Food Research International 245 (2026) 120841



Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres



Physiological, genomic, and methylomic alterations during formation and resuscitation of viable but non-culturable *Salmonella* Infantis under prolonged desiccation and antibiotic stress in soil

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ARTICLE INFO

Keywords:
Salmonella
Viable but non-culturable state
Artificial soil
Stress response
Single nucleotide polymorphisms
Methylation

ABSTRACT

Salmonella enterica can enter a viable but non-culturable (VBNC) state in soil under environmental stresses, thereby persisting in the environment and posing a potential risk to public health. This study investigated the formation and resuscitation mechanism of VBNC-state *Salmonella* Infantis under combined desiccation and antibiotic stress in soil. *S.* Infantis was inoculated into artificial soil and subject to desiccation stress (low water activity) and antibiotic stress (tetracycline or doxycycline amendment) for 226 days. Prolonged stress promoted VBNC state formation, accompanied by enhanced stress resistance, increased accumulation of reactive oxygen species, and reduced synthesis of quorum sensing autoinducer-2. Recovery of culturability in VBNC-state cells was observed following treatment with free radical scavengers, quorum sensing autoinducers, and nutrient supplements. VBNC-state cells from doxycycline-amended soil and subsequently catalase-resuscitated cells were selected for genomic and methylomic analyses based on their distinct physiological responses, with stationary-phase cells as the control. Genomic and methylomic analyses revealed genetic and epigenetic variations in genes associated with key biological functions such as response to stimulus, phosphotransferase system, oxidative stress system, and quorum sensing system during VBNC transition and resuscitation. Notably, genome-wide DNA demethylation and dynamic methylation changes in stress- and metabolism-related genes may be associated with *S.* Infantis adaptation to environmental stress and its transition between the VBNC and resuscitated states. These findings provide new insights into the molecular changes associated with VBNC state transitions under environmental stress and may inform future approaches for the monitoring and control of VBNC-state pathogens in natural environments.

1. Introduction

Foodborne salmonellosis remains one of the most pressing public health challenges globally, with fresh produce identified as a leading vehicle for *Salmonella* outbreaks in recent years (Alegbeleye et al., 2018; Alegbeleye & Sant'Ana, 2021; Schierstaedt et al., 2020). Unlike ready-to-eat processed foods, fresh produce is often consumed raw, and current post-harvest washing and disinfection protocols cannot eliminate pathogens that have been internalized during pre-harvest production or persist in adherent soil particles, creating an unmet need for effective pre-harvest control strategies to safeguard the fresh produce supply chain. One major challenge is that *Salmonella* can enter a viable but non-culturable (VBNC) state when exposed to common agricultural soil stresses, including low water activity (a_w) (Giagnoni et al., 2018; Se et al., 2021), antibiotic residues (Kim et al., 2024; Li et al., 2023), oxidative stress (Liao, Jiang, & Zhang, 2018; Morishige et al., 2013; Oliver, 2010), nutrient deficiency (Gupte et al., 2003; Liu et al., 2009), and pH changes (Tholozan et al., 1999).

The VBNC state is increasingly recognized as an important adaptive strategy that enables microorganisms to survive adverse environmental conditions through reduced metabolic activity and deeper dormancy (Ayrapetyan, Williams, Baxter, & Oliver, 2015; Ayrapetyan et al., 2018). Recent evidence from environmentally relevant microorganisms has further shown that complex environmental stresses can induce the VBNC

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https://doi.org/10.1016/j.foodres.2026.120841
Received 25 July 2026; Received in revised form 12 September 2026; Accepted 14 September 2026
Available online 21 September 2026
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state, while cells retain the potential for resuscitation under favorable conditions (Su et al., 2026; Yu et al., 2025). This stress-adaptive capacity is particularly concerning for *Salmonella*, which may enter and persist in the VBNC state in response to environmental stresses encountered along the food chain. VBNC-state *Salmonella* remains viable despite its low metabolic activity and inability to grow on standard culture media (Liu et al., 2009; Liu et al., 2010; Morishige et al., 2017). Consequently, conventional microbiological methods may fail to detect VBNC-state *Salmonella* in contaminated produce, allowing contamination to go undetected and potentially contributing to foodborne salmonellosis.

Soil serves as a persistent reservoir for *Salmonella* spp., which can survive in agricultural soils for days to years (Alegbeleye & Sant'Ana, 2023; Baloda et al., 2001; Holley et al., 2006; Islam et al., 2004) and contaminate crops via root uptake and soil splash during pre-harvest growth. The soil environment exposes *Salmonella* to multiple concurrent stresses, including prolonged desiccation and antibiotic residues, both of which have been shown to induce VBNC formation (Kim et al., 2024; Se et al., 2021). Multiple mechanisms underlying VBNC formation and resuscitation have been identified, including the toxin-antitoxin (TA) system (Ayrapetyan, Williams, & Oliver, 2015), oxidative stress responses (Liao, Zhang, et al., 2018; Nie et al., 2019), quorum sensing pathways (Ayrapetyan et al., 2014), and global stress regulators such as RpoS (Li, Yan, et al., 2014) and OxyR (Dong et al., 2020). Nevertheless, their roles in mediating *Salmonella* persistence and adaptation under the complex and concurrent stresses encountered in agricultural soils have not been well characterized. Recent research on the VBNC state of microorganisms in polluted environments has shown that combined environmental stresses, such as salinity and phenol exposure, can markedly accelerate VBNC formation and affect quorum-quenching activity (Su et al., 2026). Meanwhile, resuscitation-promoting factors have emerged as important mediators of VBNC resuscitation, facilitating the recovery of cellular activity under stressful environmental conditions (Su et al., 2026; Yu et al., 2025). These findings further highlight the dynamic nature of the VBNC state and its importance in microbial adaptation to complex environmental stresses.

Moreover, recent studies have shown that environmental stress can drive adaptive genetic changes that enhance *Salmonella* survival under adverse conditions. For example, adaptive laboratory evolution under acid stress led to genome-wide mutations that enhanced stress tolerance (Ghoshal et al., 2023). Likewise, desiccation-resistant *Salmonella* strains were found to harbor accessory stress-resistance genes that are induced during desiccation and contribute to enhanced tolerance to dry heat (Wang et al., 2021). In addition to genetic adaptation, DNA methylation is an important epigenetic mechanism regulating bacterial gene expression. In bacteria, DNA methylation has been shown to modulate virulence gene expression and pathogenicity (Heusipp et al., 2007; Sánchez-Romero et al., 2015), while more recent studies have demonstrated its broader role in regulating phenotypic heterogeneity and adaptation to changing environments (Sánchez-Romero et al., 2020). However, the contributions of genetic and epigenetic adaptations to *Salmonella* survival under agricultural soil-associated stresses, such as low water activity, nutrient limitation, and residual antibiotics, remain poorly understood.

To address this knowledge gap, we investigated VBNC-state *Salmonella* under two agriculturally relevant stresses, prolonged desiccation and antibiotic exposure, using a simulated soil system. By integrating whole-genome mutation and DNA methylation analyses, we characterized genetic and epigenetic changes associated with VBNC formation and resuscitation of *Salmonella* Infantis. This approach extends previous studies focused primarily on culturability recovery or metabolic characterization by providing a genome-wide perspective on the VBNC state under environmental stresses relevant to *Salmonella* persistence in agricultural soils and fresh produce. These molecular changes may provide potential signatures for identifying VBNC populations that escape conventional culture-based surveillance and improve our understanding of their persistence under agriculturally relevant stresses, thereby supporting more comprehensive surveillance and risk assessment of *Salmonella* contamination in pre-harvest agricultural environments.

2. Materials and methods

2.1. Bacterial inoculum

Salmonella Infantis CICC 21482, obtained from the China Center of Industrial Culture Collection, was pre-enriched in buffered peptone water (BPW, Hope, Qingdao, China) containing 40% glycerol and was stored at -20 °C. The strains were cultured in 5 mL of tryptic soy broth with yeast extract (TSB-YE, Hope) at 37 °C for 24 h. Then, 1 mL of the bacterial culture was enriched on tryptic soy agar (TSA, Hope) at 37 °C for 24 h. The colonies were collected using 5 mL of BPW to obtain the bacterial stock solution. The bacterial concentration was adjusted to 9.50 ± 0.50 Log CFU/mL for subsequent experiments.

2.2. Soil types and fertility assessment

Artificial soil, phaeozem, and laterite were used to evaluate the effect of soil type on the survival of *S.* Infantis. Artificial soil was prepared according to the method described by Giagnoni et al. (2011) (Text S1). Soil fertility was assessed by determining soil pH, organic matter, microbial biomass carbon, and microbial biomass nitrogen according to NY/T 1121.6-2006, DB 22/T 3078-2019, and the method described by Wu et al. (2006).

2.3. Soil treatments

Artificial soil was divided into 0.1-g aliquots in sterile centrifuge tubes and subjected to three treatment conditions: low-a_w artificial soil, high-a_w artificial soil and antibiotic-amended artificial soil. Low-a_w artificial soil was prepared by adjusting the a_w to 0.30 in a desiccator (Weike, Jiangsu, China) using silica gel self indicator (Zhiyuan, Tianjin, China), whereas high-a_w artificial soil was prepared by adjusting the a_w to 0.90 using saturated potassium sulfate solution (Zhang et al., 2017). Antibiotic-amended artificial soil was prepared by supplementing artificial soil with tetracycline (TC, Macklin, Shanghai, China) and doxycycline (DOC, Macklin) at final concentrations of 59.44 and 7.12 μg/kg, respectively, followed by adjustment of the a_w to 0.30. Phaeozem and laterite soils were also sterilized (Text S1) and adjusted to an a_w of 0.30. All a_w values were verified using a water activity meter (Weike, Jiangsu, China) at 25 °C. The concentration of antibiotics was selected based on the average residual concentrations of TC and DOC reported for farmland soils (Zhang et al., 2021).

For each soil treatment, a 10-μL aliquot of the bacterial inoculum was added to the centrifuge tube. The inoculated soils were adjusted to a_w 0.30 or 0.90, and stored in the corresponding desiccators. Culturable and viable cell counts, along with a_w, were measured on days 0, 1, 3, 6, 10, 24, 40, 60, 80, 100, 130, 170, and 226. At each time point, all experiments were performed in triplicates using three independent soil samples.

