Pakistan J. Zool., vol. 54(6), pp 2755-2764, 2022 **DOI**: https://dx.doi.org/10.17582/journal.pjz/20200120060106

Prokaryotic Expression, Purification, and Functional Characterization of the Large Yellow Croaker (*Larimichthys crocea*) Mannose Receptors Subunits (MRC1 and MRC2)

Xiangli Dong[1,2], Shilin Mikhail Borisovich[2], Jiji Li[1,*], Jianyu He[1], Zeqin Fu[1], Yingying Ye[1], Julia N. Lukina[3], Olga V. Apalikova[4] and Jianshe Zhang[1]

[1]*National Engineering Research Center for Marine Aquaculture, Zhejiang Ocean University, Haida South Road 1, 316022, Zhoushan, Zhejiang, PR China*
[2]*Department of Ecology, Russian State Hydro-meteorological University, Petersburg, Malookhtinskij Street 98, 195196, St. Petersburg, Russia*
[3]*Saint-Petersburg State Academy of Veterinary Medicine, Chernigovskaya Street 5, 196084, St. Petersburg, Russia*
[4]*State Research Institute of Lake and River Fisheries, Embankment Makarova Street 26, 199004, St. Petersburg, Russia*

Article Information
Received 20 January 2020
Revised 01 March 2020
Accepted 09 March 2020
Available online 22 January 2022 (early access)
Published 25 August 2022

Authors' Contribution
DX performed the experiments and wrote the article. LJ supervised and guided the experimental work, also provided project support. ZJ, SMB, JNL and OVA helped in designing the experimental scheme. HJ, FZ and YY helped in experimental work.

Key words
***Larimichthys crocea*, Mannose receptor, *Vibrio anguillarum*, Protein, Expression, Purification.**

ABSTRACT

The mannose receptor (MR) is an important receptor for the innate immune response. It is a member of the C-type lectin domain family, and has two subunits, MRC1 and MRC2. The MRC1 and MRC2 cDNA sequences have been analyzed and characterized in the large yellow croaker, a highly valued, farm raised fish that is vulnerable to many infections. Quantitative real-time PCR (qRT-PCR) analysis indicated that MRC1 and MRC2 mRNAs were expressed in eight different large yellow croaker tissues, and that their expression was up-regulated by *Vibrio anguillarum* challenge. Here, we performed membrane protein analysis and epitope analysis to select MRC1 and MRC2 protein fragments suitable for antibody production. We then PCR amplified *L.c*-MRC1 and *L.c*-MRC2 and cloned them into prokaryotic protein expression vectors (MRC1 (1044bp)-pET32A and MRC2 (993bp)-pET32A). We performed SDS-PAGE analysis of the expressed *L.c*-MRC1 and *L.c*-MRC2 proteins and demonstrated high protein expression levels and purity. This study generates some essential molecular biology tools for the study of *L. crocea* MRC1 and MRC2 protein structure and function. These tools will enable us to better understand the biological functions of MRC1 and MRC2 in defending against pathogenic bacteria challenge and the innate immune response in the large yellow croaker. These findings also provide a foundation for the preparation of a *Vibrio* vaccine.

INTRODUCTION

Marine fish play a significant role in maintaining the stability of marine ecosystems and provide a high quality protein source for human beings (He *et al*., 2014). There are many marine fish species with high economic value and methods have been developed to grow them in artificial marine culture conditions (He *et al*., 2016). For instance, the large yellow croaker (*Larimichthys crocea*), which belongs to the *Larimichthys* genus and Sciaenidae family, is a common commercial marine-cultured fish highly valued in China since the 1960s due to its high nutritional quality, palatability, and abundance. Because of overfishing in the 1970s, its population collapsed and it nearly became an endangered species (Oldham, 1982). With the development of artificial culture conditions, *L. crocea* has been raised in the southeast region of China, including Fujian, Zhejiang, Guangdong, and Guangxi provinces. However, population expansion using artificial breeding and high density farming during the 2000s lead to a decline in its immunity, causing the fish to face more threats to their survival with less resistance to aquatic environmental diseases (Dong *et al*., 2016). Many biological and non-biological factors, such as pathogenic bacteria, heavy metals, parasites, and

* Corresponding author: lijiji@zjou.edu.cn
0030-9923/2022/0006-2755 $ 9.00/0

viruses, lead to increased mortality and contribute to tremendous economic losses in the fish farming industry during serious disease outbreaks (He *et al*., 2016). The farmed croaker population is easily infected by *Vibrio anguillarum* (Dong *et al*., 2016). Previously, we sought to understand the anti-infective immune response in croaker by characterizing mannose receptor genes and their expression profiles (Dong *et al*., 2016). According to the experimental results, before the *V. anguillarum* infection, mRNAs of the mannose receptor gene were expressed in 8 tissues including liver, kidney, spleen, intestine, and heart, especially in liver, kidney and spleen. After *V. anguillarum* infection, the expression levels in liver, kidney and spleen were significantly higher than before infection, which proves that it is possible that the mannose receptor gene can regulate the infection of *V. anguillarum*.

The mannose receptor (MR) is part of the C-type lectin glycoprotein superfamily, which has three other members: the M-type receptor for secretory phospholipases A_2 (PLA_2R), DEC-205/gp200-MR6, and Endo180/uPARAP (Boskovic *et al*., 2006). All 4 members are type I transmembrane receptors, which contain an N-terminal cysteine-rich domain and a single fibronectin type II (FNII) domain. They differ from other superfamily members in that they have multiple C-type lectin-like domains (CTLDs) within a single polypeptide backbone (eight in the case of MR (Miron, 1992; Taylor *et al*., 1990)), PLA_2R, and Endo180, and ten in the case of DEC205 (Zheng *et al*., 2015; Jiang *et al*., 1995). These proteins are able to cycle between the plasma membrane and the endosomal apparatus due to discrete motifs present within their cytoplasmic domains. Despite their overall structural similarity, these four receptors have evolved to use different domains to interact with discrete ligands (East and Isacke, 2002). In addition, they differ in their ability to mediate endocytic and phagocytic events and in their intracellular destinations. Together, they represent a unique group of multi-domain, multi-functional receptors (East and Isacke, 2002).

