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

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Running Head: *Salmonella* mounts an antioxidative response using de-novo and host-acquired spermidine

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Abstract

Salmonella infection involves a cascade of attacks and defence measures. After breaching the intestinal epithelial barrier, *Salmonella* is phagocytosed by the macrophages, inside which, the bacteria face multiple stresses and, consequently, employ appropriate countermeasures. We show 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 is known to prevent 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 lesser 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. Interestingly, *Salmonella* upregulates polyamine biosynthesis in the host through its effectors from SPI-1 and SPI-2, which also solves the mystery of 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* colonization 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 strategy of the bacteria in modulating host metabolism to promote their intracellular survival and shows the potential of DFMO to curb S*almonella* infection.

Introduction

A condition with ample nutrients, and desired temperature, pH, oxygen concentration, and osmolarity is usually considered optimal for the growth of microbes. An imbalance or any alteration in these parameters impedes the growth and survival of the microbes, considered as stress conditions. However, these parameters keep fluctuating in nature [1]. Hence, to persist and survive in the natural environment, with unforeseen stressful or lethal conditions, they must be adept in sensing, responding and adapting to them [2]. In the case of food-borne pathogens, they face an array of various stresses in multiple environments in which they dwell, such as from the natural habitat to commercial settings and inside the host system [3-5]. *Salmonella* is a food-borne pathogen that enters the host system through contaminated food and water. In the host system, as it traverses 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. Macrophages are phagocytic immune cells where *Salmonella* encounters a very hostile environment. Entry of the pathogen into the cell cytoplasm leads to a burst of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [6, 7]. In macrophages, *Salmonella* resides in a specialised niche called the *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 the stresses it faces within the host macrophages.

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

67 response. In *Shigella spp.* the polyamine spermidine accumulates during its infection into
68 macrophages, which increases the expression of *katG* and helps in bacterial antioxidant response
69 [9]. Moreover, spermidine localises to the surface of *Pseudomonas aeruginosa* and bind to the
70 lipopolysaccharide (LPS) to protect the cells from oxidative damage [10]. In the Gram-positive
71 bacteria, *Streptococcus pyogenes,* extracellular spermidine enhance the survival of the bacteria by
72 upregulating oxidative response genes [11]. A research group has shown that polyamines are vital
73 in resistance against nitrosative stress in *Salmonella* Typhimurium. Further, the group showed that
74 spermidine is required for the systemic infection of *Salmonella* Typhimurium in mice [12]. Thus,
75 it is conceivable that *Salmonella* Typhimurium utilises polyamines, such as spermidine, as a stress
76 response molecule; however, the mechanism remains elusive.

77 Here, we show that spermidine biosynthesis and transport mutants of *Salmonella* Typhimurium
78 exhibit reduced survival upon infection in RAW264.7 cells. This diminished proliferation is also
79 observed in mice models of *Salmonella* infection, which is rescued in *gp91phox*$^{-/-}$ mice. We
80 demonstrate that spermidine orchestrates the various arms of antioxidative response and aids in
81 tightly regulating intracellular ROS levels. We further identify a novel antioxidative enzyme, Gsp,
82 in *Salmonella* Typhimurium, which is regulated by spermidine. The fascinating question that arises
83 is why the transporter mutant shows reduced survival. To this, we find that *Salmonella*
84 Typhimurium harnesses the host polyamine biosynthesis for its survival. Furthermore, for the first
85 time, we show that an FDA-approved chemopreventive and anti-African Human Trypanosomiasis
86 drug that inhibit the polyamine biosynthesis in the host, is able to curb *Salmonella* infection in
87 mice models.

88 **Material and methods**

89 **Bacterial strains and growth condition**

90 *Salmonella enterica* serovars Typhimurium (STM WT) wild type strain ATCC 14028s was used in
91 all experiments which was a kind gift from Prof. Michael Hensel, Abteilung Mikrobiologie,
92 Universität Osnabrück, 273 Osnabrück, Germany. The bacterial strain was cultured in Luria broth
93 (LB-Himedia) with constant shaking (175rpm) at 37°C orbital-shaker. Kanamycin,
94 Chloramphenicol and Ampicillin antibiotics (Kanamycin-50µg/ml, Cholramphenicol-20µg/ml and
95 Ampicillin-50µg/ml) were used wherever required. Strains were transformed with pFPV-m-cherry
96 plasmid for immunofluorescence assays. (Bacterial strain list in Supplementary table **S-Table 1**)

97 **Bacterial gene knock-out and strain generation**

98 The generation of gene knock-out in bacteria was done using the One-step chromosomal gene
99 inactivation protocol [13]. Briefly, the Kanamycin resistance gene and the Chloramphenicol
100 resistance gene amplified products were purified using chloroform-ethanol precipitation. Followed
101 by electroporation into the STM WT cells (expressing PKD-46 plasmid which provides the λ-Red
102 recombinase system) by a single pulse of 2.25 kV separately for the Kan^R and $Chlm^R$. The
103 transformant colonies were selected and patched on fresh plates and confirmed for knock-out using
104 PCR with primers designed corresponding to the ~100bp upstream and downstream of the genes
105 (knocked out) for the knock-out strains to observe a difference in PCR product size upon STM
106 *ΔpotCD* and STM *ΔspeED* knockout generation. For the generation of the double knock-out strain
107 (STM *ΔpotCDΔspeED*), the STM *ΔpotCD* (resistant to Kanamycin) was first transformed with the
108 plasmid pKD46 which provides the λ-Red recombinase system. To this transformed strain, the
109 purified PCR product to knock-out *speED* was electroporated to generate the STM
110 *ΔpotCDΔspeED* (resistant to Kanamycin and Chloramphenicol). For the generation of STM *Δgsp,*
111 Kanamycin resistance gene was amplified from pKD4 plasmid, and a similar protocol was used,
112 followed by selection on Kanamycin containing LB agar plates. For the generation of double gsp

spermidine mutants, STM *Δgsp* was electroporated with purified PCR product to knock-out *speED* and *potCD* (both Chloramphenicol resistance cassette). (Knockout generation Primer list in Supplementary table **S-Table 2**)

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 treated cell culture well plate at a confluency of 80% for intracellular survival assay, and expression studies.

Gentamicin protection assay

The cells were infected with STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG* at 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 700-900 rpm for 10 minutes to facilitate the proper adhesion. The plate was then incubated for 25 minutes at 37°C 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 again incubated for 60 minutes at 37°C and 5% CO_2. The media was then removed, cells were washed with 1X PBS twice, and fresh media containing 25µg/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, and for qRT-PCR three time points were considered 2 hours, 6 hours and 16 hours. For phagocytosis assay upon opsonisation, all the strains were washed

with 1XPBS and incubated at 37°C with mouse complement sera for 1 hour before gentamicin protection assay

Intracellular survival assay and phagocytosis assay

At the appropriate time post-infection, the cells were lysed using 0.1% Triton X followed by addition of 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. 12 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 hours post-infection)/(CFU at 2 hours post-infection)

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

RNA isolation and qRT-PCR

RNA isolation was performed from infected cells after appropriate hours of infection with STM WT, STM *ΔpotCD,* STM *ΔspeED* by RNA isolation was performed using TRIzol (Takara) reagent according to manufactures' protocol RNA was quantified using Thermo-fischer scientific Nano Drop followed by running on 2% agarose gel for checking the quality. For cDNA synthesis, first DNase I treatment with 3µg 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 15 seconds. Quantitative real-time PCR was done using SYBR green RT-PCR kit in BioRad qRT-PCR system. A 384 well plate with three replicates for each sample was used. The expression levels of the gene of interest were measured using specific RT primers. Gene expression levels were normalised to 16SrDNA primers of *S.*

156 Typhimurium. Gene expression levels of eukaryotic gene of interest were normalised to beta-actin
157 of mouse/human as required. For expression studies in bacteria grown in LB media, the bacterial
158 samples were harvested at 3 hours, 6 hours, 9 hours and 12 hours post subculture in fresh LB media
159 in 1:100 ratio and 1mM H_2O_2 was added to the broth, to study the gene expression in the presence
160 of oxidative stress. Then similar protocol was used to isolate total RNA using TRIzol (Takara)
161 reagent according to manufactures' protocol (Expression Primer list in Supplementary table **S-**
162 **Table 2**)

163 **Primary macrophages isolation and infection**

164 Primary macrophages were isolated from C57BL/6 mice (male, 5-6 weeks old). The mice were
165 intraperitoneally injected with 8% Brewer's Thioglycolate (HiMedia). Five days post injection the
166 primary macrophages were aseptically isolated by injecting ice-cold 1xPBS into the peritoneal
167 cavity the peritoneal lavage was collected. Any residual erythrocytes were lysed using RBC lysis
168 buffer (Sigma- R7757) , and the isolated cells were maintained in complete RPMI 1640 media for
169 further experiments.

170 **Intracellular Reactive oxygen species determination using H_2DCFDA staining**

171 Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then
172 10^8 CFU/ml of each strain was incubated with 10µM of 2',7'-dichlorodihydrofluorescein diacetate
173 (H2DCFDA) (Sigma) in 1xPBS (pH 7.2) at 37°C for 30 minutes. The bacterial cells were
174 centrifuged and the cells were resuspended in 1xPBS (pH 7.2) with Hydrogen peroxide of different
175 concentrations (0mM- 10mM) , and incubated 37°C (orbital shaker) for 2 hours. The samples were
176 transferred to a 96 well ELISA plate and fluorescence was determined in Tecan-ELISA plate reader
177 Infinite series 200 (Ex- 490nm/ Em- 520nm).

178 **Intracellular redox status determination**

179 The STM WT, STM *ΔpotCD,* STM *ΔspeED* and STM *Δgsp* were transformed with pQE60-Grx1-
180 roGFP2 (a gift from Prof. Amit Singh, CIDR, IISc). Overnight cultures were sub-cultured in fresh
181 LB media. Once the cultures reached OD 0.1 then 10^8 CFU/ml of each strain was incubated with
182 Hydrogen peroxide (0mM-5mM) of different concentrations in 1xPBS (pH 7.2) with, and
183 incubated 37°C (orbital shaker) for 2 hours. The samples were centrifuged and resuspended in
184 fresh 1xPBS (pH 7.2). And tubes were analysed for GFP fluorescence at 408nm and 488nm
185 respectively using BD-FACS Verse flow cytometer (total 10,000 events for each sample). For
186 determination of intracellular redox status upon infection into RAW264.7 cells, 10^5 RAW264.7
187 cells seeded in a 24 well tissue culture plate, were infected with each of the strains harboring the
188 roGFP2 plasmid using gentamicin protection assay. After 16 hours post infection, the macrophages
189 were washed with 1X PBS and scrapped off using cell scraper and analysed for GFP fluorescence
190 at 408nm and 488nm respectively using BD-FACS Verse flow cytometer (total 10,000 events for
191 each sample).

