

From gene to disease: the virulence amplification of *Plesiomonas shigelloides* via *lipa* overexpression

Lian Yang, Fanglian Lu, Deqin Luo, and Ranran Dong*

College of Animal Science, Guizhou University, Guiyang 550025, China

* Correspondence: rrdong@gzu.edu.cn (Dong R)

Abstract

Frequent bacterial outbreaks in aquaculture threaten commercial fish, with *Plesiomonas shigelloides* being notably common due to its *lipa* gene, a critical virulence factor. This study overexpressed the *lipa* gene in fish-derived *P. shigelloides* to investigate its impact on biofilm formation, LIPA activity, and bacterial adhesion. We infected *Silurus meridionalis* for 7 d to assess pathogenicity, observing symptoms, and collected the head kidney for transcriptome and proteomic analysis. Results were confirmed by qPCR and ELISA, and we performed GO classification, KEGG pathway, and protein interaction network analysis on differentially expressed immune proteins. The findings revealed that *lipa* overexpression increased biofilm formation ($p < 0.01$), lipoyl synthase activity ($p < 0.01$), and bacterial adhesion compared to wild-type strains, offering novel insights into its function. In *Silurus meridionalis*, this overexpression led to increased hemorrhaging in the anal and caudal fins compared to the wild-type and control groups. Furthermore, q-PCR and ELISA results demonstrated that the expression levels of NACHT/LRR/PYD domain-containing protein 4 (NLRP4), complement component 6 (C6), and lysozyme C (LZMC) correlated with the transcriptome and proteomic data. Overall, *lipa* overexpression enhances *P. shigelloides* virulence and intensifies the immune responses in *Silurus meridionalis*, providing insights into its pathogenic mechanism and supporting healthy breeding of *Silurus meridionalis*.

Keywords: *lipa* gene overexpression, *Plesiomonas shigelloides*, Proteomics, *Silurus meridionalis*

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Introduction

The high-density aquaculture sector encounters considerable challenges due to frequent disease outbreaks, with bacterial infections presenting particularly severe threats owing to their insidious onset and the substantial economic losses they incur^[1]. *Plesiomonas shigelloides*, the sole species within the genus *Plesiomonas* of the Enterobacteriaceae, is a common pathogen in fish and can be transmitted to humans via contaminated water or seafood. *P. shigelloides*, isolated from rainbow trout displaying ulcerative skin lesions and hemorrhage^[2], exhibits a high degree of pathogenicity and has the potential to induce severe inflammation, hemorrhage, and necrosis^[3]. The infection of *Silurus meridionalis* by *P. shigelloides* is marked by significant epidermal ulceration and intestinal inflammation, underscoring its virulence^[4]. Virulence is associated with a diverse array of pathogenic genes, including *acrb*, *kata*, *csgg*, *lipa*, *htpb*, *msba*, *arcb*, and *baca*^[3–5]. However, the influence of the *lipa* gene on the host's physiological functions, especially immune and metabolic regulation during the pathogenic process of *P. shigelloides*, has not yet been systematically elucidated in fish models^[6].

When facing bacterial infections, fish initiate a series of physiological and immune defense responses, including the activation of the coagulation and hemostasis system, the expression of acute-phase proteins, and the regulation of various immune effector molecules^[7,8]. The coordination and intensity of these responses directly affect the outcome of the infection. Alterations in the lipoic acid synthesis pathway influence both the sensitivity of pathogens to host-derived reactive oxygen species and their surface structures^[9,10], thereby potentially regulating immune recognition and inflammatory response by modifications to the pathogen-host interface. Therefore, elucidating

the impact of the *lipa* gene on the physiological and biochemical responses of fish hosts during *P. shigelloides* infection will not only contribute to a deeper understanding of the pathogen's virulence mechanism, but also provide an entry point for exploring the metabolic immune regulation against infection from the perspective of host physiology.

This study aimed to elucidate the influence of the *lipa* gene on the pathogenicity of *P. shigelloides*, using *S. meridionalis* as a model host. We generated a *lipa*-overexpressing bacterial strain and assessed its virulence-associated phenotypes. Concurrently, we analyzed the dynamic host response by integrating transcriptomic and data-independent acquisition (DIA) proteomic data from infected head kidney tissue. *lipa* overexpression enhanced bacterial biofilm formation, adhesion, and enzymatic activity. In *S. meridionalis*, infection with this strain triggered significant increases in the expression of immune- and coagulation-related proteins, notably lysozyme C (LZMC), NACHT/LRR/PYD domain-containing protein (NLRP4), and complement component C6. Collectively, these data suggest that *lipa* regulates both bacterial virulence and the subsequent host immune response. The specific modulation of C6, A2ML1, and LZMC offers novel clues for deciphering the physio-biochemical mechanisms underlying the host's defense against this infection.

Materials and methods

Bacterial strains and plasmid construction

The *P. shigelloides* strain utilized in this study was previously isolated and purified from diseased *Silurus meridionalis* in our laboratory. The *lipa* target gene and its promoter (19-p-*lipa*) were amplified

and seamlessly cloned into the pUC19 vector, resulting in the formation of the recombinant plasmid *lipa*-pUC19. Utilizing *lipa*-pUC19 as a template, both the *lipa* gene and its promoter were amplified, while the pBBR1MCS vector underwent inverse amplification. These fragments were subsequently ligated through seamless cloning and introduced into *Escherichia coli* DH5a competent cells via transformation. Positive clones were identified through sequencing to obtain the *P. shigelloides lipa* overexpression vector. The primer sequences employed in this study are detailed in Table 1.

Phenotypic assays

For the lipoyl synthase activity assay, overnight cultures of *P. shigelloides* underwent freeze-thaw cycles to lyse the cells. The lysate was centrifuged, and the supernatant was collected. Lipoyl synthase activity was subsequently quantified using a commercial ELISA kit (Shanghai Bohu Biotechnology Co., Ltd, Shanghai, China), with absorbance measured at 450 nm.

For the biofilm formation assay, overnight LB cultures were diluted in YP medium and dispensed into 96-well plates. Following 48-h incubation at 37 °C, biofilms were washed, fixed, stained with crystal violet, and quantified by measuring absorbance at 575 nm.