MR was first recognized as a receptor involved in the clearance of endogenous glycoproteins in the late 1970s (Pontow, 1991). It obtained its name because its lectin activity terminated in mannose, fucose, or N-acetyl glucosamine (Kilpatrick, 2010). Since its initial description, it has been the focus of significant structural and functional characterization. It has been implicated in the recognition of exposed mannose residues on the surface of certain pathogens, and the internalization of mannosylated antigens results in enhanced T cell presentation (Taylor *et al*., 2005). MR is a pattern recognition receptor (PRR), a group of receptors that play a significant role in innate immunity responses through binding to pathogen-associated molecular patterns (PAMPs) (Gazi and Pomares, 2009). MR is primarily expressed in macrophages and dendritic cells (East and Isacke, 2002).

Two subunits (MRC1 and MRC2) of MR were isolated in the large yellow croaker (*Larimichthys crocea*) (Dong *et al*., 2016). Both subunits' structures contain extracellular, transmembrane, and cytoplasmic regions. MRC1 of *L. crocea* (*L.c*-MRC1) has an extracellular region that consists of three domains: an N-terminal cysteine-rich (CR) domain, a fibronectin type II (FN II) domain, and eight tandemly arranged C-type lectin-like domains (CTLDs). *L.c*-MRC2 is similar to *L.c*-MRC1, but lacks the CR domain (Dong *et al*., 2016). Based on their secondary structures, the tertiary structures of the two proteins could be different, suggesting that each subunit plays different functional roles in the croaker. We previously performed theoretical analysis of MRC1 and MRC2 gene expression levels (Dong *et al*., 2016). Here, we expressed the two proteins in prokaryotes and purified them. Our findings could help to develop an antibacterial vaccine, which would avoid the side effects of antibiotics and provide a greener treatment for farmed fish.

MATERIALS AND METHODS

Expression plasmid construction

MRC1-CTLD and MRC2-CTLD sequence fragments were cloned and isolated using primers designed based on the previously cloned croaker MRC1 and MRC2 cDNA (Dong *et al*., 2016) (Table I). Target fragments were ligated into the pMD18-T vector. DH5α-positive clones were identified as DH5α-pMD18-T-MRC1-C and DH5α-pMD18-T-MRC2-C. The expression sequences were then cloned into recombinant plasmids pET32α (+)-MRC1-C and pET32α (+)-MRC2-C. DH5α, *E. coli* BL21 (DE3) (Transduction Biotechnology, Co., Ltd, Wuhan, China), and pET-32α (+) competent cells were purchased from Transduction Biotechnology Co. Ltd. (Wuhan, China).

Table I.- Primers used to amplify MRC1-C and MRC2-C gene fragments.

Primer name	Primer sequence (5'-3')
MRC1-F	CCGAATTCGAATTTCGTCTGTACAAC
MRC1-R	TGCTCGAGGTAACGACGCGGTTTACCGT
MRC2-F	CCGAATTCGGTACCTCTTCTCCGGAA
MRC2-R	TGCTCGAGACCACCCAGTTCAACCGGTTTAG

PCR reaction conditions

The PCR reaction conditions were established using DH5α-pMD18-T-MRC1-C and DH5α-pMD18-T-MRC2-C as templates, and the MRC1-C and MRC2-C gene fragments were amplified. The reaction was performed in a 50 μL volume, including 25 μL PreMix

Taq, 1.6 μL dNTPs, 1 μL primer-F, 1 μL primer-R, 1 μL template cDNA, and 22 μL H_2O. The PCR amplification was conducted in a thermal cycler (Bio-Rad, USA) using the following amplification conditions: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C, with a final extension of 5 min at 72°C. After double digestion with EcoRI/XhoI, the target fragment was recovered using a DNA gel recovery kit.

Preparation of competent *E. coli* BL21 (DE3) using $CaCl_2$ and transformation of *E. coli* BL21 (DE3) were performed using methods published in the "Guidelines for Molecular Cloning" (Nguyen *et al*., 2002). One microliter of culture was used to screen for positive clones by PCR. The PCR was performed in a 15 μL volume, including 1 μL template pMD18-T-MRC1 / 2-C, 1 μL MRC-R, 1 μL MRC-F, 7.5 μL PreMix Taq, and 4.5 μL H_2O. The PCR amplification was conducted in a thermal cycler (Bio-Rad, USA) using the following conditions: 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 60 s at 72°C, with a final extension of 10 min at 72°C. Plasmids were extracted from PCR-positive bacterial cultures using a small volume extraction kit (Kehaojia Biological Technology Co., Ltd., OMEGA, Wuhan) and subjected to double restriction digestion. Electrophoresis was performed and the results were examined under UV light. Positive clones were identified by PCR and restriction enzyme digestion. The recombinant plasmids were then sequenced.