192 ***In vitro* sensitivity assays**

193 Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then
194 10^8 CFU/ml of each strain was incubated with Hydrogen peroxide or sodium nitrite of different
195 concentrations (0mM- 10mM) in 1xPBS(pH 7.2) (1xPBS of pH 5.4 was used for nitrite sensitivity
196 assay) with, and incubated 37°C (orbital shaker) for 2 hours. The samples were plated on SS agar
197 to enumerate the CFU and the percentage survival was determined as:

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

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

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

Immunoblotting

The bacterial strains were grown in LB media with added 1mM 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, followed by estimation of total protein using the Bradford protein estimation method. 50µg of protein was loaded onto a Polyacrylamide Gel Electrophoresis (PAGE) without β-mercaptoethanol (prevent di-sulphde bond breakage as glutathionyl-spermidine modifies Cysteine residues through a disulphide bond), then transferred onto 0.45µm PVDF membrane (GE Healthcare). 5% skimmed milk (Hi-Media) in TTBS was used to block for 1hour at room temperature and then probed with Anti-Spermidine primary (Novus Biologicals) and the secondary HRP-conjugated antibodies. ECL (Biorad) was used for developing the blot, and images were captured using Chemi-Doc(Biorad). All densitometric analysis was performed using the Image J. The normalisation was done with respect to Ponceau S stained blot.

Transfection for knockdown

222 RAW 264.7 cells were seeded at a confluency of 50-60% 12 hours prior to transfecting using either
223 PEI (1:2 -DNA: PEI). We used two different constructs for knock-down of Odc1, A7 and F8 and
224 both as a mixed construct (A7:A8 at 1:1 ratio) and similarly for Srm , E9 and F1 and both as a
225 mixed construct (E9:F1 at 1:1 ratio) from the Sigma Mission shRNA library. 400ng of plasmid
226 DNA/well (ratio 260/280 ~1.8- 1.9) was used for transfection in a 24well plate. Cells were then
227 incubated for 8 hours at 37°C in a humidified incubator with 5% CO2; after that, the media
228 containing transfecting DNA and reagents were removed, and cells were further incubated for 48
229 hours in complete media DMEM +10% FBS. Cells were harvested for further analysis or infected
230 with the required MOI using the gentamicin protection assay. (shRNA sequence list in
231 Supplementary table **S-Table 3**)

232 **Immunofluorescence**

233 After the appropriate incubation time after gentamicin protection assay, the media was removed,
234 and the cells were washed with 1X PBS and fixed with 3.5% Paraformaldehyde for 10 minutes.
235 The cells were then washed with 1X PBS, followed by incubation with the required primary
236 antibodies (anti-mouseLAMP1 and anti-Spermidine) in a buffer containing 0.01% saponin and 2%
237 BSA, and incubated at room temperature for 45-60 minutes. After washing with 1X PBS, the
238 secondary antibody tagged to a fluorochrome was added and incubated (anti-rat-Alexafluor488 for
239 LAMP1, anti-rabbit-Alexafluor647 for spermidine). The coverslips were then washed with PBS
240 and mounted on a clean glass slide using mounting media containing an anti-fade reagent and
241 observed under the confocal microscope (Zeiss 710 microscope, at 63X oil immersion, 2x319 3x
242 zoom, and 100X zoom for studying only bacterial samples, Zeiss 880 microscope, at 63X oil
243 immersion, 2x319 3x zoom).

The histopathological sections were deparaffinized and then incubated with the required primary antibody (anti- *Salmonella* LPS) in a buffer containing 0.01% saponin and 2% BSA and incubated at room temperature for 45-60 minutes. The primary antibody was then removed by washing with 1X PBS and then incubated with the appropriate secondary antibody tagged to a fluorochrome. The sections were then washed with PBS and cover 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. For studying histopathology samples Zeiss 880 microscope 40X oil immersion 2x319 3x zoom was used.

Intracellular RNS determination

For determination of intracellular RNS, a cell permeable nitric-oxide probe, 4, 5-diaminofluorescein diacetate (DAF_2A) (Sigma) was used. The protocol has been followed as Roy Chowdhury A et. al. [14]. Briefly, After 16 hours of infection of RAW264.7 cells (transiently knock down of *Odc1*) at MOI 10 with STM WT, the cells were incubated with fresh DMEM containing 5µM 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 and the cells were washed with 1XPBS and the cells were acquired immediately for flow-cytometry (BD FACS Verse) (Ex-491nm/Em- 513nm).

Intracellular Glutathione determination

The intracellular reduced Glutathione (GSH) concentration was determined by modification of a pubmished protocol [15]. Briefly, a standard curve with known concentration of GSH (Sigma) was prepared. Reaction mixture for each contained 600µL of phosphate buffer (0.1M, pH7), 40µL 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

1000µL.The mixture was incubated at room temperature for 5 minutes and absorbance was measured at 412nm using tecan plate reader. The bacterial strains were subcultured in fresh LB media and grown till OD 0.1 (exponential phase) and washed with buffer (Tris, Sodium chloride and EDTA) and lysed using sonication. The supernatant after sonication was used as the sample for GSH detection. As previously described for standard curve. From the standard curve the intracellular concentrations were interpolated.

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 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.

In vivo animal experiment

5-6weeks old C57BL/6 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, 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/6 mice were infected by intraperitoneally injecting 10^3 CFU of STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG.* To study the colonisation in organs, spleen and liver were isolated aseptically 3 days post-infection. 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 histopathogical sample preparation.

290 For inhibitor treatment, 5-6weeks old C57BL/6 mice were infected by orally gavaging 10^7 CFU
291 of STM WT. The inhibitor DFMO (Sigma D193) was intraperitoneally injected every alternate
292 day from day 1 at two doses 2mg/kg and 1mg/kg of body weight of mice. To study the colonisation
293 in organs, the intestine (Peyer's patches), MLN, spleen and liver were isolated aseptically, 5 days
294 post-infection, and the CFU was enumerated on differential and selective SS agar by serial dilution.
295 For survival assay of mice 5-6weeks old C57BL/6 mice were infected by orally gavaging 10^8 CFU
296 of STM WT. The inhibitor DFMO was intraperitoneally injected every alternate day from day 1 at
297 two doses 2mg/kg and 1mg/kg of body weight of mice. The survival was monitored till 10 days.
298 Likewise, organs were stored in 3.5% PFA before histopathogical sample preparation.

299 **Mass Spectrometry to determine intracellular GS-sp levels**

300 The bacterial strains were grown in LB media with added 1mM hydrogen peroxide until the log
301 phase of growth. The cells were centrifuged to remove the media, and the cells were resuspended
302 in the lysis buffer (Sodium chloride, Tris, EDTA, 10% protease inhibitor cocktail) after washing
303 with 1XPBS. The cells were lysed using sonication and centrifuged at 4°C to collect the cell lysate.
304 Protein was precipitated using ice-cold acetone (Sigma, MS grade), 4 times the volume of the cell
305 lysate and by incubating at -20°C overnight. The precipitated proteins were removed by
306 centrifugation and the supernatatnt was used for analysis. Samples were analysed by ESI MS Q-
307 TOF, impact HD (Bruker Daltonics Germany) connected to Agilent HPLC 1260. Samples were
308 passed through Agilent C18, 4.6X150mm column. Mobile phase used was water and Acetonitrile
309 with 0.1% formic acid. Linear gradient was used with flow rate 0.2ml/min. Data was analysed
310 using Bruker Daltonics software Data analysis 4.1. Mass of GS-sp (Glutathionyl spermidine) is
311 434g/mol, and $(GS\text{-}sp)_2$ (oxidized form, Di-glutathionyl spermidine) is 866g/mol.

312

313 **Statistical Analysis**

314 Statistical analyses were performed with GraphPad Prism software. The Student's t-test
315 (parametric, two-tailed, unpaired) was performed as indicated. For animal experiments Non-
316 parametric Mann-whitney (two-tailed) test was performed. The results are expressed as mean ±
317 SD or mean ± SEM. Group sizes, experiment number, and p values for each experiment are
318 described in figure legends.

319 **Results**

320 **Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium**
321 **compromises its ability to be phagocytosed by the macrophages**

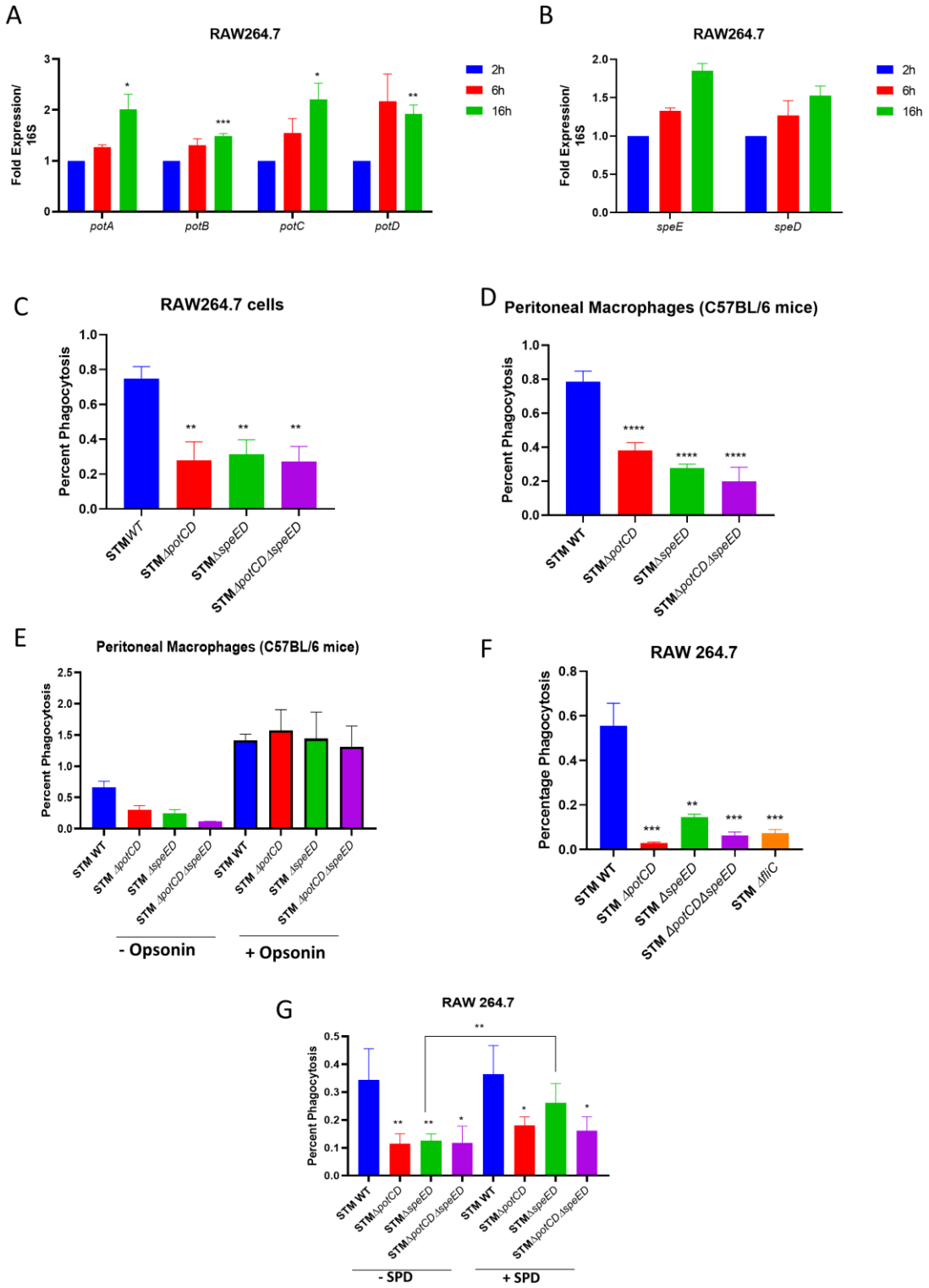
322 The pathoadaptation in *Salmonella* involves multiple players, which counteracts the stressful
323 condition it encounters in the host macrophages. Polyamines being a group of well-studied stress
324 response molecules, we were interested in determining the expression of the spermidine transporter
325 and biosynthesis genes in *Salmonella* Typhimurium. Our previous study shows that *Salmonella*
326 upregulates the spermidine transporter genes (*potA, potB, potC* and *potD*) and the biosynthesis
327 genes (*speE* and *speD*) during the log phase of growth *in vitro* [16]. Here we assessed the mRNA
328 levels of *potA, potB, potC* and *potD* in STM WT upon infection into the RAW264.7 macrophage
329 cell line. We noted that all the genes showed a 1.5-2 fold upregulation post 6 hours of infection
330 into macrophages till 16 hours (**Fig 1 A)**. Further, our results showed that the spermidine
331 biosynthesis enzymes *speE* and *speD* were upregulated 1.5 to 2 folds post 6 hours to 16 hours post-
332 infection into macrophages (**Fig 1 B)**. These results indicate that *Salmonella* Typhimurium
333 enhances its intracellular spermidine biosynthesis and imports from the extracellular milieu. It

