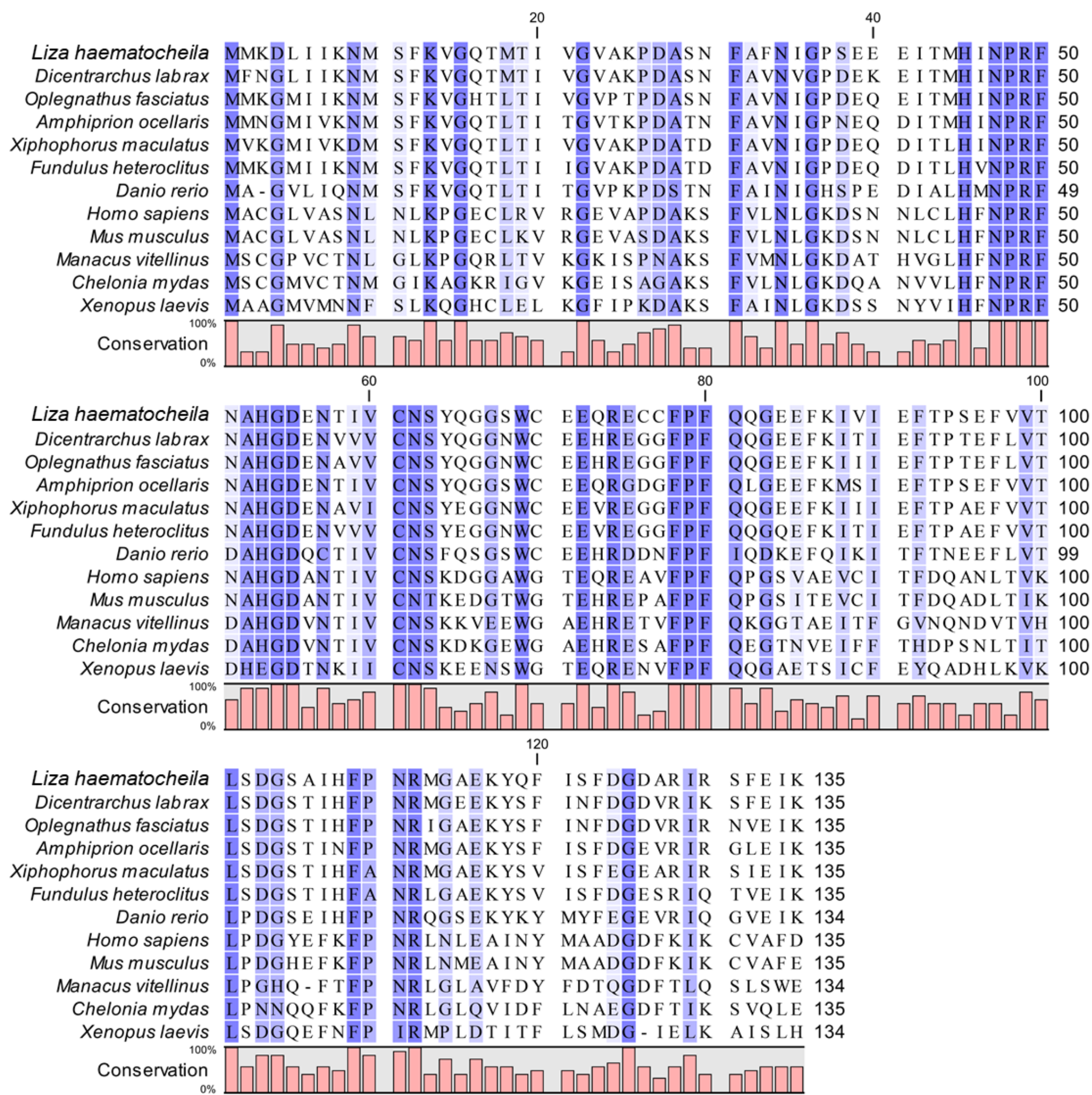

Figure 5. Multiple sequence alignment of LhGal-1 with other known galectin-1 amino acid sequences. The residues of sugar binding pocket are indicated by the '★' symbol. The residues of putative alternate dimerization interfaces are indicated by the '◆' symbol.

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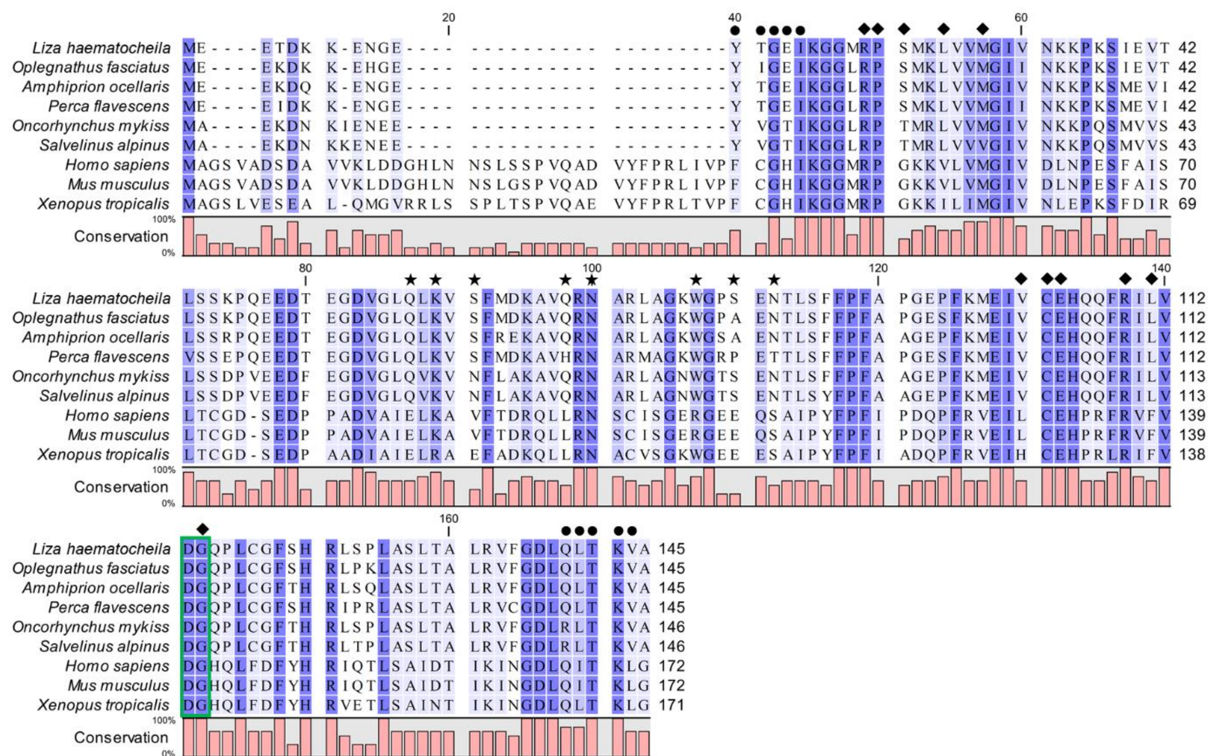