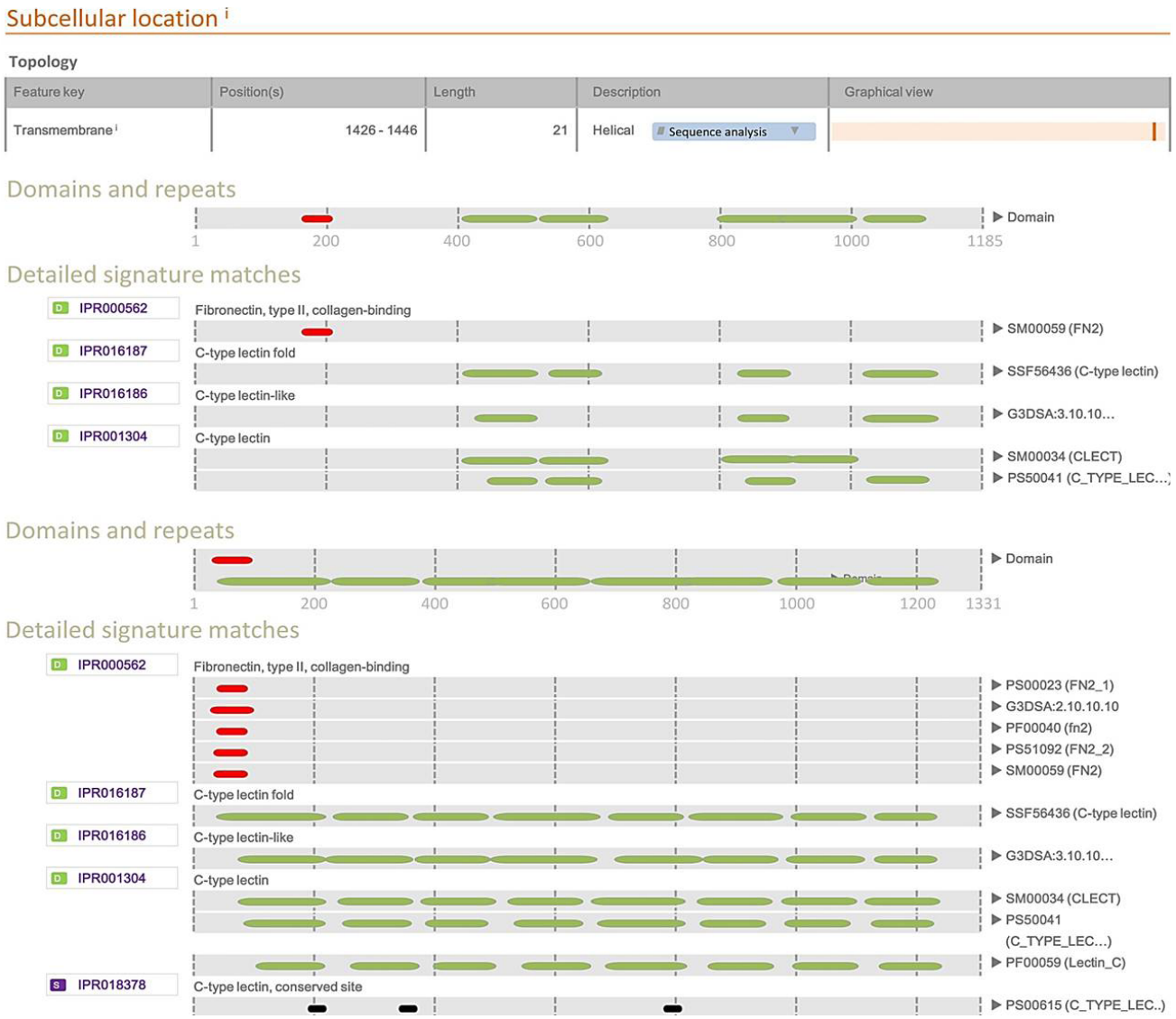

Fig. 1. *L.c*-MRC1and *L.c*-MRC2 membrane protein analysis.

Expression of L.c-MRC1-C and L.c-MRC2-C in E. coli *BL21 (DE3)*

We chose *E. coli* as the host bacterium. The recombinant plasmids MRC1 (1044 bp) -pET32A and MRC2 (993 bp) -pET32A were transformed into BL21 (DE3) *E. coli*. Protein expression was induced by incubating at 37°C on a 220 rpm shaker until the OD600 = 0.4−0.6. IPTG was added to a final concentration of 0.5 mM and the cells were incubated at 18°C for 10 h. The bacteria were precipitated and collected.

Protein purification and detection

A fresh recombinant colony was inoculated in 10 mL of LB medium and grown on a shaker to obtain a saturated culture. 500 µL of the overnight culture was then inoculated into 50 mL of LB medium, shaking at 37°C. When the culture OD600 reached 0.6, it was centrifuged at 10000 × g at room temperature. The supernatant was discarded and the samples were heated for SDS-PAGE analysis. SDS-PAGE was performed to analyze the expression of the exogenous genes in *E. coli.* The identified BL21 bacteria were inoculated into 40 mL of LB medium and expression was induced with 0.5 mM IPTG at 18°C for 10 h. After the medium was induced, the bacteria were collected by centrifugation. The cell pellet was resuspended and ultrasonic cracking was conducted. After centrifuging at 10000 rpm for 20 min at 4°C, the supernatant and precipitate were collected and sampled. The cells were resuspended in 40 mL of inclusion body solution. The supernatant was collected and filtered.

RESULTS

Target fragment determination

Membrane protein analysis showed two transmembrane domains in MRC1 and MRC2: a fibronectin type-II domain and a C-type lectin domain (Fig. 1). We then used Epitlot software (http://tools.iedb.org/bcell/) for epitope analysis; a higher amino acid score indicates that the epitope is more likely to form antibodies. Figure 2 shows regions with high antigenic epitope scores (yellow). We found two epitope concentrated areas, which corresponded to the Fibronectin type-II domain and the C-terminal side of the C-type lectin domain.

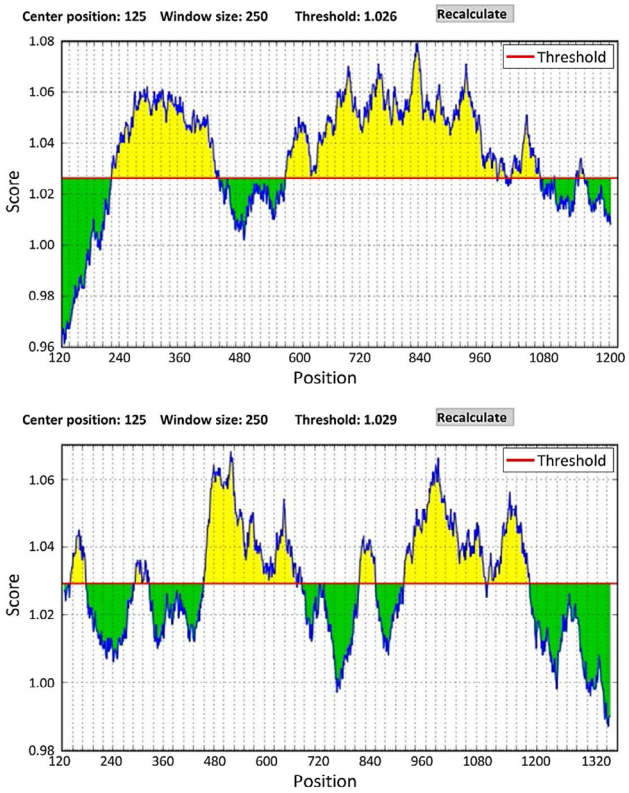

Fig. 2. Epitope analysis of *L.c*-MRC1and *L.c*-MRC2.

Table II.- *L.c*-MRC1 sequence: amino acids 795~1143, and *L.c*-MRC2 sequence: amino acids 681~991.

