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Unraveling the Impact of Short-Chain Fatty Acids on Virulence Genes in *Salmonella* Typhi: A Gene Expression Profiling Study under SCFAs-Induced Stress

[1]Fadak M. Abdulridha
[2]Bassam K. Kudhair

[1,2] Department of Laboratory Investigations, Faculty of Science, University of Kufa, Najaf/ Iraq

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**Corresponding Author:*

Emails:

Bassamk.sharuza@uoukufa.edu.iq

Abstract: *Salmonella typhi is a highly pathogenic bacterium that causes typhoid fever, a serious systematic infectious disease with significant global health implications. Short-chain fatty acids (SCFAs) influence gene expression in other Salmonella strains but their impact on Salmonella typhi is unclear. This study investigates the impact of specific SCFAs (Sodium Butyrate and Sodium Propionate) on the expression of T3SS-1 virulence-associated genes (hilC, hilD, sipC) and a regulatory gene (ropE) in Salmonella Typhi. Sub-inhibitory concentrations of SCFAs were determined (50mg/ml), allowing analysis of their effects on bacterial behavior without inhibiting growth. Gene expression analysis using RT-qPCR revealed that both Sodium Butyrate and Sodium Propionate significantly downregulated hilC, hilD, and sipC genes, essential for activating virulence factors. However, the ropE gene remained unaffected. These findings suggest that SCFAs play a role in regulating virulence in Salmonella typhi, consistent with previous research on other Salmonella strains. Understanding SCFAs' influence on Salmonella typhi virulence could lead to targeted interventions for combatting Salmonella infections and improving public health.*

***Keywords*:** *Salmonella Typhi, SCFAs, T3SS-1, hilC, hilD, sipC*

1. Introduction

Type 3 secretion system-1 (T3SS-1) associated with invasion is a significant virulence determinant that contributes to the induction of severe inflammation of the intestine through infection with Non-typhoidal strains. *Salmonella* Typhimurium is a bacterial pathogen that belongs to the genus *Salmonella* [1]. The gut microbiota produces fermentation end products known as short-chain fatty acids (SCFAs), which have the ability to impact the expression of T3SS-1 genes in enteric pathogens [2,3,4]. Studies have demonstrated that Butyrate and Propionate reduce the *Salmonella* invasion gene expression, Acetate, however, enhances their expression [3,5]. Short-chain fatty acid mixtures that mimic colonic concentrations, with elevated levels of "Butyrate and Propionate" demonstrate a stronger inhibitory impact when compared to mixtures resembling ileum concentrations,

where acetate is present in higher proportions [4]. Despite encountering similar SCFAs concentrations during infection, *Salmonella* serovars of Typhoidal and Non-typhoidal have different abilities to induce severe intestinal inflammation within a short incubation period, suggesting differences in SCFAs-mediated regulation.

One significant genetic distinction between *Salmonella* serovars is the significant presence of a lack of functional mutations in the genomes of *salmonella* Typhoidal distinguishes them from Non-typhoidal serovars *Salmonella* Typhi and *Salmonella* Typhimurium share gene order conservation but have rearrangements near rRNAs and a large inversion around the terminus in *Salmonella* Typhimurium. The genetic differences include single-gene insertions, with *Salmonella* Typhi having 601 unique genes in 82 blocks and *Salmonella Typhimurium* having 479 unique genes in 80 blocks. *Salmonella* Typhi also contains various insertions, such as chaperone usher fimbrial systems and toxin genes. Pseudogenes contribute to phenotypic and host range differences, with *Salmonella* Typhi having 204 pseudogenes, of which 145 are intact genes in *Salmonella* Typhimurium and only 23 are pseudogenes [6,7]. Many missed functions in *Salmonella* serovars Typhi are associated with central anaerobic metabolism, which plays a crucial role in gastrointestinal disease caused by Non-typhoidal *Salmonella* serovars in the gut [8]. However, further research is needed to fully understand the precise functions of these metabolic pathways that are absent in Typhoidal *Salmonella* serovars For example, the ydiQRSTD operon, which is present in other *Salmonella* genomes but absent in *Salmonella typhi* and *Salmonella* ParatyphiA genomes, is predicted to encode a pathway that enables the beta-oxidation of fatty acids when nitrate, an electron acceptor found in the intestinal lumen at the time of gastrointestinal disease, is present [9,10,11]. The absence of this operon suggests that Typhoidal serovars no longer require metabolic pathways that facilitate bacteria growth in the inflamed intestine, as these serovars do not cause gastrointestinal disease. While there have been several studies examining the impact of SCFAs on the hils and sips expression in non-Typhoidal serovars, limited knowledge exists regarding their effect on Typhoidal serovars. Given the aforementioned genetic variations between serovars, it is crucial to investigate the influence of SCFAs on *Salmonella* Typhi. The main objective of the study is to gain insights into the significance of SCFAs on the virulence of *Salmonella Typhi.*

2. Methodology

Bacterial identification

The isolate of *Salmonella* enterica subspecies enterica serovar Typhi isolated from a human fecal specimen was identified as previously described [12].

