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获奖证书

获奖项目：Curcumin is an efficacious therapeutic agent against *Chilodonella uncinata* via interaction with tubulin alpha chain as protein target

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检索报告

根据委托人刘玉军委托,通过网络检索,刘玉军发表的 1 篇论文被《科学引文索引》扩展版(SCI-Expanded)数据库收录。数据库具体检索结果如下:

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Full length article

An LNP-encapsulated mRNA vaccine targeting the major capsid protein of largemouth bass virus confers protective immunity and reduces RIG-I pathway activation in *Micropterus salmoides*

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ABSTRACT

Largemouth bass virus (LMBV) infection poses a significant threat to largemouth bass farming in China, yet effective and safe vaccines remain scarce. In this study, we designed and evaluated four mRNA vaccine candidates against LMBV, all encoding the viral major capsid protein (MCP). The fully configured construct (LMUM) contained a 5' cap1, untranslated regions (UTRs), and a poly(A) tail, and was produced by in vitro transcription. Each mRNA was encapsulated into lipid nanoparticles (LNPs), yielding homogeneous particles (~110 nm diameter, ~54 mV zeta potential) with >96% encapsulation efficiency that provided substantial protection against RNase degradation (43% of mRNA remained intact in LNP-encapsulated groups versus 10% in unprotected controls). Intramuscular immunization of largemouth bass with LNP-encapsulated LMUM (0.2 µg/g body weight) induced MCP-specific serum antibodies after primary immunization, with a strong booster response after the second dose. In the LMUM-immunized group, spleen mRNA levels of IgM and CD4 were upregulated 3.4-fold and 3.2-fold, respectively, by day 14, while the groups lacking UTR or cap/poly(A) tail failed to induce these adaptive immune markers. Interestingly, the incomplete constructs triggered stronger activation of the RIG-I pathway (RIG-I, MAVS, TBK1) than the fully modified LMUM, suggesting that the combination of cap1, UTR, and poly(A) tail helps dampen excessive innate sensing. Following a lethal LMBV challenge, the LMUM vaccine conferred 45–50% relative survival after primary immunization, which increased to 53–57% after the booster dose. In contrast, the incomplete vaccine constructs and naked mRNA gave only 10–15% survival, and control groups experienced nearly complete mortality. These results demonstrate that a fully modified mRNA-LNP vaccine against LMBV induces robust humoral and cellular immune responses and provides significant protective efficacy in largemouth bass.

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1. Introduction

Since the global pandemic caused by SARS-CoV-2 in 2019, messenger RNA (mRNA) technology has gained significant attention in the field of vaccines [1]. mRNA vaccines, such as the Pfizer-BioNTech and Moderna vaccines for the prevention of SARS-CoV-2 infection [2,3], leverage the fundamental biological principle that mRNA transfers genetic information from DNA to proteins [4]. These vaccines contain mRNA that encodes specific viral antigens, such as the spike protein of SARS-CoV-2. When these mRNA molecules are taken up by human cells, the cells produce antigenic proteins that stimulate the immune system to produce antibodies and memory cells, thereby preventing infection [5–7]. The main advantages of mRNA vaccines are their safety and flexibility [8]. After the viral gene sequence has been obtained, the corresponding mRNA molecule can be designed [9–11]. mRNA vaccines can be synthesized in vitro and quickly adjusted to respond to new virus variants. This process does not require specialized laboratory equipment or biosafety level facilities, avoiding the potential allergens and infection risks posed by complex substrates from cell and animal sources, while also reducing dependence on live viruses. This also eliminates the risk of insertional mutagenesis that may occur with DNA or viral vector vaccines. Furthermore, nucleotide and delivery system modifications enhance tissue compatibility, thereby reducing the immune rejection triggered by mRNA vaccines [12,13]. Furthermore, the potential of mRNA technology extends far beyond this. In other medical fields, mRNA has also demonstrated significant potential for application. For instance, mRNA vaccines are being developed to create personalized cancer treatments by instructing the immune system to recognize and attack cancer cells by encoding tumor-specific antigens. Additionally, mRNA is used to treat certain rare genetic diseases, such as cystic fibrosis and muscular dystrophy. By providing cells with mRNA that encodes missing or functionally abnormal proteins, their normal function can be restored [14,15].

Encouraged by the successful application of mRNA vaccines in human medicine, recent pioneering studies have begun to explore their feasibility in aquaculture species. For instance, an mRNA-LNP vaccine encoding the glycoprotein of infectious hematopoietic necrosis virus (IHNV) protected rainbow trout from lethal challenge, providing the first empirical demonstration of mRNA-LNP vaccine efficacy in fish [16]. Mechanistically, research comparing modified and unmodified mRNA vaccines against a fish rhabdovirus revealed that, unlike in mammalian systems, N1-methyl-pseudouridine (N1MPU) modification does not suppress innate immune activation in teleost fish [17]. Instead, it retains immunostimulatory capacity and triggers distinct transcriptional dynamics, including the enrichment of autophagy and ubiquitination pathways. Beyond antiviral applications, an mRNA vaccine against tuberculosis has demonstrated both prophylactic and therapeutic efficacy in a zebrafish model of infection, with protection correlating to the activation of DNA damage repair systems and autophagy within granulomas [18]. These emerging studies highlight the vast potential of mRNA technology for disease control in aquaculture, while also underscoring the need for species-specific optimization and further validation across different pathogens and fish species.

Among the many viral threats to aquaculture, largemouth bass virus (LMBV) stands out as a particularly challenging pathogen. LMBV is a type of large DNA virus that can infect both invertebrates and vertebrates, causing severe infections in various fish species and resulting in significant economic and environmental losses for the aquaculture industry [19]. Similar to coronaviruses, LMBV possesses an envelope that helps the virus evade recognition by the host immune system and facilitate cell entry [20]. The viral capsid is composed of eight different proteins, with the MCP accounting for more than 90% and playing a crucial role in capsid assembly, DNA packaging and release [21,22]. Viral proteins, including MCP, are often used as antigens in vaccine design [23–25]. Several vaccines targeting the MCP of LMBV have been developed to address these challenges, including inactivated, genetically engineered and DNA vaccines [26]. Despite offering more than 60% pro-

tection in some cases, these vaccines have significant limitations. Their protracted preparation cycles and elevated biosafety requirements make it difficult to rapidly produce vaccines tailored to specific regional viral strains, leading to variable efficacy across different geographical areas. In contrast, no reports have been published on using mRNA to prevent or control LMBV infection in aquaculture [27,28]. Therefore, developing an mRNA vaccine against LMBV is of great importance to overcome the limitations of current approaches.

Building on this emerging knowledge, our study aims to validate the potential of mRNA vaccines encapsulated in lipid nanoparticles for preventing LMBV infection in aquatic vertebrates. To this end, we designed vaccine components, including the 5' cap1, untranslated regions (UTRs), poly(A), and LNPs [29], which effectively inhibit mRNA recognition through the RIG-I signaling pathway, reduce mRNA immunogenicity, improve mRNA stability, and promote mRNA translation in fish. The vaccine triggered a specific antibody response and protected fish from LMBV infection, increasing the survival rate of *Micropterus salmoides* to 57%. These observations provide an important theoretical basis for elucidating the protective mechanisms of mRNA vaccines against viral pathologies. In addition, our findings contribute to the development of safe and effective vaccines, as well as the enhancement of disease prevention and control in aquatic animals.