Figure 6. Multiple sequence alignment of LhGal-B like with other known galectin-B like amino acid sequences. The residues of sugar binding pocket are indicated by the '★' symbol. The residues of putative alternate dimerization interfaces are indicated by the '◆' symbol. The residues of dimerization Interface are indicated by the '●' symbol.

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In order to comprehend the evolutionary relationship of mullet Galectin-1 and Galectin related protein B like in the galectin family, phylogenetic analysis was performed with the registered amino acid sequence of galectin-1, 2, 3, 9 and GRP in NCBI. As a result of phylogenetic tree using neighbor-joining method, LhGal-1 and LhGal B like are clustered in two different clades but belong to the fish group. According to the output data of pairwise comparison and phylogenetic analysis, it revealed that both genes have closely related with each gene from rock bream as well as the identified sequence of GRP from redlip mullet was designated galectin related protein B like.

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Figure 7. The Phylogenetics tree of LhGal-1 and LhGal-B like was constructed using MEGA version 6.0 with 5,000 bootstrap repeats and neighbor-joining method.

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Table 1. Percentage of identity and similarity value of LhGal-1 with homologs.

No	Species	Taxon	GenBank Accession No	Amino acids	Identity (%)	Similarity (%)
1	*Dicentrarchus labrax*	Bony fishes	ACF77003.1	135	83.7	89.6
2	*Oplegnathus fasciatus*	Bony fishes	ADV35589.1	135	81.5	89.6
3	*Amphiprion ocellaris*	Bony fishes	XP_023123473.1	135	81.5	88.9
4	*Xiphophorus maculatus*	Bony fishes	XP_005806370.1	135	77.8	91.1
5	*Fundulus heteroclitus*	Bony fishes	XP_012737057.1	135	76.3	90.4
6	*Danio rerio*	Bony fishes	AAR84190.1	134	63	80
7	*Homo sapiens*	Primates	NP_002296.1	135	41.6	57.7
8	*Mus musculus*	Rodents	NP_032521.1	135	38.7	56.9
9	*Manacus vitellinus*	Birds	XP_008929802.2	134	36.3	51.9
10	*Chelonia mydas*	Turtles	XP_027680905.1	135	37.8	55.6
11	*Xenopus laevis*	Frogs	AAK11514.1	134	38.6	50

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Table 2. Percentage of identity and similarity value of LhGal-B like with homologs.

No	Species	Taxon	GenBank Accession No	Amino acids	Identity (%)	Similarity (%)
1	*Oplegnathus fasciatus*	Bony fishes	AMR74959.1	145	93.8	96.6
2	*Amphiprion ocellaris*	Bony fishes	XP_023142396.1	145	91	95.9
3	*Perca flavescens*	Bony fishes	XP_028422504.1	145	88.3	93.1
4	*Oncorhynchus mykiss*	Bony fishes	XP_021430595.1	146	82.2	89.7
5	*Salvelinus alpinus*	Bony fishes	XP_023996989.1	146	79.5	90.4
6	*Homo sapiens*	Primates	NP_054900.2	172	35.5	53.5
7	*Xenopus laevis*	Frogs	NP_001001900.1	171	34.1	57.2

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3.2. Tissue-specific distribution of *LhGal-1* and *LhGal B like* expression

The mRNA expression analysis was revealed that *LhGal-1* and *LhGal B* like were omnipresent expressed genes in all the tested tissues. In the case of LhGalectin-1, the stomach has the highest mRNA expression level ($p < 0.05$), followed by heart, muscle, brain, intestine, spleen, head kidney, gill, skin, kidney, liver, and blood. Previous studies were reported that galectin-1 from *Ctenopharyngodon Idella* and *Epinephelus coioides* were highly expressed in muscle and heart (Zhu et al., 2019); (Chen et al., 2016). Galectins were widely known to be abundant in muscle tissues, some neurons, thymus and epithelial tissues. Galectin-1 was involved in countless biological phenomena by interacting with multifarious receptors in different cell types (Elola, Chiesa, Alberti, Mordoh, & Fink, 2005). Above all, galectin-1 bind to polylactosaime chains of laminin and promote cell detachment and attachment in the extracellular matrix which influences muscle development. However, according to tissue distribution analysis in the present study, galectin-1 was mostly expressed in the stomach followed by heart and muscle. As for stomach, it was already reported in previous research that the fundic mucin and epithelial cell surface glycocalyces were intensely recognized by galectin-1 from wistar rats in the gastrointestinal (GI) tract (Wasano & Hirakawa, 1997). Whereas, LhGal B like has significantly high expression levels in the brain ($p < 0.05$), followed by gill, muscle, stomach, intestine, kidney, heart, spleen, skin, head kidney, liver and blood. In the previous study, galectin related protein B like from rock bream also highly expressed in the brain and gill (Thulasitha, Whang, et al., 2016).