For the *in vitro* adhesion assay, fish surface mucus was collected, homogenized, and filtered. Bacterial adhesion was assessed following^[8] and quantified using ImageJ software.

For the growth curve assay, overnight cultures were diluted to an OD₆₀₀ of 0.05 in TSB broth and incubated at 37 °C. OD₆₀₀ was measured every 2 h for 24 h using a microplate reader.

Animal infection experiment

S. meridionalis, each approximately 18 cm in length, were acclimated over an 8-week period at a temperature of 26 °C with a dissolved oxygen concentration of 7 mg/L. During this time, they were fed twice daily with a commercially available fish feed, purchased online (Shandong Shengsuo Feed Technology Co., Ltd.), which

contained 50% crude protein and 7% crude fat. The fish were subsequently allocated into three experimental groups, each consisting of 20 individuals with three replicates per group: (1) a control group (control) receiving 0.2 mL of sterile saline; (2) a wild-type group (WT) administered 0.2 mL of WT *P. shigelloides* at a concentration of 1 × 10⁹ CFU/mL; and (3) an overexpression group (OE) receiving 0.2 mL of *lipa*-overexpressing *P. shigelloides* at the same concentration. After a period of 7 d, head kidney samples were collected from sick fish, rapidly frozen in liquid nitrogen, and stored at -80 °C for subsequent data-independent acquisition (DIA) proteomic analysis, conducted by Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China).

Sample collection and RNA extraction

Head kidney tissues were ground with liquid nitrogen and lysed in TRIzol. Following chloroform extraction and isopropanol precipitation, RNA pellets were washed with 75% ethanol, air-dried, and dissolved in DEPC-treated water.

Transcriptome and proteome analyses

For transcriptome analysis, total RNA (RIN ≥ 6.5, ≥ 1 µg) was subjected to poly(A) enrichment. Libraries were constructed and sequenced on an Illumina NovaSeq 6000 platform. Raw reads were quality-controlled using fastp and aligned to the reference genome using HISAT2. Differentially expressed genes were identified using DESeq2 (|log2FC| ≥ 1, FDR < 0.05), followed by GO and KEGG enrichment analyses.

For proteome analysis, head kidney tissues were lysed, and protein concentrations were determined by BCA assay. Proteins were digested with trypsin, and the resulting peptides were analyzed on a timsTOF Pro2 instrument in DIA-PASEF mode. Differentially expressed proteins were defined as FC ≥ 1.2 and *p* < 0.05. GO, KEGG, and protein-protein interaction analyses were performed using the Major platform and STRING database.

Validation experiments

For ELISA validation, five immune-related differentially expressed proteins (HA, NLRP4, C6, LZMC, A2ML1) were quantified using commercial ELISA kits.

For qRT-PCR validation, eight differentially expressed genes were selected. Reverse transcription was performed using the TransScript All-in-One kit. qRT-PCR was conducted with PerfectStart Green qPCR SuperMix on a real-time PCR system. Gene expression levels were calculated using the 2^{-ΔΔCt} method with ACTB as the internal control.

Statistical analysis

All experiments were biologically replicated three times independently, each with three technical replicates. Data are presented as mean ± standard deviation. Statistical differences were evaluated using one-way ANOVA (*p* < 0.05 considered significant). All analyses were performed using IBM SPSS Statistics 27.0.1.

Results

PCR verification of *P. shigelloides lipa* overexpression

Colony PCR analysis, conducted using 1% agarose gel electrophoresis, identified a target band of approximately 1,400 base pairs (Fig. 1).

Table 1. Primer information.

Primer name	Sequence information
<i>lipa</i> -F	CCTGATAATCACAACTTTATGAGTAAACCTATTGAGATTG
<i>lipa</i> -his-R	TGGTGGTGGTGGTGGTGGTAACTTCTTCGCCTTTGGCCT
puc19-his-F	CACCACCACCACCACTAAGGCGTAATCATGGTCATAGCTG
19- <i>lipa</i> -P-R	TGAATAGGTTTACTCATAAAGTTGTGATTATCAGGTGCAT
<i>lipa</i> -pBBR-F	CACCACCACCACCACCACTAAGGCGTAATCATGGTCATAGCTG
pBBR-his-F	TACGATATCGATGGCACCACCGGTTTATTGACTACCGGAA
ACTB-S	CCAGCACTCCCACTTCACTTT
ACTB-A	GCTTTAGATTTTGGGTTTGTCTG
<i>ccl20a.3</i> -S	GCAACAATAAATCCAGACATGACC
<i>ccl20a.3</i> -A	ACACCAGACTTTATACCTCCCATGA
LOC124381009-S	TGAGGACGCCATTTCCACTG
LOC124381009-A	CAATTACAGAGTTGTGTGCTGATGC
<i>lgi1a</i> -S	GTGAAGACTCGTTTGGCAGCA
<i>lgi1a</i> -A	GAATGGAGGACGGTGCAATAA
<i>akap12b</i> -S	GGTCACCACAGAAAGCGAAGA
<i>akap12b</i> -A	GCTGGCTTTCTCTCTCAACA
<i>nlrp4</i> -S	CAACACATGAATTATCTCCATCTGC
<i>nlrp4</i> -A	ACAGCAGAACTTACCTGGTTACTT
<i>c6</i> -S	AGACTGGAACCTTCGGAGACA
<i>c6</i> -A	CTCGCATCACAAACACTCCA
<i>lzmc</i> -S	TCAACCGAAACACTGATGGATCT
<i>lzmc</i> -A	GTGAGATGTTATCGGAAAGAAGTGC
<i>hpxa</i> -S	CATACCCATTCTGAGGCAAAACC
<i>hpxa</i> -A	ACAGCCGAGCAGTTCTTTGG

Subsequent sequencing confirmed a 99.53% identity with *P. shigelloides lipa*, thereby indicating successful overexpression.

Lipoyl synthase activity assay

The linear regression analysis of the standard curve absorbance was employed to quantify lipoyl synthase levels. The wild-type (WT) strains exhibited an average activity of 50.5 ng/L. In contrast, the *lipa*-overexpressing (OE) strains demonstrated a significantly elevated activity level, averaging 61.2 ng/L ($t = 9.418$, $p < 0.001$).