Sequence name	Sequence interval	Sequence
L.c-MRC1 sequence	795~1143 Domain	EFRLYNWDSAGSWNDVNCESYNDWICQIRADMEKLGLDFVLLTRQLVMFGVMVARLFNFQH-WQEGEPNNHNNDESCAEFRLYNWDSAGSWNDVNCESYNDWICQIRAGVTPHPPPNNTAVDY-NITSDGWLEWRGKQYYINRNSMPMEDAQHFCKQRHGNLVSILSKEENTFLWKQISRTYGSYY-IGMSVDLDGSSWWMDNSLIGLQRWDENQPSSESFDKNCVVMTYYMGFWRTCNCGQEEYSICK-RGNNPPVNTTAAPTVPLKGGCLPGWKKFDSMCYSIKTQKIRWEDARKQCYSIGGSLVSIPTR-RVQAFLITTLAETAAGDTWIGLNSLKESGFYWTDGKPRRY
L.c-MRC2 sequence:	681~991 Domain	GTSSPEWITFQEADYKFFDHRTTWDQAQRICSWFDSSLASVHSAEEEAFLANTLRKMP-KVEGDNWWLGLHTYENDGRFRWSDHSVLNYVSWALGRPHPLSRDRRCVHLSASKADWAD-QKCHSDLPYICKRVNVTGTIPPTPSSPHPPAGCPDGWSSYQHKCFRVFDHSYKVTWSAAK-LKCESQRGVLAVVSNHLEEAFVTTLLNNASIDLWVGLTSDSKGHFQWAKPGLLSYTNWAP-GEPLDNSGPHHNKTPGNCVVMIHGNPQKNTGMWASRACEMESNGYICQRPQDSERPPAP ALIPATLSKPVELGG

The later region is highly specific and would not lead to cross reactivity between MRC1 and MRC2. Thus, the C-type lectin domain region was selected as the target immunogenic region. In the above analysis, two fragments were selected for MRC1 and MRC2 (Fig. 3A; Table II). Cross analysis of the two fragments identified MRC1 amino acids 795 to 1143 and MRC2 amino acids 681 to 991 for the generation of antibodies. For MRC1 and MRC2 (681 ~ 991), the fragment homology was 6.11%. For MRC2 (681 ~ 991) and MRC1 (795 ~ 1143), the fragment homology was 14.07%. The corresponding antibodies produced by these two antigens would not lead to cross recognition.

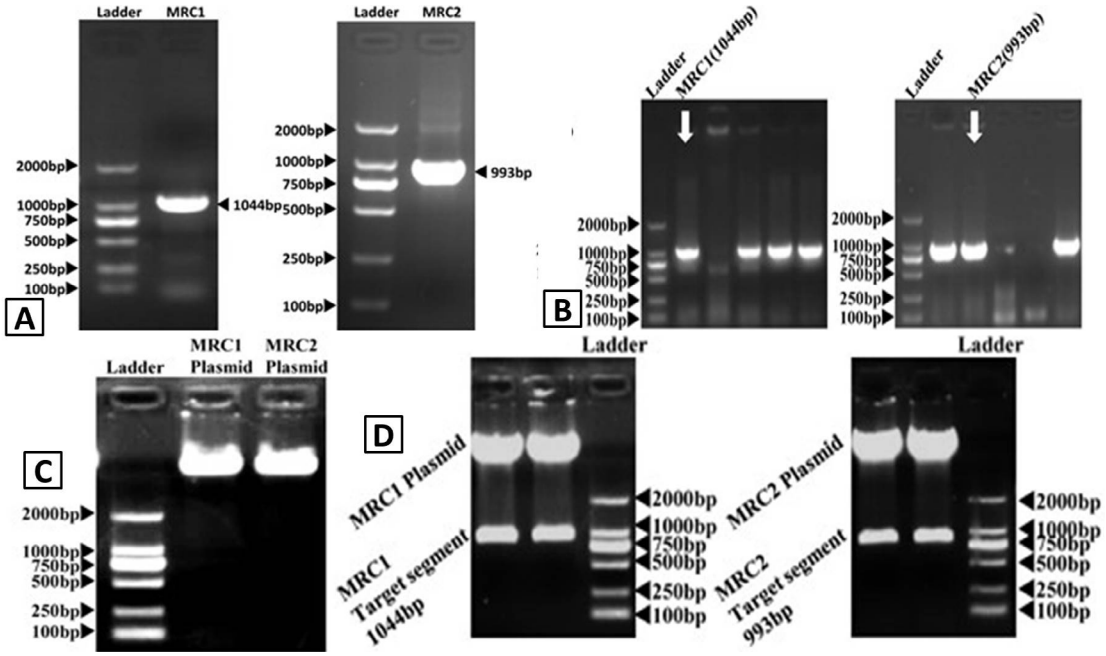

Fig. 3. DNA electrophoresis figures. A, PCR identification of *L.c*-MRC1and *L.c*-MRC2; B, colony PCR analysis of MRC1 and MRC2 pET32A vector clones; C, MRC1-pET32A and MRC2-pET32A plasmid electrophoresis; D, MRC1-pET32A and MRC2-pET32A vector restriction digests.

Table III.- Complete *L.c*-MRC1 and *L.c*-MRC2 protein sequences. The underlined part is the target sequence, and the black is the fusion protein tag. The theoretical size of the tag is 20kD, and the theoretical size of the fusion protein is 60kD.

Sequence name	Protein sequence
L.c-MRC1	MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKY-GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAK-FERQHMDSPDLGTDDDDKAMADIGSEFEEFRLYNWDSAGSWNDVNCESYNDWICQIRADMEKLGLDFVLL-TRQLVMFGVMVARLFNFQHWQEGEPNNHNNDESCAEFRLYNWDSAGSWNDVNCESYNDWICQIRAGVTPH-PPPNNTAVDYNITSDGWLEWRGKQYYINRNSMPMEDAQHFCKQRHGNLVSILSKEENTFLWKQISRTYGSYY-IGMSVDLDGSSWWMDNSLIGLQRWDENQPSSESFDKNCVVMTYYMGFWRTCNCGQEEYSICKRGNNP-PVNTTAAPTVPLKGGCLPGWKKFDSMCYSIKTQKIRWEDARKQCYSIGGSLVSIPTRRVQAFLITTLAETAAG-DTWIGLNSLKESGFYWTDGKPRRY
L.c-MRC2	MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKY-GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAK-FERQHMDSPDLGTDDDDKAMADIGSEFEGTSSPEWITFQEADYKFFDHRTTWDQAQRICSWFDSSLASVH-SAEEEAFLANTLRKMPKVEGDNWWLGLHTYENDGRFRWSDHSVLNYVSWALGRPHPLSRDRRCVHLSAS-KADWADQKCHSDLPYICKRVNVTGTIPPTPSSPHPPAGCPDGWSSYQHKCFRVFDHSYKVTWSAAKLK-CESQRGVLAVVSNHLEEAFVTTLLNNASIDLWVGLTSDSKGHFQWAKPGLLSYTNWAPGEPLDNSGPHHNKTP-GNCVVMIHGNPQKNTGMWASRACEMESNGYICQRPQDSERPPAP ALIPATLSKPVELGG

