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Impact of nafcillin and diosmin on the attachment, invasion, and stress survival of *Salmonella* Typhimurium

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Salmonella **Typhimurium is an invasive intracellular pathogen that employs various factors for its survival within host cells. To mitigate *S*. Typhimurium survival, it is crucial to identify factors that influence bacterial survival and to develop drugs that inhibit these factors. In this study, we investigated the effects of nafcillin and diosmin, both of which have been identified as inhibitors of Lon protease, on the intracellular survival of *S*. Typhimurium and its survival under various stress conditions. Additionally, we examined the expression of genes associated with the type II toxin-antitoxin system to enhance our understanding of the impact of these systems on the bacterium's survival. Our findings indicate that while nafcillin and diosmin did not affect *S*. Typhimurium attachment, they significantly reduced bacterial intracellular survival, particularly in Hep2 cells after 16 h. These inhibitors were also effective in decreasing bacterial survival under oxidative and acidic stress conditions. Furthermore, gene expression analysis revealed that although there were variations in the expression of TA system genes in *S*. Typhimurium across different cell lines, the *relEB* system emerged as the most effective among those studied, exhibiting the highest increase in expression. This study highlights the efficacy of nafcillin and diosmin in reducing the intracellular survival of *S*. Typhimurium as well as its survival under stress conditions. These findings suggest potential new strategies for developing therapies aimed at preventing *S*. Typhimurium infections.**

Keywords *Salmonella* Typhimurium, Toxin-antitoxin systems, Lon protease, Diosmin, Nafcillin

Salmonella enterica serovar Typhimurium is a Gram-negative, facultative intracellular bacterium that serves as a significant enteric pathogen affecting both humans and animals[1]. It poses a substantial threat to food-producing animals, including cattle, pigs, and poultry, and is primarily transmitted through the consumption of contaminated food or water[2].

After being ingested, *S*. Typhimurium attaches to the epithelial cells of the ileum and colon, initiating gastrointestinal infection[3]. The bacterium targets specialized epithelial cells known as M cells in the small intestine, prompting intracellular changes that facilitate their entry via the secretion of effector proteins[3,4]. The Type III secretion system (T3SS-1) plays a pivotal role in the invasion of intestinal epithelial cells by enabling bacterial entry and survival within the host[4]. *S*. Typhimurium uses two main strategies for cell entry: the "trigger" mechanism, which involves manipulation of the host's cytoskeleton, and the receptor-mediated "zipper" mechanism[5]. Successful adhesion and invasion are critical for establishing infection. Once inside, *Salmonella* resides within specialized vacuoles that shield it from the host's immune defenses[6]. Thus, blocking bacterial adhesion, invasion, and survival is crucial for managing infections, but delivering effective antimicrobial agents to target cells presents significant challenges[7].

In both human and animal health settings, various antimicrobial agents, including fluoroquinolones, are used to treat intracellular infections like those caused by *S*. Typhimurium[8,9]. However, increasing antibiotic resistance among these bacteria has become a pressing concern[8]. Moreover, some antibiotics fail to eradicate

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intracellular bacteria even at inhibitory concentrations[10]. Therefore, it is imperative to identify the factors influencing bacterial adhesion and invasion while exploring new compounds that can effectively inhibit these processes as well as intracellular survival in *S.* Typhimurium. Such efforts are essential for developing innovative strategies to combat infections caused by this significant pathogen[11].

The coordinated expression of genes within the *Salmonella* Pathogenicity Islands (SPI-1 and SPI-2) is crucial for the initial invasion and subsequent establishment of infection in host organisms[12]. SPI-1 encodes proteins that include a *Salmonella* type III secretion system (TTSS), which plays a key role in injecting effector molecules that manipulate the host's cytoskeletal structure, facilitating bacterial internalization. Once inside the host cell, genes from SPI-2 are activated, encoding another TTSS that secretes effectors aimed at redirecting endocytic trafficking[13]. This process prevents the recruitment and fusion of lysosomes with *Salmonella*-containing vacuoles, allowing the bacteria to survive within infected cells[14].

In its natural life cycle, *Salmonella* faces numerous environmental challenges, including increased temperatures, acidic gastric conditions, elevated salt levels from bile, and oxidative stress stemming from the host's immune response[15,16]. Within this framework, Lon protease—a well-conserved ATP-dependent serine protease—has a crucial role in the physiology of bacteria[17]. It contributes to the degradation of misfolded proteins, the regulation of gene expression, and the modulation of stress responses[18]. Furthermore, Lon protease is capable of degrading antitoxins within toxin-antitoxin systems, which leads to the activation of these systems[19].

By selectively degrading damaged or regulatory proteins, Lon protease helps maintain cellular homeostasis and enables adaptation to fluctuating environmental stressors[19]. Previous research has indicated the involvement of Lon in the expression of SPI-1 and SPI-2 genes in *S.* Typhimurium[20], making it essential to further investigate its role in modulating virulence during *S.* Typhimurium attachment and invasion.

Type II toxin-antitoxin systems add another layer of regulatory complexity to bacterial survival and virulence[21]. These systems consist of a stable toxin paired with a labile antitoxin, which together govern cell fate under stress conditions[22,23]. When faced with environmental challenges—such as nutrient deprivation or oxidative stress—the antitoxin may be degraded by Lon protease, allowing the toxin to exert its effects[23]. This can result in growth arrest or programmed cell death, potentially serving as a survival strategy for the bacterial population under stressful conditions[24,25].

In a prior investigation, we examined strategies for drug repositioning aimed at Lon protease in *S.* Typhimurium, successfully identifying diosmin and nafcillin as potent inhibitors of this enzyme[26]. This study focuses on evaluating how these Lon protease inhibitors, diosmin and nafcillin, affect the attachment, invasion, and survival of *S.* Typhimurium when exposed to different stress conditions.

Materials and methods

Bacterial strains and cell culturing

This research utilized the bacterial strain *S.* Typhimurium ATCC 14028, which was preserved in Brain Heart Infusion (BHI) broth (Merck, Germany) with 20% glycerol at -80 °C. The strain was subsequently grown on Luria-Bertani (LB) agar plates at 37 °C.

Three cell lines were employed: the human colorectal cancer SW480 cell line, the human laryngeal Hep2 cell line, and mouse macrophage leukemia RAW264.7 cell. These cell lines were obtained from the Cell Bank Department of the Pasteur Institute of Iran.

The SW480 line was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium, while the Hep2 line was cultured in Dulbecco's Modified Eagle Medium (DMEM)-F12. RAW264.7 cells were grown in DMEM. All media contained 10% fetal bovine serum (FBS) and 100 U/ml of penicillin-streptomycin, and the cells were incubated at 37 °C in a humidified environment with 5% CO2.