Additional experiments demonstrated that lipid nanoparticles significantly reduced RIG-I pathway recognition of the mRNA and extended its duration in vivo, thereby enhancing both protein translation and the immune response to the vaccine. In conclusion, compared with inactivated and genetically engineered vaccines [30,31], mRNA vaccines offer greater convenience and faster manufacturing, as they do not require lengthy antigen screening and expression processes. Provided that safety and immunogenicity are ensured, mRNA vaccines can be used to more quickly and effectively prevent viral diseases in largemouth bass. The effectiveness of mRNA vaccines depends critically on several key components, including the 5' cap1, UTRs, poly(A) tail and lipid nanoparticles. For the first time, we demonstrate that mRNA vaccines can effectively prevent viral disease in aquaculture, providing a new strategy for preventing and controlling fish diseases. Furthermore, we have explored the potential biological pathways engaged by mRNA vaccines in fish, paving the way for future research and optimization in this emerging field.

2. Material and methods

2.1. Reagents and materials

The T7 High Yield RNA Transcription Kit, N1-methyl-pseudouridine sodium, cap1 Capping System, and *E. coli* poly(A) Polymerase were obtained from Novoprotein Co., Ltd. (Suzhou, China). 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), sorbitan monooleate (Span 80), D- α -tocopherol polyethylene glycol succinate (TPGS), and calcium acetate were obtained from Macklin Chemical Reagent Co., Ltd. (Shanghai, China). N1-Methyl-pseudouridine was purchased from MedChem Express Co., Ltd. (Shanghai, China). The pCDNA3.1 (+) plasmid (containing the restriction sites EcoR I and BamH I) and the pET-28a (+) vector were constructed by Sangon Biotechnology Co., Ltd. (Shanghai, China). Isopropyl β -D-thiogalactopyranoside (IPTG) was obtained from Aladdin Chemical Reagent Co., Ltd. (Shanghai, China). Anti-His tag mouse monoclonal antibody and HRP-conjugated goat anti-mouse antibody were purchased from Sangon Biotechnology Co., Ltd. (Shanghai, China).

2.2. Cell, virus, and experimental fish

The largemouth bass virus isolate LMBV-FS001 (GenBank: ON936874.1) was sourced from the Aquatic Disease Laboratory, College of Animal Science and Technology, Northwest A&F University, and propagated in *Micropterus salmoides* brain (MSBr) cells. These cells were cultured in Medium 199 (Hyclone, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Zeta Life, Menlo

Park, CA, USA), streptomycin (100 U/mL), penicillin (100 U/mL), and cultured at 25°C under a humidified atmosphere of 5% CO₂ in air. Two-month-old largemouth bass (1.20 ± 0.2 g in weight, 4.0 ± 0.22 cm in length) were purchased from Yuxi District Aquaculture Company (Chongqing, China). Before the experiment, all fish were kept in an aquarium (temperature 25 ± 0.5°C, pH 6.7–7.5) for two weeks and fed with largemouth bass fodder (Yuxi District Aquatic Co., Ltd.) twice daily (morning and mid-afternoon). The fish were healthy and did not exhibit any physical injuries or abnormal swimming behavior. Before the experiment, 24 randomly selected fish from the batch were pooled into 8 samples (3 fish per pool) and tested by PCR for LMBV, and all eight pools tested negative, as shown in the nucleic acid electrophoresis image in Fig. S1. The fish were then randomly divided into eight groups in triplicate (120 fish per group) for subsequent immunization experiments. All experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Experiment Committee of Northwest A&F University (NWAUFU-2025314020038).

2.3. Sequence origin and construction

The MCP sequence, along with the 5' and 3' untranslated regions (UTRs), was selected from the largemouth bass virus isolate LMBV-FS001 (GenBank: ON936874.1) available at NCBI. This reference sequence was used to isolate and identify the virus in our laboratory. The MCP sequence of this strain is identical to that of the first identified largemouth bass virus. Two plasmids (pCDNA3.1 (+)) were synthesized. The MCP gene sequence was codon-optimized for largemouth bass (*Micropterus salmoides*). Both plasmids contained the upstream T7 RNA polymerase promoter and the MCP gene as common elements, but they differed in the presence or absence of the 5' and 3' UTRs. These plasmids were obtained from Sangon Bioengineering (Shanghai) Co., Ltd.

2.4. MCP antigen expression in *E. coli* and purification

The MCP gene sequence of the LMBV antigen was obtained by PCR amplification and then cloned into the pET-28a (+) vector. The resulting plasmid was transformed into BL21 competent cells by electroporation. Expression of the MCP protein was induced by IPTG. The MCP protein was purified using a HisPrep™ FF 16/10 column (Cytiva Co., Ltd., USA).

2.5. In vitro transcription and modification of mRNA

The linearized plasmids were transcribed in vitro using the T7 High Yield RNA Transcription Kit. During the reaction, UTP was replaced by N1-methyl-pseudouridine sodium. The vector construction is described in the Supplementary Methods. The mRNA was purified by phenol/chloroform extraction and ethanol precipitation, and then resuspended in RNase-free water. The 5' end of the mRNA was capped using the cap1 Capping System according to the manufacturer's instructions. Poly(A) tails were added to the 3' end using *E. coli* poly(A) Polymerase, followed by purification. After purification, the mRNA was dissolved in calcium acetate buffer (18.75 mM, pH 6.8). The integrity of the mRNA was analyzed by agarose gel electrophoresis (Fig. 1B).

2.6. Encapsulation and characteristic analysis of mRNA vaccines

The LNPs were composed of Span 80, DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), and D-α-tocopherol polyethylene glycol 1000 succinate (TPGS), and were prepared by vortexing with calcium acetate to form nano-lipid particles. Briefly, span 80, DOTAP, and TPGS were dissolved in 100% ethanol at a concentration of 50 mg/mL to prepare stock solutions. mRNA was dissolved in calcium acetate buffer (18.75 mM, pH 6.8). DOTAP, span 80, and TPGS were pre-mixed at a molar ratio of 50:45:5 to produce mixture A (total 40 μL). Mixture A was quickly injected into 860 μL of calcium acetate buffer containing 100 μL of mRNA (1.6 μg/μL). The resulting mixture was placed in a 1.5 mL centrifuge tube and vortexed rapidly at 2000 rpm for 45 s. It was then left

