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sRNAs Regulate Biofilm Formation via Quorum Sensing and Chemotaxis systems in *Listeria monocytogenes*

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ABSTRACT: *Listeria monocytogenes* (*L. monocytogenes*) is a highly concerning food-borne pathogen, and its biofilm formation has a significant impact on food safety. Small RNAs (sRNAs) play a crucial role in the biofilm formation of *L. monocytogenes*. We identified three sRNAs potentially associated with biofilm formation: ssrS, sRNA003, and sRNA006. Notably, sRNA003 and sRNA006 were newly discovered. This study explored the effects of three sRNAs on biofilm formation in *L. monocytogenes* and their regulatory mechanisms. Deletion of these sRNAs resulted in a 40% reduction in biofilm formation, affecting all stages of the biofilm-forming process. Real-time quantitative PCR (RT-qPCR) results showed significant differences in the transcription levels of key genes in the quorum sensing and chemotaxis systems at various growth phases compared to the wild-type. These sRNAs alter gene expression by affecting promoter activity and bind to the 5' untranslated region (UTR) of key genes in quorum sensing and chemotaxis systems, thus influencing mRNA stability and regulating biofilm formation at the post-transcriptional level. Additionally, we predicted and validated the binding sites between these sRNAs and their target mRNAs. This study elucidated the pathways through which sRNAs regulate biofilm formation in *L. monocytogenes* at the post-transcriptional level. These findings highlight the potential of sRNAs as targets for novel antibacterial strategies, contributing to the control of *L. monocytogenes* and the reduction of biofilm-related issues in the food industry.

Key words: *Listeria monocytogenes*; biofilm; sRNA; quorum sensing; chemotaxis system

1. Introduction

L. monocytogenes, a common foodborne pathogen, is widely distributed in environments such as soil and water (Wei et al., 2020). The primary hazard of *L. monocytogenes* stems from its capacity to cause foodborne infections. These infections can lead to severe symptoms of food poisoning, including life-threatening conditions, and significantly impact public health. *L. monocytogenes* has become one of the most prevalent foodborne pathogens, causing hospitalization and deaths (Finn et al., 2023). Biofilms are complex structures formed by bacteria that adhere to diverse surfaces and form stable microbial communities (Behbahani et al., 2024). Approximately one-third of the biofilm dry weight is composed of bacterial cells. The remaining weight is attributed to bacterial-produced molecules such as polysaccharides, proteins, and DNA, which constitute the extracellular polymeric substances (EPS) (Mazaheri et al., 2021; Nadell et al., 2016). These EPS promote the adhesion of bacterial cells to food and production-equipment

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surfaces, leading to persistent contamination. Data from the National Institutes of Health (NIH) and the Centers for Disease Control and Prevention (CDC) indicate that over 65% of foodborne illnesses are associated with the presence of biofilms (Mazaheri et al., 2021). For *L. monocytogenes*, biofilm formation not only enhances its survival rate in food processing equipment and refrigerated environments but also provides a stable ecological niche for bacterial cells. The extracellular matrix protects cells from environmental stresses and antimicrobial agents, which increases the risk of its spread in food (Fagerlund et al., 2021). Therefore, understanding the mechanisms of *L. monocytogenes* biofilm formation is crucial for developing effective control strategies.

Biofilm formation in *L. monocytogenes* is a dynamic and complex process that involves the coordinated action of multiple regulatory factors and signaling pathways (Rouhi et al., 2024). Quorum sensing, as an important bacterial communication mechanism, regulates the expression of genes related to biofilm formation by producing signaling molecules. In *L. monocytogenes*, two quorum sensing systems, LuxS/autoinducer 2 (AI-2) and Agr/autoinducing peptide (AIP), assume crucial functions in the regulation of biofilm formation (Banerji et al., 2022). The flagella assembly and chemotaxis systems are essential for *L. monocytogenes* motility, with this motility influencing initial adhesion and biofilm formation (Guo et al., 2023; Lemon et al., 2007). Second messengers play a significant role in regulating extracellular matrix production. Cyclic di-AMP (c-di-AMP), a key second messenger, directly binds to multiple molecular targets, thereby regulating various cellular pathways and affecting biofilm formation (Massa et al., 2020; Zhang et al., 2023). Moreover, the stress response regulator SigB is crucial for biofilm development in *L. monocytogenes* (van et al., 2010). Biofilm formation in *L. monocytogenes* is regulated by multiple regulatory factors that interact via different signaling pathways, collectively affecting biofilm formation (Rouhi et al., 2024). However, many details regarding the regulatory mechanisms involved require further study. Therefore, elucidating these regulatory mechanisms is crucial for developing effective strategies to control biofilm formation in *L. monocytogenes*.

Small RNA (sRNA) is a widespread type of noncoding RNA in bacteria that participates in bacterial responses to environmental stress, metabolic pathways, and quorum sensing (Jørgensen et al., 2020; Thorsing et al., 2018). In *L. monocytogenes*, several sRNAs have been identified, including *Rli31*, *Rli33*-1, *Rli47*, *Rli95*, *Rli60*, and *Rli87*, which are related to the virulence, stress response, and biofilm formation of *L. monocytogenes* (Bergholz et al., 2012; Sievers et al., 2014; Wurtzel et al., 2012). The deletion of sRNA *Rli60* significantly impaired the tolerance to adverse environments and reduced biofilm formation. Furthermore, the transcription levels of the *gltB* and *gltC* genes, which are associated with biofilm formation and the oxidative stress response, are reduced in the *Rli60* deletion mutant strain (Peng et al., 2016). Additionally, *lhrC* is a multicopy sRNA in *L. monocytogenes.* It directly binds to the mRNA *lapB*. The *lapB* mRNA encodes a cell wall-associated protein, and *lhrC* inhibits the translation of this protein, thereby affecting the virulence of *L. monocytogenes* (Sievers et al., 2014). These findings demonstrate the indispensable role of sRNAs in the regulatory mechanism of biofilm formation in *L. monocytogenes*.

It has been demonstrated that multiple regulatory factors can influence biofilm formation by modulating the quorum sensing system and chemotaxis system. As sRNAs play a pivotal role in regulatory networks, their potential regulatory effects on the quorum sensing and chemotaxis systems, as well as the underlying mechanisms, warrant further investigation. The purpose of this study was to elucidate the interaction networks between biofilm-associated sRNAs and key genes in the quorum sensing and chemotaxis systems, thereby uncovering the regulatory mechanisms governing *L. monocytogenes* biofilm formation. Through RNA-seq analysis of *L. monocytogenes* biofilm formation (unpublished data), our laboratory identified three sRNAs (ssrS, sRNA003, and sRNA006) that are potentially involved in this process. We constructed gene knockout mutants for each sRNA to evaluate their impact on biofilm formation capacity. On the one hand, we analyzed the transcriptional levels and promoter activity of key genes in the quorum sensing and chemotaxis systems to reveal the regulatory role of sRNAs at the transcriptional level, while assessing the mRNA stability of key genes to investigate their regulatory role at the post-transcriptional level. Electrophoretic mobility shift assays (EMSA) were used to validate the interaction between sRNAs and mRNAs and thus clarify the functional mechanisms. This research establishes a sRNA-mediated regulatory network for biofilm formation in *L. monocytogenes.*

2. Methods

2.1 Strains and plasmids

L. monocytogenes strain LMB 33426 was stored at -80 ℃ in brain heart infusion medium (BHI) (Solarbio Science and Technology Co., Ltd. Beijing, China) in our laboratory. The *Escherichia coli* (*E. coli*) strains JM109 and JM110 (Beijing Tsingke Biotech Co., Ltd. China) were utilized as host strains for the plasmid pKSV7 in the cloning experiments. The plasmids and mutant strains constructed in this study are listed in Table S1. All strains were rejuvenated in sterile Luria-Bertani broth (LB broth), with 100 mL each containing 1 g of tryptone, 0.5 g of yeast extract powder, and 1g of NaCl.

2.2 Prediction of sRNA secondary structure and construction of an evolutionary tree

The three sRNA sequences are presented in Table S2. The RNAfold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was used to predict the secondary structure of sRNA sequences based on their minimum free energy (Gruber et al., 2008; Peterson et al., 2020). Subsequently, these sequences were aligned using ClustalW, and a phylogenetic tree was constructed using the Maximum Likelihood method in MEGA 11 software. Additionally, bootstrap analysis with 1000 replicates was conducted to assess the reliability of the tree.