Cytotoxicity assays

The impact of nafcillin and diosmin on the viability of SW480, Hep2, and RAW264.7 cells was evaluated using the MTT assay. The cell lines were seeded in 96-well plates at a density of 1.0 × 10^4 cells per well and incubated for 24 h at 37 °C in a 5% CO2 atmosphere. Once a monolayer was established, the cells were washed three times with phosphate-buffered saline (PBS). Following this, 200 µL of nafcillin or diosmin at various concentrations was added to each well for an additional 24-hour incubation. After this period, 10 µL of MTT solution dissolved in PBS was introduced, and the cells were incubated for another 4 h. Subsequently, the media were removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well before measuring the absorbance at 570 nm using a microplate reader (Thermo Fisher Scientific, USA). All experiments were conducted in triplicate. Cell viability was calculated using the following formula[27]:

$$\text{Percentage viability} = (\text{OD value of treated cells} / \text{OD value of control cells})$$

Cell adhesion and invasion assays

Based on the MTT test results, concentrations of 256 µg/mL and 128 µg/mL of diosmin were selected for further experiments, as these levels did not exhibit cytotoxicity to the cell lines. Furthermore, although none of the assessed nafcillin concentrations showed cytotoxic effects on the cells, our previous study established the minimum inhibitory concentration (MIC) of this antibiotic against *S.* Typhimurium at 16 µg/mL[26]. Consequently, sub-MIC concentrations of 8 µg/mL and 4 µg/mL of nafcillin were chosen for additional investigation.

Adhesion and invasion assays were performed according to previously described methods, with slight modifications[28,29]. The SW480, Hep2, and RAW264.7 cell lines were cultured in 24-well plates at a density of 1 × 10^5 cells per well for 24 h. Following this initial incubation, the monolayers were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 100 and incubated for 1–4 h at 37 °C in a 5% CO_2 atmosphere. This was done for both untreated and treated groups, which included diosmin at concentrations

of 256 and 128 µg/mL, and nafcillin at concentrations of 4 and 8 µg/mL. After incubation, the medium was discarded, and the cells were washed twice with 1× PBS to eliminate non-adherent bacteria. The cell lines were then lysed using 1% Triton X-100, and the lysates were serially diluted and plated on LB agar for viable bacterial counts after overnight incubation.

For the invasion assay, SW480, Hep2, and RAW264.7 cell lines were also plated in 24-well plates at a density of 1 × 10^5 cells per well for 24 h. Following this initial incubation, the monolayers were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 100. In addition to the control group (cells infected with bacteria alone), diosmin at doses of 256 and 128 µg/mL and nafcillin at doses of 4 and 8 µg/mL were added to the treatment group. Infected cells from the untreated and treated groups were washed twice with PBS after 1 and 16 h of incubation. Gentamicin (100 µg/mL) was added to prevent the growth of extracellular bacteria, and the cells were incubated with 1% Triton X-100 for 1 h before lysis. The resulting suspensions were serially diluted and plated on LB agar plates for counting viable bacteria after overnight incubation.

Microscopy

Giemsa staining was performed to visualize the adherence of *S.* Typhimurium to SW480, Hep2, and RAW264.7 cells in both control and treated groups with some modifications[30]. The cell lines were seeded in 6-well plates with glass cover slips at a density of 1 × 10^5 cells per well and incubated for 24 h at 37 °C with 5% CO2. Then, they were infected with a 2 mL bacterial suspension (1 × 10^7) in cell culture medium, either alone or with 8 µg/mL nafcillin or 256 µg/mL diosmin along with 10% FBS for 3 h. After washing with PBS, the cells were fixed with 100% methanol for 20 min and stained with 10% Giemsa for 30 min, followed by three rinses with PBS. Adherence of *S.* Typhimurium to the cells was assessed using a light microscope (Olympus, Japan) at a magnification of 1000x.

Bacterial growth under stress conditions

The bacterial cultures were exposed to different stress conditions with minor modifications[20]. The *S.* Typhimurium strain was grown on LB agar and incubated for 24 h at 37 °C. A single colony from this culture was inoculated into 5 mL of LB broth, followed by another 24-hour incubation at the same temperature. Next, a second subculture was created by diluting this culture 100-fold in 50 mL of LB broth, which was then incubated at 37 °C while shaking at 200 rpm until an optical density of 0.4 at 600 nm (OD600) was reached. The bacterial cells were harvested using centrifugation at 4000 × g for 15 min. The cells were then re-suspended in pre-warmed LB broth as a control and in various conditions: LB broth adjusted to pH 3.0 (acid stress using hydrochloric acid, pre-chilled LB broth at 4 °C (cold stress), pre-warmed LB broth at 42 °C (heat stress), LB broth with 5 mM H_2O_2 (oxidative stress), and LB broth with 5% NaCl (osmotic stress). The bacteria were exposed to these stressors for 4 h, either in LB broth alone or supplemented with diosmin at concentrations of 256 and 128 µg/mL, and nafcillin at 4 and 8 µg/mL. Following the exposures, the cultures were washed twice with sterile normal saline (0.85%) before undergoing serial dilutions and plating on LB agar, with colony counts taken after a 24-hour incubation at 37 °C.

Quantitative real-time PCR (qRT-PCR)

The expression of genes related to Lon protease and type II toxin-antitoxin (TA) systems (*gnat/rhh*, *vapC/vapB*, *parB/parE*, *phd/doc*, *relE/relB*) in *S.* Typhimurium was examined using qRT-PCR under cell line experiments and different stress conditions. For the cell line studies, about 10^6 SW480, Hep2, and RAW264.7 cells were seeded in 6-well plates and incubated at 37 °C in a 5% CO2 for 24 h. These cells were then infected with 2 mL of a *S.* Typhimurium suspension (1 × 10^8 CFU/mL) in either cell culture medium or that supplemented with 8 µg/mL nafcillin or 256 µg/mL diosmin, plus 10% FBS for 3 h. RNA extraction was performed using a TRIzol-based method[31]. For stress conditions, total RNA from the bacteria was extracted via the TRIzol method after a 3-hour exposure to stressors during logarithmic-phase growth in LB broth alone or with nafcillin or diosmin.

RNA yield and purity were evaluated with a spectrophotometer at 260/280 nm and 260/230 nm (NanoDrop, Thermo Fisher Scientific, USA). DNase I treatment was done per the manufacturer's protocol (Thermo Fisher). RNA was reverse-transcribed using the AddScript cDNA synthesis kit (AddBio, South Korea) according to manufacturer instructions. Primer sequences used in the study are listed in Table 1.

qRT-PCR was conducted using a Rotor-Gene thermal cycler with the SYBR Green method (Ampliqon Co, Denmark). The total volume for reactions was 20 µL, containing 1 µL of cDNA, 10 µL of SYBR Green master mix, 7 µL of nuclease-free water, and 1 µL of each primer. The cycling protocol included an initial denaturation step at 95 °C for 12 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 25 s. The *invA* gene was used as a reference for normalizing gene expression levels, with relative fold changes calculated using the $2-\Delta\Delta Ct$ method[32].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was utilized, followed by Tukey's range test, with a significance level set at $p < 0.05$.