Construction of MRC1 and MRC2 cloning vectors

L.c-MRC1 and *L.c*-MRC2 were PCR amplified and run on an agarose gel. The *L.c*-MRC1 amplicon was about 1000 bp, which was consistent with the predicted size of *L.c*-MRC1 (1044 bp). The *L.c*-MRC2 amplicon was also around 1000 bp, which was also consistent with the predicted size of *L.c*-MRC2 (933bp) (Fig. 3). The target fragments were then ligated into the pET32A cloning vector. MRC1 (1044bp) -pET32A and MRC2 (993bp) -pET32A clones were preliminarily verified by bacterial PCR and double restriction digests (Fig. 3B). Bacterial PCR analysis showed 1000 bp and 2000 bp bands from the MRC1 (1044 bp) -pET32A culture and a 1000 bp band from the MRC2 (993 bp) -pET32A culture. The 1000 bp bands were likely the target fragments (Fig. 4). The MRC1-pET32A and MRC2-pET32A plasmids were then extracted and subjected to double restriction digests. As shown in Figure 3C and D, there were bands between 2000 bp and 1000 bp, which correspond to the pET32A fragment and the MRC1 (1044 bp) or MRC2 (993 bp) fragments. The preliminary results showed successful construction of the MRC1 (1044 bp) -pET32A and MRC1 (993 bp) -pET32A cloning vectors. Subsequent digestion verified that the insert was correct.

Protein expression and purification

As shown in Figure 4A, protein expression from the recombinant MRC1 and MRC2 vectors was induced and yielded high expression of proteins between 45 and 66.2 kDa. This was consistent with the predicted MRC1 (1044 bp) protein size (60 kDa) and the predicted MRC2 (993 bp) protein size (54 kDa) (Table III). An empty vector-induced control showed no such protein (Fig. 4B). We also tested for protein in the precipitate (Table III), further confirming protein expression. After purification, the protein fragments were between 45 kDa and 66.2 kDa (Fig. 4C), this result is consistent with the predicted protein length.

We analyzed the purity of the recovered proteins and found single bands in between 45 kDa and 66.2 kDa (Fig. 4C). *L.c*-MRC1 and *L.c*-MRC2 proteins were predicted to be 60 kDa and 54 kDa, respectively. This indicated that the proteins were of excellent purity and could be used for immunization and preparation of antibodies.

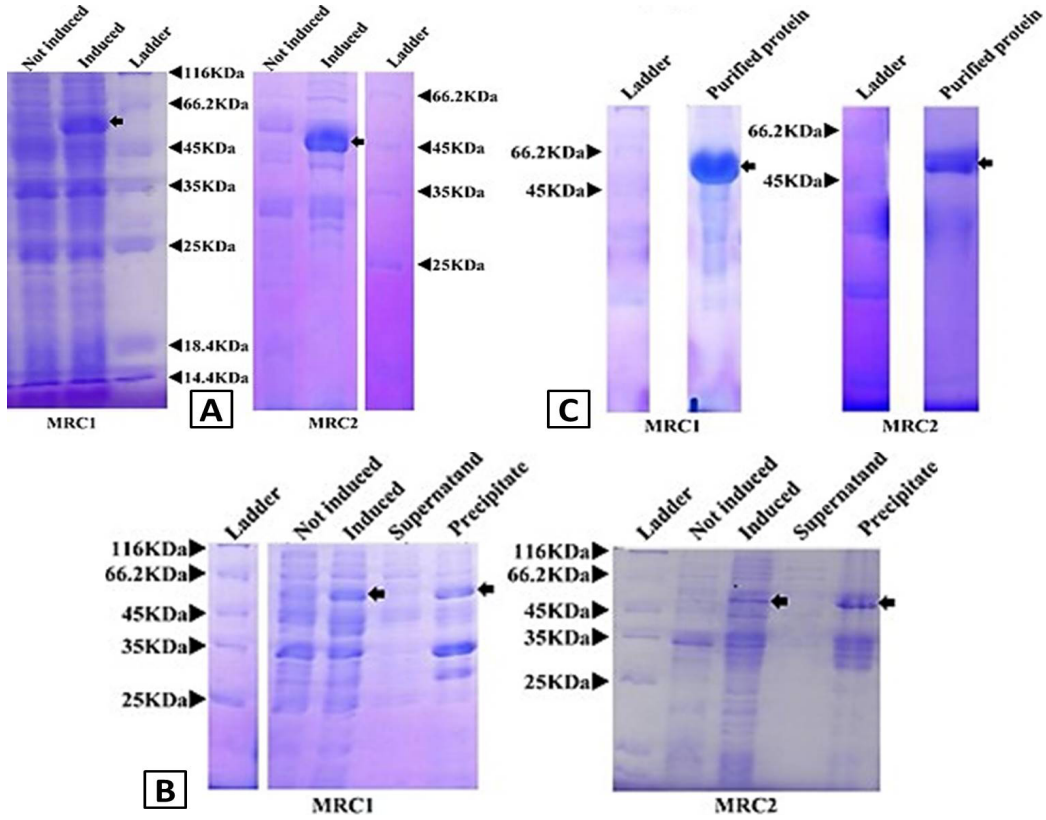

Fig. 4. Protein electrophoresis figures. A, SDS-PAGE analysis of *L.c*-MRC1 and *L.c*-MRC2 proteins; B, SDS-PAGE analysis of precipitated *L.c*-MRC1 and *L.c*-MRC2 proteins (the black arrows indicate the target protein bands. The target protein is detected in the precipitate); C, Purified *L.c*-MRC1 and *L.c*-MRC2 proteins. The black arrows indicate the target protein bands.