2.3 Gene knockout and complementary

The construction of sRNA deletion mutant strains and complementary strains of *L. monocytogenes* was based on previous methods (Shi et al., 2023). Briefly, genomic DNA from *L. monocytogenes* was extracted, and the upstream and downstream fragments of the target gene were amplified. These fragments were fused using overlap extension Polymerase Chain Reaction (PCR), and used to construct the pKSV7 recombinant

plasmid, which was subsequently introduced into *L. monocytogenes*. Homologous recombination was carried out to construct the mutant strains. The primers used for constructing *L. monocytogenes* sRNA deletion mutants and complementary strains were synthesized by GenScript Biotech Corporation and are listed in Table S3.

2.4 Construction of growth kinetic curves

The growth kinetic curve was determined according to previous methods with slight modifications (Fan et al., 2020). *L. monocytogenes* wild-type and mutant strains were inoculated into sterile LB medium and incubated at 37 ℃ for 12 h. The value of OD_{600} was adjusted to 0.5 ± 0.02. The bacterial suspension was then inoculated into a microtiter plate containing 200 μL LB medium at a 1% inoculation rate and incubated with shaking at 37 ℃. The value of OD_{600} was measured hourly using an automatic growth curve analyzer (Bioscreen, Finland). The data were plotted and fitted using SigmaPlot 15.

2.5 Quantifying L. monocytogenes biofilm

The quantification of *L. monocytogenes* biofilms followed previous methods with minor modifications(Shi et al., 2023). *L. monocytogenes* seed cultures were inoculated at a concentration of 1% into a 96-well polyethylene microtiter plate containing 200 μL of LB medium and incubated at 37 ℃. For quantification, the culture was removed, and the wells were washed three times with sterile PBS before being air-dried. Subsequently, the plate was stained with 1% crystal violet for 30 min, followed by three washes with sterile phosphate buffered saline buffer (PBS). Then, 200 μL 95% ethanol was added, and the mixture was incubated at room temperature for 5 min. The OD_{595} value was measured using an Absor-bance Reader (FLx800, Biotech, USA). Measurements were taken every 12 h until 72 h of incubation.

Confocal laser scanning microscopy (CLSM) was performed according to a previous method with minor modifications (Wei et al., 2020). A single colony was picked into sterile broth and cultured overnight. The OD_{600} was adjusted to 0.5 ± 0.02. 2 mL LB medium was added to sterile Glass Bottom Dish (Cellvis), and 1% of the bacterial culture was inoculated. The cultures were incubated at 37 ℃ for 36 h. After incubation, the culture medium was discarded, and the dishes were washed three times with sterile PBS. The biofilms were then stained with 20 mmol/L SYTO 9 and Propidium Iodide (PI) at room temperature for 30 min. Images were captured using CLSM (TCS SP8, Leica, Germany) with 488 nm settings for the green signal and 630 nm settings for the red signal.

2.6 Cell surface characteristics and adhesion capacity

The cell surface hydrophobicity (CSH) and auto-aggregation capacity were conducted according to previous methods (Shi et al., 2023). The adhesion capacity was assessed followed previous methods with minor modifications (Kang et al., 2019). Lettuce was purchased from a market in Nanjing and cut into 4 cm × 4 cm pieces. The lettuce was washed with sterile water and exposed to ultraviolet light for 15 min on each side. The cultured bacterial suspension was adjusted to an OD_{600} of 0.5 ± 0.02, and 100 μL of the suspension was added to the surface of the lettuce. The lettuce was then incubated at 37 ℃ for 36 h. After incubation, the

lettuce was washed three times with sterile water. For benzalkonium bromide treatment, the lettuce was soaked in 0.1% benzalkonium bromide solution for 5 min and then washed three times with sterile water. The washed lettuce was homogenized, serially diluted, and plated on agar plates. The plates were incubated at 37 ℃ for 24 h, after which the microbial counts were determined.

2.7 RT-qPCR analysis

RNA extraction and cDNA reverse transcription: Total RNA from *L. monocytogenes* was extracted using the Bacteria RNA Extraction Kit (Vazyme, Nanjing, China), and quantified for concentration and purity with a Nanodrop spectrophotometer (Thermo). The extracted RNA was stored at -80 ℃. cDNA synthesis was carried out using the HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China).

We quantified the transcription levels of seven key genes involved in quorum sensing and chemotaxis systems at different logarithmic growth phases (early, mid, and late logarithmic phases) in both the wild-type and mutant strains of *L. monocytogenes*. RT-qPCR data were analyzed using the relative quantification method ($2^{-\Delta\Delta Ct}$), and the details of the genes and their corresponding primers are listed in Table S3.

2.8 Determination of mRNA half-life

The stability of mRNA was evaluated by measuring the mRNA half-life according to previous methods with slight modifications (Geyer et al., 2016; Rodrigues et al., 2023). *L. monocytogenes* wild-type and deletion mutant strains were inoculated at 1% in LB medium, and incubated at 37 ℃ for 12 h. Subsequently, 2 mL of the bacterial culture was collected and centrifuged at 12,000 × g for 2 min, and the resulting pellet was stored at -80 ℃ after discarding the supernatant. Rifampicin (Solarbio Science and Technology Co., Ltd. Beijing, China) (at a final concentration of 200 μg/mL) was added to inhibit mRNA synthesis in the remaining bacterial culture. Bacterial samples were collected every 15 min, mixed with 400 μL of RNAwait (Solarbio Science and Technology Co., Ltd. Beijing, China), incubated at room temperature for 5 min, and then centrifuged at 12,000 × g for 2 min. After discarding the supernatant, the pellet was stored at -80 ℃. Total RNA extraction and relative expression levels were measured as described in section 2.7. Data analysis was performed using SigmaPlot 15.

2.9 Construction of eGFP fusion plasmid and measurement of the fluorescence intensity of GFP

The promoter-eGFP (green fluorescent protein) fusion plasmids were constructed according to previous method (Ma et al., 2021). The sequences of the eGFP gene and promoter gene were cloned, and inserted into the plasmid using the ClonExpress® II One Step Cloning Kit (Vazyme, China), resulting in a series of promoter-eGFP fusion plasmids named pEL-Pgene. All primers used are listed in Table S3.

The constructed fusion plasmids were transformed into wild-type and gene deletion mutant strains of *L. monocytogenes* through electroporation, respectively. After successful confirmation by PCR, the *L. monocytogenes* strains with the fusion plasmids were cultured in BHI medium at 37 ℃ for 12 h.

Subsequently, 1 mL of the bacterial culture was centrifuged at 12,000 × g for 1 min, washed with sterile PBS, centrifuged again at 12,000 × g for 1 min, after which the supernatant was discarded. The bacterial pellet was resuspended in sterile PBS, and the OD_{600} value was adjusted to 0.9 ± 0.02. The fluorescence intensity was measured using a Multimode Plate Reader (EnSight, PerkinElmer, USA) with excitation/emission wavelengths of 535/465 nm.

2.10 Validation of sRNA-mRNA interactions via EMSA

Transcription in vitro and EMSA experiments were performed according to previous methods with minor modifications (Lillebæk et al., 2018; Ross et al., 2019). DNA samples containing a T7 RNA polymerase promoter at the 5'-end were amplified using the LMB 33426 genome as a template. In vitro transcription was performed using the T7 High Yield RNA Transcription Kit (Vazyme, China), and the biotin-labeled sRNA was generated with Biotin-16-UTP (APExBIO, USA). All primers used are listed in Table S3.

For the EMSA, 1 μL of biotin-labeled sRNA, 1 μL of promoter mRNA, and 1 μL of REMSA binding buffer were combined, and RNase-free H_2O was added to a final volume of 10 μL. The samples were incubated at 56 ℃ for 5 min, followed by incubation at 37 ℃ for 10 min, and then placed on ice for 5 min. Gel shift assays were performed according to the Chemiluminescent RNA EMSA Kit (Beyotime, China), including gel electrophoresis, membrane transfer, and detection using a chemiluminescence gel imaging system.

