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Identification of a camelid-derived nanobody as a potential therapeutic agent against *Streptococcus agalactiae* infection

Ai-Guo Huang [a,b,c], Wei-Hao He [b,c], Lin-Jun Su [d], Fa-Li Zhang [b,c], Ying-Hui Wang [b,c,*]

[a] College of Life Science and Technology, Guangxi University, Nanning 530004, China
[b] Guangxi Laboratory on the Study of Coral Reefs in the South China Sea, Guangxi University, Nanning 530004, China
[c] School of Marine Sciences, Guangxi University, Nanning 530004, China
[d] China-ASEAN College of Marine Sciences, Xiamen University Malaysia, 43900, Malaysia

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ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) is a severe pathogen that has resulted in enormous economic losses in the global tilapia sector. GBS can be effectively controlled by antibiotics to some extent, but antibiotic resistance limits the application of antibiotics. It is urgent to find eco-friendly novel strategies against GBS. As the smallest antibody, nanobodies (Nbs) are novel effective biologics for treating bacterial and viral infections, as they can identify conserved epitopes of hypervariable pathogens. In the study, GBS-specific Nbs were screened from a naive phage-displayed Nb library, and the anti-GBS activity of Nbs was explored *in vivo*. The results showed that high-affinity Nbs were obtained from a naive phage-displayed Nb library after three rounds of biopanning. A novel nanobody Nb01 was expressed in the *Escherichia coli* expression system and purified using the Ni-NTA Agarose column. The results of indirect ELISA showed that purified recombinant protein Nb01 still had a high affinity against GBS. Furthermore, we found that Nb01 could effectively inhibit GBS infection *in vivo*. The copy numbers of GBS in the zebrafish brains were significantly reduced after treatment with Nb01. The survival rate of GBS-infected zebrafish treated with Nb01 was 70.0%, while it was only 43.3% for the controls. The expression of proinflammatory cytokine genes (*tnf-α*, *il-6* and *inf-γ*) and Wnt-signaling related genes (*wnt2*, *wnt3a* and *fzd5*) in the GBS-infected zebrafish brain were significantly decreased after treatment with Nb01. In conclusion, Nb01 could protect zebrafish from GBS infection, and these results highlighted the potential therapeutic activity of Nbs against GBS in fish.

1. Introduction

Streptococcus agalactiae, also known as Group B streptococcus (GBS), is a severe pathogen that can infect various freshwater and marine fishes worldwide, especially tilapia (Zhang et al., 2022b). GBS has been found in global tilapia major aquaculture areas, including China, Thailand, Vietnam, the USA, Israel, Brazil, and Colombia (Su et al., 2019; Zhang, 2021). Due to the high morbidity and mortality (30–90%), GBS has resulted in serious economic losses in the global tilapia industry every year (Cao et al., 2022; Su et al., 2019; Xie et al., 2021). GBS has become a hindrance to the development of global tilapia farming. At present, antibiotics have been used to prevent and control GBS (Dong et al., 2021). However, frequent and improper usage has resulted in antibiotic residue and antimicrobial resistance, which possibly affect food safety and accelerate the genetic mutation of GBS (Wang et al., 2020; Zhang et al., 2022b; Zhang, 2021). Vaccination is an alternative strategy to prevent GBS infection (Nurani et al., 2020; Ramos-Espinoza et al., 2020). However, vaccines aim to activate the immune system to trigger a specific response, so vaccines must be administered before infection to produce a strong immune response and cannot be used to treat infected fish (Liu et al., 2017; Sari et al., 2021). Moreover, there are no commercial vaccines available for the control of GBS infection. Therefore, it is urgent to identify eco-friendly novel strategies against GBS in aquaculture.

GBS can penetrate the blood-brain barrier (BBB) and lead to meningitis in tilapia (Cao et al., 2022). However, BBB can hinder drug molecules from transferring into the brain and thus consumingly decrease the therapeutic efficacy (Wang et al., 2018b; Zou et al., 2019). Thus, drugs that can cross the BBB are more likely to have a therapeutic effect than those that do not. Interestingly, nanobodies (Nbs) can cross

* Corresponding author at: Guangxi Laboratory on the Study of Coral Reefs in the South China Sea, Guangxi University, Nanning 530004, China.
E-mail address: wyh@gxu.edu.cn (Y.-H. Wang).

