List of Publications

2024

Functional OmpA of *Salmonella* Typhimurium provides protection from lysosomal degradation and inhibits autophagic processes in macrophages

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Roy Chowdhury, A., Hajra, D., Mukherjee, D., **Nair, A. V**., & Chakravortty, D. (2024). *The Journal of Infectious Diseases*, jiae376.

2024

Salmonella Typhimurium employs spermidine to exert protection against ROS-mediated cytotoxicity and rewires host polyamine metabolism to ameliorate its survival in macrophages

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Nair, A. V., Singh, A., Rajmani, R. S., & Chakravortty, D. (2024). *Redox biology*, 72, 103151.

2024

Spermidine constitutes a key determinant of motility and attachment of *Salmonella* Typhimurium through a novel regulatory mechanism

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Nair, A. V., Singh, A., Devasurmutt, Y., Rahman, S. A., Tatu, U. S., & Chakravortty, D. (2024). *Microbiological research*, 281, 127605.

2023

Decoding the invasive nature of a tropical pathogen of concern: The invasive non-Typhoidal *Salmonella* strains causing host-restricted extraintestinal infections worldwide.

Nair, A. V.*, Hajra, D.*, & Chakravortty, D. (2023). *Microbiological research*, *277*, 127488. (**equal contribution*)

2023

Absence of proline-peptide transporter YjiY in *Salmonella* Typhimurium leads to secretion of factors which inhibits intra-species biofilm formation.

Nair, A. V.*, Chandra, K.*, Chatterjee, R.*, Muralidhara, P., Singh, A., Kamanna, S., Tatu, U. S., & Chakravortty, D. (2023). Microbiological research, 273, 127411 (**equal contribution*)

2023

Syntaxin 3 SPI-2 dependent crosstalk facilitates the division of *Salmonella* containing vacuole.

Chatterjee, R., **Nair, A. V**., Singh, A., Mehta, N., Setty, S. R. G., & Chakravortty, D. (2023). *Traffic (Copenhagen, Denmark)*, *24*(7), 270–283.

2023

Salmonella Typhimurium PgtE is an essential arsenal to defend against the host resident antimicrobial peptides.

Chatterjee, R., Chowdhury, A. R., **Nair, A. V**.*, Hajra, D.*, Kar, A., Datey, A., Shankar, S., Mishra, R. K., Chandra, N., & Chakravortty, D. (2023). *Microbiological research*, 271, 12735 (**equal contribution*)

2022

Salmonella Typhimurium U32 peptidase, YdcP, promotes bacterial survival by conferring protection against in vitro and in vivo oxidative stress.

Nair, A. V.*, Hajra, D.*, Roy Chowdhury, A.*, Mukherjee, S., Chatterjee, R., & Chakravortty, D. (2022). *Microbial pathogenesis*, *173*(Pt B), 105862. (**equal contribution*)

2021

An elegant nano-injection machinery for sabotaging the host: Role of Type III secretion system in virulence of different human and animal pathogenic bacteria.

Nair, A. V.*, Hajra, D.*, & Chakravortty, D. (2021). *Physics of life reviews*, 38, 25–54. (**equal contribution*)

2019

Rhizospheric life of *Salmonella* requires flagella-driven motility and EPS-mediated attachment to organic matter and enables cross-kingdom invasion.

Karmakar, K., **Nair, A. V**., Chandrasekharan, G., Garai, P., Nath, U., Nataraj, K. N., N B, P., & Chakravortty, D. (2019). *FEMS microbiology ecology*, *95*(8), fiz107.

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Salmonella Typhimurium employs spermidine to exert protection against ROS-mediated cytotoxicity and rewires host polyamine metabolism to ameliorate its survival in macrophages

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ABSTRACT

Salmonella infection entails a cascade of attacks and defence measures. After breaching the intestinal epithelial barrier, *Salmonella* is phagocytosed by macrophages, where the bacteria encounter multiple stresses, to which it employs relevant countermeasures. Our study shows that, in *Salmonella*, the polyamine spermidine activates a stress response mechanism by regulating critical antioxidant genes. *Salmonella* Typhimurium mutants for spermidine transport and synthesis cannot mount an antioxidative response, resulting in high intracellular ROS levels. These mutants are also compromised in their ability to be phagocytosed by macrophages. Furthermore, it regulates a novel enzyme in *Salmonella*, Glutathionyl-spermidine synthetase (GspSA), which prevents the oxidation of proteins in *E. coli*. Moreover, the spermidine mutants and the GspSA mutant show significantly reduced survival in the presence of hydrogen peroxide *in vitro* and reduced organ burden in the mouse model of *Salmonella* infection. Conversely, in macrophages isolated from *gp91phox*$^{-/-}$ mice, we observed a rescue in the attenuated fold proliferation previously observed upon infection. We found that *Salmonella* upregulates polyamine biosynthesis in the host through its effectors from SPI-1 and SPI-2, which addresses the attenuated proliferation observed in spermidine transport mutants. Thus, inhibition of this pathway in the host abrogates the proliferation of *Salmonella* Typhimurium in macrophages. From a therapeutic perspective, inhibiting host polyamine biosynthesis using an FDA-approved chemopreventive drug, D, L-α-difluoromethylornithine (DFMO), reduces *Salmonella* colonisation and tissue damage in the mouse model of infection while enhancing the survival of infected mice. Therefore, our work provides a mechanistic insight into the critical role of spermidine in stress resistance of *Salmonella*. It also reveals a bacterial strategy in modulating host metabolism to promote their intracellular survival and shows the potential of DFMO to curb *Salmonella* infection.

1. Introduction

An optimal microbial growth condition has signatures of ample nutrients and ambient temperature, pH, oxygen concentration, and osmolarity. Any disturbances in one or more of these parameters can be tagged as a stress condition, detrimental to microbial survival and growth. Given the fluctuations of these parameters are nature-driven [1], the microbes need to be adept at sensing, responding, and adapting to unprecedented situations [2]. Food-borne pathogens deal with an array of stresses in their dwelling environments, stemming from natural to commercial causes and host systems [3–5]. *Salmonella* is a food-borne pathogen that enters the host system through contaminated food and water. While traversing to the intestine, *Salmonella* faces multiple stressful conditions such as low pH, nutrient deprivation, bile salt stress, competition with the resident microbes of the gut and immunoglobulins, etc. Once it breaches the epithelial barrier, it is taken up by the macrophages at the lamina propria, through which it disseminates throughout the host system. Entry of the pathogen into the macrophage leads to a burst of reactive oxygen species (ROS) generated by the NADPH phagocytic oxidase (Nox2) and reactive nitrogen species (RNS) generated majorly by inducible Nitric Oxide synthase (Nos2) [6,7]. In macrophages, *Salmonella* resides in a specialized niche called the

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Salmonella-containing vacuole (SCV), which presents multiple other stresses to the bacteria, such as acidification, nutrient limitation, and attack by the antimicrobial peptides. However, *Salmonella* employs numerous weapons from its arsenal to counteract those stresses.

Polyamines are a group of primordial stress response molecules in prokaryotes and eukaryotes [8]. Multiple research groups have elucidated the link between polyamines and bacterial stress response. In *Shigella spp.* the polyamine spermidine accumulates during its infection into macrophages, which increases the expression of *katG* and helps in bacterial antioxidant response [9]. Another study demonstrated that spermidine localises to the surface of *Pseudomonas aeruginosa* and binds to the lipopolysaccharide (LPS) to protect the cells from oxidative damage [10]. In the Gram-positive bacteria *Streptococcus pyogenes,* extracellular spermidine enhances the survival of the bacteria by upregulating oxidative response genes [11]. Espinel IC *et al.*, has shown that polyamines are vital in resistance against nitrosative stress in *Salmonella* Typhimurium. Further, the group showed that spermidine is required for the systemic infection of *Salmonella* Typhimurium in mice [12]. Our previous studies further indicated that *Salmonella* upregulates the spermidine transporter genes (*potA, potB, potC* and *potD*) and the biosynthesis genes (*speE* and *speD*) during the log phase of growth *in vitro* [13,14]. Thus, it can be inferred that *Salmonella* Typhimurium utilizes polyamines, such as spermidine, as a stress response molecule; however, the mechanism remains elusive.

Here, we show that spermidine biosynthesis and transport mutants of *Salmonella* Typhimurium exhibit reduced survival upon infection in RAW264.7 cells. This diminished proliferation is also observed in mice models of *Salmonella* infection, which is rescued in *gp91phox*$^{-/-}$ mice. We demonstrate that spermidine manipulates the various arms of antioxidative response and tightly regulates intracellular ROS levels. We further identify a novel antioxidative enzyme, GspSA, in *Salmonella* Typhimurium, which is regulated by spermidine. The primary question that remains is why the transporter mutant shows reduced survival. To this, we find that *Salmonella* Typhimurium harnesses the host polyamine biosynthesis for its survival. Furthermore, for the first time, we show that an FDA-approved chemopreventive and anti-African Human Trypanosomiasis drug that inhibits polyamine biosynthesis in the host can curb *Salmonella* infection in mice.

2. Material and methods

2.1. Bacterial strains and growth condition

Salmonella enterica serovars Typhimurium (STM WT) wild-type strain ATCC 14028s was used in all experiments, which was a kind gift from Prof. Michael Hensel, Abteilung Mikrobiologie, Universität Osnabrück, 273 Osnabrück, Germany. The bacterial strain was cultured in Luria broth (LB-Himedia) with constant shaking (175 rpm) at 37 °C orbital-shaker. Kanamycin, Chloramphenicol and Ampicillin antibiotics (Final working concentrations of Kanamycin-50 μg/ml, Chloramphenicol-20 μg/ml and Ampicillin-50 μg/ml) were used wherever required. Strains were transformed with a pFPV-m-cherry plasmid for immunofluorescence assays. For the supplementation study, the bacterial strains were grown in Luria broth with 100 μM spermidine (Sigma) addition with constant shaking (175 rpm) at 37 °C orbital-shaker overnight (Bacterial strain list in Supplementary table S- Table 1).

2.2. Bacterial gene knock-out and strain generation

The generation of gene knockout in bacteria was done using the One-step chromosomal gene inactivation protocol based on the principle of homologous recombination [15]. The plasmids pKD4 and pKD3 serve as a template for amplifying the Kanamycin resistance gene (Kan^R) and the Chloramphenicol resistance gene (Chlm^R). Briefly, the Kanamycin resistance gene (Kan^R) and the Chloramphenicol resistance gene (Chlm^R) amplified products were purified using chloroform-ethanol precipitation. The Kan^R was used to generate STM *ΔpotCD* (*pot*-spermidine transporter mutant), and Chlm^R was used to generate STM *ΔspeED* (*spe*-spermidine biosynthesis mutant). This was followed by electroporation into the STM WT cells expressing pKD-46 plasmid, which provides the λ-Red recombinase system required for the homologous recombination. The electroporation was carried out by applying a single pulse of 2.25 kV for each sample. The transformant colonies were selected and patched on fresh plates with the required antibiotic for a further selection of the transformants and confirmed for knockout using PCR with primers designed corresponding to the ~100bp upstream and downstream of the genes (knocked out) for the knockout strains to observe a difference in PCR product size upon STM *ΔpotCD* and STM *ΔspeED* knockout generation. For the generation of the double knock-out strain (STM *ΔpotCDΔspeED,* spermidine transporter and biosynthesis double mutant), the STM *ΔpotCD* (resistant to Kanamycin) was first transformed with the plasmid pKD-46, which provides the λ-Red recombinase system. To this transformed strain, the purified PCR product to knock-out *speED* was electroporated to generate the STM *ΔpotCDΔspeED* (resistant to Kanamycin and Chloramphenicol). For the generation of STM *Δgsp* (Glutathionyl-spermidine synthetase mutant), the Kanamycin resistance gene was amplified from pKD4 plasmid, and a similar protocol was used, followed by selection on Kanamycin containing LB agar plates. To generate double *gsp* spermidine mutants, STM *Δgsp* was electroporated with purified PCR product to knock out *speED* and *potCD* (both Chloramphenicol resistance cassettes) (Knock-out generation Primer list in Supplementary table S- Table 2).

2.3. Cell culture and maintenance

RAW264.7 cells (murine macrophage cell line) were cultured in DMEM - Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% FBS (Gibco) and 1% Penicillin-streptomycin (Sigma- Aldrich) at 37 °C humidified chamber (Panasonic) with 5% CO_2. For each experiment, the cells were seeded onto the appropriate cell culture well plate at a confluency of 80% for intracellular survival assay and expression studies.

2.4. Gentamicin protection assay

The cells were infected with STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG* (all strains were grown overnight in LB medium) at Multiplicity of Infection (MOI) of 10 (for intracellular survival assay) and MOI 25 (for qRT-PCR). After infecting the cell line with STM WT and the mutants, the plate was centrifuged at 900 rpm (100×*g*) (Rota-Superspin R–NV swing bucket centrifuge) for 10 min to facilitate the proper adhesion. The plate was then incubated for 25 minutes at 37 °C in a humidified chamber and 5% CO_2. Then, the media was removed from the wells and washed with 1X PBS. Fresh media containing 100 μg/mL gentamicin was added and incubated for 60 minutes at 37 °C and 5% CO_2. The media was removed, cells were washed with 1X PBS twice, and fresh media containing 25 μg/mL gentamicin was added. The plate was incubated at 37 °C and 5% CO_2 till the appropriate time. For the intracellular survival assay, two time points were considered 2 hours and 16 hours; for qRT-PCR, three time points were considered 2 hours, 6 hours, and 16 hours.

For the Opsonisation assay, the overnight grown bacterial strains were washed with 1X PBS. The washed cells (for each strain) were treated with the opsonin, here mouse complement sera, for 1 hour at 37 °C. Mouse complement-sera acts as an opsonin and thus potentiates the interaction of the bacteria with the macrophages. The treated bacterial strains were then used for infection into RAW264.7 macrophages at MOI of 50 as per the described gentamicin protection assay above and the percentage phagocytosis was determined [16].

Also, for studying the effect of exogenous spermidine in the recovery of the phenotype of mutant strains, the strains were grown *in vitro* with supplementation of 100 μM spermidine (described previously in the

"Bacterial strains and growth" section). The grown cultures were then used for infection of RAW264.7 cells using the gentamicin protection assay.

2.5. Intracellular survival assay and phagocytosis assay

At the appropriate time post-infection, the cells were lysed using 0.1% Triton X, followed by adding more 1X PBS and samples were collected. The collected samples were plated at the required dilutions on LB agar plates and kept at 37 °C. Twelve hours post incubation, the Colony forming units (CFU) were enumerated for each plate.

The fold proliferation and invasion were determined as follows:

Fold Proliferation = (CFU at 16 h post-infection)/(CFU at 2 h post-infection)

Percentage Phagocytosis = [(CFU at 2 h post-infection)/(CFU of the Pre-inoculum)] × 100.

2.6. RNA isolation and qRT-PCR

RNA isolation was performed from infected cells after appropriate hours of infection with STM WT, STM *ΔpotCD, and* STM *ΔspeED* by RNA isolation using TRIzol (from TaKaRa, RNA isoPlus-9109) reagent according to the manufacturer's protocol. RNA was quantified using Thermo-fisher scientific Nano Drop followed by running on 2% agarose gel to check the quality. For cDNA synthesis, the first DNase I treatment with 3 μg of isolated RNA was done at 37 °C for 60 minutes, which was then stopped by heating at 65 °C for 10 minutes. Then RNA (free of DNA) was subjected to Reverse transcription using Random hexamer, 5X RT buffer, RT enzyme, dNTPs, and DEPC treated water at 37 °C for 15 minutes, followed by heating at 85 °C for 15seconds as per manufacturer's protocol (from TaKaRa, PrimeScript RTKit-RR037A). Quantitative real-time PCR was done using the SYBR green RT-PCR kit (from TaKaRa, TB Green Premix Ex Taq (Tli RNaseH Plus)-RR420A) in the BioRad qRT-PCR system (BioRad CFX96 Touch Real-Time PCR Detection System). A 384-well plate with three replicates for each sample was used. The gene expression levels of interest were measured using specific RT primers. Gene expression levels were normalized to 16SrRNA primers of *S*. Typhimurium. Gene expression levels of the eukaryotic gene of interest were normalized to beta-actin of mouse/human as required. (Expression Primer list in Supplementary table S-Table 2).

For expression studies in bacteria grown in LB media, the bacterial samples were harvested at 3 hours, 6 hours, 9 hours, and 12 hours post subculture in fresh LB media in a 1:100 ratio, and 1 mM H_2O_2 was added to the broth to study the gene expression in the presence of oxidative stress. Then, a similar protocol was used to isolate total RNA using TRIzol (from TaKaRa, RNA isoPlus-9109) reagent according to the manufacturer's protocol (Expression Primer list in Supplementary table S-Table 2).

For expression studies in the liver and spleen, the tissues from the respective organs were collected in TRIzol (from TaKaRa, RNA isoPlus-9109), five days post oral gavage of 10^7 CFU of STM WT bacteria. The tissues were homogenized using 1 mm Glass beads in a Bead-beater (BioSpec products). The homogenized lysate was collected, and total RNA isolation and cDNA preparation were performed as previously described (Expression Primer list in Supplementary table S-Table 2).

2.7. Primary macrophage isolation and infection

Primary macrophages were isolated using a previously established protocol [17,18] from C57BL/6J mice (The Jackson's Laboratory, USA, male, 5–6 weeks old), *gp91phox* knockout mice and *iNOS* knockout mice (all knock-out mice are in C57BL/6J background procured from The Jackson's Laboratory, USA). The mice were intraperitoneally injected with 8% Brewer's Thioglycolate (from HiMedia). Five days post-injection, the primary macrophages were isolated by injecting ice-cold 1X PBS into the peritoneal cavity, and the peritoneal lavage was collected. Any residual erythrocytes were lysed using an RBC lysis buffer (Sigma- R7757), and the isolated cells were maintained in a complete RPMI 1640 medium for further experiments.

2.8. Intracellular reactive oxygen species determination using H_2DCFDA staining

Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then, 10^8 CFU/ml of each strain was incubated with 10 μM of 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Sigma) in 1X PBS (pH 7.2) at 37 °C for 30 minutes. The bacterial cells were centrifuged, and the cells were resuspended in 1X PBS (pH 7.2) with Hydrogen peroxide of different concentrations (0- 10 mM) and incubated at 37 °C (orbital shaker) for 2 hours. The samples were transferred to a 96-well ELISA plate, and fluorescence was determined in Tecan-ELISA plate reader Infinite series 200 (Ex- 490nm/Em- 520 nm).

2.9. Intracellular redox status determination

The STM WT, STM *ΔpotCD,* STM *ΔspeED* and STM *Δgsp* were transformed with pQE60-Grx1-roGFP2 (a gift from Prof. Amit Singh, CIDR, IISc). roGFP2 is a genetically modified form of GFP (GFP ex 488 nm) that harbours two cysteine residues, and upon oxidation, the cysteine residues form a disulphide bond, and the excitation shifts to 408 nm [19,20]. Briefly, overnight cultures were subcultured in fresh LB media (with 500 μM IPTG to induce roGFP2). Once the cultures reached OD 0.1 then, 10^8 CFU/ml of each strain was incubated with Hydrogen peroxide (0mM–5mM) of different concentrations in 1XPBS (pH 7.2) with and incubated 37 °C (orbital shaker) for 2 hours. The samples were centrifuged and resuspended in fresh 1X PBS (pH 7.2). Tubes were analysed for GFP fluorescence at 408 nm and 488 nm, respectively, using a BD-FACS Verse flow cytometer (total of 10,000 events for each sample). We have measured fluorescence intensity at 405 nm and 488 nm for each sample. During quantification, we gated for the live cells (P1), followed by the singlet population (P2). Finally, we enumerated the ratio of the mean intensity at 405 nm–488 nm from the final gated population (P2) for each sample. The higher intensity at 405 nm represents an oxidative environment. Thus, the ratio of 405/488 determines the degree of oxidation. Higher intensity at 405 nm indicates an oxidative environment. To determine intracellular redox status upon infection into RAW264.7 cells, 10^5 RAW264.7 cells seeded in a 24-well tissue culture plate were infected with each strain harbouring the roGFP2(induced with IPTG similarly) plasmid using gentamicin protection assay. After 16 hours post-infection, the macrophages were washed with 1X PBS and scrapped off using a cell scraper and analysed for GFP fluorescence at 405 nm and 488 nm, respectively, using BD-FACS Verse flow cytometer (total 10,000 events for each sample). As previously explained, we quantified the mean fluorescence intensity at 488 nm and 405 nm for each sample. Then we enumerated the ratio of the mean intensity at 405 nm–488 nm from the final gated population (P2) for each sample. Higher intensity at 405 nm indicates an oxidative environment.

2.10. In vitro sensitivity assays

Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then 10^8 CFU/ml of each strain was incubated with Hydrogen peroxide of different concentrations (0 mM–10 mM) in 1XPBS (pH 7.2) and incubated 37 °C (orbital shaker) for 2 hours. The samples were plated on SS agar to enumerate the CFU, and the percentage survival was determined as follows:

Percentage Survival: [[CFU/ml for treated with H_2O_2/ $NaNO_2$]/ [CFU/ml for untreated]] × 100

The bacterial samples were prepared to determine the nitrite sensitivity, similar to the hydrogen peroxide sensitivity assay described

above. Each strain was incubated with sodium nitrite (0 mM–10 mM) in 1X PBS of pH 5.4 to facilitate dissociation into nitrite ions and incubated 37 °C (orbital shaker) for 2 hours. The samples were plated on SS agar to enumerate the CFU, and the percentage survival was determined as previously described for hydrogen peroxide sensitivity assays. For dual hydrogen peroxide and sodium nitrite assays, the strains were incubated with both hydrogen peroxide and sodium nitrite (0 mM–10 mM concentrations of each stress molecule).

Resazurin assay was used to determine the viable cells and was performed in 96 well plates (triplicate for each sample and concentration. Briefly, after incubation for 2 hours as previously performed, Resazurin (0.2 mg/ml in 1X PBS) was added (1:10 ratio) to each well of 96 well plates. The plate was incubated at 37 °C in a shaker incubator for 2 hours. The fluorescence was measured using a Tecan plate reader infinite series 200, with Ex-520nm and Em- 590 nm. The values were obtained as Relative fluorescence units (RFU), and the percentage survival was determined as.