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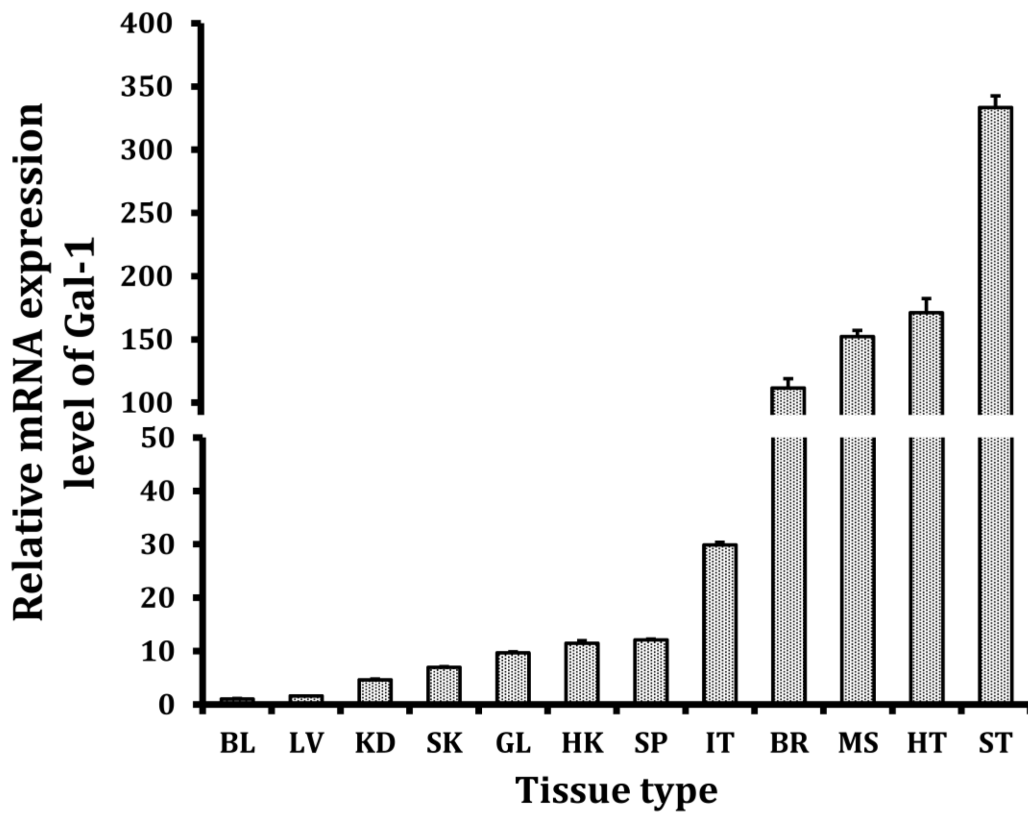

Figure 8. The relative expression levels of *LhGal-1* in different tissues.

The expression levels of *LhGal-1* was measured using *EF1α* gene as the reference gene compared with the expression level of blood. BL: blood, LV: liver, KD: kidney, SK: skin, GL: gill, HK: head kidney, SP: spleen, IT: intestine, BR: brain, MS: muscle, HT: heart, ST: stomach.

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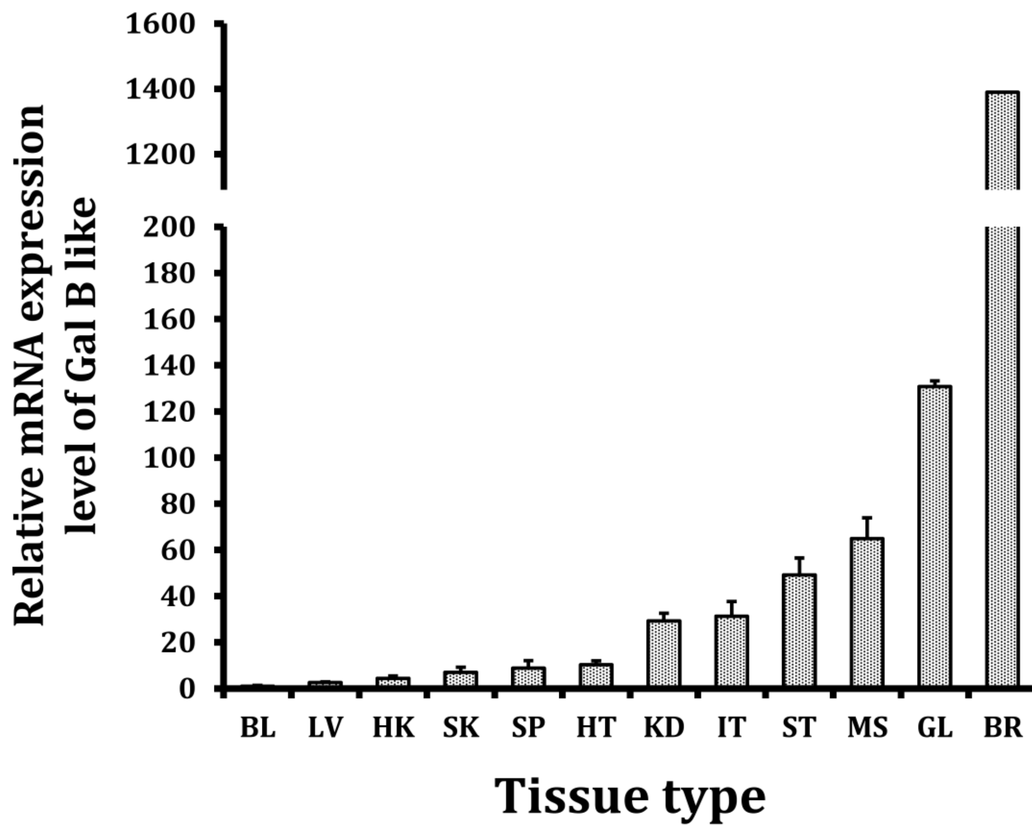

Figure 9. The relative expression levels of *LhGal-B like* in different tissues.

