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Sulforaphane Is Not Only a Food Supplement: It Diminishes the Intracellular Survival and Colonization of *Salmonella enterica*

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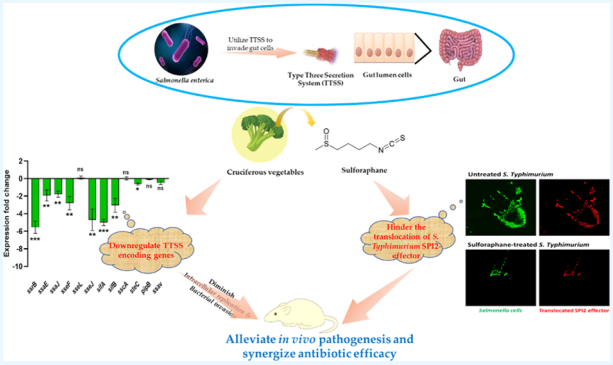
ABSTRACT: Sulforaphane is a main bioactive component in several edible cruciferous vegetables. It acquires several benefits to health in addition to its considered antibacterial and antivirulence activities. Herein, we aimed at evaluating the antivirulence activity of sulforaphane against the worldwide clinically important enteric pathogen *Salmonella enterica* serovar Typhimurium. The influence of sulforaphane on bacterial adhesion, invasion, biofilm formation, and intracellular replication was assayed. Additionally, the effect of sulforaphane on the type III secretion system (TTSS) in *S. enterica* was quantified. The outcome of the combination with different antibiotics was assessed, and an in vivo protection assay was conducted to assess the influence on *S. enterica* pathogenesis. The results showed the significant antibiofilm activity of sulforaphane at subinhibitory effect in addition to its significant reduction in bacterial invasion and intracellular replication inside the host cells. The in vivo findings emphasized the decreased capacity of *S. enterica* to induce pathogenesis in the presence of sulforaphane. Our finding attributed these antivirulence activities to the interference of sulforaphane with TTSS-type II and the downregulation of its encoding genes. In a nutshell, the edible cruciferous vegetable bioactive sulforaphane is a safe adjunct therapy that can be administrated alongside traditional antibiotics for treating clinically significant enteric pathogens as *S. enterica*.



1. INTRODUCTION

Sulforaphane, a naturally occurring isothiocyanate found in cruciferous vegetables such as cabbage, broccoli, and kale, has garnered interest for its potential health benefits.[1] These benefits include antioxidant and anti-inflammatory properties and the ability to stimulate detoxification enzymes.[2,3] Additionally, sulforaphane has demonstrated noteworthy antibacterial effects against Gram-negative bacteria such as *Pseudomonas aeruginosa*,[4] *Haemophilus influenzae*,[5] and enteric pathogens such as *Escherichia coli*,[6] *Shigella sonnei*,[5] and *Helicobacter pylori*,[7−9] as well as Gram-positive bacteria, including *Streptococcus pyogenes* and *Staphylococcus aureus*.[10,11] Owing to the aforementioned health benefits of sulforaphane and its safety at higher concentrations, it is used as a food supplement.[1] Interestingly, L-sulforaphane has garnered significant attention, as several studies, reviewed by Mazarakis et al.,[12] have focused exclusively on the clinical evidence of the L-form activity. That inspired us to investigate the antibacterial activity of L-sulforaphane against one of the most clinically significant gut pathogens, *Salmonella enterica*.

S. enterica, belonging to the *Enterobacteriaceae* family, is a nonlactose fermenting, facultatively anaerobic, peritrichous motile, intracellular Gram-negative bacterium.[13,14] Infections caused by *S. enterica* can range from localized gastroenteritis to severe systemic illness such as typhoid fever.[14,15] Importantly, and due to easy transmission via contaminated food and water, *S. enterica* continues to pose substantial challenges to public health owing to its ability to cause widespread and severe epidemics.[16] For instance, the observed increased incidence of salmonellosis in Ha'il, Saudi Arabia, where the cases were increasingly recorded in the time period 2013−2017 due to the contamination and improper preservation of food materials.[17] *Salmonella* employs complex type III secretion systems (TTSS) to translocate effector proteins into the cytoplasm of the host cells.[18,19] Two separate TTSSs encoded in Salmonella pathogenicity islands, namely, SPI1 and SPI2 are present.[20,21] In the early stages of the *Salmonella* invasion, SPI1-TTSS translocates effector proteins into the cytoplasm of the host cell to remodel the G-proteins easing the bacterial invasion.[18,22]

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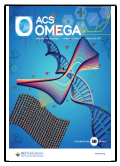