Results

Cytotoxicity of nafcillin and diosmin

The MTT cytotoxicity assay was conducted on SW480, Hep2, and RAW264.7 cell lines to evaluate the cytotoxic concentrations of nafcillin and diosmin. For all tested concentrations of nafcillin across the three cell lines, cell viability exceeded 70%, indicating non-cytotoxic effects according to international standard protocols[33]. In contrast, while most concentrations of diosmin were also non-cytotoxic, notable exceptions included 1024 µg/

Primer Name	Primer Sequence	Product size (bp)	References
gnat	F: TGTTATCTGCCGGAAAGCGT R: TGGTACTGCGCCAAATCCTT	119	50
rhh	F: AGCAAACCAACCTGACGGAT R: GCGCTCCGTCAGATATACCC	95	50
vapC	F: CGAAATCATTGCGGTTGGCA R: TCCTGTACTGCGGGTTGTTC	126	50
vapB	F: AACACCTGTCGGGCCTTATG R: ATGCGTTCAAATTCGCGGAG	96	50
parB	F: CGCTTCTGATTCACCACCCT R: TCGCAGGCATTCATCCGTTA	140	50
parE	F: ATGTCCTGGCTGAAATGGGG R: ATCAACGCCAGCTTCGCTAT	149	50
invA	F: AGCGTACTGGAAAGGGAAAG R: ATACCGCCAATAAAGTTCACAAAG	116	51
phd	F: TGGTAACAAACGTACCGCCT R: GGATTCGCAGCGAGTGAGAT	75	26
doc	F: GAAGTCCTGGAAAGTGCGGT R: GCTGATGATCACTGCGGACT	78	26
relE	F: GAACGGCTTGAAAACCCTCG R: CGCTGTACACCAGCCTGTAT	103	26
relB	F: TGGGAACTTTCAATGCCGGA R: TGAGCATAAAGTGCCGGAGG	94	26
lon protease	F: AATAACCTCAACCAGCGCGA R: TAGGTCAGGCAAACACGGAC	116	26
hilA	F: CGACAGAGCTGGACCACAAT R: CGTTTGTAAGGCCTCCTCCA	194	This study
ssrA	F: AACCGGAGGGATACGTCTGA R: ACCGCCCATCATTTTAGCCA	193	This study

Table 1. Primers used for qRT-PCR.

mL (64.74% viability) and 512 µg/mL (64.66% viability) for SW480 cells; 1024 µg/mL (65.31% viability) for Hep2 cells; and 1024 µg/mL (48.97% viability) for RAW264.7 cells (Fig. 1).

Impact of nafcillin and diosmin on *S.* Typhimurium adhesion and invasion

The effects of nafcillin and diosmin on the adhesion, invasion, and intracellular survival of *S.* Typhimurium in SW480, Hep2, and RAW264.7 cells were examined. Results indicated that none of the tested concentrations of either drug produced a statistically significant reduction in bacterial adhesion after 1 and 4 h of incubation in the respective cell lines (Fig. 2).

In terms of bacterial invasion, assessed via the gentamicin method, after 2 h of incubation with nafcillin and diosmin, there was no significant decrease in invasion observed in SW480 and Hep2 cells. However, in RAW264.7 cells treated with 4 µg/mL nafcillin, a significant reduction of 1.56 logarithms in the *S.* Typhimurium count was recorded ($p = 0.0016$).

After 16 h of incubation, there was no significant reduction in bacterial invasion for RAW264.7 cells. Nevertheless, in SW480 cells, treatment with 8 µg/mL nafcillin resulted in a notable 2.08 log reduction in bacterial count ($p = 0.0014$). Additionally, in Hep2 cells, a significant reduction in bacterial count was observed at 256 µg/mL diosmin, with a 1.25 log reduction ($p = 0.0075$). Further reductions were seen with nafcillin, including a 3.95 log reduction at 8 µg/mL ($p < 0.0001$) and a 2.95 log reduction at 4 µg/mL ($p < 0.0001$), (Fig. 3).

Giemsa staining

To assess the attachment of *S.* Typhimurium to the tested cells in the presence of 8 µg/mL nafcillin or 256 µg/mL diosmin, a comparison was made to the control group. Following three hours of bacterial infection, the cells were washed, fixed, and stained using Giemsa stain. Our observations indicated that the number of bacteria adhering to SW480, Hep2, and RAW264.7 cells in the treated group did not remarkably differ from that of the control group (Fig. 4).

Survival of *S.* Typhimurium under stress conditions

To investigate the impact of various stressors on the survival of *S.* Typhimurium in the presence of different concentrations of nafcillin and diosmin, colony count assays were conducted. The results indicated that treatment with diosmin and nafcillin had no significant effect on bacterial survival under cold stress. At 42 °C, the administration of 8 and 4 µg/mL nafcillin resulted in a 3.08 ($p = 0.0003$) and a 2.3 ($p = 0.0030$) log reduction in bacterial counts, respectively, when compared to the control group.

Under osmotic stress, a 2.74 ($p = 0.0002$) log reduction was observed in the presence of 8 µg/mL nafcillin compared to the control group. In conditions of acidic stress, a 2.88 ($p < 0.0001$) and a 1.7 ($p = 0.0047$) log reduction was noted for the 8 and 4 µg/mL nafcillin treatments, respectively, while a 2.36 ($p = 0.0004$) and a 1.4 ($p = 0.0167$) log reduction was observed for 256 and 128 µg/mL diosmin, respectively. Additionally, during oxidative stress, a 2.03 ($p = 0.0003$) and a 1.82 ($p = 0.0007$) log reduction was recorded with 8 and 4 µg/mL