2.4. Measurement of culturable and viable cell counts

2.4.1. Culturable cell counts

For each of the inoculated soil samples, 9.9 mL of BPW was added, followed by thorough shaking. 1 mL of soil bacterial suspension was pipetted into a new centrifuge tube containing 9 mL of BPW, vortex mixed for 1 min, and then subjected to tenfold series dilution. Then, 100 μL of the bacterial suspension of each dilution gradient was plated on TSA. The plates were incubated at 37 °C for 24 h, and the number of colonies was counted.

2.4.2. Viable cell counts

Each of the inoculated soil samples was washed twice with 0.5 mL of 0.85% sodium chloride (NaCl) solution, and resuspended in 0.5 mL of fresh 0.85% NaCl solution. 5 μL of 2 mmol/L PMAxx™ dye (Biotium, San Francisco, USA) (García-Fontana et al., 2016; Nocker et al., 2006) was added to 0.5 mL of soil bacterial suspension. It was incubated at room temperature in dark for 10 min after vortex mixing. Then, the soil bacterial suspension was exposed to light for 15 min using PMA-Lite™ LED Photolysis Device (Biotium), washed twice, and pelleted by centrifugation at 7500 ×g for 10 min (Zhao et al., 2020). The genomic DNA was extracted using the Ezup Column Soil DNA Purification Kit (Sangon, Shanghai, China). Quantitative polymerase chain reaction (qPCR) was performed on the genomic DNA using primers and probe of *invA* (Sangon), and AceQ qPCR Probe Master Mix (Vazyme, Jiangsu, China) in the CFX96 Real-Time PCR Detection System (Bio-Rad, Delaware, USA) (Text S2). The PMAxx-qPCR standard curve was established by using serial-diluted DNA solution of PMAxx-treated samples on day 0 as the reaction template of qPCR. The standard curve demonstrated a linear relationship between threshold cycle (Ct) values and Log_{10} CFU/Sample. The standard curves of PMAxx-qPCR were shown in Fig. S1.

VBNC-state cells were obtained when the ratio of culturable cell counts to viable cell counts was less than 0.01. When the culturable count was below the detection limit (< 1.39 Log CFU/Sample), it could be considered as completely entering the VBNC state. In this study, the VBNC state was operationally defined based on the loss of culturability relative to PMAxx-qPCR-based viability assessment, as previously applied for the detection of viable and VBNC *Salmonella* (Li et al., 2022; Li & Bae, 2024; Severo et al., 2025), together with subsequent resuscitation ability.

2.5. Resistance assay

The resistance assays were preformed on soil samples containing VBNC-state cells after 226 days of treatment, with soil samples containing untreated stationary-phase cells serving as negative controls. The samples collected on day 226 were used for all subsequent assays.

The soil samples were subject to different stress conditions: (1) heat treatment in a 55 °C water bath for 3 h (Cui et al., 2022); (2) ultraviolet (UV) treatment under 254 nm, 20 W UV radiation for 1 h (Zhang et al., 2018); (3) acid treatment in 50 μL of 12% hydrochloric acid (HCl) solution for 1 h (Ji et al., 2009); (4) alkali treatment in 50 μL of 8% sodium hydroxide (NaOH) solution for 1 h (Ji et al., 2009); (5) soil disinfectant treatment in 0.0105 g of calcium cyanamide (Yuzhongao, Henan, China) for 1 h (Shi et al., 2021).

Following each stress treatment, genomic DNA was extracted and cell populations were quantified using PMAxx-qPCR. The population decrease rate was calculated using Eq. 1:

$$\text{Decrease rate}\ (\%) = \frac{N_i - N_f}{N_i} \times 100 \quad (1)$$

where N_i and N_f represent the cell populations before and after stress treatment, respectively. A lower decrease rate indicated greater stress resistance.

2.6. Resuscitation assay

The resuscitation assay was performed using different resuscitation reagents, including 1000 U/mL catalase (CAT) (Aladdin, Shanghai, China), 100 mmol/L sodium pyruvate (Aladdin), logarithmic cell-free supernatants (L-CFS), stationary CFS (S-CFS), TSB-YE, logarithmic non-nutrition cell-free supernatants (L-NCFS), stationary NCFS (S-NCFS) and 0.85% NaCl solution. 0.5 mL of each resuscitation reagent was added to each soil sample, followed by incubation at 37 °C for 6 h and 24 h. The mixture was serially diluted in BPW and plated on TSA to measure the culturable cell counts.

In this study, CFS of logarithmic- and stationary-phase *S.* Infantis were used as resuscitation reagents because they contained quorum sensing autoinducer-2 (AI-2) associated with bacterial resuscitation (Ayrapetyan et al., 2014). Here, CFS and NCFS referred to supernatants prepared using TSB-YE and 0.85% NaCl solution, respectively. Detailed preparation steps were provided in Text S3.

To distinguish true resuscitation of VBNC-state cells from regrowth of residual culturable cells and to compare the effects of different resuscitation reagents under various soil conditions, theoretical generation time (TGT), a previously established approach for validating VBNC resuscitation (Ayrapetyan et al., 2014), was calculated using Eq. 2, where C_i and C_f represent the CFU values of samples without resuscitation and with 1-h resuscitation, respectively. The TGTs of stationary- and logarithmic-phase cells were used as culturable-cell references. TGTs shorter than those reference values were considered indicative of VBNC resuscitation rather than cell proliferation alone.

$$\text{Theoretical generation time}\ (\text{min}) = \frac{60}{\left[\frac{\log(C_f) - \log(C_i)}{\log 2}\right]} \quad (2)$$

2.7. Reactive oxygen species (ROS) detection assay

Soil bacterial suspensions were prepared using 0.85% NaCl solution. To ensure comparability, the viable cell counts of stationary-phase and VBNC-state cells in soil bacterial suspensions were adjusted to 6.19 ± 0.11 and 6.53 ± 0.28 Log CFU/Sample respectively. In addition, the viable cell count of samples after 24-h resuscitation was adjusted to 6.34 ± 0.31 Log CFU/Sample. These bacterial suspensions were used for both the ROS detection assay and subsequent bioluminescence-based quorum sensing assay.

The intracellular ROS levels were measured using Reactive Oxygen Species Assay Kit (Biosharp, Hefei, China). The stationary-phase, VBNC-state and resuscitated cell samples were washed with 1 mL of 0.85% NaCl solution and resuspended in 1 mL of 10 μmol/L H_2DCFDA. After incubation under darkness at 37 °C for 15 min, the pellets were washed and resuspended in 1 mL of 0.85% NaCl solution. Then, 100 μL of suspension was added to each hole of 96-well sterile plate. The fluorescence intensity (FI) of the suspension was detected immediately with a TriStar2S multifunctional enzyme marker (Berthold, Bad Wildbad, Germany) at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. Each sample was performed using eight technical replicates.

2.8. Bioluminescence-based quorum sensing assay

AI-2 was measured using the *Vibrio harveyi* BB170 bioluminescence assay as previously described (Bassler et al., 1997) with slight modifications. Briefly, 200 μL of working solution (Text S4) was mixed with 20 μL of CFS of stationary-phase, VBNC-state and resuscitated *S.* Infantis from soil samples (Text S3), respectively. The mixture was transferred to a 96-well sterile plate and the bioluminescence value was read using a TriStar2S multifunctional enzyme marker (Berthold) at a wavelength of 490 nm. Background luminescence was assayed by quantifying control samples that contained 20 μL of TSB-YE and 200 μL of working solution. Data were presented as luminescence levels (arbitrary units) with the average background luminescence subtracted. Each sample was performed using eight technical replicates.

2.9. Genomic analysis

In this study, DOC caused the most significant changes in the culturability and stress resistance of *S.* Infantis, while CAT demonstrated the highest efficacy in resuscitating the VBNC-state cells. Therefore, VBNC-state cells from DOC-amended soil and the subsequent CAT-resuscitated cells were selected for the subsequent genomic and