After the internalization of the *Salmonella* cells, the immune cells are contained in a specialized phagosome known as Salmonella-containing vacuoles (SCV). In SCV, immune cells produce diverse oxidizing agents and enzymes to eradicate the bacterial cells.[18,23] However, SPI2-TTSS translocates a wide array of effectors that guarantee the survival of *Salmonella* and secure the delivery of nutrition.[24] Importantly, TTSS interplays with other systems to control the bacterial adhesion, invasion, and establishment of serious infections in the host tissues.[18,21,25]

The number of new antibiotics developed and approved has declined steadily over the past few decades, leaving fewer options for combating resistant bacteria.[26−29] Consequently, innovative solutions and alternative therapies are urgently needed to address this critical gap.[28,30−33] The use of natural products to discover new antibacterials is a promising avenue for addressing antibiotic resistance.[32−35] These compounds offer diverse and novel mechanisms of action that could lead to the development of effective new treatments.[27,36,37] Interestingly, the antivirulence activity of sulforaphane at subinhibitory concentration against *P. aeruginosa* was approved. The antivirulence activity of sulforaphane was owed to its ability to antagonize the bacterial quorum sensing (QS) systems that control the expression of diverse virulence factors in *P. aeruginosa*.[4] Furthermore, sulforaphane inactivated the *H. pylori* urease that resulted in diminishing the pathogenesis.[8] In addition to the previously suggested mechanism of the sulforaphane' s antivirulence activity, it inhibited the intracellular survival of *H. pylori*[7] and *S. aureus*.[11] These data encourage us to further explore the antivirulence properties of sulforaphane against another clinically important pathogen *S. enterica* serovar Typhimurium (*S. Typhimurium*), evaluating its effect on TTSS and its influence on the *Salmonella* pathogenesis.

2. MATERIALS AND METHODS

2.1. Bacteria, Media, and Chemicals. L-Sulforaphane was obtained from Cayman Chemical (Michigan). The antibacterial experiments were performed against *S. enterica* serovar Typhimurium NCTC 12023. The used microbiological media were obtained from Oxoid Ltd. (United Kingdom). Dimethyl sulfoxide (DMSO) was used to dissolve sulforaphane at a concentration of 0.5% v/v (2.8 mM). All other chemicals and solvents are of pharmaceutical grade.

2.2. Determination of Minimum Inhibitory Concentration (MIC). MIC of sulforaphane against *S. typhimurium* was determined using the broth microdilution method according to the guidelines of the Clinical Laboratory and Standards Institute (CLSI, 2015) as previously detailed.[35]

2.3. Influence of Subinhibitory Concentrations on Bacterial Growth. To eliminate any influence of sulforaphane on *S. typhimurium* growth, viable bacterial counts were performed on *S. typhimurium* cultured in Luria−Bertani (LB) broth supplied or not with sulforaphane at sub-MIC.[38]

2.4. Adhesion and Biofilm Assay. Fresh cultures of *S. typhimurium* were grown in Tryptic Soy Broth (TSB) supplied or not with sulforaphane at sub-MIC. Bacterial suspensions, at a cell density of 2×10^6 CFU/mL, were then transferred to polystyrene microtiter plates and exposed to 0.001 μM *N*-hexanoyl-DL-homoserine lactone at 37 °C for either 2 or 24 h to assess bacterial adhesion or biofilm formation, respectively.[20,39] Following the incubation periods, nonadherent bacterial cells were rinsed out, and the remaining stained cells were fixed with ethanol 95% at 60 °C for 25 min and then stained with 0.1% crystal violet for 15 min. Excess dye was removed, and the crystal violet was extracted with methanol before the optical density was measured at 590 nm.[31,40]

2.5. Internalization in the Host Cells. The invasion and intracellular replication assays were conducted to assess the ability of *S. typhimurium* to invade host cells and replicate within them, respectively.[20,22,41] For the invasion assay, host cells (HeLa cells) were seeded in 24 well plates at a density of 5×10^5 cells/well and infected with *S. typhimurium* treated or not treated with sulforaphane at a sub-MIC at a multiplicity of infection (MOI) of 1. After a specified incubation period (30 min), noninternalized bacteria were washed out, and the remaining nonadhered extracellular *Salmonella* were killed with gentamicin (100 μg/mL) for 1 h. The invaded bacteria were then quantified by lysing host cells with Triton X-100 (0.1%) for 20 min at 20 °C, and the cell lysates were serially diluted and counted.

In the intracellular replication assay, infected host cells (raw macrophages (RAW264.7)) were incubated for specific durations to allow bacterial replication. At predetermined time points (2 and 16 h postinfection), the macrophages were lysed with 0.1% Triton X-100, and the intracellular bacteria were quantified by plating lysates on appropriate agar plates. The ratio of intracellular bacteria at later time points compared with the initial inoculum was calculated to determine the fold increase in intracellular replication.