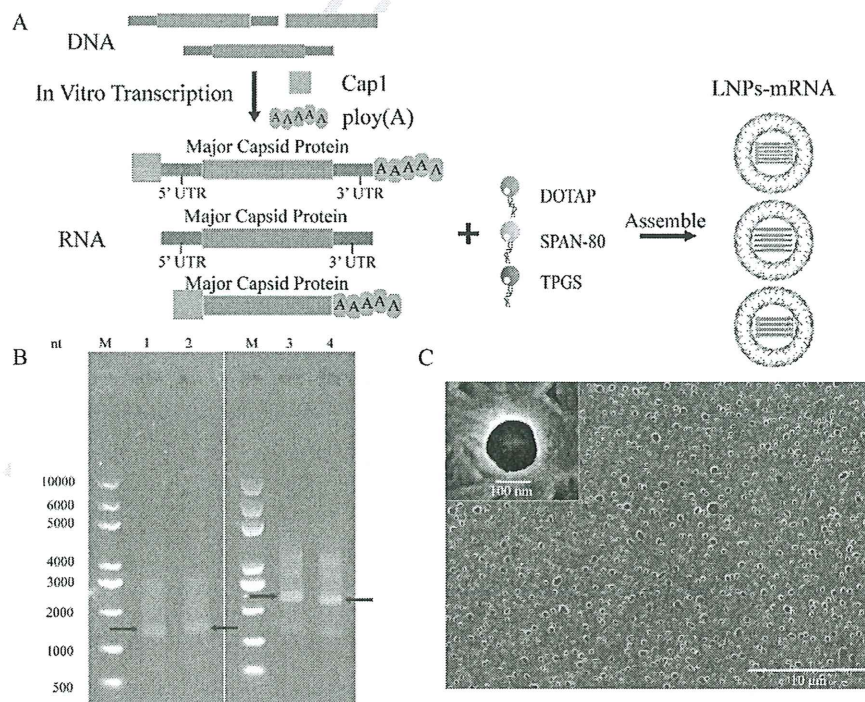


Fig. 1. Design and production of an mRNA vaccine against LMBV. A, schematic representation of mRNA preparation and lipid nanoparticle (LNP) encapsulation, including MCP, UTR sequences, a 5' cap, and a 3' poly(A) tail. B, gel electrophoresis analysis demonstrating the integrity of the mRNA sequence and the presence of the 3' poly(A) tail. C, scanning electron microscopy images of the LNPs showing a uniform particle size with an average diameter of approximately 100 nm.

Table 1

Design and source of primers for RT-qPCR and PCR test.

Genes		Accession no.	Primer sequence (from 5' to 3')	Cycling condition
β-actin	Forward	XM_038695351	CTGGTTGTGACAACGGATCC	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		GGCAACTCTCAGTCGTTGT	
IgM	Forward	MN871984	CAACGTCTGACCATCACC	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		GTCCCGTCTGGCACAGTA	
CD4	Forward	XM_038711094	GGAAGAAGATGCTGGACCG	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		GGTCCACAGGTCTGAGTTG	
TBK1	Forward	XM_038708273	GATCCCTTGACGCCGTCAG	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		TACAGGTCCCAGTTTCTTGTG	
RIG-I	Forward	XM_038704577	TTCATTITCGTTTCGTCGCTC	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		CATCCTACGCTTCTCCAGTCA	
MAVS	Forward	XM_038728262	TGTGTGCGAGAGGAAGACAG	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		CCCTGGCAAACGACATCTTTC	
IFNα	Forward	XM_038719795	CAATGGATCTTGTAGTTGCGGT	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		GCCTGTAAGCAGCCACGG	
MCP	Forward	ON418985	CCAACTACACTTGCCTCACT	95°C-1 min, 53°C-30 s, 72°C-10 s
	Reverse		GAAGGGTGAGGATCGTTGAG	

to stand at room temperature for 10 min before use or stored at -80°C for later use. The prepared mRNA vaccine contained 18 mM calcium acetate buffer and had a total lipid/surfactant concentration of 2 mg/mL. The amount of RNA in the solution was quantified using the Quant-iT RiboGreen RNA Quantification kit according to the manufacturer's instructions [32]. The mRNA samples were analyzed by fluorescence (excitation ~ 480 nm, emission ~ 520 nm) using a microplate reader to determine the content of unencapsulated mRNA. The mRNA encapsulation efficiency (EE) was calculated using the following formula:

$$\text{Encapsulation efficiency} = \left(\frac{\text{Total mRNA} - \text{Unencapsulated mRNA}}{\text{Total mRNA}} \right) \times 100\%$$

The particle size and zeta potential of the LNP/mRNA complexes were measured by dynamic light scattering (Zetasizer Nano 3600, Malvern Instruments Limited, UK).

2.7. Largemouth bass immunization

The largemouth bass were randomly divided into eight groups in triplicate (120 fish per group): L-LMUM groups at three different concentrations (0.1, 0.2, and 0.4 $\mu\text{g/g}$), and the L-LMU, L-LMM, and L-LM groups at the same concentration (0.2 $\mu\text{g/g}$), along with a blank group (calcium acetate buffer group) and a negative control group (LNPs group). All injections were administered at a fixed volume of 20 μL per fish. For the experimental groups, the injection solutions were prepared by diluting a stock mRNA-LNP formulation at 0.16 $\mu\text{g}/\mu\text{L}$ and adjusting to a final volume of 20 μL , such that each 20 μL dose delivered the required mRNA amount per gram of body weight. Specifically, the LNP control group received 20 μL of empty LNPs (2.5 μg lipid), and the buffer control group received 20 μL of calcium acetate buffer (18 mM). On day 1, the initial immunization was performed by intramuscular injection at the base of the dorsal fin. On day 28, a booster immunization was given by the same method [33].

2.8. Enzyme-linked immunosorbent assay

Specific antibody titers against the MCP antigen were determined using ELISA. Briefly, 96-well plates (Thermo Fisher) were coated with 100 μL of recombinant MCP antigen (5 $\mu\text{g}/\text{mL}$) in coating buffer and incubated overnight at 4°C . The plates were then washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST, pH 7.4). Next, the wells were blocked with 200 μL of 5% skim milk in PBST for 1 h at 37°C , followed by washing with PBST. Subsequently, 100 μL of serially diluted largemouth bass serum samples were added to each well and incubated for 1 h at 37°C . After washing with PBST, 100 μL of HRP-

conjugated goat anti-fish IgM (diluted 1:1000 in PBST) was added and incubated for 1 h at 37°C . The plates were washed again, and then 100 μL of TMB One Solution (HRP substrate) was added to each well, followed by incubation for 15 min at 37°C in the dark. The reaction was terminated by adding 50 μL of 1 M sulfuric acid. The absorbance at 450 nm was recorded using a multifunctional microplate reader (BioTek Synergy H1).

2.9. Specific antibody detection and gene quantification

Blood samples were collected from largemouth bass on days 1, 7, 14, 21, and 35 post-vaccinations. After coagulation at room temperature, serum was obtained, and antibody titers were measured by ELISA. Total RNA was extracted from the spleen using the MiniBEST Universal RNA Extraction Kit (TaKaRa), and cDNA was synthesized by reverse transcription of total mRNA using RevertAid RT (Thermo Scientific™). The primers used for RT-qPCR are listed in Table 1. RT-qPCR was performed to detect relevant gene expression according to the manufacturer's instructions. β-Actin was selected as the housekeeping gene. Each sample was analyzed in three biological replicates, and gene expression levels were calculated relative to β-actin using the $\Delta\Delta\text{Ct}$ method.