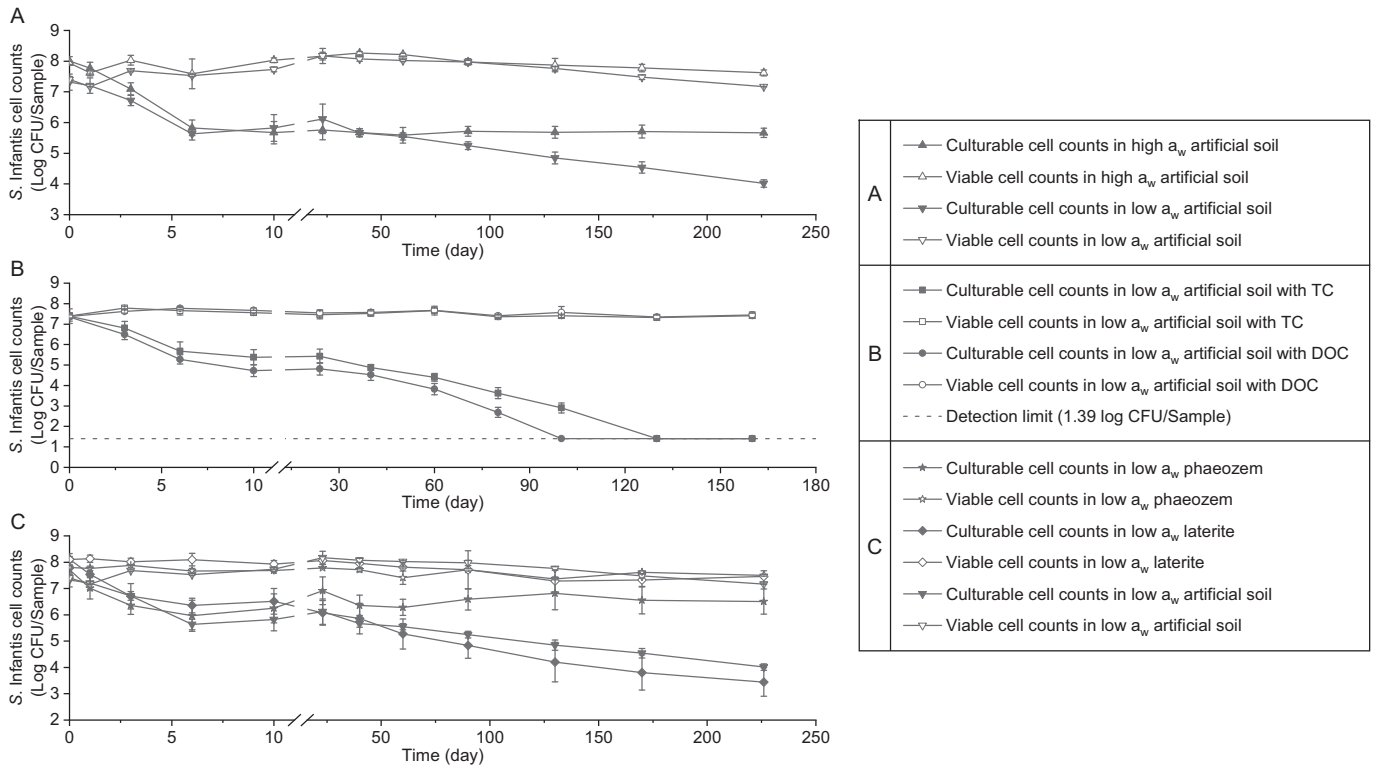


Fig. 1. Survival curves of *S.* Infantis in different treatments during the long-term storage. (A) High water activity (a_w) and low a_w artificial soils. (B) Low a_w artificial soils with antibiotics. (C) Different types of soils with low a_w. The culturable and viable cell counts were quantified by plate counting and PMAxx-qPCR, respectively. The data presented the means and standard deviations of three biological replicates. The x-axis was displayed with a break between 10 and 24 days, allowing clearer visualization of early sampling points (0-10 days) while retaining the long-term sampling period (24–226 days). TC: tetracycline; DOC: doxycycline.

methylomic analyses, with stationary-phase cells as the control. A single sample from each condition was subjected to genomic and methylomic sequencing. Genomic DNA was extracted using the Ezup Column Soil DNA Purification Kit (Sangon).

The genome sequencing of *S.* Infantis was carried out using the Illumina sequencing platform (Illumina, California, USA), with detailed methods shown in Text S5. The sequencing reads were trimmed using Trimmomatic v0.39 (Bolger et al., 2014) to remove barcode sequences and low-quality bases, then subject to taxonomic classification using Kraken2 v2.1.3 (Wood et al., 2019) to confirm the absence of contamination. Genomes were assembled with SPAdes v3.15.5 (Bankevich et al., 2012) and annotated with Prokka v1.14.6 (Seemann, 2014). Single nucleotide polymorphism (SNP) calling was performed using CFSAN SNP Pipeline v2.2.1 (Davis et al., 2015), with a minumum variant allele frequency of 0.6. The assembled genome of stationary-phase cells was used as the reference genome for the identification of SNPs in VBNC-state and resuscitated cells. The genome annotation diagram of *S.* Infantis was shown in Fig. S2.

2.10. Methylomic analysis

The methylome sequence analysis was performed using the Oxford Nanopore Technologies sequencing platform (ONT, Oxford, UK), with detailed methods shown in Text S5. The raw electrical signals obtained after sequencing were basecalled using Guppy (Wick et al., 2019) to obtain the original sequence files, which were evaluated for quality and filtered to obtain clean reads. The sequencing data was re-squiggled using Tombo (Marcus et al., 2017), and then the methylation sites of 5mC, CpG, 6mA, dcm, and dam were measured using Alternative Model. The methylation level was calculated as the number of methylation sites/number of methylation and non-methylation sites.

The circos diagrams, distribution map, and level of various methylation modification sites in the whole genome of *S.* Infantis were shown in Fig. S3, Fig. S4, and Table S1, respectively. The methylation level characteristics of each transcript region were shown in Fig. S5. Differential DNA methylation during the VBNC transition (VBNC-state cells *vs.* stationary-phase cells) and the resuscitation transition (resuscitated cells *vs.* VBNC-state cells) was then analyzed.

Differential methylation loci (DMLs) were analyzed using Methylkit (Akalin et al., 2012), followed by identification of differential methylation using Fisher's exact test. The difference level was set to 0.1, and the *q* value was set to 0.05. The *P*-value was corrected using the sliding linear model (SLIM) method (Wang et al., 2011). Differential methylation regions (DMRs) were identified using Metilene (Jühling et al., 2016) and corrected through multiple tests. The threshold settings were as follows: (1) Methylation difference (C or A sites) $\geq$ 0.1; (2) Number of DMLs (C or A sites in the region) $\geq$ 5; (3) Distance between adjacent DMLs (C or A sites) $\leq$ 50 bp; (4) The Mann-Whitney U (MWU) test's *P*-value $<$ 0.05. Finally, the differential modified genes associated with DMR were analyzed for Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment using Allenricher (Zhang et al., 2020).

2.11. Statistical analysis

Experiments were performed in three biological replicates, unless indicated otherwise. Data were presented as the mean $\pm$ standard deviation (SD). The figures were plotted using OriginPro 2018 and ChiPlot platform (https://www.chiplot.online). Data were analyzed by one-way ANOVA followed by Fischer's LSD post-hoc test in SPSS Statistics 21.0. Significance was determined at $P < 0.05$.

Table 1
The soil fertility index of three soil types.

Indicator Name	Artificial soil	Phaeozem	Laterite
Soil pH	9.833 ± 0.150	8.503 ± 0.015	6.543 ± 0.012
Organic matter (g/kg)	0.645 ± 0.017	1.401 ± 0.023	0.360 ± 0.017
Biomass carbon (mg/kg)	378.947 ± 21.071	1365.789 ± 18.330	94.737 ± 9.165
Biomass nitrogen (mg/kg)	77.066 ± 15.413	107.342 ± 21.468	19.267 ± 3.854

The water activity of the soils was adjusted to 0.30 ± 0.05. The data presented the means and standard deviations of three biological replicates.

3. Results and discussion

3.1. Low water activity, antibiotics, and low soil fertility promoted the formation of VBNC-state Salmonella

3.1.1. Effect of water activity on the survival of S. Infantis in soil

In either low or high a_w artificial soil (Fig. 1A), culturable *S.* Infantis populations decreased significantly ($P < 0.05$) within 6 days after inoculation, with more than 98% of cells entering the VBNC state, indicating rapid loss of culturability during early storage. The culturable population then remained around 6 Log CFU/Sample from day 6 to day 60, suggesting adaptation to the artificial soil environment. After 60 days, the two treatments diverged. In low a_w soil, culturable populations declined further, with 99.93% of cells entering the VBNC state by day 226, whereas no significant change was observed in high a_w soil ($P > 0.05$). In contrast, viable cell counts quantified by PMAxx-qPCR remained stable throughout storage under both conditions, indicating sustained cell viability during soil storage. Overall, low a_w conditions promoted VBNC state formation. Consistent with the present findings, Se et al. (2021) reported that low a_w soil impaired the culturability of *Escherichia coli* and promoted VBNC state formation.

3.1.2. Effect of antibiotics on the survival of S. Infantis in soil

Upon antibiotic exposure (Fig. 1B), culturable populations decreased rapidly during the first 6 days and remained relatively constant until day 24. The culturable populations then gradually declined, falling below the detection limit (<1.39 Log CFU/Sample) on days 130 and 100 in TC- and DOC-amended low-a_w soils, respectively, indicating complete loss of culturability. In contrast, viable cell counts quantified by PMAxx-qPCR remained stable throughout soil storage in both treatments. Compared with TC, DOC exhibited a stronger VBNC-inducing effect in *S.* Infantis.

Antibiotic stress can induce different dormant or stress-tolerant states, including VBNC and persister cells, across various bacterial species (Gou et al., 2024; Kim et al., 2024; Li et al., 2023; Mangiaterra et al., 2023). These states share characteristics such as reduced metabolic activity and enhanced stress tolerance. Specifically, DOC has been reported to induce the VBNC state in *Burkholderia cenocepacia* (Sarekoski, 2018), whereas TC can induce persister cells in *Vibrio splendidus* (Li