2.6. Assay of the SPI2 Effector Translocation. The translocation of the SPI2 effector SseJ was used to assess the effect of sulforaphane at sub-MIC on the effectiveness of SPI2-T3SS in *S. typhimurium* survival and induction of infection in host cells. The plasmid pWsk29 P_{sseJ}*sseJ*::hSurvivin that encodes the hemagglutinin (HA)-tagged SPI2 effector protein SseJ was constructed and transformed to *S. typhimurium* as detailed.[20,22] The obtained *S. typhimurium* carrying the plasmid was cultivated with or without sulforaphane at subinhibitory concentration. These bacterial suspensions were then used to infect macrophages or HeLa cells in the presence of 0.001 μM *N*-hexanoyl-DL-homoserine lactone at an MOI of 100.[20,22,41] The infected cells were immune-stained to assess the translocated effector proteins after 16 h, using antibodies against Salmonella LPS (rabbit anti-Salmonella O1,4,5, Difco, BD) and the HA epitope tag (Roche, Basel, Switzerland). Secondary antibodies were used as follows: antirabbit antibodies tagged with GFP (green fluorescent protein) to stain *Salmonella* cells (Abcam), Cyanine5 (Cy5) dye to stain the translocated SseJ::HA (Invitrogen, MA), and diamidino-2-phenylindole (DAPI) dye as a counter stain for macrophages (Thermo Fisher Scientific).[19,20,22] A Leica laser scanning confocal microscope was used to capture the images of infected cells and translocated SseJ::HA. The fluorescence intensities of SseJ::HA were quantified using the J-image program.

2.7. Expression of the TTSS-SPI2 Genes. SPI2-inducing minimal medium (PCN-P, pH 5.8) was used to induce the SPI2 effectors' expression in the presence of sulforaphane at sub-MIC.[24] RNA was isolated from the bacterial cultures using RNAeasy Mini Kit (Qiagen, Germany), kept and stored at −80 °C.[42,43] cDNA was acquired through the utilization of the high-capacity cDNA reverse transcriptase kit (Applied Biosystem), and subsequently, it was amplified using the Syber Green I PCR Master Kit (Fermentas) according to the provided protocol.[42,44] Relative expression of tested genes was

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calculated using the $2^{-\Delta\Delta CT}$ method and normalized to housekeeping gene *gyrB*, the used primers were previously listed.[41]

2.8. Combination with Antibiotics. The outcome of sulforaphane at sub-MIC in combination with antibiotics, at their MICs, was assessed using the checkerboard method.[38,45] The antibiotics chosen for evaluation represent different classes and include chloramphenicol, cefotaxime, ciprofloxacin, gentamicin, amoxycillin-clavulanic acid, and tetracycline. The effectiveness of the combinations was evaluated using the fractional inhibitory concentration (FIC) index, determined by dividing MIC of the antibiotic in combination with MIC of the antibiotic solely. Optical densities were recorded at 600 nm following a 24 h incubation. FIC index values falling between 0.5 and 4 signified an indifferent effect; those exceeding 4 indicated antagonism, while values below 0.5 suggested synergy.

2.9. Protection of Mice. To investigate the in vivo suppressive effect of sulforaphane at sub-MIC on *S. typhimurium* pathogenesis, albino mice (3 weeks old) received intraperitoneal injections (I.P) of bacteria that had been treated with sulforaphane at sub-MIC, dissolved in DMSO (0.5% v/v).[36,46] The experiment included four groups with 5 mice in each. Group I received *S. typhimurium* (1×10^6 CFU/mL) treated with sulforaphane at sub-MIC. Group II served as positive control and was injected with *S. typhimurium*. Groups III and IV were left uninfected or injected with DMSO (0.5%, v/v) and served as negative controls. Following 1 week of monitoring, the mice were euthanized, and their liver and kidney tissues were dissected and homogenized. Bacterial loads were precisely determined through serial dilution in PBS and plating onto Salmonella Shigella agar (SSA) plates. The bacterial loads within the organs were evaluated in terms of the number of colony-forming units (CFU)/ g tissue.

2.10. Statistical Evaluations. The reported data represent mean values ± standard deviation (SD) in comparison to untreated controls for all assays, except for the evaluation of the effect of sulforaphane on the downregulation of involved genes, where mean values ± standard error (SE) were calculated. Unless specified otherwise, significance was assessed using the student's *t* test ($p < 0.05$).

3. RESULTS

3.1. Effect of Sulforaphane at Sub-MIC on the Bacterial Growth. Sulforaphane prevented the *S. typhimurium* growth at 8 μg/mL. Accordingly, to investigate the antivirulence activity of sulforaphane while excluding its effect on bacterial growth, all of the next experiments were conducted using sulforaphane at sub-MIC (1/4 MIC = 2 μg/mL). Additionally, viable counts of *S. typhimurium* cultured in the media provided with sulforaphane at sub-MIC (2 μg/mL) were conducted and compared with those of untreated control bacteria (Figure 1). A one-way ANOVA test was used to calculate the statistical significance.