2.10. Virus challenge

On day 14 after both primary and secondary immunizations, largemouth bass were challenged with 20 μL of 1×10^3 TCID₅₀ LMBV-FS001 (NCBI: GCA_029269695.1) at the base of the pelvic fin using a 25 μL LC manual syringe (0.028 in OD). Clinical signs of LMBV and the number of deceased fish were recorded daily for 14 days, with dead largemouth bass being collected promptly. And through PCR, it was determined whether the deceased individuals died due to LMBV infection. The detection primers are shown in Table 2, and the detection results are presented in Fig. 6D. The relative survival rate (RSR) was calculated after this period using the following formula [24]:

$$\text{RSR} = \left(\frac{1 - \text{Mortality of experimental group}}{\text{Control group mortality}} \right) \times 100\%$$

Table 2

Design and source of primers for PCR test.

Genes		Accession no.	Primer sequence (from 5' to 3')	Cycling condition
MCP	Forward	ON418985	ATGTCTTCTGTTACGGGTT	95°C-1 min, 55°C-30 s, 72°C-10 s
	Reverse		TTACAGGATGGGGAACCCA	

2.11. Statistical analysis

Statistical analysis was performed using Origin 9 software (Northampton, USA) and GraphPad Prism (GraphPad, Inc., USA). All data are presented as mean $\pm$ SD. Statistical analyses were performed using GraphPad Prism 9.0. One-way ANOVA followed by Tukey's honest significant difference (HSD) post hoc test was used to compare all groups at each time point. Different lowercase letters (a, b, c, d, etc.) above the bars indicate statistically significant differences ($p < 0.05$), while the same letter indicates no significant difference.

3. Results

3.1. Design and characterization of LMBV mRNA vaccines

To evaluate the contribution of individual mRNA structural elements to vaccine efficacy, four mRNA constructs encoding the MCP of largemouth bass virus (LMBV) were designed. The basic construct containing only the MCP coding sequence was designated LM (LMBV-MCP). Addition of the untranslated region (UTR) to LM yielded LMU (LMBV-MCP-UTR). Modification with a 5' cap1 structure and a poly(A) tail produced LMM (LMBV-MCP-modified). The fully configured construct containing UTR, 5' cap1 and poly(A) tail was designated LMUM (LMBV-MCP-UTR-Modified) (Fig. 1A and B). All four mRNAs were successfully synthesized by in vitro transcription and purified for subsequent encapsulation. Each mRNA construct was encapsulated in lipid nanoparticles (LNPs) to generate the corresponding vaccine formulations: L-LM, L-LMU, L-LMM and L-LMUM. Naked LMUM without LNP encapsulation served as an additional control. Dynamic light scattering (DLS) analysis revealed that all LNP-formulated vaccines exhibited similar physicochemical properties, with an average particle diameter of approximately

110 nm and a zeta potential of approximately +54 mV (Fig. 2B). Scanning electron microscopy (SEM) confirmed the spherical morphology of the nanoparticles, with diameters consistently around 100 nm (Fig. 1C). These characteristics are considered favorable for cellular uptake and vaccine delivery.

3.2. High encapsulation efficiency and protection against RNase degradation

Encapsulation efficiency was determined using the RiboGreen assay. All four LNP-formulated vaccines demonstrated encapsulation efficiencies exceeding 96%, indicating that the vast majority of mRNA was successfully packaged within the lipid nanoparticles (Fig. 2A). DLS analyzer was used to measure the particle size and zeta potential of LNPs. The LNPs encapsulating RNA had an average particle size increase of approximately 10%, while the average zeta potential decreased by 8% (Fig. 2B). To assess the protective capacity of LNPs against nuclease degradation, mRNA was exposed to RNase A in the presence or absence of LNP encapsulation. After 6 h of exposure, the proportion of intact mRNA in the unprotected group was 10%, while that in the LNP encapsulation group was 43% (Fig. 2C and D), demonstrating that LNPs effectively protect mRNA from enzymatic degradation.

3.3. In vitro expression and immunogenicity in fish

To verify that the mRNA vaccines could be translated into antigenic protein, MSBr cells were transfected with the constructs. Protein expression was detected only in cells receiving LNP-encapsulated mRNAs, confirming the functionality of the vaccines (Fig. 3A–D). Largemouth bass were immunized intramuscularly at the base of the dorsal fin on day 1 and boosted on day 28 with the following treatments: L-LMUM at three

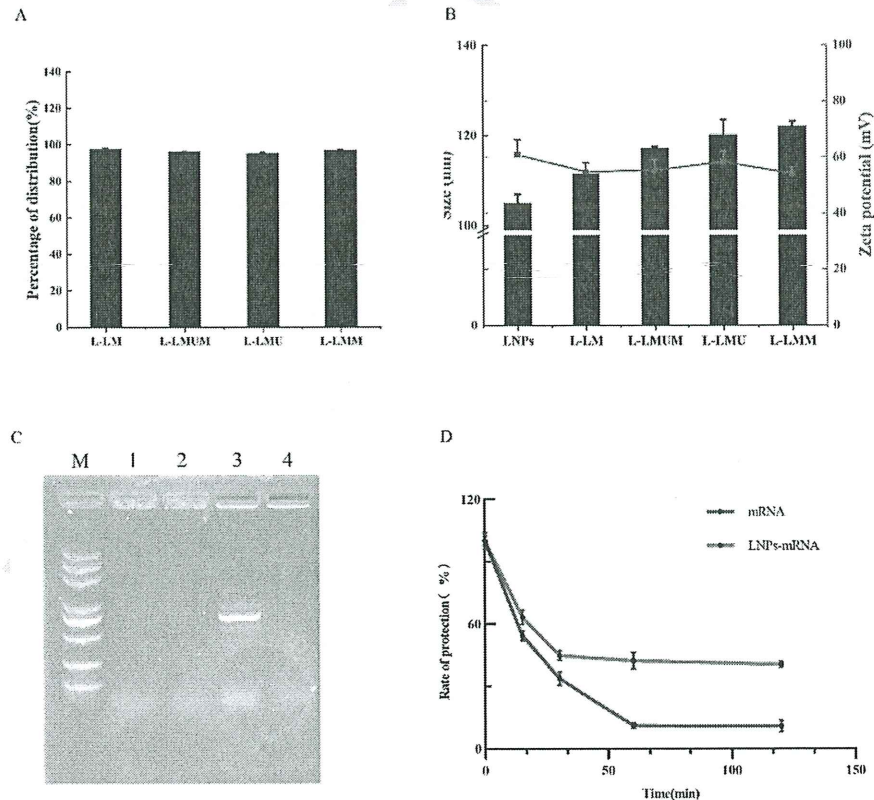


Fig. 2. Encapsulation efficiency, particle size distribution, zeta potential, and protective effect of mRNA-loaded LNPs. A, encapsulation efficiency of mRNA in LNPs exceeded 95%. B, the mRNA vaccine exhibited an average diameter of approximately 110 nm and a zeta potential of approximately +54 mV. C and D, ribonuclease protection assay. Lanes 1–4 correspond to the LNP control, naked LMUM, L-LMUM, and blank control groups.