2.11 Statistical analysis

All the above experiments were conducted in triplicate. Data were analyzed using ANOVA, followed by Duncan's test and Student's t test to determine the significant differences between the means using SPSS 20.0 software. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Characterization of sRNAs in L. monocytogenes

Our laboratory identified and validated two novel sRNAs in *L. monocytogenes*, namely sRNA003 and sRNA006. Additionally, ssrS was identified as an RNA polymerase modulator in previous research (Mandin et al., 2007). In this study, PCR amplification was performed to validate the three sRNAs, confirming that they are sequences within the genome of *L. monocytogenes* LMB33426 (Fig. S1). Additionally, the Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values of the three sRNAs determined by RNA-seq are provided (Table S2), indicating their expression in *L. monocytogenes* LMB33426. These sRNAs are in intergenic regions and do not encode proteins (Fig. 1A). The secondary structures of ssrS, sRNA003, and sRNA006 were predicted by the RNAfold server with the minimum free energy (MFE) algorithm. The most stable conformations of ssrS, sRNA003, and sRNA006 were predicted to feature multiple stem-loop structures enriched with UC motifs (Fig. 1B). Phylogenetic analysis conducted using Mega 11 revealed that these three sRNAs are exclusive to the *Listeria* genus, with highly conserved

sequences among most *L. monocytogenes* strains. Furthermore, these sRNAs were also identified in a few nonpathogenic *Listeria* species such as *Listeria innocua*, *Listeria welshimeri*, and *Listeria seeligeri* (Fig. 1C). sRNA003 and sRNA006 exhibited greater conservation than did ssrS (Fig. S2). The conservation and structural analysis of sRNAs are crucial for understanding the role of sRNAs in the survival and adaptability of *L. monocytogenes*.

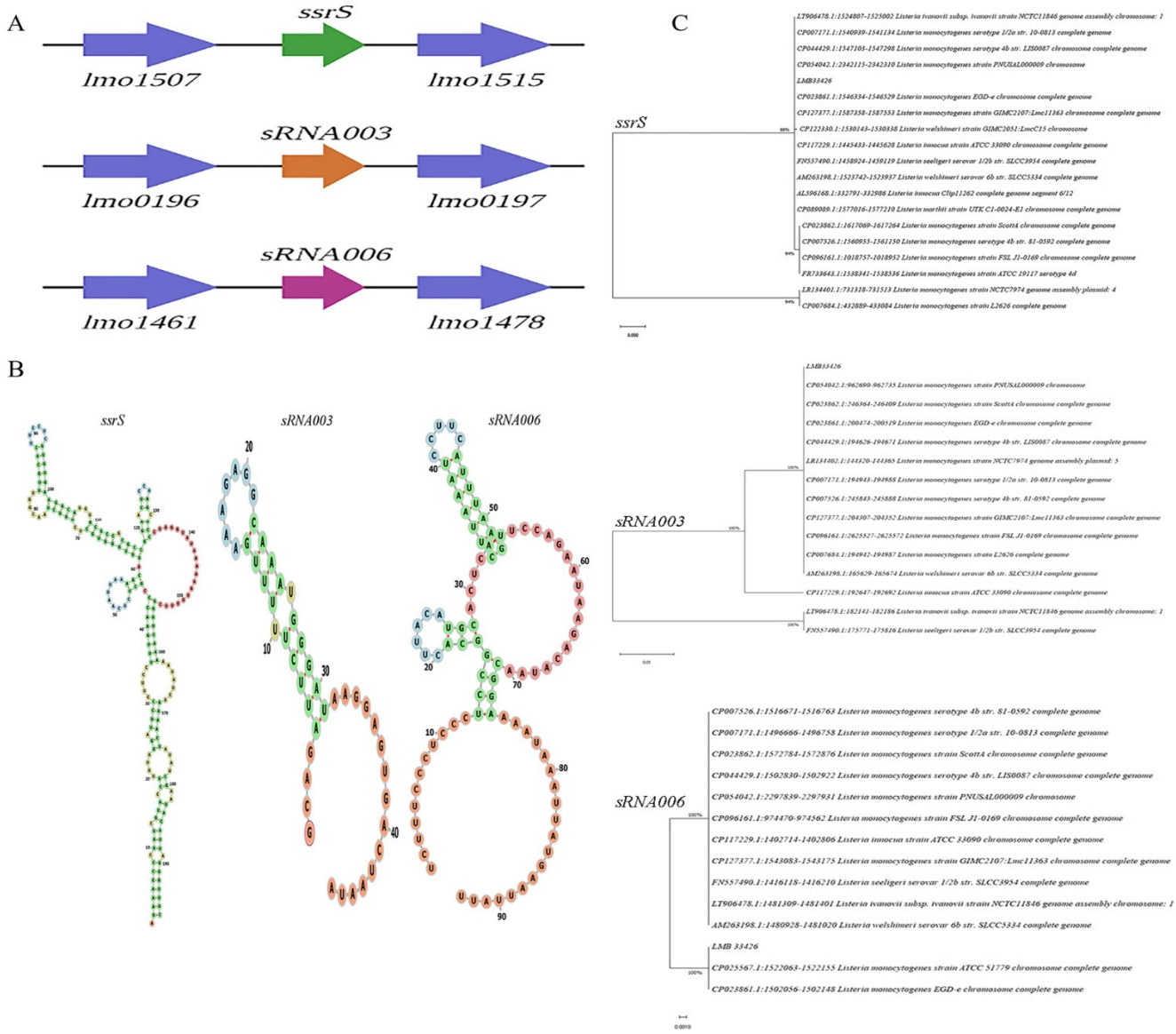


Figure 1 Bioinformatic analysis of ssrS, sRNA003, and sRNA006. (A) Localization of ssrS, sRNA003, and sRNA006 in the intergenic regions of *L. monocytogenes*. (B) Secondary structures of ssrS, sRNA003, and sRNA006. Nucleotide colors represent different types of structures: green for stems, red for multiloops, yellow for interior loops, blue for hairpin loops, and orange for 5' and 3' unpaired regions. (C) Phylogenetic tree of ssrS, sRNA003, and sRNA006.

3.2 sRNAs mutant did not affect L. monocytogenes growth at 37 ℃

We evaluated the growth capacity of the wild-type strain, sRNA deletion mutant strains, and complementary strains at 37 ℃ (Fig. S3A and Fig. S3B) and 30 ℃ (Fig. S3C and Fig. S3D), and fitted the data with a sigmoidal model ($R^2 > 0.99$). At 37 ℃, there were no significant differences in OD_{600} values both the sRNA deletion mutants (Fig. S3A) and complementary strains (Fig. S3B) compared to the wild-type ($P > 0.05$). From 3 h to 8 h, the cultures entered the logarithmic growth phase, followed by a

stable phase. The results indicate that sRNA deletion does not affect the growth of *L. monocytogenes* at 37 ℃. At 30 ℃, during the early growth phase (0 h to 4 h), there were no significant differences in growth between the sRNA deletion mutant strains and the wild-type *L. monocytogenes* ($P > 0.05$). However, after entering the logarithmic growth phase, the OD_{600} values of *LM-ΔssrS*, *LM-ΔsRNA003*, and *LM-ΔsRNA006* were significantly lower than that of the wild-type ($P < 0.05$) (Fig. S3C), indicating a delay in the growth of the sRNA deletion mutant strains. There were no significant differences between the three sRNA deletion mutant strains. After 10 h, the OD_{600} values of the sRNA deletion mutant strains reached wild-type levels, with no significant differences ($P > 0.05$). There was no significant growth delay in the growth of the complementary strains during the logarithmic growth phase compared to that of the wild-type ($P > 0.05$) (Fig. S3D).