3.2. Antiadhesive and Antibiofilm Activities of Sulforaphane at Sub-MIC. To evaluate the efficacy of sulforaphane at a sub-MIC level (2 μg/mL) in disrupting *S. typhimurium* adhesion and biofilm formation, spectrophotometric measurement of crystal violet intensity on adhered cells was conducted after 1 and 24 h (Figure 2). Sulforaphane at sub-MIC levels significantly attenuated bacterial adhesion and biofilm formation. Specifically, it decreased bacterial adhesion

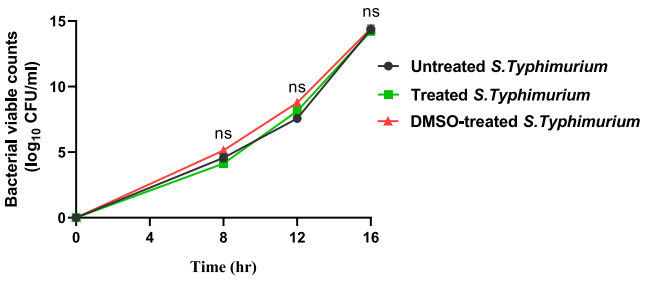

Figure 1. Impact of sulforaphane at sub-MIC on bacterial growth. The viable counts of *S. typhimurium* cultured in media supplied or not with sulforaphane were performed. There were no significant differences between cultures treated with or not with sulforaphane. ns: nonsignificant, $p > 0.05$.

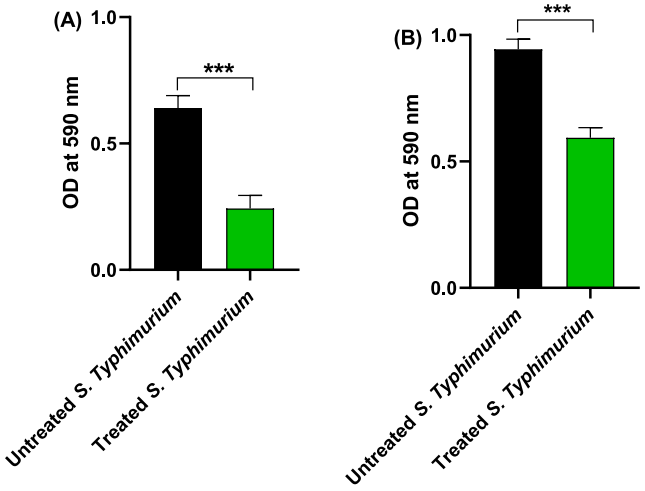

Figure 2. Sulforaphane at sub-MIC significantly lessened the (A) adhesion and (B) biofilm formation of *S. typhimurium* to abiotic surfaces. ***$p < 0.001$.

by 62% and biofilm formation by 37% at subinhibitory concentrations.

3.3. Effect of Sulforaphane at Sub-MIC on the *Salmonella* Internalization. *Salmonella* utilizes TTSS for initiating infection and survival intracellularly within the phagosomes of host immune cells. To investigate the potential of sulforaphane to disrupt *S. typhimurium* invasion and intracellular replication, viable bacteria were quantified in HeLa cells and macrophages, respectively, in the presence and absence of sulforaphane at sub-MIC levels (2 μg/mL) (Figure 3). The bacterial counts of invading bacterial cells in HeLa cells and the intracellularly replicated bacteria were significantly reduced in the presence of sulforaphane at sub-MIC. Sulforaphane at sub-MIC reduced the bacterial invasion by 46% and intracellular replication by 55%.

3.4. Interference of Sulforaphane at Sub-MIC with TTSS-SPI2. Bearing in mind that TTSS plays a crucial role in the survival of *S. typhimurium* in host cells, the functionality of TTSS in the presence of sulforaphane at sub-MIC (2 μg/mL) was assessed by quantifying the translocation of the SPI2 effectors into the cytoplasm of the host cells. The intensity of the red fluorescence resulting from cy5-stained translocated SPI2 effector (SseJ::HA) was quantified using HeLa cells and macrophages (Figure 4).