Determining the SIC of Short chain fatty acids

The SIC of sodium propionate and sodium butyrate was determined by culturing the target bacteria in different concentrations. A single colony was picked and incubated for 18 hours. The culture was then diluted to an OD of 0.002 at 600 nm. For each sub-inhibitory concentration, 12 wells were prepared with varying concentrations of SCFAs. After incubation, the growth of *Salmonella* Typhi was measured, and the two highest concentrations of sodium butyrate that did not inhibit bacterial growth were identified as the SICs for this study.

Culture Conditions for gene expression screening

Bacterial cultures of *Salmonella typhi* were grown on TSB broth in the presence or absence of SCFAs to study their impact on gene expression. Two sets of cultures were prepared:

the control group without SCFAs and the treatment groups with either 10 mM or 50 mM of either Sodium Propionate or Sodium Butyrate individually. The cultures were incubated overnight and transferred to new flasks for further growth. After 4 hours, SCFAs were added to facilitate bacterial growth, and the cultures were allowed to grow for an additional 12 hours. The bacterial pellets were collected, stored, and used for RNA extraction the next day.

Bacterial RNA extraction

To isolate total RNA from *Salmonella*, a modified extraction procedure was employed, incorporating both manual modifications based on Kim [13] and a commercially available kit supplied by Solarbio (China). The process involved preparing a lysis buffer, heating it, and adding it to the pre-prepared sample. After various steps of vortexing, boiling, and incubation, the RNA was extracted and concentrated using specific solutions and centrifugation.

RNA concentration measurement

UV-visible spectroscopy was used for this purpose, evaluating purity regarding protein contamination and confirming the absence of chromophore materials. Absorbance readings greater than 0.15 were considered significant, and an OD260 nm absorbance of 1 corresponded to 40 µg of RNA per ml. The RNA concentration was calculated using the formula: RNA concentration = 40 x A260 x dilution factor, and pure RNA typically has an OD260/OD280 ratio of approximately 2.0, while lower ratios suggest contamination with proteins or phenols.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

This assay was performed using Solarbio Universal RT-qPCR Kit and Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Cat no. RP1100). The cDNA synthesis involved two steps: first, combining total RNA with Oligo(dT)16 and then adding specific components of M-MLV buffer, dNTPs, Rnasin, and M-MLV enzyme for the second step as described by the manufacturer. The reaction was incubated at 42°C for 60 minutes followed by 95°C for 5 minutes, and a cDNA dilution of 1:30 was chosen for further experiments. The qPCR reaction mixture was prepared using 2X SYBR Green PCR Master mix, and the DNA primers used here along with program conditions were set exactly as described by [12] with fluorescence acquisition set at the extension step using the Fam channel. The quantitative PCR was performed with triplicates for each sample. As for controls, one reaction was conducted in the presence of RNA instead of cDNA, and another control had no nucleic acid template to validate the primer specificity and the contamination state. Relative gene expression was calculated using the Livak method, involving the calculation of ΔCT and ΔΔCT values, and fold changes were determined using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.3.0. One-way analysis of variance (ANOVA) and Tukey's post hoc test were conducted to assess the significance between groups. The standard deviations are represented as error bars in all figures to indicate the variability of the data points. The threshold for statistical significance was set at a P value < 0.05.

3. Results and Discussion

SCFAs sub-inhibitory concentration

The results indicate that a concentration of 50 mM had no discernible effect on the growth OD600 nm for Sodium Propionate or Sodium Butyrate. Consequently, the concentrations of both 10 mM and 50 mM were selected for investigating gene expression. Notably, these

concentrations fall within the range of 5-45 mM, which is recommended for studying sub-inhibitory effects on different *Salmonella* serovars, as indicated by previous research [2,14,15,16,17,18].