Percentage survival = [RFU of the sample with added hydrogen peroxide]/[RFU of the sample without hydrogen peroxide]

2.11. Immunoblotting

The bacterial strains were grown in LB media with added 1 mM hydrogen peroxide until the log phase of growth. The cells were centrifuged to remove the media, and the cells were resuspended in the lysis buffer (Sodium chloride, Tris, EDTA, 10% protease inhibitor cocktail (Roche, 04693116001)) after washing with 1X PBS. The cells were lysed using sonication and centrifuged at 4 °C to collect the cell lysate, followed by estimation of total protein using the Bradford protein estimation method. 50 μg of protein was loaded onto a Polyacrylamide Gel Electrophoresis (PAGE) (12% Resolving gel) without β-mercaptoethanol (prevent di-sulphide bond breakage as glutathionyl-spermidine modifies Cysteine residues through a disulphide bond), then transferred onto 0.45 μm PVDF membrane (GE Healthcare). 5% skimmed milk (Hi-Media) in TBST was used to block for 1 hour at room temperature and then probed with Anti-Spermidine primary antibody (Novus Biologicals, NB100-1847, 1:2000 dilution in 2.5% BSA) and secondary HRP-conjugated antibody (anti-rabbit IgG HRP linked Cell Signaling Technology #7074, 1:2000 dilution in 2.5% BSA). ECL (BioRad) was used to develop the blot, and images were captured using Chemi-Doc (BioRad). All densitometric analysis was performed using Image J. The normalisation was done with respect to the Ponceau S stained blot.

For studying the expression on iNOS, the RAW264.7 macrophages were washed with PBS and collected using cell scrapper, after infection with STM WT (MOI 10). The cells were centrifuged at 300g at 4 °C for 10 minutes. The cells were resuspended in RIPA lysis buffer supplemented with 10% protease inhibitor cocktail (Roche, 04693116001) and incubated on ice for 30 minutes. Total protein was estimated using Bradford estimation. 100 μg of the protein samples were loaded onto a Polyacrylamide Gel Electrophoresis (PAGE) (8% Resolving gel for larger size of iNOS). Then transferred onto 0.45 μm PVDF membrane (GE Healthcare). 5% skimmed milk (Hi-Media) in TBST was used to block for 1 hour at room temperature followed by probing with Anti-iNOS antibody (Sigma, SAB4502011, 1:1000 dilution in 2.5% BSA) and HRP-conjugated antibody (anti-rabbit IgG HRP linked Cell Signaling Technology #7074, 1:2000 dilution in 2.5% BSA). ECL (BioRad) was used to develop the blot, and images were captured using Chemi-Doc (BioRad). All densitometric analysis was performed using Image J.

2.12. Transfection for knockdown

To ensure maximum knockdown, we have targeted the 3'-UTR of Odc1 and Srm from mouse. We used CLUSTAL OMEGA to align and identify the 3'-UTR complementary regions. We chose two sequences for each gene. For transfection, RAW 264.7 cells were seeded at a 50–60% confluency 12 hours before transfecting using PEI (1:2 -DNA: PEI) [18, 21]. We used the two different constructs for the knockdown of Odc1, A7 and A8 and both as a mixed construct (A7:A8 at 1:1 ratio) and similarly for Srm, E9 and F1 and both as a mixed construct (E9:F1 at 1:1 ratio) from the Sigma Mission shRNA library. 400 ng of plasmid DNA/well (ratio 260/280–1.8–1.9) was used for transfection in a 24well plate. Cells were then incubated for 8 hours at 37 °C in a humidified incubator with 5%CO_2; after that, the media containing transfecting DNA and reagents were removed, and cells were further incubated for 48 hours in complete media DMEM +10% FBS. Cells were harvested for further analysis or infected with the required MOI using the gentamicin protection assay (shRNA sequence list in Supplementary table S-Table 3).

2.13. Immunofluorescence

After the appropriate incubation time after the gentamicin protection assay, the media was removed, and the cells were washed with 1X PBS and fixed with 3.5% Paraformaldehyde(PFA) for 10 min. The cells were washed with 1X PBS, incubated with the required primary antibodies [anti-mouseLAMP1(1D4B, DSHB,1:100 dilution) and anti-Spermidine (anti-spermidine Novus Biologicals, NB100-1847, 1:100 dilution)] in a buffer containing 0.01% saponin and 2% BSA, and incubated at room temperature for 45–60 min. After washing with 1XPBS, the secondary antibody tagged to a fluorochrome was added and incubated [anti-rat-Alexafluor488(#112-547-003 Jacksons Immunoresearch Lab Inc. 1:200 dilution) for LAMP1, anti-rabbit-Alexafluor647 (SIGMA-ALDRICH Antibody-CF647, 1:200 dilution) for spermidine]. The coverslips were then washed with 1X PBS and mounted on a clean glass slide using mounting media containing an anti-fade reagent and observed under the confocal microscope (Zeiss 710 microscope, at 63X oil immersion, 2X319 3X zoom, and 100X zoom for studying only bacterial samples, Zeiss 880 microscope, at 63X oil immersion, 2X319 3x zoom).

The histopathological sections were deparaffinized by washing in Xylene for 10 min, followed by absolute ethanol for 5 min, followed by washes in 95% ethanol and 70% ethanol for 2 min each. Finally, after a wash in water, the slides were heated in Tris-EDTA buffer (pH 9.0, 10 mM Tris, 1 mM EDTA) for 2 minutes (microwave used) for antigen retrieval. The dried slides were then incubated with the required primary antibody (anti- *Salmonella* LPS, Sigma #SAB4200862, 1:200) in a buffer containing 0.01% saponin and 2% BSA (for blocking) and incubated at room temperature for 45–60 minutes. The primary antibody was removed by washing with 1X PBS and then incubated with the appropriate secondary antibody tagged to a fluorochrome. The sections were washed with 1X PBS and covered with coverslip after using mounting media containing an anti-fade reagent. The coverslips were sealed with clear nail polish and observed under the confocal microscope. Zeiss 880 microscope 40X oil immersion 2X319 3X zoom was used to study histopathology samples.

2.14. Intracellular RNS determination

To determine intracellular RNS, a cell-permeable nitric-oxide probe, 4, 5- Diaminofluorescein diacetate (DAF_2A) (Sigma, #D225), was used. The protocol has been modified from Roy Chowdhury *et al.,* 2022 [22]. RAW264.7 cells were infected with STM WT at MOI-10 as per the gentamicin protection assay. Here, D, L-α-difluoromethylornithine (DFMO Sigma #D193, at 5 μM) and IFNγ (Invitrogen, # BMS326, at 20 ng/μL) and 1400W dihydrochloride (iNOS inhibitor, Sigma #W4262, at 5 μM) treatment was given with 25 μg/mL gentamicin. After 16 hours of infection and incubation, the cells were then incubated with fresh DMEM containing 5 μM of DAF_2DA. The cells were incubated at 37 °C in a humidified incubator with 5% CO_2 for 30 minutes. The media containing dye was removed, the cells were washed with 1X PBS, and the cells were acquired immediately for flow cytometry (BD FACS Verse) (Ex- 491nm/Em- 513 nm).

2.15. Intracellular glutathione determination

The intracellular reduced Glutathione (GSH) concentration was determined by modification of a published protocol [23]. Briefly, a standard curve with the known concentration of GSH (Sigma) was prepared. The reaction mixture for each contained 600 μL of phosphate buffer (0.1 M, pH7), 40 μL of 0.4% w/v 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, from Sigma), 100 μL of the standard solutions of GSH (0mM–1mM range) and autoclaved MilliQ water to make up the volume to 1000 μL. The mixture was incubated at room temperature for 5 minutes, and absorbance was measured at 412 nm using a tecan plate reader. The bacterial strains were subcultured in fresh LB media and grown till OD 0.1 (exponential phase), washed with buffer (Tris, Sodium chloride and EDTA) and lysed using sonication. The supernatant was used as the sample for GSH detection after sonication. As previously described for the standard curve. From the standard curve, the intracellular concentrations were interpolated.

2.16. In silico analysis

The *in silico* protein structure determination was performed using the SWISS-MODEL software (https://swissmodel.expasy.org/), where we supplied the protein sequence of GspSA of *Salmonella* Typhimurium from UniProt. We analysed the model with the highest sequence identity and maximum coverage with the Gsp from *E. coli*. The structure depicts a Homo dimer with a GMQE of 0.93 and QMEANisCo Global of 0.88 ± 0.05.

2.17. In vivo animal experiment

5–6weeks old C57BL/6J mice were infected by orally gavaging 10^7 CFU of STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG*. To study the colonisation in organs, the intestine (Peyer's patches), MLN, spleen and liver were isolated aseptically (in Biosafety level −2 cabinet), 5 days post-infection, and the CFU was enumerated on differential and selective SS agar by serial dilution. For intraperitoneal infection, 5–6weeks old C57BL/6J mice were infected by intraperitoneally injecting 10^3 CFU of STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG.* The spleen and liver were isolated aseptically 3 days post-infection to study the colonisation in organs. Blood was isolated by heart puncture 3 days post-infection. The CFU was enumerated on differential and selective SS agar by serial dilution. Organs were stored in 3.5%PFA before histopathological sample preparation.

For inhibitor treatment, 5–6 weeks of C57BL/6J mice were infected by orally gavaging 10^7 CFU of STM WT. The inhibitor DFMO (Sigma, D193) was intraperitoneally injected every alternate day from day 1 at two doses 2 mg/mice and 1 mg/mice. To study the colonisation in organs, the intestine (Peyer's patches), MLN, spleen and liver were isolated aseptically, 5 days post-infection, and the CFU was enumerated on differential and selective SS agar by serial dilution. For survival assay of mice 5–6 weeks old, C57BL/6J mice were infected by orally gavaging 10^8 CFU of STM WT. The inhibitor D, L-α-difluoromethylornithine (DFMO) was intraperitoneally injected every alternate day from day 1 at two doses 2mg/mice (125 mg/kg of body weight) and 1mg/mice (62.5 mg/kg of body weight). The doses were chosen and modified depending on previous studies on the treatment of tumours in mice [24,25]. However, the dose here in the case of infection scenario, has been reduced to two doses, 2mg/mice and 1mg/mice. The survival was monitored for 10 days. Likewise, organs were stored in 3.5% PFA before histopathological sample preparation.

For disease scoring, a small part of the liver was removed and fixed in 3.5% PFA. The 5 μm thick sections from fixed and paraffin-embedded tissues were cut onto glass slides and stained with hematoxylin and eosin (H&E) for histopathological examination. The comparison of pathological changes in the tissues was evaluated under light microscopy by a veterinary pathologist and scored with a scientific method using Mitchison's virulence/pathology scoring system with some modifications, considering the aggregations of inflammatory cells, vascular congestion, and necrosis [26]. The histopathology/disease scores were graded as 0–3: 0 for normal pathology, 1 for mild/minor pathology, 2 for moderate pathology, and 3 for severe pathological changes.

All experiments are done as per the norms of IEAC of the Indian Institute of Science, Bangalore. The approved protocol number is CAF/Ethics/852/2021.

2.18. Mass spectrometry to determine intracellular GS-sp levels

The bacterial strains were grown in LB media with added 1 mM hydrogen peroxide until the log phase of growth. The cells were centrifuged to remove the media, and the cells were resuspended in the lysis buffer (Sodium chloride, Tris, EDTA, 10% protease inhibitor cocktail) after washing with 1XPBS. The cells were lysed using sonication and centrifuged at 4 °C to collect the cell lysate. Protein was precipitated using ice-cold acetone (Sigma, MS grade), 4 times the volume of the cell lysate, and by incubating at −20 °C overnight. The precipitated proteins were removed by centrifugation, and the supernatant was used for analysis. Samples were analysed by ESI MS Q-TOF, impact HD (Bruker Daltonics Germany), and connected to Agilent HPLC 1260. Samples were passed through Agilent C18, 4.6 × 150mm column. The mobile phase used was water and acetonitrile with 0.1% formic acid. A linear gradient was used with a flow rate of 0.2 ml/min. Data was analysed using Bruker Daltonics software Data analysis 4.1. Mass of GS-sp (Glutathionyl spermidine) is 434 g/mol, and $(GS\text{-}sp)_2$ (oxidized form, Di-glutathionyl spermidine) is 866 g/mol.

2.19. Statistical analysis

Statistical analyses were performed with GraphPad Prism software. All data was tested for normal distribution using Shapiro-Wilk test. The Student's t-test (parametric, two-tailed, unpaired) and One-way ANOVA with Dunnet's post-hoc test were performed as indicated. Two-way ANOVA with Tukey's post-hoc test was used for grouped data. For animal experiments, a Non-parametric One-way ANOVA (Kruskal Wallis) test with Dunn's post-hoc test was performed. The results are expressed as mean ± SD from three independent experiments ($N \geq 3$). Group sizes and p values for each experiment are described in figure legends.

3. Results

3.1. Loss of spermidine transporter and biosynthesis genes in Salmonella Typhimurium compromises its ability to be phagocytosed by the macrophages

The pathoadaptation in *Salmonella* involves multiple players, which counteracts the stressful condition it encounters in the host macrophages. Polyamines are a group of well-studied stress response molecules, and we were interested in determining the expression of the spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium. Here, we assessed the mRNA levels of *potA, potB, potC* and *potD* in STM WT upon infection into the RAW264.7 macrophage cell line. We noted that all the genes showed a 1.5–2 fold upregulation post 6 h of infection into macrophages till 16 h (Fig. 1 A). Further, our results showed that the spermidine biosynthesis enzymes *speE* and *speD* were upregulated 1.5–2 folds, post 6 h–16 h post-infection into macrophages (Fig. 1 B). These results indicate that *Salmonella* Typhimurium enhances its intracellular spermidine biosynthesis and imports from the extracellular milieu. It might be a strategy of the pathogen to increase the intracellular pool of stress response molecules like the polyamine spermidine, as it encounters the hostile environment of host macrophages.

We then determined how the spermidine transporter and biosynthesis mutants (STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeED*)

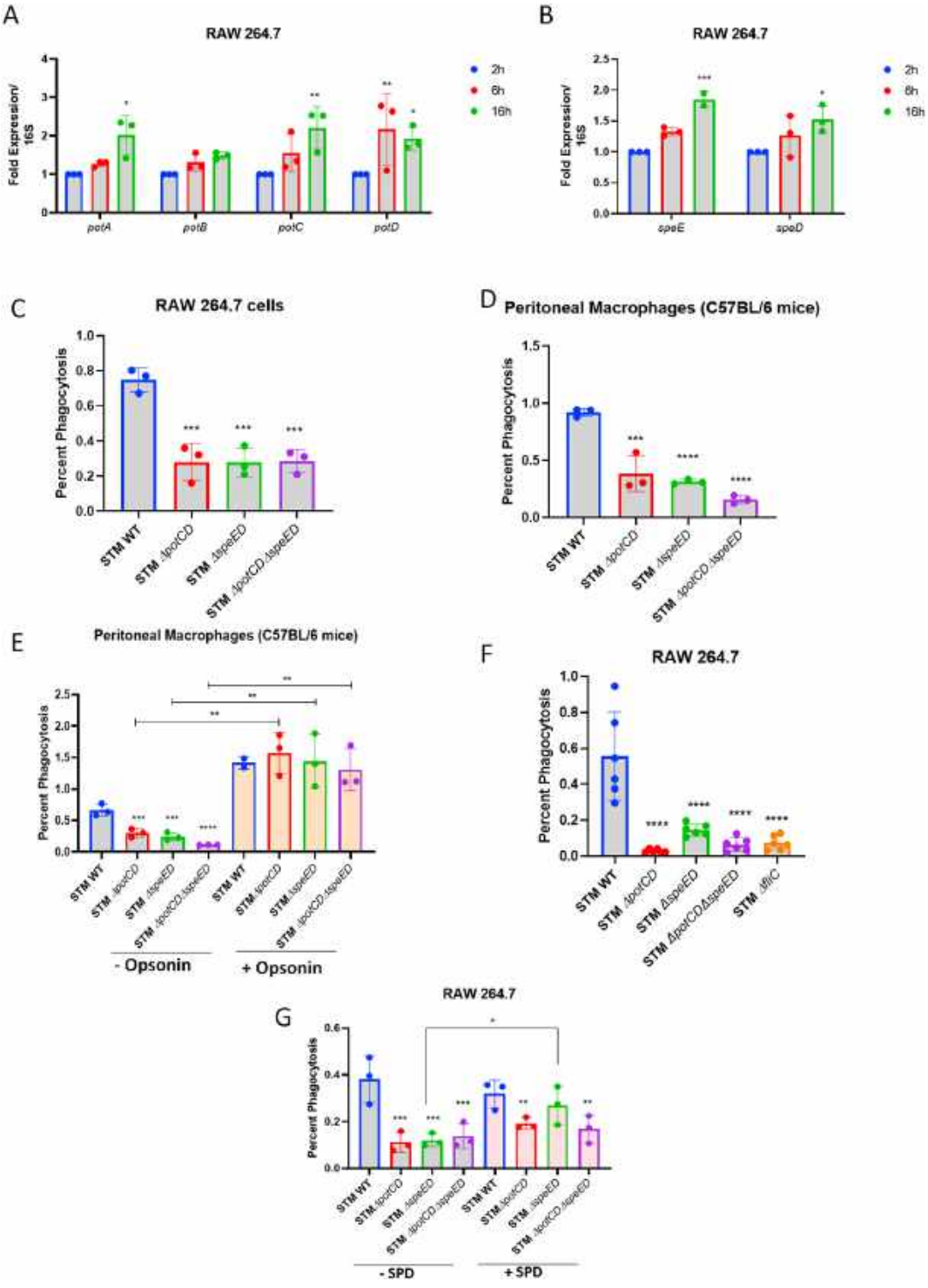

Fig. 1. Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium compromises its ability to be phagocytosed by the macrophages
A. The mRNA expression of spermidine transporter genes *potA, potB, potC* and *potD* in STM WT upon infection into RAW264.7 cells, B. The mRNA expression of spermidine biosynthesis genes *speE* and *speD* in STM WT upon infection into RAW264.7 cells, C. The percentage phagocytosis of the spermidine mutants in RAW264.7 cells, D. The percentage phagocytosis in primary macrophages isolated from peritoneal lavage of C57BL/6 mice, E. The percentage of phagocytosis upon pre-treatment with mouse-complement sera, which act as an opsonin, F. The percentage phagocytosis in RAW264.7 cells with flagellin mutant (STM *ΔfliC*) (data is from one experiment representative of 3 independent experiments), G. The percentage phagocytosis in RAW264.7 cells with the spermidine mutants grown in media supplemented with 100 μM spermidine (SPD). One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way ANOVA with Tukey's post-hoc test was used to analyze the grouped data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N=3).

behave upon infection into RAW264.7 macrophages. We infected STM WT, STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeED* into RAW264.7 cells and observed that STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeED* showed reduced phagocytosis by the macrophages compared to the wild type (Fig. 1C). Interestingly, STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeED* showed a compromised ability to proliferate intracellularly in the RAW264.7 cells, further validating a previous study [12](Fig. S1 A). To further validate our results, we assessed the behaviour of the spermidine transporter and biosynthesis gene mutants in primary macrophages isolated from the peritoneal lavage of C57BL/6 mice. Likewise, the mutants showed attenuated proliferation and uptake by phagocytosis into the peritoneal macrophages (Fig. 1 D and S1 B). These results suggest that spermidine is a critical molecule in *Salmonella* Typhimurium to infect and survive in macrophages. To further investigate the reason behind the reduced ability to be taken up by macrophages upon loss of spermidine biosynthesis and transport, we treated STM WT, STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeED* with mouse complement-sera. Upon pre-treatment with mouse complement-sera, we noted a rescue in the reduced uptake of the mutants by peritoneal macrophages isolated from C57BL/6 mice (Fig. 1 E). A study on *Salmonella* Typhimurium revealed that the non-flagellate and non-motile *Salmonella* show reduced phagocytosis by macrophages. The authors explained that non-flagellate and non-motile *Salmonella* collide less frequently with macrophages and get less time to maintain contact with the macrophage surface, thereby showing decreased phagocytosis [27]. Our group previously showed that loss of spermidine production and import in *Salmonella* Typhimurium results in the loss of flagella formation on the bacterial cell surface [14]. Thus, we determined the percentage phagocytosis for a flagellin-deficient strain of *Salmonella* Typhimurium, STM *ΔfliC* and observed that STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* and STM *ΔfliC* similarly exhibited a significantly decreased ability to be taken up by the RAW264.7 macrophages (Fig. 1 F). Furthermore, we observed a rescue of the attenuated percentage phagocytosis and fold proliferation of only STM *ΔspeED* upon supplementation of spermidine (100 μM) during the *in vitro* growth of bacteria prior to infection (Fig. 1 G and S1C). We generated single gene mutants for abrogating the spermidine transport (STM *ΔpotA*) and spermidine biosynthesis (STM *ΔspeE*) function and further complemented the genes through a vector (STM *ΔpotA: potA* and STM *ΔspeE:speE*); we observed a recovery of the fold proliferation and percentage phagocytosis nearly to STM WT in the complemented strains (Fig S1 D-E). Hence, the plausible explanation is that the reduced ability to form flagella in the spermidine mutants causes less frequent interaction with the macrophages and provides minimal contact time for infection, leading to reduced phagocytosis by the macrophages.

3.2. *Spermidine provides stress resistance in Salmonella Typhimurium by regulating the expression of numerous antioxidative enzymes*

The loss of spermidine transport and biosynthesis function in *Salmonella* Typhimurium renders it incapable of proliferation and survival in macrophages. In the host macrophages, the bacteria encounter numerous threats, the foremost of which is the rapid oxidative burst mediated by NOX2. The reactive oxygen species superoxide radical can easily diffuse through the bacterial membrane and pose a major threat to the pathogen. ROS acts on multiple molecules such as nucleic acids, proteins and lipids, thus damaging the cell membranes, DNA and proteins within the bacteria [28]. Spermidine has been linked to stress response against oxidative stress and protects bacteria in *E. coli* and *Pseudomonas aeruginosa.* Thus, we were intrigued to understand the role of spermidine in antioxidative response in *Salmonella* Typhimurium. We examined the survival of STM WT, STM *ΔpotCD*, STM *ΔspeED*, and STM *ΔpotCDΔspeE* upon exposure to the oxidative agent hydrogen peroxide *in vitro.* We noticed that at high concentrations of hydrogen peroxide, 5 mM and 10 mM, the STM *ΔpotCD*, STM *ΔspeED* and STM *ΔpotCDΔspeE* showed significantly lesser survival than STM WT (Fig. 2 A). Moreover, the complemented strains for spermidine transport and synthesis mutants showed a rescue in attenuated survival in the presence of high concentrations of hydrogen peroxide *in vitro* (Fig. S2 A). We further determined the expression of *potA, potB, potC, potD*, *speE* and *speD* in STM WT upon exposure to 1 mM hydrogen peroxide. There was a 2–3 fold upregulation in the mRNA expression of the transporter genes over the untreated during the early log phase of growth (6 h) and the late log phase of growth (12 h) *in vitro* (Fig. S2 B-E). Similarly, the biosynthesis genes *speE* and *speD* were 4–6 fold upregulated in their corresponding mRNA expressions during their early log phase of growth (6 h) and the late log phase of growth (12 h) *in vitro* (Fig. S2 F and G). Our results show that *Salmonella* Typhimurium upregulates spermidine transport and biosynthesis upon oxidative stress, suggesting that spermidine mounts a protective function to aid bacterial survival in such a stressful condition.