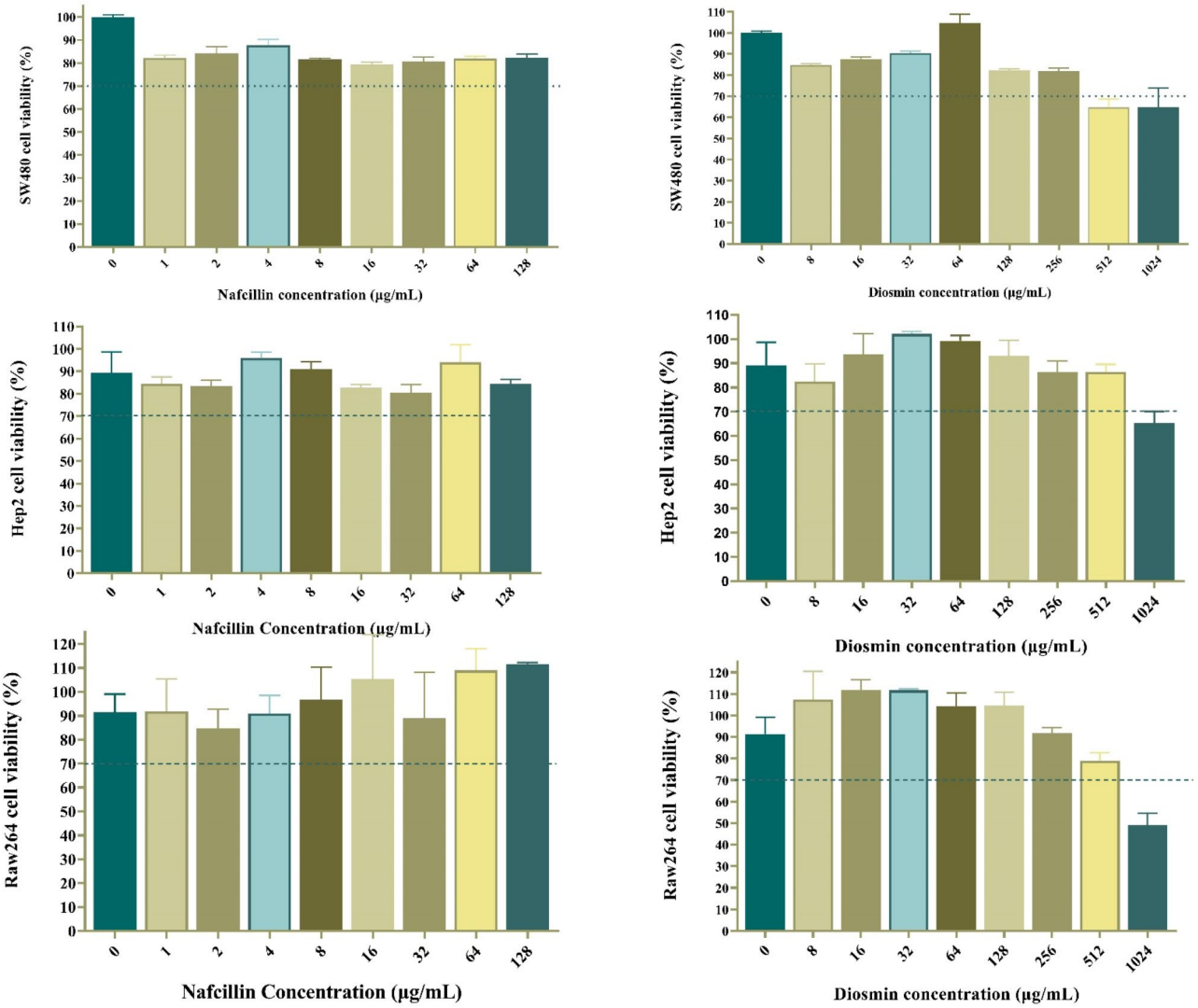

Fig. 1. Cytotoxicity of nafcillin and diosmin in SW480, Hep2 and RAW264.7 cell lines.

nafcillin, respectively, and a 0.96 ($p = 0.0488$) and a 1.38 ($p = 0.0052$) log reduction was noted with 256 and 128 µg/mL diosmin, respectively, in comparison to the control group (Fig. 5).

Quantitative real-time PCR analysis

This study aimed to evaluate the expression levels of the *lon protease*, type II toxin-antitoxin (TA) system, *ssrA*, and *hilA* in *S.* Typhimurium infecting SW480, Hep2, and RAW264.7 cell lines. Additionally, we investigated the expression of *lon protease* and the type II TA system under various stress conditions through real-time PCR.

Our results indicated that among the systems examined in *S.* Typhimurium infecting Hep2 cells, the *relE/relB* system exhibited the most significant increase in expression, with values of 21.4 and 43.4, followed by the *gnat/rhh* system at 5.8 and 6.63 ($p < 0.0001$). For *S.* Typhimurium infecting SW480 cells, the *relE/relB* system again showed the highest expression increase, recorded at 18.64 and 27.85, along with the *phd/doc* system at 9.49 and 19.49 ($p < 0.0001$). In *S.* Typhimurium infecting RAW264.7 cells, there was an elevation in expression for most systems analyzed, with the *relE/relB* system showing a peak increase of 30.46 and 41.43 ($p < 0.0001$). In groups treated with diosmin and nafcillin, a decrease in expression was noted across all studied genes compared to the untreated group; however, the presence of diosmin and nafcillin increased the expression of the *hilA* gene (Fig. 6).

Regarding gene expression under different stress conditions, we observed an increase in *lon protease* expression across all four stress types: heat, oxidative, osmotic, and acidic. Notably, the *gnat/rhh* genes experienced the highest increase in expression during the heat stress conditions, with increases of 3.21 and 8.1 ($p < 0.0001$). Under osmotic stress, most genes studied demonstrated elevated expression, with the *gnat/rhh* genes showing increases of 4.02 and 1.88 ($p < 0.0001$). In acidic stress, only the *parB/parE* system exhibited a significant increase, recorded at 2.18 and 4.14 ($p < 0.0001$). Conversely, during oxidative stress, none of the