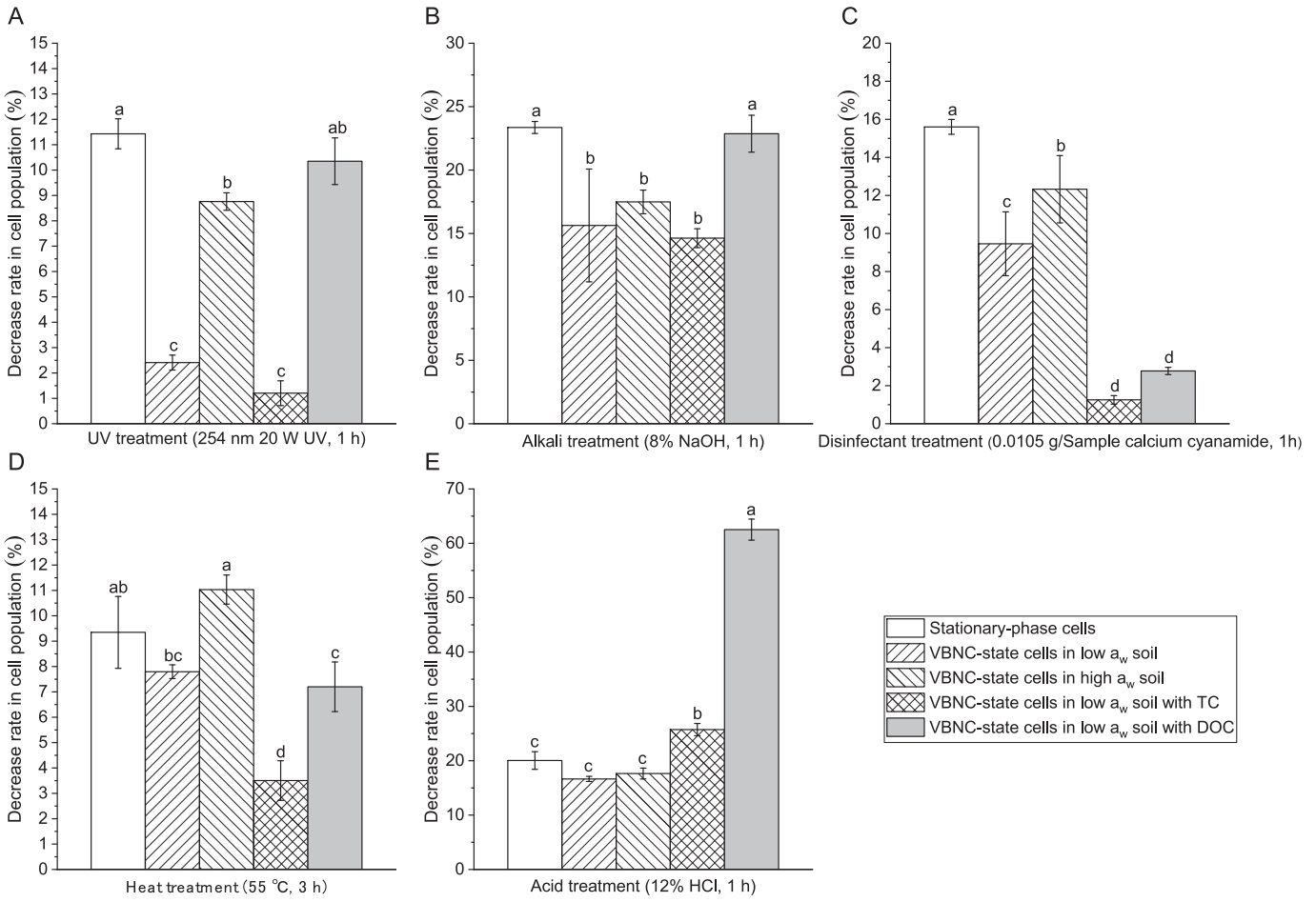


Fig. 2. Reduction in population of stationary-phase and viable but non-culturable (VBNC)-state *S.* Infantis under different environmental stresses. (A) Ultraviolet (UV) treatment. (B) Alkali treatment. (C) Disinfectant treatment. (D) Heat treatment. (E) Acid treatment. Cell populations were determined using PMAxx-qPCR, with stationary-phase cells serving as the control. The decrease rate represents the reduction in cell populations following stress treatment. Different letters indicate significant differences at $P < 0.05$. The data presented the means and standard deviations of three biological replicates. a_w: water activity; TC: tetracycline; DOC: doxycycline.

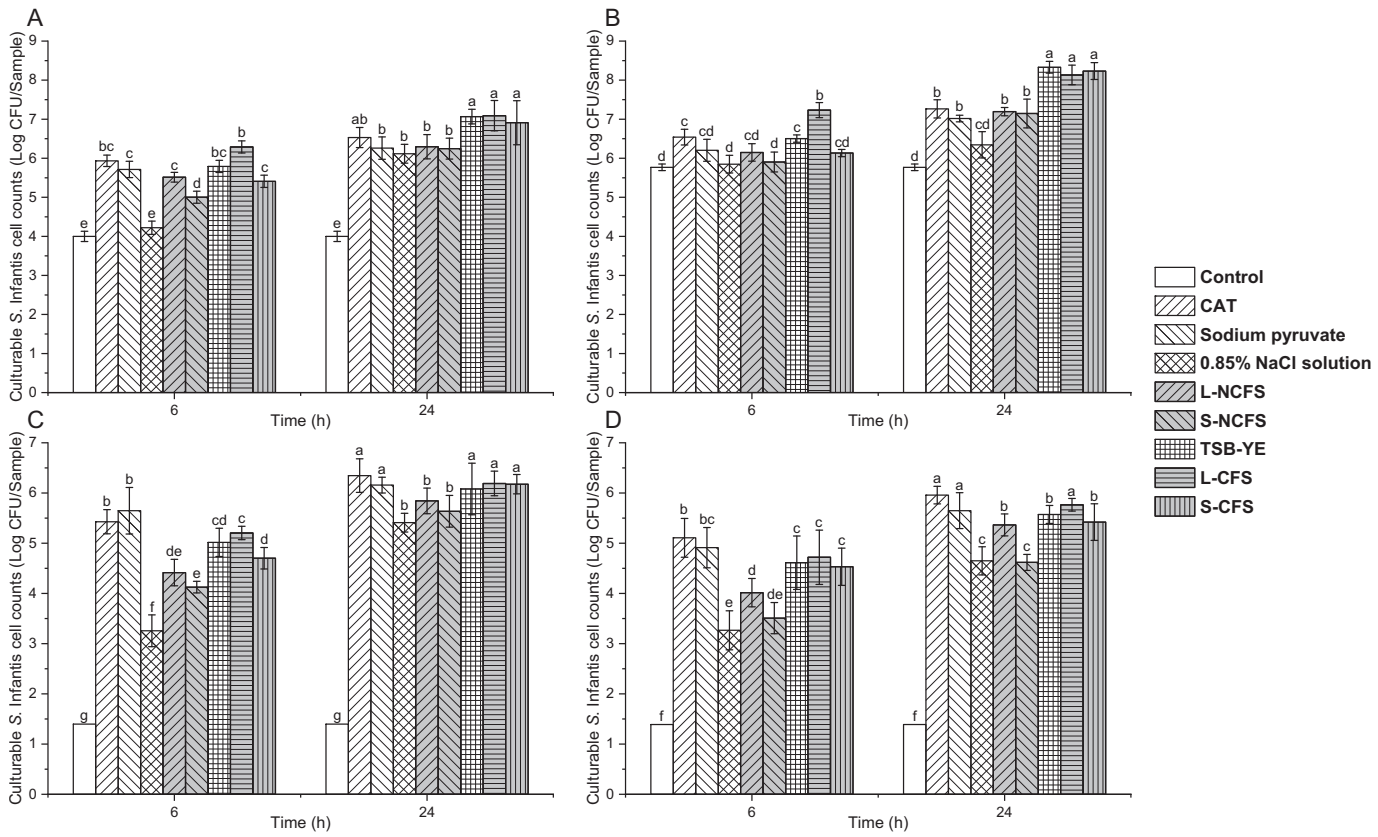


Fig. 3. Resuscitation of viable but non-culturable (VBNC)-state *S.* Infantis under different treatments in artificial soil. (A) Low water activity (a_w) soil. (B) High a_w soil. (C) Low a_w soil with tetracycline (D) Low a_w soil with doxycycline. The recovery of culturability induced by different reagents was assessed using culturable cell counts at 6 h and 24 h. The number of culturable cells was obtained by plate counting method, and VBNC-state cells without resuscitation treatment were used as negative control. Different letters indicate significant differences at $P < 0.05$. The data presented the means and standard deviations of three biological replicates. CAT: catalase; NaCl: sodium chloride; L-NCFS: logarithmic non-nutrition cell-free supernatants; S-NCFS: stationary non-nutrition cell-free supernatants; TSB-YE: tryptic soy broth with yeast extract; L-CFS: logarithmic cell-free supernatants; S-CFS: stationary cell-free supernatants.

et al., 2021) and the VBNC state in *Staphylococcus epidermidis* (Carvalhais et al., 2018). Despite their similarities, persister cells generally resume growth rapidly after antibiotic removal, whereas VBNC-state cells exhibit deeper dormancy and often require specific conditions for resuscitation (Ayrapetyan et al., 2018). The two populations may also coexist along a continuum of bacterial dormancy (Ayrapetyan, Williams, Baxter and Oliver, 2015). In this study, the prolonged loss of culturability together with the populations detected by PMAxx-qPCR was consistent with the development of the VBNC state under combined desiccation and antibiotic stress.

3.1.3. *Effect of soil types on the survival of S. Infantis*

Culturable cell counts in laterite and artificial soil showed a similar decreasing trend, with 99.99% and 99.93% of cells entering VBNC state by day 226, respectively. Nevertheless, phaeozem showed the smallest reduction in culturability, with only 89.88% of cells entering VBNC state by day 226 (Fig. 1C). The higher persistence of culturability in phaeozem was consistent with its higher soil fertility (Table 1), suggesting that VBNC state formation was associated with soil fertility. Previous studies have shown that *Salmonella* survives better in fertile soils. Organic-rich loam and compost-amended soils enhance *Salmonella* survival compared with sandy or unamended soils (Alegbeleye & Sant'Ana, 2023; Shah et al., 2019).

No significant differences in viable cell counts were observed among the three soil types (Fig. 1C). However, the greater variability observed in phaeozem and laterite likely reflected their more complex compositions. Therefore, artificial soil was selected for subsequent experiments because of its more standardized composition.

3.2. *VBNC-state S. Infantis in antibiotics-amended soils exhibited enhanced stress resistance*

In this study, over 99% of cells entered the VBNC state on day 226 under the four abovementioned soil conditions. At this time point, the average cell population determined by PMAxx-qPCR across the four soil treatments was 7.53 ± 0.28 Log CFU/Sample, comparable to 7.19 ± 0.11 Log CFU/Sample for stationary-phase cells. As PMAxx-qPCR primarily reflects membrane integrity and may not fully represent physiological viability after stress exposure, stress resistance was assessed by comparing reductions in viable counts across stationary-phase and VBNC-state cells following identical stress treatments (Fig. 2).

Under treatments of UV and alkali (Fig. 2A and B), VBNC-state cells, except those derived from DOC-amended soil, exhibited significantly stronger resistance than stationary-phase cells ($P < 0.05$). VBNC-state cells induced by combined stress of low a_w and TC showed the highest resistance, with only 1.20% and 14.63% reductions in cell populations following UV and alkaline treatments, respectively. Under treatment of soil disinfectant (Fig. 2C), VBNC-state cells from antibiotics-amended soils also exhibited greater resistance than stationary-phase cells.

Under heat treatment (Fig. 2D), VBNC-state cells in TC- and DOC-amended soils exhibited significantly enhanced thermotolerance ($P < 0.05$). VBNC-state cells induced under low a_w soil also showed slightly higher thermotolerance than those from high a_w soil ($P < 0.05$). Previous studies have similarly reported enhanced heat resistance of *Salmonella* under low a_w conditions (Xu et al., 2019), possibly because reduced water activity stabilizes cellular proteins during heat stress (Li & Gänzle, 2016). In addition, the addition of antibiotics might induce cross

Table 2
Theoretical generation times (TGT) required for the observed increase in culturability during the resuscitation of *S.* Infantis.