3.3 sRNAs mutants reduced biofilm formation by L. monocytogenes

The effects of ssrS, sRNA003, and sRNA006 on *L. monocytogenes* biofilm formation were evaluated using the crystal violet staining method. The biofilm formation in the sRNA deletion mutant strains was significantly lower than in the wild-type *L. monocytogenes* ($P < 0.05$), with a reduction of approximately 40% compared to the wild-type (Fig. 2A). During the formation stage of biofilm (12 h to 24 h), *L. monocytogenes* showed a rapid biofilm generation rate. Compared to the wild-type, in which the biofilm generation rate reached 40% through our calculation, the sRNA deletion mutant strains had a lower biofilm generation rate. During the maturation (24 h to 36 h) and degradation stages (36 h to 48 h), the biofilm dissolution rates were greater for *LM-ΔssrS*, *LM-ΔsRNA003*, and *LM-ΔsRNA006*. The biofilm amount in the sRNA deletion mutant strains decreased by more than 60%, which was higher than the biofilm dissolution rate of the wild-type *L. monocytogenes*. The biofilm formation capacity of sRNA complementary strains was restored to the wild-type level (Fig. S4). The CLSM images showed that the wild-type *L. monocytogenes* formed a uniform and dense biofilm enveloping the bacterial cells. In the sRNA gene deletion mutant strains, biofilm formation decreased, and the biofilm distribution became sparse and uneven. Furthermore, the increased red signals indicated more dead cells, suggesting a weakened protective effect of the biofilm on bacterial cells (Fig. 2B). Cell surface hydrophobicity and auto-aggregation contribute to the adhesion of bacterial cells to material surfaces, which is critical for biofilm formation. Deletion of sRNAs significantly decreased the cell surface hydrophobicity and auto-aggregation capacity (Fig. 2C). We also conducted adhesion experiments on lettuce, and the results showed that sRNA deletion reduced the adhesion of *L. monocytogenes* and made the bacteria more susceptible to removal by disinfectants such as benzalkonium bromide (Fig. 2D).

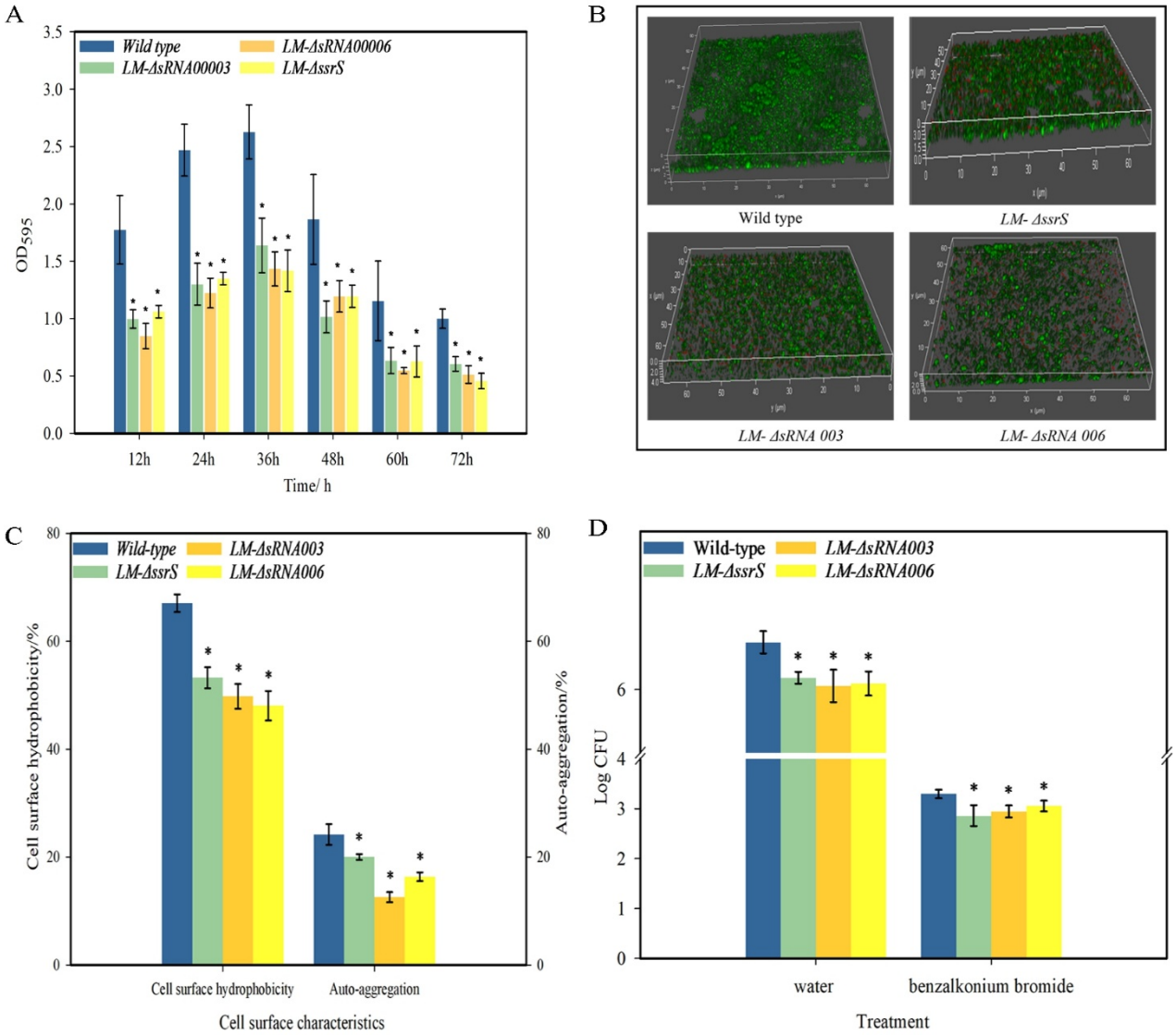


Figure 2 Impact of sRNA deletion on biofilm formation and adhesion. (A) Biofilm formation over 12-72 h for the wild-type and sRNA deletion strains of *L. monocytogenes*. (B) CLSM images of biofilms formed by wild-type and sRNA deletion strains, with green signals indicating live cells, and red signals indicating dead cells. (C) Measurements of the cell surface hydrophobicity and auto-aggregation capacity of the wild-type and sRNA deletion strains of *L. monocytogenes*. (D) Adhesion of the wild-type and sRNA deletion strains of *L. monocytogenes* to lettuce. "Water" indicates washing with sterile water, and "Benzalkonium bromide" indicates soaking with benzalkonium bromide for 5 min followed by washing with water. * indicates significant differences compared to the wild-type ($P < 0.05$).

3.4 sRNAs mutation affected the transcription levels of genes related to biofilm formation in L. monocytogenes

Based on the determined growth curve, the transcription levels of biofilm formation related genes (involving quorum sensing systems and chemotaxis systems) were determined at different logarithmic growth phases (4 h, 6 h, and 8 h) (Fig 3).