334 might be a strategy of the pathogen to increase the intracellular pool of stress response molecules
335 like the polyamine spermidine, as it encounters the hostile environment of host macrophages.

336 We then determined how the spermidine transporter and biosynthesis mutants (STM Δ*potCD*, STM
337 Δ*speED* and STM Δ*potCD*Δ*speED*) behave upon infection into RAW264.7 macrophages. We
338 infected STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* into RAW264.7 cells
339 and observed that STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* showed reduced
340 phagocytosis by the macrophages compared to the wild type (**Fig 1 C**). Interestingly, STM
341 Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* showed a compromised ability to proliferate
342 intracellularly in the RAW264.7 cells further validating a previous study [12](**Fig S1 A)**. To further
343 validate our results, we assessed the behaviour of the spermidine transporter and biosynthesis gene
344 mutants in primary macrophages isolated from the peritoneal lavage of C57BL/6 mice. Likewise,
345 the mutants showed attenuated proliferation and uptake by phagocytosis into the peritoneal
346 macrophages (**Fig 1 D and S1 B)**. Collectively these results suggest that spermidine is a critical
347 molecule in *Salmonella* Typhimurium to infect and survive in macrophages. To further investigate
348 the reason behind the reduced ability to be taken up by macrophages upon loss of spermidine
349 biosynthesis and transport, we treated STM WT, STM Δ*potCD*, STM Δ*speED* and STM
350 Δ*potCD*Δ*speED* with mouse complement-sera. Mouse complement-sera acts as an opsonin and
351 thus potentiates the interaction of the bacteria with the macrophages. Upon pre-treatment with
352 mouse complement-sera, we noted a rescue in the reduced uptake of the mutants by peritoneal
353 macrophages isolated from C57BL/6 mice (**Fig 1 E)**. A study on *Salmonella* Typhimurium
354 revealed that the aflagellate and non-motile *Salmonella* collide less frequently with macrophages
355 and gets merest time to maintain contact with the macrophages, thereby showing decreased
356 phagocytosis [17]. Our group previously showed that loss of spermidine production and import in

357 *Salmonella* Typhimurium results in the loss of flagella formation on the bacterial cell surface [16].
358 Thus, we determined the percentage phagocytosis for a flagellin-deficient strain of *Salmonella*
359 Typhimurium, STM Δ*fliC* and observed that STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE*
360 and STM Δ*fliC* exhibited a significantly decreased ability to be taken up by the RAW264.7
361 macrophages (**Fig 1 F**). Furthermore, we observed a rescue of the attenuated percentage
362 phagocytosis and fold proliferation of only STM Δ*speED* upon supplementation of spermidine
363 (100μM) during the growth of bacteria prior to infection (**Fig 1 G and S1C**). We generated single
364 gene mutants for abrogating the spermidine transport (STM Δ*potA*) and spermidine biosynthesis
365 (STM Δ*speE*) function and further complemented the genes through a vector (STM Δ*potA:potA*
366 and STM Δ*speE:speE*), we observed a recovery of the fold proliferation and percentage
367 phagocytosis nearly to STM WT in the complemented strains (**Fig S1 D-E)** . Hence, the plausible
368 explanation is that the reduced ability to form flagella in the spermidine mutants causes less
369 frequent interaction with the macrophages and provides minimal contact time for infection, leading
370 to reduced phagocytosis by the macrophages.

Figure 1: Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium compromises its ability to be phagocytosed by the macrophages



371

372 **Figure 1: Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium**
373 **compromises its ability to be phagocytosed by the macrophages**

374 A.The mRNA expression of spermidine transporter genes *potA, potB, potC* and *potD* in STM WT
375 upon infection into RAW264.7 cells, B. The mRNA expression of spermidine biosynthesis genes
376 *speE* and *speD* in STM WT upon infection into RAW264.7 cells, C. The percentage phagocytosis
377 of the spermidine mutants in RAW264.7 cells, D. The percentage phagocytosis in primary
378 macrophages isolated from peritoneal lavage of C57BL/6 mice, E. The percentage phagocytosis
379 upon pre treatment with mouse-complement sera which act as an opsonin, F. The percentage
380 phagocytosis in RAW264.7 cells with flagellin mutant (STM *ΔfliC*), G. The percentage
381 phagocytosis in RAW264.7 cells with the spermidine mutants grown in media supplemented with
382 100μM spermidine (SPD). Student's t-test was used to analyze the data; p values ****<0.0001,
383 ***<0.001, **<0.01, *<0.05. Two-way Anova was used to analyze the grouped data; p values
384 ****<0.0001, ***<0.001, **<0.01, *<0.05.

385

386 **Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of the**
387 **expression of numerous antioxidative enzymes**

388 The loss of spermidine transport and biosynthesis function in *Salmonella* Typhimurium renders it
389 incapable of proliferation and survival in macrophages. In the host macrophages, the bacteria
390 encounter numerous threats, of which the foremost is the rapid oxidative burst mediated by the
391 NOX2. The reactive oxygen species superoxide radical can easily diffuse through the bacterial
392 membrane and pose a major threat to the pathogen. ROS acts on multiple molecules such as nucleic
393 acids, proteins and lipids, thus damaging the cell membranes, DNA and proteins within the bacteria

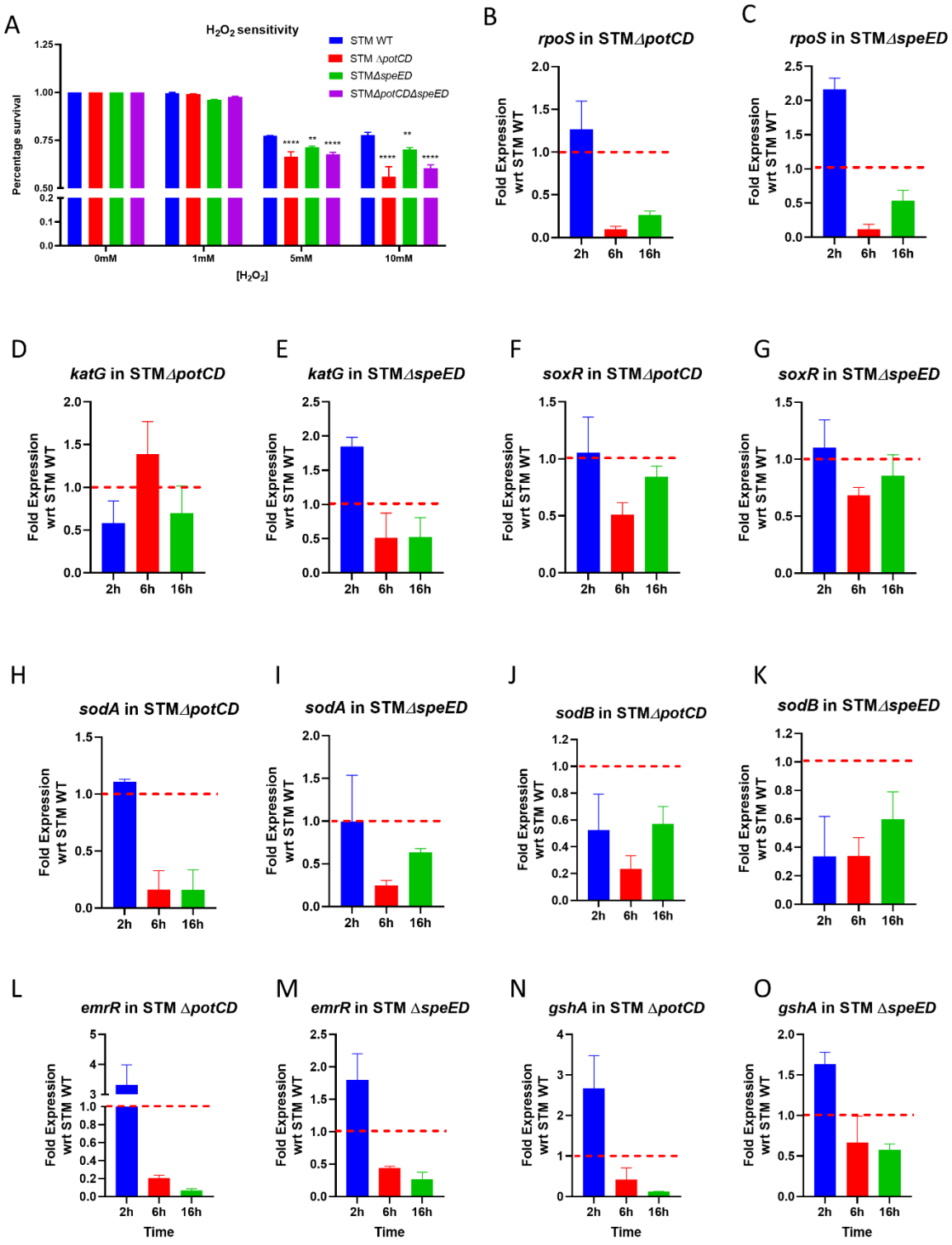
394 [18]. Spermidine has been linked to stress response against oxidative stress and protects bacteria
395 in *E. coli* and *Pseudomonas aeruginosa.* Thus, we were intrigued to understand the role of
396 spermidine in antioxidative response in *Salmonella* Typhimurium. We examined the survival of
397 STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE* upon exposure to the oxidative agent
398 hydrogen peroxide *in vitro.* We noticed that at high concentrations of hydrogen peroxide, 5mM
399 and 10mM, the STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speE* showed significantly lesser
400 survival than STM WT(**Fig 2 A**). Moreover, the complemented strains for spermidine transport
401 and synthesis mutants showed a rescue in attenuated survival in the presence of high
402 concentrations of hydrogen peroxide *in vitro* (**Fig S2 A)**. We further determined the expression of
403 *potA, potB, potC, potD*, *speE* and *speD* in STM WT upon exposure to 1mM hydrogen peroxide.
404 There was a 2-3 fold upregulation in the mRNA expression of the transporter genes over the
405 untreated during the early log phase of growth (6 hours) and the late log phase of growth (12 hours)
406 *in vitro* (**Fig S2 B-E**). Similarly, the biosynthesis genes *speE* and *speD* were 4-6 fold upregulated
407 in their corresponding mRNA expressions during their early log phase of growth (6 hours) and the
408 late log phase of growth (12 hours) *in vitro* (**Fig S2 F and G**). Our results show that *Salmonella*
409 Typhimurium upregulates spermidine transport and biosynthesis upon oxidative stress suggesting
410 that spermidine mounts a protective function in such a stressful condition to aid bacterial survival.