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the BBB freely (Gettemans and De Dobbelaer, 2021; Ruiz-Lopez and Schuhmacher, 2021; Zhu et al., 2022). Nbs, referred to as the variable domain of *Camelidae* heavy-chain only antibodies, are the smallest antibody (about 15 kDa) with intact antigen-binding fragments (Hamers-Casterman et al., 1993; Hu et al., 2021). Nbs retain full antigen-binding properties as traditional antibodies with high affinity and specificity (Zhang et al., 2022a). Compared with traditional antibodies, Nbs possess several unique advantages, such as good antigenicity towards cryptic epitopes, weaker immunogenicity, stronger penetration, highly stable under extreme conditions and easily expressed (Ji et al., 2021; Muyldermans, 2021; Zhang et al., 2022a). Currently, Nbs have been widely used in the fields of environmental monitoring, diagnosis and therapy (Dulal et al., 2022; Ji et al., 2020a; Li et al., 2021; Wilken and McPherson, 2018). However, there are few reports on the applications of Nbs in fish diseases, especially in the therapy of GBS.

In the study, we aimed to obtain anti-GBS Nbs from a naive phage-displayed Nb library. Firstly, GBS-specific Nbs were screened from a naive phage-displayed Nb library, and 3 GBS-specific Nbs were obtained from the Nb library. Subsequently, Nb01 was selected for further expression in the prokaryotic expression system based on the binding activity and abundance. Furthermore, the affinity of purified Nb01 to GBS was verified by indirect ELISA. Finally, the anti-GBS activity of the purified Nb01 was evaluated in zebrafish. This work would lay foundations that Nbs may serve as potential agents for the prevention and therapy of GBS.

2. Materials and methods

2.1. Zebrafish and bacterial strain

Zebrafish (total length: 3.53 ± 0.22 cm, body weight: 0.41 ± 0.08 g, mean ± SD) were purchased from an Aquarium Market at Nanning, Guangxi, China. Zebrafish were randomly collected before the start of the experiment to verify GBS-free status by PCR detection. Zebrafish were acclimatized in 200 L aquariums at 28 °C for 2 weeks, and fed twice daily with a commercial feed. GBS strain GXYL7, isolated from diseased tilapia, was kindly given by Dr. Meiling Yu from the same school. The serotype of the strain has been proven to be Ia.

2.2. Biopanning of GBS-specific Nbs

Three rounds of biopanning of GBS-specific nanobodies were performed. Briefly, the immunotubes were coated with 2 mL of inactivated GBS bacteria (about 1×10^8 CFU/mL) overnight at 4 °C. After blocking with 2 mL of 3% (*w/v*) nonfat powder milk, 2 mL of naive phage-displayed Nb library (Kangti Life, Shenzhen, China) was added to reach around 1×10^{12} pfu/tube and incubated for 1 h at 37 °C. Then the unbound phages were removed by washing with 2 mL of PBST for 10 times. The remaining bound phage particles were eluted with 1 mL of 0.25 mg/mL trypsin for 30 min, and immediately neutralized with 10 μL of 10% AEBSF. Finally, eluted phage was added to infect *Escherichia coli* SS320 strains and amplified overnight at 37 °C after infecting with M13K07 helper phages. Then the amplified phages were purified using PEG 4000/2.5 M NaCl precipitation for the next round of selection. Three rounds of biopanning were conducted with gradually decreasing concentration of coated inactivated GBS bacteria.

2.3. Phage ELISA

After three rounds of panning, 48 individual clones from the agar plate of the third round of eluted phages were picked randomly for identification by phage ELISA. Briefly, 96-well plates were coated with inactivated GBS bacteria (about 1×10^8 CFU/mL) overnight at 4 °C. After washed with PBST, the plates were blocked with 3% nonfat milk powder for 1 h at 37 °C. Then the plates were washed and the amplified phages were added to each well, followed by incubation for 2 h at 37 °C. After washed with PBST for 6 times, the HRP-conjugated anti-M13 mouse monoclonal antibody was added and incubated for 1 h at 37 °C. Subsequently, 100 μL of TMB solution (Solarbio, China) was added and incubated for color development. The reaction was stopped with 50 μL of 2 M H_2SO_4, and absorbance at 450 nm was detected by a microplate reader. The positive clones were sequenced with phiS3/psiR3 primers (Table S1) and the Nbs were grouped based on their complementary determining regions (CDRs) of amino acid sequence.