Treatment	TGT (min)			
	Low a_w[a] soil	High a_w soil	TC[b]-amended soil	DOC[c]-amended soil
CAT[d]	10.83 ± 0.95	28.83 ± 4.62	14.12 ± 2.64	18.83 ± 3.63
Sodium pyruvate	12.48 ± 1.79	41.08 ± 5.72	12.06 ± 3.67	23.67 ± 6.17
0.85% NaCl[e] solution	257.74 ± 39.23	287.65 ± 42.13	232.58 ± 45.96	235.41 ± 48.54
L-NCFS[f]	14.47 ± 1.44	77.22 ± 8.19	67.85 ± 13.45	70.57 ± 16.98
S-NCFS[g]	24.53 ± 5.10	153.31 ± 18.47	91.84 ± 20.65	106.49 ± 19.36
TSB-YE[h]	11.85 ± 1.02	30.68 ± 4.81	20.82 ± 1.93	39.02 ± 2.73
L-CFS[i]	8.93 ± 0.69	13.70 ± 1.99	17.12 ± 2.16	31.63 ± 7.66
S-CFS[j]	15.80 ± 1.94	82.66 ± 8.58	32.68 ± 6.69	47.30 ± 8.72

The data presented the means and standard deviations of three biological replicates. *S.* Infantis during logarithmic and stationary growth in TSB-YE were used as the control, with TGT of 57.89 ± 4.31 min and 216.74 ± 37.08 min, respectively.
[a] a_w: water activity.
[b] TC: tetracycline.
[c] DOC: doxycycline.
[d] CAT: catalase.
[e] NaCl: sodium chloride.
[f] L-NCFS: logarithmic non-nutrition cell-free supernatants.
[g] S-NCFS: stationary non-nutrition cell-free supernatants.
[h] TSB-YE: tryptic soy broth with yeast extract.
[i] L-CFS: logarithmic cell-free supernatants.
[j] S-CFS: stationary cell-free supernatants.

protection in bacteria, thereby further enhancing thermotolerance (Liao et al., 2020).

Under treatment of acid (Fig. 2E), there was no significant change in the resistance of VBNC-state cells induced under high and low a_w conditions ($P > 0.05$). In contrast, VBNC-state cells from TC- and DOC-amended soils exhibited significantly reduced acid resistance ($P < 0.05$). Previous studies have shown that acidification enhances the antibacterial activity of tetracyclines, with DOC exhibiting substantially greater activity than TC under acidic conditions (Granados-Chinchilla & Rodríguez, 2017), possibly by increasing bacterial membrane permeability (Delcour, 2009; Nelson & Levy, 2011).

3.3. Free radical scavengers exhibited stronger resuscitation effect on VBNC-state S. Infantis in antibiotics-amended soils

Fig. 3 illustrated the resuscitation of VBNC-state cells induced by divergent soil conditions using free radical scavengers (CAT and sodium pyruvate), AI-2-containing supernatants (L-NCFS, S-NCFS, L-CFS and S-CFS), and the solvent controls (TSB-YE and 0.85% NaCl solution). Resuscitation was evaluated based on the recovery of culturability, with TGT analysis providing additional supporting evidence. Except for 0.85% NaCl, all resuscitation treatments resulted in significantly shorter TGTs than stationary-phase cells and, in most cases, logarithmic-phase cells (Table 2), further supporting the resuscitation of VBNC-state cells (Bari et al., 2013; Whitesides & Oliver, 1997). Overall, VBNC-state cells induced under low a_w conditions showed the greatest resuscitation capacity, followed by TC-amended soil, DOC-amended soil, and high a_w soil.

Under low and high a_w soil conditions (Fig. 3A and B), the resuscitation effect of different reagents differed markedly after 6-h treatment, with L-CFS outperforming most other reagents ($P < 0.05$). After 24 h, L-CFS, S-CFS, and TSB-YE yielded the highest populations of resuscitated *S.* Infantis, likely because their nutrient-rich compositions supported post-resuscitation growth. In addition, L-NCFS and S-NCFS generally showed greater recovery of culturability than 0.85% NaCl, suggesting that quorum-sensing autoinducers promoted VBNC resuscitation (Jiang et al., 2010; Ma et al., 2024). This is consistent with previous studies showing that AI-2-containing CFS facilitates VBNC resuscitation in *Salmonella*, potentially through activation of the LuxPQ-mediated signal transduction cascade and enhanced antioxidant capacity (Ayrapetyan et al., 2014; Bari et al., 2013; Lenz et al., 2004; Liao et al., 2019; Reissbrodt et al., 2002).

For VBNC-state cells induced in antibiotic-amended soils (Fig. 3C and D), recovery of culturability varied among the reagents. After 6 hours of treatment, CAT and sodium pyruvate recovered significantly more *S.* Infantis cells than most other treatments ($P < 0.05$). The only exception was observed in DOC-amended soil, where sodium pyruvate showed comparable efficacy to L-CFS, S-CFS, and TSB-YE ($P > 0.05$). After 24 h, resuscitated populations became similar among most treatments, except for 0.85% NaCl, L-NCFS and S-NCFS. These findings suggest that antibiotic exposure may increase intracellular oxidative stress, allowing CAT and sodium pyruvate to promote early resuscitation by scavenging reactive oxygen species (ROS).

Nevertheless, while the recovery of culturability and TGT analysis support the resuscitation of VBNC-state cells, the contribution of residual culturable cell regrowth cannot be completely excluded. Further validation using additional culture-based approaches, single-cell analysis, or fluorescence-based lineage tracking is therefore warranted.

3.4. Combined stress of low a_w and antibiotics led to increased ROS accumulation in VBNC-state and resuscitated cells

The intracellular ROS levels in stationary-phase, VBNC-state and resuscitated *S.* Infantis under various soil conditions are shown in Fig. 4. In all treatments, intracellular ROS increased significantly upon entry into the VBNC state and decreased after resuscitation ($P < 0.05$), but remained much higher than in stationary-phase cells ($P < 0.05$).

ROS levels were markedly higher in VBNC-state cells from TC- and DOC-amended soils (Fig. 4C and D) than in those from soils without antibiotics (Fig. 4A and B). Together with the greater recovery of culturability of free radical scavengers CAT and sodium pyruvate in antibiotic-induced VBNC-state cells (Fig. 3C and D), these findings suggest that antibiotic exposure promoted intracellular ROS accumulation, making ROS scavenging particularly important for VBNC resuscitation (Cuny et al., 2005; Pienaar et al., 2016; Se et al., 2021).

3.5. Combined stress of low a_w and antibiotics led to inhibition of AI-2 synthesis in VBNC-state and resuscitated cells

The intercellular AI-2 levels in stationary-phase, VBNC-state and resuscitated *S.* Infantis under different soil conditions was shown in Fig. 5. Overall, the stationary-phase cells exhibited the highest AI-2 production ($P < 0.05$). AI-2 levels decreased significantly upon entry into the VBNC state and increased after resuscitation ($P < 0.05$). As a key quorum sensing signal molecule, AI-2 has been widely reported to be involved in VBNC state formation and resuscitation (Ayrapetyan et al., 2014; Bari et al., 2013; Liu et al., 2020).

Compared with low and high a_w soils (Fig. 5A and B), VBNC-state cells from TC- and DOC-amended soils exhibited markedly lower AI-2 levels (Fig. 5C and D), suggesting that combined low a_w and antibiotic stress more strongly suppressed AI-2 production. Similar to the ROS results, AI-2 levels increased significantly after resuscitation ($P < 0.05$), but recovered to only about half of those in stationary-phase cells and remained significantly lower than those in soils without antibiotics ($P < 0.05$). This incomplete recovery may reflect persistent antibiotic-induced cellular damage (Harms et al., 2016; Wesche et al., 2009) or incomplete restoration of cellular functions, such as oxidative stress

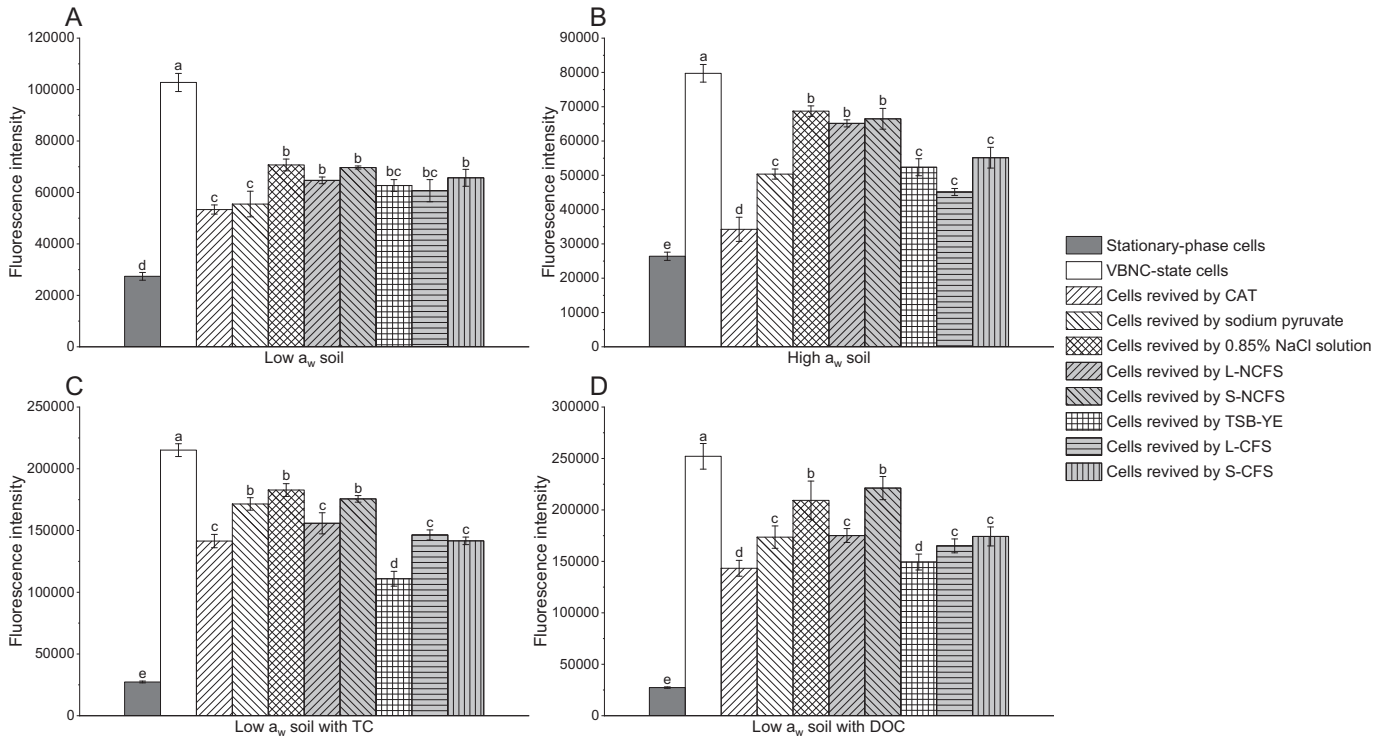


Fig. 4. Reactive oxygen species of *S.* Infantis under different artificial soil conditions. (A) Low water activity (a_w) soil; (B) High a_w soil; (C) Low a_w soil with tetracycline (TC); (D) Low a_w soil with doxycycline (DOC). The fluorescence intensity of *S.* Infantis was measured using a multifunctional enzyme marker. Different letters indicate significant differences at $P < 0.05$. The data presented the means and standard deviations of three biological replicates. VBNC: viable but non-culturable; CAT: catalase; NaCl: sodium chloride; L-NCFS: logarithmic non-nutrition cell-free supernatants; S-NCFS: stationary non-nutrition cell-free supernatants; TSB-YE: tryptic soy broth with yeast extract; L-CFS: logarithmic cell-free supernatants; S-CFS: stationary cell-free supernatants.