Bacteria sense the environmental changes and cues to respond and adapt to the altered environment. They use the two-component systems, transcriptional activators and repressors, to alter gene expression in response to a stimulus [29]. Polyamines in *E. coli* regulate multiple genes at the transcription and translation together, referred to as the "Polyamine modulon". These involve the numerous mRNAs, tRNAs, sigma factors, translational factors, and two-component systems during bacterial growth and in stress conditions [30–32]. *Salmonella* harbours multiple antioxidative enzymes to detoxify the ROS intracellularly [33]. Our study so far shows that STM *ΔpotCD*, STM *ΔspeED,* and STM *ΔpotCDΔspeED* are attenuated in survival under *in vitro* oxidative stress more than STM WT. To gain mechanistic insight into the attenuated survival of *Salmonella* Typhimurium, we determined the mRNA expression of the critical transcription factor *rpoS*, which activates the expression of the catalase enzymes (*katG and katE*) to detoxify hydrogen peroxide to water in the bacteria enzymatically [34]. In both STM *ΔpotCD* and STM *ΔspeED,* the mRNA expression of *rpoS* is significantly downregulated from 6 h post-infection in RAW264.7 cells (Fig. 2 B and C, S3 A). Further, its downstream target *katG* was also downregulated in STM *ΔpotCD* and STM *ΔspeED* 6 h post-infection into RAW264.7 cells (Fig. 2 D and E, S3 B). We further assessed the mRNA expression of the transcription factor *soxR*, which regulates the expression of superoxide dismutases (*sodA* and *sodB*) [35]. Upon infection in RAW264.7 cells, expression of *soxR* was significantly downregulated in STM *ΔpotCD* and STM *ΔspeED* with respect to STM WT (Fig. 2 F and G, S3 C). Superoxide dismutases act on superoxide radicals, the potent ROS encountered in macrophages, converting to hydrogen peroxide. The mRNA expression of both *sodA* and *sodB* were downregulated in STM *ΔpotCD* and STM *ΔspeED* upon infection into RAW264.7 macrophages (Fig. 2 H–K, S3 D and E). A significant antioxidant in most living organisms is glutathione (GSH), which directly acts as a quencher of ROS [36]. GSH is synthesized by Glutathione synthase (GshA), which is regulated by EmrR transcription factor. We observed that the mRNA expression of *emrR* is downregulated in STM *ΔpotCD* and STM *ΔspeED* upon infection into RAW264.7 macrophages (Fig. 2 L and M, S3 F). Similarly, *gshA* transcript expression is downregulated (Fig. 2 N and O, S3 G). To further validate the down regulation of the glutathione synthesis arm in spermidine transport and biosynthesis mutants, we determined the intracellular GSH levels and noted that in STM *ΔpotCD,* STM *ΔspeED* and STM *ΔpotCDΔspeED* the levels were significantly less (Figs. S3H and I). Taken together, these results indicate that spermidine regulates the transcription of multiple transcription factors involved in oxidative stress response in *Salmonella* Typhimurium. Importantly, we found a mechanism of oxidative stress resistance in *Salmonella* Typhimurium regulated by spermidine, potentiating the survival of the bacteria.

3.3. *Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in Salmonella Typhimurium, and together mount an intracellular antioxidative response*

The spermidine synthesized from putrescine has two fates. It is either acetylated by the enzyme SpeG or covalently conjugated to GSH to form

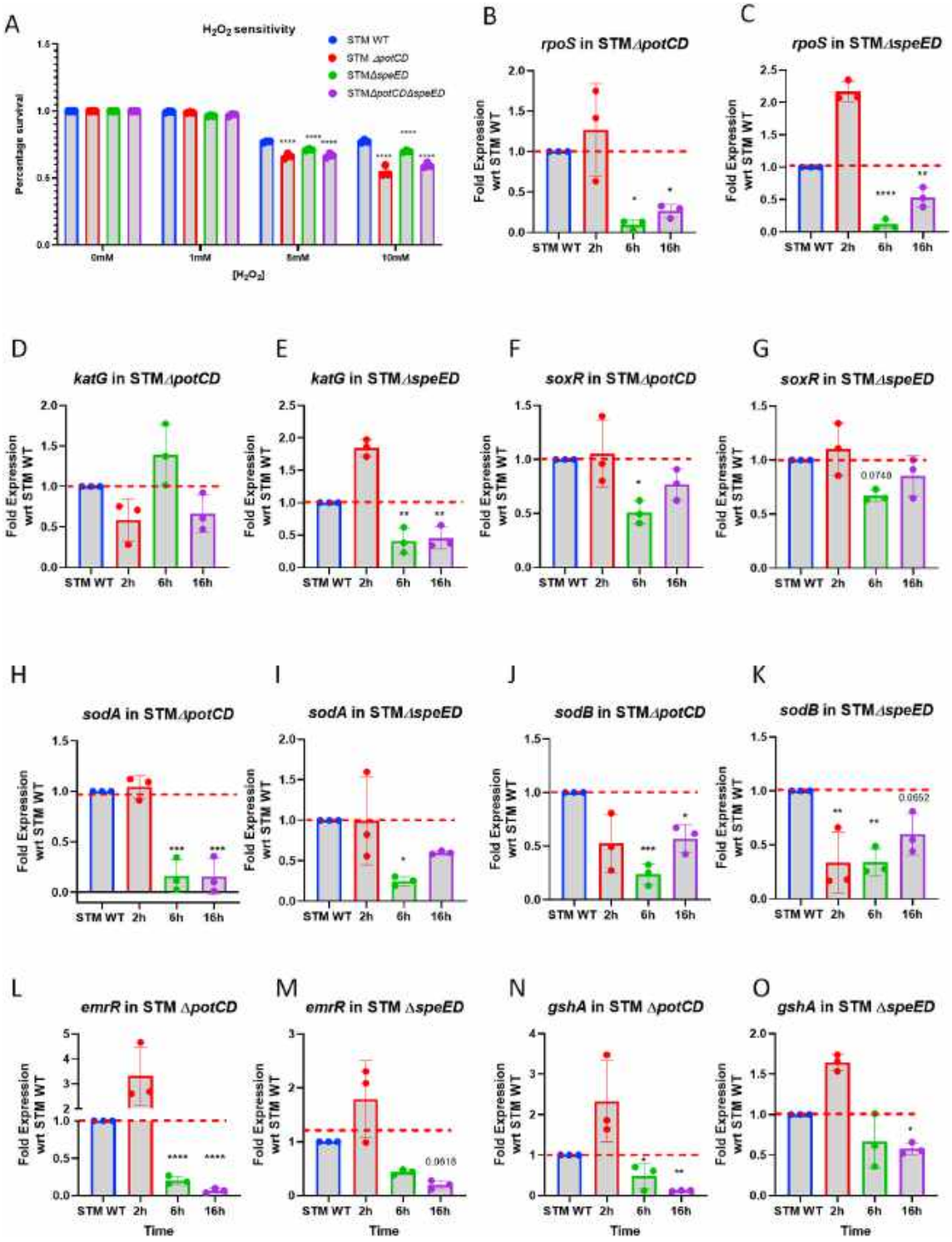

Fig. 2. Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of the expression of numerous antioxidative enzymes
A. The *in vitro* hydrogen peroxide sensitivity assay with the spermidine transport and biosynthesis mutants ((data is from one experiment representative of 3 independent experiments)), B. The mRNA expression of stress-responsive transcription factor *rpoS* in STM *ΔpotCD* upon infection into RAW264.7 cells, C. The mRNA expression of stress-responsive transcription factor *rpoS* in STM *Δwas speED* upon infection into RAW264.7 cells, D. The mRNA expression of *katG* in STM *ΔpotCD* was upon infection into RAW264.7 cells, E. The mRNA expression of *katG* in STM *ΔspeED* upon infection into RAW264.7 cells, F. The mRNA expression of oxidative stress-responsive transcription factor *soxR* in STM *ΔpotCD* upon infection into RAW264.7 cells, G. The mRNA expression of oxidative stress-responsive transcription factor *soxR* in STM *ΔspeED* upon infection into RAW264.7 cells, H. The mRNA expression of *sodA* in STM *ΔpotCD* upon infection into RAW264.7 cells, I. The mRNA expression of *sodA* in STM *ΔspeED* upon infection into RAW264.7 cells, J. The mRNA expression of *sodB* in STM *ΔpotCD* upon infection into RAW264.7 cells, K. The mRNA expression of *sodB* in STM *ΔspeED* upon infection into RAW264.7 cells, L. The mRNA expression of glutathione synthetase specific transcription factor *emrR* in STM *ΔpotCD* upon infection into RAW264.7 cells, M. The mRNA expression of glutathione synthetase-specific transcription factor *emrR* in STM *Δwas speED* upon infection into RAW264.7 cells, N. The mRNA expression of *gshA* in STM *ΔpotCD* upon infection into RAW264.7 cells, O. The mRNA expression of *gshA* in STM *ΔspeED* upon infection into RAW264.7 cells. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data, p values****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N ≥ 3).

Glutathionyl-spermidine (GS-sp) catalysed by Glutathionyl-spermidine synthetase (Gss). Tabor (1974) first discovered the existence of this enzyme in *E. coli* [37]. In *E. coli* GS-sp is generated at a higher level in the stationary phase and very less in the late exponential phase. It also interacts and modifies the thiol-containing proteins under high H_2O_2-containing media, forming Gsp-thiolated proteins (PS-Gsp). Certain *in vitro* experiments with dehydro-ascorbate suggested that GS-sp might have higher antioxidant properties than GSH [38] and may be more effective in protecting against DNA damage by free radicals [39]. However, in *E. coli,* Gss could not be linked to its pathogenicity [40]. Among *Enterobacteriaceae*, *Salmonella* was found to possess this unique enzyme. Our *in silico* analysis suggested that the enzyme in *Salmonella* Typhimurium (GspSA, encoded by *gsp*) has 90% identity with *E. coli gss,* and the SWISS-MODEL predicts it to be a homo-dimeric protein (Fig. S4 A-C). Also, the spermidine synthesized by SpeE in *Salmonella* Typhimurium is directly fed into the pathway to synthesize GS-sp. Thus, we investigated this novel enzyme's biological role in *Salmonella* Typhimurium. We noted that the mRNA expression of *gsp* is significantly upregulated at the late-log phase of growth of STM WT in LB media in the presence of H_2O_2 (Fig. S4 D). Upon infection into RAW264.7 cells, STM WT upregulates the mRNA expression of *gsp* at 6 h and 16 h post-infection into RAW264.7 cells (Fig. 3 A). Further, the *Salmonella* Typhimurium mutant of *gsp* (STM *Δgsp*) showed attenuated proliferation in RAW264.7 cells similar to STM *ΔkatG,* which has reduced capability to detoxify ROS, while upon complementation of *gsp* in the mutant (STM *Δgsp:gsp*) partially rescues the phenotype and we observe a higher fold proliferation than the mutant (Fig. 3 B). Thus, our results suggest that *gsp* is important in *Salmonella* Typhimurium to survive and cope with the oxidative stress and hostile environment of macrophages. Interestingly, the mRNA expression of *gsp* was downregulated in both STM *ΔpotCD* and STM *ΔspeED* at 16 h post-infection into RAW264.7 macrophages (Fig. 3C and D). These findings show that spermidine regulates GspSA enzyme and thereby controls the flux of spermidine into the GspSA pathway. Also, it further potentiates the ability of *Salmonella* Typhimurium to mount an antioxidative response by regulating *gsp* expression.

GspSA enzyme in Trypanosomes was named Trypanothione synthetase, while the conjugate is called Trypanothione (TSH). In trypanosomes, TSH is essential, and these organisms rely entirely on TSH and possess no GSH reductase. Also, they have evolved to use TSH reductase instead of GSH reductase, Glutaredoxins or Thioredoxins [41]. In *E. coli* GS-sp forms bonds with the Cysteine thiol groups of numerous proteins and protect them from oxidation under oxidative stress. Cysteine thiol groups are highly prone to attack by ROS and get oxidized to sulphinic and sulphonic acids. In *Salmonella* Typhimurium, we observe that loss of *gsp* results in attenuated proliferation and survival in macrophages. Thus, we investigated the survival of STM *Δgsp* upon exposure to hydrogen peroxide *in vitro*. STM *Δgsp* exhibited reduced survival in high 5 mM and 10 mM concentrations of H_2O_2 (Fig. S4 E). Likewise, upon exposure to agents of oxidative stress and nitrosative stress H_2O_2 and $NaNO_2$ together, we observe that STM *Δgsp* exhibited reduced survival at higher concentrations such as 5 mM and 10 mM compared to STM WT (Fig. 4 E). Thus, *gsp* is critical in *Salmonella* Typhimurium to shield the bacteria from the action of ROS and RNS. As we observed, spermidine transporter and biosynthesis mutants and *gsp* mutants of *Salmonella* Typhimurium are compromised in their survival under oxidative stress and in macrophages; thus, we were interested in assessing the intracellular ROS detoxification abilities of the strains. We determined the intracellular ROS in STM WT, STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG*. Interestingly, STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG* showed significantly higher intracellular ROS levels when they were exposed to 5 mM and 10 mM H_2O_2 (Fig. 3 F). Further, the complemented strains for spermidine transport and synthesis mutants showed reduced intracellular ROS in the presence of high concentrations of hydrogen peroxide *in vitro* (Fig. S4 F). These results suggest that lower intracellular levels of spermidine correlate to higher intracellular ROS levels in *Salmonella* Typhimurium. To validate our observed results, we utilised a genetically engineered tool to sense the redox status of the bacterial cytosol. We used pQE60-Grx1-roGFP2 plasmid that contains roGFP2, a genetically modified form of GFP. The glutaredoxin (Grx1) fused to the roGFP2 reversibly transfers electrons between the cytosolic pool of GSH/GSSG and the thiol group of roGFP2, and the ratio of fluorescence ratio at 405 nm and 488 nm determine the redox status of bacterial cytoplasm [22]. We observed that STM *ΔpotCD*, STM *ΔspeED* harbouring the pQE60-Grx1-roGFP2 showed a higher ratio of 405nm/488 nm compared to STM WT in the presence of 5 mM H_2O_2 *in vitro* and upon infection into RAW 264.7 macrophages (Fig. 3 G and H). However, STM *Δgsp* did not show a significantly higher 405nm/488 nm ratio. Moreover, upon supplementation of the growth media with 100 μM spermidine, only in STM *ΔspeED* we observed a lower intracellular ROS and lesser 405nm/488 nm in higher concentrations of H_2O_2 (Fig. S4 G and H). Thus, our results indicate that spermidine is critical in mounting an antioxidative response to detoxify the intracellular ROS by regulating multiple antioxidant genes in *Salmonella* Typhimurium. To validate our observed results, we used mass spectrometry to determine the intracellular levels of glutathionyl-spermidine (Gs-sp) in STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*gsp*. Our study qualitatively shows that the synthesis of GS-sp and $(GS\text{-}sp)_2$ (oxidized form, di-glutathionyl-spermidine) in STM WT upon exposure to 1 mM hydrogen peroxide, which is absent in the spermidine mutants and STM *Δgsp* (Fig. S5 A and B). We further determined the presence of PS-Gsp and showed that STM WT modifies proteins by GS-sp (detected by anti-spermidine antibody), which is reduced upon treatment with beta-mercaptoethanol. The spermidine mutants show less modification, which is almost negligible in STM *Δgsp* (Fig. S5 C-E).

We observe that STM *ΔpotCD* STM *ΔspeED* shows a higher intracellular ROS than STM *Δgsp*. Thus, to understand does STM *Δgsp* phenocopy STM *ΔpotCD* STM *ΔspeED*, we generated double mutants STM *ΔgspΔpotCD* and STM *ΔgspΔspeED*. We find that the double mutants show a similar reduced fold proliferation and percentage phagocytosis in RAW 264.7 cells (Fig S6 A-B). However, they show an enhanced intracellular ROS upon exposure to hydrogen peroxide (Fig. S6 C). Upon co-infection of the double mutants with STM *Δgsp* in C57BL/6 mice, we see that STM *Δgsp* competes with the double mutants in colonizing the liver (Fig S6 D-E). To further dissect the role of spermidine in the protection of *Salmonella* Typhimurium from oxidative stress, we infected STM WT, STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG* in primary macrophages isolated from the peritoneal lavage of *gp91phox* $^{-}/^{-}$ mice. Gp91Phox is the major subunit of the Nox2 complex that aids in the catalysis of oxygen to superoxide radicals. Interestingly, we observe a rescue in the attenuated fold proliferation of STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG* in peritoneal macrophages isolated from *gp91phox* $^{-}/^{-}$ mice (Fig. 3 I). Our results demonstrate the vital role of spermidine in oxidative stress resistance in *Salmonella* Typhimurium.

3.4. Spermidine is critical for Salmonella Typhimurium to colonise the primary and secondary sites of infection in mice

Salmonella infects the host and breaches the epithelial cells at the Peyer's patches in the distal ileum, it disseminates to the secondary sites of infection, namely the Mesenteric Lymph node (MLN), spleen and liver. *Salmonella* is taken from the basolateral surface of the epithelial cells at the lamina propria by the macrophages and polymorphonuclear cells (PMN). We observed that the spermidine transporter and biosynthesis mutants show attenuated survival in macrophages and under oxidative stress *in vitro*. We were intrigued to study the behaviour of the mutants during *in vivo* colonisation in the mouse model of *Salmonella* infection. We infected C57BL/6J mice by orally gavaging with STM WT, STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG* at a CFU of 10^7 per mouse. We noted that STM *ΔpotCD*, STM *ΔspeED*,

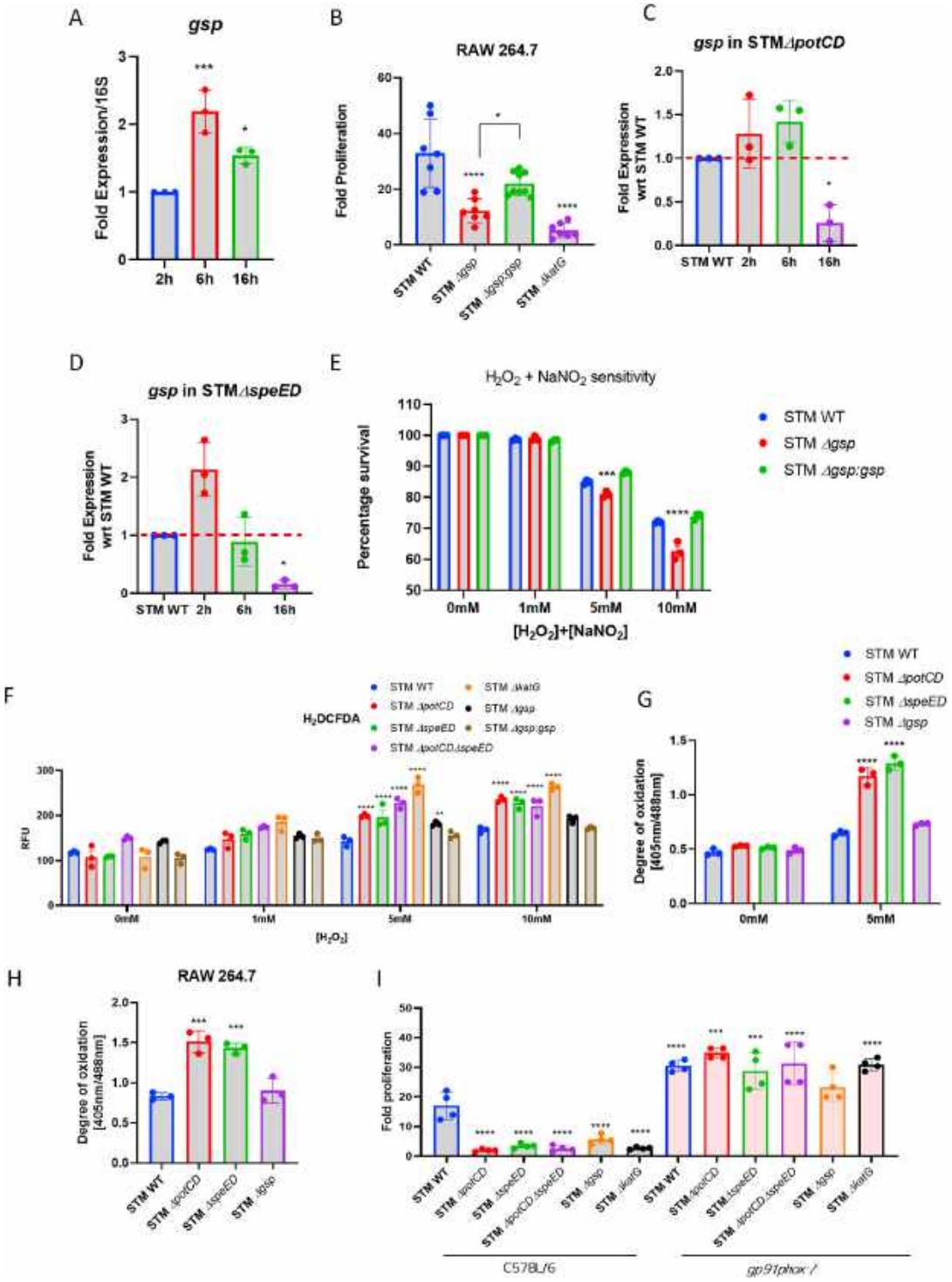

Fig. 3. Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in *Salmonella* Typhimurium, and together mount an intracellular antioxidative response

A.The mRNA expression of *gsp* in STM WT upon infection into RAW264.7 cells, B The fold proliferation of STM WT, STM *Δgsp* and STM *Δgsp:gsp* in RAW264.7 cells, (data is from one experiment representative of 3 independent experiments) C. The mRNA expression of *gsp* in STM *ΔpotCD* upon infection into RAW264.7 cells, D. The mRNA expression of *gsp* in STM *ΔspeED* upon infection into RAW264.7 cells, E The *in vitro* Hydrogen peroxide and nitric oxide sensitivity assay (data is from one experiment representative of 3 independent experiments), F. Intracellular reactive oxygen species determination using the cell-permeable H_2DCFDA dye, G. The intracellular degree of oxidation in spermidine and *gsp* mutants using pQE60-grx1-roGFP2 construct *in vitro*, H. The intracellular degree of oxidation using pQE60-grx1-roGFP2 construct in spermidine and *gsp* mutants upon infection into RAW 264.7 cells, I. The fold proliferation in primary macrophages isolated from wild-type C57Bl/6 mice and *gp91phox*$^{-}$/$^{-}$ mice (data is from one experiment representative of 3 independent experiments). One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way ANOVA with Tukey's post-hoc test was used to analyze the grouped data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N ≥ 3).