2.4. Expression and purification of nanobody Nb01

The expression and purification of nanobody Nb01 were performed as described in a previous study (Wang et al., 2019). Briefly, the Nb01 fragments were amplified by PCR using VHH-1F/VHH-1R primers (Table S1) and cloned into the EcoR I and Hind III sites of pET-28a(+) vector. For Nb01 expression, the recombinant plasmid pET28a-Nb01 was transformed into *E. coli* BL21(DE3), and protein expression was induced by 0.8 mM IPTG at 37 °C for 5 h. To determine recombinant protein Nb01 expressed as soluble or inclusion body protein, the cells were ruptured and both supernatant and pellet fractions were detected using SDS-PAGE. Recombinant proteins in inclusion bodies were dissolved in 8 M urea and then purified using the Ni-NTA Agarose column. The denatured proteins were refolded by dilution in base refolding buffer (0.88 mM KCl, 21 mM NaCl, 55 mM Tris, 880 mM L-arginine, pH 8.2) with 15 mM oxidized glutathione, 150 mM reduced glutathione and 10 mM EDTA. The refolded proteins were dialyzed in PBS. The purity of recombinant protein Nb01 was detected by SDS-PAGE. The purified recombinant proteins were concentrated in ultrafiltration tubes (Millipore, USA). The concentration was tested using a BCA protein assay kit.

2.5. Affinity verification

To detect the binding activity of Nb01 to GBS, indirect ELISA was performed as described in previous reports (He et al., 2020; Wang et al., 2019). Briefly, the 96-well microtiter plates were coated with inactivated GBS (about 1×10^8 CFU/mL). After washed 3 times with PBST, the plate was blocked for 1 h at 37 °C with 3% nonfat milk powder in PBS. The dilution series (from 1.25 to 20 mg/L) of purified recombinant protein Nb01 was added to wells followed by 1 h incubation at 37 °C. After washed 3 times with PBST, the Nb01 was detected using the anti-His tag monoclonal antibody (Solarbio, China). Subsequently, HRP-conjugated goat anti-mouse IgG (Solarbio, China) was used to detect the bound antibodies. Color was developed using TMB solution, and the reaction was stopped by addition of 50 μL of 2 M H_2SO_4. The optical density at 450 nm was measured by means of a microplate reader.

2.6. Anti-GBS activity of Nb01 in zebrafish

The zebrafish survival assay was conducted to evaluate the anti-GBS activities of Nb01. Zebrafish were randomly divided into two groups. Groups of zebrafish were intraperitoneally injected with 20 μL GBS solutions (about 1.0×10^8 CFU/mL) only or a mixture containing GBS and Nb01 (100 mg/kg body weight, preincubated for 30 min). Each treatment was performed in triplicate, and each replicate contains 10 zebrafish. Changes in the survival of GBS-infected zebrafish were closely monitored for 168 h. In addition, zebrafish were euthanized at 24 h post-infection, and brain tissues were removed for quantified the copy numbers of GBS.

2.7. Genomic DNA extraction and absolute quantification of the copy numbers of GBS

The brain tissues of GBS-infected zebrafish were taken at 24 h indicated above. All tissues were triturated into a fine powder using a tissue homogenizer. And the genomic DNA was extracted as described previously (Cao et al., 2022). The genomic DNA concentrations were