UV-visible characteristics of isolated RNA

The bacterial cultures were grown with and without SCFAs. The bacterial pellets were then used for RNA isolation. The concentration of RNA was measured based on the absorbance, which ranged from 0.6 to 1.5 µg/µL. The UV-visible spectrophotometer indicated that the samples were free of protein contaminations, as the $OD_{260/280\ nm}$ ratio was 1.94 ± 0.05 (n: 10). Furthermore, the spectrophotometer confirmed the absence of chromophore substances responsible for light absorption and emission in the visible range (380-700 nm) as in Figure(1).

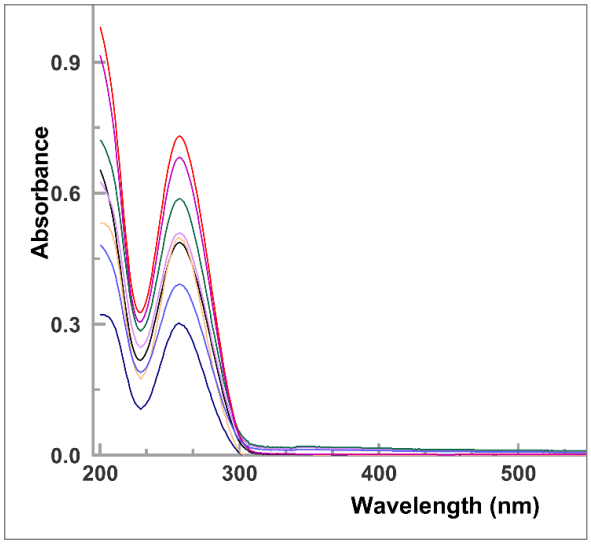
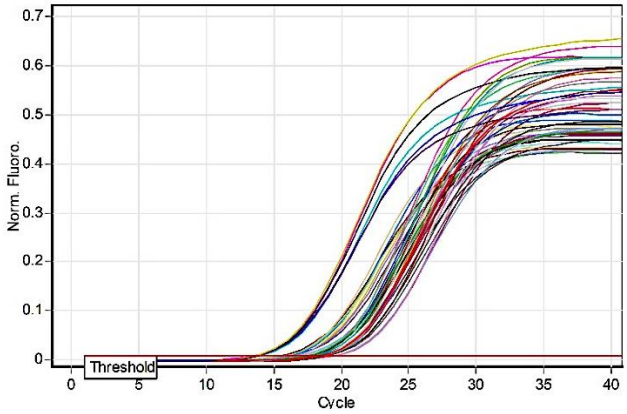


Figure (1): Uv-visible spectrum (200 -550 nm) of 50 folds RNA diluted samples. The isolated RNA was diluted with nuclease-free water, and the absorbance was measured after blanking the spectrophotometer with nuclease-free water only.

Gene expression

Amplification was observed in the samples, and no amplification was noticed for controls Figure (2). Melting curve analysis of each gene, including the housekeeping gene (*16S rRNA*), demonstrated distinct and specific peaks at specific melting points for each amplicon, indicating the absence of non-specific amplicons resulting from the non-specific binding of primers (Figure 3)

The study revealed that Sodium Butyrate and Sodium Propionate significantly downregulated the gene expression of *hilC*, *hilD*, and *sipC*, while no such effect was observed for the *ropE* gene. When comparing the fold changes in *hilC* and *hilD* expression at different concentrations of SCFAs (10 and 50 mM), no significant difference was found. In contrast, *sipC* exhibited concentration-dependent downregulation, with the higher concentration showing a greater effect Figure (4). This indicates that the response of *sipC* to Sodium Butyrate and Sodium Propionate might vary depending on the concentration.



Figure(2): A graph depicting a qPCR experiment conducted on *Salmonella* Typhi under the stress of SCFA.

In this comprehensive gene expression profiling study conducted under short-chain fatty acid (SCFAs)-induced stress, we delve into the intricate interplay between SCFAs and virulence genes in *Salmonella* Typhi. Unraveling the impact of SCFAs on the pathogenicity of this notorious bacterium holds significant importance for understanding its adaptive responses and virulence mechanisms. The current study's findings align with previous