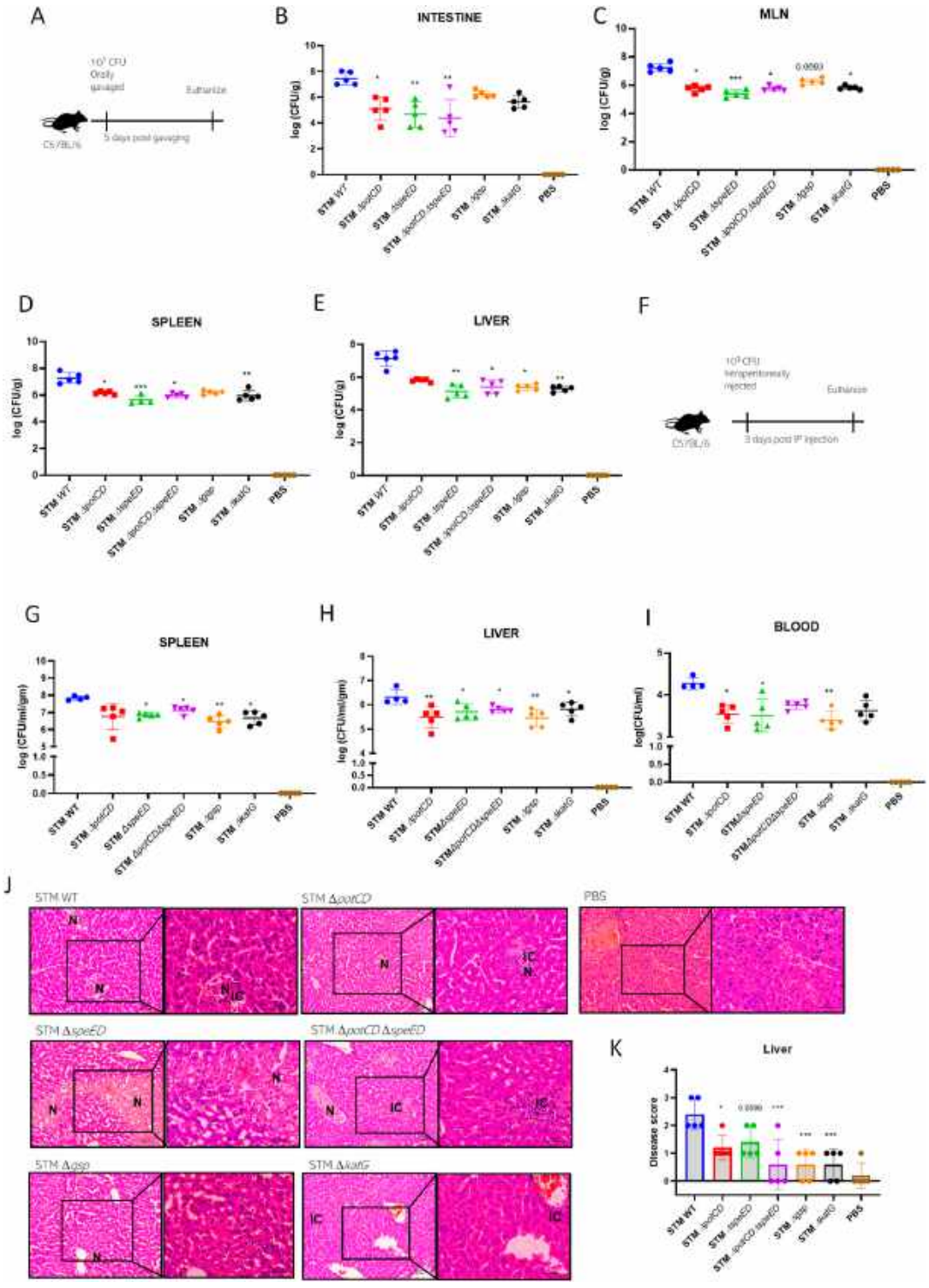

Fig. 4. Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and secondary sites of infection in mice
A. The experimental protocol for organ burden in C57BL/6 mice by orally gavaging 10^7 CFU per mice, B. The organ burden post 5 days of oral gavage in the intestine, C. in the Mesenteric lymph node (MLN), D. in the spleen, E. in the liver, F. The experimental protocol for organ burden in C57BL/6 mice upon intraperitoneal (I.P) infection with 10^3 CFU per mice, G. The organ burden 3 days post I.P infection in the spleen, H. in the liver, I. dissemination in blood, J. The hematoxylin and eosin staining of the sections of the liver 3 days post I.P infection of C57Bl/6 mice (N=5, scale bar- 50 μM), K. Disease score from the histopathological sections of the liver. Here, (IC) Multiple aggregations of inflammatory cells dispersed in the liver parenchyma, (N) shows several small necrotic areas, (C) congestion and damage to the endothelial lining of the central vein, (HPV) congestion of the hepatic portal vein. The disease score is 0 for normal pathology, 1 for mild/minor pathology, 2 for moderate pathology, and 3 for severe pathological changes. Non-parametric One-way ANOVA (Kruskal Wallis) with Dunn's post-hoc test was used to analyze organ burden in mice; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. One-way ANOVA with Dunnet's post-hoc test was used to analyze the rest of the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N ≥ 3).

STM *ΔpotCDΔspeE,* and STM *ΔkatG* showed a significantly lower organ burden in Peyer's patches, MLN, spleen, and liver upon oral gavage (Fig. 4A–E). Meanwhile, STM Δ*gsp* shows significantly lower colonisation compared to STM WT in MLN and liver upon oral gavage (Fig. 4A–E). Our previous study showed that the spermidine transporter and biosynthesis mutants exhibit poor invasiveness into IECs, which can explain our *in vivo* colonisation upon oral gavage [13]. Oral gavage mimics the physiological route of *Salmonella* infection into its host. Hence, it requires to be able to breach the intestinal barrier successfully. Incapability to invade the IECs in STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE* and STM *Δgsp* explains the diminished colonisation in the organs. To dissect the role of spermidine in the *in vivo* colonisation, we infected C57Bl/6J mice intraperitoneally by bypassing the entry and breaching the epithelial barrier. We observed that upon infecting intraperitoneally, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG* exhibited reduced colonisation in the spleen and liver and less dissemination in blood compared to STM WT (Fig. 4F–I). Meanwhile, STM *ΔpotCD* was found to colonise less in the liver and showed less dissemination in blood upon intraperitoneal injection (Fig. 4F–I). Also, the histopathological sections show significantly less liver tissue damage with STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeE,* STM *Δgsp* and STM *ΔkatG*, which is validated by disease scoring of the same (Fig. 4 J and K, S7 A-G). Our results thus show that spermidine aids in the *in vivo* pathogenesis and virulence of *Salmonella* Typhimurium.

3.5. *Salmonella rewires host polyamine metabolism to potentiate its survival within host macrophages*

Most of the intracellular pathogens establish their persistence in the phagocytic cells and are often found to be associated with different populations of macrophages. Like *Brucella abortus,* it preferentially resides in the Alternatively activated macrophages (AAM), where it survives and replicates by exploiting the host polyamines. A research group has shown that the metabolism of AAM is shifted to increase polyamine biosynthesis by *Brucella abortus* and thereby promote bacterial survival [42]. Similarly, *Salmonella* also resides in macrophages, a way to lead chronic infections. We were interested to know whether *Salmonella* exploits the host polyamines and leads to a rewiring of host cell metabolism. To understand the host-pathogen relationship in depth, we assessed the mRNA expression of Ornithine decarboxylase 1 (*mOdc,* mouse Ornithine decarboxylase), which catalyses the rate-determining step of polyamine biosynthesis and Spermidine synthase (*mSrm,* mouse Spermidine synthase) that synthesises spermidine from putrescine by transferring the aminopropyl group from decarboxylated-S-adenosyl methionine in RAW264.7 cells upon infection with STM WT. We observed that mRNA expression of *mOdc1* and *mSrm* were upregulated at 6- and 16-h post-infection (Fig. 5 A and B). Further, the mRNA expression of *mOdc1* and *mSrm* were enhanced in the spleen and liver of C57BL/6 mice 5 days post oral gavage with STM WT (**Fig C– F**). Our results show that *Salmonella* Typhimurium enhances the expression of host polyamine biosynthesis genes upon infection into the host. Moreover, we have previously observed that the *Salmonella* Typhimurium that cannot import spermidine cannot survive and proliferate as much as STM WT in macrophages. To delve further into the role of host-acquired polyamines, we knocked down *mOdc1* in RAW264.7 cells (Fig. S8 A). Upon knockdown of *mOdc1*, *Salmonella* Typhimurium showed significantly attenuated proliferation in RAW264.7 cells (Fig. 5 G). However, the knockdown of *mOdc1* did not alter the percentage of phagocytosis by the macrophages (Fig. S8 C). Similarly, we knocked down *Srm* in RAW264.7 cells and observed that the knockdown of spermidine synthase in the host compromises the ability of STM WT to proliferate and get phagocytosed by the macrophages (Fig. S8 B, D and E).

The question that arises is how *Salmonella* regulates the host polyamine metabolic pathways. *Salmonella* utilizes pathogenicity island 1 and 2 (SPI-1 and SPI-2) encoded effectors to enter and survive in the specialized host niches [43,44]. Although SPI-1 is well studied in the initial process of invasion, recent studies suggest that both SPI-1 and SPI-2 effectors are required for SCV maturation and *Salmonella* survival in the host cells [45–47]. Thus, to investigate how *Salmonella* modulates host cell polyamine metabolism, we infected RAW264.7 cells with an SPI-1 mutant (STM Δ*invC*) and a SPI-2 mutant (STM Δ*ssaV*), both of which cannot export effectors of SPI-1 and SPI-2 respectively. We observed that the mRNA expression of *mOdc1* and *mSrm*, were significantly downregulated in macrophages infected with STM Δ*invC* and STM Δ*ssaV* compared to in STM WT (all normalized to Uninfected) (We further determined the intracellular spermidine by immunofluorescence and found that macrophages infected with STM Δ*invC* and STM Δ*ssaV* showed reduced spermidine production compared to STM WT and uninfected cells (Fig. 5J and K, S8 F). Thus, our data suggests that *Salmonella* utilizes effectors from SPI-1 and SPI-2 to modulate the host cell polyamine metabolic pathways. Furthermore, a stronger effect was observed with STM Δ*invC* in regulating host polyamine metabolism.

3.6. *The chemopreventive drug DFMO reduces Salmonella Typhimurium burden in the host by enhancing nitric oxide production*

Polyamines are essential molecules in eukaryotes, with multiple roles in differentiation, proliferation, and development. Many studies have shown that polyamine levels are upregulated in cancer cells, and elevated levels of polyamines are associated with breast cancer, neuroblastoma, hepatocellular carcinoma, prostate cancer, lung cancer, colorectal cancer, and leukaemia [48–52]. D, L-α-difluoromethylornithine (DFMO), an inhibitor of ODC, was developed as a potent drug to treat cancer in 1970 [53]. DFMO irreversibly binds to the active site of ODC and acts as a suicide inhibitor, thereby reducing polyamine levels and having a cytostatic effect. It was effective only in neuroblastoma as a single therapeutic agent and clinical trials for other cancer types were unsatisfactory [54,55]. However, DFMO has been successfully developed as a chemopreventive drug, with FDA approval for treating Human African Trypanosomiasis (HAT) [56,57]. To test whether DFMO can be used as a therapeutic drug against *Salmonella* infection, we treated RAW 264.7 cells with DFMO during the *Salmonella* Typhimurium infection. We observed a significant attenuation in the fold proliferation of STM WT (Fig. 6 A). Studies have shown that DFMO binds to ODC to prevent further putrescine production from ornithine, acts on Arginase1, and reduces the available pool of ornithine for polyamine biosynthesis [58,59]. This ensures the arginine flux is fed into the nitric oxide synthase (Nos2) pathway and leads to elevated levels of nitric oxide in the cell [60]. We assessed the mRNA expression of *mNos2* upon knockdown of *mOdc1* in RAW264.7 cells, followed by infection with STM. We observed an upregulation of *mNos2* mRNA levels at 6- and 16-h and an upregulation of mNOS2 protein at 16 h post-infection in RAW264.7 cells (Fig. 6 B S9 A and B). Further, we determined nitric oxide levels using a cell-permeable dye DAF_2DA and noted that upon treatment with DFMO there was higher production of nitric oxide (Fig. 6C and D). While when we inhibited mNos2 using 1400W, the nitric oxide levels did not increase on further treatment of DFMO (Fig. S9 C and D). Furthermore, infecting primary macrophages isolated from *iNOS*$^{-}$/$^{-}$ mice we observed that the fold proliferation is not altered upon treatment of DFMO from the untreated cells (Fig. 6 E, S9 E). Taken together, our results demonstrate the role of DFMO in diminishing the survival and proliferation of *Salmonella* Typhimurium by blocking Odc1 and enhancing nitric oxide production in murine macrophages.

To further address whether DFMO also acts as a potent anti-*Salmonella* drug in mice model of infection, we infected C57BL/6 mice with 10^7 CFU per mouse by oral gavage. Every alternate day, the mice were injected with DFMO in two different doses for two cohorts [2mg/mice (125 mg/kg of body weight) and 1mg/mice (62.5 mg/kg of body weight)]. Five days post oral gavage, we observed that DFMO treatment of 2 mg/kg body weight of mice significantly reduced the colonisation of

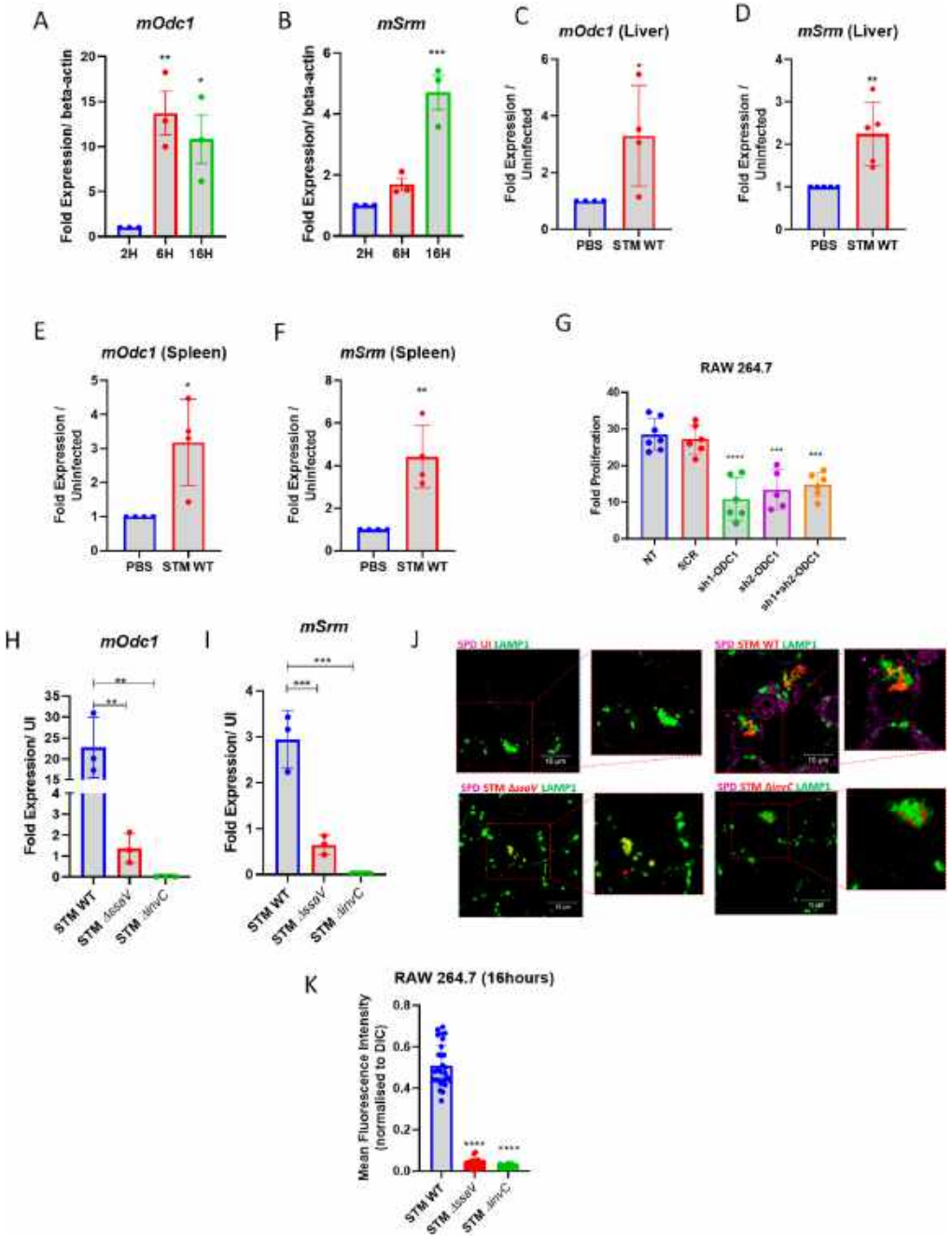

Fig. 5. *Salmonella* rewires host polyamine metabolism to potentiate its survival within host macrophages

A. The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in RAW264.7 cells upon infection with STM WT, B. The mRNA expression of *mSrm* (mouse spermidine synthase) in RAW264.7 cells upon infection with STM WT, C. The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in the liver of C57BL/6 mice 5 days post-infection with STM WT by oral gavage, D. The mRNA expression of *mSrm* (mouse spermidine synthase) in the liver of C57BL/6 mice 5 days post-infection with STM WT by oral gavage, E. The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in the spleen of C57BL/6 mice 5 days post-infection with STM WT by oral gavage, F. The mRNA expression of *mSrm* (mouse spermidine synthase) in the spleen of C57BL/6 mice 5 days post-infection with STM WT by oral gavage, G. The fold proliferation of STM WT in RAW264.7 cells upon transient knockdown of *mOdc1,* (data is from one experiment representative of 3 independent experiments) H. The percentage phagocytosis of STM WT in RAW264.7 cells upon transient knockdown of *mOdc1*. Here, SCR is Scrambled (no target for knockdown), and two different targeted shRNA were used for knockdown purposes: Sh1 is shRNA-1 for knockdown, Sh2 is shRNA-2 for knockdown, and Sh1+Sh2 indicates where both the shRNAs were used to obtain the knockdown, NT is untransfected, H. The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in RAW264.7 cells upon infection with STM WT, STM Δ*ssaV,* and STM Δ*invC*, normalized to the expression in uninfected macrophages, I. The mRNA expression of *mSrm* (mouse spermidine synthase) in RAW264.7 cells upon infection with STM WT, STM Δ*ssaV,* and STM Δ*invC*, normalized to the expression in uninfected macrophages, J. Immunofluorescence imaging to study spermidine in RAW 264.7 cells upon infection with STM WT, STM Δ*invC,* STM Δ*ssaV,* K. The quantification of (J) (data is from one experiment representative of 3 independent experiments). Here, green is Anti-mouse LAMP1(Alexa fluor 488), Red is pFPV-M-cherry expressing *Salmonella* strains, magenta is anti-Spermidine (Alexa fluor 647), and UI- uninfected. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data; p values****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N ≥ 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