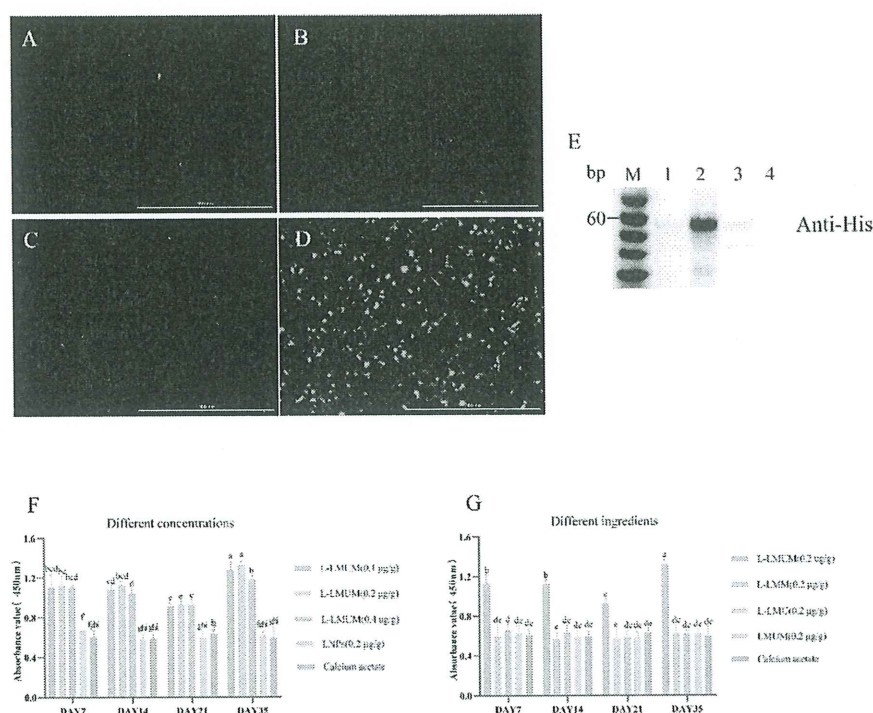


Fig. 3. In vitro and in vivo verification of MCP protein expression. A-D, transfected LM, LMU, LMM and LMUM, respectively. E, the Western blot result of MCP, Lane 1-4 was transfected with LMU, LMUM, LMM and LM. F-G ELISA detection of specific antibody. F, expression levels of specific antibody at different concentrations. G, expression levels of specific antibody under different modifications.

doses (0.1, 0.2 and 0.4 µg/g body weight), L-LMU (0.2 µg/g), L-LMM (0.2 µg/g), naked LMUM (0.2 µg/g), empty LNPs, or calcium acetate buffer (control). No adverse effects on feeding or behavior were observed in any group. Blood was collected on days 7, 14, 21 and 35. Serum antibody levels against MCP were measured by ELISA using recombinant MCP protein as the coating antigen (Fig. 3E and F). Fish immunized with L-LMUM at all three doses produced detectable MCP-specific antibody by day 7, with levels remaining elevated at day 14 and declining by day 21. After the booster (day 35), antibody titers rose sharply and were significantly higher than after the primary immunization. In contrast, no specific antibodies were detected in the L-LMU, L-LMM, naked LMUM, empty LNPs or buffer groups throughout the experiment.

3.4. Expression of immune-related genes

To further evaluate immune responses, spleens were collected from immunized fish on days 7, 14, 21 and 35, and the mRNA levels of IgM, CD4 and IFNα were quantified by RT-qPCR (Fig. 4). In the L-LMUM (0.2 µg/g) groups, IgM expression increased 3.4-fold within 14 days post primary immunization; CD4 expression was upregulated 3.2-fold; and IFNα showed a modest increase of 0.5-fold. No significant upregulation of IgM or CD4 was observed in the L-LMU, L-LMM or naked LMUM groups. Interestingly, in these three groups IFNα expression increased approximately 3-fold on day 7, returned to baseline by day 21, and again increased after the booster. In all experimental groups except controls, gene expression levels after the booster were significantly higher than after the first immunization.

3.5. Involvement of the RIG-I pathway

To investigate whether mRNA vaccine components modulate innate immune recognition, we examined the expression of RIG-I pathway genes (RIG-I, MAVS, TBK1) in the spleen by RT-qPCR (Fig. 5). On day 7, in fish receiving empty LNPs or naked LMUM no significant expression of these genes was detected. In the L-LMUM (0.2 µg/g) group (containing

all three elements), RIG-I (4.9-fold), MAVS (2.2-fold) and TBK1 (2.5-fold) were also elevated, but to a much lower extent. In contrast, the L-LMU and L-LMM groups (lacking either UTR or cap/poly(A)) showed strong activation: RIG-I expression was 4-fold higher than that in the L-LMUM (0.2 µg/g), MAVS and TBK1 were upregulated separately 3.5-fold and 2-fold, respectively after primary immunization. These data indicate that the presence of both the 5' cap1/poly(A) and the UTR dampens activation of the RIG-I pathway.

3.6. Protective efficacy against LMBV challenge

To evaluate protection, immunized fish were challenged with a lethal dose of LMBV (1×10^3 TCID₅₀) on day 14 after primary or secondary immunization, and mortality was monitored for 14 days (Fig. 6). Clinical signs typical of LMBV infection were observed in moribund fish, and viral DNA was detected in multiple tissues by PCR (Fig. 6C and D). Following primary immunization, survival rates in the three L-LMUM groups ranged from 45% to 50%, whereas L-LMU, L-LMM and naked LMUM groups had survival rates of only 10-15%. The LNP control group showed 2% survival, and all fish in the buffer control died. After the booster immunization, survival in the L-LMUM groups increased slightly to 53-57%, while the other vaccine groups remained below 15% and controls again experienced complete mortality. Deaths began around day 4 post-challenge and ceased by day 13 in all groups (See Fig. 7).

4. Discussion

In this study, we developed an mRNA vaccine against LMBV and systematically evaluated the contribution of its structural elements-5' cap1, UTR, poly(A) tail and LNP delivery-to immune activation and protection in largemouth bass. Our results demonstrate that the fully configured mRNA vaccine (L-LMUM) elicits specific antibody responses, upregulates key immune genes, and provides significant protection against lethal viral challenge, with survival rates reaching 57% after boosting.