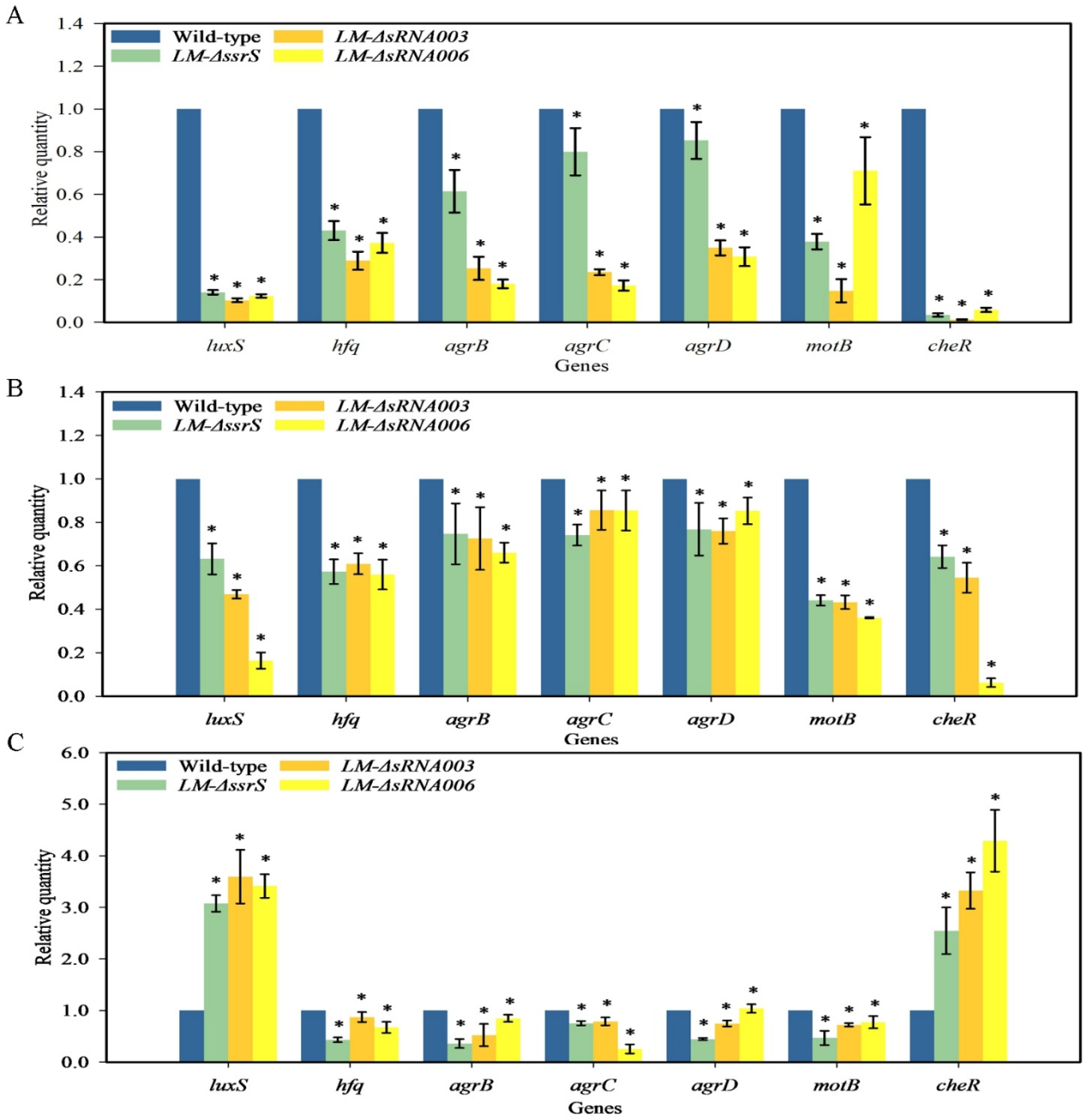


Figure 3 Transcription levels of key biofilm formation genes (*luxS* and *hfq* are related to the lux quorum sensing system; *agrB*, *agrC*, and *agrD* are key genes in the agr quorum sensing system; *motB* and *cheR* are key genes in the chemotaxis system). (A) Transcription levels at the early logarithmic growth phase (4 h). (B) Transcription levels at the mid-logarithmic growth phase (6 h). (C) Transcription levels at the late logarithmic growth phase (8 h). * indicates significant differences compared to the wild-type.

Compared to that of the wild-type, the transcription level of *hfq* was significantly lower at different logarithmic phases ($P < 0.05$) in *LM-ΔssrS*, *LM-ΔsRNA003*, and *LM-ΔsRNA006*. Deletion of ssrS, sRNA003, and sRNA006 significantly decreased the transcription levels of *luxS* during the early and mid-logarithmic growth phases ($P < 0.05$). However, in the late logarithmic growth phase, the transcription levels of *luxS* in the sRNA deletion mutant strains were significantly higher than wild-type strain ($P < 0.05$). In the late logarithmic phase, the transcription level of *luxS* was significantly lower compared to the mid-logarithmic phase (Fig. S4). We suggest that the transcription level of *luxS* is closely related to the growth phase. Additionally, the deletion of sRNA led to a significant decrease in the transcription levels of

agrB, agrC, and *agrD* compared to the wild-type ($P < 0.05$). The three sRNAs play significant regulatory roles in the lux and agr quorum sensing systems of *L. monocytogenes*.

In the chemotaxis system, the transcription levels of the *motB* in sRNA deletion strains were significantly lower than wild-type *L. monocytogenes* at different logarithmic growth phases ($P < 0.05$). The transcription level of *cheR* was significantly decreased in the early and middle logarithmic growth phases but significantly increased ($P < 0.05$) in the late logarithmic growth phase in the sRNA deletion mutant strains. By comparing *cheR* transcription levels between the late and middle logarithmic phases (Fig. S5), we found that in both the wild-type and sRNA deletion mutant strains, *cheR* transcription was lower in the late logarithmic phase than in the middle logarithmic phase.

3.5 sRNAs mutant affected the mRNA stability of genes related to biofilm formation in L. monocytogenes

We studied the effects of sRNAs on mRNA stability by determining the mRNA half-life of key genes in *L. monocytogenes*. The results were fitted using an Exponential Decay model (Fig. S6). The equation, coefficient of determination (R^2), and mRNA half-life values are presented in Table 1. All R^2 values for the mRNA degradation curve equations exceeded 0.95, indicating the mRNA degradation curve equations could accurately predict the mRNA half-life.

The results showed that in *LM-ΔssrS*, the half-life of agr system genes (*agrB*, *agrC*, *agrD*) were significantly reduced by more than 45% compared to the wild-type (Table 1). The half-life of the *hfq* gene was slightly reduced by approximately 10%. Similarly, the deletion of sRNA006 resulted in a substantial decrease in the half-life of genes involved in quorum sensing and chemotaxis compared to that of the wild-type. The results indicate that sRNA deletion leads to reduced stability of key genes in these systems, underscoring the importance of sRNAs in maintaining mRNA stability. Additionally, in *LM-ΔsRNA003*, only the *agrB* mRNA half-life was significantly reduced by 65%. However, in *LM-ΔsRNA003*, a unique case was observed. Compared with those in the wild-type strain, the half-life of *luxS* and *cheR* mRNAs in the *sRNA003* deletion strain were 17.23 and 10.84 min, respectively, increasing of 12% and 28%. These findings suggest that *sRNA003* affects the stability of *luxS* and *cheR* mRNAs, with their stability being enhanced in the absence of sRNA003.

3.6 sRNAs mutant affected the promoter activity in L. monocytogenes

We constructed promoter-eGFP fusion plasmids and introduced into the wild-type and sRNA deletion mutant strains of *L. monocytogenes* to evaluate promoter activity by measuring fluorescence intensity. Fluorescence microscopy (Leica, Germany) revealed green fluorescence in the *L. monocytogenes* strains with the fusion plasmid (Fig. 4A). The wild-type strain showed significantly higher fluorescence intensity with the pEL-Pagr plasmid, indicating higher activity of the agr promoter (Fig. 4B). The results showed that the fluorescence intensity was significantly lower in the sRNA deletion mutant strains compared to the wild-type ($P < 0.05$), indicating reduced promoter activity due to sRNA deletion.