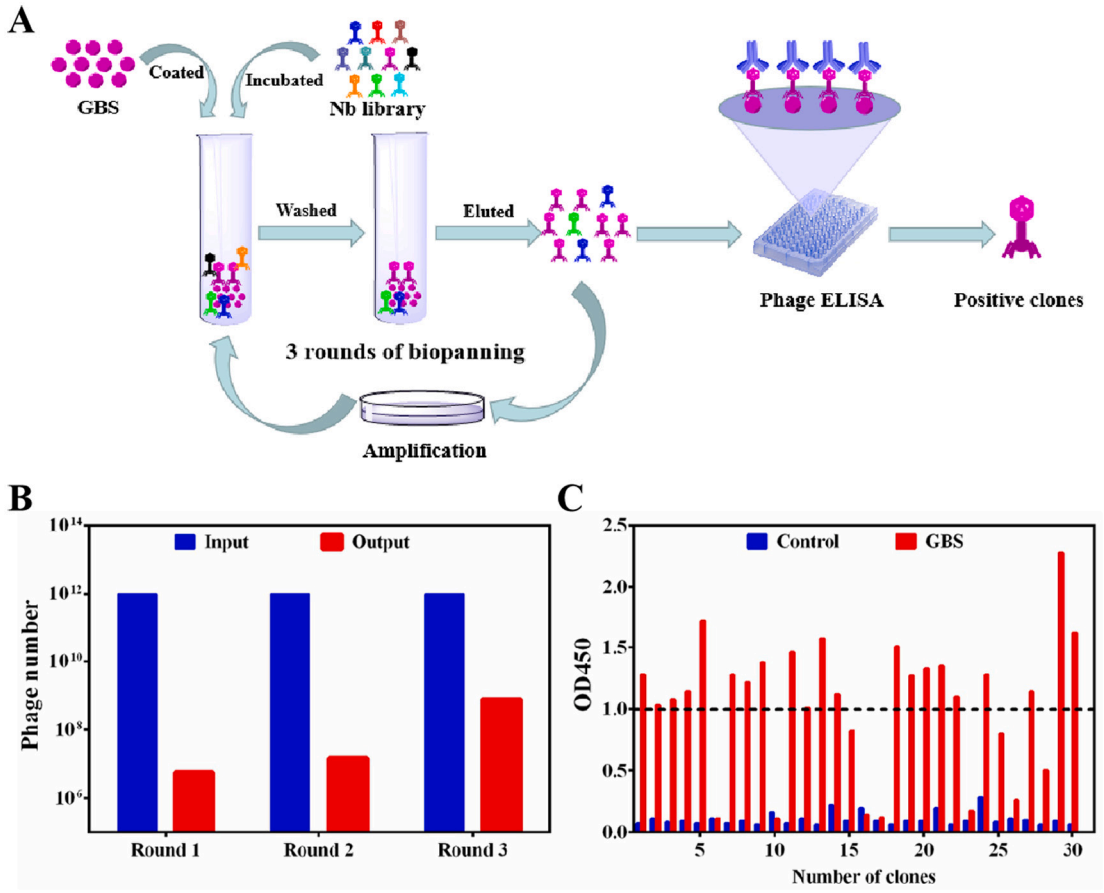

Fig. 1. Biopanning of GBS-specific Nbs. (A) Schematic diagram of biopanning of GBS-specific Nbs; (B) The enrichment of eluted phage during the biopanning cycles; (C) Screening and identification of the GBS-specific Nbs *via* indirect phage-ELISA.

subjected to quantification by NanoDrop spectrophotometer.

The copy numbers of GBS genomic DNA were quantified using quantitative real-time PCR (qPCR) and standard curve as a previous study (Cao et al., 2022). Briefly, the *cfb* gene, encoded the CAMP factor, was cloned using *cfb* primers (Table S2) and inserted into the pMD19-T simple vector (Takara, China). Then the vector was transferred into *Escherichia coli* strain DH5α. The concentration of the plasmid was measured by NanoDrop spectrophotometer to quantify the copy numbers of the target amplicon. The plasmid was serially diluted 10-fold and used as the template for qPCR. The qPCR was performed in ABI 7500 Fast Real-Time PCR Detection System using UltraSYBR Mixture (CWBIO, China). The PCR cycling conditions were: 95 °C for 10 min, then 40 cycles at 95 °C denaturation for 15 s, and 60 °C annealing for 30 s. To verify the amplification of a single product, a melt curve analysis of 5 s per step from 65 to 95 °C was performed at the end of each PCR thermal profile. Regression of the log of *cfb* gene copy numbers and the corresponding cycle threshold (Ct) value was used as a standard curve to determine the copy numbers of GBS.

2.8. RNA extraction and relative quantification of the expression of zebrafish genes

Total RNA from zebrafish brain tissues was extracted with TRIzon Reagent (CWBIO, China). The quality and concentration of extracted RNA were determined by 1.5% agarose gel electrophoresis and NanoDrop spectrophotometer. Then extracted RNA was reverse-transcribed into cDNA using HiFiScript cDNA Synthesis Kit (CWBIO, China). The expression levels of zebrafish genes were determined using qPCR according to the description above. The primers for qPCR in this study were shown in Table S2.

2.9. Statistical analysis

Statistical analyses were performed using SPSS 18.0 statistical software (SPSS Inc., USA). The significance was determined by analyzing the data using one-way ANOVA and post-hoc Tukey test. All data were presented as mean ± SD.