411 Bacteria sense the environmental changes and cues to respond and adapt to the altered
412 environment. They use the two-component systems, transcriptional activators and repressors to
413 alter gene expression in response to a stimulus [19]. Polyamines in *E. coli* regulate multiple genes
414 at the transcription and translation together, referred to as the "Polyamine modulon". These involve
415 the numerous mRNAs, tRNAs, sigma factors, translational factors and two-component systems
416 during the bacterial growth as well as in stress conditions [20-22]. *Salmonella* harbors multiple

417 antioxidative enzymes to detoxify the ROS intracellularly [23]. Our study so far show that STM
418 Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speE* is attenuated in survival under *in vitro* oxidative
419 stress than STM WT. To gain mechanistic insight into the attenuated survival of *Salmonella*
420 Typhimurium we determined the mRNA expression of the critical transcription factor *rpoS*, which
421 activates the expression of the catalase enzymes (*katG and katE*) in order to detoxify hydrogen
422 peroxide to water in the bacteria enzymatically [24]. In both STM Δ*potCD* and STM Δ*speED* the
423 mRNA expression of *rpoS* is significantly down regulated from 6 hours post infection in
424 RAW264.7 cells (**Fig 2 B and C, S3 A**). Further, its downstream target *katG* was also down
425 regulated in STM Δ*potCD* and STM Δ*speED* from 6 hours post infection into RAW264.7 cells
426 (**Fig 2 D and E, S3 B**). We further assessed the mRNA expression of the transcription factor *soxR*,
427 which regulates the expression of superoxide dismutases (*sodA* and *sodB*) [25]. Upon infection in
428 RAW264.7 cells, expression of *soxR* was significantly downregulated in STM Δ*potCD* and STM
429 Δ*speED* with respect to STM WT (**Fig 2 F and G, S3 C**). Superoxide dismutases act on superoxide
430 radicals, the potent ROS encountered in macrophages, converting to hydrogen peroxide. The
431 mRNA expression of both *sodA* and *sodB* were likewise downregulated in STM Δ*potCD* and STM
432 Δ*speED* upon infection into RAW264.7 macrophages (**Fig 2 H-K, S3 D and E**). A major
433 antioxidant in most living organisms is glutathione (GSH), which directly acts as a quencher of
434 ROS [26]. GSH is synthesized by Glutathione synthase (GshA) which in turn is regulated by EmrR
435 transcription factor. We observed that the mRNA expression of *emrR* is downregulated in STM
436 Δ*potCD* and STM Δ*speED* upon infection into RAW264.7 macrophages (**Fig 2 L and M, S3 F**).
437 Similarly, *gshA* transcript expression is downregulated as well (**Fig 2 N and O, S3 G)**. To further
438 validate the down regulation of the glutathione synthesis arm in spermidine transport and
439 biosynthesis mutants, we determined the intracellular GSH levels and noted the in STM Δ*potCD,*

440 STM Δ*speED* and STM Δ*potCD*Δ*speED* the levels were significantly less (**Fig S3H and I).** Taken
441 together these results indicate that spermidine regulates the transcription of multiple transcription
442 factors involved in oxidative stress response in *Salmonella* Typhimurium. Importantly, we found
443 a mechanism of oxidative stress resistance in *Salmonella* Typhimurium regulated by the
444 spermidine, potentiating the survival of the bacteria.

Figure 2: Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of the expression of numerous antioxidative enzymes



446 **Figure 2: Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of**
447 **the expression of numerous antioxidative enzymes**

448 A.The *in vitro* hydrogen peroxide sensitivity assay with the spermidine transport and biosunthesis
449 mutants, B. The mRNA expression of stress responsive transcription factor *rpoS* in STM *ΔpotCD*
450 upon infection into RAW264.7 cells, C. The mRNA expression of stress responsive transcription
451 factor *rpoS* in STM *ΔspeED* upon infection into RAW264.7 cells, D. The mRNA expression of
452 *katG* in STM *ΔpotCD* upon infection into RAW264.7 cells, E. The mRNA expression of *katG* in
453 STM *ΔspeED* upon infection into RAW264.7 cells, F. The mRNA expression of oxidative stress
454 responsive transcription factor *soxR* in STM *ΔpotCD* upon infection into RAW264.7 cells, G. The
455 mRNA expression of oxidative stress responsive transcription factor *soxR* in STM *ΔspeED* upon
456 infection into RAW264.7 cells, H. The mRNA expression of *sodA* in STM *ΔpotCD* upon infection
457 into RAW264.7 cells, I. The mRNA expression of *sodA* in STM *ΔspeED* upon infection into
458 RAW264.7 cells, J. The mRNA expression of *sodB* in STM *ΔpotCD* upon infection into
459 RAW264.7 cells, K. The mRNA expression of *sodB* in STM *ΔspeED* upon infection into
460 RAW264.7 cells, L. The mRNA expression of glutathione synthetase specific transcription factor
461 *emrR* in STM *ΔpotCD* upon infection into RAW264.7 cells, M. The mRNA expression of
462 glutathione synthetase specific transcription factor *emrR* in STM *ΔspeED* upon infection into
463 RAW264.7 cells, N. The mRNA expression of *gshA* in STM *ΔpotCD* upon infection into
464 RAW264.7 cells, O. The mRNA expression of *gshA* in STM *ΔspeED* upon infection into
465 RAW264.7 cells.

466

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

469 The spermidine synthesized from putrescine has two fates. It is either acetylated by the enzyme
470 SpeG or it is covalently conjugated to GSH to form Glutathionyl spermidine (GS-sp) catalysed by
471 the enzyme Glutathionyl-spermidine synthetase (Gss). Tabor (1974) first discovered the existence
472 of this enzyme in *E. coli* [27]. In *E. coli* GS-sp is generated at a higher level in the stationary phase
473 and very less in the late exponential phase. It also interacts and modifies the thiol containing
474 proteins under high H_2O_2-containing media leading to the formation of Gsp-thiolated proteins (PS-
475 Gsp). Certain *in vitro* experiments with dehydro-ascorbate suggested that GS-sp might have higher
476 antioxidant properties than GSH [28] and may be more effective in protecting against DNA
477 damage by free radicals [29]. However, in *E. coli* Gss could not be linked to its pathogenicity [30].
478 Among *Enterobacteriaceae*, *Salmonella* was found to possess this unique enzyme. Our *in silico*
479 analysis suggested that the enzyme in *Salmonella* Typhimurium (GspSA, encoded by *gsp*) has 90%
480 identity with *E. coli gss* and the SWISS MODEL predicts it to be a homo-dimeric protein (**Fig S4**
481 **A-C**). Also, the spermidine synthesised by SpeE in *Salmonella* Typhimurium is directly fed into
482 the pathway to synthesise GS-sp. We thus, investigated biological role of this novel enzyme in
483 *Salmonella* Typhimurium. We noted that the mRNA expression of *gsp* is significantly upregulated
484 at the late-log phase of growth of STM WT in LB media in the presence of H_2O_2 (**Fig S4 D**). Upon
485 infection into RAW264.7 cells, STM WT upregulates the mRNA expression of *gsp* at 6 hours and
486 16 hours post-infection into RAW264.7 cells (**Fig 3 A**). Further, the *Salmonella* Typhimurium
487 mutant of *gsp* (STM Δ*gsp*) showed attenuated proliferation in RAW264.7 cells similar to STM
488 Δ*katG,* which has reduced capability to detoxify ROS (**Fig 3 B)**. Thus, our results suggest that *gsp*
489 is important in *Salmonella* Typhimurium to survive and cope with the oxidative stress and hostile

490 environment of macrophages. Interestingly, the mRNA expression of *gsp* was found to be
491 downregulated in both STM Δ*potCD* and STM Δ*speED* at 16 hours post-infection into RAW264.7
492 macrophages (**Fig 3 C and D)**. These findings show that spermidine maintains its intracellular
493 levels by regulating the flux of spermidine into other pathways, such as the GspSA pathway. Also,
494 it further potentiates the ability of *Salmonella* Typhimurium to mount an antioxidative response by
495 regulating *gsp* expression.

