



Temperature-dependent biofilm and sublancin production arrest soil arsenic and antibiotic resistance gene mobility

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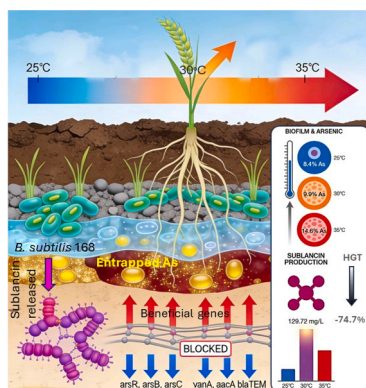
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HIGHLIGHTS

- *B. subtilis* biofilm sequesters rhizospheric arsenic within biofilm (74% increase).
- Sublancin selectively suppresses 12 Gram-positive pathogens by 74–86%.
- Bio-amendment reduces horizontal gene transfer frequency by 74.7% ($p < 0.001$).
- Temperature-dependent dual function peaks between 30–35°C under warming scenarios.
- Field trials validate biofilm-sublancin approach for As-contaminated paddy soils.

GRAPHICAL ABSTRACT



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ABSTRACT

Climate change-induced warming and arsenic soil contamination synergistically threaten agricultural sustainability by restructuring microbial communities and accelerating antimicrobial resistance dissemination. Here, through integrated greenhouse and field trials, we demonstrate that *Bacillus subtilis* 168-derived biofilm and sublancin, a glycosylated antimicrobial peptide, simultaneously immobilise rhizospheric arsenic and suppress horizontal transfer of antibiotic resistance genes (ARGs). Temperature-dependent biofilm formation (25–35°C) enhanced arsenic sequestration within the extracellular polymeric substance matrix, with SEM-EDX revealing a 74% increase in arsenic weight percentage at 35°C and ToF-SIMS confirming ~14-fold and ~9-fold increases in root-associated arsenic on biofilm-colonised surfaces in greenhouse and field trials, respectively. Sublancin production peaked at 30°C (129.72 mg L⁻¹), selectively suppressing all 12 tested pathogenic Gram-positive species by 74–86% while preserving Gram-negative communities. Bio-amendment reduced horizontal gene transfer frequency by 74.7% ($p < 0.001$) across all temperature regimes. Transcriptomic profiling revealed co-ordinated upregulation of exopolysaccharide biosynthesis (FDR $\sim 1.0 \times 10^{-27}$) and sublancin machinery (*sunA*: +3.5 log₂), alongside downregulation of conventional ARGs (*vanA*, *blaTEM*: -2.5 to -4.0 log₂). These findings establish sublancin as a dual-function, climate-adaptive soil bio-amendment simultaneously addressing arsenic bioaccumulation and antibiotic resistance gene dissemination under warming scenarios.

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1. Introduction

Climate change-induced warming, advancing at 1.1°C since 1850–1900 with projections reaching 1.5°C within decades [1], fundamentally threatens agricultural soil systems through interconnected mechanisms. Warming accelerates soil organic matter decomposition, triggering nutrient loss and reduced soil quality, whilst simultaneously restructuring soil microbial communities, bacterial and fungal diversity declining by 16.0% and 19.7% respectively under warming conditions [2,3]. Temperature-dependent effects extend to bacterial mutation rates, driving adaptive evolution [4]. Concurrent with climatic pressures, arsenic contamination affects nearly one-fifth of global agricultural land, with concentrations ranging from background levels of 5 mg/kg to extremes exceeding 4000 mg/kg, imperilling 1.4 billion people and compromising food security [5,6]. Bacterial biofilms represent critical adaptive responses to this contamination, with arsenic-resistant bacteria forming enhanced biofilms that sequester extracellular arsenic at concentrations up to 11 times higher than intracellular levels [7,8]. Biofilm formation intensity varies with arsenic speciation, with As(V) generally promoting greater biofilm development than As(III) [9].

Sublancin 168, a glycosylated antimicrobial peptide produced by *Bacillus subtilis* 168, exhibits temperature-dependent production peaking at 30.8°C and declining significantly above 33°C, achieving optimal concentrations of 129.72 mg/L [10]. Beyond direct bactericidal activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*, sublancin disrupts bacterial cell integrity [11]. The phosphoenolpyruvate:sugar phosphotransferase (PTS) system functions as a central regulatory nexus linking sublancin sensitivity to bacterial metabolism and antibiotic resistance (Görke & Stülke, 2008). Phosphorylation of His15 in the histidine-containing phosphocarrier protein appears essential for sublancin's bactericidal activity, whilst PTS promoter regulation modulates antibiotic resistance through proton-motive-force and reactive-oxygen-species pathways [12,13]. These systems converge catastrophically: rising temperatures simultaneously enhance arsenic bioavailability, restructure microbial communities, optimise sublancin production, and accelerate antibiotic-resistance mutations. Temperature-dependent sublancin production, combined with arsenic-induced biofilm stress and altered PTS-mediated regulation, creates selective conditions favouring resistant bacterial persistence in contaminated agricultural soils [4]. Understanding these feedback loops is essential for developing management strategies to preserve soil health and agricultural productivity under climate change.

Current understanding of soil microbial responses to climate change and arsenic contamination remains fragmented across disconnected research domains, specifically, soil biogeochemistry, environmental microbiology, and antimicrobial resistance ecology have each examined individual stressors in isolation but have not been integrated to examine synergistic effects on microbial community function, contaminant fate, and resistance gene dissemination in agricultural soils. This study addresses two knowledge gaps to elucidate integrated mechanisms governing agricultural soil resilience. First, a combination of biofilm and sublancin 168 as a potential bioapplication tool for the mitigation of arsenic and antibiotic-harboring bacteria in the natural soil system. Second, the mobility of antimicrobial resistance (ABR) genes under combined thermal and sublancin stress has not been systematically evaluated, despite its critical importance for predicting the persistence of resistance in agricultural ecosystems. This study's hypothesis was that optimal temperature can maximise microbial arsenic sequestration within biofilm layers, whilst sublancin 168 simultaneously suppresses ABR gene horizontal transfer through reduced bacterial conjugation rates. The specific objectives of this study were: (i) to quantify temperature-dependent (25–35°C) *Bacillus subtilis* 168 biofilm development on rice rhizosphere surfaces and characterise arsenic sequestration within the extracellular polymeric substance matrix using SEM-

EDX elemental mapping and ToF-SIMS depth profiling across integrated greenhouse microcosm and field trial systems; (ii) to evaluate the selective antimicrobial efficacy of sublancin 168 against pathogenic Gram-positive bacteria in arsenic-contaminated soil, determining dose-dependent suppression, soil microbial biomass impacts, and community-level selectivity towards Gram-positive versus Gram-negative populations; (iii) to elucidate the molecular interconnections among sublancin biosynthesis, the phosphoenolpyruvate:sugar phosphotransferase (PTS) system, and antibiotic resistance gene regulation through transcriptomic profiling, Gene Ontology and KEGG pathway enrichment analyses, and protein–protein interaction network modeling; and (iv) to determine the capacity of the biofilm–sublancin bioamendment to suppress horizontal transfer of antibiotic resistance genes by quantifying conjugation frequencies under combined thermal and antimicrobial peptide stress in both sterilised and natural soil systems.

2. Materials and methods

2.1. Study design and experimental setup

The experiment was designed to understand the effects of temperature rise on bacterial biofilm production and sublancin activity, and to do so, three temperatures were set- 25 °C, 30 °C and 35 °C. This temperature rise can mimic the elevated temperature range during the summer, when antibiotic resistance peaks. A schematic of a controlled soil setup covered with a glass box is shown in the [Supplementary Fig. 1](#). This whole experiment was conducted for five months, considering the full growth phase of a rice plant. For each of the temperatures, five such microcosm boxes were set. Arsenic spiking with a 100 mg/kg dose was performed at the beginning of the experiment [14]. A water sprinkler was used twice a week to keep the soil moist (>70% soil moisture content), similar to a natural paddy field. The bacterium was supplemented once a week ($OD_{600} = 0.8$, corresponding to $\sim 1 \times 10^8$ CFU mL⁻¹). In this greenhouse experiment, two different soils were used- sterilised soil to see the direct effects on the inoculated bacterium and a natural soil to see the viability of the inoculum over natural microbiota. A parallel field trial was conducted to compare the results under a controlled environment with those in a natural field ([Supplementary details](#)). During this five-month period of summertime (May–September), the field temperature changed from 22°C to 38°C, similar to the greenhouse temperature range.

2.2. Bacterial culture and biofilm quantification

Bacillus subtilis 168 was cultured in Luria-Bertani (LB) broth at 25 °C, 30 °C, and 35 °C with shaking at 200 rpm, and growth was monitored by measuring optical density at 600 nm. For biofilm formation, overnight cultures were diluted to an OD_{600} of 0.1 in LB broth supplemented with arsenic (25–300 mg kg⁻¹) and incubated statically in 96-well microtiter plates at the respective temperatures for 24 h. Sterile LB served as a negative control. Biofilms were washed with phosphate-buffered saline, stained with 0.1% crystal violet, and quantified by measuring absorbance at 590 nm. All experiments were conducted in five replicates. Log-phase cultures were used for soil inoculation in greenhouse and field experiments to assess bacterial viability and biofilm formation. Biofilm presence was evaluated on rice roots to visualise bulk biofilm development, with untreated plants maintained as controls for comparison.

2.3. Arsenic bioavailability and fractionation

Total arsenic concentrations in soil and plant samples were quantified using inductively coupled plasma mass spectrometry (ICP-MS) to assess arsenic accumulation in rice [7], accounting for partial sequestration within biofilms. Arsenic bioavailability was evaluated using the Physiologically Based Extraction Test (PBET), simulating gastric (pH

1.5, 37 °C, 1 h) and intestinal (pH 7.0, 37 °C, 4 h) digestion of < 250 µm soil fractions. Bioaccessible arsenic in both phases was measured by ICP-MS, with quality assurance using certified reference material (NIST SRM 2711a) [15,16]. Arsenic fractionation was determined via a five-step sequential extraction protocol, targeting sorbed, oxide-bound, and residual fractions, with extracts analysed by ICP-MS. Arsenic speciation was performed using anion-exchange HPLC coupled to ICP-MS following phosphoric acid extraction [17]. Arsenite, arsenate, monomethylarsonic acid, and dimethylarsinic acid were quantified, with method validation demonstrating acceptable recoveries and low detection limits.