The differential outcomes among vaccine groups clearly demon-

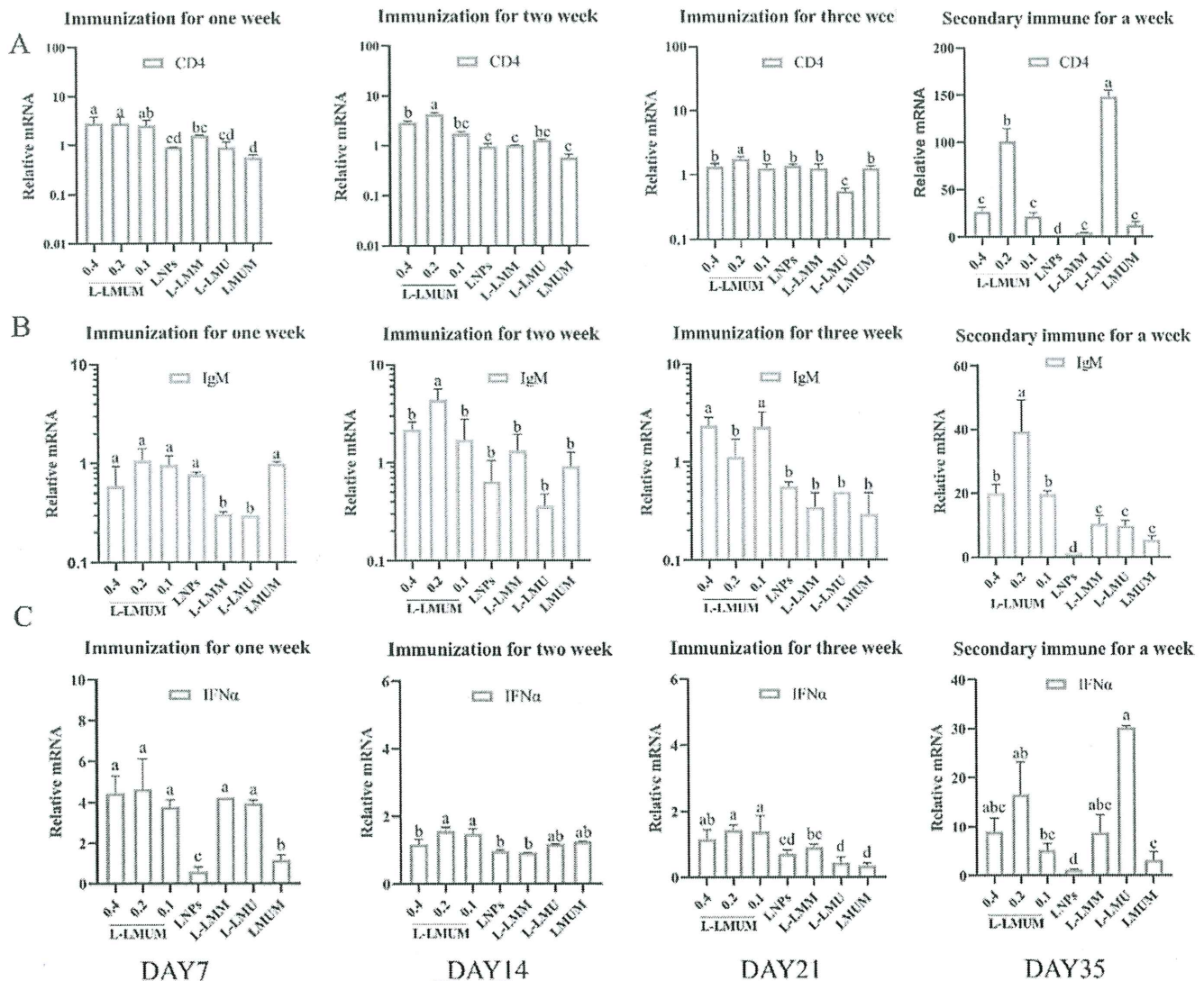


Fig. 4. The results of Quantitative Real-time PCR for CD4, IFN α and IgM gene expression were presented. A-C, showed the gene expression of CD4, IgM and IFN α respectively from day 7 to day 35. CD4 and IFN α gene expression increased on day 7 after the first immunization, then gradually decreased and returned to normal by day 21, while IgM gene expression slightly increased on day 7, remained elevated until the day 21. Significantly increased expression of all genes in each group was observed 7 days after the second immunization, indicating that the second immunization could induce stronger humoral immunity. The blank control group received calcium acetate buffer only, and all gene expression levels were normalized to this group.

strated the indispensable role of each structural element. Only fish immunized with L-LMUM produced detectable MCP-specific antibodies (Fig. 3F and G), indicating that both the UTR and the cap 1/poly(A) modifications are required for efficient *in vivo* translation of the antigen. This finding aligns with the established roles of these elements in enhancing mRNA stability and translation efficiency in eukaryotic cells [34–36]. Together, these results confirm that the complete set of mRNA structural elements is critical for efficient antigen expression and the induction of specific antibodies.

The pattern of exogenous RNA recognition by the RIG-I pathway is central to understanding both antiviral immunity and mRNA vaccine design. The role of the RIG-I pathway in RNA virus infection and mRNA vaccine entry into cells has been well demonstrated. When viral RNA, foreign RNA, or aberrantly transcribed RNA lacks a proper 5' cap1 structure, it can be recognized by RIG-I, which then undergoes ubiquitination and activation. Activated RIG-I binds to the downstream adaptor protein MAVS on the mitochondrial membrane, leading to the recruitment and activation of TBK1 and NF- κ B, together with IKK ϵ , form a signaling complex that drives the expression of IFN α , ultimately clearing the

abnormal RNA. Meanwhile, the activation of NF- κ B can promote inflammation, which is detrimental to the survival of the host [37]. However, the presence of a complete set of structural elements including a 5' cap1, untranslated regions, and a poly(A) tail enables exogenous mRNA to evade excessive RIG-I activation [38,39]. The poly(A) binding protein (PABP) can form a closed-loop structure through the interaction of the translation initiation factors eIF4G and eIF4E with the 5' cap1 and is synergistically involved in the regulation of mRNA stability and translation activity [40].

Analysis of immune gene expression revealed that L-LMUM induced moderate upregulation of IgM, CD4 and IFN α , consistent with a balanced adaptive immune response [41]. Notably, the L-LMU and L-LMM groups exhibited strong but transient IFN- α induction after primary immunization, accompanied by minimal IgM/CD4 responses. This pattern suggests that these incomplete mRNAs are recognized as “non-self” by the innate immune system, leading to IFN α production, but they are unable to sustain antigen expression necessary for robust adaptive immunity [42]. The IFN α spike observed on day 7 in these groups likely reflects engagement of the RIG-I pathway, as confirmed by the profound upregulation