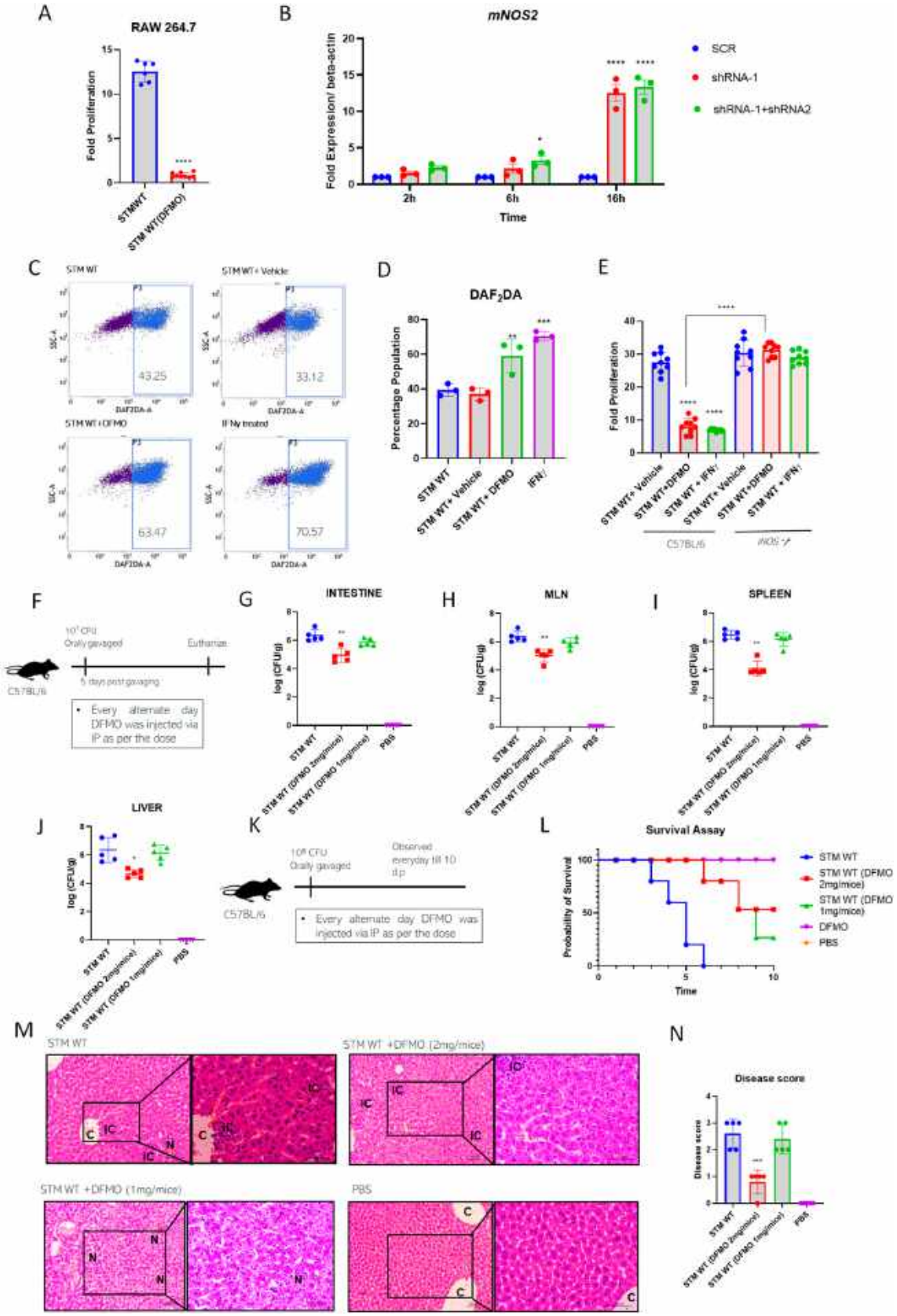

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Fig. 6. The chemopreventive drug DFMO reduces *Salmonella* Typhimurium burden in the host by enhancing nitric oxide production
A. The intracellular fold proliferation of STM WT in RAW264.7 cells upon treatment with Difluoromethyl ornithine (DFMO) (5 μM), quantified by Gentamicin protection assay, B. The mRNA expression of *mNos2*(mouse Nitric oxide synthase) in RAW264.7 cells, upon transient knockdown of *Odc1* followed by infection with STM WT, is normalized to beta-actin as an internal control, C. The representative scatter plot for the DAF2DA dye-positive population of RAW264.7 cells, upon transient knockdown of *Odc1* followed by infection with STM WT, the data is determined by flow-cytometry post-staining the infected macrophages with the dye, D. The quantification of (C), E. The fold proliferation of STM WT upon infection into primary macrophages isolated from *iNOS*$^{-/-}$ mice followed by treatment with DFMO (data is from one experiment representative of 3 independent experiments), F. The experimental procedure to study the organ burden of STM WT in C57BL/6 mice upon treatment with DFMO, G-J. The organ burden of STM WT in the Intestine, MLN, Spleen and Liver of C57BL/6 mice upon intraperitoneal treatment of DFMO (2mg/mice and 1mg/mice) as mentioned in (E), K. Experimental procedure used to study the survival of C57BL/6 mice upon infection with STM WT and treatment with DFMO, L. The survival of C57BL/6 mice upon infection with STM and intraperitoneal treatment of DFMO (2mg/mice and 1mg/mice) as mentioned in (J), M. Hematoxylin and eosin staining of histopathological sections of liver upon DFMO treatment to C57BL/6 mice (N=5, scale bar- 50 μM). Here, (IC) Multiple aggregations of inflammatory cells dispersed in the liver parenchyma, (N) shows several small necrotic areas, (C) congestion and damage to the endothelial lining of the central vein, (HPV) congestion of the hepatic portal vein. The disease score index for liver tissue damage upon STM WT infection in C57BL/6 mice with DFMO treatment. The disease score is as:0 for normal pathology, 1 for mild/minor pathology, 2 for moderate pathology, and 3 for severe pathological changes. One-way ANOVA with Dunnet's post-hoc test was used to analyze the data, non-parametric One-way ANOVA (Kruskal Wallis) with Dunn's post-hoc test was used to analyses organ burden; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way ANOVA with Tukey's post-hoc test was used to analyze the grouped data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. All data are represented as mean ± SD from three independent experiments (N ≥ 3). The survival curve has been analysed using Mantel-Cox log rank method.

STM WT in the intestine, MLN, spleen and liver than in the untreated mice (Fig. 6F–J). Further, using immunofluorescence, we observed that DFMO significantly reduced the colonisation of STM and the levels of spermidine in mouse ileum (Fig S10 A-D). Moreover, treatment of mice with DFMO at a dose of 2mg/mice increased the survival of mice upon infection with STM WT (Fig. 6 K and L). Also, the weight reduction in mice treated with DFMO at a dose of 2mg/mice was less upon infection with STM WT (Fig. S10 E). Next, we evaluated the tissue damage upon infection of STM in mice liver. The results show that DFMO treatment of mice at a dose of 2mg/mice significantly lowered the disease score, suggesting lesser tissue damage than untreated (Fig. 6 M and N, S10 F). Thus, DFMO serves as a potential drug to treat *Salmonella* infection in mice by reducing the bacterial burden and tissue damage in mice and enhancing the survival of mice upon infection with STM.

4. Discussion

Salmonella is often referred to as a smart pathogen. Over the years, it has developed multiple strategies to combat host-derived stresses [60,61]. A significant part of *Salmonella's* life cycle during its pathogenesis involves residing in the macrophages. This Gram-negative bacteria experience multiple host-induced environmental stress conditions inside the macrophages [62]. Rapid oxidative burst and ROS production are critical mechanisms by which the host macrophages try to limit the invading pathogen. ROS includes superoxide radicals, hydroxyl radicals, peroxy-nitrites, peroxy-chlorides, and hydrogen peroxide. ROS can pass through the bacterial cell wall and act on lipids, proteins, and nucleic acids by oxidizing them and leading to cellular damage. To combat the oxidative burst generated by Nox2 in macrophages, *Salmonella* carries multiple antioxidant enzymes such as the catalases KatE and KatG, the superoxide dismutases SodA and SodB, the Alkyl hydroperoxide reductase, the glutaredoxins and thioredoxins and Hrg transcriptional regulator [35]. Polyamines assist in *Salmonella* virulence and aid in stress resistance. However, the mechanism behind the role of polyamines in *Salmonella* stress resistance and virulence remains less appreciated.

Our study identifies spermidine as a stress-responsive regulatory molecule in *Salmonella* Typhimurium. We show spermidine is critical for the survival and proliferation of STM in macrophages and in the presence of oxidative stress *in vitro*. The spermidine transporter and biosynthesis mutants display significantly reduced capability to be phagocytosed by the macrophages. Findings from our previous study showed that the intracellular level of spermidine is substantially less in both the spermidine transport and biosynthesis mutants, which further explains the attenuated proliferation of both the mutants in macrophages [13]. The previous findings also showed that in the absence of the transport genes in *Salmonella* Typhimurium, the synthesis genes are downregulated and vice versa. The lack of spermidine transport and biosynthesis diminishes the mRNA expression of multiple arms of oxidative stress response in *Salmonella*, such as those regulated by RpoS, SoxR/S and EmrR, respectively. Numerous studies show the role of polyamines in regulating the transcription of multiple genes by interacting with DNA in eukaryotes. They bind to the DNA and change conformation as in C-MYC transcription, and in other cases, enhance DNA-protein binding affinities like for Estrogen-response elements [63,64]. Thus, in *Salmonella* Typhimurium, *rpoS*, *soxR,* and *emrR* similarly fall under the "Polyamine modulon".

We further characterise a novel enzyme, GspSA, in *Salmonella* Typhimurium, which synthesises a conjugated product of glutathione (GSH) and spermidine called GS-sp. GspSA is critical for *Salmonella* Typhimurium to survive and proliferate in macrophages. Our study shows that the absence of GspSA attenuates the survival of STM under oxidative stress conditions *in vitro,* suggesting a vital role of GspSA in protecting STM from oxidative damage. GS-sp in *E. coli* carries out its function by modifying protein thiol groups under oxidative stress to protect the proteins from getting oxidized and damaged. In *Salmonella* Typhimurium, we further show that the spermidine regulates the *gsp* expression and the subsequent production of GS-sp. We expect that GS-sp generated under oxidative stress conditions and inside the macrophages likewise interact with cysteine-thiol groups in proteins to shield them from oxidative damage. MK Chattopadhyay (2013) showed that this enzyme (GspSA) is specifically present in two groups of organisms, namely bacteria and kinetoplastids, respectively, and completely absent in other organisms such as humans, rats, drosophila, etc., among the bacteria group in all *enterobacteria* showed 27–100% homology and >65% identity in around 50% of the species, with *E. coli* [40]. Thus, the absence of GspSA in eukaryotes makes it a potent drug target for treating *Salmonella* infections. Thus, spermidine exerts pleiotropic effects in *Salmonella* by manipulating the multiple arms of antioxidative response. Our study gives a mechanistic insight into spermidine's regulation under oxidative stress. Spermidine regulates *rpoS, soxR* and *emrR* transcription factors and their downstream antioxidative enzymes. However, the absence of spermidine has a prominent effect on the expression of superoxide dismutases (*sodA* and *sodB*) and glutathione synthase enzymes. It strengthens the bacteria to cope with hostile host environments, such as the high ROS levels generated by the Nox2 enzyme in macrophages. Our studies in the *in vivo* model of *Salmonella* Typhimurium infection further dissect the role of spermidine in assisting in the pathogenesis by potentiating its ability to cope with the oxidative stress and thus enhancing its virulence strategies. The observation of higher fold proliferation of the spermidine mutants in peritoneal macrophages isolated from Nox2 knockout mice (*gp91phox*$^{-/-}$ mice) also corroborates with the role of spermidine in oxidative stress response. Considering our multiple mutants, we find that the spermidine transporter mutant does not show statistically significant reduced colonisation compared to the

other mutants in the liver upon oral gavage. However, the trend shows lesser colonisation by this mutant. On the other hand, the *gsp* mutant also does not have a statistically significant difference in its ability to disseminate in blood, while the trend shows less dissemination than STM WT. Thus, a subtle difference is observed among the mutants through our *in vivo* study, suggesting the minute variance among the mutants themselves. Moreover, it is at the nexus of multiple oxidative stress response arms in *Salmonella*, thereby assisting in mounting an anti-oxidative response to promote its survival in macrophages.

Studies in the past have offered insights into the function of polyamines in the pathogenesis of multiple virulent bacteria. Many human pathogenic bacteria have developed ways to exploit, interfere and manipulate the polyamine metabolism of the host to enhance their fitness within the niche. As in *Shigella* and *Vibrio* spp. polyamines produced by the bacteria are critical in determining virulence [65,66]. Meanwhile, bacteria such as *Legionella* spp., which does not synthesize polyamines, depend on the host-acquired polyamines for their pathogenesis [67]. Another unique mechanism is observed in *H. pylori,* which activates polyamine oxidation, thereby dysregulating the innate immune response [68]. Also, in response to *H. pylori* infection, the host macrophages increase the arginase activity and ornithine decarboxylase activity to produce polyamines [69]. A recent study showed that SARS-Cov-2 hijacks the host polyamines for reproduction and infection [70].

Our findings also reveal that *Salmonella* Typhimurium enhances the polyamine production in the host upon infection using its specialized pathogenicity island encoded effectors from SPI-1 and SPI-2, which might be a strategy to hijack the host polyamines for its survival. This further explains another reason for the reduced proliferation observed for the spermidine transport mutant in macrophages. Also, the knockdown of host ornithine decarboxylase attenuates *Salmonella* proliferation in macrophages. The upregulation of Odc1 activity manages to feed the amino acid, L-arginine, into the polyamine biosynthesis and prevent nitric oxide production, which otherwise would be detrimental for the pathogen. Polyamines have been well-studied as a drug target for the treatment of multiple cancers. The oncogene MYC is upregulated in 70 per cent of the cancer types. Ornithine decarboxylase (Odc1), a rate-limiting enzyme of polyamine biosynthesis, is transcriptionally activated directly by MYC, thereby increasing Odc1 levels in cancer [71]. RAS is another essential factor in cell growth and cancer development, and it acts on Odc1 to activate Odc1 in cancer cells translationally [72]. Polyamine biosynthesis is also associated with other pathways, such as AKT and mTORC pathways [73,74]. Previous studies show that D, L-α-difluoromethylornithine (DFMO) can be useful in treating various cancers [54]. It acts as a suicide inhibitor of the rate-limiting enzyme Odc1 in the polyamine biosynthesis pathway [58]. Studies suggest that it restricts the use of arginine for polyamine biosynthesis. Arginine is the common precursor to polyamine biosynthesis and nitric oxide synthesis. Thus, blocking polyamine biosynthesis enhances nitric oxide (NO°) production [75]. Nitric oxide is generated from the inducible Nitric oxide synthase (Nos2) enzyme in macrophages as an innate immune response to invading pathogens or inflammation [76]. The NO° radical is a labile molecule that reacts with peroxides and thiols to produce highly reactive species of peroxynitrites and nitrosothiols to control the infection by the invading pathogen, including *Salmonella* [7,77]. It also acts on nucleic acids, leading to deamination and mutagenesis [76]. Our study shows that upon using mOdc1 suicide inhibitor, DFMO, *Salmonella* proliferation could be diminished, and it reduces the bacterial burden in mice. The FDA-approved chemo-preventive drug, DFMO, for Human African Trypanosomiasis treatment is a potential drug to cure *Salmonella* infection by blocking host polyamine biosynthesis and moreover enhancing the NO° production in macrophages. In the devastating age of increasing antibiotic resistance, such a drug promises to combat deadly pathogens like *Salmonella* effectively.

Though this study underlines the role and mechanism of spermidine's anti-oxidative role in *Salmonella*, it bears a few limitations. Firstly, our study shows the changes in the expression of various antioxidative genes at the mRNA levels in the spermidine transport and biosynthesis mutants in *Salmonella.* However, it does not reveal precisely how spermidine regulates expression. We speculate in regard to the published literature on spermidine's interaction with nucleic acids and its role in the regulation of transcription and translation, that the observation from our study might also be from a similar mode of regulation by spermidine [78]. Future studies on understanding the mechanism of interaction of spermidine with these genes will provide further mechanistic depth to our observation. Secondly, our study confirmed that *Salmonella* employs the effectors encoded by the SPI-1 and SPI-2 to modulate the host polyamine metabolic pathway. However, which effectors are critical in the modulation of the host remains unanswered. In order to unravel the critical effectors involved in the upregulation of the host polyamine biosynthesis pathway further research is necessary.

5. Conclusion

A substantial duration of the infection cycle of *Salmonella* involves the macrophages, which present a very hostile environment to the bacteria. However, *Salmonella* can survive and proliferate within host macrophages and utilize it to disseminate to secondary sites of infection. Our study identifies a novel strategy employed by *Salmonella* Typhimurium to counteract oxidative and nitrosative stress within the host. We demonstrate that spermidine is a critical regulatory molecule in *Salmonella* that regulates multiple antioxidative pathways along with a novel antioxidative enzyme (GspSA) in *Salmonella* to prevent oxidative damage and assist in its virulence in mice. It further rewires host polyamine metabolism in a SPI-1 and SPI-2-dependent manner to prevent nitric oxide production and enhance survival. In the era of antimicrobial resistance, this study further recognizes an FDA-approved chemo-preventive drug, DFMO, which inhibits the host-polyamine metabolism, as a prospective antidote to treat *Salmonella* infection.

Availability of data and materials

All data generated and analysed during this study, including the supplementary information files, are incorporated in this article. The data is available from the corresponding author on request.

Ethics statement

All the animal experiments were approved by the Institutional Animal Ethics Committee, and the guidelines provided by The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, a statutory Committee, established under Chapter 4, Section 15 (1) of the Prevention of Cruelty to Animals Act 1960) and by National Animal Care were strictly followed during all experiments. (Registration No: 435 48/1999/CPCSEA). The approved protocol number is CAF/Ethics/852/2021.

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CRediT authorship contribution statement

Abhilash Vijay Nair: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Conceptualization. **Anmol Singh:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **R.S. Rajmani:** Writing – review & editing, Writing – original draft, Validation, Methodology. **Dipshikha Chakravortty:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization, Project administration, Resources, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

All the data are shared as main figures and supplementary figures

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2024.103151.

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Spermidine constitutes a key determinant of motility and attachment of *Salmonella* Typhimurium through a novel regulatory mechanism

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ABSTRACT

Spermidine is a poly-cationic molecule belonging to the family of polyamines and is ubiquitously present in all organisms. *Salmonella* synthesizes, and harbours specialized transporters to import spermidine. A group of polyamines have been shown to assist in *Salmonella* Typhimurium's virulence and regulation of *Salmonella* pathogenicity Inslad 1 (SPI-1) genes and stress resistance; however, the mechanism remains elusive. The virulence trait of *Salmonella* depends on its ability to employ multiple surface structures to attach and adhere to the surface of the target cells before invasion and colonization of the host niche. Our study discovers the mechanism by which spermidine assists in the early stages of *Salmonella* pathogenesis. For the first time, we report that *Salmonella* Typhimurium regulates spermidine transport and biosynthesis processes in a mutually inclusive manner. Using a mouse model, we show that spermidine is critical for invasion into the murine Peyer's patches, which further validated our in vitro cell line observation. We show that spermidine controls the mRNA expression of fimbrial (*fimA*) and non-fimbrial adhesins (*siiE*, *pagN*) in *Salmonella* and thereby assists in attachment to host cell surfaces. Spermidine also regulated the motility through the expression of flagellin genes by enhancing the translation of sigma-28, which features an unusual start codon and a poor Shine-Dalgarno sequence. Besides regulating the formation of the adhesive structures, spermidine tunes the expression of the two-component system BarA/SirA to regulate SPI-1 encoded genes. Thus, our study unravels a novel regulatory mechanism by which spermidine exerts critical functions during *Salmonella* Typhimurium pathogenesis.

1. Introduction

Salmonella enterica is considered a primary foodborne pathogen and the most pathogenic species of the genus *Salmonella* (Crump et al., 2015). Upon ingestion through contaminated food and water, *Salmonella* survives the acidic pH in the stomach and successfully reaches the small intestine. *Salmonella* harbours multiple virulence-associated genes, most clustered into 23 *Salmonella* pathogenicity islands (SPIs) (Espinoza et al., 2017). The primary cell type encountered by *Salmonella* is the epithelial cell lining the intestinal lumen at the Peyer's patches. The first step to a successful infection is passing through the thick mucous lining and adhering to the epithelial cell surface, followed by its subsequent invasion (Wagner and Hensel, 2011a). To infect the host epithelial cells, it utilizes a highly elegant nanomachine, the Type 3 secretion system(T3SS) encoded by the SPI-1, which transports effectors into the host cytosol, leading to actin cytoskeletal rearrangement and uptake of the bacteria (Hajra et al., 2021). Once inside the host cell, it activates another set of virulence genes encoded by SPI-2, which aid in the survival and replication of *Salmonella* in the specialized niche called the *Salmonella* containing vacuole(SCV) (Hajra et al., 2021; Eswarappa et al., 2010). It is then taken up by the CD18 + expressing macrophages and dendritic cells and disseminates through the reticuloendothelial system (Leung and Finlay, 1991; Das et al., 2009).

Polyamines are polycationic compounds associated with several functions in eukaryotes, including cell growth, cell division, stress response gene regulation, etc (Igarashi and Kashiwagi, 2010). They are ubiquitously present in all life forms, including bacteria. *Salmonella* can anabolically metabolize putrescine and spermidine from arginine or

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ornithine as the precursor and cadaverine from lysine (Igarashi and Kashiwagi, 2010; Álvarez-Ordóñez et al., 2010). Besides its synthesis ability, it has three transport systems: PotABCD, PotFGHI and PotE. PotABCD imports preferentially spermidine and putrescine, while PotFGHI and PotE import and export only putrescine, respectively (Jelsbak et al., 2012) (Fig. 1 A).

In bacteria, polyamines are involved in biofilm formation, virulence, and motility (Chattopadhyay et al., 2003; Nesse et al., 2015). In *Shigella sp.,* the accumulation of spermidine shields it from oxidative stress within the host macrophages, which is essential for its invasion (Barbagallo et al., 2011). *Streptococcus pneumoniae* upregulates spermidine transporter PotD levels during infection, and its loss leads to attenuated pneumococcal virulence in mice (Shah et al., 2011). Also, in *Vibrio cholerae* and *Yersinia pestis,* polyamines are crucial players regulating biofilm formation. Studies in *Salmonella* Typhimurium, show that spermidine is required for stress resistance. The double mutant of spermidine transport and biosynthesis shows reduced virulence in competition with the wild-type *Salmonella* in mouse spleen (Espinel et al., 2016). Further, *C. elegans,* upon infection with *Salmonella* mutant for synthesis or transport of spermidine and putrescine, survive better than wild-type infected ones (Jelsbak et al., 2012). Moreover, the study showed that putrescine and spermidine regulate SPI-1 in *Salmonella* to invade the epithelial cells (Jelsbak et al., 2012). Another study from the same group further illustrated that putrescine and spermidine modulate the translation of HilA, the master regulator of SPI-1 genes (Guerra et al., 2020). At the same time, the deletion of *speG* that functions in the catabolism of spermidine in *Salmonella* Typhimurium reduces the intracellular replication in multiple human cell lines (Fang et al., 2017). Studies to date show that putrescine and spermidine are critical in *Salmonella* pathogenesis. Spermidine is a major polyamine in bacteria and functions to regulate the expression of numerous genes in prokaryotes by interacting directly with the negatively charged nucleic acids. However, the molecular mechanism and the players involved or regulated by spermidine to assist in the virulence of *Salmonella* remains a mystery.

We were intrigued to understand whether the essential polyamine in bacteria, spermidine, alone plays a role in the pathogenesis of *Salmonella* Typhimurium. Also, to unravel the molecular mechanism by which spermidine modulates the virulence of *Salmonella* Typhimurium. We show spermidine is a crucial player during the infection cycle of *Salmonella* Typhimurium. Spermidine is critical in each of the early stages of the infection cycle of *Salmonella* and its survival within host epithelial cells. We delineate a novel regulatory network involving spermidine in modulating the surface adhesive and motility structures in *Salmonella* Typhimurium.