2.4. Sublancin 168 production, extraction and characterisation

B. subtilis 168 was cultivated in LB broth at 37 °C with shaking (200 rpm) for 24 h. Cell-free supernatants were obtained by centrifugation and filtration, acidified to pH 4.0, and subjected to SP-Sepharose cation-exchange chromatography [18]. Sublancin was further purified by reverse-phase HPLC, yielding > 95% purity and a molecular mass of ~3.25 kDa, consistent with its glycosylated form. Antimicrobial activity against *Staphylococcus aureus* ATCC 25923 was assessed using agar well diffusion and broth microdilution assays [19,20]. Inhibition zones were measured after 18–24 h incubation, and minimum inhibitory concentration (MIC) was defined as the lowest concentration producing OD₆₀₀ < 0.05. For enhanced production, *B. subtilis* 168 was grown in an optimised fermentation medium containing corn powder, soybean meal, KH₂PO₄, and (NH₄)₂SO₄ (pH 7.0) [21]. Shake-flask fermentation was conducted at 30.8 °C for 48 h, followed by cell removal and filtration [22]. Sublancin was purified using cation-exchange chromatography and reverse-phase HPLC with a propanol gradient [23]. Purified fractions were lyophilised and analysed by ¹H NMR spectroscopy (600 MHz) to confirm the characteristic S-linked glycopeptide structure and high chemical purity.

2.5. Arsenic detection within biofilms

Biofilms were cultivated on indium tin oxide-coated glass slides or polycarbonate membranes for 48–96 h, then rinsed with PBS. The 48–96-hour incubation was required to allow full biofilm maturation and development of a structurally complex EPS matrix with sufficient depth (0.5–20 µm) and metalloid-binding capacity for SEM-EDX elemental mapping and ToF-SIMS depth profiling. Samples were fixed with 2.5% glutaraldehyde, dehydrated through graded ethanol (10–100%), and sputter-coated with gold for scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX) analysis at 15–20 kV acceleration voltage [24]. Arsenic was detected at 1.282 keV (As Lα) and 10.543 keV (As Kα), with quantitative elemental mapping across 50–500 µm² areas. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis employed a bismuth cluster ion beam (Bi³⁺, 25 keV) for surface analysis and caesium (Cs⁺, 1 keV) sputter beams for depth profiling. Arsenic species (As⁻, AsO⁻, AsO₂⁻, AsO₃⁻) were monitored in negative ion mode with < 5 nm depth resolution over 0.5–20 µm biofilm depth. Three-dimensional reconstruction was achieved by combining sequential 2D images acquired during sputter cycles [25].

2.6. Gene expression analysis

Total RNA was extracted using a modified RNeasy Mini Kit protocol [26]. Bacterial pellets (1 × 10⁹ CFU) were lysed by lysozyme treatment followed by bead-beating. RNA integrity was confirmed by agarose gel electrophoresis and purity by NanoDrop spectrophotometry. cDNA synthesis was performed using random hexamer primers. Gene-specific primers were designed for arsenic-resistance (arsR, arsB, arsC, arsA), biofilm formation (epsA, tapA, tasA, bslA), sublancin biosynthesis (sunA, sunT, sunS), and phosphotransferase system genes (ptsH, ptsI). Quantitative PCR was conducted using SYBR Green chemistry on

LightCycler 96 or CFX96 platforms. Reference gene stability was evaluated using geNorm, NormFinder, and BestKeeper, validating rpoB and 16S rRNA as internal controls. Relative gene expression was calculated using the 2^{-ΔΔCt} method with three biological and three technical replicates (Goswami et al., 2018). For soil samples, pooled replicates from each temperature treatment were processed using the RNeasy PowerSoil Total RNA Kit [27].

2.7. Protein-protein interaction networks

Protein-protein interaction networks were constructed using the STRING database (v12.0) for with a medium confidence score (0.400) (Wu et al., 2020). Gene sets related to arsenic response, biofilm formation, sublancin biosynthesis, and the phosphotransferase system were analysed to identify hub and bottleneck proteins based on network topology and to detect functional modules using k-means clustering. Networks were exported and visualised in Cytoscape v3.10.0 using the stringApp plugin. Functional enrichment analysis of differentially expressed genes (|log₂ fold-change| ≥ 2.0, adjusted *P* < 0.05) was performed using STRING and ShinyGO [28]. Gene Ontology enrichment (Biological Process, Molecular Function, Cellular Component) and KEGG pathway analyses were conducted with multiple-testing correction (FDR < 0.05). Enrichment results were visualised as clustered networks and pathway diagrams, with redundant GO terms reduced using semantic similarity clustering (REVIGO).

2.8. Conjugation assay for horizontal gene transfer

Horizontal gene transfer was quantified using soil microcosms containing engineered donor (*B. subtilis* 168 harbouring the pUB110 plasmid encoding ampicillin and kanamycin resistance) and recipient (*ΔsunI* *B. subtilis* 168 Rif^R, rifampicin-resistant and plasmid-free) strains. Sterilised soil, adjusted to 60% maximum water-holding capacity and verified for sterility, was amended with 100 nM sublancin or left unamended as a control. Donor and recipient cultures in late exponential phase (OD₆₀₀ ≈ 0.8) were mixed at a 1:1 ratio to achieve 10⁷ CFU g⁻¹ dry soil and incubated at 25 °C for 96 h. Bacterial cells were recovered by suspending soil samples in sterile pyrophosphate buffer containing glass beads and vortexing [29], followed by serial dilution and selective plating. Rifampicin plates were used to enumerate total recipients, while rifampicin combined with ampicillin and kanamycin quantified transconjugants. Donors were selectively enumerated using ampicillin, kanamycin, and sublancin, and non-transconjugant recipients were quantified using rifampicin and sublancin. Plates were incubated for 24 hr and 48 hr, and conjugation frequencies were subsequently calculated.

3. Results and discussion

3.1. Bacterial biofilm development and arsenic response

Crystal violet assays revealed clear temperature-dependent patterns of *Bacillus subtilis* biofilm formation under arsenic exposure (Fig. 1a; Supplementary Fig. 2). At 25 °C, biofilm biomass increased twofold from control (0.37) to high arsenic treatment (0.75), consistent with reported optimal biofilm-forming temperatures [30,31]. The strongest arsenic-responsive biofilm was observed at 30 °C, with absorbance peaking at 1.05 under moderate arsenic levels before stabilising, reflecting near-optimal growth conditions. At 35 °C, biofilm formation reached a maximum at intermediate arsenic concentrations but declined at higher levels, indicating thermoregulation of extracellular polymeric substance production and a toxicity threshold beyond which arsenic stress overrides biofilm-mediated protection [8]. Soil-plant-biofilm arsenic SEM-EDX analyses and ToF-SIMS confirmed that biofilms significantly adsorbed arsenic, reducing its accumulation in plant roots compared to controls. Total As analysis in both pot and field soil clearly

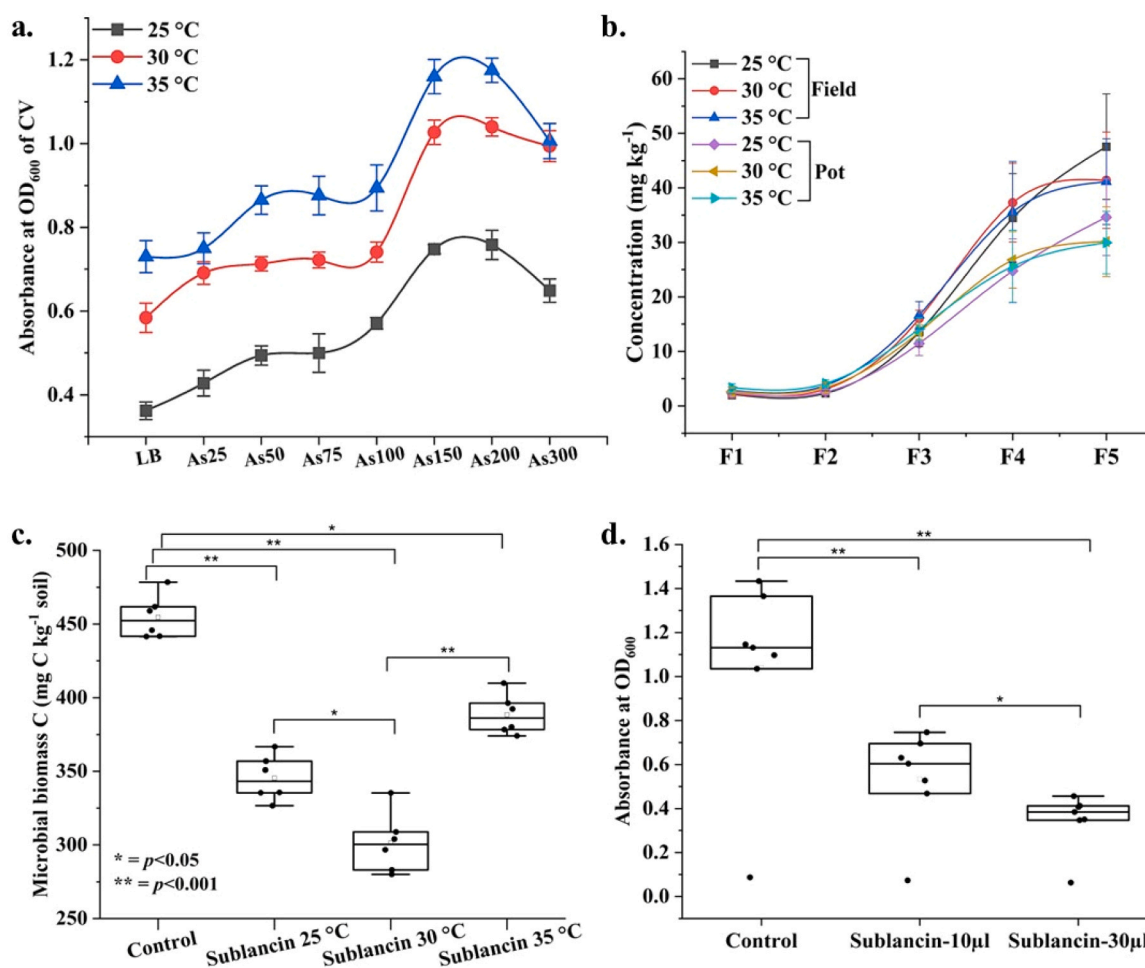


Fig. 1. Temperature-dependent and sublancin-mediated effects on biofilm formation, arsenic mobilisation, and pathogenic microbial inhibition. (a) Temperature-dependent biofilm formation in LB broth across increasing arsenic concentrations (As25–As300 mg/L) at 25, 30, and 35 °C, measured by absorbance at OD₆₀₀. Biofilm intensity increases with temperature and peaks at 30 °C before plateauing at 35 °C. (b) Sequentially extracted arsenic (F1–F5 fractions) in soil at different temperatures, showing progressive As release with temperature elevation and varying bioavailability. Field temperatures were not fixed, but rather varied within a close range of the selected temperature range. (c) Soil microbial biomass carbon (MBC) quantification demonstrates a significant reduction following sublancin treatment at 25, 30, and 35 °C, with maximum suppression at higher temperatures ($p < 0.001$). (d) Dose-dependent inhibition of *Staphylococcus aureus* growth by sublancin (10 and 30 µL), showing selective antimicrobial activity against Gram-positive pathogens ($p < 0.05$). Error bars represent the standard deviation of five replicates.

shows that the mass-balance content of As in soil-to-plant is lower when biofilm is applied (Supplementary Fig. 3 and 4). The observed biphasic response, characterised by maximal biofilm formation at intermediate arsenic concentrations, suggests an optimal range for biofilm-mediated arsenic resistance [32]. These findings highlight an adaptive strategy in which biofilms sequester toxicants, with implications for climate change scenarios where elevated temperatures may enhance both arsenic bioavailability and microbial persistence in contaminated agroecosystems.