3. Results and discussion

GBS has a high transmission and mortality rate in the global tilapia industry, and currently GBS infections can be controlled by antibiotics to some extent (Wang et al., 2018a). However, the widespread use of antibiotics in aquaculture has led to the emergence of antibiotic residue and resistance (Liu et al., 2019). Vaccination has been considered as an effective strategy in preventing GBS infection in aquaculture (Ke et al., 2021). Nevertheless, the development of a broadly protective and likely multicomponent/multivalent vaccine against all GBS strains is one of the most important challenges in vaccine design. An effective vaccine may be multicomponent to provide coverage of GBS strains of distinct serotypes with genetic variabilities. Moreover, there remains no licensed vaccine against GBS. Nbs are successful new biological products in the fields of diagnosis and therapy, as they can identify conserved epitopes on hypervariable pathogens (Amcheslavsky et al., 2021; Zhu et al., 2022). Due to smaller paratope diameters and a longer CDR3, Nbs can penetrate deeply into cryptic cavities or dense clefts on antigens (Ji et al., 2020b). The highly stable of Nbs under extreme conditions such as thermal and chemical denaturation has been demonstrated (Hu et al., 2021), which are suitable for oral administration in aquaculture. Furthermore, Nbs can be easily expressed in large quantities in different systems at a low production cost (Ji et al., 2021). Consequently, Nbs may

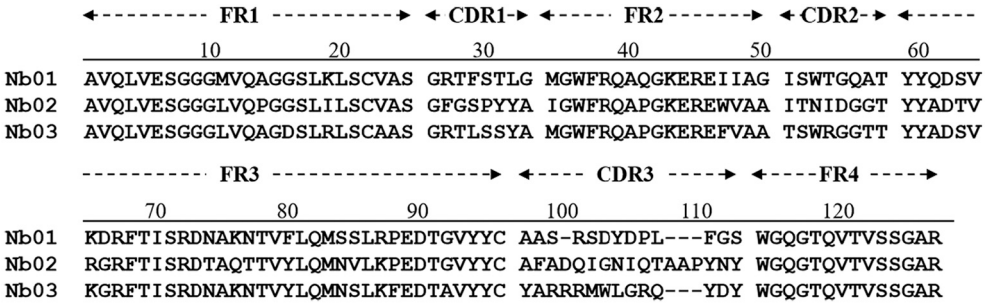

Fig. 2. Alignment of the amino acid sequence of GBS-specific Nbs.

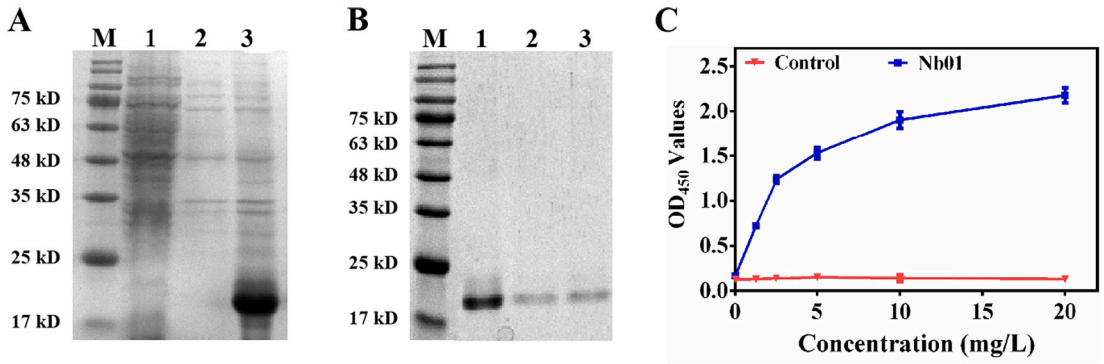

Fig. 3. Characterization of GBS-specific Nb01. (A) SDS-PAGE analysis of the expression of Nb01. M: protein marker, Lanes 1: empty pET-28a control, Lanes 2: supernatant, Lanes 3: precipitate; (B) SDS-PAGE analysis of the purified Nb01; (B) Determination of the binding activity of Nb01 to GBS by indirect ELISA. The values are presented as means ± SD ($n = 3$).

be suitable in aquaculture for the treatment of a wide range of diseases by oral administration. In the study, a naive phage-displayed Nb library was used to screen potent anti-GBS Nbs. Notably, Nb01 had a significant anti-GBS activity by reducing the copy numbers of GBS in zebrafish brains and improving the survival rate of GBS-infected zebrafish.