The expression levels of *LhGal-B like* was measured using *EF1α* gene as the reference gene compared with the expression level of blood. BL: blood, LV: liver, HK: head kidney, SK: skin, SP: spleen, HT: heart, KD: kidney, IT: intestine, ST: stomach, MS: muscle, GL: gill, BR: brain

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3.3. Expression pattern analysis of *LhGal-1* and *LhGal B like* after immune challenge

Transcriptional regulation of LhGal-1 and LhGal B like was hourly examined in gill and blood after challenging immune stimulants with poly I:C, LPS or *L. garvieae* in order to estimate submerged involvement of LhGal-1 and LhGal B like in immune response. The gill of aquatic organisms is play important role as the first line of host defense and constantly exposed to danger of pathogen invasion from the external environment. Moreover, the blood cells are involved in the surveillance of diverse infectious microbes and cellular immune responses. Accordingly, these candidates were selected to establish the temporal mRNA expression profiling of LhGal-1 and LhGal B like post-injection of immune stimulants. In the gill, after injection of LPS, *L. garvieae*, the mRNA expression of LhGal-1 and LhGal B like were significantly up-regulated at 48 h. The poly I:C treatment in the gill was shown significant up-regulation at 48 h and 72 h but down-regulation at 6 h and 24 h. As for the relative mRNA expression level of target genes from the blood, LhGal-1 was dramatically up-regulated at 6 h post-injection of LPS, poly I:C and *L. garvieae*. Whereas LhGal B like was down-regulated at 6 h and subsequently upregulated at 24 h after stimulation with all selected treatment. The previous study reported that the mRNA expression pattern of galectin-1 from rock bream in the gill significantly down-regulated in the beginning but up-regulated at 48 h p.i. by *E. tarda* and *S. iniae* (Thulasitha, Umasuthan, Whang, Nam, & Lee, 2016). Galectin from crab in the hemocyte was up-regulated in early time point by *V. anguillarum*, *M. luteus* and *P. pastoris* (Wang et al., 2016). Compared to the control group (0 h), mRNA expression of LhGal-1 and LhGal B like after injection were significantly up-regulated in the gill and blood. Hence, it is considered that LhGal-1 and LhGal B like might have a potential correlation with PAMPs and gram-positive bacteria. All these results are suggested that LhGal-1 and

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LhGal B like might affect to the host as a regulator of immune cells and to the irrupted pathogen as PRR in two different tissues of red-lip mullet.

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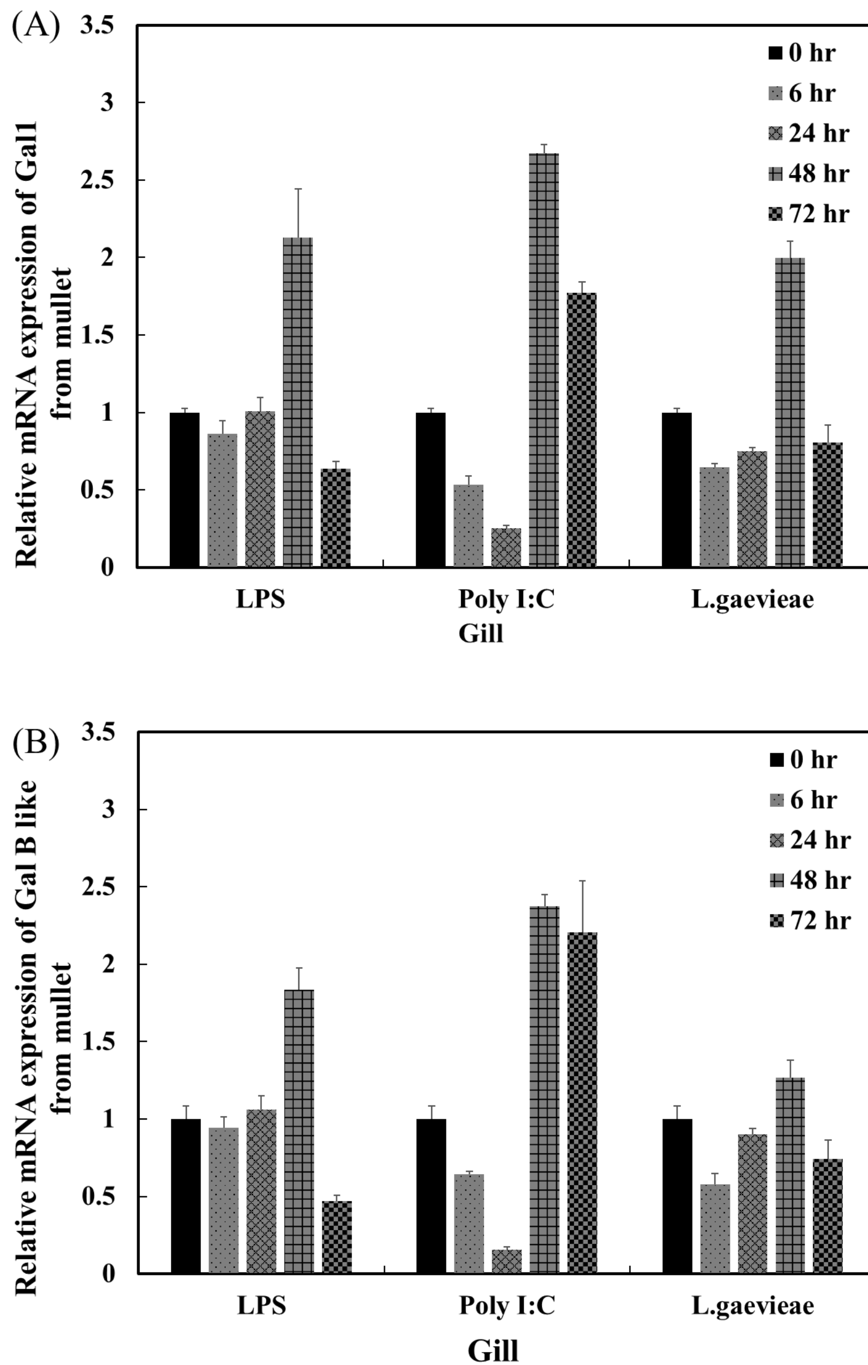

Figure 10. The mRNA expression of (A) LhGal-1 and (B) LhGal B like in the gill upon challenge experiment with LPS, poly I:C and *L. garvieae.* The mRNA expression of LhGal-1 and LhGal B like was calculated by $2^{-\Delta\Delta CT}$ method using LhEF1α as the reference gene. The relative mRNA level was compared with PBS-injected control at each time point. Error bars represent the standard deviation (SD, n=3)