2. Material and methods

2.1. Bacterial strains and growth condition

Salmonella enterica serovars Typhimurium (STM WT) wild type strain ATCC 14028S was used in all experiments which was a kind gift from Prof. Michael Hensel, Abteilung Mikrobiologie, Universität Osnabrück, 273 Osnabrück, Germany. The bacterial strain was cultured in Luria broth (Luria broth, Himedia) with constant shaking (175 rpm) at 37 °C orbital-shaker. Growth kinetics experiment was performed in LB media, M9 minimal media and F-media (Allam et al., 2012; Chandra et al., 2023; Sridhar and Steele-Mortimer, 2016). The growth was monitored using Bioscreen C (Labsystems, Finland). S1 Table (Supplementary Information).

2.2. Bacterial gene knockout and strain generation

The generation of gene knockout in bacteria was done using the One-step chromosomal gene inactivation protocol by Datsenko and Wanner (2000) (Datsenko and Wanner, 2000). Briefly, primers were designed to amplify the antibiotic resistance genes from pKD4 and pKD3 plasmids, respectively. The primers were designed such that the amplified antibiotic resistance genes have a 60-base pair of homologous regions corresponding to flanking regions of the gene to be knocked-out in bacteria. The purified PCR amplified Kanamycin and Chloramphenicol resistance cassettes were electroporated into the STM WT cells (expressing pKD-46 plasmid, which provides the λ-Red recombinase system) by a single pulse of 2.25 kV separately for the Kan^R and $Chlm^R$. This ensures the homologous recombination between the PCR product and the corresponding region of the gene of interest in the bacterial chromosomal DNA. The transformant colonies were selected and patched on fresh plates and confirmed for knockout using PCR with primers designed for confirmation. For the generation of the double knockout strain (STM Δ*potCD*Δ*speED*), the STM Δ*potCD* (resistant to Kanamycin) was first transformed with the plasmid pKD46. To this transformed strain, the purified PCR product to knockout *speED* was electroporated to generate the STM Δ*potCD*Δ*speED* (resistant to Kanamycin and Chloramphenicol). For generation of chromosomal *fliA*-FLAG in STM WT, STM Δ*potCD,* and STM Δ*speED* the same protocol of homologous recombination using pKD-46 plasmid was used. The amplification of FLAG-Kan^R was done from pSUB11 plasmid (a generous gift from Prof. Umesh Varshney, MCB, IISc). The purified PCR product were electroporated into the respective strains, and transformants were selected in Kanamycin containing LB agar plates. For generation of the complemented strains, pQE60(low copy number) plasmid was used. The *potA* and *speE* genes were amplified using PCR and cloned into the vector under T5 promoter, at *Bam*HI and HinDIII sites of the multiple cloning site of the vector. The cloned pQE60 plasmids were then transformed into STM Δ*potA* to generate STM Δ*potA:potA* (complemented strain) and STM Δ*speE* to generate STM Δ*speE:speE* (complemented strain). S2 Table (Supplementary Information) for primer list.

2.3. Cell culture and maintenance

Caco-2 cells (human intestinal epithelial cell line) were cultured in DMEM - Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% FBS (Gibco), 1% Non-essential amino acids (Sigma- Aldrich), 1% Sodium pyruvate (Sigma- Aldrich) and 1% Penicillin-streptomycin (Sigma- Aldrich) at 37 °C humidified chamber (Panasonic) with 5% CO_2. HeLa cells (human epithelial cell line) were cultured in DMEM - Dulbecco's Modified Eagle Medium (Lonza) supplemented with 10% FBS (Gibco) at 37 °C humidified chamber (Panasonic) with 5% CO_2. For each experiment, the cells were seeded onto the appropriate treated cell culture well plate at a confluency of 80% either without coverslips (for intracellular survival assay, adhesion assay and qRT-PCR) or with coverslips (for immunofluorescence microscopy).

2.4. Gentamicin protection assay

The cells were infected with STM WT, STM Δ*potCD,* STM Δ*speED* and STM Δ*potCD*Δ*speED* at MOI of 10 (for intracellular survival assay, adhesion assay, and immunofluorescence microscopy) and MOI 25 (for qRT-PCR). After infecting the cell line with STM WT and the mutants, the plate was centrifuged at 700–900 rpm for 10 min to facilitate the proper adhesion. The plate was then incubated for 25 min at 37 °C humidified chamber and 5% CO_2. After washing with 1X PBS fresh media containing 100 μg/mL gentamicin was added and again incubated for 60 min at 37 °C and 5% CO_2. Similarly, fresh media containing 25 μg/mL gentamicin was added. The plate was incubated at 37 °C and 5% CO_2 till the appropriate time. For the intracellular survival assay, two time points were considered 2 h and 16 h, and for qRT-PCR three time points were considered 2 h, 6 h and 16 h.

2.5. Intracellular survival assay and invasion assay

At the appropriate time post-infection, the cells were lysed using 0.1% Triton X followed by the addition of more 1X PBS and samples

were collected. The collected samples were plated at the required dilutions on LB agar plates and incubated at 37 °C for 12 h followed by CFU enumeration.

The fold proliferation and invasion were determined as follows.

Fold Proliferation = (CFU at 16 h post-infection)/(CFU at 2 h post-infection).

Percentage invasion = [(CFU at 2 h post-infection)/(CFU of the Pre-inoculum)]× 100.

2.6. Adhesion assay

Modified from the gentamicin protection assay. Here the plate was then incubated for 10 min at 37 °C humidified chamber and 5% CO_2 post-infection. Then the media was removed, and the cells were washed with 1X PBS twice to remove the loosely adhered bacteria. The mammalian cells were then lysed with 0.1% Triton-X 100 to release the adhered bacteria into the solution, and the samples were collected. The collected samples were plated at the required dilutions on LB agar plates and incubated at 37 °C for 12 h, followed by CFU enumeration.

The percentage adhesion was determined as follows:

Percentage adhesion = [(CFU at 10 min post-infection)/ (CFU of the Pre-inoculum)]× 100.

2.7. Immunofluorescence

After the appropriate incubation time, the media was removed, and the cells were washed with 1X PBS and fixed with 3.5% Paraformaldehyde for 10 min. The cells were then washed with 1X PBS, incubated with the required primary antibody in a buffer containing 0.01% saponin and 2% BSA, and incubated at room temperature for 45–60 min. After washing with 1X PBS, the secondary antibody tagged to a fluorochrome was added and incubated. The coverslips were then washed with PBS and mounted on a clean glass slide using mounting media containing an anti-fade reagent and observed under the confocal microscope (Zeiss 710 microscope, at 63X oil immersion, 2 ×319 3x zoom, and 100X zoom for studying only bacterial samples, Zeiss 880 microscope, at 63X oil immersion, 2 ×319 3x zoom). For studying histopathology samples 40X oil immersion 2 × 319 3x zoom was used. For the FliC study, strains were subcultured and grown in LB media, with or without supplementation of 100 μM spermidine till the log phase of growth (OD 0.1). After washing with 1X PBS, the samples were smeared on a clean glass slide, air dried and stained as previously explained, using specific antibody in the buffer. For the invasion assay, the buffer contained only 2% BSA.

2.8. RNA isolation and qRT-PCR

RNA isolation was performed from infected cells after appropriate hours of infection with STM WT, STM Δ*potCD,* STM Δ*speED* by RNA isolation was performed using TRIzol (TaKaRa) reagent according to manufacturers' protocol RNA was quantified using Thermo-fischer scientific Nano Drop. For cDNA synthesis, 3 μg of isolated RNA was treated with DNase1 at 37 ℃ for 60 min, followed by heat inactivation at 65 ℃ for 10 min. Then RNA (free of DNA) was subjected to Reverse transcription using the manufacturer's protocol (RT kit TaKaRa). Quantitative real-time PCR was done using SYBR green RT-PCR kit in BioRad qRT-PCR system. The gene expression levels of interest were measured using specific primers. Gene expression levels were normalized to 16SrRNA primers of *S*. Typhimurium. For expression studies in bacteria grown in LB media, the bacterial samples were harvested at 3 h, 6 h, 9 h and 12 h post subculture in fresh LB media in 1:100 ratio. Then a similar protocol was used to isolate total RNA using TRIzol (TaKaRa) reagent according to manufacturers' protocol. S2 Table (Supplementary Information) for primer list.

2.9. Immunoblotting

The bacterial strains were grown in LB media until the log phase of growth. The cells were centrifuged to remove the media, and the cells were resuspended in lysis buffer (Sodium chloride, Tris, EDTA, 10% protease inhibitor cocktail) after washing with 1XPBS. The cells were lysed using sonication and centrifuged at 4 °C to collect the cell lysate, followed by estimation of total protein using the Bradford protein estimation method. 50 μg of protein was loaded onto a Polyacrylamide Gel Electrophoresis (PAGE), then transferred onto 0.45 μm PVDF membrane (GE Healthcare). 5% skimmed milk (Hi-Media) in TTBS was used to block for 1 h at room temperature and then probed with Anti-FLAG primary and secondary HRP-conjugated antibodies. ECL (Biorad) was used for developing the blot, and images were captured using Chemi-Doc GE healthcare. All densitometric analysis was performed using the Image J.

2.10. Swimming assay

2 μl of bacterial samples were spotted on the 0.3% agar plates supplemented with 0.5% yeast extract, 1% casein enzyme hydrolysate, 0.5% NaCl and 0.5% glucose (swim agar plates). The plates were incubated at 37°C for 6 h, and then images were taken using a BioRad-chemidoc. The diameters of the motility halos were measured. At least five replicate plates were used for each condition.

2.11. Beta-galactosidase assay

Salmonella Typhimurium harboring lacZ transcriptional fusions to *hilA* and *spiC* were used for the assay. The transcriptional activity of each gene was determined by performing a β-galactosidase assay, as described previously (Marathe et al., 2016).

2.12. Transmission electron microscopy

Flagella were visualized by slightly modifying the protocol described in Garai et al. (2016). Briefly, overnight bacterial cultures were subcultured in LB media and incubated until it reached an OD of 0.1. The bacterial cultures were centrifuged at 2000 rpm for 10 min at 4°C. The bacterial cells were washed with 1XPBS twice and finally, the cells were resuspended in 100 μl of 1X PBS. 10 μl of the cell suspension was added to the copper grid, air dried, stained with 1% uranyl acetate for 30 s, and visualized under the transmission electron microscope.

2.13. Mass-spectrometry for determination of intracellular spermidine and putrescine

The sample preparation was done as explained Feng Y et.al; previously (Feng et al., 2022). Briefly, STM WT, STM Δ*potCD,* STM Δ*speED* and STM Δ*potCD*Δ*speED* were grown in LB media until log phase of growth. The cells were centrifuged to remove the media and the cells were resuspended in 80% methanol (Thermo-fischer) after washing with 1XPBS. The cells were lysed using sonication and centrifuged at 4 °C to collect the cell lysate. The methanol was evaporated in vacuum at low temperatures and then lyophilized at − 40 °C. Dried metabolite extracts were dissolved in 1.0 mL of 0.1% Formic acid in ddH2O, vortexed briefly, followed by centrifugation at 5000 rpm, 4ºC for 5 min. 0.5 mL of supernatant was transferred to a HPLC vial (Amber coloured) for LC–MS/MS analysis on a Agilent 1260 HPLC system coupled to an Agilent QQQ 6460 mass spectrometer. An Agilent Eclipse Plus C18 (50 mm × 4.6 mm, 1.8 μm) column at 30 °C was utilized for LC separation. Samples were injected (10 μl) from the auto sampler kept at 5 °C. The isocratic elution was as follows: 20% mobile phase B was maintained for 5 min at a flow rate of 0.5 mL min− 1. Mass spectra were acquired on a 6460 QQQ mass spectrometer (Agilent, USA) equipped with an electrospray ionization (ESI) source in positive ion mode. The

Mass parameters and multiple reactions monitoring spermidine and putrescine transition ions are shown in Table S3 (Supplementary Information). Peak identification and amounts of spermidine and putrescine were evaluated using Agilent MassHunter Data Acquisition, Agilent MassHunter QQQ Qualitative Analysis and Agilent MassHunter QQQ Quantitative Analysis softwares on the basis of the known amounts of spermidine and putrescine.

2.14. In vivo animal experiment

5–6weeks old C57BL/6 mice were infected by orally gavaging 10^7 CFU of STM WT, STM Δ*potCD,* STM Δ*speED* and STM Δ*potCD*Δ*speED*. For invasion assay intestine was isolated 6 h post-infection, and CFU was enumerated on differential and selective SS agar by serial dilution followed by plating.

2.15. Availability of data and materials

All data generated and analyzed during this study, including the supplementary information files, are incorporated in this article. The data is available from the corresponding author on request.

3. Ethics statement

All the animal experiments were approved by the Institutional Animal Ethics Committee, and the Guidelines provided by National Animal Care were strictly followed during all experiments. (Registration No: 435 48/1999/CPCSEA).

4. Results

4.1. Salmonella Typhimurium concurrently regulates the spermidine import and biosynthesis processes to maintain the intracellular spermidine homeostasis

Salmonella Typhimurium can synthesize and import spermidine; the outstanding question that arises is how *Salmonella* regulates the two processes and maintain an intracellular homeostasis of spermidine. We initially accessed the expression of the ATP-dependent transport apparatus genes *potA*, *potB*, *potC*, and *potD* that localize to the cell wall passing through the periplasmic space (IGARASHI and KASHIWAGI, 1999). We observed that during in vitro growth of *Salmonella,* all the genes encoding the transporter show a bimodal mRNA expression, with a higher expression at the mid-log phase(6 h) and at the early stationary phase(12 h) compared to the early-log phase(3 h) (Fig. 1 B). Similarly, we observed that *speE* and *speD,* the genes encoding the two enzymes that catalyse spermidine synthesis, show a bimodal pattern of mRNA expression (Fig. 1 C). In prokaryotes, spermidine plays a pivotal role in stress response, nutrient starvation, etc., which explains the higher mRNA expression of the transport and biosynthesis genes during the mid-log phase in *Salmonella*. To investigate how *Salmonella* regulates the transport system and its intracellular spermidine biosynthesis during its growth, we generated chromosomal knockout strains of *Salmonella* Typhimurium, namely STM Δ*potCD* that cannot import spermidine, STM Δ*speED* that cannot synthesize spermidine and a double knockout STM Δ*potCD*Δ*speED* that lacks both the functions. We studied the mRNA expression of biosynthesis genes in transporter mutants and vice-versa. We observed that *potA*, *potB*, *potC* and *potD* showed significant downregulation in mRNA expression post the early-log phase in STM Δ*speED* (Fig. 1 D). Likewise, both *speE* and *speD* show downregulation post the early-log phase in STM Δ*potCD* (Fig. 1 E). Thus, in *Salmonella,* spermidine import and biosynthesis are concurrently regulated.

To further verify our observation, we determined the intracellular spermidine levels in the different strains. We observed that the spermidine levels in the three mutants were significantly lower than STM WT, and there was no compensation in the transport or the biosynthesis mutants (Figs. 1 F, S1 A and S1 B). However, inhibition of spermidine synthesis might cause an accumulation of its precursor putrescine. We further determined the intracellular putrescine levels in *Salmonella* Typhimurium, and we observed that the levels are higher in STM Δ*potCD*, STM Δ*speED*, and STM Δ*potCD*Δ*speED* than STM WT (S1C-E Fig). Accumulation of spermidine in bacteria is toxic and it relies on acetylation by SpeG for removal. Studies show that polyamine toxicity is due to spermidine oxidation to acrolein and aldehydes that produce ROS as a by-product (Kumar et al., 2022; Sakamoto et al., 2020). However, putrescine is not reported to be metabolised to release these toxic by-products and depends only on removal by the specialised exporter PotE in *Salmonella.* Our findings, thus, indicated that *Salmonella* Typhimurium enhances spermidine import and synthesis during the mid-log phase of growth and that the bacterium concurrently regulates both processes to maintain the cellular spermidine levels.

Further, we analyzed how the loss of spermidine import and biosynthesis affects the growth of *Salmonella* in F-media that mimics the intravacuolar acidic environment of SCV, and we observed that all the strains showed similar growth kinetics, which did not alter upon exogenous spermidine supplementation (Figs. 1 G and H, S1 F). Similarly, we did not observe any difference in the in vitro growth in rich LB media and the minimal M9 media (Fig. S1 G and H), further supporting previous findings (Espinel et al., 2016). We further supplemented spermidine and putrescine in minimal M9 media during the in vitro growth; however, we also did not observe any significant difference upon supplementation (Fig. S1 I and J). Hence, loss of spermidine import and biosynthesis does not alter the growth kinetics of *Salmonella* in vitro.

4.2. The loss of spermidine synthesis and transport is sufficient to limit Salmonella Typhimurium infection in the host

The primary site of infection for *Salmonella* is the intestinal epithelial cells(IECs) in the large intestine (Galán, 2021). Previously, a group had shown that *Salmonella* strains deficient in putrescine and spermidine transport or biosynthesis exhibited a reduced invasion and proliferation in epithelial cells, while spermidine is required for proliferation into macrophages (Jelsbak et al., 2012; Espinel et al., 2016; Schroll et al., 2014). However, it is interesting to understand whether the loss of only spermidine transport or biosynthesis function impacts the virulence trait of *Salmonella* in epithelial cells. Thus, we explored the ability of spermidine mutants to invade the human intestinal cell line Caco-2 cells and proliferate within the cells. Upon infection into Caco-2 cells, we observed that STM Δ*potCD*, STM Δ*speED*, and STM Δ*potCD*Δ*speED* invaded significantly less and exhibited a substantially lower fold proliferation than STM WT (Fig. S2A−C). We further complemented the single gene mutants for spermidine transport (STM Δ*potA*) and biosynthesis (STM Δ*speE*) and observed a recovery in the fold proliferation and percentage invasion of the complemented strains upon infection into Caco-2 cells (S2 D and S2 E Fig.). We also performed the infection into HeLa cells, and likewise, we observed a similar lesser invasion and lower fold proliferation of the spermidine transport and biosynthesis gene mutants (Fig. S2F and G). *Salmonella* employs multiple strategies to survive and proliferate within its host niche (Steele-Mortimer et al., 2002; Lahiri et al., 2010). To understand the link between spermidine and *Salmonella* Typhimurium survival in epithelial cells, we determined the expression of the spermidine transporters and the biosynthesis genes upon infection into Caco-2 cells. The transporters *potA*, *potB*, *potC* and *potD* showed a gradual upregulation of their corresponding mRNA expression post 2 h of infection into Caco-2 cells (Fig. 2 A). Furthermore, the *speE* and *speD* showed a gradual upregulation of mRNA expression post 2 h of infection in Caco-2 cells with a significant upregulation at 16 h post-infection (Fig. 2 B). As we previously studied, we further determined the regulation of the two sets of genes during infection into Caco-2 cells. We observed a downregulation of *speE* and *speD* mRNA expression in STM Δ*potCD* and downregulation of *potA*, *potB*, *potC* and *potD* mRNA expression in STM Δ*speED* upon infection into Caco-2 cells

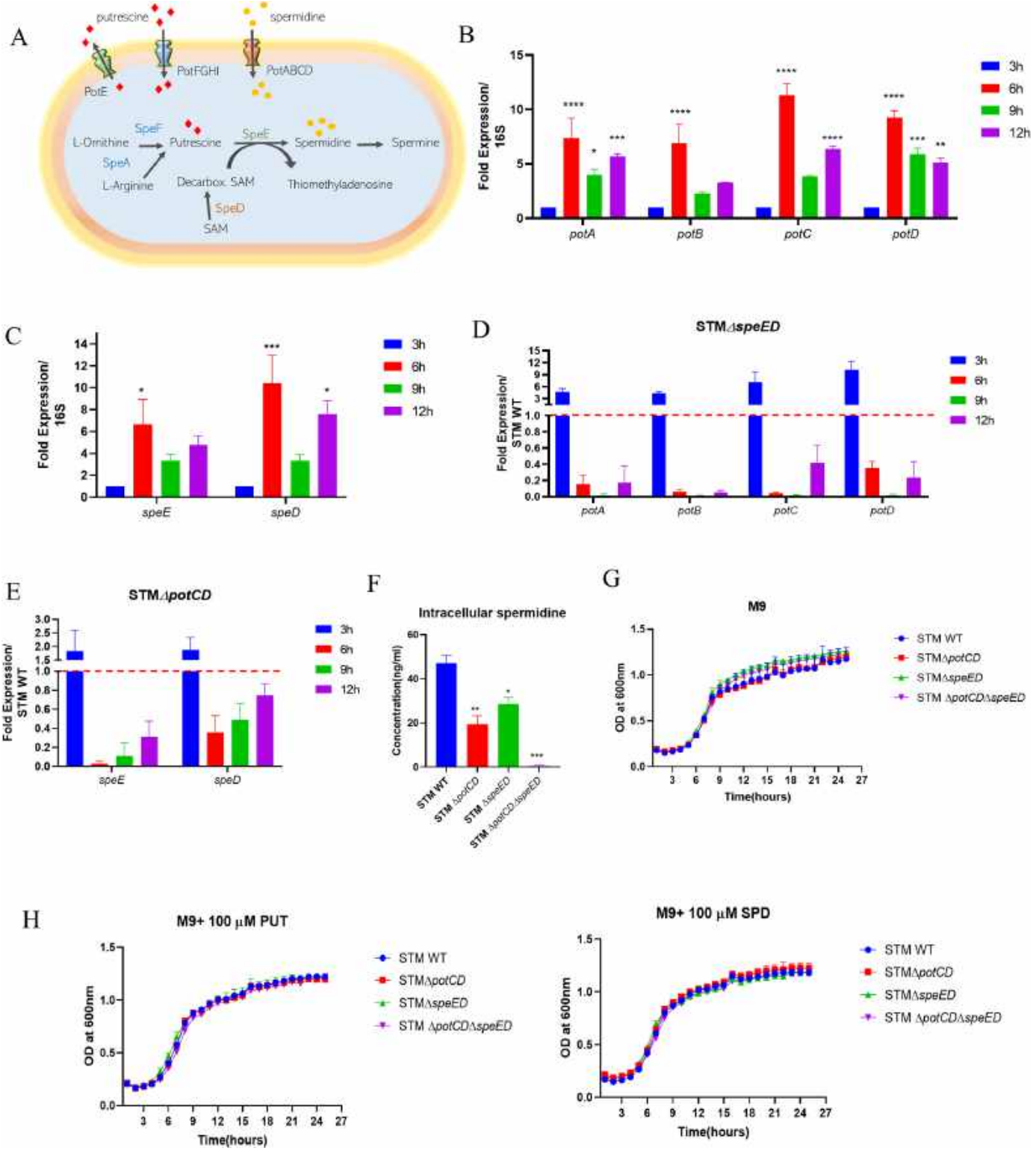

Fig. 1. *Salmonella* Typhimurium concurrently regulates the spermidine import and biosynthesis processes to maintain the intracellular spermidine homeostasis. A. Schematic of Polyamine transport and biosynthesis in *Salmonella* Typhimurium, B.The mRNA expression of pot-transporter genes in STM WT during in vitro growth in LB media, C. The mRNA expression of *speE* and *speD* genes in STM WT during in vitro growth in LB media, D. The mRNA expression of *pot*-transporter genes in STM Δ*speED* during in vitro growth in LB media, E. The mRNA expression of *speE* and *speD* genes in STM Δ*potCD* during in vitro growth in LB media, F. Intracellular spermidine determination using Mass spectrometry, G. Growth kinetics of STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* in acidic F-media, H. In Acidic F-media supplemented with 100 μM Spermidine (SPD). Student's t-test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05.