Biofilm formation assay

Crystal violet staining revealed a significant increase in biofilm formation in the OE strains ($OD_{575} = 0.19 \pm 0.02$) compared to the wild-type (WT) strains ($OD_{575} = 0.17 \pm 0.01$; $t = 4.5$, $p < 0.001$), thereby corroborating the role of *lipa* in virulence.

Bacterial adhesion assay

Microscopic analysis demonstrated enhanced mucus adhesion in OE strains, characterized by purple-stained aggregates (Fig. 2b), in contrast to the dispersed, red-stained wild-type (WT) bacteria (Fig. 2a). Quantitative analysis revealed an average of 903 bacteria per field in the WT group, compared to 1,339 bacteria per field in the OE group. This observation suggests a modification in membrane permeability, potentially attributable to the overexpression of *lipa*.

Growth characteristics of the *lipa*-overexpressing strain

To exclude the possibility that enhanced virulence was due to altered bacterial fitness, we compared the growth curves of wild-type and *lipa*-overexpressing *P. shigelloides*. The results showed that overexpression of the *lipa* gene causes a 2-h growth delay in *P. shigelloides*, but it exhibits a higher plateau period (Fig. 3).

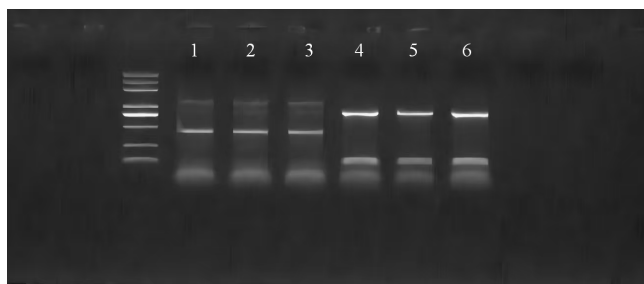


Fig. 1 PCR identification of Shiga-*lipa*-PBBR overexpressing colonies. Serial numbers 1–3 are wild-type *P. shigelloides*, and serial numbers 4–6 are *P. shigelloides* with overexpression of the *lipa* gene.

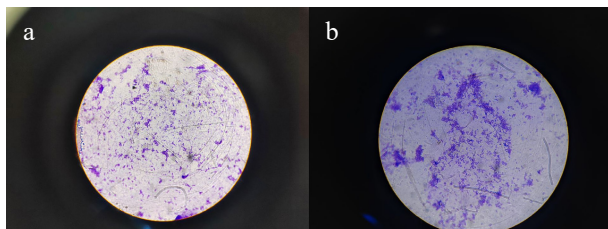


Fig. 2 The adhesion of wild-type *P. shigelloides* and *lipa* gene overexpressed *P. shigelloides* to the mucus on the body surface of the *Silurus meridionalis*. (a) staining of wild-type *P. shigelloides*, and (b) staining of *P. shigelloides* overexpressing the *lipa* gene.

Clinical symptoms post-infection

Seventy-two hours post-infection, no lesions were observed in the control group (Fig. 4a). In contrast, the wild-type group demonstrated hemorrhaging in the anal and tail fins, accompanied by skin darkening (Fig. 4b). The overexpression group presented with severe symptoms, including excessive mucus secretion, fin hemorrhage, cloacal swelling, and necrotic lesions (Fig. 4c, d).

Venn diagram analysis

A total of 10,911 genes (77.56% of total) were commonly expressed between WT and OE groups, and 7,675 proteins (99.22% of total) were shared between the two groups (Fig. 5a, b).

Differentially expressed genes (DEGs) and proteins (DEPs)

Differential expression analysis between OE and WT groups identified 476 DEGs (179 up, 297 down) and 350 DEPs (164 up, 186 down), including 66 immune-related DEPs (Fig. 6). GO and KEGG enrichment analyses were performed to explore the potential functional roles of these differential molecules.

Integrated transcriptomic and proteomic analysis

By integrating DIA proteomic and transcriptomic data, the expression levels of immune-related proteins and genes were analyzed respectively through clustering heat maps. Among them, C6, NRPL4, and LZMC showed consistent expression trends in the transcriptome

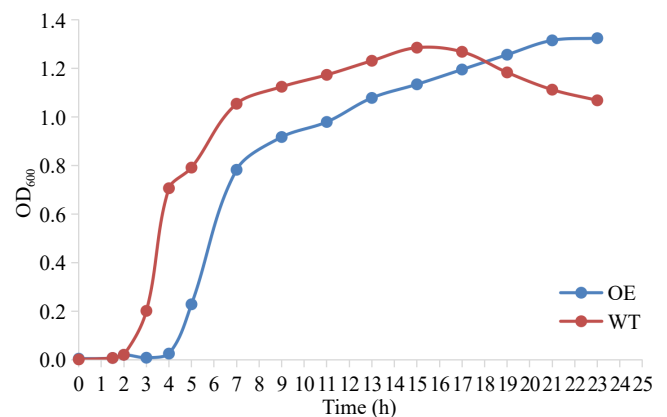


Fig. 3 Growth curves of wild-type (WT) and *lipa*-overexpressing (OE) *P. shigelloides*.

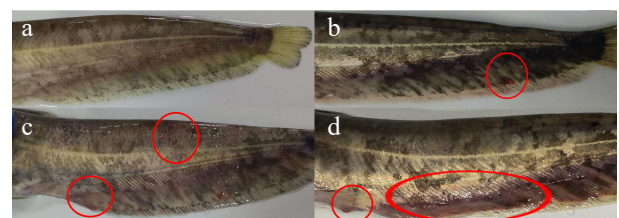


Fig. 4 Symptoms of *P. shigelloides* infection for 72 h. (a) asymptomatic *Silurus meridionalis* injected with sterile saline in the control group; (b) bleeding on the anal fin and tail fin with blackness on the body surface in the wild group; (c) obvious lesions on the body surface with bleeding on the tail fin in the overexpression group; and (d) redness and swelling of the genital orifice, black skin and bleeding on the anal fin and the tail fin in the overexpression group.