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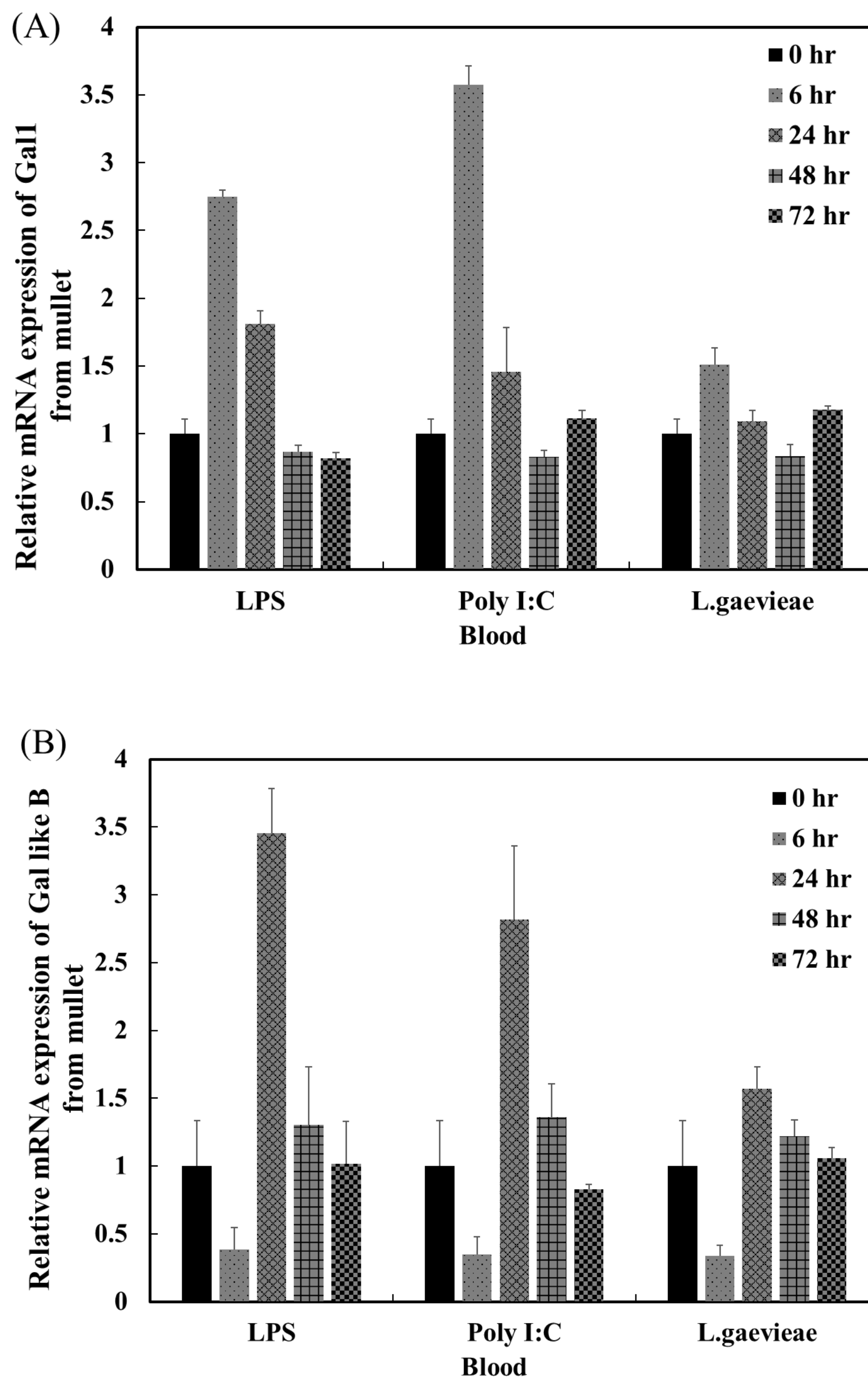

Figure 11. The mRNA expression of (A) LhGal-1 and (B) LhGal B like in the blood cell upon challenge experiment with LPS, poly I:C and *L. garvieae.* The mRNA expression of LhGal-1 and LhGal B like was calculated by $2^{-\Delta\Delta CT}$ method using LhEF1α as the reference gene. The relative mRNA level was compared with PBS-injected control at each time point. Error bars represent the standard deviation (SD, n=3)

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3.4. Sugar binding ability of rLhGal-1 and rLhGal B like

In order to investigate the carbohydrate specificity and binding ability of rLhGal-1 and rLhGal B like, sugar binding assay was performed with three different materials; α-lactose, galactose and glucose. Although all of the materials have the specificity of LhGal-1 and LhGal B like, disaccharide α-lactose, has a higher specificity of both galectins than monosaccharide galactose and glucose. Moreover, the result has shown the different binding ability of LhGal-1 and LhGal B like according to carbohydrate. In the case of lactose, LhGal-1 has a higher binding capacity at all concentrations of proteins than LhGal B like. While both galectins have similar binding ability of galactose and glucose at 1.25, 2.5, and 5 μg of protein, LhGal-1 has a higher binding capacity at 10 and 20 μg of protein. The result has shown differences in binding ability of LhGal-1 and LhGal B like between lactose and galactose and it is based on their structural feature. Their CRDs are beta-sandwich with two skewed sheets and shape the part of carbohydrate binding site. Six strands in beta sheet form a groove and it is long enough to capture linearized polysaccharide (Poirier et al., 2004). Even if both galactose and lactose have a common part of beta-galactoside, LhGal-1 and LhGal B like have a higher affinity with lactose which is longer than galactose. Thus, it is suggested that the binding ability of galectins is concerned with the source of affinity as well as the length of their ligands.