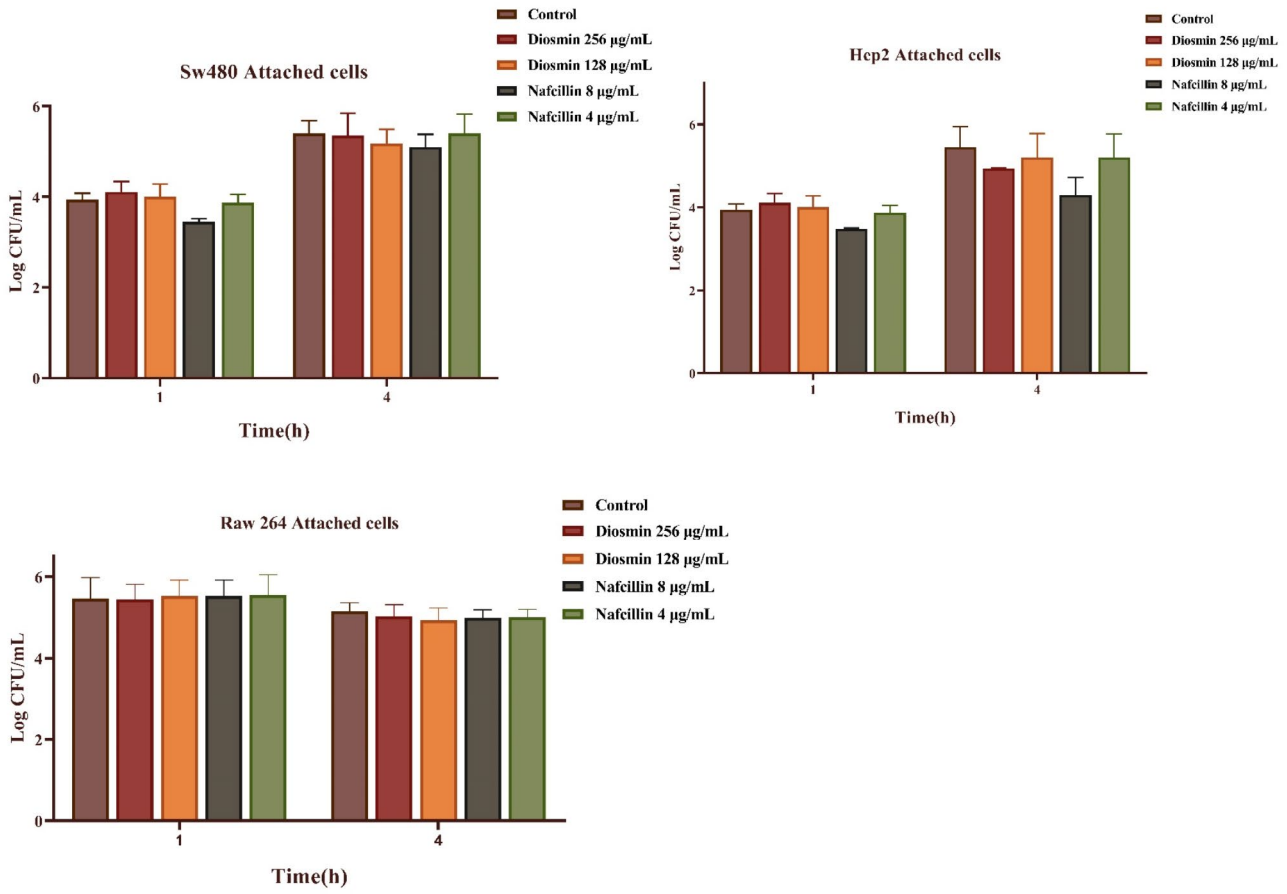

Fig. 2. Attachment of *S*. Typhimurium ATCC 14028 to SW480, Hep2 and RAW264.7 cell lines. Error bars indicate the standard deviation (SD) of three independent experiments.

systems analyzed displayed a considerable increase ($p < 0.0001$). Furthermore, treatment with diosmin and nafcillin resulted in reduced expression of the type II toxin-antitoxin systems (Fig. 7).

Discussion

S. Typhimurium is a foodborne pathogen responsible for diseases in humans and animals. Its ability to attach to and invade host cells while responding to various environmental stresses is crucial for its virulence[34]. The ability of *S*. Typhimurium to endure within macrophages and other host cells, as well as in various stressful conditions, is closely associated with its pathogenicity[15,35]. This bacterium adapts to challenging environments while maintaining its ability to cause disease[36]. When *S*. Typhimurium successfully evades the host's immune response and persists within cells, it can result in prolonged infections, heightened disease severity, and complications like systemic infections[37]. By reducing its intracellular survival, we may mitigate these adverse outcomes, leading to improved infection management[37]. Furthermore, intracellular bacteria often evade the effects of antibiotics because their location within host cells limits antibiotic penetration. By targeting the mechanisms that enable *S*. Typhimurium to thrive inside cells, we could enhance the efficacy of existing antibiotics or develop new therapeutic approaches[38]. Among the various molecular mechanisms that regulate cell invasion and responses to stress, the Lon protease and type II antitoxin (TA) systems have been implicated in bacterial survival under these conditions, according to the literature[39]. This study investigates the role of nafcillin and diosmin as previously identified as inhibitors of Lon protease[26] in *S*. Typhimurium intracellular survival, and resilience against various stresses.

Adhesion and invasion assays were conducted using SW480, Hep2, and Raw 264.7 cells. The adhesion of *S*. Typhimurium to these cells remained relatively unchanged at different concentrations of nafcillin and diosmin during the study period. However, nafcillin and diosmin, as inhibitors of Lon protease, effectively reduced the survival of *S*. Typhimurium in Hep2 cells after 16 h. In the case of SW480 cells, an 8 µg/ml concentration of nafcillin significantly reduced the survival of *S*. Typhimurium after 16 h, while a decrease in survival was also observed with 4 µg/ml nafcillin and 256 µg/ml diosmin; however, these reductions were not statistically significant. In the macrophage cell line Raw 264.7, bacterial invasion was also reduced in the presence of nafcillin and diosmin during the initial two hours. Notably, 8 µg/ml nafcillin significantly decreased *S*. Typhimurium survival during this period, with a subsequent decrease in relative survival observed at 16 h, although this reduction was not statistically significant. Kirthika et al.[20] previously investigated the adhesion and invasion

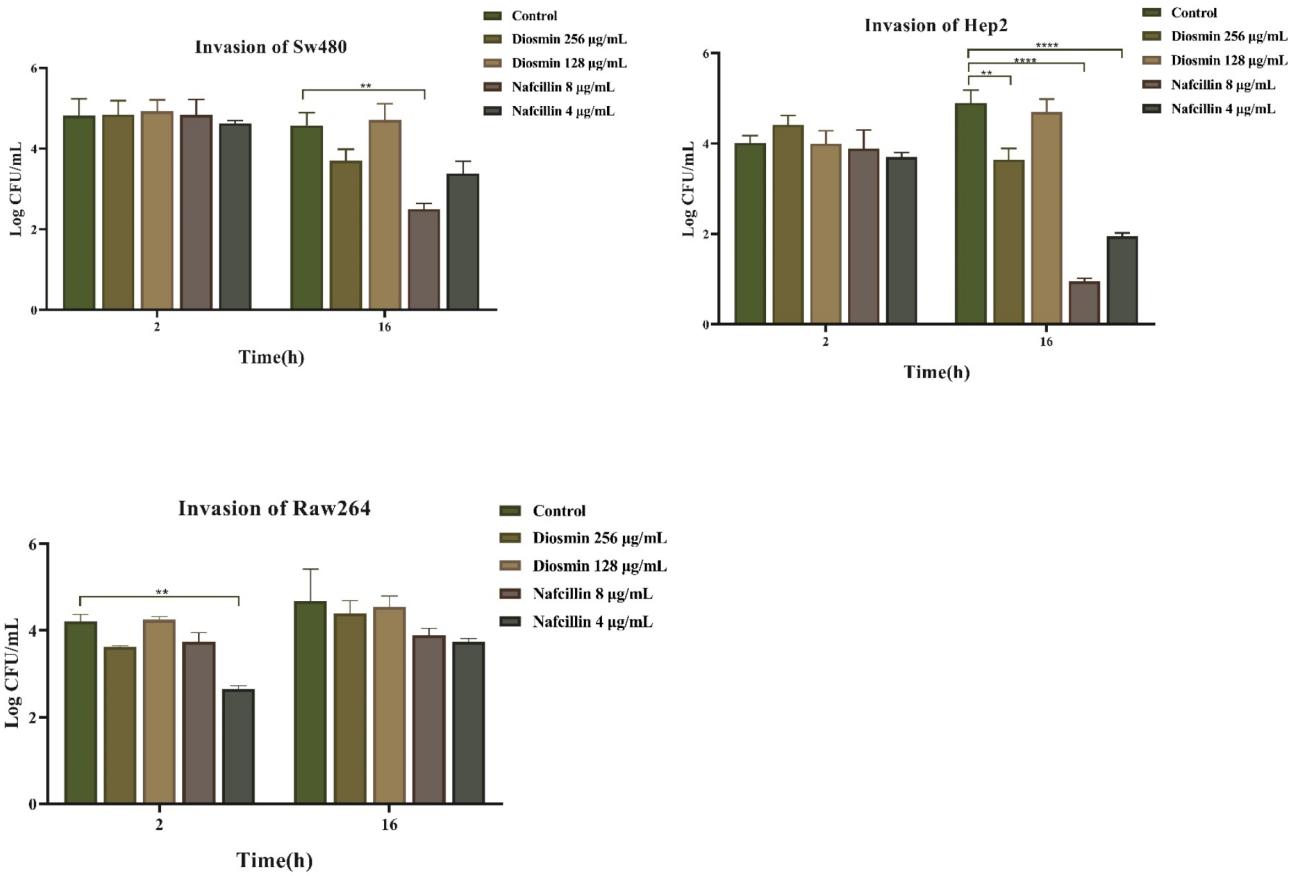

Fig. 3. Invasion of *S*. typhimurium ATCC 14028 to SW480, Hep2 and RAW264.7 cell lines. Error bars indicate the standard deviation (SD) of three independent experiments.

of *S*. Typhimurium strains with the Lon gene deleted in HeLa, HepG2, and Caco-2 cells, comparing them to wild-type strains. Their findings indicate that the deletion of the *lon* gene significantly enhanced both adhesion and invasion into host cells. However, the intracellular survival of the Lon mutant strains was notably impaired compared to that of the wild-type strains. In our study, we observed reduced intracellular survival in epithelial cells at 16 h, while in macrophages, survival was diminished during the first two hours. These findings suggest that the effects of Lon protease may vary depending on the cell type.