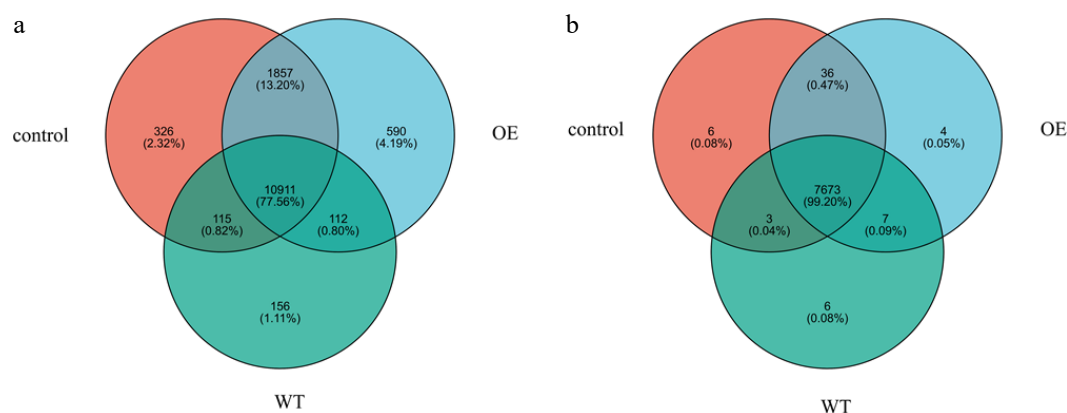


Fig. 5 Venn diagram of genes and proteins. The overlapped part of the figure represents the number of genes and proteins shared between groups; the part without overlap represents the number of genes and proteins unique to the group, and the numbers represent the corresponding gene and protein numbers. Among them, WT represents the wild-type strain group, and OE represents the overexpressed strain group.

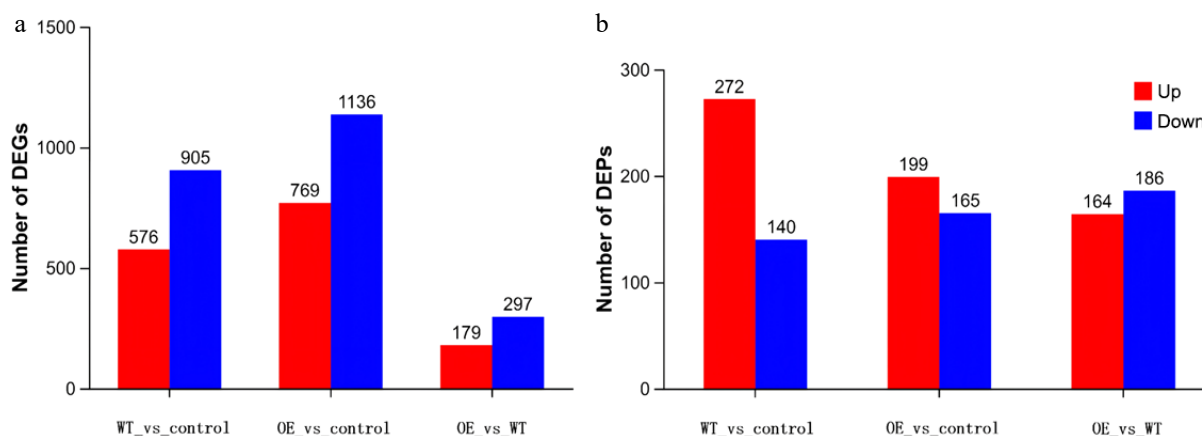


Fig. 6 Statistics of differential proteins and genes. Note: The horizontal coordinate is each difference comparison group. By default, red represents the upregulated gene and protein in the difference comparison group, and blue represents the downregulated gene and protein. Among them, WT represents the wild-type strain group, and OE represents the overexpressed strain group.

and proteome, and were all significantly upregulated in the OE group. The color gradient from blue to red represents the Spearman correlation coefficient (ρ) ranging from -1 to 1 , with blue indicating negative correlation, white indicating no correlation, and red indicating positive correlation. X-axis denotes the identified proteins, and the Y-axis denotes the corresponding genes. Only significant correlations ($p < 0.05$) are displayed. In these visualizations, color intensity corresponds to expression level, facilitating the visual identification of molecular clusters potentially pivotal to the immune response and their expression dynamics (Fig. 7).

Functional annotation of immune-related DEPs

GO enrichment analysis of immune-related DEPs indicated significant enrichment in biological processes such as negative regulation of wound response, hemostasis, and coagulation, as well as in the cellular component membrane attack complex (Fig. 8). Subsequent KEGG pathway analysis further revealed that these DEPs were predominantly involved in NOD-like receptor signaling, regulation of the actin cytoskeleton, and neuroactive ligand-receptor interaction (Fig. 9). Immune-related DEPs were enriched in negative regulation of wound response, hemostasis, and coagulation (biological processes), and membrane attack complex (cellular component) (Fig. 9).

ELISA validation

When comparing the OE and WT groups, PHA exhibited no significant change, whereas LZMC demonstrated a significant difference ($p < 0.05$). Notably, NLRP4, C6, and A2ML1 showed highly significant differences ($p < 0.01$). The ELISA results were consistent with the DIA proteomic data, thereby confirming the accuracy of our proteomic analysis (Fig. 10). This validation substantiates that *lipa* overexpression significantly altered the expression of key immune-related proteins in infected *Silurus meridionalis*.

qRT-PCR validation

To confirm RNA-Seq reliability, we analyzed the mRNA levels of the following eight differentially expressed genes (*ccl20a.3*, *loc124381009*, *lgi1a*, *akap12B*, *c6*, *nlrp4*, *lzmc*, and *hpxa*) by qRT-PCR. The results showed significant differences in *ccl20a.3*, *akap128*, *c6*, and *lzmc* genes among the three groups ($p < 0.05$). *loc124381009* and *lgi1a* showed no differences between the WT and OE groups but differed significantly from the control group ($p < 0.01$). NLRP4 and *hpxa* showed no significant difference between the control *p* and WT groups but differed significantly from the OE group ($p < 0.05$) (Fig. 11).