3.1. Biopanning and identification of GBS-specific Nbs

Compared to the immune libraries, naive libraries are able to recognize a wider diversity of binders of any potential antigen (Liu et al., 2018; Yan et al., 2015). Phage display is a selection technique, which allows the display of foreign proteins on the phage surface (Liu et al., 2018). Currently, phage display is the most extensive and standard technique for biopanning target-specific Nbs. In the study, a naive phage-displayed Nb library was used to biopanning the GBS-specific Nbs. The schematic diagram of biopanning of GBS-specific Nbs is present in Fig. 1A. The numbers of input phages in three rounds of biopanning were all 1×10^{12} pfu, while the numbers of output phages were 5.6×10^{6}, 1.44×10^{7}, and 7.6×10^{8}, respectively (Fig. 1B). The numbers of output phages had a gradually increasing trend, indicating the high enrichment of GBS-specific phages (He et al., 2020). Subsequently, 48 individual clones were selected randomly from the agar plate of third rounds of biopanning for phage ELISA. As shown in Fig. 1C, 24 out of 48 clones were identified as positive compared with the negative controls. All 24 positive clones were sequenced, and ultimately 3 different VHH families were obtained, named Nb01, Nb02 and Nb03 (Fig. 2). By alignment, the amino acid sequence of Nbs consisted of 7 regions, including 4 framework regions (FRs) and 3 CDRs (Zhu et al., 2022). Among these sequences, different CDR3 were identified. In addition, Nb01 is the most abundant and contained 16 clones (66.7%), followed by Nb02 contained 6 clones (25.0%) and Nb03 contained 2 clones (8.3%). Considering the binding activity and numbers, Nb01 is selected for further study.

3.2. Expression, purification and verification of GBS-specific Nb01

Nbs can be easily expressed in different expression systems and retained the antigen-combining properties (Hu et al., 2021; Ji et al., 2020b). In the study, the recombinant plasmid pET28a-Nb01 contained N-terminal His tag was constructed (Fig. S1). The recombinant plasmid pET28a-Nb01 was confirmed by PCR detection (Fig. S1B), restriction enzyme digestion (Fig. S1C) and sequence analysis. Subsequently, the recombinant plasmid pET28a-Nb01 was transformed into *E. coli* BL21 (DE3) for the expression of 6 × His-tagged Nb01 by inducing with 0.8 mM IPTG. SDS-PAGE analysis showed that Nb01 could be expressed in BL21 cells and mainly expressed as inclusion bodies (Fig. 3A). The molecular size of recombinant protein Nb01 was approximately 18 kDa, which is consistent with the theoretical values and other study (Zhu et al., 2022). The recombinant protein Nb01 was purified using Ni-NTA resin under denaturing conditions (Wang et al., 2019). Then the purified Nb01 was refolded, dialyzed and examined by SDS-PAGE. The results showed that the recombinant protein Nb01 was obtained with high purity (Fig. 3B). The binding ability of recombinant protein Nb01 was determined by indirect ELISA against GBS (He et al., 2020). The results showed that the binding ability of Nb01 to GBS increased with the increase of Nb01 concentration, indicating that recombinant protein Nb01 retains an excellent affinity against GBS (Fig. 3C).

3.3. Anti-GBS activity of Nb01 in zebrafish

The potential therapeutic activity of Nbs has been widely reported in previous studies (Amcheslavsky et al., 2021; Dulal et al., 2022; Pymm et al., 2021; Zhu et al., 2022). For example, Nbs had antibacterial activity against enterotoxigenic *Escherichia coli* (ETEC) *in vitro* and *in vivo* by targeting a highly-conserved epitope within the putative receptor binding region of ETEC adhesins (Amcheslavsky et al., 2021). Nbs (T2E7, T2G9 and T4E5) could effectively neutralize typhoid toxin