research, such as that of Gantois [5], which demonstrated that exposure to butyric acid led to a reduction in the invasion of epithelial cells of intestinal via *Salmonella enterica* [5]. They investigated the molecular mechanisms behind the reduced invasion epithelial cells of intestinal via *Salmonella* serovar Enteritidis and Typhimurium when cultivated in a medium supplemented with butyrate. They conducted a comparative transcriptomic analysis to gain insights into this phenomenon. The research findings showed that the presence, of 10 mM. butyrate resulted from in-the downregulation of 19 genes that were common to both *Salmonella* Enteritidis and Typhimurium. Among these genes, 17 were located within the *Salmonella* pathogenicity island 1 (SPI1) [5]. Notably affected by this downregulation were *hilD*, *sipA*, *sipC*, and *sipD*, which are genes regulated by SPI1. Both *hilC* and *hilD* have distinct functions in the activation of hilA expression. They act independently by binding to the upstream repressing area of *hilA* gene and neutralizing the repressive influence [19,20,21].

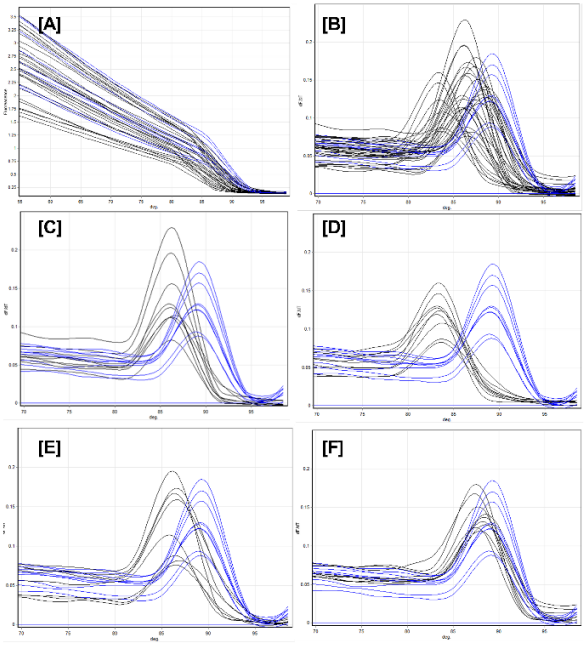


Figure (3): Melting curve data of qPCR. The melting curves [A] and the analysis [B] of all genes were obtained. Subfigures [C], [D], [E], and [F] represent the individual melting curve analysis of *hilC*, *hilD*, *sipC*, and *ropE*, respectively. The black trace represents the gene of interest, while the blue trace represents the housekeeping gene (*16S rRNA*) trace.

HilD directly influences the expression of *hilA* through binding to the repressing sequence located upstream of the gene, resulting in its de-repression. HilA, in turn, activates *invF* and *prgH*, which further activate the sip genes responsible for encoding TTSS effector proteins [22,23,24,25,26].

Hence it is highly probable that butyrate disrupts the *hilA*-dependent control of SPI1 by impacting the regulation of *hilD* transcription. In addition, Liu, [18] also reported the downregulation of *hilD*, *sipA*, *sipC*, and *sipD* in *Salmonella* Typhimurium grown in laboratory media and food models such as milk or chicken juice. They observed this downregulation in response to both propionate and butyrate at concentrations of 0.5 and 2.0 mg/ml, which is equivalent to approximately 5 and 20 mM [18].

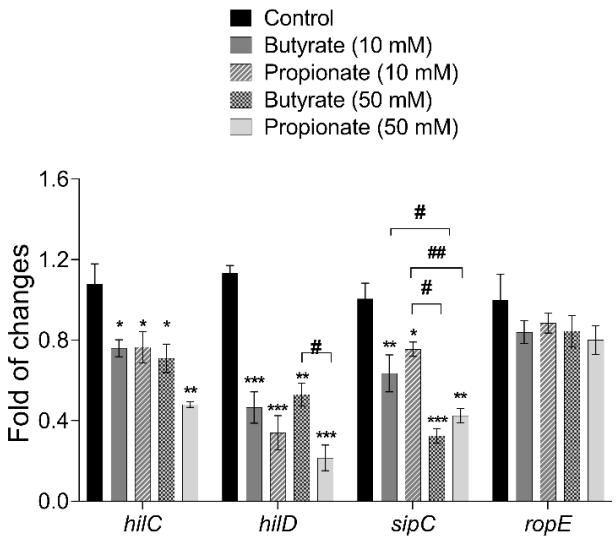


Figure (4): Fold changes in virulence and stress-related genes in *Salmonella* Typhi under the influence of Sodium Propionate and Sodium Butyrate (10 and 50 mM), normalized to the housekeeping gene (*16S rRNA*). The data were compared to the

control condition of TSB growth without SCFAs. The asterisk (*) symbol indicates the presence of statistically significant differences compared to the control condition, while the hashtag (#) denotes differences between treatments. The number of symbols corresponds to the significance level of the p-value: *, # for p < 0.05; **, ## for p < 0.01; and * for p < 0.001. The variances were analyzed using one-way ANOVA, and post-hoc analysis was performed using Tukey's honestly significant difference test (p < 0.05).**