3.5. Downregulation Effect of Sulforaphane on the TTSS-SPI2 Genes at Sub-MIC. Sulforaphane at sub-MIC

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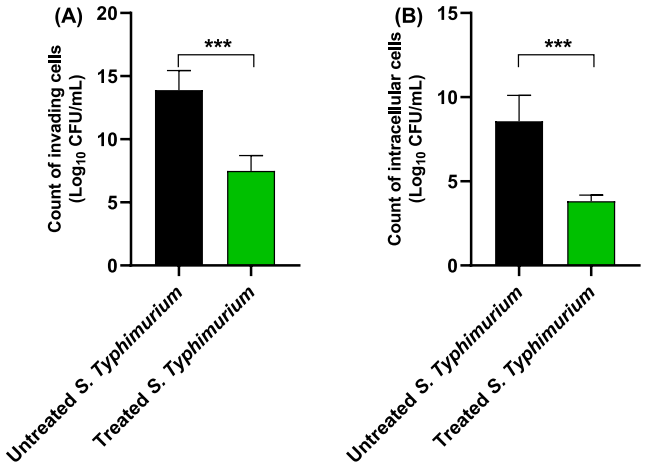

Figure 3. Sulforaphane at sub-MIC significantly diminished the (A) invasion and (B) intracellular replication of *S. typhimurium* into HeLa and raw macrophages, respectively. ***$p < 0.001$.

significantly downregulated the expression of several SPI2 effectors, and more downregulation was observed in genes *ssrB*, *sseJ*, *sifA*, and *sifB* (3−5-fold). While the downregulation effect was 2-fold in genes *ssaE*, *ssaJ*, and *sseF*, the expression of *steC* and *ssaV* genes did not exceed one-fold. On the other hand, sulforaphane has no influence on the expression of *sseL*, *sscA*, and *pipB* genes (Figure 5).

3.6. Outcome of the Sulforaphane Combination with Antibiotics. The MIC values for combinations of sulforaphane at subminimal inhibitory concentration (2 μg/mL) with the antibiotics under examination (ciprofloxacin, streptomycin, ceftazidime, amoxycillin/clavulanic acid, and chloramphenicol) were determined, and the FIC index was computed to assess the effects of these combinations (Table 1). As depicted in Table 1, sulforaphane reduced MICs of the antibiotics tested, indicating synergistic effects. The calculated FIC values (in all combinations) were consistently less than 0.5, clearly indicating the synergistic effect of combining sulforaphane with other antibiotics.

MICs of antibiotics such as ciprofloxacin, streptomycin, ceftazidime, amoxycillin/clavulanic acid, and chloramphenicol was detected against *S. typhimurium*. MIC of combinations of sulforaphane at subminimal inhibitory concentration (2 μg/mL) with the antibiotics was detected. The FIC index was calculated to evaluate the outcome of these combinations. Sulforaphane lessened MICs of the tested antibiotics showing synergistic outcomes.

3.7. Protection of Sulforaphane to the Mice against *Salmonella* Infection. Sulforaphane at sub-MIC conferred 50% protection to the mice infected with *S. typhimurium*. There were four deaths observed among the positive control group injected with *S. typhimurium*, while this number was reduced to two in the group injected with *S. typhimurium* pretreated with sulforaphane. The survival curve (Figure 6A) clearly revealed a significant reduction in the *S. typhimurium* pathogenesis and its ability to induce infection in the presence of sulforaphane ($p = 0.0114$) (Figure 6A). Of note, no deaths were observed in the negative control groups. Most importantly, the counting of bacterial loads in the isolated kidney and live tissues revealed significant reductions in the bacterial counts upon treatment with sulforaphane (Figure 6B,C).

4. DISCUSSION

S. enterica is a common cause of foodborne illness worldwide that ranges from mild gastroenteritis to severe enteric fever.[13,47] During the past decade, there was a notable increase in the incidence of salmonellosis in Ha'il, Saudi Arabia, probably due to the contamination and improper preservation of food materials.[17] Sulforaphane is a prominent component in the cruciferous vegetables and acquires considerable antimicrobial activities against several enteric pathogens.[3,4] Furthermore, the safety of sulforaphane has been approved at high concentrations and can be provided as a food supplement.[8,48] Sulforaphane showed significant antivirulence activities employing several mechanisms targeting urease in *H. pylori*[7,8] and QS systems in *P. aeruginosa*,[33,49] importantly, it inhibits the intracellular survival of *S. aureus*[11] and *H. pylori*.[7] *Salmonella* is an intracellular bacterium that can survive inside the phagosome of host immune cells recruiting specialized injectosome "TTSS" to cope the adverse conditions.[22,50] Bearing in mind these findings, it was hypothesized that sulforaphane could acquire activity against *S. typhimurium*, placing particular emphasis on examining its impact on TTSS as well as bacterial internalization inside host cells.