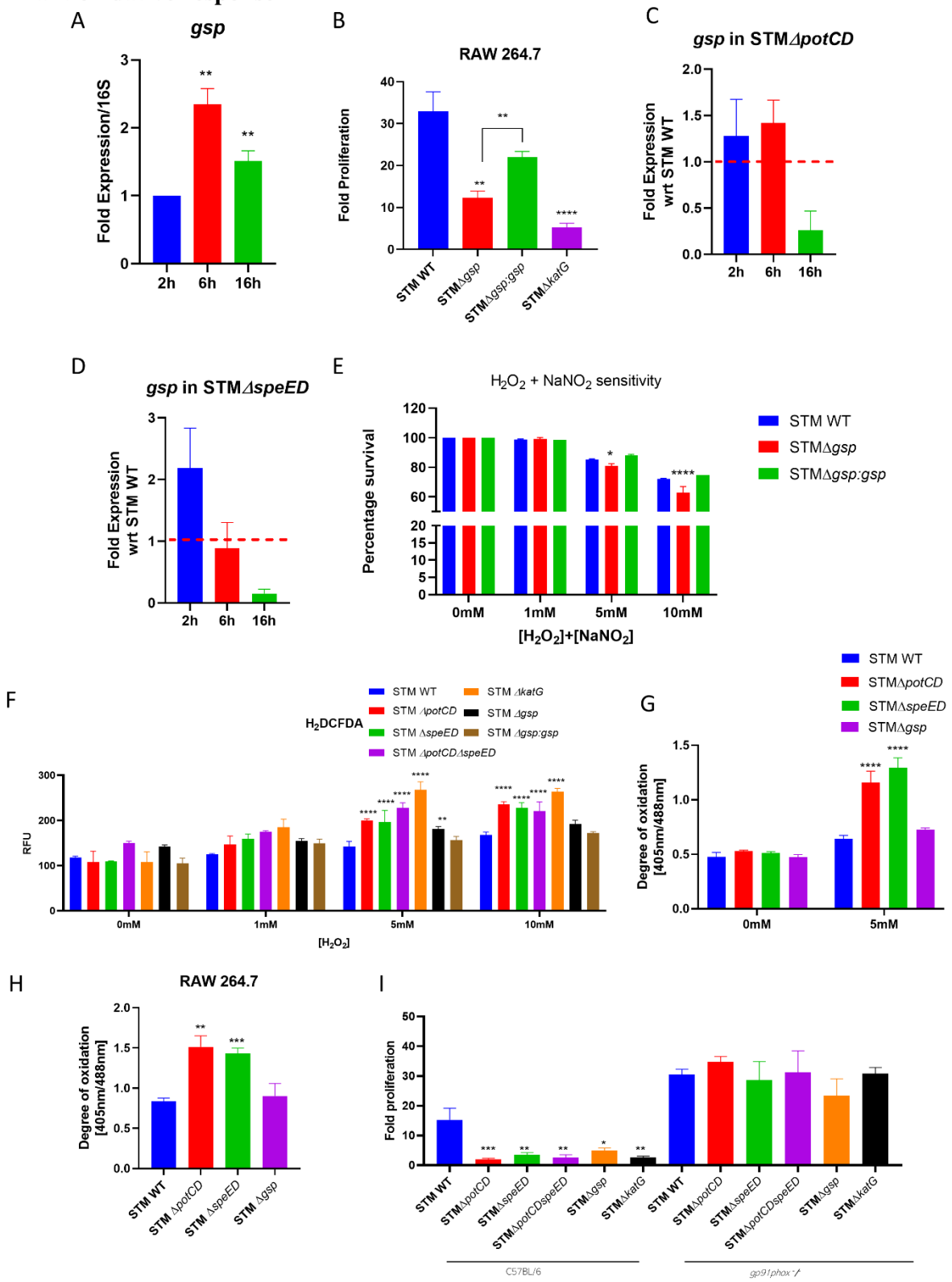
496 GspSA enzyme in Trypanosomes was named Trypanothione synthetase, while the conjugate is
497 called Trypanothione (TSH). In trypanosomes TSH is essential, and these organisms entirely rely
498 on TSH and possess no GSH reductase. Also, they have evolved to use TSH reductase instead of
499 GSH reductase, Glutaredoxins or Thioredoxins [31]. In *E. coli* GS-sp forms bonds with the
500 Cysteine thiol groups of numerous proteins and protect them from oxidation under oxidative stress.
501 Cysteine thiol groups are highly prone to attack by ROS and get oxidized to sulphinic and
502 sulphonic acids. In *Salmonella* Typhimurium we observe that loss of *gsp* results in attenuated
503 proliferation and survival in macrophages. Thus, we investigated the survival of STM Δ*gsp* upon
504 exposure to hydrogen peroxide *in vitro*. STM Δ*gsp* exhibited reduced survival in the presence of
505 high 5mM and 10mM concentrations of H_2O_2 (**Fig S4 E**). Likewise, upon exposure to agents of
506 oxidative stress and nitrosative stress H_2O_2 and $NaNO_2$ together, we observe that STM Δ*gsp*
507 exhibited reduced survival at higher concentrations such as 5mM and 10mM (**Fig 4 E**). Thus, *gsp*
508 is critical in *Salmonella* Typhimurium to shield the bacteria from the action of ROS and RNS. As
509 we observed that spermidine transporter and biosynthesis mutants and *gsp* mutant of *Salmonella*
510 Typhimurium are compromised in their survival under oxidative stress and in macrophages thus,
511 we were interested to assess the intracellular ROS detoxification abilities of the strains. We
512 determined the intracellular ROS in STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*

513 STM Δ*gsp* and STM Δ*katG*. Interestingly, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*
514 STM Δ*gsp* and STM Δ*katG* showed significantly higher intracellular ROS levels when they were
515 exposed to 5mM and 10mM H_2O_2 (**Fig 3 F**). Further the complemented strains for spermidine
516 transport and synthesis mutants showed a reduced intracellular ROS in the presence of high
517 concentrations of hydrogen peroxide *in vitro* (**Fig S4 F)**. These results suggest that lower
518 intracellular levels of spermidine correlate to higher intracellular ROS levels in *Salmonella*
519 Typhimurium. To validate our observed results, we utilised a genetically engineered tool to sense
520 the redox status of the bacterial cytosol, roGFP2 is a genetically modified form of GFP, and we
521 use pQE60-Grx1-roGFP2 plasmid. The glutaredoxin (Grx1) fused to the roGFP2, reversibly
522 transfers electrons between the cytosolic pool of GSH/GSSG and the thiol group of roGFP2, and
523 the ratio of fluorescence ratio at 408nm and 488nm determine the redox status of bacterial
524 cytoplasm [14]. We observed that STM Δ*potCD*, STM Δ*speED* harbouring the pQE60-Grx1-
525 roGFP2 showed higher ratio of 405nm/488nm compared to STM WT in the presence of 5mM
526 H_2O_2 *in vitro* and also upon infection into RAW 264.7 macrophages (**Fig 3 G and H**). However,
527 STM Δ*gsp* did not show a significantly higher ratio of 405nm/488nm. Moreover upon
528 supplementation of the growth media with 100 μM spermidine, only in STM Δ*speED* we observed
529 a lower intracellular ROS and lesser 405nm/488nm in higher concentration of H_2O_2 (**Fig S4 G**
530 **and H)**. Thus, our results indicate that spermidine is critical in mounting an antioxidative response
531 to detoxify the intracellular ROS, by regulating multiple antioxidant genes in *Salmonella*
532 Typhimurium. To validate our observed results we determined the intracellular levels of
533 glutathionyl-spermidine in STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*gsp* using mass
534 spectrometry. Our study qualitatively shows that the synthesis of GS-sp and $(GS\text{-}sp)_2$ (oxidized
535 form, di-glutathionylspermidine) in STM WT upon exposure to 1mM hydrogen peroxide, which

536 is absent in the spermidine mutants and STM Δ*gsp* (**Fig S5 A and B**). We further determined the
537 presence of PS-Gsp and show that STM WT shows modifications of proteins by GS-sp (detected
538 by anti-spermidine antibody), which is reduced upon treatment with beta-mercaptoethanol. The
539 spermidine mutants show very less modification, while it is almost negligible in STM Δ*gsp* (**Fig**
540 **S5 C-E**).

541 We observe that STM Δ*potCD*, STM Δ*speED* show a higher intracellular ROS than STM Δ*gsp.*
542 Thus to understand does STM Δ*gsp* phenocopy STM Δ*potCD*, STM Δ*speED*, we generated double
543 mutants STM Δ*gsp*Δ*potCD* and STM Δ*gsp*Δ*speED.* We find that, the double mutants show a
544 similar reduced fold proliferation and percentage phagocytosis in RAW 264.7 cells (**Fig S6 A-B)**.
545 However, they show an enhanced intracellular ROS upon exposure to hydrogen peroxide (**Fig S6**
546 **C)**. Upon co-infection of the double mutants with STM Δ*gsp* in C57BL/6 mice, we see that STM
547 Δ*gsp* out competes the double mutants in colonizing the liver (**Fig S6 D-E)**. To further dissect the
548 role of spermidine in protection of *Salmonella* Typhimurium from oxidative stress, we infected
549 STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* in
550 primary macrophages isolated from the peritoneal lavage of *gp91phox -/-* mice. Gp91Phox is the
551 major subunit of the NOX2 complex, that aids in the catalysis of oxygen to superoxide radical.
552 Interestingly we observe a rescue in the attenuated fold proliferation of STM Δ*potCD*, STM
553 Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* in peritoneal macrophages isolated
554 from *gp91phox -/-* mice (**Fig 3 I**). Our results demonstrate the vital role of spermidine in oxidative
555 stress resistance in *Salmonella* Typhimurium.

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



557 **Figure 3: Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in**
558 ***Salmonella* Typhimurium, and together mount an intracellular antioxidative response**

559 A.The mRNA expression of *gsp* in STM WT upon infection into RAW264.7 cells, B The fold
560 proliferation of STM WT, STM *Δgsp* and STM *Δgsp:gsp* in RAW264.7 cells, C. The mRNA
561 expression of *gsp* in STM *ΔpotCD* upon infection into RAW264.7 cells, D. The mRNA expression
562 of *gsp* in STM *ΔspeED* upon infection into RAW264.7 cells, E The *in vitro* Hydrogen peroxide
563 and nitic oxide sensitivity assay, F. Intracellular reactive oxygen species determination using the
564 cell permeable H_2DCFDA dye, G. The intracellular degree of oxidation in spermidine and *gsp*
565 mutants using pQE60-grx1-roGFP2 construct *in vitro*, H. The intracellular degree of oxidation
566 using pQE60-grx1-roGFP2 construct in spermidine and *gsp* mutants upon infection into RAW
567 264.7 cells , I. The fold proliferation in primary macrophages isolated from wild type C57Bl/6
568 mice and *gp91phox-/-* mice, Student's t-test was used to analyze the data; p values ****<0.0001,
569 ***<0.001, **<0.01, *<0.05. Two-way Anova was used to analyze the grouped data; p values
570 ****<0.0001, ***<0.001, **<0.01, *<0.05.