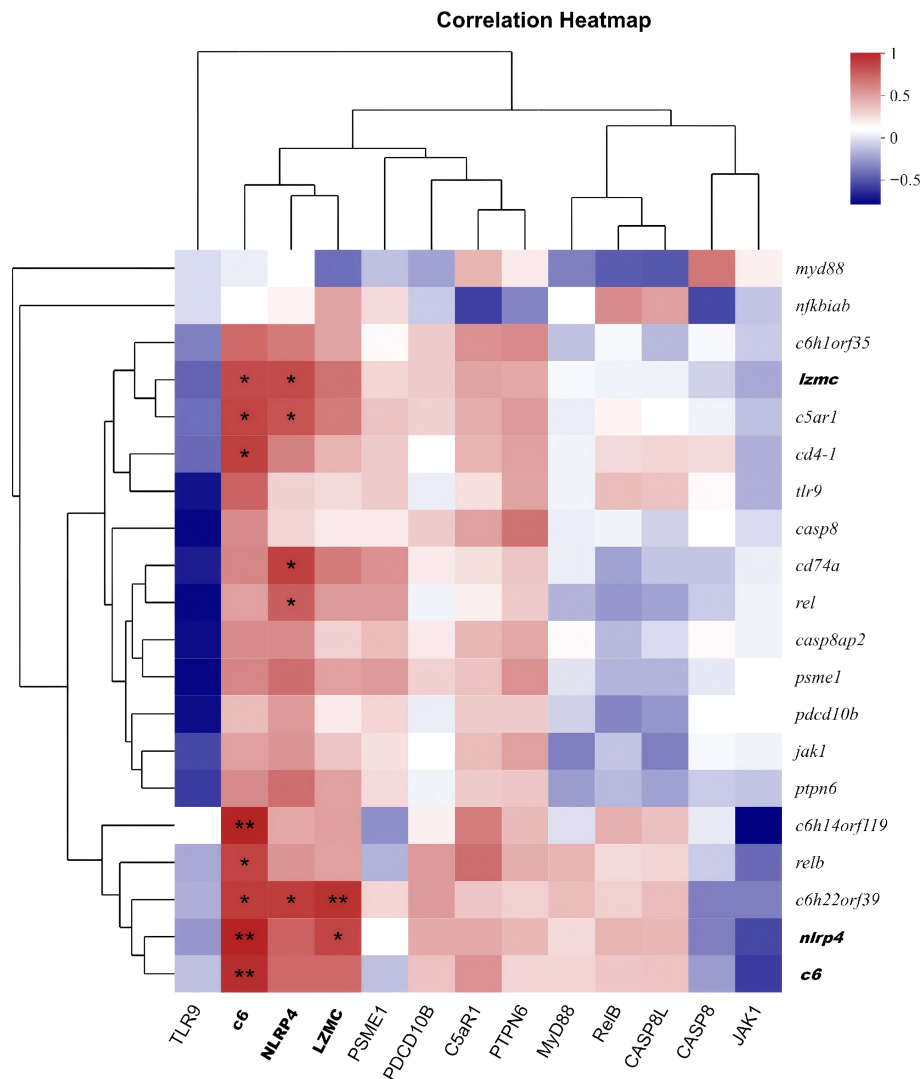


Fig. 7 Spearman correlation heatmap. Spearman correlation heatmap of immune-related molecules between the WT and OE groups. The color gradient from blue to red represents the Spearman correlation coefficient (ρ) ranging from -1 to 1 ; red indicates positive correlation, white indicates no correlation, and blue indicates negative correlation. The X-axis denotes proteins, and the Y-axis denotes genes. Only significant correlations ($p < 0.05$) are shown. Data are from three biological replicates per group.

Discussion

PPI network

STRING analysis of immune-related DEPs revealed interconnected nodes (e.g., complement C6, A2ML, NLRP) with high interaction scores (Fig. 12). The results indicate that C6 primarily interacts with C5 and C7, collectively participating in the assembly of the membrane attack complex (MAC). A2ML, an alpha-2-macroglobulin homolog, may synergize with complement proteins like C6 in immune signal transduction and shares a common immune pathway with HPXB. NLRP1, a member of the NLR family, forms inflammasome complexes with proteins such as IRAK4, contributing to the release of inflammatory cytokines.

Coordinated regulation of complement C6 and Alpha-2-Macroglobulin Like-1

The interaction between the complement and coagulation cascades is illustrated (Fig. 13). During infection with *lipa*-overexpressing

strains, both C6, crucial for MAC assembly, and the thrombin inhibitor A2ML1 were upregulated, suggesting a coordinated immune response. The elevation of C6 may be driven by two non-exclusive mechanisms: increased complement recognition of bacterial surfaces altered by *lipa*, or pathogen-induced interference with host regulatory pathways. Importantly, A2ML1 might help prevent thrombosis by enhancing plasminogen activator activity^[11]. While acute infection models likely capture peak activation states, research on different infection periods is necessary to elucidate the timing between initial complement activation and subsequent coagulation changes.

NLRP4 inflammasome activation in anti-bacterial defense

The NOD-like receptor (NLR) pathway (Fig. 14) triggers inflammation through caspase-1-dependent interleukin-1 β (IL-1 β) maturation. Notably, NLRP4 is significantly upregulated ($p < 0.001$) in infections with lipoyl synthase overexpression, suggesting that bacterial lipoyl acid synthesis may modulate inflammasome responses. Emerging evidence indicates that the lipoylated metabolic protein lipoyl-E2-