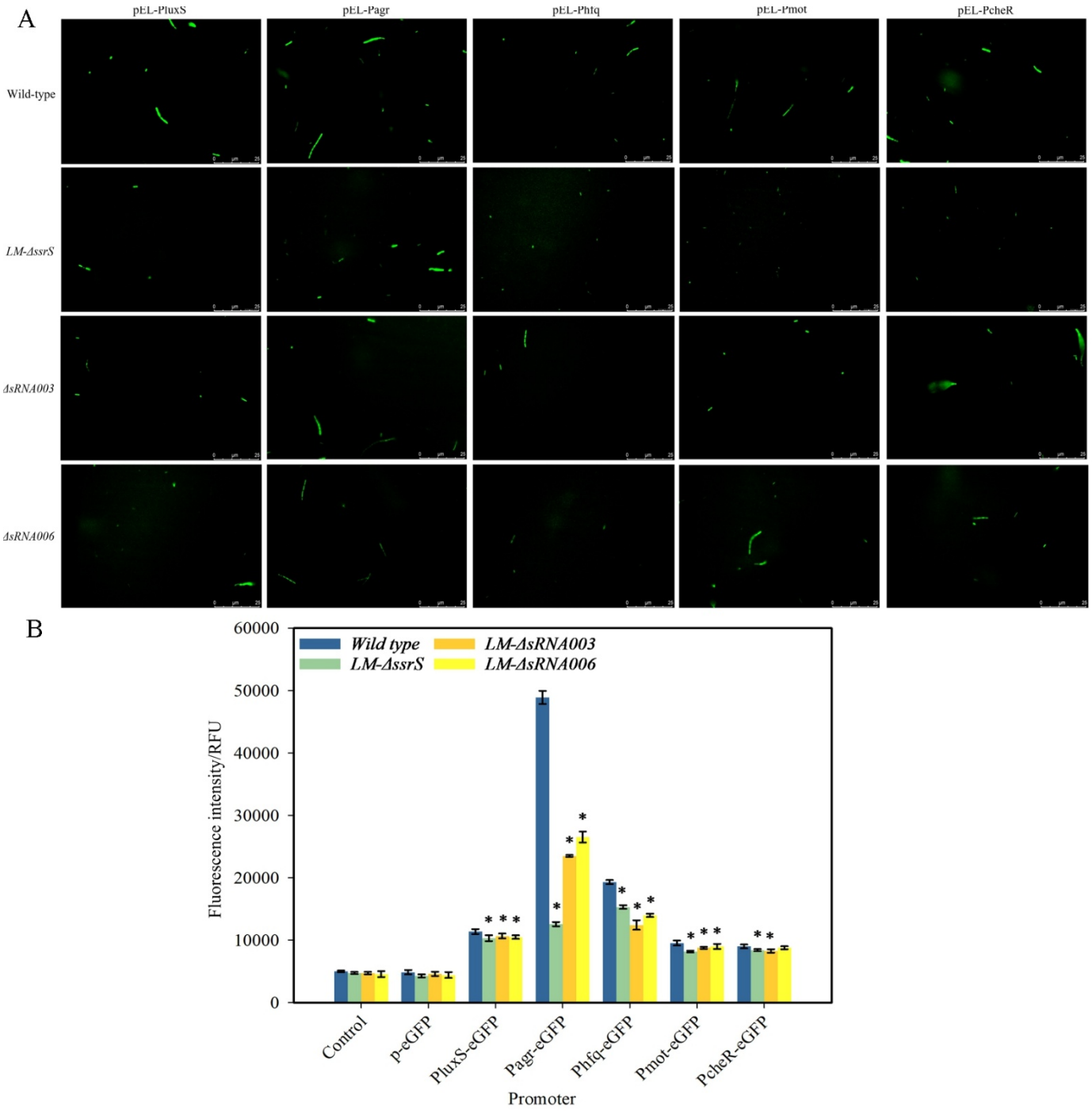
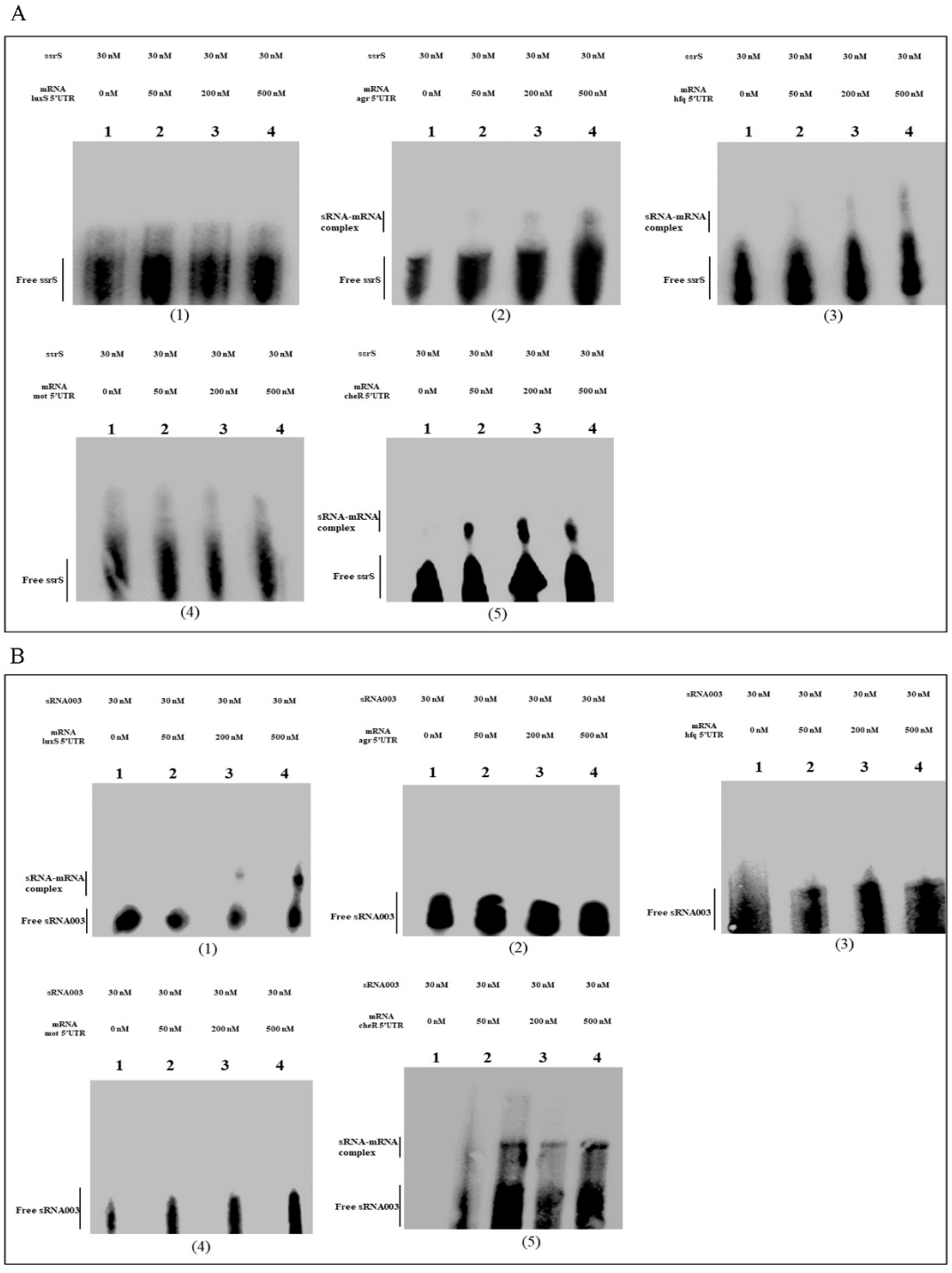


Figure 4 Determination of promoter activity. (A) Fluorescence microscopy observation of wild-type and sRNA deletion strains of *L. monocytogenes* (transformed with the pEL plasmid fused with the promoter sequence). Green signals indicate eGFP-expressing *L. monocytogenes*. (B)The intensity of fluorescence. "Control" represents *L monocytogenes* without a plasmid. "LM-pEL" represents *L monocytogenes* with the eGFP plasmid (without the promoter sequence). "pEL-Pgene" represents *L monocytogenes* with the eGFP plasmid fused with the target gene promoter sequence. * indicates significant differences compared to the wild-type ($P < 0.05$).

3.7 In vitro validation of sRNA binding to key gene 5'UTR mRNAs

We performed sRNA-mRNA EMSA using biotin-labeled sRNAs and in vitro transcribed target gene 5'UTR mRNA. The sRNAs were incubated with mRNA at concentrations of 0, 50, 200, and 500 nmol/L to determine the combination between sRNAs and mRNAs (Fig. 5). ssrS, sRNA003, and sRNA006 bind to the 5'UTRs mRNA of key genes in quorum sensing and chemotaxis systems, leading to differential shifts in migration. The results showed that ssrS bound to the 5'UTR mRNA of the *agr*, *hfq*, and *cheR* genes (Fig.

5A), that sRNA003 bound to the mRNA of *luxS* and *cheR* (Fig. 5B), and that sRNA006 bound to the mRNA of *agr*, *mot*, and *cheR* 5'UTR (Fig. 5C). Both sRNAs could bind to the *cheR* 5'UTR to regulate the transcription level of the *cheR* gene. Using the IntaRNA server (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp), we predicted the binding sites between sRNA and mRNA (Fig. 6). The binding sites of sRNAs are predominantly rich in cytosine (C) and uracil (U), with some sites also containing adenine (A). Subsequently, the mutant 5'UTR mRNA sequences were generated to disrupt sRNA binding, and validated through EMSA. The results showed that the mutant 5'UTR mRNA was failed to bind to sRNA (Fig. S7), confirming these predicted sites are the actual binding sites between sRNA and mRNA.