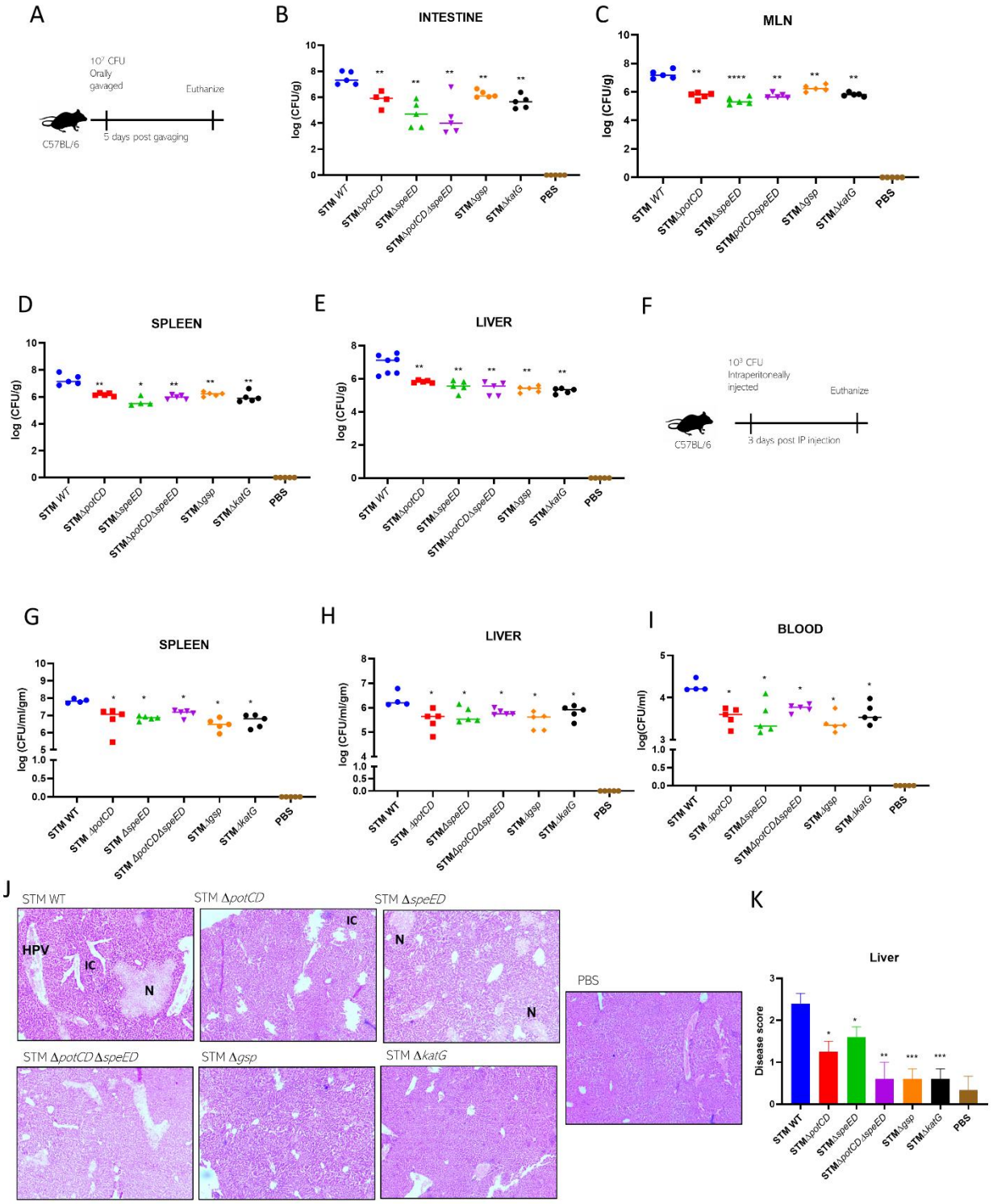
571

572 **Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and secondary**
573 **sites of infection in mice**

574 *Salmonella* infects the host and breaching the epithelial cells at the Peyer's patches in the distal
575 ileum, it disseminates to the secondary sites of infection namely the Mesentric Lymph node
576 (MLN), spleen and liver. From the basolateral surface of the epithelial cells at the lamina propria,
577 *Salmonella* is taken by the macrophages and polymorphonuclear cells (PMN). We observed that
578 the spermidine transporter and biosynthesis mutants show attenuated survival in macrophages and

579 under oxidative stress *in vitro.* We were intrigued to study the behaviour of the mutants during *in*
580 *vivo* colonisation in the mouse model of *Salmonella* infection. We infected C57BL/6 mice by orally
581 gavaging with STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM
582 Δ*katG* at a CFU of 10^7 per mice. We noted that STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*
583 STM Δ*gsp* and STM Δ*katG* showed a significantly lower organ burden in Peyer's patches, MLN,
584 spleen and liver upon oral gavage (**Fig 4 A-E)**. Our previous study showed that the spermidine
585 transporter and biosynthesis mutants exhibit a poor invasiveness into IECs, which can explain our
586 *in vivo* colonisation upon oral gavage [16]. Oral gavage mimics the physiological route of
587 *Salmonella* infection into its host. Hence, it requires to be able to breach the intestinal barrier
588 successfully. Incapability to invade the IECs in STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE*
589 and STM Δ*gsp* explains the diminished colonisation in the organs. To dissect the role of spermidine
590 in *in vivo* colonisation we infected C57Bl/6 mice intraperitoneally, by bypassing the entry by
591 breaching epithelial barrier. We observed that upon infecting intraperitoneally, STM Δ*potCD*, STM
592 Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* exhibited reduced colonisation in
593 spleen and liver and less dissemination in blood compared to STM WT (**Fig 4 F-I)**. Also, the
594 histopathological sections show significantly less liver tissue damage with STM Δ*potCD*, STM
595 Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* , which is validated by disease scoring
596 of the same(**Fig 4 J and K)**. Our results thus show that spermidine aids in the *in vivo* pathogenesis
597 and virulence of *Salmonella* Typhimurium.

Figure 4: Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and secondary sites of infection in mice



598

599 **Figure 4: Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and**
600 **secondary sites of infection in mice**

601 A.The experimental protocol for organ burden in C57BL/6 mice by orally gavaging 10^7 CFU per
602 mice, B. The organ burden post 5 days of oral gavage in intestine, C. in Mesentric lymph node
603 (MLN), D. in spleen, E. in liver, F. The experimental protocol for organ burden in C57BL/6 mice
604 upon intraperitoneal (I.P) infection with 10^3 CFU per mice, G. The organ burden 3 days post I.P
605 infection in spleen, H. in liver, I. dissemination in blood, J. The hematoxylin and eosin staining of
606 the sections of liver 3 days post I.P infection of C57Bl/6 mice, K. Disease score from the
607 histopathogical sections of liver, Here, Small necrotic areas (N), Congestion and damage to the
608 endothelial lining of the central vein (C), Congestion of the hepatic portal vein and poor hepatic
609 architecture (HPV), Inflammatory immune cells (IC). The disease score is as :0 for normal
610 pathology, 1 for mild/ minor pathology, 2 for moderate pathology, and 3 for severe pathological
611 changes. Mann Whitney test was used to analyse organ burden in mice; p values ****<0.0001,
612 ***<0.001, **<0.01, *<0.05. Student's t-test was used to analyze the data; p values ****<0.0001,
613 ***<0.001, **<0.01, *<0.05.

614

615 ***Salmonella* rewires host polyamine metabolism to potentiate its survival within host**
616 **macrophages**

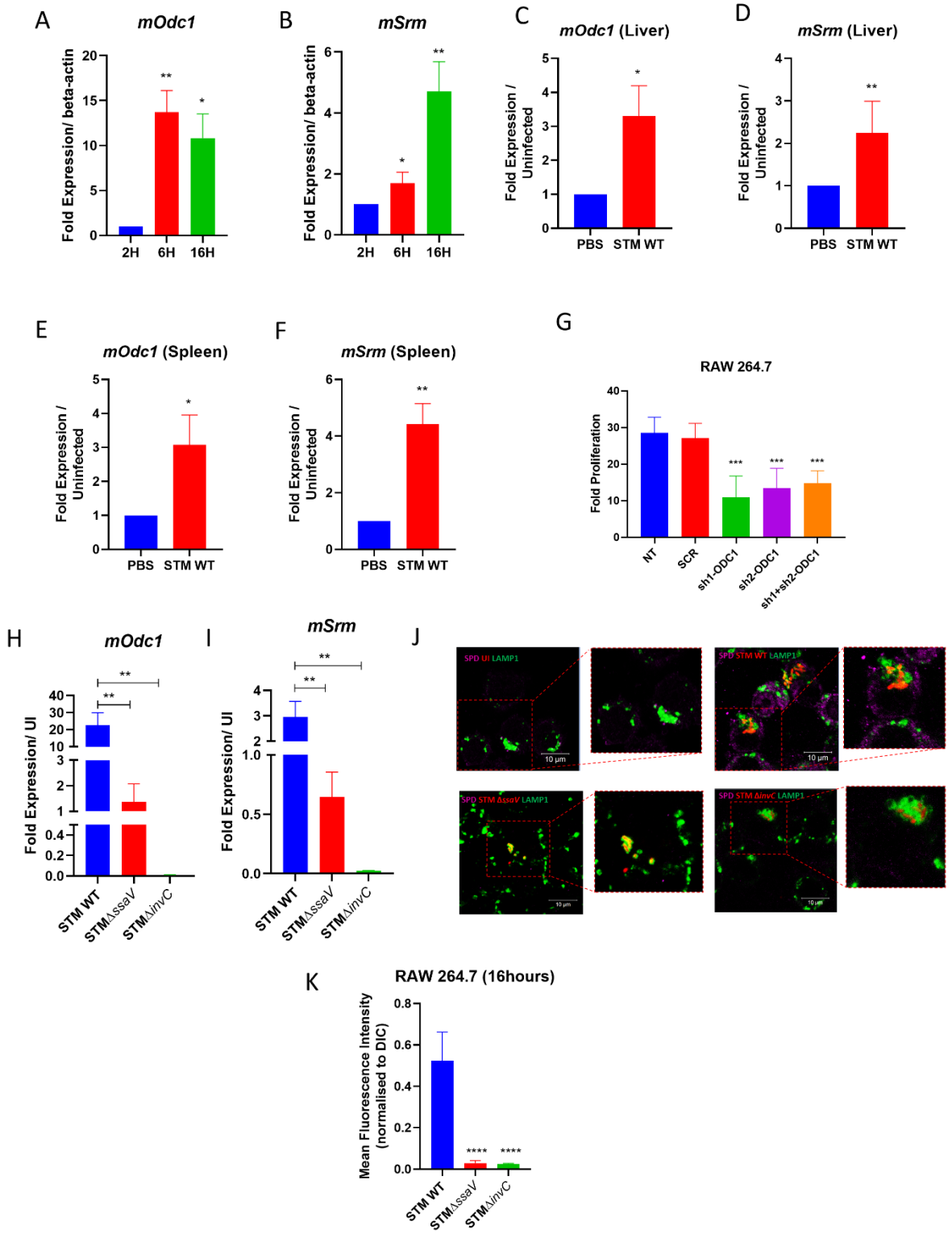
617 Most of the intracellular pathogens establish their persistence in the phagocytic cells and are often
618 found to be associated with different populations of the macrophages. Like *Brucella abortus*
619 preferentially resides in the Alternatively activated macrophages (AAM), where it survives and
620 replicates by exploiting the host polyamines. A research group has shown that the metabolism of

621 AAM is shifted to increase polyamine biosynthesis by *Brucella abortus* and thereby promote the
622 bacterial survival [32]. Similarly, *Salmonella* also resides in macrophages, a way to lead chronic
623 infections. We were interested to know whether *Salmonella* exploits the host polyamines and leads
624 to a rewiring of host cell metabolism. To understand the host-pathogen relationship in depth, we
625 assessed the mRNA expression of Ornithine decarboxylase 1 (*mOdc, mouse Ornithine*
626 *decarboxylase*) which catalyses the rate-determining step of polyamine biosynthesis and
627 Spermidine synthase (*mSrm, mouse Spermidine synthase*) that synthesises spermidine from
628 putrescine by transferring the aminopropyl group from decarboxylated-S-adenosyl methionine in
629 RAW264.7 cells upon infection with STM WT. We observed that mRNA expression of *mOdc1*
630 and *mSrm* were upregulated at 6 and 16 hours post-infection (**Fig 5 A and B**). Further, the mRNA
631 expression of *mOdc1* and *mSrm* were enhanced in the spleen and liver of C57BL/6 mice 5 days
632 post oral gavage with STM WT (**Fig C- F**). Our results show that *Salmonella* Typhimurium upon
633 infection into the host enhances the expression of host polyamine biosynthesis genes. Moreover,
634 we have previously observed that the *Salmonella* Typhimurium that cannot import spermidine
635 cannot survive and proliferate as much as STM WT in macrophages. To further delve into the role
636 of host acquired polyamines, we knocked down *Odc1* in RAW264.7 cells (**Fig S6 A**). Upon
637 knockdown of *mOdc1*, *Salmonella* Typhimurium showed significantly attenuated proliferation in
638 RAW264.7 cells (**Fig 5 G**). However, the knockdown of *mOdc1* did not alter the percentage of
639 phagocytosis by the macrophages (**Fig S7 C)**. Similarly, we knocked down *Srm* in RAW264.7 cells
640 and observed that knock-down of spermidine synthase in the host compromises the ability of STM
641 WT to proliferate and get phagocytosed by the macrophages (**Fig S7 B, D and E)**.