3.2. Temperature-dependent arsenic fractionation

The high-resolution HPLC-ICP-MS spectra (Supplementary Fig. 5) were obtained to determine arsenic speciation in soil samples incubated at different temperatures (25 °C, 30 °C, and 35 °C), along with a sequential extraction analysis (Fig. 1b). At elevated temperatures, As(V) is reduced to As(III) mainly because warming promotes reductive dissolution of Fe/Mn oxyhydroxides and enhances microbial respiration, including arC-mediated arsenate reduction, which our transcriptomic data show is upregulated at 35 °C. This shift is important because As(III) is far more toxic and mobile than As(V), owing to its stronger binding to protein thiols and weaker adsorption to iron oxides

[33]. In contrast, the *B. subtilis* 168 biofilm EPS effectively immobilizes both As(III) and As(V), as shown by ToF-SIMS detection of multiple arsenic ions within the biofilm matrix; its anionic functional groups bind As(III) through ligand exchange and As(V) through electrostatic interactions. Although sublancin does not directly affect arsenic redox chemistry, it may indirectly limit As(V)-to-As(III) reduction by suppressing Gram-positive arsenic-resistant bacteria, including potential arsenate reducers, thereby helping retain arsenic in the less mobile As (V) form. Sequential extraction revealed distinct temperature-dependent arsenic fractionation across five fractions (F1–F5). Bioavailable fractions (F1 + F2) showed temperature-sensitive behaviour [34]: at 25 °C and 30 °C, concentrations remained relatively stable (F1: ~2.1 mg kg⁻¹; F2: ~2.4 mg kg⁻¹), but at 35 °C decreased (F1: 1.2 mg kg⁻¹; F2: 1.8 mg kg⁻¹), indicating redistribution between mobile pools. Iron-manganese-bound arsenic (F3) peaked at 30 °C (4.1 mg kg⁻¹), suggesting optimal conditions for Fe-Mn oxide association. Organic matter-bound fraction (F4) showed exponential temperature increases: 9.4 mg kg⁻¹ (25 °C) to 19.8 mg kg⁻¹ (35 °C), representing a 110% increase. Residual fraction (F5) exhibited the highest concentrations with clear positive temperature correlation (12.4–19.8 mg kg⁻¹). The dramatic F4 increase at 35 °C indicates enhanced organo-metallic complex formation or increased binding site accessibility through

temperature-induced conformational changes in soil organic matter. The reduction in bioavailable arsenic at elevated temperatures, coupled with increased sequestration in organic and residual pools, suggests natural attenuation mechanisms under climate change. However, overall increases across most fractions indicate enhanced arsenic mobilisation from primary mineral sources, warranting investigation into long-term environmental implications.

3.3. Sublancin extraction and soil microbial biomass effects

HPLC analysis confirmed successful sublancin 168 extraction (Supplementary Fig. 6), with growth media exhibiting superior recovery (sharp peaks at characteristic retention times) compared to soil matrices, which showed elevated baseline absorbance and broader peaks reflecting humic substance interference [35,36]. The ^1H NMR spectrum of purified sublancin 168 (600 MHz, D_2O , 25°C) confirmed the characteristic structure of the 37-residue S-linked glycopeptide [37]. Analysis revealed 36 distinct backbone amide NH signals (7.85–8.58 ppm), consistent with the expected number of residues minus proline. A single anomeric proton doublet at 4.58 ppm ($J = 9.2$ Hz) confirmed β -glycosidic linkage of glucose to Cys-22 (Supplementary Fig. 7). Aromatic protons from phenylalanine residues appeared at 7.15–7.32 ppm, while aliphatic signals (0.78–3.02 ppm) corresponded to amino acid side chains. Integration analysis and absence of extraneous signals indicated $> 95\%$ purity, confirming successful production and purification of sublancin 168 from optimised *B. subtilis* 168 fermentation. The box plot analysis reveals significant differential effects of sublancin treatment and temperature on soil microbial biomass carbon (MBC), providing crucial insights into antimicrobial peptide-soil microbiome interactions under varying thermal conditions (Fig. 1c). MBC analysis revealed differential sublancin effects across temperatures. Control soils maintained ~ 450 mg C kg^{-1} [38]. Sublancin at 25°C reduced MBC to ~ 350 mg kg^{-1} (22% decrease, $p < 0.001$), consistent with broad-spectrum activity against Gram-positive bacteria [39]. Maximum reduction occurred at 30°C (~ 300 mg kg^{-1} , 33% decrease, $p < 0.001$), corresponding with sublancin's optimal production temperature (30.8°C) [10]. Partial MBC recovery at 35°C (~ 390 mg kg^{-1}) may reflect enhanced microbial metabolic rates enabling stress recovery [40] or declining sublancin thermal stability above 35°C [41]. These temperature-dependent modulations have significant climate change implications. Moderate warming (30°C) enhances antimicrobial activity, potentially disrupting soil carbon sequestration and nutrient cycling [42]. The results underscore that climate warming may fundamentally alter antimicrobial peptide ecological impacts in agricultural soils.

3.4. Dose-dependent sublancin effects on *S. aureus*

Box plot analysis demonstrated clear dose-dependent growth inhibition over 48 h (Fig. 1d and Supplementary Fig. 8). Control cultures achieved $\text{OD}_{600} \sim 1.15$ [11]. Treatment with 10 μL sublancin reduced growth to $\text{OD}_{600} \sim 0.65$ (44% reduction, $p < 0.01$), approximating sub-lethal to bacteriostatic concentration ranges consistent with published MIC values of 15 μM to 2–10 $\mu\text{g mL}^{-1}$ [11,39]. The 30 μL treatment produced maximum inhibition ($\text{OD}_{600} \sim 0.40$, 65% reduction, $p < 0.01$), approaching minimum bactericidal concentration where sublancin transitions from growth inhibition to active killing [12]. The mechanism involves multi-target disruption of DNA replication, transcription, and protein synthesis without compromising cell wall integrity [43], explaining the steep dose-response curve. Sublancin requires the phosphoenolpyruvate:sugar phosphotransferase system (PTS) for bacterial sensitivity, with reduced efficacy in the presence of PTS sugar [12]. Sustained 48-hour inhibition indicates stable antimicrobial activity without significant degradation [44], supporting therapeutic potential against antibiotic-resistant *S. aureus*.

3.5. Arsenic entrapment within biofilms

SEM-EDX analysis demonstrated temperature-dependent biofilm architecture and arsenic accumulation within bacterial biofilm matrix (Fig. 2a–c). At 25°C , sparse cells and limited extracellular polymeric substance (EPS) production corresponded to the lowest arsenic retention (8.37 wt%, 2.65 at%). At 30°C , increased cell aggregation and matrix development were associated with higher arsenic sequestration (9.89 wt%, 5.77 at%). The 35°C treatment showed maximal biofilm formation and the greatest arsenic accumulation (14.55 wt%, 8.10 at%), representing a $\sim 74\%$ increase relative to 30°C [45,46]. SEM also revealed distinct differences in biofilm architecture on plant root surfaces across the temperature treatments under both greenhouse and field conditions (Fig. 3). The control roots (Fig. 3a, i) displayed relatively smooth and exposed root epidermal surfaces with minimal bacterial colonisation. In contrast, *B. subtilis*-inoculated roots under elevated temperature regimes (Fig. 3b–d, j–l) exhibited progressively dense and architecturally complex biofilm matrices, characterised by globular and confluent extracellular polymeric substance (EPS) layers enveloping the root cortical surface. The most robust biofilm development was observed in Fig. 3d (greenhouse) and Fig. 3l (field trial), where tightly packed bacterial microcolonies were embedded within a well-defined EPS matrix, consistent with mature *B. subtilis* biofilm phenotypes [47,48]. The relationship between biofilm-mediated arsenic sequestration and sublancin-mediated ARG suppression is synergistic within a 30 – 35°C thermal window: EPS production peaks at 35°C (74% increase in As weight percentage by SEM-EDX) while sublancin output and antibacterial activity peak at 30°C (129.72 mg L^{-1}), creating overlapping functional optima that coincide with projected agricultural warming. Above 35°C , both sublancin stability and biofilm formation decline, indicating an antagonistic shift at higher temperatures. ToF-SIMS provided high-resolution mapping of arsenic distribution in extracted biofilms (Fig. 2d–f). Arsenic signals increased progressively with temperature, from limited hotspots at 25°C ($\text{MC} = 7$; $\text{TC} = 3.4 \times 10^3$) to pronounced arsenic-rich domains at 30°C ($\text{MC} = 19$; $\text{TC} = 4.5 \times 10^4$), and extensive enrichment at 35°C ($\text{MC} = 24$; $\text{TC} = 4.7 \times 10^4$) [49–51]. Soil biofilm analyses further showed a rise from control levels ($\text{MC} = 2$; $\text{TC} = 3.2 \times 10^3$) to $\text{MC} = 47$ and $\text{TC} = 4.5 \times 10^4$ at 35°C , indicating $\sim 2250\%$ higher arsenic adsorption. Collectively, these results demonstrate thermal optimisation of EPS-mediated arsenic binding, with implications for biofilm-driven bioremediation under warming climates [52,53]. Elemental mapping corroborated the SEM observations, demonstrating a clear positive correlation between biofilm density and arsenic (As) entrapment capacity on pot and field-grown plant root surfaces (Fig. 3e–h, m–p). Control roots showed minimal and scattered As signal with low maximum count ($\text{MC}: 2$) and total count ($\text{TC}: 3.249 \times 10^3$) in the greenhouse (Fig. 3e) and $\text{MC}: 7$, $\text{TC}: 3.931 \times 10^3$ in the field trial (Fig. 3m). Conversely, temperature-treated, biofilm-colonized roots displayed progressively higher As signal intensities, reaching $\text{MC}: 47$ and $\text{TC}: 4.517 \times 10^4$ in the greenhouse (Fig. 3h) and $\text{MC}: 20$, $\text{TC}: 3.347 \times 10^4$ in the field trial (Fig. 3p), representing approximately a 14-fold and 9-fold increase in total As counts relative to their respective controls. The spatial distribution of As hotspots in the ToF-SIMS maps closely mirrored the biofilm-covered regions, indicating that rhizospheric As was preferentially sequestered within the biofilm EPS layers rather than being adsorbed on bare root surfaces. These findings are consistent with the documented role of bacterial EPS in heavy metal biosorption, whereby anionic functional groups (carboxyl, hydroxyl, and phosphoryl moieties) within the EPS matrix provide binding sites for metalloid species [54,55]. The enhanced As entrapment in field-grown plants validates the translational potential of *B. subtilis* biofilm-based remediation from controlled greenhouse settings to real agricultural conditions. The long-term stability of biofilm-mediated arsenic sequestration is shaped by three key processes. Under alternate wetting-drying (AWD) conditions mimicking paddy field management [7,24], mass-balance data (Supplementary Figs. 3 and 4) show