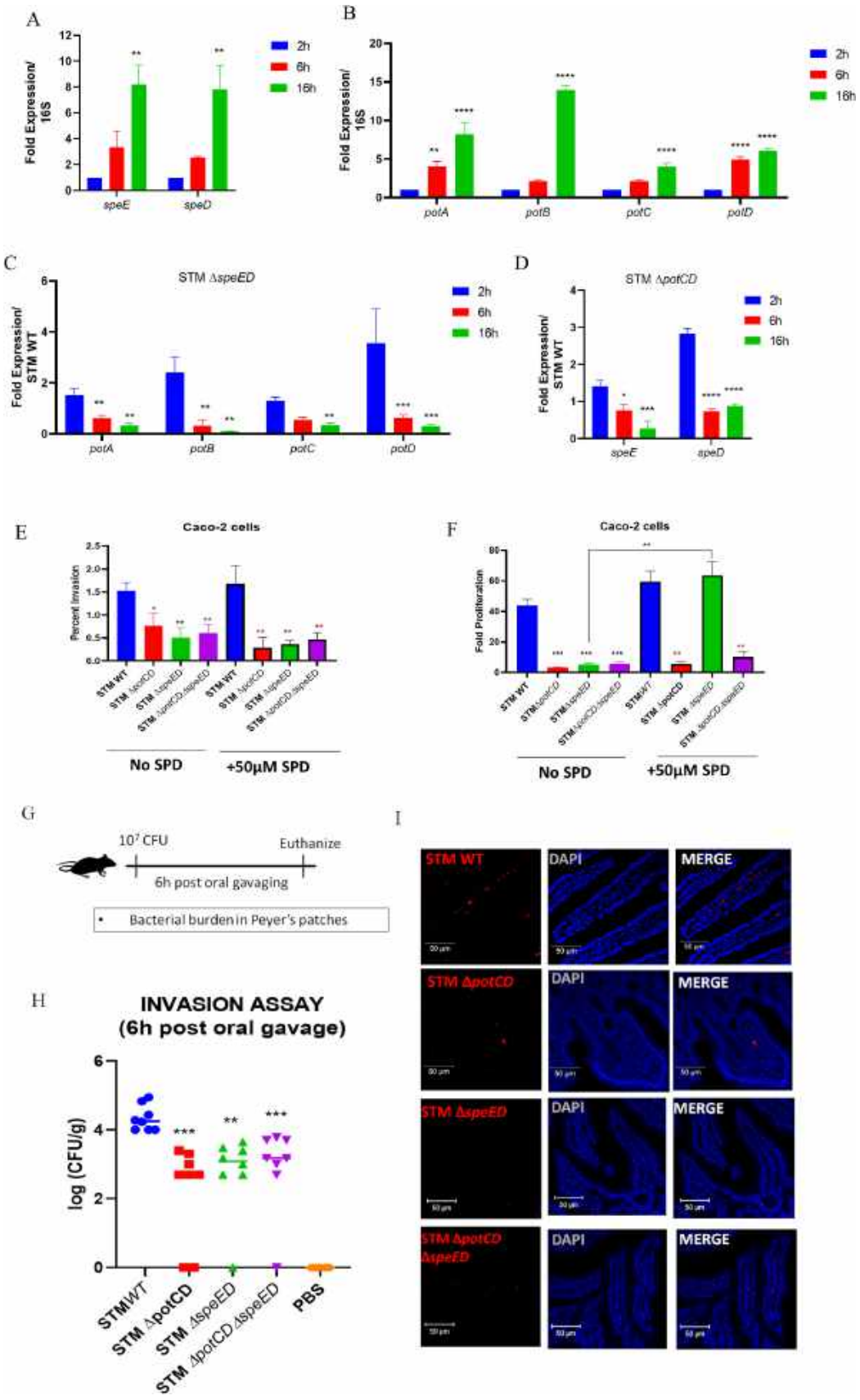

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Fig. 2. The loss of spermidine synthesis and transport is sufficient to limit *Salmonella* Typhimurium infection in the host. A. The mRNA expression of *speE* and *speD* in STM WT post infection into Caco-2 cells, B. The mRNA expression of *pot*-transporter genes in STM WT post infection into Caco-2 cells, C. The mRNA expression of *pot*-transporter genes in STM Δ*speED* post infection into Caco-2 cells, D. The mRNA expression of *speE* and *speD* genes in STM Δ*potCD* post infection into Caco-2 cells, E. The percentage invasion of the STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* in Caco-2 cells with supplementation of exogenous spermidine during infection, F. The fold proliferation in Caco-2 cells of STM WT and the mutants with supplementation of exogenous spermidine during infection, G. Experimental procedure for studying invasion of STM into mice Peyer's patches, H. Burden of STM in Peyer's patches post 6 h of oral gavage to assess invasion, I. Immunofluorescence of histopathological sections of mice intestine (Peyer's patches) to study invasion, DAPI is used to stain the nucleic acids in the cells, and *Anti-Salmonella* (LPS) (Cy3 tagged secondary antibody used-Red). Student's t-test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05. Two-way Anova was used to analyze the grouped data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05. For in-vivo studies Mann-Whitney test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05.

(Fig. 2 C and D). The observed downregulation of the genes explain the lower fold proliferation of both the individual mutants even though they lack just one of the two functions (transport/ biosynthesis). Taken together, these results suggest that loss of spermidine transport and biosynthesis functions are sufficient to limit the ability of *Salmonella* Typhimurium to infect IECs. Also, the bacterium maintains the intracellular spermidine by tuning both functions mutually inclusively, even within the host cells.

We further assessed whether supplementation of exogenous spermidine rescues the lower fold proliferation and invasion. The supplementation of Caco-2 cells with 50 μM spermidine during infection did not rescue the reduced invasion for any of the mutants (Fig. 2 E). However, it only rescued the lower fold proliferation for STM Δ*speED* (Fig. 2 F). Although in STM Δ*speED* the spermidine transport system genes are down-regulated, bacteria still manage to accumulate basal levels of spermidine in STM Δ*speED* (Fig. 1F), which suggests that the transporter activity might still be there to rescue the phenotype. However, when supplemented in minimal M9 media during the in vitro growth of the different strains before infection, it rescued the lower fold proliferation and the lesser invasion of the STM Δ*speED* only (Fig. S2H and I). To further validate our in vitro cell line results, we then studied the invasion of the mutants into the intestine of C57BL/6 mice (Fig. 2 G). Interestingly, STM Δ*potCD*, STM Δ*speED*, and STM Δ*potCD* Δ*speED* invaded significantly less into the Peyer's patches of the C57BL/6 mice (Fig. 2H). The immunofluorescence study with the histopathological sections of mouse ileum further validated the reduced invasion and colonisation of the spermidine mutants in the intestine (Fig. 2 I). Thus, *Salmonella* requires spermidine to invade successfully, subsequently survive, and proliferate within the IECs in vitro and in vivo.

4.3. Adhesion of Salmonella Typhimurium to epithelial cells is aided by spermidine by regulation of fimbrial and non-fimbrial adhesins

Most bacterial pathogens must first reach the site of infection, followed by sequential steps of adhesion, invasion, multiplication and proliferation to infect and colonize the host tissues successfully (Pizarro-Cerdá and Cossart, 2006). During the pathogenesis of *Salmonella*, a crucial step towards infection into the IECs is its ability to adhere to the surface of the IECs (Wagner and Hensel, 2011b). Our study so far shows that the loss of spermidine transport or synthesis capability of *Salmonella* reduces the invasiveness of the bacteria into human epithelial cells. During the pathogenesis of the respiratory tract pathogen *S. pneumoniae,* the polyamine cadaverine is critical for adhesion, leading to colonization in the nasopharynx (Nakamya et al., 2018). Also, exogenous spermidine increases the adhesion of *Bifidobacterium animalis subs. lactis* Bb12 in the mucous of infants (Mantziari et al., 2021). To understand the role of spermidine in the adhesion of *Salmonella* Typhimurium to epithelial cells, we performed an adhesion assay in Caco-2 cells with various strains. All three mutants showed significantly lower adhesion than the wild-type ones as the *fimA* (Type1 fimbriae) mutant control (Fig. 3 A, C and D). Upon complementation of *potA* and *speE* in the single gene mutants of spermidine transport and biosynthesis, we observe a partial recovery of the adhesion as of the STM WT (Fig. S3A). A similar observation was seen in HeLa cells (Fig. S3B–D). The addition of exogenous polyamine to the bacteria before infection reversed the phenotype in STM Δ*speED* (Fig. 3 B, C and D).

Salmonella employs multiple systems ranging from monomeric structures to highly complex and giant structures to adhere to the host cells. *Salmonella* possesses multiple fimbrial gene clusters that encode fimbrial appendages to bind to the host cell surfaces (Rehman et al., 2019; Bäumler et al., 1996). Apart from fimbrial proteins, its cell surface is decorated with various non-fimbrial proteins like PagN, outer membrane proteins (Omps) and the type 1 secreted giant adhesin SiiE etc (Wagner and Hensel, 2011b; Gerlach et al., 2007; Li et al., 2019). We observed that the fimbrial and non-fimbrial adhesins in both mutants show lower mRNA expression than in the wild type during the exponential phase of growth (Fig. 3 E and F). Furthermore, growth in supplementation of exogenous spermidine showed increased mRNA expression in the mid-log growth phase for the non-fimbrial *siiE* and *pagN* and the fimbrial *fimA* genes (Fig. S3E). Overall, our observations confirm that spermidine assists in the adhesion of *Salmonella* Typhimurium to host cell surfaces by controlling the expression of fimbrial and non-fimbrial genes.

4.4. Spermidine regulates flagellar gene expression by enhancing the translation of FliA, which otherwise has a poor Shine-Dalgarno sequence and an unusual start codon

Gram-negative and Gram-positive bacteria express flagella on their surfaces, primarily as a motility structure (Haiko and Westerlund-Wikström, 2013). However, many studies have shown flagella to act as an appendage to adhere to host cell surfaces, such as the chromosomal mutation of the flagellar cap protein(*fliD)* in *Pseudomonas aeruginosa* resulted in the loss of adhesion to mucin on epithelial cells (Arora et al., 1998). Similarly, in *Vibrio cholerae,* non-motile variants exhibited reduced virulence due to poor adsorption onto the cells (Guentzel and Berry, 1975). Likewise, in *Salmonella,* the importance of flagella as an adhesive structure has been shown by many researchers (Dibb-Fuller et al., 1999; Salehi et al., 2017). Researchers from our group have previously demonstrated that the loss of flagella in *Salmonella* Typhimurium led to reduced adhesion to Caco-2 cells and lesser colonization in the gut of *C. elegans* (Garai et al., 2016). Thus, we were interested in deciphering the role of spermidine in regulating flagella. We carried out a swimming motility assay and observed that STM Δ*potCD* and STM Δ*potCD*Δ*speED* showed highly attenuated movement on soft agar (Figs. 4 A, S4A). In contrast, STM Δ*speED* exhibited a 40% reduction in movement compared to the wild type (Fig. 4 A, S4 A). Furthermore, the complemented strains for STM Δ*potA* and STM Δ*speE* showed recovery in their swimming motility (Fig. S4B and C). Hence, we determined the mRNA expression of the *fliC and fljB* that encode flagellin protein in *Salmonella* Typhimurium and found that both the genes show significant downregulation in STM Δ*potCD* and STM Δ*speED* (Fig. 4 B and C). However, the growth of STM Δ*speED* in the presence of spermidine increased the swimming motility similar to the wild type and the mRNA expressions of *fliC* and *fljB* during the mid-log phase of growth (Fig. 4 A and D).

We performed immunofluorescence to study the flagellin FliC expression on the surface of *Salmonella* Typhimurium. The presentation of the flagellin FliC was significantly less on the surface in all the mutants than in the wild type (Figs. 4 E and F, S4 D). The result was further

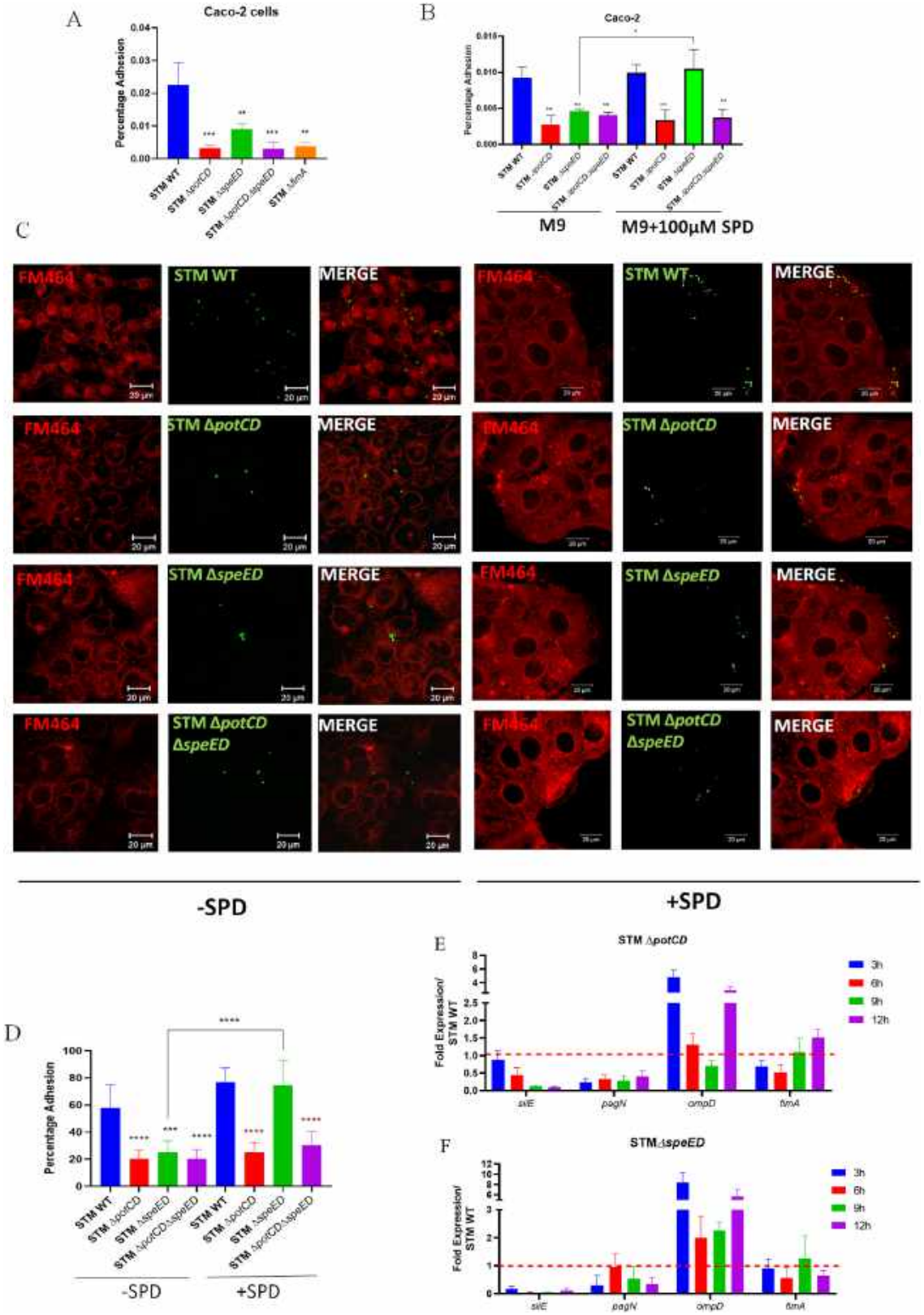

Fig. 3. Adhesion of *Salmonella* Typhimurium to epithelial cells is aided by spermidine by regulation of fimbrial and non-fimbrial adhesins. A. Adhesion assay of STM WT and the mutants Caco-2 cells, B. Adhesion assay in Caco-2 of STM WT and the mutants grown in M9 minimal media supplemented with spermidine, C. Immunofluorescence imaging to study the adhesion to Caco-2 cells of STM WT and the three mutants grown in M9 minimal media with and without supplementation of spermidine, here FM464(red) is used to stain the lipids for Caco-2 and Anti-*Salmonella*(LPS) (Alexafluor-488 tagged secondary antibody used-Green) for STM, D. Quantification of C, E. The mRNA expression of non-fimbrial adhesins such as *siiE*, *pagN* and *ompD* and fimbrial adhesin *fimA* genes in STM Δ*potCD* during in vitro growth in LB media, F. The mRNA expression of non-fimbrial adhesins such as *siiE*, *pagN* and *ompD* and fimbrial adhesin *fimA* genes in STM Δ*speED* during in vitro growth in LB media. Student's t-test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05.

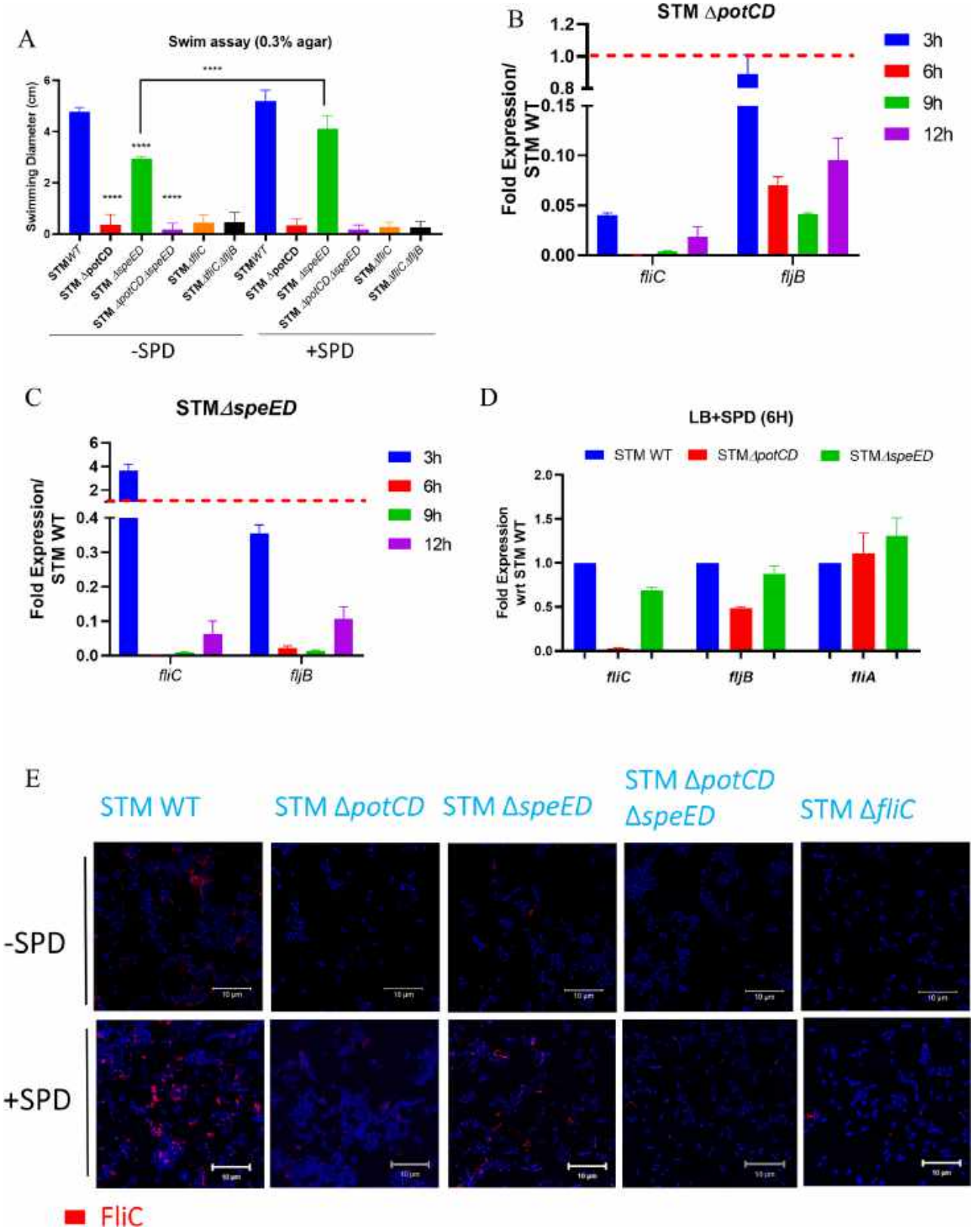

Fig. 4. Spermidine regulates flagellar gene expression by enhancing the translation of FliA, which otherwise has a poor Shine-Dalgarno sequence and an unusual START codon. A. Swimming motility of STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* grown in M9 minimal media supplemented with and without spermidine on 0.3% agar, B. The mRNA expression of the genes *fliC* and *fljB*, coding for flagellin in STM Δ*potCD* during in vitro growth in LB media, C. The mRNA expression of the genes *fliC* and *fljB*, coding for flagellin in STM Δ*speED* during in vitro growth in LB media, D. The mRNA expression of the genes *fliC* and *fljB* coding for flagellin and *fliA*, coding for the sigma-factor-28 in STM Δ*potCD* and STM Δ*speED* during log phase of growth (6 h) in LB media supplemented with spermidine, E. Immunofluorescence imaging to study the expression of FliC (flagella) on the surface of STM WT and the three mutants grown with and without supplementation of spermidine, here DAPI (blue) is used to stain the nucleoid of STM and Anti-FliC(Cy3-tagged secondary antibody used-Red) for STM flagella, F. The quantification of

E, G. TEM images of STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speED* and STM Δ*fliC* grown till log phase of growth, with and without supplementation of spermidine (red arrow heads in zoom-in images show the flagella), H. The mRNA expression of the gene *fliA*, coding for sigma-factor-28 in STM Δ*potCD* during in vitro growth in LB media, I. The mRNA expression of the gene *fliA*, coding for sigma-factor-28 in STM Δ*speED* during in vitro growth in LB media, J. Western blot of FliA-FLAG in STM WT, STM Δ*potCD* and STM Δ*speED* grown till log phase of growth, with and without supplementation of spermidine. Student's t-test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05. Two-way ANOVA was used to analyze the grouped data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05.

validated using Transmission electron microscopy. Likewise, we observed reduced numbers of flagella on the surface of STM Δ*speED*, while no flagella on the surface of STM Δ*potCD* and STM Δ*potCD*Δ*speED* similar to STM Δ*fliC* (Fig. 4 G). The surface presentation of FliC was found to be more upon the growth of STM Δ*speED* in the presence of exogenous spermidine (Fig. 4 E-G).