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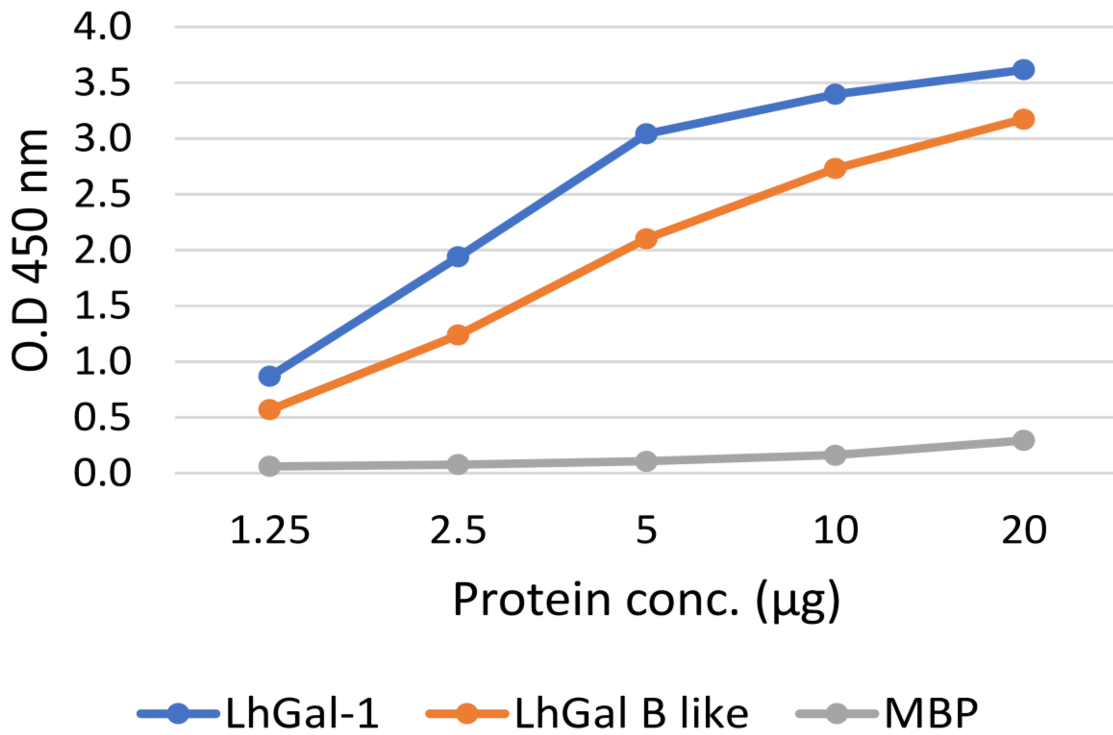

Figure 12. Sugar binding activity of rLhGal-1 and rLhGal B like for α-lactose

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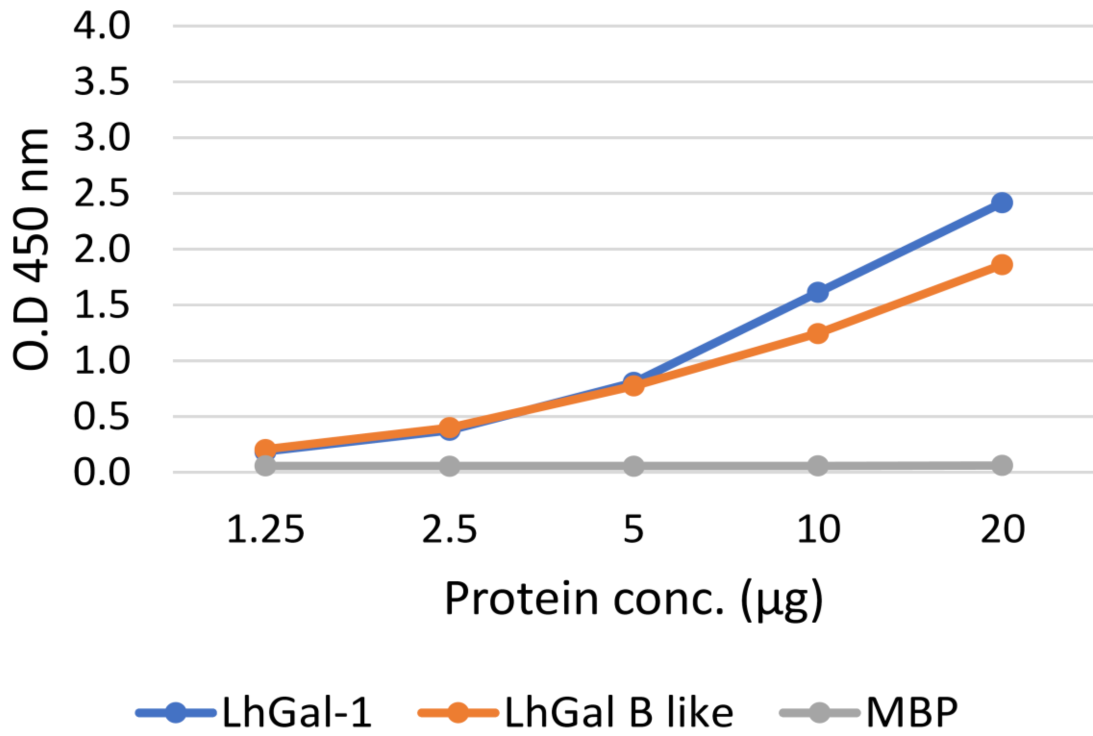

Figure 13. Sugar binding activity of rLhGal-1 and rLhGal B like for galactose.