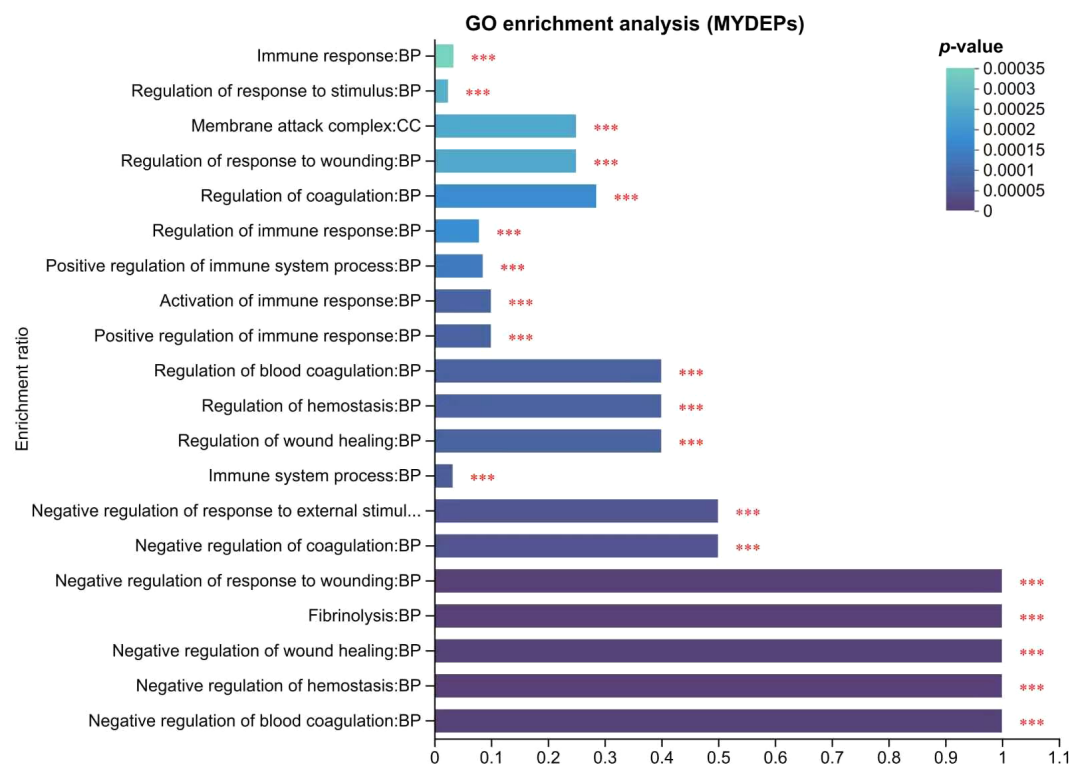


Fig. 8 Analysis of immune-related DEPs GO. Note: The abscissa represents the GO term, and the ordinate represents the enrichment rate (refers to the ratio of the number of proteins enriched in the GO term to the number of proteins annotated to the GO term; the greater the ratio, the greater the degree of enrichment). The column color gradient indicates the significance of enrichment, with p or FDR < 0.001 marked as ***, p or FDR < 0.01 marked as **, and p or FDR < 0.05 marked as *.

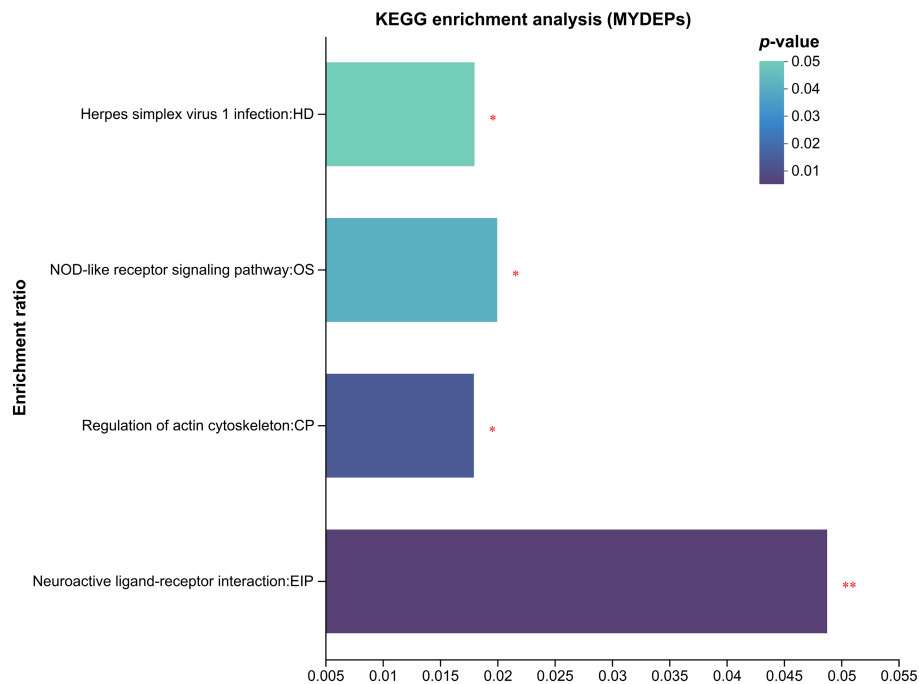


Fig. 9 Enrichment analysis of immune-related DEPs in KEGG. The abscissa represents the pathway name, and the ordinate represents the enrichment rate (refers to the ratio of the number of proteins enriched in the pathway to the number of proteins annotated to the pathway; the larger the ratio, the greater the enrichment). The column color gradient indicates the significance of the enrichment, and the darker the default color, the more significant the enrichment of the KEGG term, where p or FDR < 0.01 is marked as **, and p or FDR < 0.05 is marked as *.

PDH, whose lipoylation depends on *lipa*, is released by bacteria and suppresses macrophage activation by reducing Toll-like receptor 1/2 (TLR1/2) recognition of bacterial lipopeptides^[12]. A *lipa*-deficient

strain induces heightened pro-inflammatory cytokine production, which is diminished in the absence of TLR2^[12]. Furthermore, lipolic acid synthesis limits macrophage production of bactericidal reactive