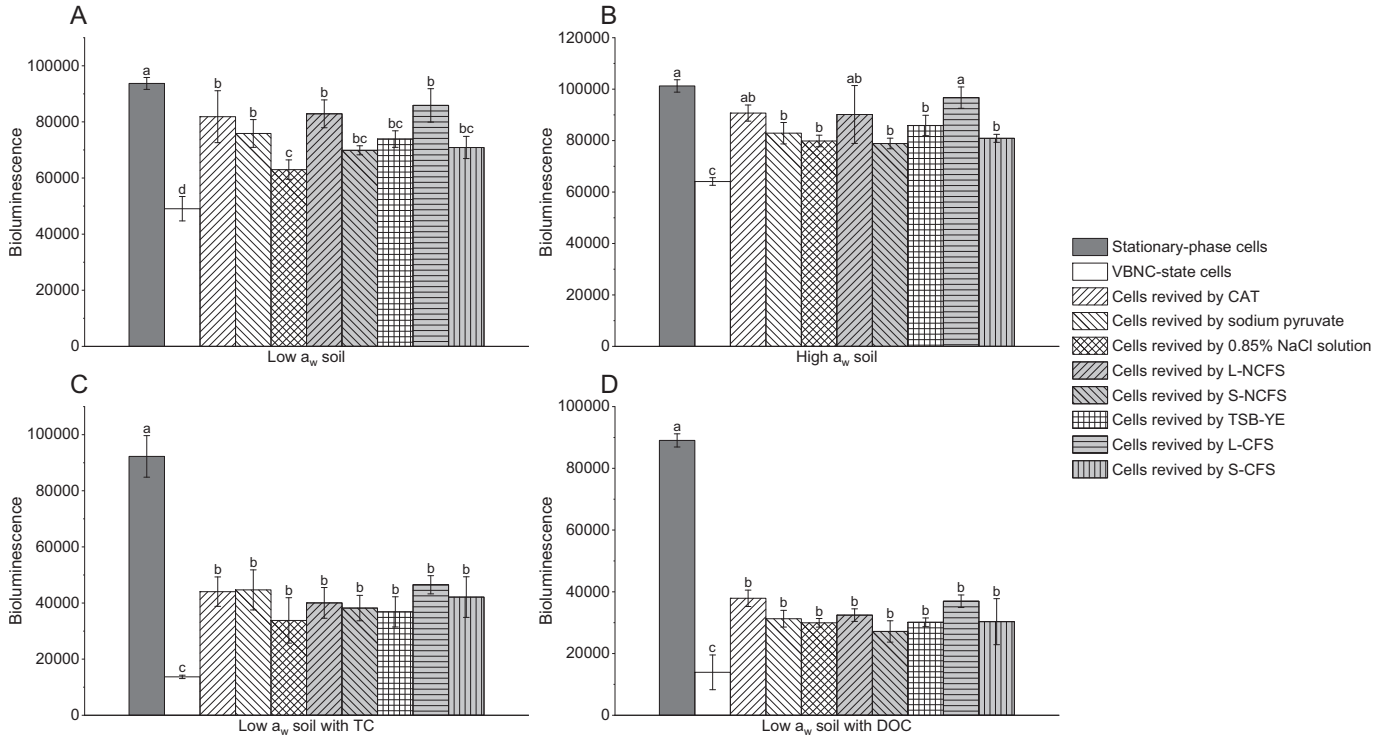


Fig. 5. Quorum sensing autoinducer-2 signal of *S.* Infantis under different artificial soil conditions. (A) Low water activity (a_w) soil; (B) High a_w soil; (C) Low a_w soil with tetracycline (TC); (D) Low a_w soil with doxycycline (DOC). The bioluminescence value of *S.* Infantis was measured using a multifunctional enzyme marker. Different letters indicate significant differences at $P < 0.05$. The data presented the means and standard deviations of three biological replicates. VBNC: viable but non-culturable; CAT: catalase; NaCl: sodium chloride; L-NCFS: logarithmic non-nutrition cell-free supernatants; S-NCFS: stationary non-nutrition cell-free supernatants; TSB-YE: tryptic soy broth with yeast extract; L-CFS: logarithmic cell-free supernatants; S-CFS: stationary cell-free supernatants.

Table 3
Single nucleotide polymorphisms (SNPs) of VBNC-state and resuscitated *S.* Infantis following treatment with doxycycline (DOC)-amended soil.

Gene	Function	Number of SNPs[a]		
		VBNC-state cells	Resuscitated cells	Shared SNPs[b]
oadA	Oxaloacetate decarboxylase α chain	1	0	0
oadB	Oxaloacetate decarboxylase β chain	6	7	6
yfdG	Prophage bactoprenol-linked glucose translocase	4	2	2
tnpA	IS200/IS605 family transposase IS200F	2	2	2
rRNA	5S ribosomal RNA, 16S ribosomal RNA	3	3	3
Others	Hypothetical protein[c], NCS[d]	56	63	55

[a] The assembled genome of stationary-phase cells was used as the reference genome for SNP calling in the VBNC-state and resuscitated cells.
[b] Shared SNPs referred to SNPs identified in both of the VBNC-state and resuscitated cells.
[c] Hypothetical protein referred to the protein with unknown function encoded by coding sequence.
[d] NCS referred to the non-coding sequence.

response, protein synthesis, and energy metabolism, during resuscitation (Giagnoni et al., 2018).

3.6. Genomic analysis revealed genetic changes associated with VBNC formation and resuscitation under combined low a_w and DOC stress

To investigate genetic changes associated with VBNC state formation and resuscitation under combined low a_w and antibiotic stress, genomic and methylomic analyses were performed on stationary-phase, VBNC-state, and resuscitated *S.* Infantis cells from DOC-amended soil. DOC treatment was selected because it induced the greatest changes in culturability and stress resistance. As the omics analyses were conducted under a single representative stress condition, whether the observed molecular changes extend to other stress conditions requires further investigation.

Compared with stationary-phase cells, 72 SNPs were identified in VBNC-state cells, including 25 located in coding sequences (CDSs). Resuscitated cells contained 77 SNPs, 23 of which were located in CDSs (Table 3 and Table S2). Most SNPs were shared between VBNC-state and resuscitated cells, suggesting that these mutations arose under combined low a_w and DOC stress and persisted after resuscitation.

Although most stress-associated SNPs occurred in non-coding sequences (NCSs) or hypothetical protein genes, mutations were also identified in the coding sequences of *oadA*, *oadB*, *yfdG*, *tnpA*, and rRNA genes. While their functional relevance remains to be established, the known functions of several affected genes provide biological context for these mutations. Among them, *oadA* and *oadB* encode oxaloacetate decarboxylase, which is involved in pyruvate metabolism (Li, Zhu, et al., 2014) and has been associated with adaptation to hypoxia and acid stress in *Vibrio cholerae* (Dahinden et al., 2005; Repizo et al., 2013). In addition, *yfdG* encodes a membrane-associated glucose translocase involved in O-antigen modification, which influences bacterial cell-surface properties (Silva et al., 2016; Slauch et al., 1996) and resistance to gastrointestinal stress and bacteriophages (Wahl et al., 2019).

3.7. Methylomic analysis revealed widespread DNA methylation changes during VBNC formation and resuscitation

3.7.1. Analysis of differential methylation regions (DMRs)

To investigate epigenetic changes associated with *Salmonella*'s adaptation to combined low a_w and DOC stress, differentially methylated regions (DMRs) were identified for the VBNC transition and resuscitation transition. DMR analysis showed that the VBNC transition contained substantially fewer DMRs than the resuscitation transition. In both transitions, the number of hypomethylated DMRs (DMR-Hypo), representing regions with decreased methylation, was substantially higher than that of hypermethylated DMRs (DMR-Hyper), representing regions with increased methylation (Table 4). Overall, DMR-associated genes exhibited widespread demethylation across 4mC, 5mC, and 6mA modification sites.

Table 4
Number of differential methylation regions (DMRs) identified in the VBNC transition and resuscitation transition.

Modification site	Process	DMRs	DMR-Hyper[a]	DMR-Hypo[b]
5mC	VBNC transition	702	60	642
	Resuscitation transition	2428	441	1987
CpG	VBNC transition	782	85	697
	Resuscitation transition	2424	538	1886
6mA	VBNC transition	898	37	861
	Resuscitation transition	1986	253	1733
dcm	VBNC transition	32	0	32
	Resuscitation transition	392	0	392
dam	VBNC transition	690	0	690
	Resuscitation transition	1090	1	1089

[a] DMR-Hyper: hypermethylated DMRs.
[b] DMR-Hypo: hypomethylated DMRs.

Although DNA demethylation is common in bacteria, its physiological significance remains poorly understood. Previous studies have shown that environmental conditions influence bacterial DNA methylation patterns (Hale et al., 1994), and that widespread 6mA demethylation during DNA repair may promote bacterial survival by delaying genome replication or cell division (Li et al., 2012; Sánchez-Romero & Casadesús, 2020). Similarly, in human lymphoblastoid cells, environmental changes have been associated with genome-wide alterations in 5mC modifications and transcriptional regulation (Chowdhury et al., 2016). Consistent with these findings, genome-wide DNA demethylation was identified in both VBNC-state and resuscitated *S.* Infantis, suggesting that DNA demethylation may be associated with an important epigenetic regulatory mechanism underlying bacterial adaptation to combined low a_w and antibiotic stress.