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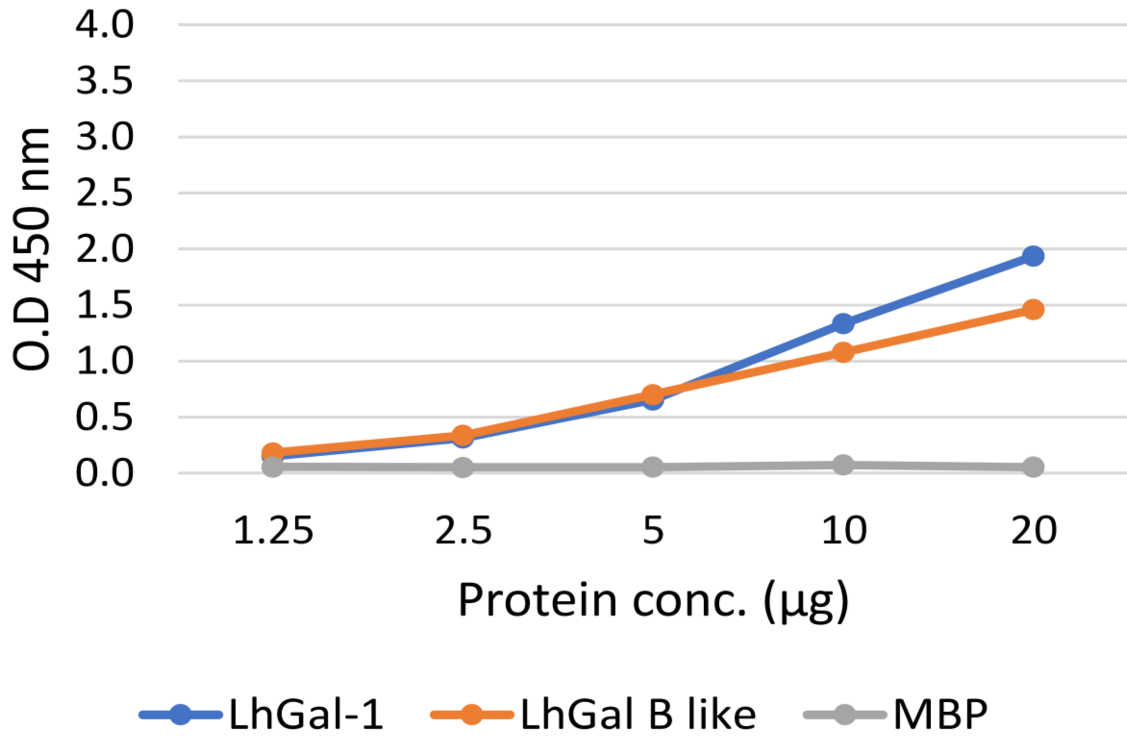

Figure 14. Sugar binding activity of rLhGal-1 and rLhGal B like for galactose.

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3.5. Microbial binding activity of rLhGal-1 and rLhGal B like

The bacterial binding ability of LhGal-1 and LhGal B like was evaluated by ELISA method with several Gram positive and negative bacteria. The result was revealed that both LhGal-1 and LhGal B like could bind to all tested G(+) and G(-) bacteria compared with elution buffer and MBP control. When reacting both galectins with *E. coli, V. harveyi, S. iniae, S. parauberis* respectively, LhGal-1 have a higher level of absorbance in comparison to LhGal B like. However, the results of the binding assay with galectins and rest of the tested bacteria (*E. tarda*, *L.garvieae*, and *V. anguilarum*) were shown that LhGal B like have similar or more affinity than LhGal-1. Although galectin-1 was known that is more conserved and has higher binding capacity than galectin related protein, and the result indicated that it depends on bacterial species. The absorbance of LhGal-1 and LhGal B like with *E.coli* has been about 4-fold and 2-fold higher than other tested microbes respectively. Binding activity of galectin from *Eriocheir sinensis* was demonstrated in the previous study that there are interactions between rEsGal and PAMPs (LPS, PGN and glycan) in dose-dependent manner. In addition, the highest binding activity was shown for interacting with LPS from *E.coli* and 200 nmol L^{-1} of galectin, (Wang et al., 2016). Among the PAMPs, lipopolysaccharides and peptidoglycan are a major component of Gram-negative and positive bacterial cell wall respectively. Moreover, both LPS and PGN contain polysaccharide in common which is the potential binding site of galectins and vary in bacterial species. Thus, these results suggested that LhGal-1 and LhGal B like could recognize the various invading pathogen and might serve as a PRR which is involved in the innate immune response of the red-lip mullet.

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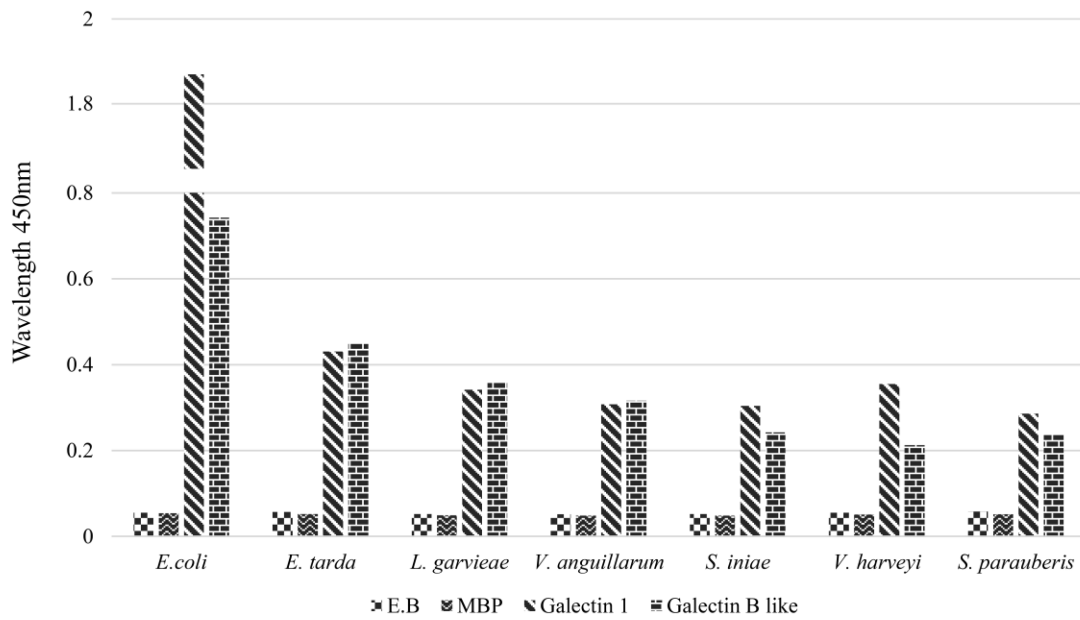

Figure 15. Microbial binding activity of LhGal-1 and rLhGal B like to several gram negative and positive bacteria; *Escherichia coli, Edwardsiella tarda, Lactococcus garvieae, Vibrio anguillarum, Streptococcus iniae, Vibrio harveyi, Streptococcus parauberis*.