DISCUSSION

The use of the large yellow croaker (*L. crocea)* has been compromised in recent years by widespread disease outbreaks due to various bacterial pathogens (Taylor *et al*., 2005; Zheng *et al*., 2015; Jiang *et al*., 1995; East and Isacke, 2002). In particular, *Vibrio anguillarum* is a major pathogen of farmed fish that causes severe damage and heavy economic losses to the fish farming industry. The most susceptible fish species include both marine and freshwater fish such as Japanese flounder, European sea bass (Aamri *et al*., 2015; Zlotkin *et al*., 1998), Asian seabass (Kayansamruaj *et al*., 2015), rainbow trout (Lahav *et al*., 2004; Safari *et al*., 2016), zebrafish (Membrebe *et al*., 2016), Nile tilapia (Membrebe *et al*., 2016), channel catfish (Xia *et al*., 2008; Wang *et al*., 2016), and large yellow croaker. In this study, we tested the prokaryotic expression and purification of two *L. crocea* mannose receptor gene subunits (MRC1 and MRC2). The mannose receptor (MR) is a pattern recognition receptor (PRR), a type of receptor that plays a significant role in innate immunity immune responses through binding to pathogen-associated molecular patterns (PAMPs) (Gazi and Pomares, 2009; Chen *et al*., 2019). MR recognizes surface polysaccharides of various pathogens, such as viruses, bacteria, yeasts, and parasites, including HIV (Nguyen and Hildreth, 2010), *Dengue virus* (Miller *et al*., 2008), *Candida albicans* (Marodi *et al*., 1991), *Mycobacterium tuberculosis* (Lailleux *et al*., 2003; Rajaram *et al*., 2010), *Pneumocystis carinii* (Ezekowitz *et al*., 1991; Swain *et al*., 2003), *Cryptococcus neoformans* (Dan *et al*., 2008), *Klebsiella pneumonia* (Zamze *et al*., 2002), *Streptococcus pneumonia* (Zamze *et al*., 2002) and *Leishmania* spp. (Chakraborty *et al*., 1998, 2001). In recruited inflammatory peritoneal macrophages, MR levels were increased in response to interleukin-4 (IL-4), IL-13, and IL-10 (Chakraborty *et al*., 2001). Owing to its crucial role in innate immunity responses, MR has been extensively studied in humans and mice. However, there have been few studies of MR in fish. Additionally, prokaryotic MR protein expression levels were undetermined. Therefore, we chose to study the mannose receptor gene. Prior to this study, we have studied the quantitative expression of the mannose receptor gene. Based on this, we continued to study the prokaryotic expression and purification of the gene of the *L. crocea*, and obtained good expression and higher purification results.

The MRC1 subunit contains four domains: an extracellular region containing a cysteine-rich (CR) domain, a domain containing fibronectin type two repeats (FNII) and multiple C-type lectin-like carbohydrate recognition domains (CTLDs), a transmembrane domain, and a short cytoplasmic tail. MRC2 is similar to MRC1, but lacks the extracellular CR domain (Dong *et al*., 2016). We performed membrane protein analysis in the process of antigen preparation and found two conserved domains of MRC1 and MRC2: the fibronectin type-II domain and the C-type lectin domain. Further, epitope analysis identified two concentrated epitope areas in the fibronectin type-II region and the C-type lectin region (Fig. 2). The C-terminal side of the C-type lectin region is highly specific, would not cause cross-reactivity between MRC1 and MRC2, and would lead to better clones. Thus, the C-type lectin region was selected as the immunogen region. We performed cross identification analysis on the selected target fragments (Fig. 2). Homology comparison of MRC1 (795 ~ 1143 bp) and MRC2 (681 ~ 991 bp) suggests that the corresponding antibodies produced by these two antigens would not be cross reactive. The MRC1 fragment (amino acids 795 to 1143) and the MRC2 fragment (amino acids 681 to 991) would generate antibodies against the C-type lectin regions of each respective protein subunit. These two regions are within the CTLDs 5−7. Although both MRC1 and MRC2 have 8 CTLDs, CTLD 4−8 have higher glycoprotein binding capacity (Dong *et al*., 2016). The 8 CTLDs domains of *L.c*-MRC1 are: residues 212 to 341, 360 to 484, 503 to 625, 645 to 770, 791 to 913, 933 to 1070, 1085 to 1203, and 1220 to 1346, and the 8 CTLDs of *L.c*-MRC2 are: residues 97 to 222, 245 to 368, 383 to 507, 531 to 666, 683 to 809, 830 to 965, 982 to 1110, and 116 to 1247) (Dong *et al*., 2016). Comparison of the tertiary structures of the two proteins (Fig. 5) indicated that the MRC1-C and MRC2-C subunits may have different functions.

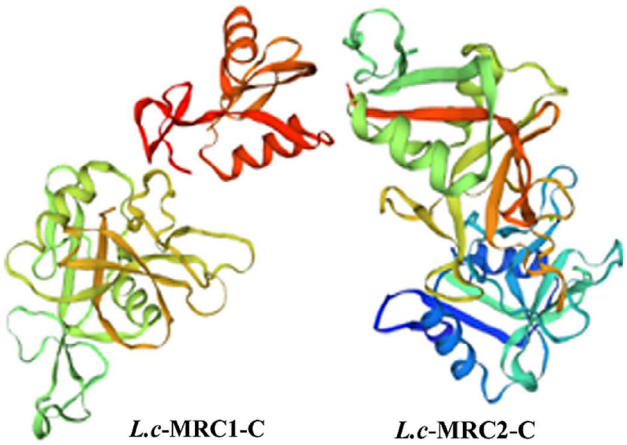

Fig. 5. 3D protein structures of *L.c*-MRC1-C and *L.c*-MRC2-C. These protein structures were predicted using SWISSMODEL. The N-terminus is blue and the C-terminus is red (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Our findings further our understanding of innate immunity in *L. crocea* and will enable future studies of its antimicrobial defense mechanisms. Our results could help to establish effective disease control measures and potentially pave the way for developing an MR-based vaccine against bacteria, viruses, and other infections in *L. crocea*. Previously, MRC1 and MRC2 genes were found to be expressed in various *L. crocea* tissues at different tissue-specific expression levels. Bacterial challenge experiments showed that MRC1 and MRC2 mRNA expression levels were upregulated in the liver, spleen, and kidney of young fish, suggesting that MRC1 and MRC2 contribute to the defense against pathogenic bacterial infections. Prokaryotic expression and antibody generation will provide necessary tools for further study of *L. crocea* MR.

As opposed to the traditional use of antibiotics and antimicrobial compounds, vaccination is an effective green intervention that can help to control *V. anguillarum* infection in aquaculture (Wang *et al*., 2016). Genetically engineered vaccines are safer and more serotype-independent (Wang *et al*., 2016). However, efforts to develop subunit vaccines are limited by the lack of the conserved protective antigens. Thus, the identification and screening of novel and conservative immunogenicity antigens is crucial for the development of an effective subunit vaccine (Tian *et al*., 2011). In this study, we evaluated the immunogenicity of *L.c*-MRC1 and *L.c*-MRC2 and determined that these two candidate immunogens could potentially be used as an effective vaccine against *V. anguillarum* infection in *L. crocea*.

CONCLUSION

Here, we report the cloning of partial *L. crocea* mannose receptor subunit (MRC1 and MRC2) cDNAs. The amino acid sequences of MRC1 and MRC2 were highly conserved with other vertebrates. Using membrane protein analysis, epitope analysis, and target fragment cross identification analysis, the C-type lectin region was identified to be highly specific and not cross reactive between MRC1 and MRC2. Thus, it was selected as the immunogen region. These fragments were then cloned into a prokaryotic expression vector. Expression was induced in *E. coli* and the proteins were purified. We achieved high protein expression levels and excellent protein purity. The purity and expression levels were suitable for antibody preparation.