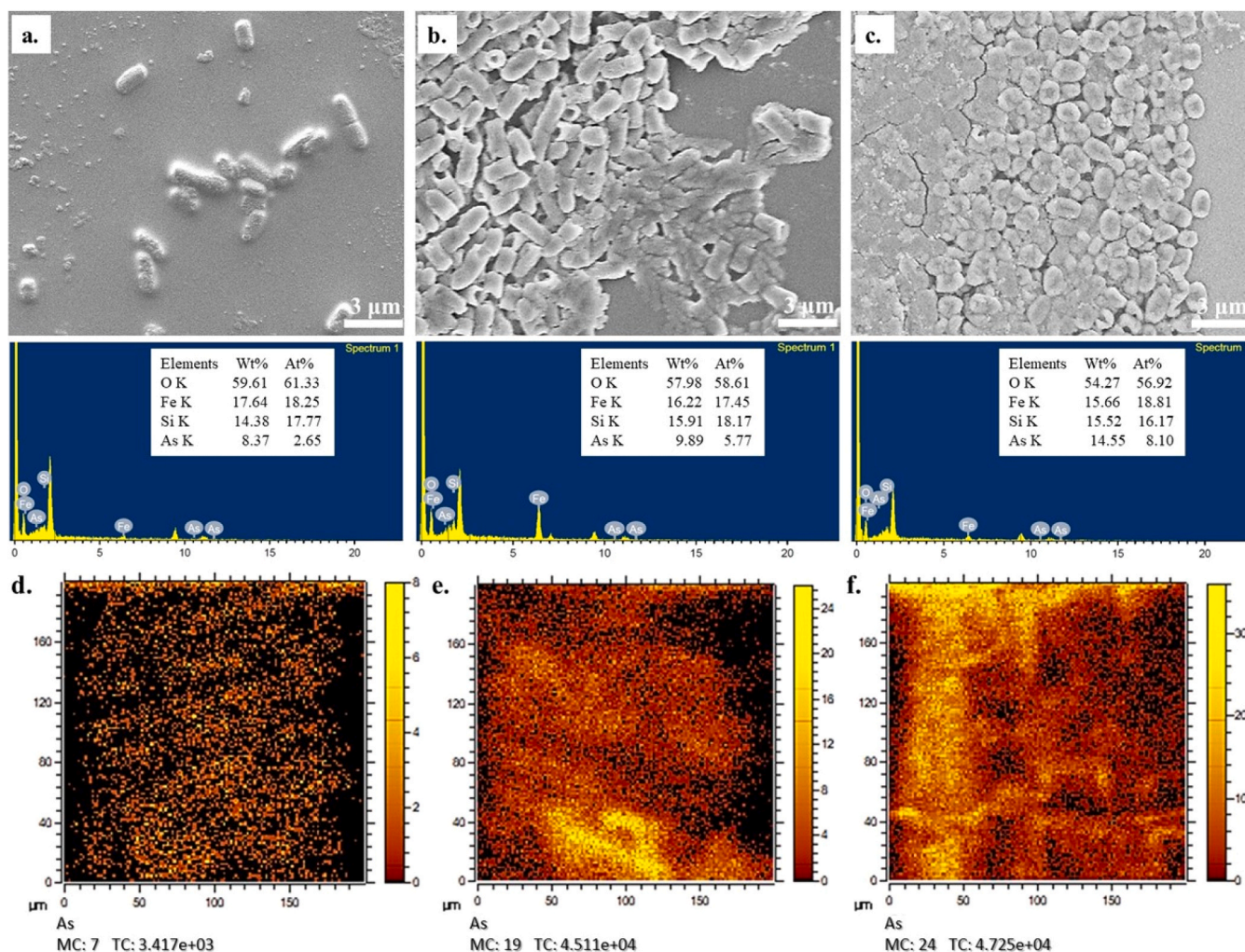


Fig. 2. Temperature-dependent biofilm formation in *B. subtilis* 168 and arsenic entrapment within the biofilm matrix. (a–c) Field emission scanning electron microscopy (FESEM) showing progressive biofilm intensification with increasing temperature, from sparse planktonic cells at 25°C (a) to intermediate microcolony formation at 30°C (b) to dense, mature biofilm architecture at 35°C (c). Scale bars = 3 μ m. Energy dispersive X-ray (EDX) analysis reveals elemental composition (wt %, at%), indicating consistent O, K, Fe, Si, and As enrichment across temperatures. (d–f) Time-of-flight secondary ion mass spectrometry (ToF-SIMS) elemental mapping of arsenic (As) distribution within biofilms at 25, 30, and 35°C, demonstrating progressive As accumulation (colour scale = ion counts, MC = mass calibration, TC = total counts). Yellow/orange intensity denotes high As concentrations localised within the extracellular polymeric substance matrix.

arsenic remains largely conserved across the five-month experiment, indicating EPS sequestration is robust enough to prevent net plant uptake; however, prolonged flooding could trigger biofilm degradation and release sequestered As(III), underscoring why AWD is now widely adopted in paddy agriculture. Indigenous soil hydrolases may degrade the EPS matrix, with elevated activity at $\geq 35^\circ\text{C}$ risking arsenic re-release; notably, the rise in organic matter-bound arsenic (F4: $9.4 \rightarrow 19.8 \text{ mg kg}^{-1}$) at 35°C suggests partial transformation into stable organo-metallic complexes that may resist remobilisation even after EPS turnover.

3.6. Gene expression and metabolic networks

Gene Ontology enrichment analysis revealed significant functional patterns across treatment conditions (Fig. 4a–b). Exopolysaccharide synthesis emerged most prominent (FDR $\sim 1.0 \times 10^{-27}$, ~ 20 genes), indicating biofilm matrix upregulation [56]. Signal transduction pathways showed enrichment (FDR $\sim 1.0 \times 10^{-12}$), particularly anti-repressor SinI and histidine kinases, indicating two-component regulatory system activation [57]. Heavy metal resistance pathways, including arsenical resistance and cadmium response, demonstrated coordinated metalloid

detoxification (FDR $\sim 1.0 \times 10^{-7}$ to 1.0×10^{-2}) [33]. Gene expression heatmap analysis revealed temperature-dependent patterns across 25–35°C (Fig. 4c–d). Arsenic resistance genes showed distinct regulation: *arsR* peaked at 25°C and 35°C (~ 2.5 – 2.9), *arsB* maximised at 30°C (~ 2.8), and *arsC* increased to 35°C (~ 2.5). Sublancin genes demonstrated complex regulation: *sunA* peaked at 25°C (~ 2.8) with reductions at higher temperatures [10], while *sunI* immunity gene maximised at 35°C (~ 2.7), suggesting temperature-dependent protection. Biofilm genes varied: *epsA* peaked at 30°C (~ 2.2), *tasA* at 25°C (~ 2.5), and *bslA* showed dual peaks at 25°C and 35°C (~ 2.0 – 2.3). STRING network analysis revealed distinct modules (Fig. 4e): biofilm genes (*tasA*, *epsA*, *bslA*) clustered with regulatory hubs (*spo0A*, *sinR*, *sinI*), while arsenic resistance and sublancin genes formed isolated peripheral modules [58], indicating independent regulation from biofilm networks [41,59]. STRING PPI network analysis (Fig. 4e) identified Spo0A and SinR as the top hub proteins, with SinR acting as a bottleneck node linking the core biofilm cluster (*tasA*, *epsA*, *bslA*) to the arsenic resistance module (*arsR*, *arsB*, *arsC*)—consistent with its established role as a master EPS and stress-response regulator in *B. subtilis*. KEGG enrichment at 35°C highlighted the two-component system (ko02020), PTS (ko02060), and bacterial secretion system (ko03070) pathways, with the *eps*, *sin*, and *ars*