Sulforaphane inhibited the *S. typhimurium* growth at a considerable low concentration; however, its antivirulence activities were assayed using 1/4 MIC (2 μg/mL) to exclude any influence on growth. Bacteria employ diverse systems to induce pathogenesis, mainly QS systems, which play diverse roles in orchestrating bacterial virulence including the production of virulence factors and biofilm formation in both Gram-negative and Gram-positive bacteria.[51−53] In *Salmonella*, QS significantly contributes to its pathogenicity playing a crucial role in regulating virulence, biofilm formation, and interaction with the host environment.[54,55] In our previous study, sulforaphane at sub-MIC diminished the production of virulence factors and biofilm formation in *P. aeruginosa*, which in turn decreased the bacteria's capability to establish infection in the mice. These antivirulence activities and antibiofilm were attributed to the sulforaphane's interference with the QS systems and downregulation of their encoding genes.[4] In compliance with these results, sulforaphane at sub-MIC diminished *S. typhimurium* adhesion and biofilm formation. That could be attributed to the anti-QS activities of sulforaphane.[4]

The intracellular accumulation of sulforaphane to high levels resulted in the eradication of the intracellular resistant *H. pylori*.[7] Additionally, sulforaphane showed considered ability to diminish the intracellular survival of *S. aureus* via inhibition of mitogen-activated protein kinase (MAPK) (p38) and c-Jun N-terminal kinase (JNK) MAPK signaling pathways in macrophages.[11] Interestingly, sulforaphane significantly decreased the *S. typhimurium* invasion and intracellular replication, which is in agreement with the others' findings mentioned above. However, in an intracellular pathogen such as *Salmonella*, the mechanism of sulforaphane's antivirulence is quite different as it depends on its interference with TTSS. TTSS is an injectosome encoded mainly by two clusters of genes localized in SPI1 and SPI2 to produce two types of TTSS.[13,18] Although the first type of TTSS is involved in the early stages of *Salmonella* invasion, the functions of the second type are more important and essential to the bacterial survival inside the host cells.[18,56] The TTSS machinery is working via translocation of effector proteins to remodel the host cells and ease the

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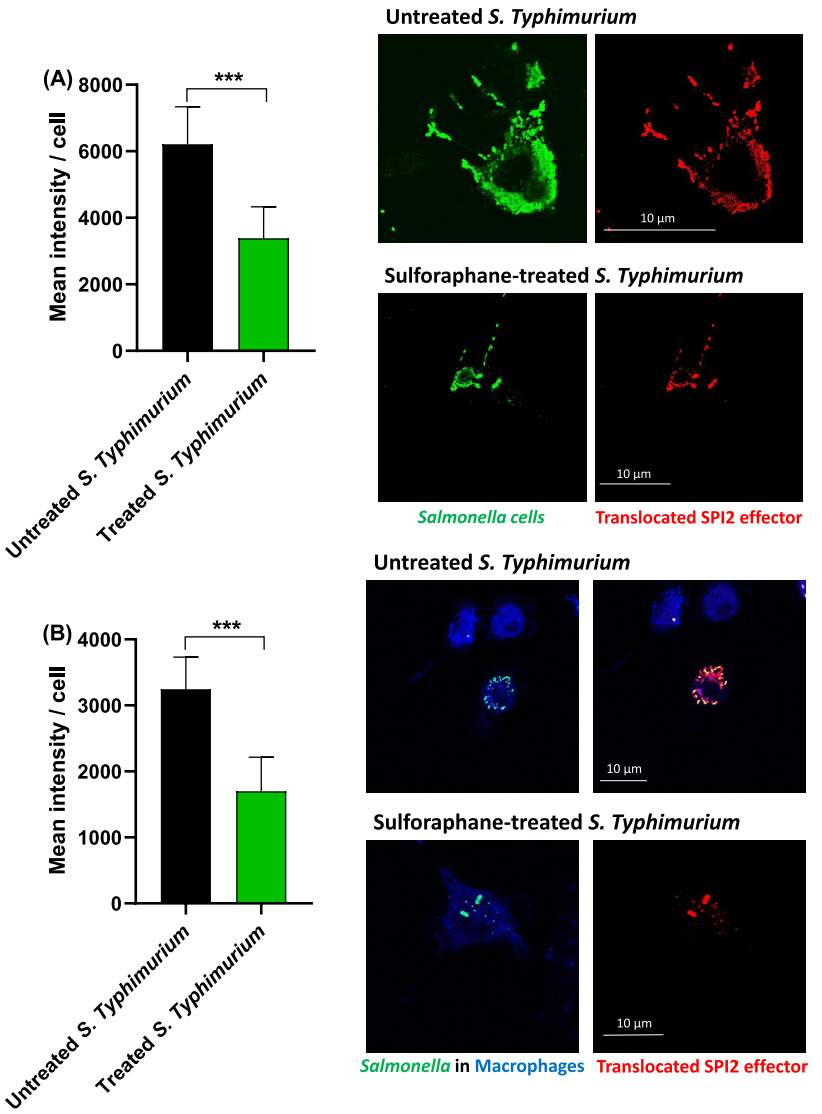

Figure 4. Sulforaphane significantly hindered the translocation of *S. typhimurium* SPI2 effector. The red fluorescence intensity (cy5) of the translocated SseJ::HA was quantified in the (A) HeLa and (B) raw macrophages infected with *S. typhimurium* treated with sulforaphane at sub-MIC. The Bacterial cells were counterstained with GFP-tagged secondary antibodies appearing green, while the macrophages were stained with DAPI. The intensity was measured at least in 30 cells infected with similar numbers of bacterial cells. Sulforaphane significantly diminished the translocation of the SPI2 effector (***$p < 0.001$).