The expression of type II toxin-antitoxin (TA) systems, the *hilA* gene as an SPI-1 activating factor[40], and *ssrA* as one of the SPI-2 activating factors in *S*. Typhimurium[41] infecting different cell lines was investigated. Although the expression levels of type II TA system genes varied across different cell lines—likely due to the distinct cellular environments affecting gene activity—the *relEB* system exhibited a significant increase in bacteria isolated from all three cell types. This finding underscores the importance of the *relEB* system in the intracellular adaptation of *S*. Typhimurium.

Upon addition of nafcillin and diosmin to the cell cultures, expression levels of these TA systems decreased, indicating that these compounds inhibit Lon protease activity, thereby affecting TA system function. Interestingly, while the presence of nafcillin and diosmin did not significantly impact the expression of *ssrA*, an increase in *hilA* gene expression was observed under these conditions. Kirthika et al.[20] also reported that deletion of Lon protease resulted in a substantial increase in the expression of candidate SPI-1 genes such as *invF* and *hilC*, while candidate SPI-2 genes like *sifA* and *sseJ* showed no such increase. This suggests a specific relationship between Lon protease and SPI-1 rather than SPI-2. Other studies have implicated type II toxin-antitoxin systems in bacterial intracellular survival. For instance, Paul et al. found that, while adhesion rates were not significantly different between wild-type (WT) and isogenic mutants, invasion rates were impaired in both Δhha and ΔtomB mutants[42]. Tiwari et al. examined the growth of wild-type and mazF-mutated strains of the type II toxin system in THP-1 macrophages, revealing that the survival of the mazF mutant strain was fourfold impaired compared to the wild-type strain at day 6 post-infection[43].

We subjected *S*. Typhimurium to various stress conditions, including temperature, osmotic, oxidative, and acidic stress, in the presence of nafcillin and diosmin as Lon protease inhibitors, and assessed the expression of *lon protease* genes and type II TA systems. Our results indicated that bacterial survival was significantly reduced under oxidative and acidic stress when nafcillin and diosmin were present. In contrast, only a significant reduction in survival was observed under heat and osmotic stress at an 8 µg/ml concentration of nafcillin, likely attributable to its antibiotic properties in addition to its role as a Lon protease inhibitor.

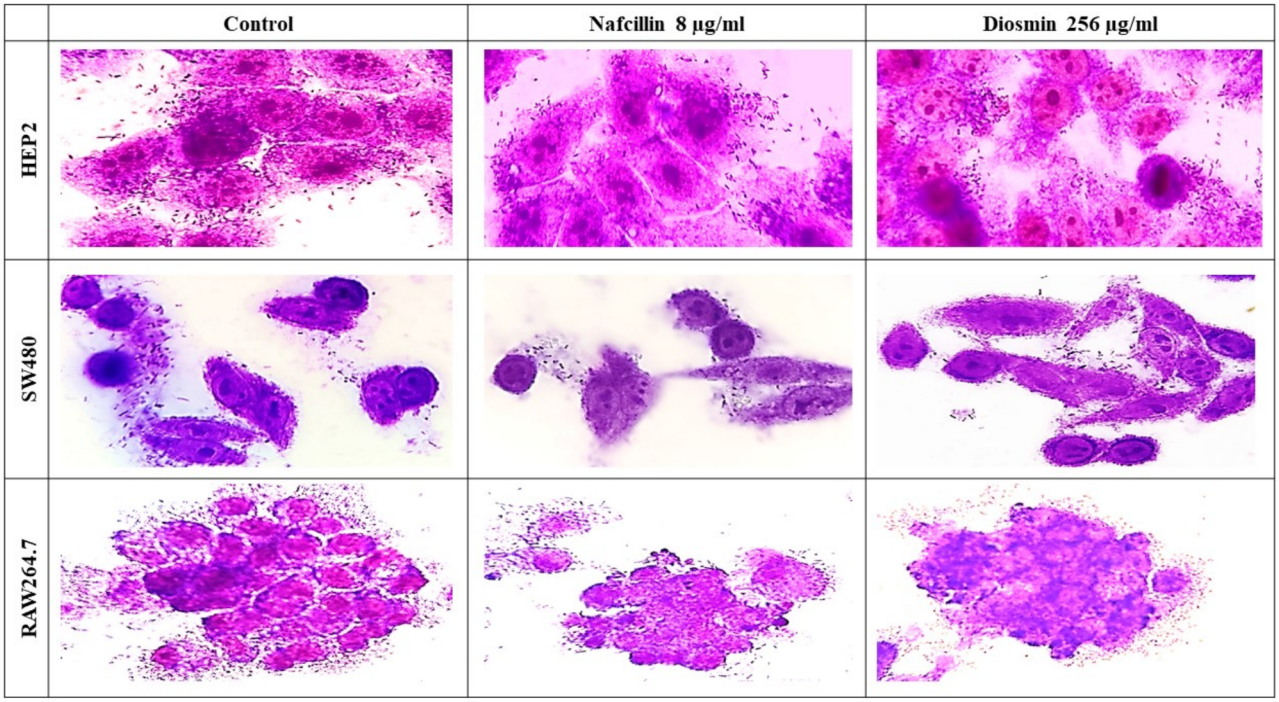

Fig. 4. Giemsa staining of *S.* typhimurium ATCC 14028 to SW480, Hep2 and RAW264.7 cell lines under light microscope at 1000x magnification.