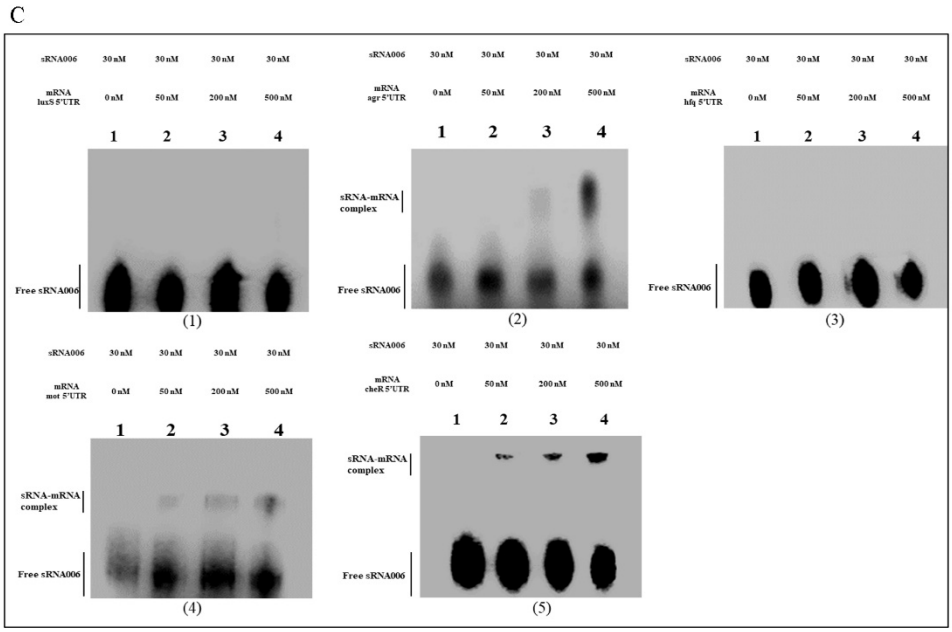
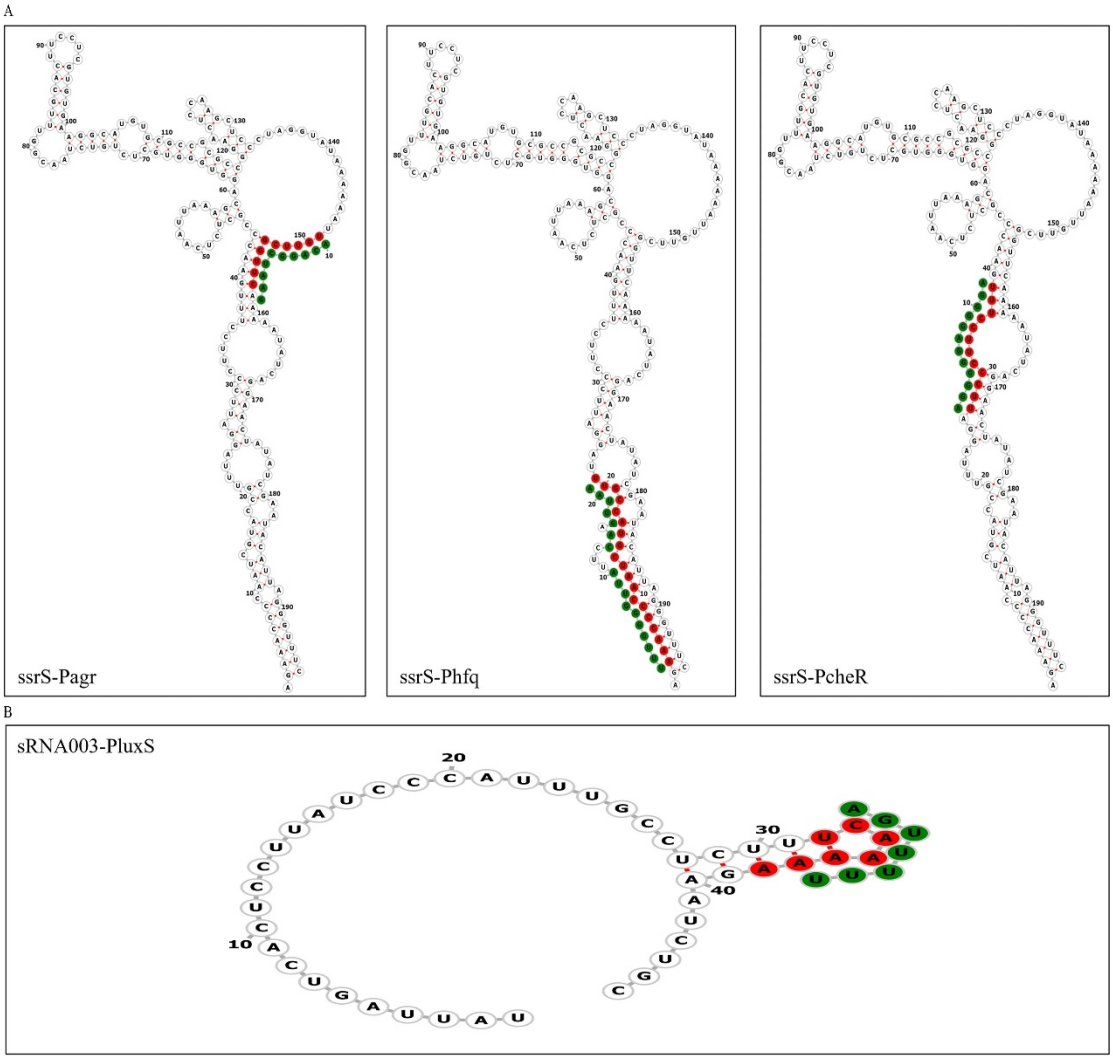


Figure 5 Detection of sRNA-mRNA interactions by EMSA. Biotin-labeled sRNA was used at a final concentration of 30 nmol/L. The mRNA targets included (1) *luxS* 5'UTR mRNA, (2) *agr* 5'UTR mRNA, (3) *hfq* 5'UTR mRNA, (4) *mot* 5'UTR mRNA, and (5) *cheR* 5'UTR mRNA. The mRNA concentrations in lanes 1 to 4 were 0, 50, 200, and 500 nmol/L, respectively. (A) Interaction of ssrS with mRNA as determined by EMSA. (B) Interaction of sRNA003 with mRNA as determined by EMSA. (C) Interaction of sRNA006 with mRNA as determined by EMSA.



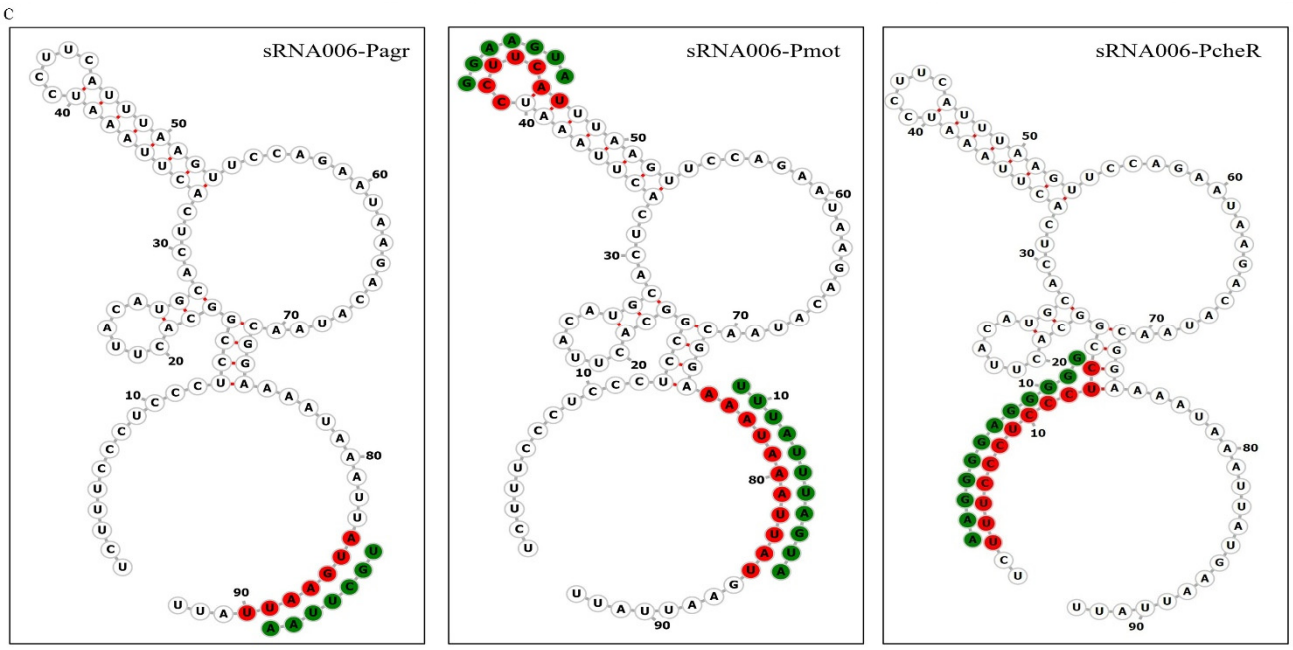


Figure 6 Prediction of sRNA binding sites on the 5'UTR of target gene mRNAs. (A) Binding sites of ssrS with mRNA. (B) Binding sites of sRNA003 with mRNA. (C) Binding sites of sRNA006 with mRNA. Red indicates sRNA binding sites, and green indicates mRNA binding sites.