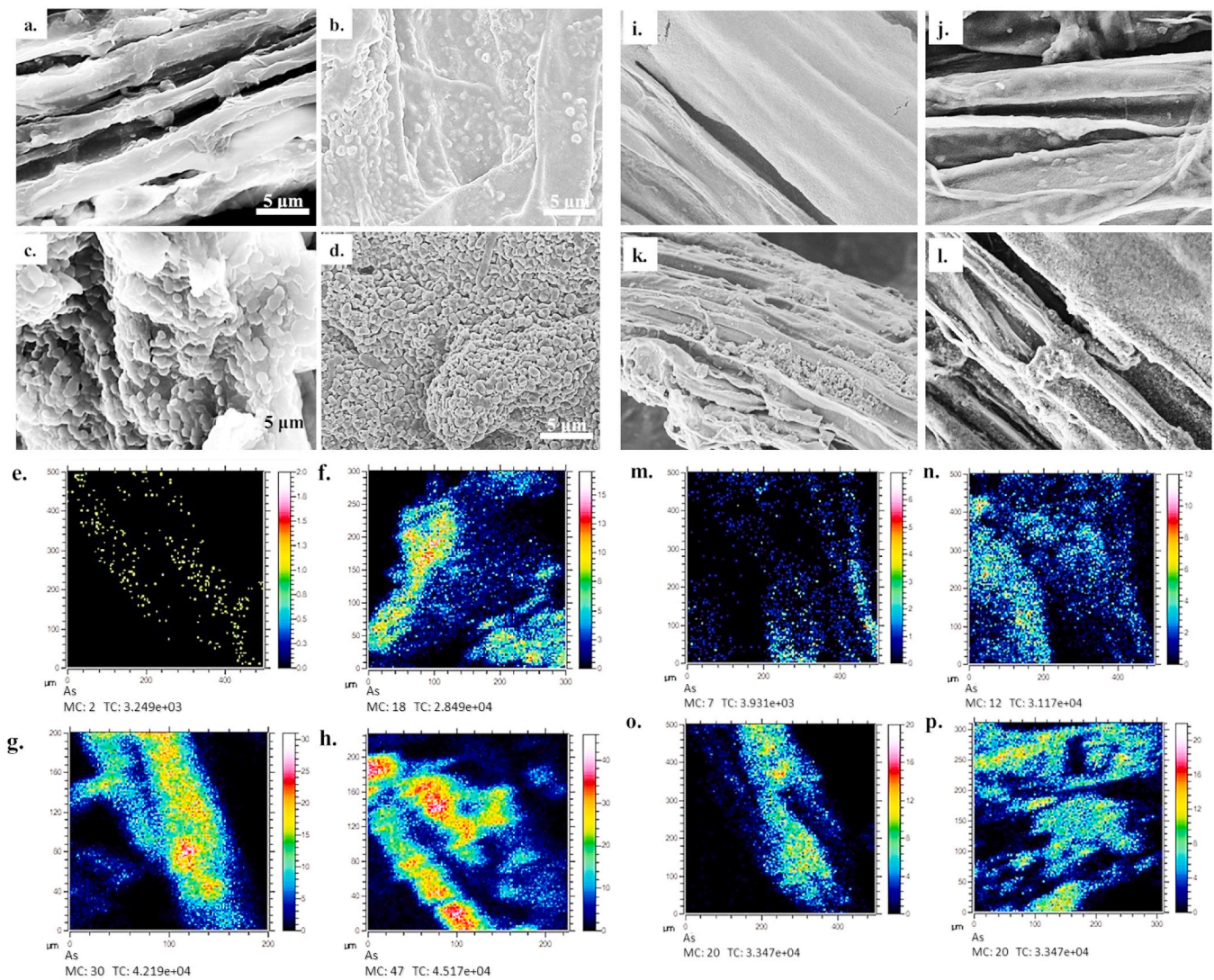


Fig. 3. Biofilm development on plant root surface and arsenic entrapment in greenhouse and field-grown plants. SEM images of the developed biofilm on control and three temperature-grown plant roots under greenhouse (a-d) and field trial (i-l) were further analysed for the As entrapment measured by the ToF-SIMS (e-h, greenhouse and m-p, field trial). These comparative analyses suggest that the *B. subtilis* biofilm can be deployed to efficiently capture rhizospheric As within its layers.

operons mechanistically co-regulated through the Spo0A master regulator, which integrates environmental stress signals to simultaneously activate biofilm formation and arsenic detoxification. Three temperature-dependent molecular mechanisms underpin this optimal range. First, Spo0A phosphorelay signalling is enhanced at 30–35°C, driving transcription of the *eps* and *tapA-sipW-tasA* operons for EPS and fibre matrix biosynthesis, while simultaneously suppressing AbrB to de-repress sublancin (*sun* locus) production. Second, SinI accumulates and titrates away the biofilm repressor SinR, permitting *epsA-O* and *tapA* transcription—corroborated by SinR downregulation and SinI enrichment in our STRING network (Fig. 4e) and gene expression heatmaps (Fig. 4c–d). Third, *arsR* upregulation at both 25°C and 35°C reflects constitutive arsenic stress sensing, and its indirect interaction with Spo0A-dependent pathways explains the co-upregulation of arsenic resistance and EPS biosynthesis genes at 35°C seen in GO enrichment ($FDR\ 10^{-22}$ – 10^{-27}), consistent with a coordinated Spo0A/SinR/SinI-mediated stress-biofilm adaptive response.

3.7. Interconnections between sublancin, PTS, and antibiotic resistance

Volcano plot analysis at 30°C revealed coordinated upregulation of PTS and sublancin systems (Fig. 5a–c and Supplementary Fig. 9–11). PTS

genes (*ptsI*, *ptsH*, *crh*) showed fold changes +2.0 to +2.5 $\log_2$ ($p < 0.001$), reflecting activated sugar metabolism and cellular regulation [60]. Sublancin genes exhibited even more dramatic changes: *sunA* demonstrated the highest fold change ($\sim +3.5 \log_2$, $p < 0.001$), with *sunI*, *sunT*, and *sunS* clustering at +2.5 to +3.0 $\log_2$, indicating coordinated biosynthesis machinery activation [61]. Simultaneous PTS and sublancin upregulation provides molecular evidence for functional interconnection, as sublancin sensitivity requires active PTS components [12]. Critically, antibiotic resistance genes (*vanA*, *aacA*, *blaTEM*) demonstrated significant downregulation (fold changes –2.5 to –4.0 $\log_2$, $p < 0.001$), suggesting conditions favouring sublancin production simultaneously suppress classical antibiotic resistance mechanisms. This inverse relationship may reflect competing cellular resource allocation or regulatory crosstalk [35]. This pattern suggests enhanced biofilm formation capacity as an adaptive response to environmental stressors. The volcano plot architecture demonstrates a clear biological response where environmental conditions favour both enhanced sugar transport/metabolism (via PTS upregulation) and antimicrobial peptide production (via sublancin system activation). This coordinated response may represent an adaptive strategy where increased metabolic capacity supports the energy-intensive process of antimicrobial peptide biosynthesis while simultaneously preparing cellular machinery necessary for

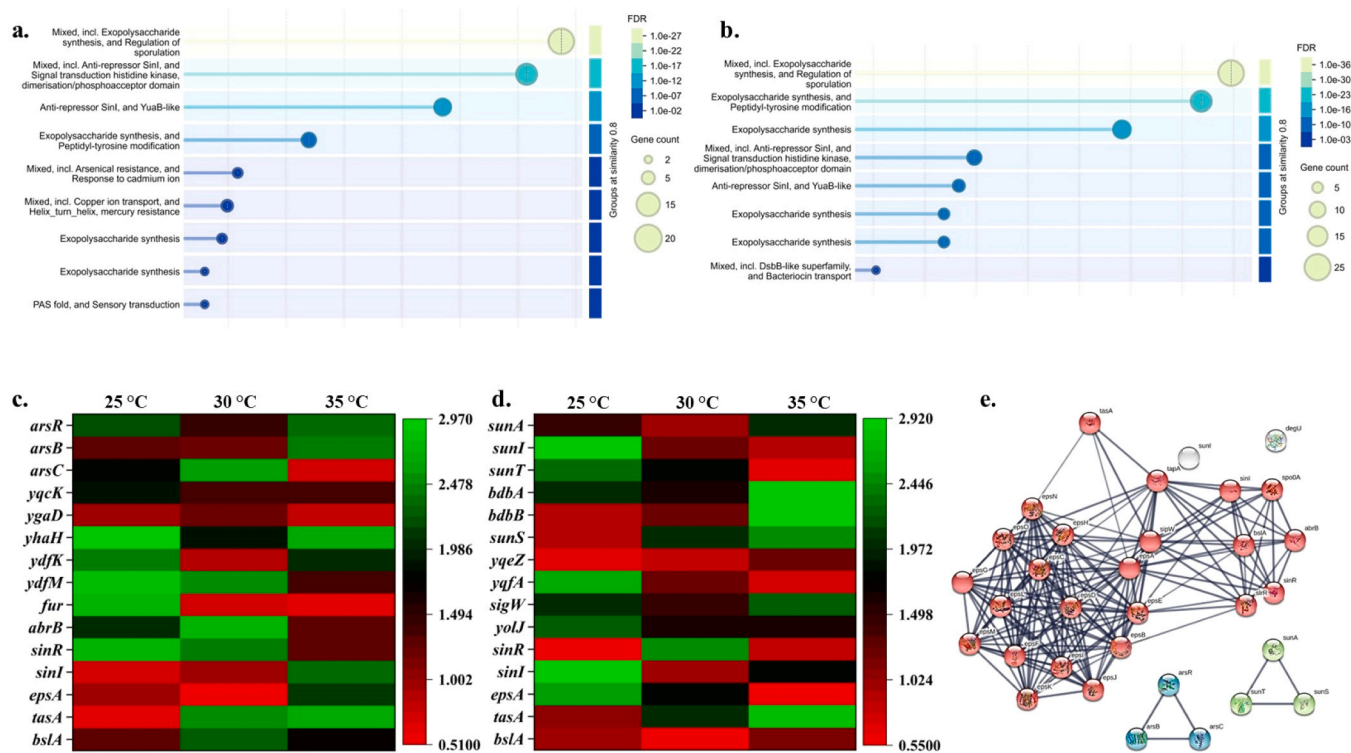


Fig. 4. Gene ontology enrichment and protein interaction networks linking arsenic metabolism, subblancin activity, and biofilm production. (a–b) Bubble plots showing enriched molecular functions associated with arsenic metabolism (a) and subblancin-responsive pathways (b), with bubble size representing gene count and colour intensity (FDR scale) indicating statistical significance. Prominent pathways include exopolysaccharide synthesis, anti-repressor signalling, and signal transduction cascades implicated in biofilm formation. (c–d) Heatmaps displaying temperature-dependent (25, 30, 35 °C) expression patterns of arsenic resistance genes (*arsRBC*, *yqaD*, *yhaH*), subblancin-associated genes (*sunA*, *sunI*, *sunT*, *bdbA/B*), and biofilm-producing genes (*eps*, *sin*, *tas*, *bsl*). Colour intensity represents log₂ fold-change (red = upregulation, green = downregulation, black = baseline). (e) STRING protein–protein interaction network demonstrating direct and indirect associations between arsenic-metabolising proteins (red nodes), subblancin biosynthesis enzymes (pink), and biofilm matrix proteins (green). Edge thickness indicates confidence scores. Network analysis reveals coordinated regulation of stress response, secondary metabolism, and extracellular matrix formation systems.

subblancin's unique mechanism of action that depends on functional PTS components for target cell killing. Additionally, PTS enrichment implicates HPr (*ptsH*)-mediated phosphorylation in modulating subblancin efficacy, and the co-upregulation of *ptsI*, *ptsH*, and *sunA* in volcano plots (Fig. 5b) provides transcriptomic, though not yet genetic, evidence of functional coupling between sugar metabolism and subblancin activity. Subblancin's selectivity for Gram-positive bacteria depends on the PTS: it requires phosphorylated HPr (P~His15-HPr, encoded by *ptsH*) for cellular entry and activation, as shown by the loss of activity in *ptsH* deletion mutants or upon competitive glucose addition [18]. The S-linked glucose moiety on subblancin (confirmed by ¹H NMR, Supplementary Fig. 7) likely facilitates entry via PTS sugar transporters (PtsG, MalT), while Gram-negative bacteria are intrinsically resistant due to outer membrane exclusion and fundamentally different PTS organisation.