As the levels of *fliC* and *fljB* decreased in both the spermidine transport and biosynthesis mutants of *Salmonella*, we next determined the expression of the sigma factor σ^{28}(FliA) that aids in the transcription of the flagellin genes. In both STM Δ*potCD* and STM Δ*speED*, the mRNA expression was not downregulated in the exponential to mid-log growth phase (Fig. 4 H and I). Polyamines are polycationic and thus interact with the negatively charged nucleic acids and often regulate the transcription and translation of multiple genes, which fall under the polyamine regulon. Multiple sigma factors, such as *rpoS*, *hns, oppA* etc., are known to be under the polyamine modulon (Igarashi et al., 2015; Igarashi and Kashiwagi, 2011). In *E. coli,* OppA has a weak and distant Shine-Dalgarno sequence, and polyamines stimulate the translation of OppA in such a case (Igarashi et al., 1997; Yoshida et al., 1999). Also, polyamines increase the translation of RpoN and H-NS whose transcript contains a poor Shine-Dalgarno (SD) sequence in *E. coli* and that of Cra, which possesses an unusual "GUG" start codon in its transcript (Terui et al., 2007). In *Salmonella,* HilA translation is also regulated by spermidine, which has a distant SD sequence (Guerra et al., 2020). Interestingly, *Salmonella* Typhimurium *fliA* contains an unusual "GTG" start codon and a poor SD sequence located farther than 6–7 bps from the START codon (Fig. S4E). We tagged *fliA* with FLAG in the chromosome of STM WT, STM Δ*potCD* and STM Δ*speED,* and observed that there was a significant downregulation of FliA in both STM Δ*potCD* and STM Δ*speED*. Further, we noted an increase in the expression of FliA when STM Δ*speED* was grown in the presence of exogenous polyamine (Figs. 4 J, S4 F). These results suggests that spermidine regulates the expression of flagellin genes by enhancing the translation of FliA (σ^{28}).

4.5. Spermidine modulates the expression of the two-component system BarA/SirA in Salmonella thereby controlling SPI-1 gene expression

Salmonella employs multiple ways to invade the IECs, an effective strategy being to induce its uptake by the otherwise non-phagocytic cells. *Salmonella* pathogenicity island-1 (SPI-1) encoded type-3 secretion system (T3SS) and effector proteins are involved in inducing the uptake by epithelial cells (Raffatellu et al., 2005). The initial attachment of the bacteria to the mucin and the cell surface activates a complex intracellular regulatory network, leading to the formation of the T3SS on the surface that penetrates the host cell membrane and translocates multiple effectors into the host cytosol (Hajra et al., 2021; Hensel, 2004). Multiple environmental signals such as osmolarity, pH, and oxygen concentration activate the SPI-1 through the master regulator HilA. Apart from these bile acids, short-chain fatty acids and magnesium ion concentration also stimulate the expression of SPI-1 genes in *Salmonella (*Lou et al., 2019*)*. We validated their findings and observed the expression of SPI-1 genes in STM WT, STM Δ*potCD* and STM Δ*speED* during their in vitro growth. All the genes were significantly downregulated, even in the spermidine mutants (Fig. 5 A and B). We further validated the results by using *lacZ* constructs under the promoter of *hilA* and *spiC*. The LacZ activity was significantly low in STM Δ*potCD* and STM Δ*speED* when cloned under the *hilA* promoter. Upon STM Δ*speED*'s growth with spermidine supplementation, the LacZ activity was recovered (Figs. S5A-S1B). Our study suggests that deleting spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium is sufficient to reduce *hilA* transcription. On the contrary, we did not observe a difference in LacZ activity when cloned under *spiC* promoter (Fig. S5C). In contrast to the accepted model of *Salmonella* infection multiple elegant studies show that some of the SPI-1 genes are also critical in intracellular replication (Giacomodonato et al., 2007; Pfeifer et al., 1999; Steele-Mortimer et al., 2002). Thus, we investigated the mRNA expression of the SPI-1 genes in both STM Δ*potCD* and STM Δ*speED* post-infection into Caco-2 cells and observed that all the genes are downregulated compared to STM WT (Fig. 5C and D). As the SPI-1 genes have a highly complex regulatory network, to gain a mechanistic insight into the regulation of SPI-1 genes by spermidine, we accessed the mRNA expression of the essential two-component system BarA/SirA, upstream of *hilA* in the regulatory network, which directly regulates *hilA* (Teplitski et al., 2003). Both *barA* and *sirA* mRNA levels were significantly low in STM Δ*potCD* and STM Δ*speED* than the wild type (Fig. 5 E and F). Taken together these findings conclude that spermidine orchestrates the expression of SPI-1 genes through the *barA/sirA* axis.

Along with SPI-1 encoded effectors, the T3SS of SPI-1 also translocate an SPI-5 encoded effector protein, SopB. SopB is a phosphoinositide-phosphatase, and researchers from our group have previously shown the role of this SPI-1 effector in *Salmonella* Typhimurium virulence (Chatterjee et al., 2023). We used HA-tagged SopB to understand the regulation of SPI-1 in STM Δ*potCD* and STM Δ*speED*. We noticed that, indeed, in the two mutants, the translocation of SopB is lesser than in the wild type (Fig. S5E). Our results give a mechanistic depth to the control of SPI-1 via the vital two-component system, thereby regulating the invasion capability of *Salmonella.*

5. Discussion

The pathogenesis of most pathogens involves entry into the host tissues and cells. However, before the entry, the pathogen must reach the site, attach and adhere to the cell surfaces and subsequently invade using multiple strategies. Over the years, *Salmonella* has emerged as a successful enteric pathogen by modulating its strategies and employing diverse arms and shields, allowing it to conquer various niches during its pathogenesis. The complexity of the arsenals and the surface structures forces us to develop combating strategies against the disease-causing pathogen. Polyamines are ubiquitously present in all living forms, including prokaryotes. In prokaryotes, polyamines regulate the expression of multiple genes and assist in growth and stress response. Studies also show that putrescine and spermidine impact DNA supercoiling by regulating DNA gyrase activation in bacteria (Duprey and Groisman, 2020). Few research groups have shown that polyamines are essential in *Salmonella's* virulence and stress response (Jelsbak et al., 2012). However, a dearth of mechanistic understanding remains in the field. Of the various polyamines, putrescine, spermidine and cadaverine are the major ones in *Salmonella*. Most studies show that putrescine and spermidine are important for *Salmonella's* virulence. Thus, we were fascinated to delve into the molecular mechanism behind the role of the vital polyamine, spermidine, in *Salmonella* pathogenesis.

We report that spermidine transport and synthesis processes are regulated concurrently in *Salmonella*. It suggests that the transporter and the synthesis genes must function together to maintain the intracellular homeostasis of spermidine. The acetylation by SpeG is vital in removing the accumulated spermidine, which might be toxic at high levels (Fang et al., 2017). However, our study shows that *Salmonella* has more complexity in maintaining the homeostasis of spermidine

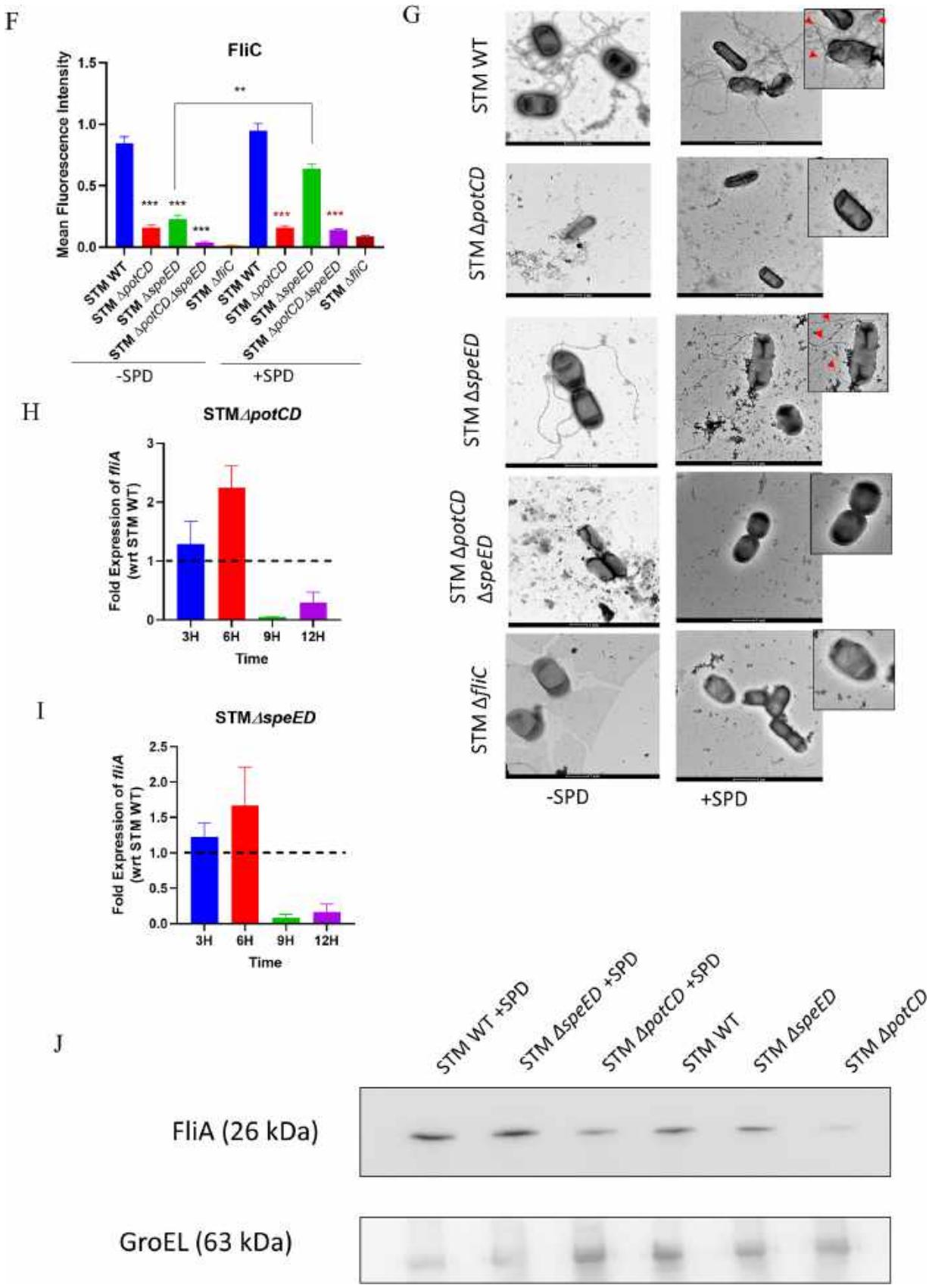

Fig. 4. (*continued*).

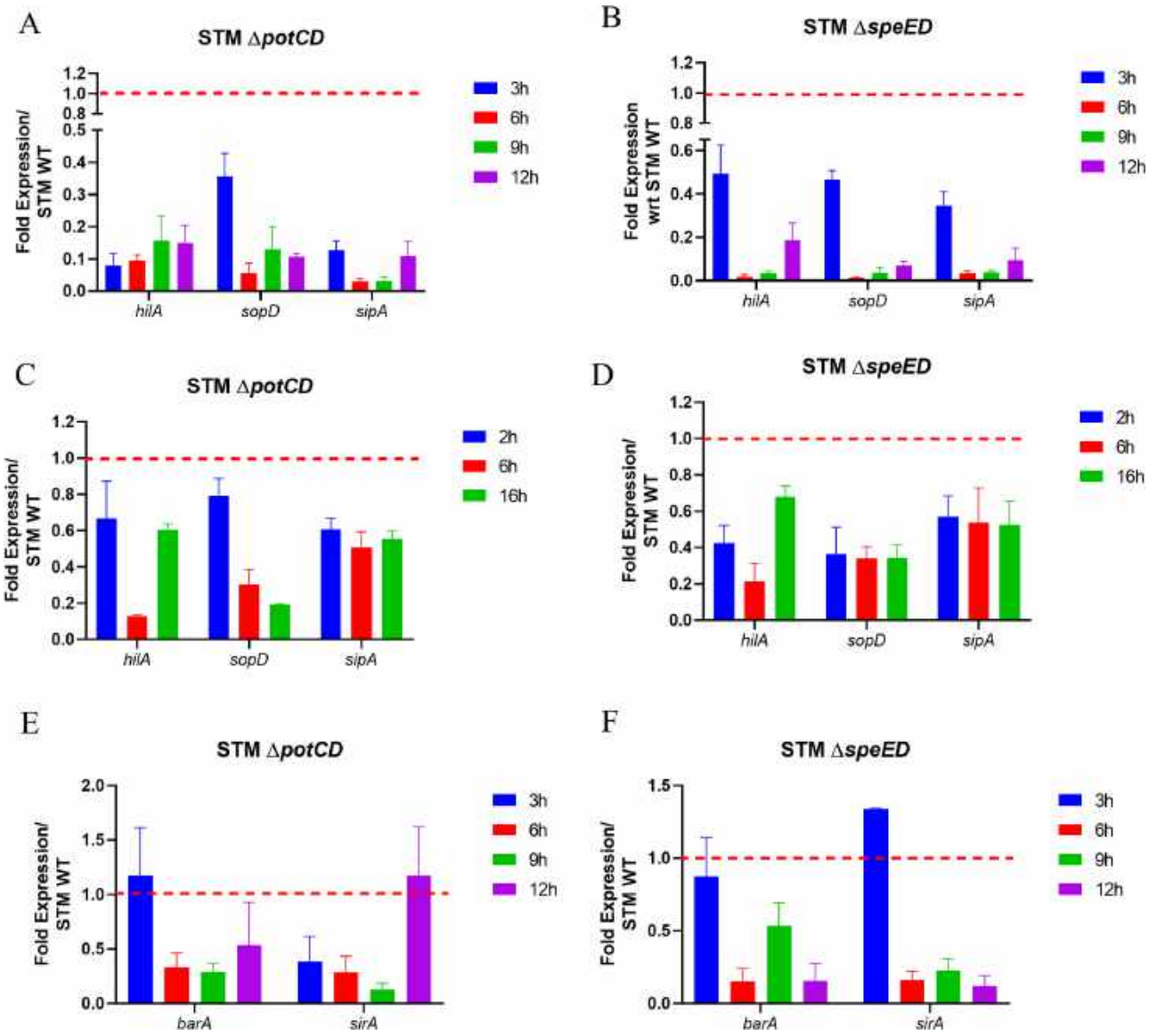

Fig. 5. Spermidine modulates the expression of the two-component system BarA/SirA in *Salmonella* thereby controlling SPI-1 gene expression. A. The mRNA expression SPI-1 master-regulator and effectors such as *hilA*, *sopD* and *sipA* respectively in STM Δ*potCD* during in vitro growth in LB media, B. The mRNA expression of the SPI-1 master-regulator and effectors such as *hilA*, *sopD* and *sipA* respectively in STM Δ*speED* during in vitro growth in LB media, C The mRNA expression of SPI-1 master-regulator and effectors such as *hilA*, *sopD* and *sipA* respectively in STM Δ*potCD* post infection into Caco-2 cells D. The mRNA expression SPI-1 master-regulator and effectors such as *hilA*, *sopD* and *sipA* respectively in STM Δ*speED* post infection into Caco-2 cells, E. The mRNA expression the two-component system *barA* and *sirA*, that regulates the SPI-4 and SPI-1, in STM Δ*potCD* during in vitro growth in LB media, F. The mRNA expression the two-component system *barA* and *sirA*, that regulates the SPI-4 and SPI-1 in STM Δ*speED* during in vitro growth in LB media, G. Spermidine is a novel regulatory molecule in *Salmonella* Typhimurium regulating expression of flagellin, adhesins and SPI-1 thereby facilitating a successful invasion into IECs. Student's t-test was used to analyze the data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05. Two-way ANOVA was used to analyze the grouped data; p values * ** *< 0.0001, * ** <0.001, * *< 0.01, * <0.05.

intracellularly. In *E. coli,* formate transporter (FocA) and biosynthesis genes (PflB) function in a concerted manner; moreover, FocA interacts with PflB to import formate into the bacteria (Kammel and Sawers, 2023; Doberenz et al., 2014). In *Salmonella*, the spermidine transporter and biosynthesis genes may also interact to function concertedly; thus, we observe a simultaneous upregulation of their mRNA expressions. We also find that putrescine levels are higher in spermidine mutants, however, the accumulation of putrescine may not be toxic as it is not reported to be converted to toxic metabolites and is removed from the cell by exporters (Sakamoto et al., 2020). On the contrary, the accumulation of spermidine in *Salmonella* adversely affects virulence by generating acrolein and ROS (Fang et al., 2017). Recent studies also show that gut commensal bacteria produce biogenic amines and contribute to the intestinal pool of polyamines (Kibe et al., 2014; Kitada et al., 2018). The genra of *Bifidobacterium, Clostridium,* and *Lactobacillus* are the ones majorly contributing to the intestinal pool of polyamines (Pugin et al., 2017; Matsumoto and Benno, 2004). *Salmonella,* an entric pathogen, might benefit from the polyamines produced by the gut commensal in the intestinal lumen. Through the PotABCD spermidine transporter, *Salmonella* may readily take up the extracellular polyamines produced by the gut microbes and from the metabolism of the dietary biogenic amines.

Our study demonstrates that *Salmonella* utilizes small molecules like spermidine to regulate the expression of multiple adhesive and non-adhesive complex surface structures, thus identifying new members of polyamine regulon in *Salmonella*. Although previous studies show that spermidine and putrescine regulate HilA translation to regulate SPI-1 genes. We delineate a molecular mechanism regulating the

transcription of SPI-1 genes. We show that spermidine tunes the expression of the two-component system BarA/SirA, which further governs the elegant nano-injection machinery of the T3SS and the effectors of the SPI-1 for its uptake and survival in the host cells. We expect spermidine to bind to the anionic nucleic acids, thereby tuning the expression of the multiple genes in *Salmonella.* However, further study is essential in unravelling spermidine's interaction mechanism with the nucleic acid in *Salmonella.* Our study also shows that the SPI-4 encoded giant adhesin *siiE* mRNA expression is downregulated in spermidine biosynthesis and transport mutants. Studies have illustrated that BarA/SirA and HilA regulate the transcription of SPI-4 genes *(*Lou et al., 2019*)*. Thus, our study delves into the complexity of networks involving spermidine in orchestrating the adhesion and invasion of *Salmonella*.

We identify a novel regulatory pathway in *Salmonella*, where our results suggest that spermidine might aid in overcoming the obstacle of a weak and poor transcript, thereby maintaining the synthesis of the elaborate surface tools required for motility and attachment. This mechanism suggests a similar regulation for numerous genes with identical attributes as FliA, across the serovars of *Salmonella*. Multiple genes might possess such distant and non-consensus SD sequences or unusual start codons, and spermidine might interact and enhance the translation of such transcripts in *Salmonella.* Studies show that spermidine interacts and increases the proximity of the SD sequence and the start codon by bringing them closer to initiate the translation of OppA and other members of the polyamine modulon (Yoshida et al., 1999; Higashi et al., 2008). Spermidine further initiates translation from inefficient start codons by fmet-tRNA for RpoN, Cra and HN-S (Igarashi et al., 2015; Terui et al., 2007). Spermidine in *Salmonella* might interact similarly to regulate the translation of the polyamine modulons. Interestingly, we observed that in *Salmonella* Typhi, which is the human-restricted serovar, *fliA* possesses a similar unusual "GTG" START codon and a poor SD sequence located 10 bp from the START codon (Fig. S4G). Thus, further research on spermidine in *Salmonella* will shed light on the mechanistic insights of the interaction of spermidine with nucleic acids to regulate the expression, as observed in our study. Also unravel the other members of the polyamine modulon in *Salmonella.*

As previously explained, pathogenic bacteria use a complex network of molecules to evade and survive. Our study solves the enigma of how spermidine regulates diverse aspects and is a critical player in the complex network regulating the virulence of *Salmonella.* As multiple pathogenic bacteria, including *Salmonella*, *Helicobacter pylori* and the Gram-positive pathogen *Staphylococcus aureus* must adhere and invade host cells, it is crucial to prevent the entry of pathogens by preventing adhesion and invasion into the host cells (Huang et al., 2016; Agerer et al., 2005). Thus, our findings prove that targeting spermidine metabolism is sufficient to impact the virulence of *Salmonella* by negatively affecting the adhesion and invasion of the pathogen and reducing infectivity. Our study opens avenues to design drugs that target *Salmonella's* spermidine metabolism, thus reducing *Salmonella's* infectivity and burden.

6. Conclusion

The critical step in the pathogenesis of *Salmonella* is its ability to reach the site of infection and adhere to the cell surface to invade and colonize the host niche. Polyamines are well studied in the context of bacterial pathogenesis and have been linked to the virulence of *Salmonella*. However, the mechanism behind the role of polyamines is less appreciated. We show that the loss of only the spermidine pool is sufficient to limit *Salmonella* infection in the host. Our study further gives a mechanistic understanding of the role of spermidine in pathogenesis, and we show that it orchestrates the expression of motility and multiple surface adhesive structures. Interestingly, we unravel a mechanism of translational regulation of sigma-28 by spermidine in *Salmonella* Typhimurium, which might be extrapolated to numerous genes in the pathogen.

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CRediT authorship contribution statement

Nair Abhilash Vijay: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation. **Chakravortty Dipshikha:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Tatu Utpal:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation. **Rahman S A:** Writing – review & editing, Writing – original draft, Methodology. **Devasurmutt Yashas:** Writing – review & editing, Writing – original draft, Validation, Methodology. **Singh Anmol:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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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.micres.2024.127605.

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