4. Discussion

L. monocytogenes biofilms protect cells from adverse conditions and provide a necessary environment for cell growth. sRNA play an important role in post-transcriptional gene regulation, which are indispensable in the regulatory network governing biofilm formation (Storz et al., 2011). For example, both sRNA *Rli87* (Kun et al., 2014) and *Rli60* (Peng et al., 2016) can regulate the environmental stress response in *L. monocytogenes*. The deletion of these sRNAs caused changes in growth under high osmolarity, oxidative conditions and temperature stress, and the biofilm formation was decreased. Similarly, the deletion of sRNA003 and sRNA006 affects the environmental adaptation capability of *L. monocytogenes* and impacts biofilm formation. Such phenotypic changes in biofilm formation are typically mediated by downstream regulatory effects on functional genes. Consistent with this, our subsequent analyses revealed that these sRNAs directly interact with key genes in quorum sensing and chemotaxis systems, which are critical for biofilm development. We suggest that these three novel sRNAs regulate biofilm formation-related target genes to impact biofilm formation.

sRNA provides a novel means to rapidly respond to environmental changes without the energy consumption associated with protein synthesis, thus reducing metabolic costs (Beisel et al., 2010; Dutcher et al., 2018). In the biofilm formation stages, sRNAs act as intermediates between quorum sensing systems and biofilm-related regulatory genes (Svenningsen et al., 2018; Zhang et al., 2023). The deletion of sRNA003 and sRNA006 reduces the transcription levels of key genes in the lux and agr systems, indicating that these sRNAs regulate quorum sensing systems in *L. monocytogenes* to affect biofilm formation. Notably, in the late logarithmic growth phase, the *luxS* gene transcription level was significantly higher in sRNA gene deletion mutant strains than in the wild-type. S-Adenosylhomocysteine (SAH), a biotoxin byproduct of

methylation processes, accumulates due to the actions of LuxS and Pfs, leading to cytotoxicity (Belval et al., 2006; Olszewska et al., 2017). We suggest that in sRNA deletion mutant strains, *L. monocytogenes* upregulates *luxS* expression to enhance the efficiency of SAH conversion and avoid cytotoxicity. MotB, a flagellar rotor anchor protein, generates torque in the flagellar motor (Ruhal et al., 2021). CheR, a methyltransferase, methylates chemoreceptor proteins on the cell membrane, allowing the cell to sense stimulus signals (Michel et al., 1998; Piercey et al., 2016). The deletion of sRNA003 and sRNA006 significantly reduces the transcription levels of *motB* and *cheR*, indicating that these sRNAs regulate the chemotaxis system in *L. monocytogenes*. Additionally, in the late logarithmic growth phase, the transcription level of *cheR* significantly increased in the sRNA deletion strains. We suggest that the deletion of sRNAs reduces motility and nutrient availability while causing the accumulation of harmful substances, resulting in adverse growth conditions. To overcome this adversity, *L. monocytogenes* promotes *cheR* expression, enhancing chemoreceptor protein methylation efficiency and increasing motility to evade unfavorable factors.

Beyond transcriptional regulation, sRNAs also affects gene expression post-transcriptionally by modulating mRNA stability. mRNA degradation is a key mechanism by which bacteria regulate gene expression to adapt to environmental changes. By rapidly degrading specific mRNAs, cells quickly reduce the synthesis of target proteins (Durand et al., 2015; Hör et al., 2018). mRNA degradation maintains RNA balance within cells, removing harmful mRNA molecules to preserve normal cellular functions (Karousis et al., 2016; Trinquier et al., 2020). sRNAs are pivotal regulators of gene expression, significantly influence mRNA stability by base-pairing with specific mRNA sequences to either promote or inhibit degradation (Durand et al., 2015). For example, in *Clostridium perfringens*, the sRNA VR-RNA binds to the *colA* mRNA 5’-UTR to increase mRNA stability, and the deletion of VR-RNA shortens *colA* mRNA's half-life by 2 min (Obana et al., 2010). The EMSA experiments demonstrated that sRNA ssrS and sRNA006 bind to the 5’UTRs of key genes in the quorum sensing and chemotaxis systems, resulting in reduced mRNA half-life. Additionally, sRNAs indirectly affect mRNA stability by regulating RNA degrading enzymes (Machado et al., 2022), affecting translation levels or RNA-binding proteins (Trinquier et al., 2020). We hypothesize that in this study, ssrS, sRNA003, and sRNA006 affect mRNA stability through these alternative mechanisms rather than by binding to the 5' UTR of the genes. Moreover, sRNAs bind to the 5’UTRs or adjacent regions of mRNAs, leading to accelerated degradation. In *E. coli*, sRNA *Spot42* binds to the translation initiation site of the *galK* gene, causing the degradation of genes sharing a 3′ end. Deletion of *Spot42* increases the mRNA half-life of the gal genes by 200% (Jeon et al., 2023). sRNA003 binds to the 5’UTRs of the *luxS* and *cheR* genes, accelerating the degradation of these mRNAs. Deletion of sRNA003 increased the half-life of the *luxS* and *cheR* mRNAs by 12% and 28%, respectively. We propose that the binding of sRNA003 to 5’UTRs increases the susceptibility of mRNAs to nuclease identification and processing, increasing mRNA instability, and that this instability is alleviated in the absence of sRNA.

sRNAs' ability to bind specific mRNAs stems from intrinsic structural motifs, which fine-tune binding specificity, strength, and dynamics to shape regulatory efficiency. The CU-rich motifs are characteristic of sRNAs in bacteria with low-GC content (Geissmann et al., 2009). Research has shown that CU sequences, particularly the UCCC motif, are crucial for sRNA binding in *Staphylococcus aureus* (Geissmann et al., 2009; Pitman et al. 2015). In *Streptococcus pneumoniae*, the CU-rich motif CCUCCU is associated with RBS (Ribosome binding site) blockade (Patenge et al., 2015; Pitman et al., 2015). In *L. monocytogenes*, the UC-rich motifs have been identified in sRNA, with *lhrC* containing three CUCCC motifs that interact with the target *lapB* mRNA (Pitman et al., 2015; Susanne et al., 2014). Sequence analysis of ssrS, sRNA003, and sRNA006 revealed that all three sRNAs contain CU-rich motifs, facilitating their mRNA binding. Interestingly, all three sRNAs bind to the 5'UTR of the *cheR* gene, with binding sites containing CU-rich motifs (UCCC or CUCCU). Additionally, some binding sites within these sRNAs are AU-rich. The AU base pairs, with only two hydrogen bonds, exhibit weaker binding strength compared to CU base pairs, enabling rapid binding and dissociation. The flexibility allows bacterial cells to promptly respond to environmental changes(Gomes et al., 2019; Mai et al., 2019). The AU-rich motifs in sRNAs recruit the Hfq protein upon mRNA binding, altering RNA stability (Kovach et al., 2014).

5. Conclusion

In this study, we shed light on the crucial role of the sRNAs ssrS, sRNA003, and sRNA006 regulating biofilm formation in *L. monocytogenes* by affecting the quorum sensing and chemotaxis systems (Fig. S8). Our findings revealed that these sRNAs bind to the 5'UTRs of key genes, altering mRNA stability and expression levels. These insights highlight the critical role of sRNAs in bacterial adaptability and pathogenicity, providing potential targets for controlling *L. monocytogenes* infections through disruption of biofilm formation mechanisms. Future research could focus on studying the potential of these sRNAs as therapeutic targets in specific in vivo models. This could involve testing the efficacy of sRNA-targeting agents in animal models of *L. monocytogenes* infection to assess their potential for treating infections and preventing biofilm related complications.

Conflict of interest statement

We declare that we have no financial or personal relationships with any individuals or organizations that could unduly influence our work. There are no professional or personal interests, including those related to products, services, or companies, that could affect the integrity of the manuscript's content or its review.

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