642 The question that arises is how does *Salmonella* regulate the host polyamine metabolic pathways?
643 *Salmonella* utilizes *Salmonella* pathogenicity island 1 and 2 encoded effectors for its entry and

644 survival in the specialized host niches [33, 34]. Although SPI-1 is well studied in the initial process
645 of invasion, recent studies suggests that both SPI-1 and SPI-2 effectors are required for SCV
646 maturation and *Salmonella* survival in the host cells [35-37]. Thus to investigate how *Salmonella*
647 modulates host cell polyamine metabolism, we infected RAW264.7 cells with SPI-1 mutant (STM
648 Δ*invC*) and a SPI-2 mutant (STM Δ*ssaV*), both of which lack the ability to import effectors of SPI-
649 1 and SPI-2 respectively. We observed that the mRNA expression of *mOdc1* and *mSrm*, were
650 significantly downregulated in macrophages infected with STM Δ*invC* and STM Δ*ssaV* compared
651 to in STM WT (all normalized to Unifected) (**Fig 5H and I)**. We further determined the
652 intracellular spermidine by immunofluorescence and found that macrophages infected with STM
653 Δ*invC* and STM Δ*ssaV* showed reduced spermidine production compared to in STM WT and
654 uninfected cells (**Fig 5J and K)**. Thus, our data suggests that *Salmonella* utilizes effectors from
655 SPI-1 and SPI-2 to modulate the host cell polyamine metabolic pathways.

Figure 5: *Salmonella* rewires host polyamine metabolism to potentiate its survival within host macrophages



656

657 **Figure 5: *Salmonella* rewires host polyamine metabolism to potentiate its survival within host**
658 **macrophages**

659 A.The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in RAW264.7 cells upon
660 infection with STM WT, B. The mRNA expression of *mSrm* (mouse spermidine synthase) in
661 RAW264.7 cells upon infection with STM WT, C. The mRNA expression of *mOdc1* (mouse
662 ornithine decarboxylase) in liver of C57BL/6 mice 5 days post infection with STM WT by oral
663 gavage, D. The mRNA expression of *mSrm* (mouse spermidine synthase) in liver of C57BL/6 mice
664 5 days post infection with STM WT by oral gavage,E. The mRNA expression of *mOdc1* (mouse
665 ornithine decarboxylase) in spleen of C57BL/6 mice 5 days post infection with STM WT by oral
666 gavage, F. The mRNA expression of *mSrm* (mouse spermidine synthase) in spleen of C57BL/6
667 mice 5 days post infection with STM WT by oral gavage, G. The fold proliferation of STM WT in
668 RAW264.7 cells upon transient knockdown of *mOdc1,* H. The percentage phagocytosis of STM
669 WT in RAW264.7 cells upon transient knockdown of *mOdc1.* Here SCR is Scrambled (no target
670 for knock-down), two different targeted shRNA were used for knock-down purposes, Sh1is
671 shRNA-1 for knock-down, Sh2 is shRNA-2 for knock-down, and Sh1+Sh2 indicates where both
672 the shRNAs were used to obtain the knock-down, H. The mRNA expression of *mOdc1* (mouse
673 ornithine decarboxylase) in RAW264.7 cells upon infection with STM WT, STM Δ*ssaV,* and STM
674 Δ*invC*, normalized to the expression in uninfected macrophages, I. The mRNA expression of *mSrm*
675 (mouse spermidine synthase) in RAW264.7 cells upon infection with STM WT, STM Δ*ssaV,* and
676 STM Δ*invC*, normalized to the expression in uninfected macrophages, J. Immunofluorescence
677 imaging to study spermidine in RAW 264.7 cells upon infection with STM WT, STM Δ*invC,* STM
678 Δ*ssaV*, here green is Anti-mouse LAMP1(Alexa fluor 488), Red is pFPV-M-cherry expressing

679 *Salmonella* strains, magenta is anti-Spermidine (Alexa fluor 647) and UI- uninfected. Student's
680 t-test was used to analyze the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05.

681

682 **The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in the host by**
683 **enhancing nitric oxide production**

684 Polyamines are essential molecules in eukarotes, with multiple roles in differentiation,
685 proliferation and development. Many studies have shown that polyamine levels are upregulated in
686 cancer cells and elevated levels of polyamines are associated with breast cancer, neuroblastoma,
687 hepatocellular carcinoma, prostate cancer, lung cancer, colorectal cancer and lukemia [38-42].
688 D,L-α-difluoromethylornithine (DFMO) , an inhibitor of ODC was developed as a potent drug to
689 treat cancer in the year 1970 [43]. DFMO irreversibly binds to the active site of ODC and acts as
690 a suicide inhibitor, thereby reducing polyamine levels and having a cytostatic effect. As a single
691 therapeutic agent it was found to be effective only in neuroblastoma , and cinical trial for other
692 cancer type was unsatisfactory [44, 45]. However, DFMO has been successfully developed as a
693 chemopreventive drug, with FDA approval for treatment of Human African Trypanosomiasis
694 (HAT) [46, 47]. To test whether DFMO can be used as a therapeutic drug against *Salmonella*
695 infection, we treated RAW264.7cells with DFMO during the infection with *Salmonella*
696 Typhimurium, and observed a significant attenuation in the fold proliferation of STM WT (**Fig 6**
697 **A)**. Studies have shown that DFMO binds to ODC to prevent further production of putrescine from
698 ornithine and also acts on Arginase1 and reduces the available pool of ornithine for polyamine
699 biosynthesis [48, 49]. This, ensures the flux of arginine to be fed into the nitric oxide synthase
700 (NOS2) pathway and leads to elevated levels of nitric oxide in the cell, which in turn negatively

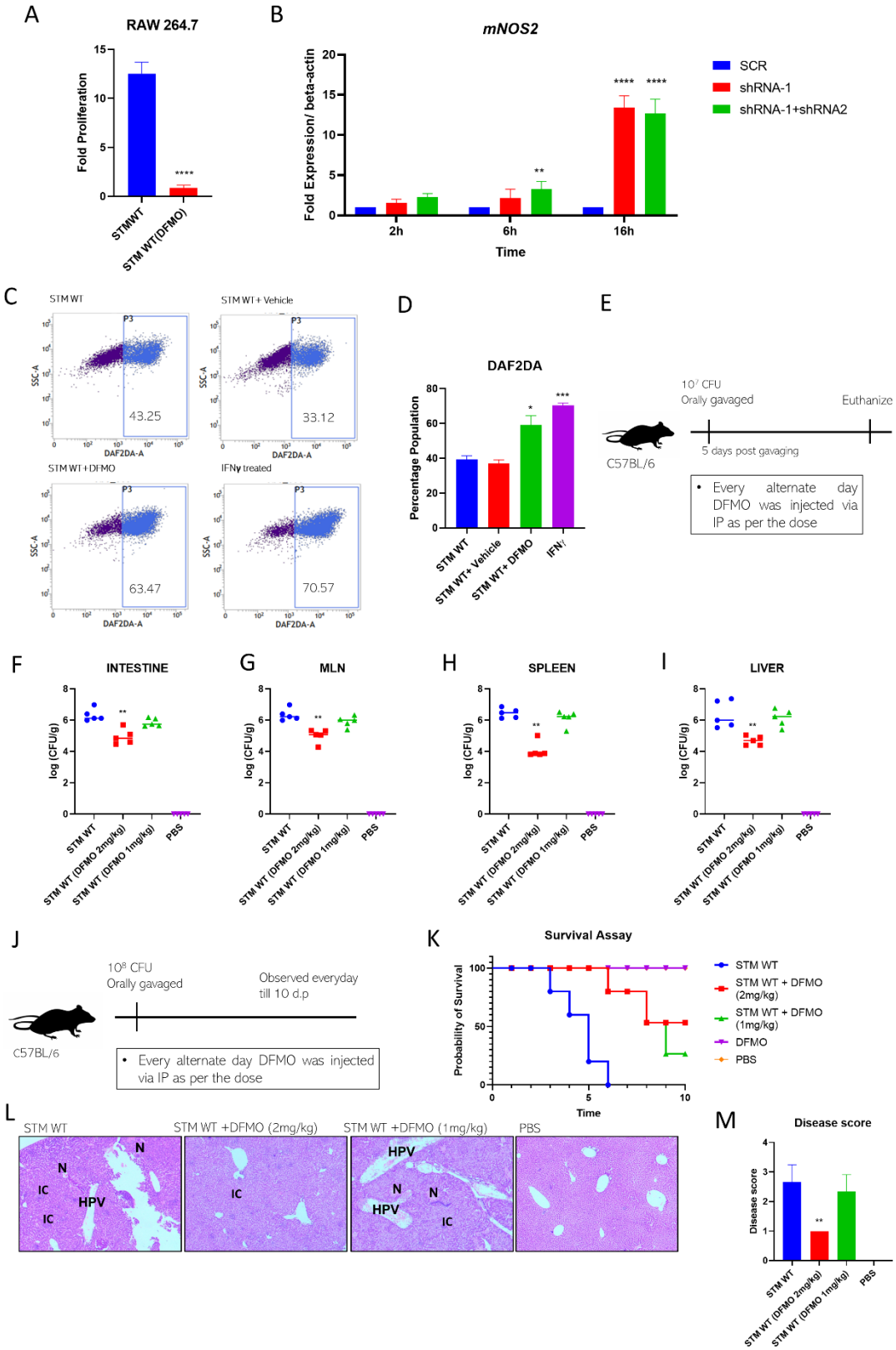
701 acts on ODC to further block polyamine synthesis [50].We assessed the mRNA expression of
702 *mNos2* upon knockdown of *mOdc1* in RAW264.7 cells followed by infection with STM. We
703 observed an upregulation of *mOdc1* mRNA levels at 6 hours and 16 hours post infection in
704 RAW264.7 cells (**Fig 6 B)**. Further we determined the levels of nitric oxide using a cell permeable
705 dye DAF-2DA, and noted that upon treatment with DFMO there was higher production of nitric
706 oxide (**Fig 6 C and D)**. Taken together our results demonstrate the role of DFMO in diminishing
707 the survival and proliferation of *Salmonella* Typhimurium by blocking Odc1 and enhanced
708 production of nitric oxide in murine macrophages.