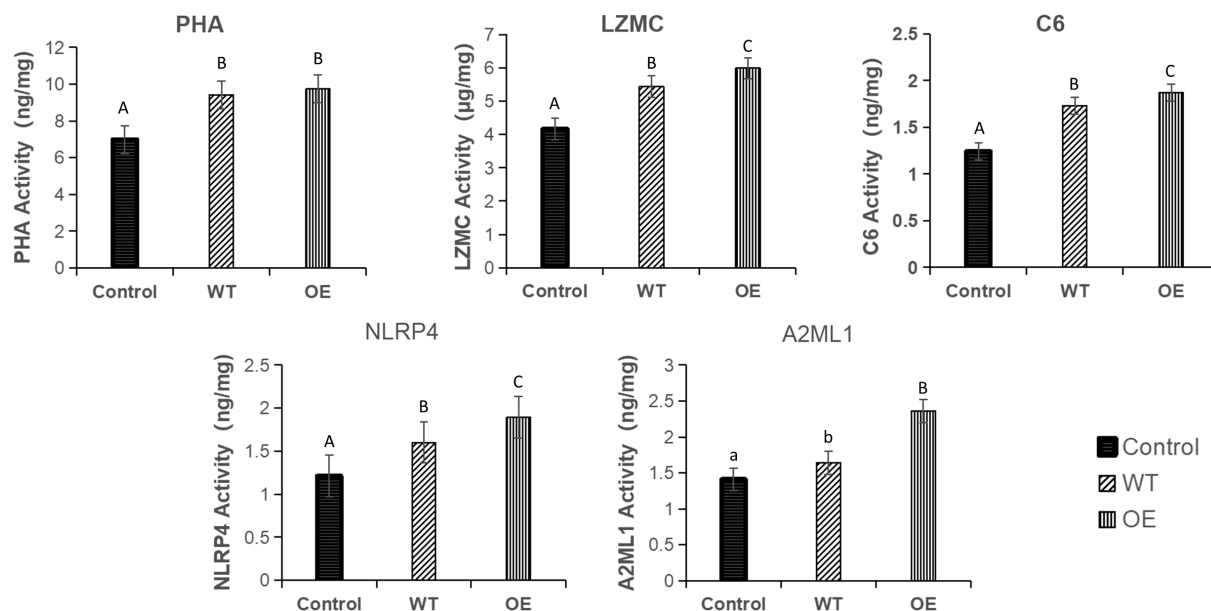


Fig. 10 Contents of PHA, LZMC, C6, NLRP4, and A2ML1 in the head kidney of *Silurus meridionalis*. There was no significant difference among groups represented by the same letter, while there were statistical differences at corresponding levels among groups represented by different letters. Among them, WT represents the wild strain group, and OE represents the overexpressed strain group.

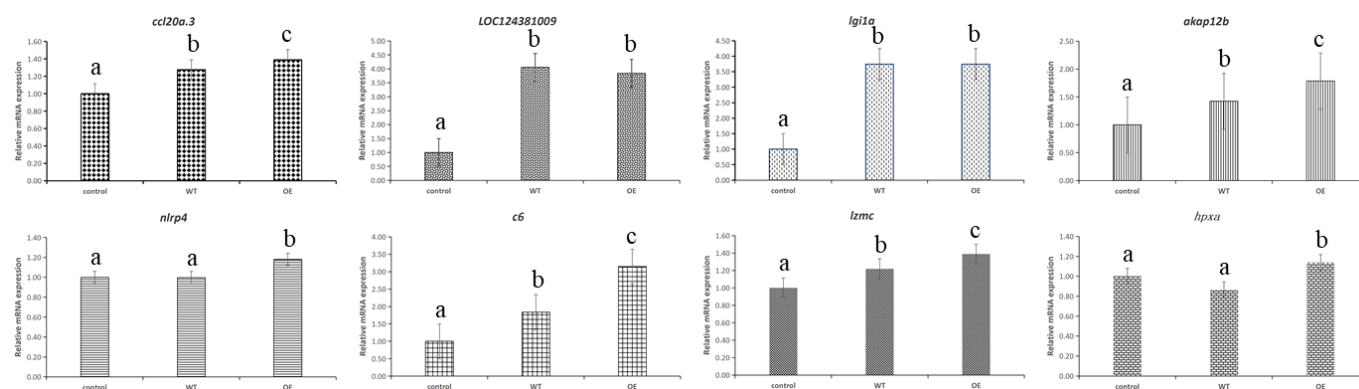


Fig. 11 Verification results of eight DEGs qRT-PCR.

oxygen species (ROS) and reactive nitrogen species (RNS), promoting bacterial persistence during infection^[13]. Although IL-1 β plays a crucial role in recruiting neutrophils to clear bacterial infections^[14], excessive inflammatory responses inadvertently benefit pathogens by generating nutrient-rich microenvironments^[15], potentially explaining severe pathology despite strong immune responses.

Enhanced biofilm formation capacity in *lipa*-overexpressing *P. shigelloides*

Our findings indicate that *lipa* overexpression significantly enhances biofilm formation in *P. shigelloides*. Bacterial biofilms, which consist of microbial communities embedded within an extracellular polymeric substance (EPS) matrix, confer resistance to environmental stressors and increase pathogenic potential^[16]. However, due to the lack of a *lipa* knockout/complementation strain (attempts to generate a knockout were unsuccessful), causality cannot be firmly established from the current data. The *lipa*-mediated enhancement of biofilm formation may hypothetically involve several mechanisms: (1) the upregulation of adhesion-related proteins, such as pili, facilitating initial surface attachment; (2) the modulation of EPS synthesis to strengthen the biofilm structural integrity; and (3) potential

interference with quorum sensing (QS) via the acyl-homoserine lactone (AHL) signaling pathway^[17]. We acknowledge that direct experimental evidence supporting these proposed mechanisms (pili, EPS, QS) is currently lacking, and these interpretations remain speculative. The resulting fortified biofilm likely provides protection against host immune clearance and antibiotic treatments^[18], highlighting *lipa* as a potential target for therapeutic intervention. Future research should aim to elucidate the precise roles of *lipa* in adhesion, EPS regulation, and QS to advance the development of anti-biofilm strategies.

lipa overexpression augments bacterial adhesion to *S. meridionalis* surface mucus

Fish epidermal mucus of fish serves as a primary immunological barrier, comprising antimicrobial peptides (AMPs), lysozymes, and immunoglobulins that neutralize invading pathogens^[19,20]. Microscopic analysis demonstrated that *lipa*-overexpressing strains formed dense, purple-stained aggregates on *S. meridionalis* mucus, in contrast to the dispersed, red-stained wild-type bacteria. This phenotypic shift suggests that *lipa* expression alters membrane permeability, leading to: (1) the upregulation of specific adhesins^[21]; and (2) modifications of metabolic pathways that facilitate sustained colonization. These

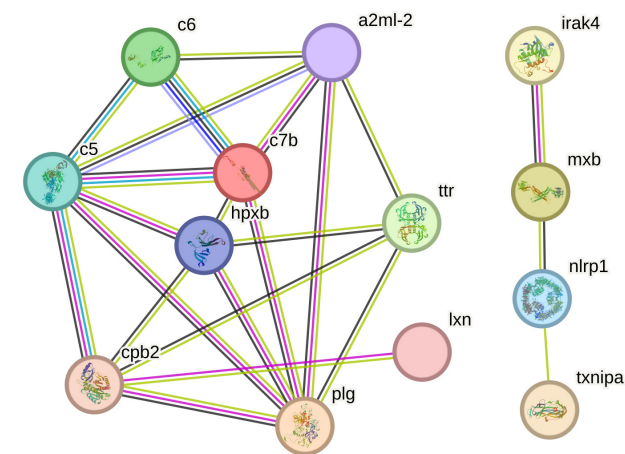


Fig. 12 Network diagram of immune-related differential protein interaction between the WT (wild) group and the OE (overexpression) group.