ACKNOWLEDGEMENTS

This work was financially supported by the Fundamental Research Funds for Zhejiang Provincial Universities and Research Institutes (No.: 2019J00038), the General Scientific Project of Zhejiang Education Department (No.: Y201840255), and the 2017 Scientific Research Startup Funds of Zhejiang Ocean University (Funding No.: 12245090418).

Statement of conflict of interest statement

The authors report no conflicts of interest and were alone responsible for the content and writing of the paper.

REFERENCES

Aamri, F., Real, F., Acosta, F., Bravo, J., Román, L., Déniz, S. and Padilla, D., 2015. Differential innate immune response of European seabass (*Dicentrarchus labrax*) against *Streptococcus iniae*. *Fish Shellf. Immunol.*, **46**: 436-441. https://doi.org/10.1016/j.fsi.2015.05.054

Boskovic, J., Arnold, J. N., Stilion, R., Gordon, S., Sim, R.B., Rivera-Calzada, A., Wienke, D., Isacke, C.M., Martinez, P.L. and Llorca, O., 2006. Structural model for the mannose receptor family uncovered by electron microscopy of Endo180 and the mannose receptor. *J. biol. Chem.,* **281**: 8780-8787. https://doi.org/10.1074/jbc.M513277200

Caroline, M.C., Priscila, V.S., Alex, S.D.M., Caroline, P.V.,D., Deborah, C.G.C., Maria, J.A.C.R., Arthur, J.S.R. and Phan, V.N.G., 2015. Hsp70 and p53 expressions and behavior of juvenile pompano, *Trachinotus carolinus* (Perciformes, Carangidae), at controlled temperature increase. *J. exp. Mar. Biol. Ecol.*, **470**: 34-42. https://doi.org/10.1016/j.jembe.2015.04.024

Chakraborty, P., Ghosh, D. and Basu, M.K., 2001. Modulation of macrophage mannose receptor affects the uptake of virulent and avirulent *Leishmania donovani* promastigotes. *J. Parasitol.*, **87**: 1023-1027. https://doi.org/10.1645/0022-3395(2001)087[1023:MOMMRA]2.0.CO;2

Chakraborty, R., Chakraborty, P. and Basu, M.K., 1998. Macrophage mannosyl fucosyl receptor: Its role in invasion of virulent and avirulent *L. donovani* promastigotes. *Biosci. Rep.*, **18**: 129-142. https://doi.org/10.1023/A:1020192512001

Chen, P., Huang, Z., Zhu, C., Han, Y., Xu, Z., Sun, G., Zhang, Z., Zhao, D., Ge, G. and Ruan, L., 2019. Complete mitochondrial genome and phylogenetic analysis of gruiformes and charadriiformes. *Pakistan J. Zool.,* **52**: 425-439. https://doi.org/10.17582/journal.pjz/20190603010623

Dan, J.M., Kelly, R.M., Lee, C.K. and Levitz, S.M., 2008. Role of the mannose receptor in a murine

model of *Cryptococcus neoformans* infection. *Infect. Immun.*, **76**: 2362-2367. https://doi.org/10.1128/IAI.00095-08

Dong, X., Li, J., He, J., Liu, W., Jiang, L., Ye, Y. and Wu, C., 2016. Anti-infective mannose receptor immune mechanism in large yellow croaker (*Larimichthys crocea*). *Fish Shellf. Immunol.,* **54**: 57-65. https://doi.org/10.1016/j.fsi.2016.04.006

East, L. and Isacke, C.M., 2002. The mannose receptor family. *Biochim. biophys. Acta*, **1572**: 364-386. https://doi.org/10.1016/S0304-4165(02)00319-7

Ezekowitz, R.A.B., Williams, D.J., Koziel, H., Armstrong, M.Y.K., Warner, A., Richards, F.F. and Rose, R.M., 1991. Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. *Nature*, **351**: 155-158. https://doi.org/10.1038/351155a0

Gazi, U. and Martinez-Pomares, L., 2009. Influence of the mannose receptor in host immune responses. *Immunobiology*, **214**: 561. https://doi.org/10.1016/j.imbio.2008.11.004

He J., Wang J., Xu M., Wu C. and Liu H., 2016. The cooperative expression of heat shock protein 70 KD and 90 KD gene in juvenile *Larimichthys crocea* under *Vibrio alginolyticus* stress. *Fish Shellf. Immunol.,* **58**: 59-69. https://doi.org/10.1016/j.fsi.2016.09.049

He, J., Liu, H. and Wu, C., 2014. Identification of SCARA3, SCARA5 and MARCO of class A scavenger receptor-like family in *Pseudosciaena crocea*. *Fish Shellf. Immunol.,* **41**: 38-49. https://doi.org/10.1016/j.fsi.2014.07.037

Jiang, W., Swiggard, W.J., Heufler, C., Peng, M., Mirza, A., Steinman, R.M. and Nussenzweig, M.C., 1995. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature*, **375**: 151-155. https://doi.org/10.1038/375151a0

Kayansamruaj, P., Dong, H.T., Nguyen, V.V., Le, H.D., Pirarat, N. and Rodkhum, C., 2015. Susceptibility of freshwater rearing Asian seabass to pathogenic *Streptococcus iniae*. *Aquacul. Res.*, **48**: 1-8. https://doi.org/10.1111/are.12917

Kilpatrick, D.C., 2010. Mannan-binding lectin and its role in innate immunity. *Transf. Med.,* **12**: 335-352. https://doi.org/10.1046/j.1365-3148.2002.00408.x

Lahav, D., Eyngor, M., Hurvitz, A., Ghittino, C. and Eldar, A., 2004. *Streptococcus iniae* type II infections in rainbow trout *Oncorhynchus mykiss*. *Dis. aquat. Organ.*, **62**: 177-180. https://doi.org/10.3354/dao062177

Lailleux, T., Maeda, N., Nigou, J., Gicquel, B. and Neyrolles, O., 2003. How is the phagocyte lectin keyboard played? Master class lesson by *Mycobacterium tuberculosis*. *Trends Microbiol.*, **11**: 263. https://doi.org/10.1016/S0966-842X(03)00102-1

Marodi, L., Korchak, H.M. and Johnston, R.B., 1991. Mechanisms of host defense against *Candida* species: I. Phagocytosis by monocytes and monocyte-derived macrophages. *J. Immunol.*, **146**: 2783-2789.