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3.6. Microbial agglutination activity of rLhGal-1 and rLhGal B like

rLhGal-1 and rLhGal B like were examined their bacterial agglutination activity using gram negative bacteria *Escherichia coli*. In the present study, the result has shown that both rLhGal-1 and rLhGal B like can aggregate the *E. coli*. In the previous study, galectin-1 and galectin related protein like B from rock bream demonstrated their agglutination activity with various gram positive, negative bacteria and parasite (Thulasitha, Umasuthan, et al., 2016; Thulasitha, Whang, et al., 2016). Agglutination activity of rLhGal-1 and rLhGal B like was indicated that both galectins were involved in immune defense against gram negative *E. coli* and might work as pathogen recognition receptors or facilitator of opsonization and phagocytosis of macrophages (Cerliani et al., 2011).

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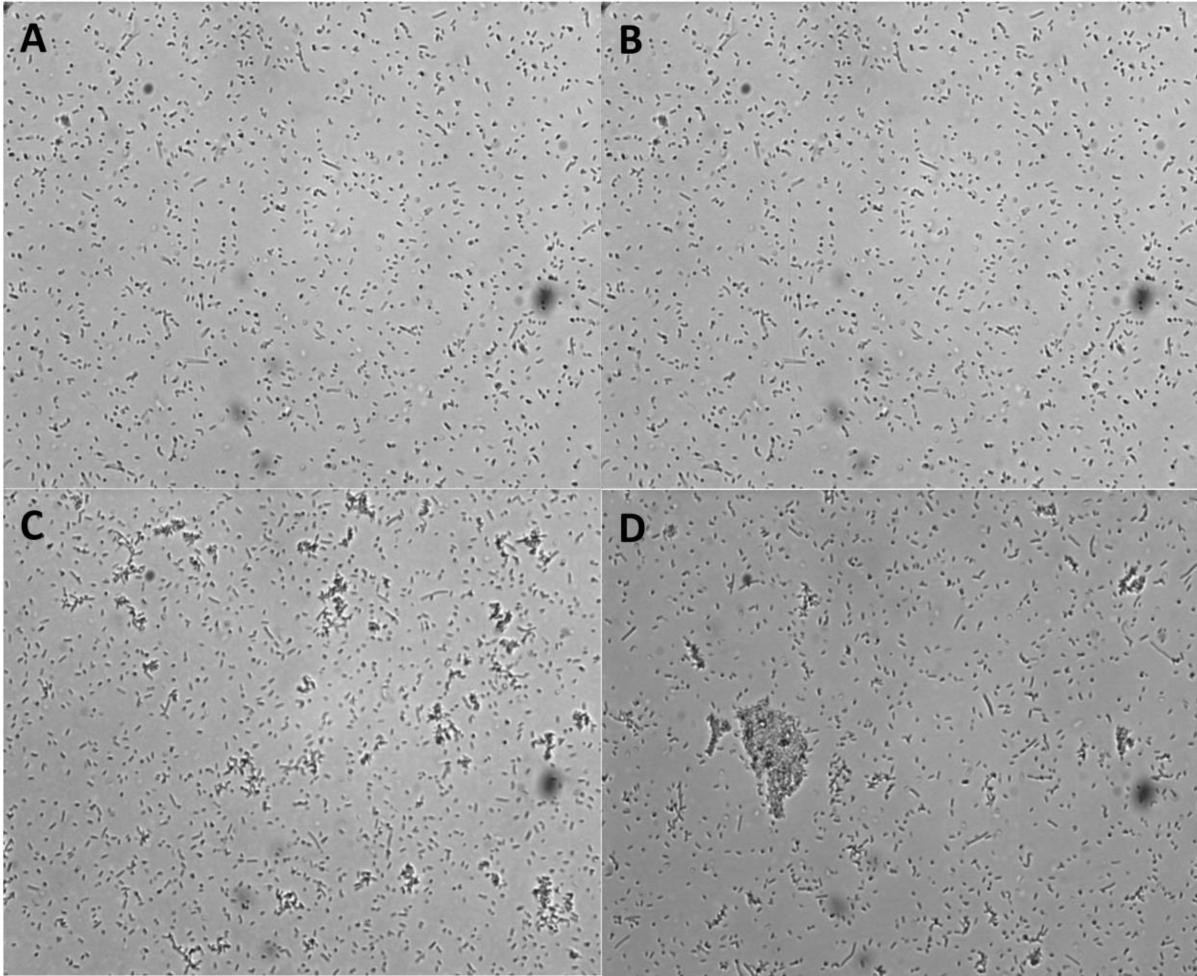

Figure 16. Agglutination of *E. coli* by rLhGal-1 and rLhGal B like.

The bacteria were grown in LB broth and harvest at 1 O.D and washed twice in TBS. Their cell number was adjusted to 5 × 10^6 CFU/ml and treated with 20 µg of rLhGal-1 and rLhGal B like. The recombinant maltose binding protein(rMBP) and TBS were used as controls. A: TBS control, B: MBP control, C: treated 20 µg of rLhGal-1, D: treated 20 µg of rLhGal B like.

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4. Conclusion

Galectin-1 and galectin related protein B like from redlip mullet, *L. haematocheila* were identified and cloned. It was revealed that both genes were evolutionarily conserved prototype galectins by several bioinformatic analyses and molecular characterization. Spatial mRNA expression profiles showed the highest expression in the stomach and brain respectively. The transcriptional regulation delineated their potential role in immunity, maintaining homeostasis and coordination in their expression. Recombinant LhGal-1 and LhGal B like proteins showed sugar and microbial binding activity and difference in their binding ability. Furthermore, they have potential involvement in bacterial agglutination in vitro. Overall, these results provided sufficient evidence to show their potential role in host-pathogen interaction and are suggested that LhGalectin-1 and LhGalectin related protein B like might have been involved in the host immune defense system.

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