Interestingly, Hung [14] found that *hilD* in *Salmonella* Typhimurium was significantly downregulated in the presence of 10 mM propionic acid. However, *hilC* remained unchanged, consistent with their microarray data. Furthermore, they observed a more than eightfold downregulation of *sipC*. Although the *sipC* down regulation observed here was not as significant as reported by [14] it is highly likely that this inconsistency is due to the variation in serovar types. This is supported by the findings of Gantois [5] who reported a sixfold downregulation of *hilA* expression in Enteritidis serovar but in the Typhimurium serovar, it was less than twofold change, suggesting the involvement of other genetic factors in specific serovars [5]. According to Gupta [17], sodium levels were (22 and 45 mm) Butyrate Significantly decreased adhesion and invasion of *Salmonella* Enteritidis in chicken enterocytes, while only the 45 mM concentration reduced invasion in macrophages. The Expression of *Salmonella* Enteritidis virulence genes was largely unaffected, However, specific genes associated with the type-3 secretion system, adherence, macrophage survival, and oxidative stress exhibited upregulation. Notably, sipA was upregulated approximately four-fold. However, the authors acknowledged the inconsistency in the RT-qPCR data.

Limited knowledge exists regarding the impact of short-chain fatty acids (SCFAs) on genes associated with the type-3 secretion system in *Salmonella* Typhi to the best of the current study author, only one study conducted by Bronner [15] has shed light on this topic. The study focused on the operon of ydiQRSTD, which is absent in the genomes of *Salmonella* Typhi and *Salmonella* Paratyphi A but present in *Salmonella* Typhimurium. This operon allows *Salmonella* Typhimurium to utilize microbiota-derived butyrate through a pathway for the beta-oxidation of fatty acids. This process is facilitated by the presence of nitrate, an electron-acceptor that becomes available in the intestinal lumen during gastrointestinal disease [9,10,11].

However, it is important to note that Bronner's study was conducted on *Salmonella typhimurium* after inducing mutations in the ydiQRSTD operon. Their findings revealed a significant downregulation of *hilD* in the *Salmonella* Typhimurium ydiD mutant when the bacteria were under anaerobic growth conditions in a media supplemented with a mixture of 20 mM Short-chain fatty acids consisting of 5 mM propionate, and 3 mM butyrate, 12 mM acetate. These results were further validated by restoring *hilD* expression through the use of a complemented *ydiD* mutant. Furthermore, when the ability to utilize butyrate was genetically removed in *Salmonella* Typhimurium, it led to diminished epithelial invasion and a decrease in intestinal inflammation. Specifically, the *ydiD* deletion made *Salmonella* Typhimurium susceptible to the inhibitory effects of butyrate on the expression of invasion genes [15]. The research findings are significant because they suggest that virulence-associated (Vi) capsule polysaccharide is present but very long O-antigen chains are absent, common features, in Salmonella Typhi, along with the genetic removal of butyrate utilization can greatly reduce the intestinal inflammation caused by *Salmonella* Typhi. These findings provide valuable insights into the evolutionary process and emergence of *Salmonella* Typhi as an

extraintestinal pathogen, as they highlight the importance of specific genetic changes in transitioning from a gastrointestinal pathogen to an extraintestinal pathogen.

Conclusion

The study investigated the impact of sub-inhibitory concentrations (10 mM and 50 mM) of Propionate and Butyrate on gene expression in *Salmonella* Typhi. Significant downregulation of *hilC*, *hilD*, and *sipC* genes in response to both SCFAs were indicated. The *ropE* gene showed no significant change in expression. The effect on *sipC* expression was concentration-dependent, with higher concentrations showing a stronger impact. Previous research supported these findings in Non-Typhoidal *Salmonella* strains.

Overall, this study sheds light on the role of SCFAs in regulating virulence in *Salmonella* Typhi. Understanding SCFA-induced stress may aid in developing targeted interventions to combat *Salmonella* infections and improve public health.

Ethical approval

The authors state that there are no competing interests present.

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