Membrebe, J.D., Yoon, N.K., Hong, M., Lee, J., Lee, H., Park, K., Seo, S., Yoon, I., Yoo, S., Kim, Y.C. and Ahn, J., 2016. Protective efficacy of *Streptococcus iniae* derived enolase against Streptococcal infection in a zebrafish model. *Vet. Immunol. Immunopathol.*, **170**: 25-29. https://doi.org/10.1016/j.vetimm.2016.01.004

Miller, J.L., Wet, B.J.M.D., Dewet, B.J.M., Martinez-Pomares, L. and Gordon, S., 2008. The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathogens*, **4**: 17. https://doi.org/10.1371/journal.ppat.0040017

Miron, S., 1992. Characterization of the murine macrophage mannose receptor: Demonstration that the downregulation of receptor expression mediated by interferon-gamma occurs at the level of transcription. *Blood,* **80**: 2363. https://doi.org/10.1182/blood.V80.9.2363.bloodjournal8092363

Nguyen, D.G. and Hildreth, J.E.K., 2010. Involvement of macrophage mannose receptor in the binding and transmission of HIV by macrophages. *Eur. J. Immunol.*, **33**: 483-493. https://doi.org/10.1002/immu.200310024

Nguyen, H.T., Kanai, K. and Yoshikoshi, K., 2002. Ecological investigation of *Streptococcus iniae* in cultured Japanese flounder (*Paralichthys olivaceus*) using selective isolation procedures. *Aquaculture,* **205**: 17. https://doi.org/10.1016/S0044-8486(01)00667-6

Oldham, C.H.G., 1982. Science in contemporary China. *China Quart.,* **89**: 99-113. https://doi.org/10.1017/S0305741000000102

Pontow, S., 1991. Mannose receptor. *Int. Rev. Cytol.,* **137**: 221-244. https://doi.org/10.1016/S0074-7696(08)62606-6

Rajaram, M.V.S., Brooks, M.N., Morris, J.D., Torrelles, J.B., Azad, A.K. and Schlesinger, L.S., 2010. *Mycobacterium tuberculosis* activates human macrophage peroxisome proliferator-activated receptor? Linking mannose receptor recognition to regulation of immune responses. *J.*

Immunol., **185**: 929-942. https://doi.org/10.4049/jimmunol.1000866

Safari, R., Adel, M., Lazado, C.C., Caipang, C.M. and Dadar, M., 2016. Host-derived probiotics *Enterococcus casseliflavus* improves resistance against *Streptococcus iniae* infection in rainbow trout (*Oncorhynchus mykiss*) via immunomodulation. *Fish Shellf. Immunol.*, **52**: 198-205. https://doi.org/10.1016/j.fsi.2016.03.020

Sun, Y. and Hu, Y.H., 2015. Cell-penetrating peptide-mediated subunit vaccine generates a potent immune response and protection against *Streptococcus iniae* in Japanese flounder (*Paralichthys olivaceus*). *Vet. Immunol. Immunopathol.*, **167**: 96-103. https://doi.org/10.1016/j.vetimm.2015.07.008

Swain, S.D., Lee, S.J., Nussenzweig, M.C. and Harmsen, A.G., 2003. Absence of the macrophage mannose receptor in mice does not increase susceptibility to *Pneumocystis carinii* infection *in vivo*. *Infect. Immun.*, **71**: 6213-6221. https://doi.org/10.1128/IAI.71.11.6213-6221.2003

Taylor, M.E., Conary, J.T., Lennartz, M.R., Stahl, D.P. and Drickamer, K., 1990. Primary structure of the mannose receptor contains multiple motifs recembling carbohydrate-recognition domains. *J. biol. Chem.*, **265**: 12156-12162.

Taylor, P.R., Gordon, S. and Martinez-Pomares, L., 2005. The mannose receptor: Linking homeostasis and immunity through sugar recognition. *Trends Immunol.*, **26**: 110. https://doi.org/10.1016/j.it.2004.12.001

Tian, H., Fu, F., Li, X., Chen, X., Wang, W., Lang, Y., Cong, F., Liu, C., Tong, G., and Li, X., 2011. Identification of the immunogenic outer membrane protein A antigen of *Haemophilus parasuis* by a proteomics approach and passive immunization with monoclonal antibodies in mice. *Clin. Vaccine Immunol.,* **18**: 1695-1701. https://doi.org/10.1128/CVI.05223-11

Wang, B., Gan, Z., Cai, S., Wang, Z., Yu, D., Lin, Z., Lu, Y., Wu, Z. and Jian, J., 2016. Comprehensive identification and profiling of Nile tilapia (*Oreochromis niloticus*) microRNAs response to *Streptococcus agalactiae* infection through high-throughput sequencing. *Fish Shellf. Immunol.*, **54**: 93-106. https://doi.org/10.1016/j.fsi.2016.03.159

Wang, E., Wang, J., Long, B., Wang, K., He, Y., Yang, Q., Chen, D., Geng, Y., Huang, X., Ouyang, P. and Lai, W., 2016. Molecular cloning, expression and the adjuvant effects of interleukin-8 of channel catfish (*Ictalurus punctatus*) against *Streptococcus iniae*. *Scient. Rep.*, **6**: 29310. https://doi.org/10.1038/srep29310

Xia, L., Xiong, D., Gu, Z., Xu, Z, Chen, C., Xie, J. and Xu, P., 2008. Recovery of *Acinetobacter baumannii* from diseased channel catfish (*Ictalurus punctatus*) in China. *Aquaculture*, **284**: 288. https://doi.org/10.1016/j.aquaculture.2008.07.038

Zamze, S., Martinez-Pomares L., Jones, H., Taylor, P.R., Stillion, R.J., Gordon, S. and Wong, S.Y.C., 2002. Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. *J. biol. Chem.*, **277**: 41613-41623. https://doi.org/10.1074/jbc.M207057200

Zheng, F., Asim, M., Lan, J., Zhao, L., Wei, S., Chen, N., LIu, X., Zhou, Y. and Lin, L., 2015. Molecular cloning and functional characterization of mannose receptor in zebra fish (*Danio rerio*) during Infection with *Aeromonas sobria*. *Int. J. mol. Sci.,* **16**: 10997-11012. https://doi.org/10.3390/ijms160510997

Zlotkin, A., Hershko, H. and Eldar, A., 1998. Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. *Appl. environ. Microbiol.*, **64**: 4056-4067. https://doi.org/10.1128/AEM.64.10.4065-4067.1998