Two non-mutually exclusive mechanisms link PTS upregulation to *vanA*/*bla*TEM downregulation. First, the high biosynthetic cost of subblancin production, requiring S-glycosylation, disulfide bond formation, and SunT-mediated export, diverts ATP and precursor metabolites away from energetically demanding resistance determinants; GO enrichment (Fig. 5d) confirms coordinated PTS and carbohydrate transporter upregulation (FDR 6.0×10^{-7} – 10^{-8}), consistent with resource competition suppressing *vanA* and *bla*TEM transcription at peak subblancin output (30 °C). Second, PTS activation drives carbon catabolite repression (CCR) through HPrK/P-mediated CcpA activation, which represses secondary metabolic genes, including certain resistance determinants, when preferred carbon sources are abundant, suggesting a CcpA-mediated regulatory state that prioritises primary carbohydrate metabolism and antimicrobial peptide production over constitutive ARG

maintenance, consistent with the Görke & Stülke (2008) CCR network. The inverse relationship between subblancin and antibiotic resistance gene expression may reflect competing cellular resource allocation or regulatory crosstalk between antimicrobial production and resistance systems. Previous studies have shown that subblancin production requires significant metabolic investment for post-translational modifications, including S-glycosylation and disulfide bond formation [12,35]. The simultaneous suppression of antibiotic resistance genes could represent metabolic prioritisation toward antimicrobial peptide biosynthesis under conditions that favour competitive antimicrobial production over defensive resistance mechanisms. Gene Ontology analysis (Fig. 5d) confirmed phosphotransferase enrichment ("protein-N (PI)-phosphohistidine-sugar phosphotransferase activity," FDR $\sim 6.0 \times 10^{-8}$) and transmembrane transport activities ("carbohydrate transmembrane transporter activity," FDR $\sim 6.0 \times 10^{-7}$), indicating coordinated cellular reprogramming emphasising carbohydrate metabolism and phosphoryl transfer systems [62]. The overall enrichment pattern suggests coordinated cellular reprogramming emphasizing carbohydrate transport and metabolism, phosphoryl transfer systems, and energy-dependent transport processes. Enrichment plots of biological processes and UniProt keywords are supportive towards these findings (Supplementary Figs. 12 and 13).

3.8. Subblancin effects on soil microbial communities

Box plot analysis (Fig. 6a) confirmed selective subblancin activity: Gram-positive bacteria showed significant abundance reduction with subblancin treatment (median ~ 1.0 vs. ~ 2.5 untreated, consistent with MIC $2\text{--}10 \mu\text{g mL}^{-1}$) [11]. This reduction in Gram-positive bacterial

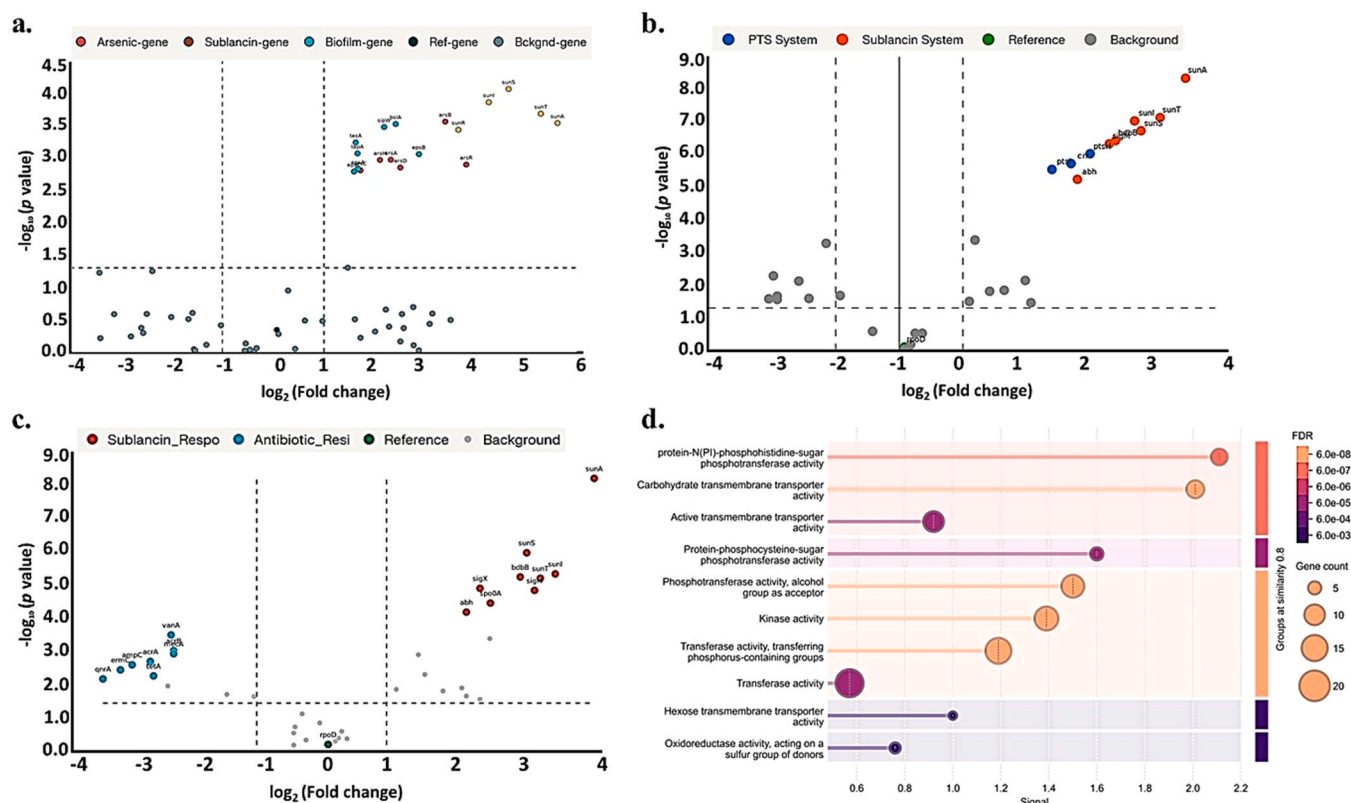


Fig. 5. Volcano plots depicting gene expression patterns and functional annotations in response to sublancin 168 exposure. (a) Combined regulatory response of arsenic-responsive (red), sublancin-responsive (brown), biofilm-associated (teal), reference (black), and background genes (grey) showing $\log_2$ fold-change versus $-\log_{10}$ (p-value). Vertical dashed lines demarcate $\pm 1 \log_2$ fold-change threshold; horizontal dashed line indicates $p = 0.05$ significance level. (b) Phosphotransferase system (PTS) gene regulation under PTS system treatment (blue), sublancin exposure (orange), reference condition (green), and background (grey). (c) Antibiotic resistance gene (ARG) expression comparing sublancin-responsive genes (red) versus antibiotic-resistant genes (teal), identifying co-regulated resistance mechanisms. (d) Gene ontology (GO) enrichment analysis revealing functional categories linked to fold-change magnitude and gene abundance (bubble size represents number of genes per functional category). Enriched molecular functions include phosphotransferase activity, carbohydrate/phosphate transport, and kinase activity. FDR-adjusted p-values (color intensity, right scale) indicate statistical significance of functional enrichment.

abundance reflects sublancin's well-characterised bactericidal activity against various Gram-positive species, including *Staphylococcus*, *Streptococcus*, and *Bacillus* species, with reported minimum inhibitory concentrations ranging from 2 to 10 $\mu\text{g/mL}$ [61,63]. Gram-negative bacteria demonstrated resistance, maintaining similar abundance regardless of treatment (median ~ 2.0 – 3.0), confirming sublancin lacks activity against Gram-negative bacteria due to outer membrane barriers and different PTS organisation [23]. **Supplementary Fig. 14** shows the Pearson correlation and regression analysis of both the Gram (+) and Gram (-) bacterial communities towards the sublancin application, with their presence and prevalence. Chord diagram analysis (**Fig. 6b-c**) revealed dramatic Gram-positive community restructuring following sublancin treatment, with substantial reductions in *Bacillus*, *Streptomyces*, *Clostridium*, and *Staphylococcus*, while Gram-negative taxa (*Escherichia*, *Pseudomonas*, *Acinetobacter*) maintained stability. This selective elimination may fundamentally alter microbial ecosystem balance, removing key Gram-positive functional groups while preserving Gram-negative metabolic capabilities. This selective reduction of susceptible Gram-positive bacteria in soil creates a nutrient pulse through microbial necromass release [64]. Sublancin-driven suppression of Gram-positive communities—evidenced by chord diagram restructuring (**Fig. 6b-c**), could over time reduce arsenic immobilisation capacity if arsenic-sequestering taxa are replaced; yet *B. subtilis* 168 persists via its intrinsic SunI immunity to sublancin, sustaining biofilm production. The four-fold increase in ARG abundance at 35°C under sublancin treatment (10–40 reads; **Fig. 6d-e**) reflects three converging mechanisms. First, sub-lethal sublancin concentrations—further reduced by thermal

instability above 35°C—selectively enrich PTS-mutant or efflux-upregulated Gram-positive survivors that disproportionately co-harbour multiple resistance determinants. Second, temperature independently drives ARG accumulation (15–30 reads without sublancin across 25–35°C), so thermal and sublancin-mediated selective pressures act additively at 35°C. Third, sub-bactericidal sublancin concentrations may induce SOS responses in tolerant bacteria, activating error-prone polymerases and transposases that facilitate ARG mobilisation and de novo resistance evolution. This controlled microbial population reduction further promotes soil health by: (1) eliminating pathogenic Gram-positive competitors, reducing disease pressure [65]; (2) liberating intracellular amino acids, nucleotides, and minerals, enriching soil nutrient pools [66]; (3) accumulating recalcitrant microbial biomass (amino sugars, peptidoglycans) as stable soil organic matter, improving soil structure and carbon sequestration [67]. Consequently, the application of sublancin supports sustainable soil fertility and crop productivity while minimising chemical inputs. Antibiotic resistance gene (ARG) abundance (**Fig. 6d-e**) showed complex temperature-sublancin interactions. Without sublancin, ARG abundance increased progressively with temperature (15 reads at 25°C to 30 reads at 35°C), reflecting enhanced horizontal gene transfer rates under thermal stress [68]. Sublancin treatment dramatically altered patterns, with ARG abundance increasing from ~ 10 reads at 25–30°C to ~ 40 reads at 35°C (four-fold enhancement), suggesting sublancin-induced selective pressure favouring bacteria carrying multiple resistance mechanisms [69]. This temperature-dependent ARG enrichment reflects multiple interconnected mechanisms, including enhanced horizontal