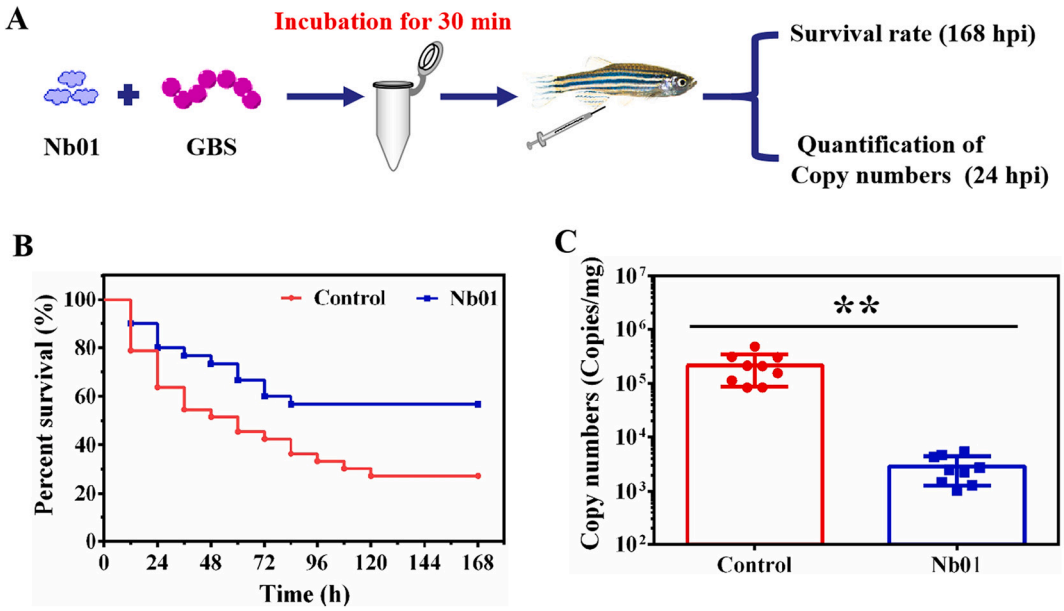

Fig. 4. The Neutralization of GBS by Nb01 in zebrafish. (A) Schematic diagram describing the workflow for (B-C); (B) Survivorship curve of GBS-infected zebrafish. Mortality of each group ($n = 30$) was recorded continuously for 168 h; (C) Copy numbers of GBS in the brain tissues. $^{**}p < 0.01$, compared to control.

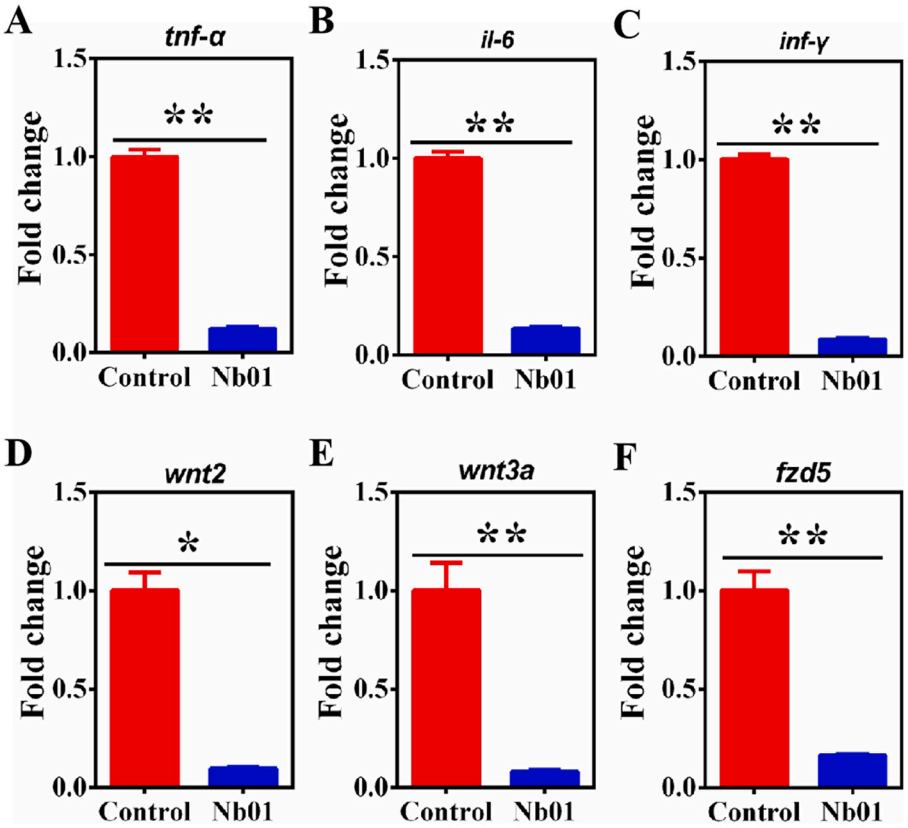

Fig. 5. Changes in the gene expression of (A) *tnf-α*, (B) *il-6*, (C) *inf-γ*, (D) *wnt2*, (E) *wnt3a*, (F) *fzd5* in the brain tissues of GBS-infected zebrafish. The values are presented as mean ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control.