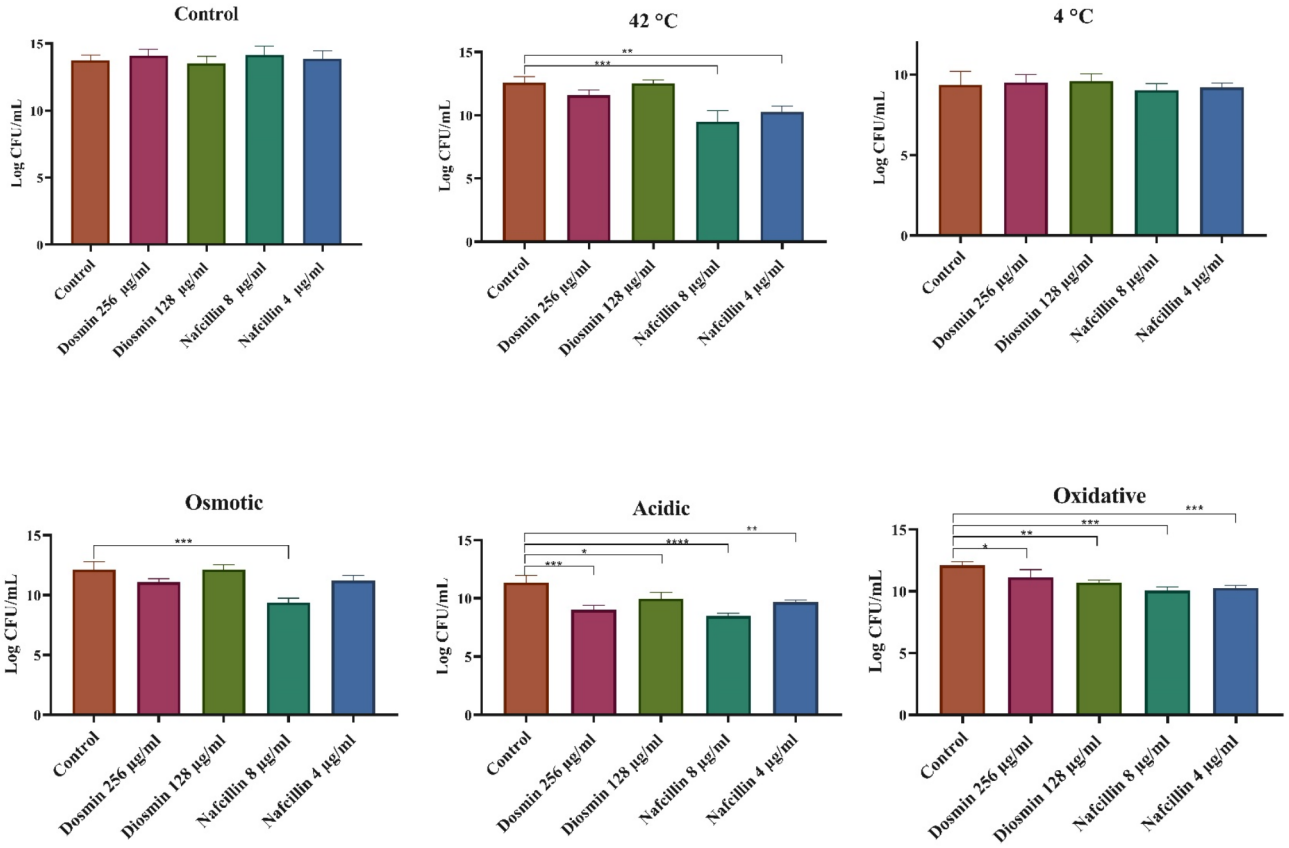

Fig. 5. Effect of diosmin and nafcillin on the survival of *S.* typhimurium ATCC 14028 in different stress condition. Error bars indicate the standard deviation (SD) of three independent experiments.

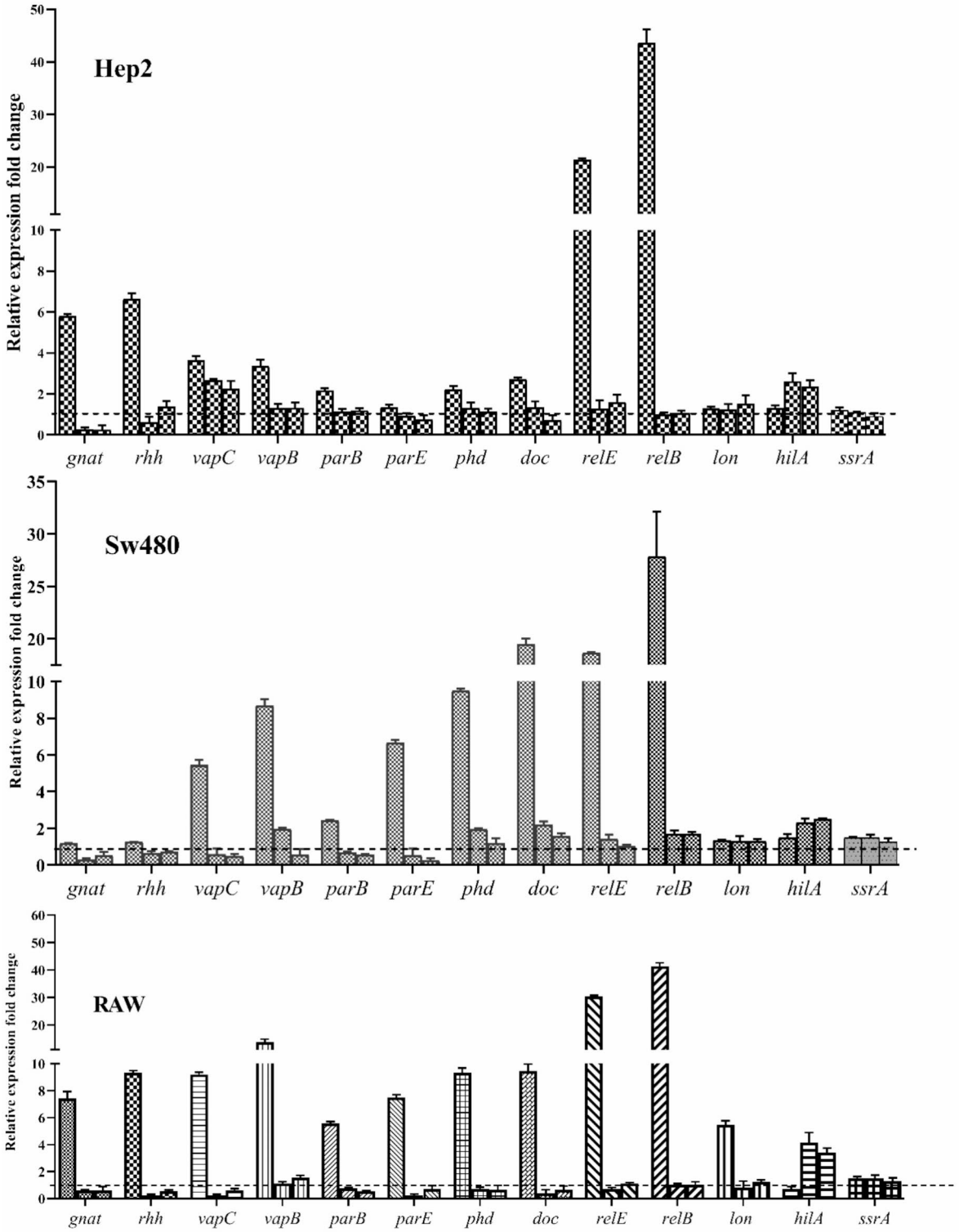

Fig. 6. Analysis of the relative expression levels of Type II TA System genes in *S*. Typhimurium ATCC 14028 infecting SW480, Hep2, and RAW264.7 cell lines. For each group of genes, three bars are presented: the first bar indicates gene expression levels in infecting cell, the second represents expression in infecting cell exposed to nafcillin (8 μg/mL), and the third shows expression in infecting cell treated with Diosmin (256 μg /mL). Relative expression normalization was conducted using the reference gene *invA* and error bars reflect the standard deviations derived from three biological replicates ($P < 0.0001$, determined by one-way ANOVA).