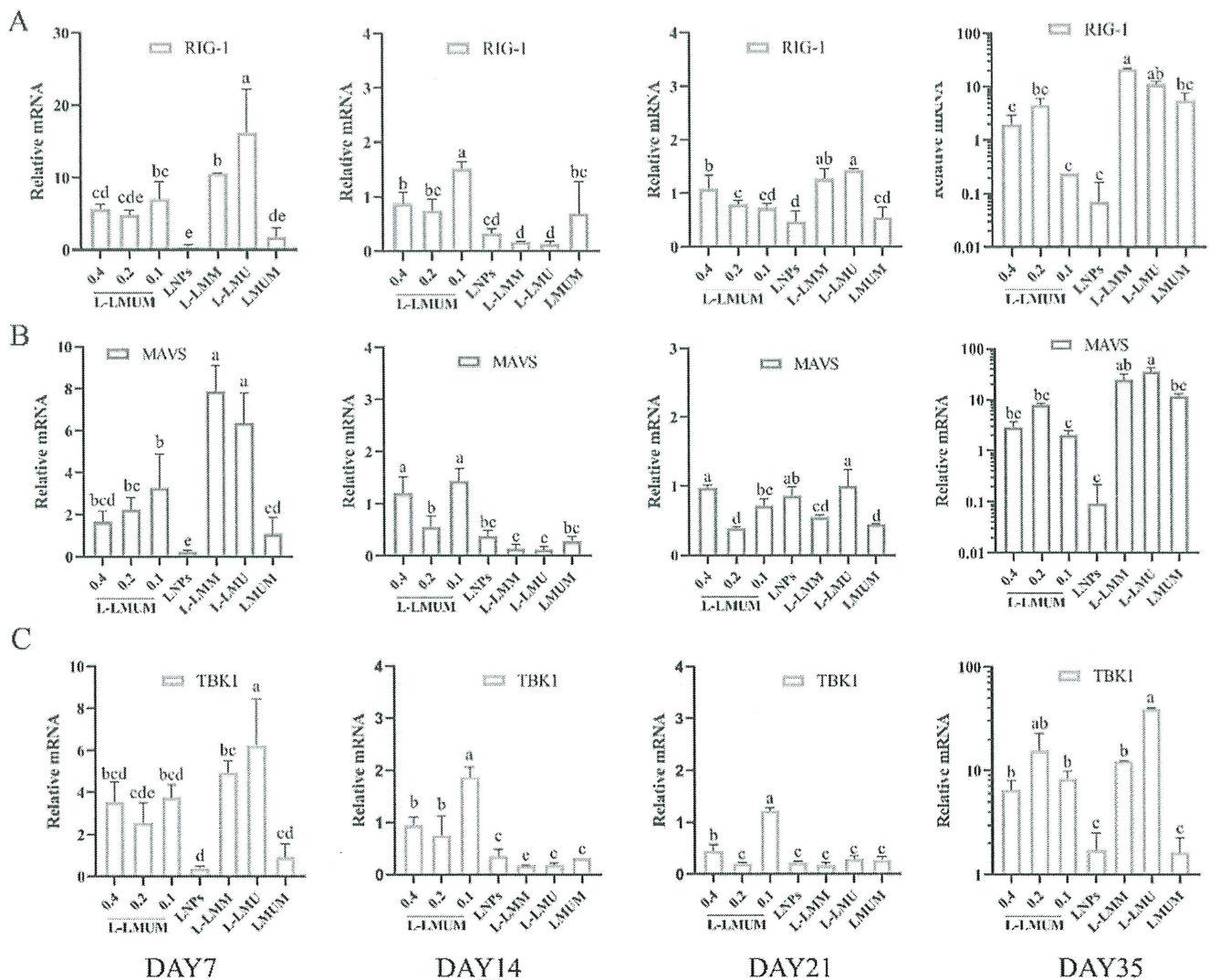


Fig. 5. This figure shows the results of Quantitative Real-time PCR for RIG-I, MAVS and TBK1. A-C, showed the gene expression of RIG-I, MAVS and TBK1 respectively from day 7 to day 35. No significant expression was detected in empty LNP or naked LMUM groups. L-LMUM (0.2 μg/g) induced moderate upregulation (4.9-fold, 2.2-fold and 2.5-fold, respectively), whereas L-LMU and L-LMM (lacking UTR or cap1/poly(A)) induced stronger activation (RIG-I 4-fold higher than L-LMUM, MAVS 3.5-fold and TBK1 2-fold), suggesting that full mRNA elements dampen RIG-I pathway activation. Expression levels are shown relative to the blank control group (calcium acetate buffer).

of RIG-I, MAVS and TBK1 in the same fish (Fig. 5). In contrast, L-LMUM triggered only modest RIG-I pathway activation, indicating that the combination of cap1, UTR and poly(A) helps the mRNA evade innate sensors. This is consistent with reports that properly capped and polyadenylated mRNAs with structured UTRs mimic endogenous transcripts and are poorly recognized by RIG-I-like receptors [38,43].

The observed dampening of RIG-I activation by complete mRNA elements may represent an important biological trade-off. While strong innate sensing (as seen with incomplete constructs) can induce type I interferons, it may also lead to rapid clearance of the mRNA and insufficient antigen accumulation. Conversely, moderate RIG-I activation by fully modified mRNA may allow prolonged antigen expression and more effective adaptive immunity. This balance is reminiscent of observations in mammalian systems and warrants further investigation in teleosts.

The critical role of LNP encapsulation was demonstrated by the complete lack of immune responses and protection in fish receiving naked LMUM. Without a delivery vehicle, mRNA is rapidly degraded by extracellular ribonucleases and cannot efficiently cross the cell membrane [44,45]. The cationic LNPs used here (zeta potential > +30 mV) fa-

cilitated cellular uptake and endosomal escape [27], as previously described [46,47]. The high encapsulation efficiency (>96%) and particle size (~110 nm) are well within the optimal range for vaccine delivery, contributing to the efficacy of the vaccine [48,49].

Protection against LMBV challenge correlated strongly with the presence of a complete mRNA construct. L-LMUM conferred 45-50% survival after primary vaccination and 53-57% after boosting, whereas all other vaccine groups afforded ≤20% survival. This protection level is comparable to that reported for some DNA and subunit vaccines against fish viruses (e.g., 61.1-78% for a genetic vaccine against LMBV) [50], but the mRNA platform offers the distinct advantages of rapid production and flexibility to respond to emerging variants. Notably, the survival rates after primary and booster immunization were similar, suggesting that a single dose may already induce near-maximal protection under the conditions tested. The absence of any adverse effects indicates good tolerability.