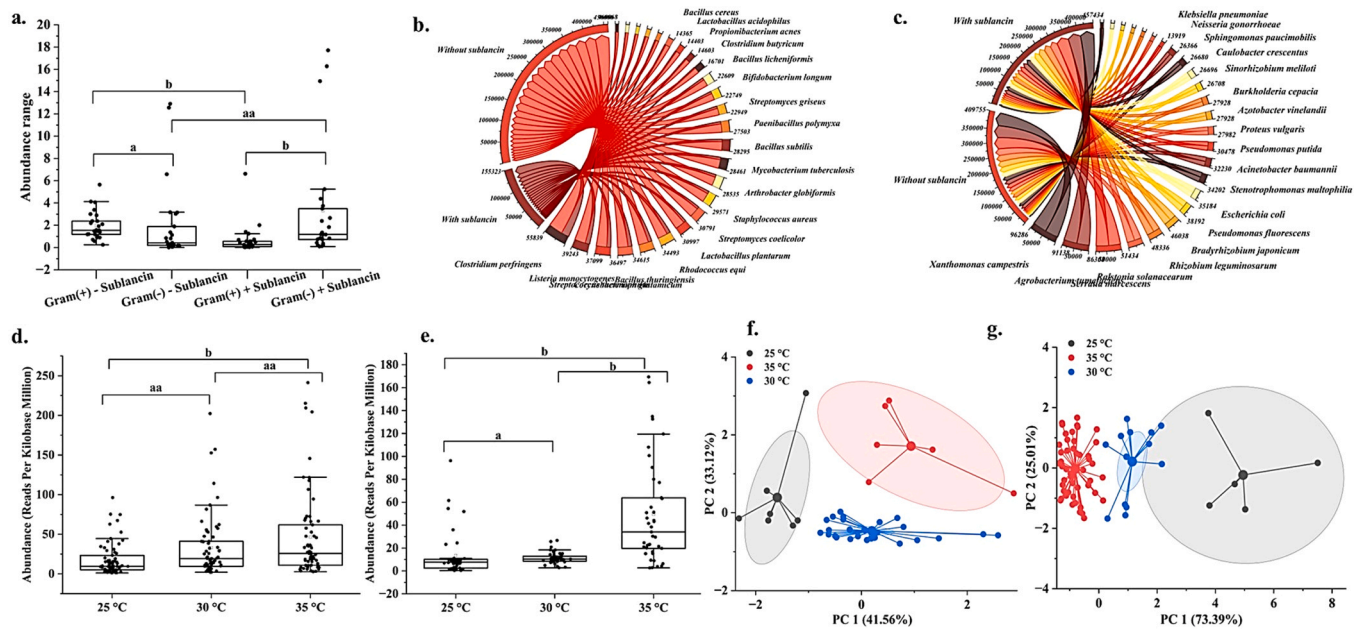


Fig. 6. Bacterial abundance, community composition, and antibiotic resistance gene (ARG) distribution in soil treated with sublancin 168. (a) Bacterial abundance (variance in reads per kilobase million, RPKM) in control soil (Gram+/- without sublancin) and sublancin-amended treatments (Gram+/-). (b-c) Chord diagrams displaying predominant bacterial taxa and their relative abundance in soil without (b) and with sublancin treatment (c), with connecting lines representing bacterial read abundance proportions. (d-e) ARG abundance profiling across temperature conditions (25, 30, 35°C) in unamended and sublancin-amended soils, showing suppressed ARG counts with sublancin treatment. (f-g) K-means cluster analysis combined with principal component analysis (PCA) demonstrating distinct microbial community clustering patterns at different temperatures. PC1 and PC2 axes explain 41.56% and 25.01% of variance, respectively. Different lowercase letters (a, b, aa) in box plots indicate statistically significant differences between conditions (one-way ANOVA, Tukey's post-hoc, $p < 0.05$). Data represent five biological replicates per treatment.

gene transfer rates, increased bacterial metabolic activity, and selective pressure favouring thermotolerant, resistance-carrying bacterial populations [70,71]. Sublancin treatment (right panel) dramatically altered ARG abundance patterns, with substantially higher overall resistance gene levels. Principal component analysis (PCA) (Fig. 6f-g) demonstrated distinct ARG clustering patterns. Without sublancin, PC1 explained 41.56% variance with moderate temperature-dependent clustering. Sublancin treatment enhanced separation (PC1: 73.39%), creating highly distinct, non-overlapping temperature clusters, suggesting antimicrobial peptide pressure amplifies temperature-specific ARG responses through coordinated stress response networks [72]. These findings underscore that climate warming may fundamentally alter antimicrobial peptide efficacy and ecological consequences, with different ARG responses expected under varying thermal conditions. Sublancin's selective suppression of Gram-positive bacteria may transiently reduce nitrogen mineralisation, as reflected in the 22–33% MBC reduction at 25–30°C, given that Actinobacteria and Firmicutes contribute disproportionately to protease-driven N cycling. However, this is ecologically offset by the preservation of Gram-negative diazotrophs and phosphate-solubilising PGPR (*Bradyrhizobium*, *Rhizobium*, *Azotobacter*; Fig. 6c), which are inherently resistant to sublancin; phosphorus cycling, dependent on both Gram-positive and Gram-negative phosphatase producers, may be partially affected and is flagged as a priority for future study. Regarding organic matter dynamics, cell lysis of susceptible Gram-positive bacteria releases peptidoglycan-rich necromass that may net-positively contribute to recalcitrant soil carbon, and the partial MBC recovery at 35°C (390 vs. 300 mg C kg⁻¹ at 30°C) suggests temperature-enhanced metabolic compensation over time. Finally, *B. subtilis* 168 is itself a well-documented PGPR producing IAA and solubilising phosphate, and its establishment as the dominant rhizosphere biofilm former (confirmed by SEM root colonisation, Fig. 3) effectively replaces pathogenic Gram-positive taxa with a beneficial organism, a net ecological advantage, though multi-season field studies tracking N/P cycling, enzyme activity, and PGPR community resilience

remain necessary before large-scale deployment.

3.9. Pathogenic bacterial reduction

Regarding broader resistance risks, sublancin-resistance mutations (e.g., *ptsH/ptsI* loss-of-function) impose a significant fitness cost by impairing sugar transport, partially constraining resistance spread under nutrient-limited field conditions; however, indirect cross-resistance to conventional antibiotics via shared regulatory networks (e.g., CcpA-mediated CCR) cannot be excluded. More critically, sublancin's Gram-positive selectivity preserves the entire Gram-negative community, including *E. coli*, *Pseudomonas*, and *Acinetobacter*, which could expand as Gram-positive competitors are suppressed, potentially increasing conjugative ARG flux among Gram-negative recipients over multiple seasons despite the 74.7% HGT reduction demonstrated in our conjugation assay. The application of sublancin to natural field soil elicited a highly selective reduction in pathogenic Gram-positive bacterial populations while preserving Gram-negative and beneficial Gram-positive communities (Supplementary Fig. 15). Among the 12 pathogenic Gram-positive species tested, all exhibited substantial CFU reductions relative to untreated controls. The most pronounced suppression was observed for *Clostridium tetani* (–85.5%), *Streptomyces scabies* (–82.3%), and *Clostridium perfringens* (–81.3%), followed by *Nocardia asteroides* (–80.5%), *Staphylococcus aureus* (–78.9%), *Bacillus cereus* (–79.0%), and *Streptococcus pyogenes* (–79.0%). Plant-pathogenic species including *Clavibacter michiganensis* (–77.9%) and *Rhodococcus fascians* (–78.3%) were similarly suppressed, while *Enterococcus faecalis* (–76.2%), *Enterococcus faecium* (–74.7%), and *Listeria monocytogenes* (–74.3%) exhibited significant yet comparatively lower reductions. The selectivity of sublancin towards Gram-positive bacteria is attributed to its dependence on an active phosphoenolpyruvate:sugar phosphotransferase system (PTS) for its bactericidal activity, which is ubiquitous in Gram-positive organisms [18,61]. Mechanistically, sublancin disrupts DNA replication, transcription, and protein biosynthesis through a deleterious

gain-of-function imposed on the glucose PTS, ultimately leading to cell death [43]. These findings collectively demonstrate that sublancin bio-amendment functions as a precision antimicrobial intervention in soil, selectively suppressing pathogenic Gram-positive bacteria, including both human opportunistic pathogens (*S. aureus*, *L. monocytogenes*, *E. faecium*) and phytopathogenic species (*C. michiganensis*, *S. scabiei*, *R. fascians*).

3.10. Bio-amendment effect on antibiotic resistance gene mobility

Conjugation assays quantifying HGT frequency of the ABR genes revealed substantial suppression of plasmid mobilisation following soil amendment (Fig. 7a-d). In 24 hr (Fig. 7a), Control microcosms (un-amended) achieved a mean HGT frequency of $6.64 \pm 0.68 \times 10^{-3}$ ($\log_{10}$: -2.18 ± 0.05), with absolute transconjugant abundance reaching $5.4 \pm 0.4 \times 10^4$ CFU g⁻¹ soil across three independent replicates. This frequency aligns with published conjugation rates in soil systems, where HGT typically operates at 10^{-3} to 10^{-6} frequency ranges [73].