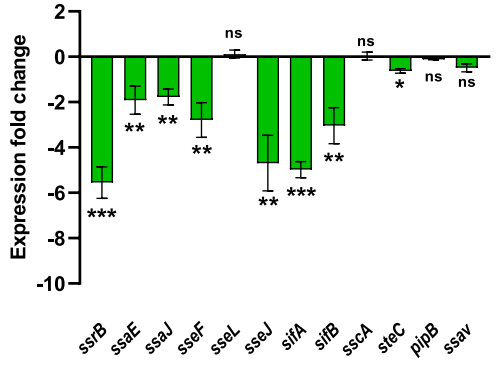

Figure 5. Sulforaphane at sub-MIC downregulated the expression of some SPI2 genes. The expression levels were normalized to the *gyrB* gene. ns: nonsignificant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Salmonella invasion, intracellular replication, and then establish an infection in host cells.[22] In this direction, the influence of sulforaphane at the sub-MIC on the effector translocation was assayed. The translocation of HA-tagged SseJ (SPI2 effector protein) was quantified in HeLA cells and macrophages; sulforaphane significantly diminished the translocation of the SPI2 effector that indicates its interference with TTSS and explains the significant reduction in the *Salmonella* intracellular replication.

Table 1. Susceptibility to Antibiotics in the Presence of Sulforaphane at Sub-MIC

antibiotic	MICs (μg/mL)	$MIC_{antibiotic+sulf}$ (μg/mL)	FIC
ciprofloxacin	4	0.5	0.25
ceftazidime	56	32	0.5
amoxycillin/clavulanic acid	256	128	0.5
streptomycin	8	2	0.25
chloramphenicol	256	32	0.125

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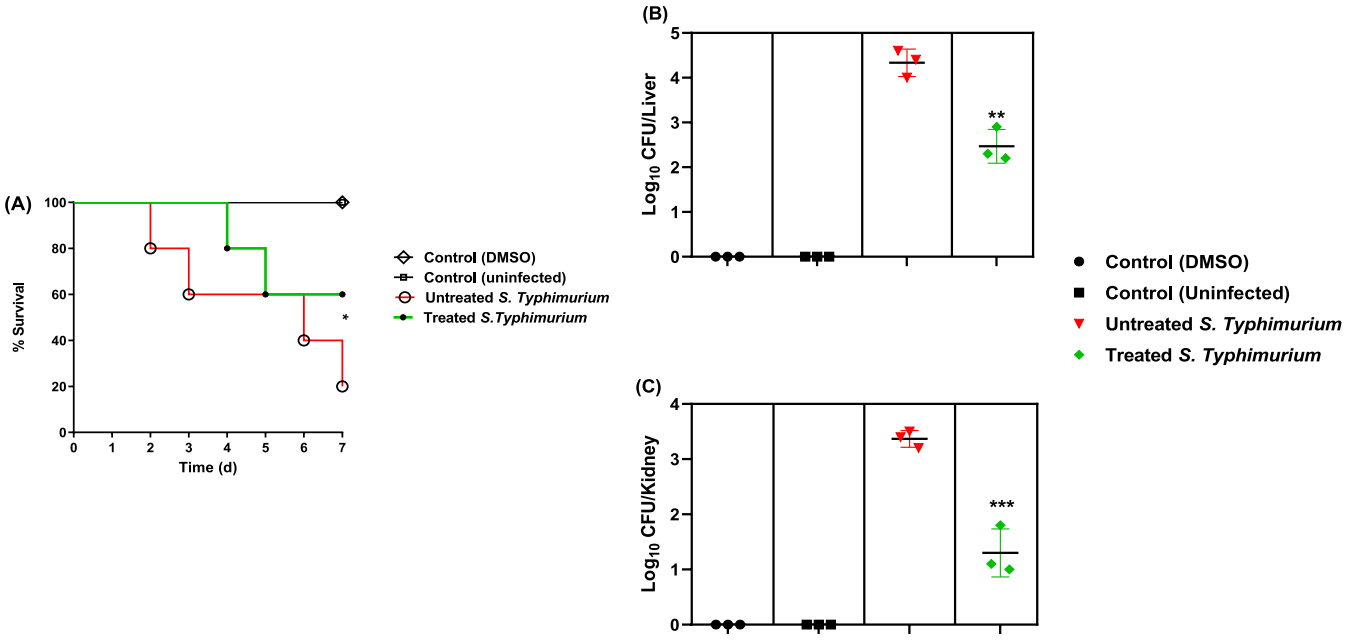

Figure 6. Sulforaphane significantly protected mice against *S. typhimurium*. (A) Survival curve for *S. typhimurium*-treated mice (n = 5) in the presence or absence of sulforaphane. The bacterial load in the isolated (B) liver and (C) kidney tissues. *p < 0.05, **p < 0.01, and ***p < 0.001.