In conclusion, the L-LMUM mRNA vaccine, incorporating all structural optimizations, successfully induced protective immunity in large-mouth bass, highlighting the potential of mRNA-LNP platforms for aqua-

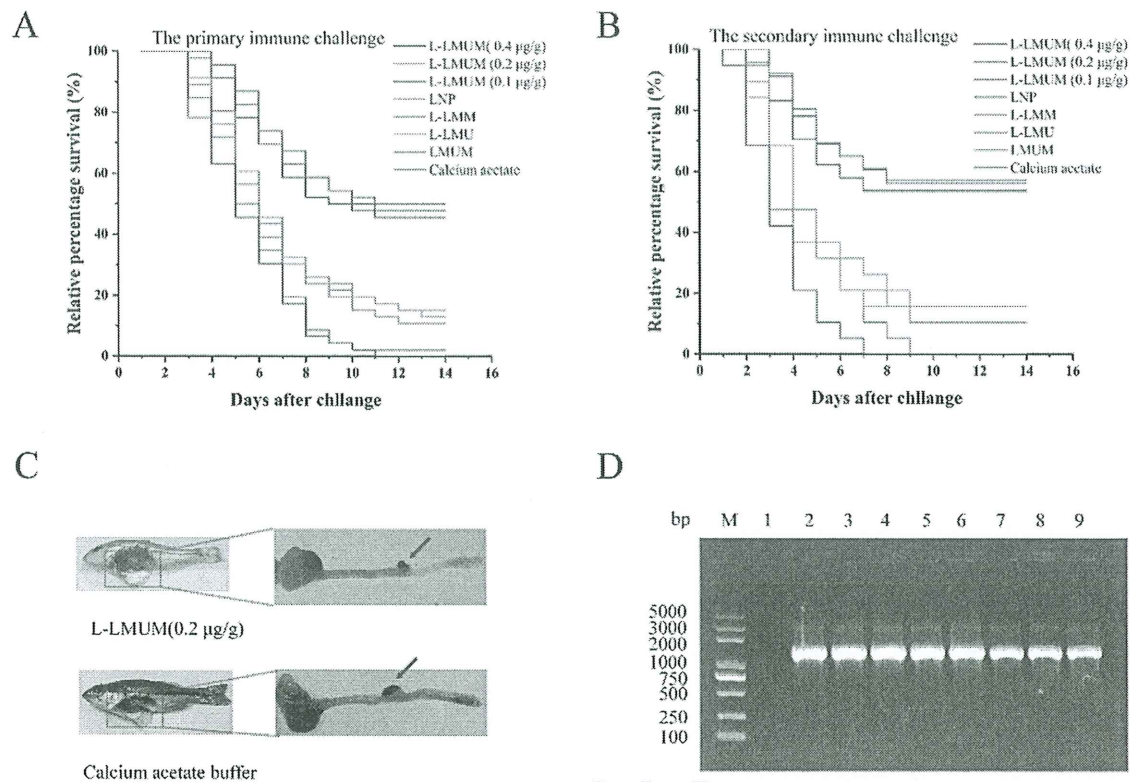


Fig. 6. The MCP mRNA vaccine protects largemouth bass from lethal doses of LMBV. Eight groups of largemouth bass ($n = 50$ per group) were immunized with different mRNA vaccines or placebos. Seven days after the primary vaccination and seven days after the booster immunization, the fish were intraperitoneally injected with a lethal dose (1×10^3 TCID₅₀) of LMBV-FS001. Mortalities were recorded daily. A, survival rate of largemouth bass within 14 days after primary challenge. B, the challenge survival rate of largemouth bass within 14 days after secondary challenge. C, the spleen in the calcium acetate buffer group was swollen and larger than that in the L-LMUM (0.2 µg/g) group. D, lane 1-9 were respectively the non-inoculated group, calcium acetate LNP, LMUM, L-LMU, L-LMM and L-LMUM (0.1, 0.2 and 0.4 µg/g).

culture disease control. Future work should focus on optimizing dose regimens, exploring longer-term protection, and testing the platform against other important aquatic viruses.

5. Conclusion

In this study, mRNA vaccines were designed that incorporated various components, including 5' cap1, untranslated regions, poly(A) tails, and LNPs. The combination of these elements demonstrated the crucial role of complete mRNA vaccine components in facilitating mRNA entry into largemouth bass. Further results indicated that 5' cap1, poly(A) tails, and untranslated regions inhibited the activation of the RIG-I signaling pathway, reduced mRNA immunogenicity, improved stability, and promoted mRNA translation in largemouth bass. The antigen expressed by the prepared mRNA vaccine exhibited good immunogenicity and provided immune protection for largemouth bass, suggesting that the application of mRNA vaccines holds significant potential in preventing diseases in aquatic economic animals.

Credit author statement

Y. Liu: Methodology, Formal analysis, Investigation, Validation, Writing-Original Draft. G. Zhang: Methodology, Formal analysis. H. Qu: Methodology, Formal analysis. Z. Hu: Methodology. M. He: Software, Resources. F. Ling: Resources. G. Wang: Supervision, Writing review & editing. T. Liu: Supervision, Project administration, Funding acquisition, Writing-Review & Editing.

Ethics statement

All experimental animals in our experiments are carried out in strict accordance with the guidelines of the Animal Experiment Committee of Northwest A&F University (IACUC, NWAFU-2025314020038), which meets the requirements of morality and ethics.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2026.111560>.

Data availability

Data will be made available on request.

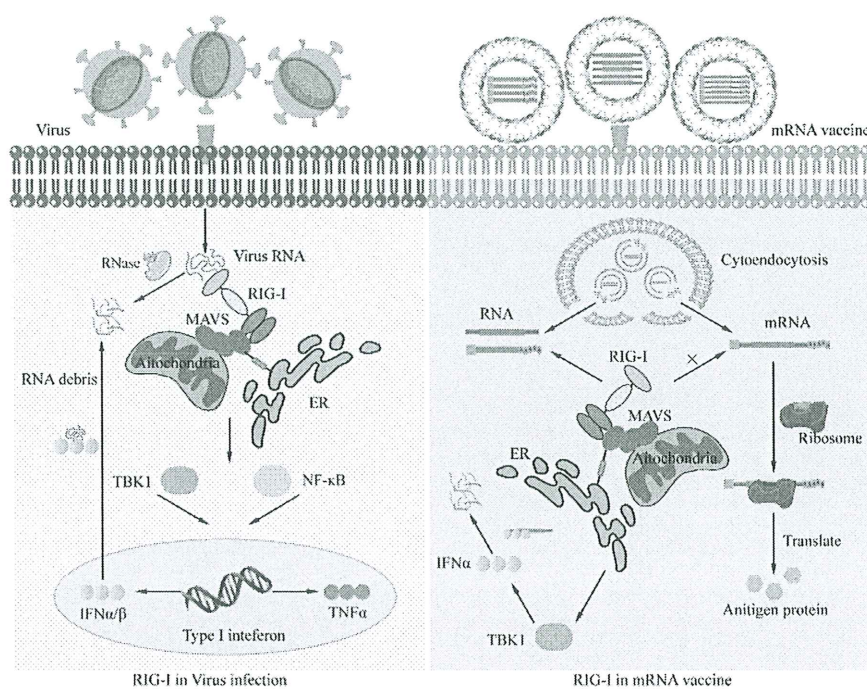


Fig. 7. The pattern of exogenous RNA recognition by the RIG-I pathway. The role of the RIG-I pathway in RNA virus infection and in the entry of mRNA vaccines into cells have been demonstrated. When viral RNA, foreign RNA, or abnormally transcribed RNA lacks a 5' cap (1), it can activate RIG-I, leading to its ubiquitination modification, then binds to downstream adaptor protein MAVS on the mitochondrial membrane, further activates TBK1 and NF-κB, and form a complex with IKKε, thereby driving IFNα expression and ultimately leading to the clearance of the abnormal mRNA.

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