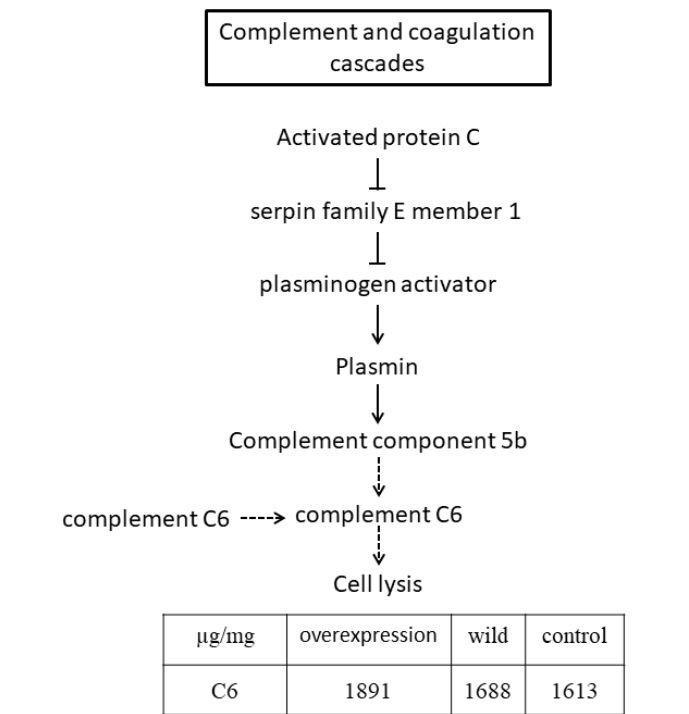


Fig. 13 Simplified complement and coagulation cascade pathway.

adaptations might counter mucus clearance mechanisms mediated by viscosity and AMPs.

Limitations and future direction

Several limitations of this study should be acknowledged. First, although *lipa* overexpression significantly enhanced adhesion and biofilm formation, we were unable to generate a *lipa* knockout strain despite multiple attempts, precluding complementation experiments necessary to establish a direct causal relationship between *lipa* and the observed phenotypes. Second, while we have discussed potential mechanisms involving pili, EPS, and quorum sensing, these interpretations remain speculative, as direct experimental evidence—such as measurements of pilin expression, EPS production, or AHL signaling activity—is currently lacking. Consequently, our findings should be interpreted as correlative rather than mechanistic. Future studies

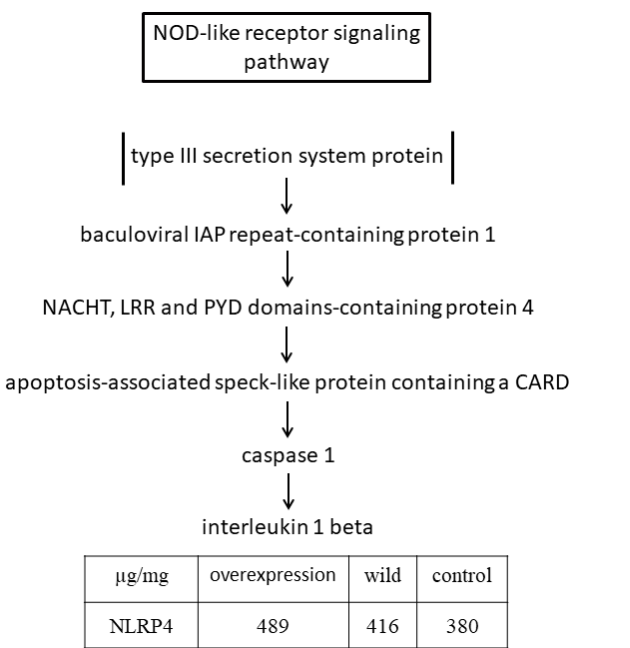


Fig. 14 Simplified diagram of the NOD-like receptor signaling pathway.

should employ alternative strategies to disrupt *lipa* function (e.g., conditional knockdown or CRISPRi) and directly quantify the proposed downstream effectors to validate these hypotheses.

Conclusions

In conclusion, our *in vitro* experiments reveal that *lipa* overexpression in *P. shigelloides* significantly augments biofilm formation, lipoyl synthase activity, and host adhesion capacity, supporting its role in virulence through modulation of bacterial surface properties and metabolism. In *S. meridionalis* infection models, *P. shigelloides* overexpressing *lipa* induced more skin ulceration and systemic inflammation. Integrated transcriptomic and proteomic analysis of head kidney tissue revealed that the immune-related factors NLRP4, C6, and LZMC were significantly upregulated in the group treated with *lipa*-overexpressing *P. shigelloides*. This finding underscores that the coordinated defense response in *S. meridionalis* is likely mediated through the complement–coagulation cascade and NOD-like receptor signaling pathways. In addition, the aberrant downregulation of lysozyme C-like protein (LZMC) suggests efficient pathogen-mediated evasion of innate immunity. Collectively, these findings suggest that targeted inhibition of lipoyl synthase activity may represent a promising therapeutic strategy against *P. shigelloides* infections.

Ethical statements

The animal study protocol was approved by the Experimental Animal Ethics Committee of Guizhou University (protocol code EAE-GZU-2024-T103 and date of approval 12 March 2024).

Author contributions

The authors confirm their contributions to this study as follows: conceptualization: Dong R, Yang L; methodology: Yang L; investigation: Lu F; resources: Dong R; data curation: Luo D; writing—original draft preparation: Yang L; writing—review and editing, project administration, funding acquisition: Dong R; visualization: Lu F; supervision:

Luo D. All authors have read and agreed to the published version of the manuscript.

Data availability

The raw sequence data generated in this study have been deposited in the OMIX database at the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession numbers OMIX011657 and CRA037443. The data are publicly accessible at <https://ngdc.cnbc.ac.cn/omix> and <https://ngdc.cnbc.ac.cn/gsa>.

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Conflict of interest

The authors declare no conflict of interest.

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