The second type of TTSS is encoded by more than 30 genes that encode the (i) structural apparatus (Ssa) of TTSS, (ii) effectors (Sse) that are translocated in the host cytoplasm easing the bacterial cope inside phagosomes, (iii) chaperones (Ssc) that are required to keep the functions of effectors, and (iv) regulatory secretion system SsrAB that is located in a separate operon.[13,56−58] Sulforaphane at sub-MIC significantly downregulated the regulatory gene *ssrB* as well as apparatus encoding genes *ssaE* and *ssaJ*; however, there was no significant effect on the expression of *ssaV*. Sulforaphane downregulated the expression of effectors encoding genes *sseF*, *steC*, and *sseJ*, but there was no effect on the expression of the *sseL* gene. The genes *sseF and sseJ* encode essential effectors for maintaining the integrity of SCV,[56,57] while SteC manipulates the host cell actin cytoskeleton and modulates signaling pathways.[59] *Salmonella* spreads a dynamic network of filaments known as Salmonella-induced filaments (SIFs) that are crucial for the bacterial to thrive intracellularly.[60] Our results showed that sulforaphane significantly decreased the expression of SIF formation and microtubule bundling encoding genes *sifA* and *sifB*.[57] On the other hand, sulforaphane has no effect on gene encodes PipB which plays a role in the SCV trafficking along microtubules securing the *Salmonella* survival.[23,61] In addition, sulforaphane has no significant effect on the expression of chaperone encoding gene *sscA*. These findings attest to the downregulating influence of sulforaphane at sub-MIC on SPI2 genes that could explain the lessened SPI2 effector translocation and in turn the reduction in the intracellular replication.

To ensure the above in vitro results, a mice protection assay was conducted to evaluate the capacity of sulforaphane to diminish the *S. typhimurium* pathogenesis. Intriguingly, sulforaphane at sub-MIC significantly saved mice against *S. typhimurium* and also significantly decreased the bacterial colonization in the isolated liver and kidney tissues. That is in great alignment with the interference of sulforaphane with TTSS and a decrease in the invasion and intracellular replication abilities. One of the significant challenges in treating *Salmonella* infections is the development of antibiotic resistance. Sulforaphane may offer a complementary approach to combat antibiotic-resistant strains by targeting different mechanisms than conventional antibiotics. In this study, sulforaphane showed significant antivirulence activities and exerted a synergistic effect with various antibiotics even at low concentrations (at sub-MIC levels). The current study proposes utilizing L-sulforaphane as an antivirulence agent at 2 μg/mL, which is a concentration readily obtainable from just a few grams of cruciferous vegetables, such as broccoli.[1] Additionally, sulforaphane is safe at high concentrations and can be easily delivered with diet in the form of edible cruciferous vegetables.[48,62] This could emphasize its possible administration as an adjunct to traditional antibiotics to overcome the resistant enteric bacterial infections such as *H. pylori*[7,8] and *S. typhimurium*.

5. CONCLUSIONS

Sulforaphane is a predominant bioactive component in edible cruciferous plants. Besides, the sulforaphane anti-inflammatory and antioxidant known activities acquire significant antibacterial effects in particular against intracellular enteric pathogens. Our findings emphasized the sulforaphane interference with TTSS, which plays a key role in the enteric pathogen *S. enterica* invasion and intracellular replication. Furthermore, sulforaphane possesses antibiofilm activity that is in compliance with its previously proved anti-QS activity. In vivo findings attested to the in vitro results, where sulforaphane protected mice against *S. typhimurium* and decreased the bacterial colonization. Sulforaphane showed synergistic outcomes when combined with different antibiotics. To sum up, sulforaphane, which is abundantly present in edible cruciferous vegetables and can be directly delivered to the stomach, can serve as a safe adjuvant to antibiotics to treat clinically important enteric pathogens. However, further pharmacological and pharmaceutical investigations are necessary before considering the potential clinical use of sulforaphane.

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Author Contributions

S.M.D.R.: Methodology. A.S.A.L., A.M., and E.-S.K.: Investigation, Visualization, Funding acquisition. A.A.H.R. and M.M.B.: Methodology, Validation, Investigation. W.A.H.H.: Conceptualization, Writing—original draft, Supervision, Administration.

Notes

The authors declare no competing financial interest.

Ethical Approval The Faculty of Pharmacy, Port Said University Ethical Committee, allowed in vivo mouse tests in this study. The experiments were conducted in agreement with the ARRIVE guidelines and the UK Animals Act of 1986 (Accession no. PSU.PHR.19).

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