secreted by *Salmonella enterica* by targeting the glycan receptor-binding PltB and nuclease CdtB *in vitro* and *in vivo* (Dulal et al., 2022). In this study, the anti-GBS activity of Nb01 was investigated in zebrafish. As shown in Fig. 4A, zebrafish were intraperitoneally administered GBS solutions only or a mixture of GBS-Nb01 for 168 h. The results showed that GBS-infected zebrafish treated by Nb01 had a higher survival rate than that in control groups (Fig. 4B). The survival rate of control groups was about 43.3%, while the survival rate of GBS-infected zebrafish treated by Nb01 was up to 70.0% at 168 hpi. Furthermore, brain tissues were separated to quantify the copy numbers of GBS. Interestingly, the copy numbers of GBS in Nb01 groups were significantly lower than that in control groups (Fig. 4C). The copy numbers of the control groups were

about 2.15 × 10^5 copies/mg. However, the copy numbers in Nb01 groups were significantly decreased to 2.85 × 10^3 copies/mg. The reduction of copy numbers of GBS in the brains might be at least due to Nb01 targeting receptor-binding epitopes, thereby inhibiting GBS entry into the brain. Taken together, these results indicated that Nb01 possessed potent anti-GBS activity *in vivo via* reducing the copy numbers of GBS and protecting zebrafish from GBS infection.

3.4. Gene expression of proinflammatory cytokines and Wnt-signaling in GBS-infected zebrafish

GBS can penetrate the BBB and cause meningitis in fish, which results in inflammatory responses in the brains (Eto et al., 2020; Palang et al., 2020; Patterson et al., 2012). Wnt-signaling is an evolutionarily conserved signaling pathway that is involved in different biological functions in organisms. Previous studies have shown that pathogens infection can also activate Wnt molecules in fish (Cavalcanti et al., 2012; Li et al., 2018). Moreover, Wnt-signaling can regulate inflammatory responses in fish (Sharma et al., 2021). In the study, the expression of proinflammatory cytokines and Wnt-signaling in GBS-infected zebrafish brains were monitored by qPCR. As shown in Fig. 5, Nb01 treatment significantly reduced the expression of proinflammatory cytokine genes (*tnf-α*, *il-6* and *inf-γ*) and Wnt-signaling related genes (*wnt2*, *wnt3a* and *fzd5*) in the GBS-infected zebrafish brains. The expression levels of *tnf-α*, *il-6*, *inf-γ*, *wnt2*, *wnt3a* and *fzd5* in Nb01 groups were 8.26-fold, 7.58-fold, 11.82-fold, 10.27-fold, 12.76-fold and 6.11-fold lower than those in control groups, respectively. The down-regulation of the expression of proinflammatory cytokine genes and Wnt-signaling related genes might be due to the binding between Nb01 and GBS, which blocked GBS infection in the brain and thereby resulted in decreasing the inflammatory responses.

4. Conclusion

In this study, GBS-specific Nbs were screened from a naive phage-displayed Nb library, and Nb01 had significantly anti-GBS activity in zebrafish models. GBS-specific Nb01 was expressed and characterized, and recombinant protein Nb01 had a good affinity for GBS. Moreover, Nb01 could reduce the copy numbers of GBS in zebrafish brains, and improve the survival rate of GBS-infected zebrafish. Furthermore, the anti-GBS activity of Nb01 might reduce the tissues damage by blocking the inflammatory responses. Our study provides a proof of concept for the use of Nbs as therapeutic molecules against GBS *in vivo*.

CRediT authorship contribution statement

Ai-Guo Huang: Funding acquisition, Methodology, Writing - original draft, Writing - review & editing. **Wei-Hao He:** Methodology, Writing - review & editing. **Lin-Jun Su:** Data curation, Methodology, Writing - review & editing. **Fa-Li Zhang:** Data curation, Writing - review & editing. **Ying-Hui Wang:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare no competing interests in this manuscript.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2022.738725.

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