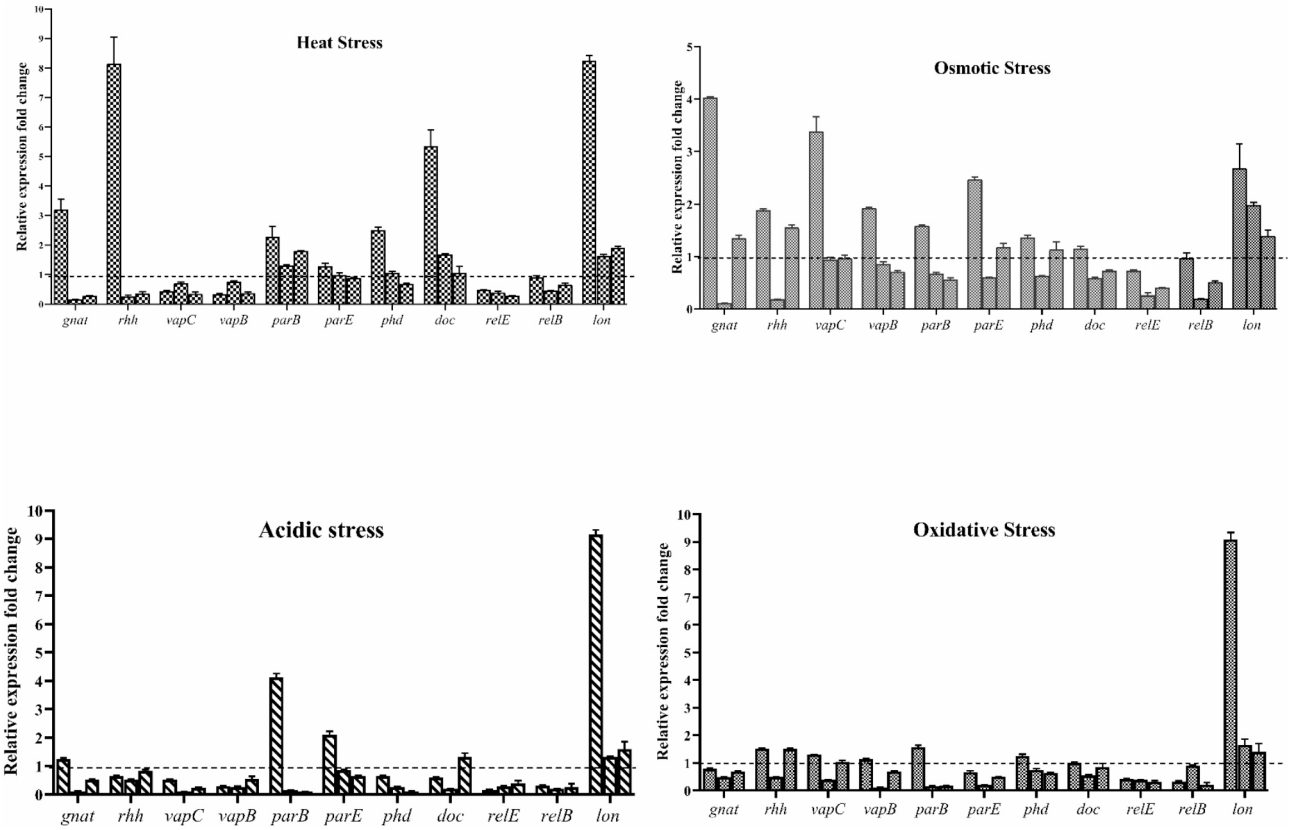

Fig. 7. Analysis of the relative expression levels of Type II TA System genes in *S.* Typhimurium ATCC 14028 in different stress conditions. For each group of genes, three bars are presented: the first bar indicates gene expression levels in stress condition, the second represents expression in stress condition exposed to Nafcillin (8 μg/mL), and the third shows expression in stress condition treated with Diosmin (256 μg /mL). Relative expression normalization was conducted using the reference gene *invA* and error bars reflect the standard deviations derived from three biological replicates ($P < 0.0001$, determined by one-way ANOVA).

Regarding gene expression profiles, *lon* protease gene expression was significantly elevated across all stress conditions examined. According to the literature, Lon protease is essential for regulating oxidative and acid stress in *S.* Typhimurium[20]. Additionally, the deletion of this protease in *Actinobacillus pleuropneumoniae* leads to inhibited growth when exposed to thermal, osmotic, and oxidative stresses[44]. Similarly, the deletion of Lon protease in *Dickeya solani* results in increased sensitivity to specific stress conditions, particularly osmotic and high-temperature stresses[45].

However, the role of toxin-antitoxin (TA) systems in response to different stresses appears to vary across studies. For instance, a study of six type II TA systems in *Klebsiella pneumoniae* found no significant changes in their expression levels following exposure to oxidative stress, indicating that these TA systems may not be involved in this particular response[46]. In contrast, it has been clearly demonstrated that the two type II TA systems, yefM-yoeB and relBE, play a role in the oxidative stress response in *Streptococcus pneumoniae*[47]. Deletion of either or both TA systems significantly reduced the survival of *S. pneumoniae* after exposure to hydrogen peroxide (H_2O_2)[47]. Furthermore, the ParDE TA system has been shown to enhance cell survival in *Escherichia coli* at 42 °C[48]. The expression of relE/RHH-like TA systems, fic/phd, and brnTA was increased following exposure of *Brucella* species to acidic conditions (pH 5.5), suggesting a potential role for these TA systems under acidic stress[49]. In our study, no significant increase in expression was observed for any of the systems examined under oxidative stress. Conversely, most systems exhibited increased expression under osmotic stress. Overall, the expression levels of type II TA systems varied depending on the specific type of stress, suggesting that each system may respond differently to distinct stress conditions.

Conclusion

The results presented in this study demonstrate that Lon protease is crucial for the survival of *S.* Typhimurium within various host cells and in response to oxidative, acidic, osmotic, and thermal stresses. Our findings also highlight the effectiveness of nafcillin and diosmin as Lon protease inhibitors in reducing intracellular survival and viability, particularly under acidic and oxidative stress conditions. This suggests their potential utility in mitigating the pathogenesis and systemic infection associated with *S.* Typhimurium.

Data availability

The datasets were analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions

N.N. contributed to the conception, methodology, design, and writing—original draft. N.N. and N.B. and A.K. performed data collection and analysis. F.M.J. and S.R. and S.G. were involved interpretation of the data, review & editing of the study. F.M.J. and S.R. contributed to study supervision. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

This study was approved by the Ethics Committee of Iran University of Medical Sciences by Ethics No. (IR.IUMS.FMD.REC.1402.262).

Additional information

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