3.7.2. KEGG enrichment analysis

KEGG enrichment analysis identified 3, 7, and 5 DMR-Hyper-enriched pathways, and 11, 9, and 10 DMR-Hypo-enriched pathways for 5mC-, CpG-, and 6mA-associated DMRs, respectively. In contrast, dcm- and dam-associated DMRs yielded only 4 and 9 DMR-Hypo-enriched pathways, respectively. Notably, most enriched pathways were identified during the resuscitation transition (Table 5). Significantly enriched KEGG pathways (corrected $P < 0.05$) are shown in Fig. 6 and Table S3.

During the VBNC transition, CpG-associated DMR-Hypo genes were enriched in galactose metabolism, whereas during the resuscitation transition, 5mC-associated DMR-Hyper genes were enriched in the same pathway, suggesting dynamic epigenetic regulation of galactose metabolism during VBNC state formation and resuscitation (Xu et al., 2020). During the resuscitation transition, 5mC-, CpG-, and 6mA-associated DMR-Hypo genes were enriched in C5-branched dibasic acid metabolism, a pathway involved in energy production and the biosynthesis of key enzymes, transporters, and regulatory factors, suggesting that DNA demethylation may be associated with metabolic reactivation during resuscitation (Huang et al., 2020; Lundgren et al., 2014; Zhou et al.,

Table 5
KEGG pathway enrichment of differential methylation region (DMR)-associated genes in the VBNC transition and resuscitation transition.

Modification site		VBNC transition	Resuscitation transition
5mC	DMR-Hyper[a]	/	Phosphotransferase system; Valine, leucine and isoleucine degradation; Galactose metabolism
	DMR-Hypo[b]	Pentose and glucuronate interconversions	Phosphotransferase system; Porphyrin and chlorophyll metabolism; C5-Branched dibasic acid metabolism; Secondary bile acid biosynthesis; Selenocompound metabolism; Nonribosomal peptide structures; Ascorbate and aldarate metabolism; Cysteine and methionine metabolism; Two-component system; Geraniol degradation
CpG	DMR-Hyper	Drug metabolism-other enzymes; Two-component system	Aminobenzoate degradation; Cysteine and methionine metabolism; Nitrotoluene degradation; Phenylpropanoid biosynthesis; Phosphotransferase system
	DMR-Hypo	Galactose metabolism; Lysine biosynthesis; Pentose and glucuronate interconversions	Phosphotransferase system; C5-Branched dibasic acid metabolism; Bacterial chemotaxis; Porphyrin and chlorophyll metabolism; Nonribosomal peptide structures; Ascorbate and aldarate metabolism; Pentose and glucuronate interconversions
6mA	DMR-Hyper	ABC transporters	Porphyrin and chlorophyll metabolism; Phosphotransferase system; C5-Branched dibasic acid metabolism; Propanoate metabolism
	DMR-Hypo	Phosphotransferase system; Biosynthesis of ansamycins; Galactose metabolism; Geraniol degradation; Styrene degradation; Porphyrin and chlorophyll metabolism	C5-Branched dibasic acid metabolism; Aminobenzoate degradation; Pentose and glucuronate interconversions; Bacterial chemotaxis; Porphyrin and chlorophyll metabolism
dcm	DMR-Hypo	/	Porphyrin and chlorophyll metabolism; Nitrotoluene degradation; Quorum sensing; Phosphotransferase system
dam	DMR-Hypo	ABC transporters; Phosphotransferase system	Pentose and glucuronate interconversions; Lysine biosynthesis; Selenocompound metabolism; Aminobenzoate degradation; Sulfur metabolism; Two-component system; Galactose metabolism; Phosphotransferase system

[a] DMR-Hyper: hypermethylated DMRs.
[b] DMR-Hypo: hypomethylated DMRs.

2021).

Notably, the phosphotransferase system (PTS) was significantly enriched for multiple methylation types during the resuscitation transition, suggesting that DNA methylation alterations may contribute to PTS regulation during resuscitation. PTS is a central metabolic and regulatory pathway involved in carbohydrate uptake, signal transduction, and bacterial adaptation to environmental stresses (Postma et al., 1993; Zúñiga et al., 2005). Previous studies have shown that PTS regulates quorum sensing, virulence, flagellar expression, and bacterial responses to nutrient limitation and other environmental stresses (Lim et al., 2019), and may contribute to the transition into dormant states under severe stress conditions (Barth et al., 2009; Wu et al., 2018). In this study, 6mA methylation levels in PTS-associated genes decreased during the VBNC transition and increased during the resuscitation transition, suggesting a potential role of DNA methylation alterations in regulating PTS-associated functions during VBNC formation and resuscitation.

In addition, during the resuscitation transition, CpG methylation was increased whereas dcm-associated methylation was decreased in *S.* Infantis genes assigned to the nitrotoluene degradation pathway. These genes included a LysR family transcriptional regulator and an alkene reductase. LysR-type transcriptional regulators control bacterial gene expression and are involved in virulence and stress responses in *Salmonella* (Lahiri et al., 2009), suggesting that methylation changes in this regulator may be associated with transcriptional adaptation during VBNC resuscitation. Notably, dcm-associated DMR-Hypo genes were enriched in the quorum sensing pathway in resuscitated cells. Together with the increased AI-2 levels after resuscitation, these findings suggest an important role of quorum sensing regulation in VBNC resuscitation.

3.7.3. GO enrichment analysis

GO enrichment analysis showed that only 5mC- and 6mA-associated DMR genes were significantly enriched in biological processes related to bacterial stress adaptation (corrected P value < 0.05) (Fig. 7). These processes were mainly associated with gene expression, oxoacid metabolism, polysaccharide and lipid biosynthesis metabolism, various stress responses, biofilm formation, and pilus assembly, with DMR-Hypo genes predominating. During the VBNC transition, 5mC-associated DMR-Hypo genes were enriched in gene expression, oxoacid metabolism, polysaccharide biosynthesis metabolism, and stress responses (Fig. 7A). During the resuscitation transition, 5mC-associated DMR-Hypo genes were associated with negative regulation of gene expression, lipid biosynthesis metabolism, and oxoacid metabolic process, while the 5mC-associated DMR-Hyper genes were primarily enriched in lipid catabolic process and oxoacid metabolic process (Fig. 7B).

Notably, oxoacid metabolic process was significantly enriched in both the VBNC and resuscitation transitions, suggesting its potential role in *S.* Infantis stress adaptation. Oxoacid metabolism has been reported to contribute to bacterial oxidative stress responses by regulating redox balance through 2-oxoacid dehydrogenase complexes and metabolites such as pyruvate and α-ketoglutarate (Bunik, 2018). As shown in the resuscitation and ROS assays (Figs. 3 and 4), ROS accumulation was closely associated with VBNC state formation and resuscitation in *S.* Infantis under antibiotic stress. Given the role of oxoacid metabolism in bacterial redox regulation, methylation changes in oxoacid metabolism-related genes may be associated with ROS homeostasis during VBNC transitions.

Compared with 5mC, 6mA-associated DMR genes showed

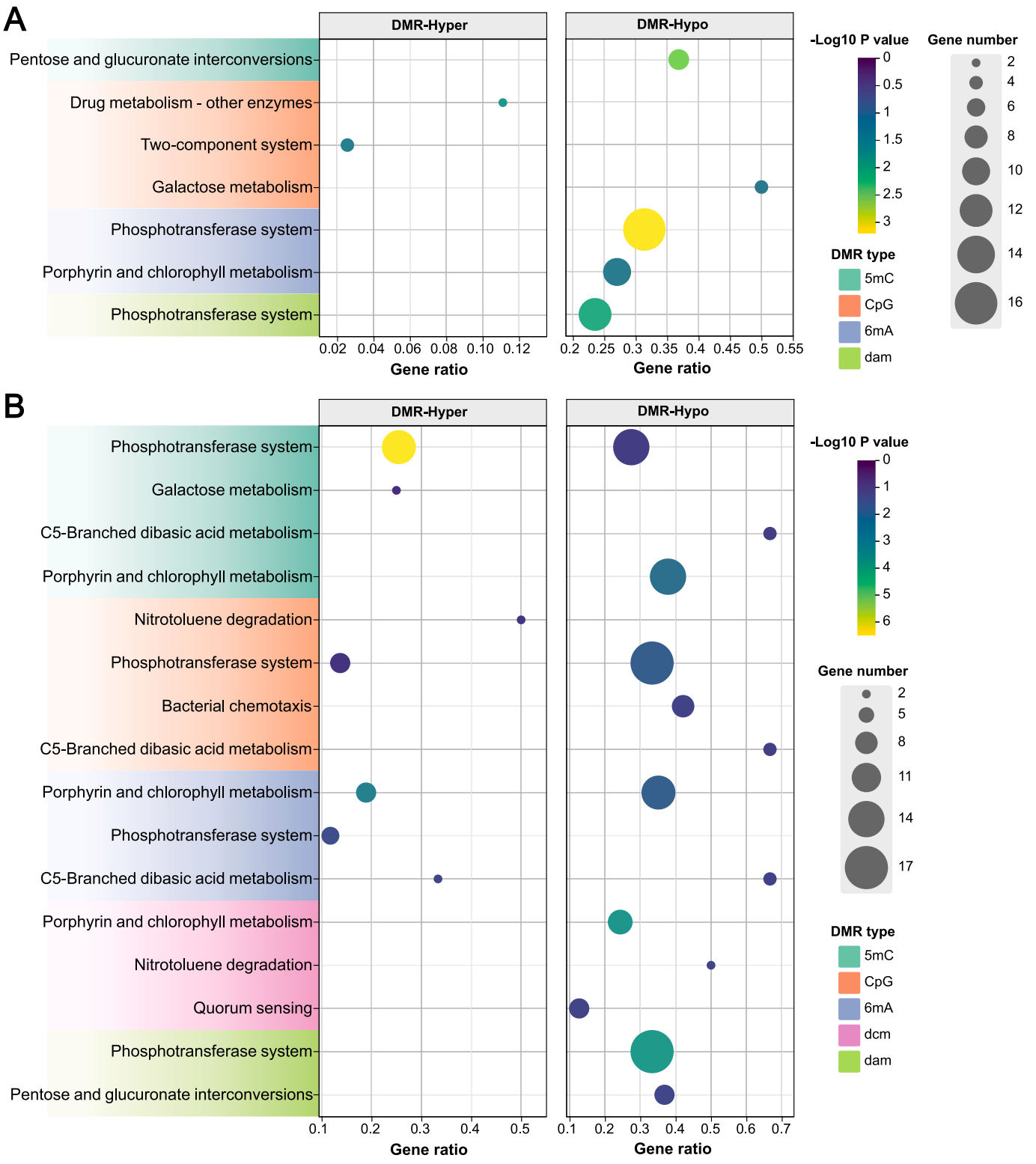


Fig. 6. KEGG pathway enrichment analysis of differential methylation region (DMR)-associated genes. (A) Enriched pathways during the viable but non-culturable (VBNC) transition. (B) Enriched pathways during the resuscitation transition. The gene ratio represents the proportion of DMR-associated genes among all genes assigned to each pathway. Dot color indicates statistical significance, defined as the negative logarithm of the corrected *P* value. Dot size represents the number of DMR-associated genes in each pathway. DMR-Hyper: hypermethylated DMRs; DMR-Hypo: hypomethylated DMRs.