709 To further address whether DFMO also act as potent anti-*Salmonella* drug in mice model of
710 infection, we infected C57BL/6 mice with 10^7 CFU per mice by oral gavage. Every alternate day
711 the mice were injected with DFMO in two different doses for two different cohorts (2mg/kg of
712 body weight of mice and 1mg/kg of body weight of mice). Five days post oral gavage we observed
713 that DFMO treatment of 2mg/kg body weight of mice significantly reduced the colonisation of
714 STM WT in intestine, MLN, spleen and liver than in the untreated mice (**Fig 6 E-I)**. Further, using
715 immunofluorescence we observed DFMO significantly reduced the colonisation of STM and and
716 the levels of spermidine in mouse ileum (**Fig S8 A-D).** Moreover, treatment of mice with DFMO
717 at a dose of 2mg/kg body weight of mice, increased the survival of mice upon infection with STM
718 WT (**Fig 6 J and K)**. Also, the weight reduction in mice treated with with DFMO at a dose of
719 2mg/kg body weight, was less upon infection with STM WT (**Fig S8 E)**. Next, we evaluated the
720 tissue damage upon infection of STM in mice liver. The results show that DFMO treatment of
721 mice at a dose of 2mg/kg of body weight significantly lowered the disease score, suggesting lesser
722 tissue damage compared to untreated (**Fig 6 L and M)**. Thus, DFMO serves as a potential drug to

723 treat *Salmonella* infection in mice by reducing the bacterial burden and tissue damage in mice and

724 enhancing the survival of mice upon infection with STM.

Figure 6: The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in the host by enhancing nitric oxide production



726 **Figure 6: The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in**
727 **the host by enhancing nitric oxide production**

728 A. The intracellular fold proliferation of STM WT in RAW264.7 cells upon treatment with
729 Difluoromethyl ornithine (DFMO) (5μM), quantified by Gentamicin protection assay, B. The
730 mRNA expression of *mNos2*(mouse Nitric oxide synthase) in RAW264.7 cells, upon transient
731 knockdown of *Odc1* followed by infection with STM WT, the expression is normalized to beta-
732 actin as an internal control, C. The representative scatter plot for DAF2DA dye positive population
733 of RAW264.7 cells, upon transient knockdown of *Odc1* followed by infection with STM WT, the
734 data is determined by flow-cytometry post staining the infected macrophages with the dye, D. The
735 quantification of (C), E. The experimental procedure to study the organ burden of STM WT in
736 C57BL/6 mice upon treatment with DFMO, F-I. The organ burden of STM WT in Intestine, MLN,
737 Spleen and Liver of C57BL/6 mice upon intraperitoneal treatment of DFMO (2mg/kg and 1mg/kg
738 of body weight) as mentioned in (E), J. The Experimental procedure to study the survival of
739 C57BL/6 mice upon infection with STM WT and treatment with DFMO, K.The survival of
740 C57BL/6 mice upon infection with STM WT and upon intraperitoneal treatment of DFMO
741 (2mg/kg and 1mg/kg of body weight) as mentioned in (J), L. Hematoxylin and eosin staining of
742 histopathological sections of liver upon DFMO treatment to C57BL/6 mice. Here, Small necrotic
743 areas (N), Congestion and damage to the endothelial lining of the central vein (C), Congestion of
744 the hepatic portal vein and poor hepatic architecture (HPV), Inflammatory immune cells (IC), M.
745 The disease score index for liver tissue damage upon STM WT infection in C57BL/6 mice with
746 DFMO treatment. The disease score is as :0 for normal pathology, 1 for mild/ minor pathology, 2
747 for moderate pathology, and 3 for severe pathological changes, Student's t-test was used to analyze

748 the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way Anova was used to
749 analyze the grouped data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05.

750

751 **Discussion**

752 A significant part of the life cycle of *Salmonella* during its pathogenesis involves its stay inside
753 the macrophages. The Gram-negative bacteria come across multiple host environmental stress
754 conditions inside the macrophages [51]. A key mechanism by which the host macrophages try to
755 limit the invading pathogen is by the rapid oxidative burst and ROS production. ROS includes
756 superoxide radicals, hydroxyl radicals, peroxy-nitrites, peroxy-chlorides and hydrogen peroxide.
757 ROS can pass through the bacterial cell wall and act on lipids, proteins and nucleic acids by
758 oxidising them and leading to cellular damage. *Salmonella* is often referred to as a smart pathogen.
759 Over the years, it has evolved to possess multiple strategies to combat the host-derived stresses
760 [52, 53]. To combat the oxidative burst generated NOX2 in macrophages, *Salmonella* possesses
761 multiple antioxidant enzymes such as the catalases KatE and KatG, the superoxide dismutases
762 SodA and SodB, the Alkyl hydroperoxide reductase, the glutaredoxins and thioredoxins and Hrg
763 transcriptional regulator [25]. Polyamines assist in *Salmonella* virulence and aid in stress
764 resistance. However, the mechanism behind the role of polyamines in *Salmonella* stress resistance
765 and virulence remains less appreciated.

766 Our study identifies spermidine as a stress-responsive regulatory molecule in *Salmonella*
767 Typhimurium. We show spermidine is critical for the survival and proliferation of STM in
768 macrophages and in the presence of oxidative stress *in vitro.* The spermidine transporter and
769 biosynthesis mutants show significant reduced capability to be phagocytosed by the macrophages.

770 Findings from our previous study showed that the intracellular level of spermidine is significantly
771 less in both the spermidine transport and biosynthesis mutants, which further explains the
772 attenuated proliferation of both the mutants in macrophages [16]. The previous findings also
773 showed that in absence of the transport genes in *Salmonella* Typhimurium the synthesis genes are
774 down regulated and vice versa. The absence of spermidine transport and biosynthesis diminishes
775 the mRNA expression of multiple arms of oxidative stress response in *Salmonella.* Multiple studies
776 show the role of polyamines in the regulation of transcription of multiple genes by interacting with
777 DNA in eukaryotes. They bind to the DNA and change conformation as in C-MYC transcription,
778 and in other cases, enhance DNA-protein binding affinities like for Estrogen-response elements
779 [54, 55]. Thus, in *Salmonella* Typhimurium *rpoS, soxR* and *emrR* fall under the "Polyamine
780 modulon".

781 We further characterise a novel enzyme GspSA in *Salmonella* Typhimurium, which synthesises a
782 conjugated product of glutathione (GSH) and spermidine called GS-sp. GspSA is critical for
783 *Salmonella* Typhimurium to survive and proliferate in macrophages. Our study shows that the
784 absence of GspSA attenuates the survival of STM under oxidative stress conditions *in vitro,*
785 suggesting a vital role of GspSA in protecting STM from oxidative damage. GS-sp in *E. coli* carries
786 out its function by modifying protein thiol groups under oxidative stress to protect the proteins
787 from getting oxidised and damaged. In *Salmonella* Typhimurium, we further show that the
788 spermidine regulates the *gsp* expression and the subsequent production of GS-sp. We expect that
789 GS-sp generated under oxidative stress conditions and inside the macrophages, likewise interact
790 with cysteine-thiol groups in proteins to shield them from oxidative damage. MK Chattopadhyay
791 (2013) showed that this enzyme (GspSA) is specifically present in two groups of organisms namely
792 bacteria and kineto-plastids respectively and completely absent in other organisms such as human,

793 rats, drosophila etc., among the bacteria group in all *enterobacteria* showed 27-100% homology
794 and >65% identity in around 50% of the species, with *E. coli* [30]. Thus, the absence of GspSA in
795 eukaryotes makes it a potent drug target for treatment of *Salmonella* infections. Thus, spermidine
796 exerts pleiotropic effects in *Salmonella*, by orchestrating the multiple arms of antioxidative
797 response. It strengthens the bacteria to cope with hostile host environments. Our studies in the *in*
798 *vivo* model of *Salmonella* Typhimurium infection further dissect the role of spermidine in assisting
799 in the pathogenesis by enhancing its virulence properties. Moreover, it is at the nexus of multiple
800 oxidative stress response arms in *Salmonella*, thereby assisting in mounting an antioxidative
801 response to promote its survival in macrophages.

802 Studies over the past years have given insights into the function of polyamines in the pathogenesis
803 of multiple virulent bacteria. Many human pathogenic bacteria have developed ways to exploit,
804 interfere and manipulate the polyamine metabolism of the host to enhance their fitness within the
805 niche. As in *Shigella* and *Vibrio spp.* polyamines produced by the bacteria are critical in
806 determining virulence [56, 57]. While bacteria such as *Legionella spp*., which does not synthesise
807 polyamines, depend on the host-acquired polyamines for their pathogenesis [58]. Another unique
808 mechanism is observed in *H. pylori* which activates polyamine oxidation, thereby dysregulation
809 the innate-immune response [59]. Also, in response to *H. pylori* infection, the host macrophages
810 increase the arginase activity and ornithine decarboxylase activity to produce polyamines [60]. A
811 recent study showed that SARS-Cov-2 hijacks the host polyamines for its reproduction and
812 infection [61].

813 Our findings also reveal that *Salmonella* Typhimurium enhances the polyamine production in the
814 host upon infection using its specialized pathogenicity island encoded effectors from SPI-1 and
815 SPI-2, which might be a strategy to hijack the host polyamines for its survival. This further explains

816 another reason for the reduced proliferation observed for the spermidine transport mutant in
817 macrophages. Also, the knockdown of host ornithine decarboxylase attenuates *Salmonella*
818 proliferation in macrophages. The upregulation of Odc1 activity manages to feed the amino acid,
819 L-arginine, into the polyamine biosynthesis and prevent nitric oxide production, which otherwise
820 would be detrimental for the pathogen. Polyamines have been a well-studied as a drug target for
821 treatment of multiple cancers. The oncogene MYC is upregulated in 70 percent of the cancer types.
822 Ornithine decarboxylase (Odc1) rate limiting enzyme of polyamine biosynthesis is
823 transcriptionally activated, directly by MYC, thereby increasing Odc1 levels in cancer [62]. RAS
824 is another essential factor in cell growth and cancer development, and it directly acts on Odc1 to
825 translationally activate Odc1 in cancer cells [63]. Polyamines biosynthesis is also associated with
826 multiple other oncogenes such as AKT1 and mTORC1 [64, 65]. We further show that upon using
827 Odc1 suicide inhibitor, DFMO, *Salmonella* proliferation could be diminished, and it reduces the
828 bacterial burden in mice. The FDA-approved chemopreventive drug, DFMO, for Human African
829 Trypanosomiasis treatment serves to be a potential drug to cure *Salmonella* infection. In the
830 devastating age of increasing antibiotic resistance, such a drug promises to effectively combat
831 deadly pathogens like *Salmonella.*

832 **Conclusion**

833 A substantial duration of the infection cycle of *Salmonella* involves the macrophages, which
834 present a very hostile environment to the bacteria. However, *Salmonella* is able to survive and
835 proliferate within host macrophages and utilizes it to disseminate to secondary sites of infection.
836 Our study identifies a novel strategy employed by *Salmonella* Typhimurium to counteract
837 oxidative and nitrosative stress within the host. We demonstrate that spermidine is a critical
838 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 its survival. In the era of anti-microbial resistance, this study further recognizes an FDA -approved chemopreventive 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 analyzed 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 National Animal Care were strictly followed during all experiments. (Registration No: 435 48/1999/CPCSEA).

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Author contribution statement

AVN and DC conceived the study. AVN and DC designed experiments. AVN and AS performed experiments. RSR analysed for the disease score of tissue samples. AVN, analyzed the data, prepared the figures and wrote the manuscript draft. AVN, AS and DC reviewed and edited the manuscript. DC supervised the work. All the authors read and approved the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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