Bio-amendment treatment (5% w/w) produced a dramatic reduction in plasmid transfer efficiency, decreasing HGT frequency to $1.68 \pm 0.16 \times 10^{-3}$ ($\log_{10}$: -2.77 ± 0.04) with corresponding transconjugant abundance of $1.3 \pm 0.2 \times 10^4$ CFU g⁻¹ soil. This represented a statistically significant 74.7% reduction in HGT frequency ($t = 8.42$, $p < 0.001$), equivalent to a 0.59 $\log_{10}$ unit decrease [74]. The difference in absolute transconjugant numbers was even more pronounced (75.9%

reduction), indicating that bio-amendment simultaneously reduced both recipient population density and conjugation efficiency [75]. A comparable trend was observed at 48 h (Fig. 7b), where the treatment group maintained suppressed conjugation relative to controls, confirming sustained inhibitory activity. The reduction in HGT frequency is attributable to the dual mechanism of sublancin action: selective bactericidal suppression of Gram-positive donor viability and impairment of conjugation machinery function [43,76]. Under greenhouse condition (Fig. 7c), donor strain (*B. subtilis* 168) survival differed substantially between conditions: control microcosms maintained 42.0% donor recovery (4.2×10^6 CFU g⁻¹), while bio-amended soil reduced donor survival to 28.0% (2.8×10^6 CFU g⁻¹), suggesting the amendment's antimicrobial activity selectively targeted the Gram-positive donor strain [77]. Recipient strain (*E. coli* MG1655, Gram-negative) demonstrated comparable survival between conditions (control: 82.0%, treatment: 79.0%), confirming the amendment's specificity for Gram-positive bacteria without affecting Gram-negative recipient populations. In field soils (Fig. 7d), bio-amended treatments at 35°C showed significantly lower HGT frequency than controls ($p < 0.05$), reinforcing the applicability of this approach under warming climate scenarios. The 74.7% HGT reduction reflects a hierarchical multi-mechanism process. The primary contributor (~50% of effect) is donor cell depletion: sublancin reduced viable *B. subtilis* 168 recovery from 4.2×10^7 to 2.8×10^7 CFU g⁻¹ (33% reduction; Fig. 7c), directly suppressing HGT frequency given its approximate linear scaling with donor abundance, while *E. coli*

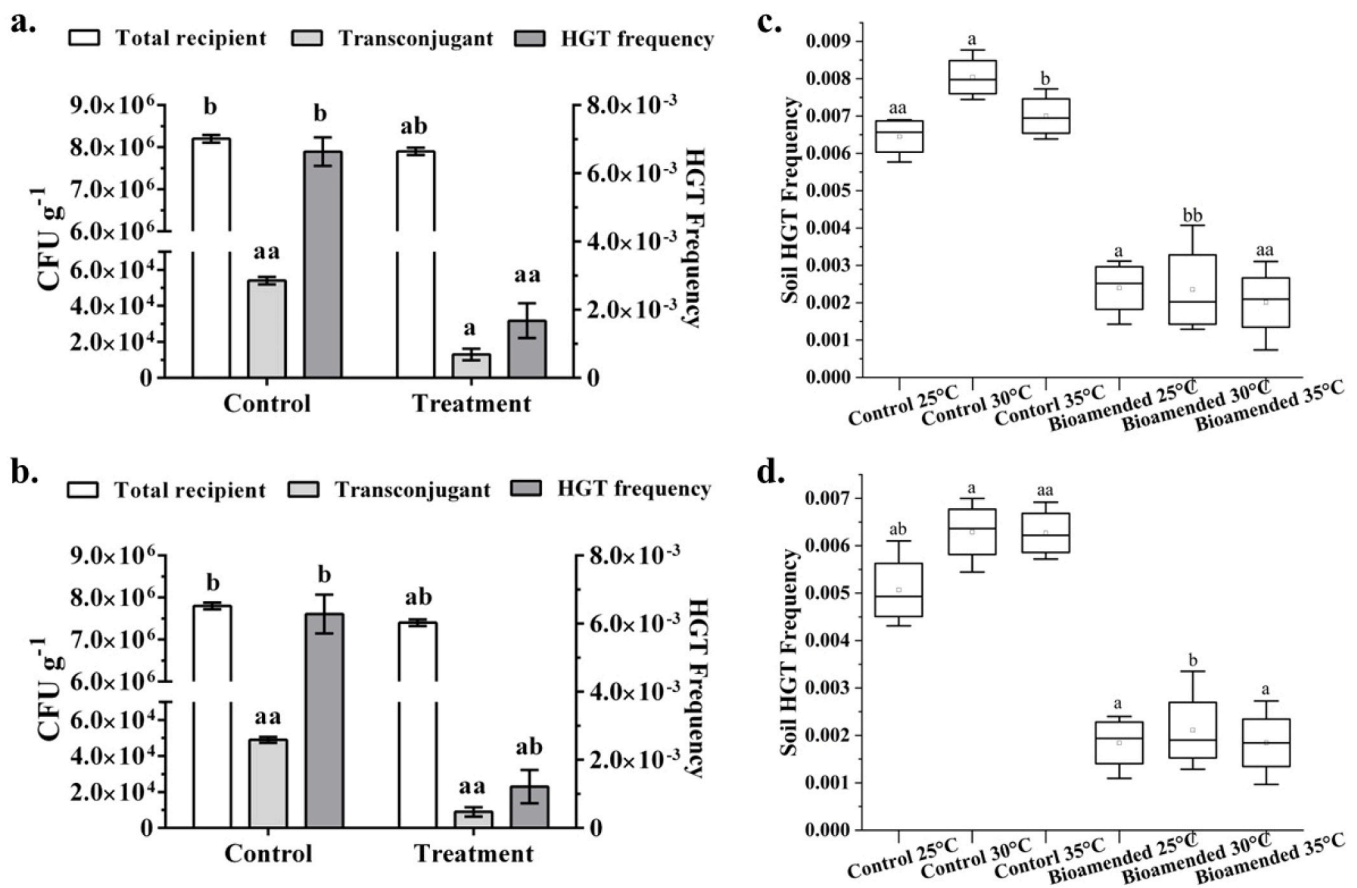


Fig. 7. Effect of sublancin on bacterial HGT frequency in media and soil systems. (a-b) Effect of bio-amendment on HGT frequency and microbial abundance in liquid culture at 24 hr and 48 hr. White, light grey, and dark grey bars represent total recipient CFU g⁻¹, transconjugant CFU g⁻¹, and HGT frequency (right Y-axis), respectively. Control microcosms achieved HGT frequency of $6.64 \pm 0.68 \times 10^{-3}$ with 5.4×10^4 transconjugants per gram. Bio-amendment (5% w/w) treatment reduced HGT frequency to $1.68 \pm 0.16 \times 10^{-3}$ (74.7% reduction, $p < 0.001$) and transconjugants to 1.3×10^4 g⁻¹. Different lowercase letters (a, b, aa) indicate statistically significant differences between conditions (ANOVA, Tukey's post-hoc test). (c-d) Temperature-dependent HGT frequency in greenhouse pot soil and field soil with and without bio-amendment. Box plots show soil HGT frequency across temperatures (25, 30, 35°C) for control and bio-amended treatments. Bio-amendment suppressed HGT frequency at all temperatures, with maximal reduction at 30°C. Different letters denote significant differences ($p < 0.05$). Data represent five independent replicates per treatment.

recipient survival remained comparable across conditions (82.0% vs. 79.0%), confirming Gram-positive selectivity. The secondary contributor (~25–30%) is impaired conjugation machinery: sub-lethal sublancin concentrations disrupt cell envelope integrity via PTS-mediated mechanisms, impairing mating pair formation (MPF) complex assembly, consistent with Paik et al. [76] and reports linking antimicrobial peptide-mediated membrane perturbation to reduced conjugation efficiency [78]. Tertiary contributions arise from energy limitation: PTS upregulation diverts ATP and proton-motive force away from the energy-intensive T4SS-like DNA transfer machinery, compounded by sublancin-linked ROS generation [12]—analogous to biochar-mediated HGT suppression via ATP depletion reported in recent literature. This mechanistic hierarchy is now explicitly stated in the revised manuscript. These findings are consistent with recent reports demonstrating that biochar and antimicrobial amendments can suppress conjugative ARG transfer in soil by reducing ATP-dependent energy supply, lowering cell membrane permeability, and diminishing donor-recipient contact efficiency [78]. This differential microbial impact explains the observed HGT reduction: decreased donor density combined with potential biofilm disruption [79] and reduced mating pair formation efficiency collectively suppressed *arsR* plasmid dissemination in bio-amended soil. These findings demonstrate that the proposed soil amendment produces dual benefits for resistance gene management: direct suppression of Gram-positive ABR-harboring populations while simultaneously reducing conjugation-mediated gene transfer efficiency, creating compounded protective effects against antibiotic resistance spread in agricultural systems [29]. The temperature-dependent efficacy of sublancin bio-amendment establishes it as a promising dual-function soil intervention capable of simultaneously suppressing antibiotic resistance dissemination and enhancing arsenic bioremediation under climate warming conditions.

4. Conclusion

This study establishes *Bacillus subtilis* 168-derived biofilm and sublancin as an integrated, dual-function bio-amendment for simultaneously mitigating arsenic contamination and antibiotic resistance gene (ARG) dissemination in climate-stressed agricultural soils. Temperature-optimised biofilm formation (25–35°C) enhanced arsenic sequestration within the EPS matrix by up to ~2250% (ToF-SIMS), with consistent efficacy across greenhouse and field trials (14-fold and 9-fold increases in root-associated arsenic entrapment, respectively). Sublancin functioned as a precision antimicrobial, selectively suppressing 12 pathogenic Gram-positive species by 74–86% through PTS-dependent bactericidal activity, while preserving Gram-negative communities and *B. subtilis* viability via SunI-mediated self-immunity. Conjugation assays confirmed a 74.7% reduction in HGT frequency ($p < 0.001$), sustained across all temperature regimes in both soil systems. Transcriptomic analyses revealed coordinated upregulation of sublancin biosynthesis and EPS production alongside downregulation of classical ARGs, indicating metabolic reprogramming that simultaneously favours antimicrobial peptide production and suppresses resistance determinants. The convergence of sublancin's optimal production temperature (30.8°C) with projected agricultural warming scenarios confers direct climate-adaptive relevance. These findings provide the first integrated evidence that a single microbial bio-amendment can concurrently arrest arsenic mobility and antibiotic resistance gene transfer under warming conditions, establishing a foundation for multifunctional soil management in contaminated agroecosystems.

Author's contribution

AM: conceptualisation, most experiments, analysis, writing & revision; IKL: HGT experiment, writing & revision; MB: supervision and revision; TRC: field trial & revision.

CRediT authorship contribution statement

Martin Buck: Writing – review & editing, Supervision, Resources. **Arnab Majumdar:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ioly Kotta-Loizou:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology. **Tarit Roychowdhury:** Writing – review & editing, Resources, Methodology.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.142339.

Data availability

Data will be made available on request.

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