enrichment in a broader range of biological processes, including biofilm formation, cell adhesion, glucuronate catabolism and metabolism, and pilus assembly. Specifically, the 6mA-associated DMR-Hypo genes were enriched in gene expression, polysaccharide biosynthesis metabolism, chemical stress response, biofilm formation, and cell adhesion in VBNC-state cells (Fig. 7C), and in positive regulation of gene expression, L-lyxose metabolic process, lipid biosynthesis metabolism, and pilus assembly in resuscitated cells (Fig. 7D). Only two 6mA-associated DMR-Hyper genes were enriched in glucuronate catabolism and metabolism.

The biological processes enriched in 5mC- and 6mA-associated DMR

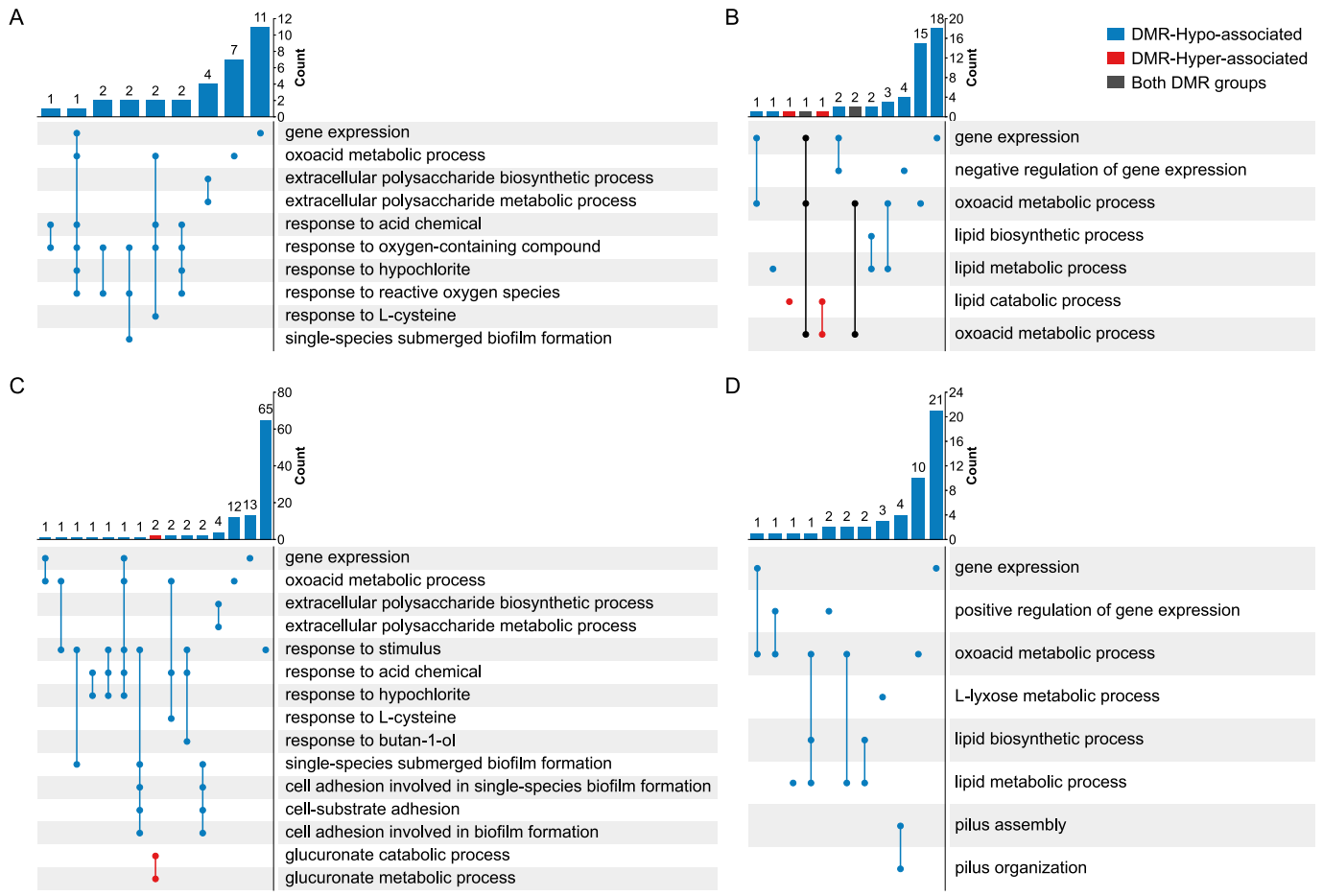


Fig. 7. UpSet plot showing gene intersections among significantly enriched GO terms associated with differential methylation regions (DMRs). (A) 5mC-associated DMRs during the viable but non-culturable (VBNC) transition. (B) 5mC-associated DMRs during the resuscitation transition. (C) 6mA-associated DMRs during the VBNC transition. (D) 6mA-associated DMRs during the resuscitation transition. Red and blue indicate GO terms associated with DMR-Hyper and DMR-Hypo genes, respectively. Gray indicates intersections involving GO terms from both DMR-Hyper and DMR-Hypo groups. Bar height represents the number of DMR-associated genes in each GO term intersection. DMR-Hyper: hypermethylated DMRs; DMR-Hypo: hypomethylated DMRs.

genes were largely consistent under the same physiological state. Genes exhibiting methylation changes during the VBNC transition were enriched in biological processes related to responses to acid, ROS and other oxygen-containing compounds, as well as additional stress adaptive responses. These findings indicate that the observed methylation changes may be associated with the adaptation of *S.* Infantis to DOC-amended soil, particularly its response to antibiotic-induced oxidative stress. Consistently, previous studies in lactic acid bacteria have shown that 6mA methylation changes can enhance resistance to oxidative stress (Jiao & Zhao, 2022).

The GO enrichment patterns showed that methylation changes during VBNC formation were mainly associated with stress responses, gene regulation, and biofilm-related processes, whereas those during resuscitation were enriched in metabolic processes and cellular structure-related functions. These patterns suggest a potential role for DNA methylation in stress adaptation and cellular recovery during VBNC transitions (Sánchez-Romero & Casadesús, 2020).

Taken together, the physiological and molecular findings suggest that VBNC transitions under combined low a_w and antibiotic stress involve changes in cellular stress responses and metabolic regulation. The accumulation of ROS and suppression of AI-2 during VBNC formation, together with genetic and methylation changes, may reflect adaptation toward oxidative stress tolerance and reduced cellular activity. During resuscitation, methylation changes involving oxoacid metabolism and the PTS may be linked to redox balance and metabolic recovery, while those associated with quorum sensing may contribute to the partial recovery of AI-2 signaling. These genetic and epigenetic changes provide potential molecular links to the observed physiological responses; however, their causal relationships require further functional validation.

Indeed, recent studies in *Salmonella* have shown that genome-wide DNA methylation can remain largely static across growth conditions and may not directly correlate with gene expression (Bourgeois et al., 2022). Therefore, further mechanistic studies, particularly targeted validation of methyltransferases or differentially methylated loci, are needed to clarify their functional roles during VBNC transitions (Payelleville & Brillard, 2021). In addition, the genomic and methylomic analyses were performed on selected samples without biological replication, and the identified changes should therefore be interpreted as associations with the VBNC transitions rather than definitive adaptive or regulatory events. Further validation will be required to establish their functional significance. Finally, the use of a single *S.* Infantis strain limits the broader applicability of these findings, warranting validation across diverse *Salmonella* serovars and strains.

4. Conclusion

In summary, this study showed that combined desiccation and antibiotic stress in soil led to elevated accumulation of ROS and inhibition of AI-2 synthesis in *S.* Infantis. These physiological changes drove *S.* Infantis into the VBNC state, resulting in enhanced resistance to various stress conditions. Genomic and methylomic analyses of DOC-

induced VBNC-state cells revealed genetic and epigenetic variations in genes associated with key biological functions such as response to stimulus, PTS, oxoacid metabolism, and oxidative stress system. During resuscitation, intracellular ROS decreased whereas AI-2 production increased, accompanied by methylation changes in genes involved in PTS, nitrotoluene degradation, oxoacid metabolism, and activation of quorum sensing system. Collectively, these findings suggest that genome-wide DNA demethylation and dynamic methylation changes in stress- and metabolism-related genes may be linked to *S*. Infantis adaptation during VBNC formation and resuscitation. However, further transcriptomic or targeted mutational analyses are needed to verify the functional roles of the identified SNPs and methylation changes.

CRediT authorship contribution statement

Zhuolin Ma: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Dinghui Xu:** Investigation. **Xiaoying Qin:** Investigation. **Shaoting Li:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Hongmei Zhang:** Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Guiying Li:** Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the Natural Science Foundation of Guangdong Province, China (No. 2024A1515012086, 2025A1515012134, and 2025A1515011652) and the National Natural Science Foundation of China (No. 42330702).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2026.120841.

Data availability

Genome and methylome data are publicly available through the NCBI BioProject PRJNA1250766.

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