Nitric Oxide Induced \textit{stx2} Expression Is Inhibited by the Nitric Oxide Reductase, NorV, in a Clade 8 \textit{Escherichia coli} O157:H7 Outbreak Strain

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Abstract: \textit{Escherichia coli} O157:H7 pathogenesis is due to Shiga toxin (Stx) production, though variation in virulence has been observed. Clade 8 strains, for instance, were shown to overproduce Stx and were more common among hemolytic uremic syndrome cases. One candidate gene, \textit{norV}, which encodes a nitric oxide (NO) reductase found in a clade 8 O157:H7 outbreak strain (TW14359), was thought to impact virulence. Hence, we screened for \textit{norV} in 303 O157 isolates representing multiple clades, examined \textit{stx2} expression following NO exposure in TW14359 for comparison to an isogenic mutant (\textit{\textDelta}norV), and evaluated survival in THP-1 derived macrophages. \textit{norV} was intact in strains representing clades 6–9, whereas a 204 bp deletion was found in clades 2 and 3. During anaerobic growth, NO induced \textit{stx2} expression in TW14359. A similar increase in \textit{stx2} expression was observed for the \textit{\textDelta}norV mutant in anaerobiosis, though it was not impaired in its ability to survive within macrophages relative to TW14359. Altogether, these data suggest that NO enhances virulence by inducing \textit{stx2} production in TW14359, and that toxin production is inhibited by NorV encoded by a gene found in most clade 8 strains. The mechanism linked to these responses, however, remains unclear and likely varies across genotypes.

Keywords: Shiga toxin; \textit{Escherichia coli}; O157; virulence; pathogenesis; nitric oxide

1. Introduction

Shiga toxin-producing \textit{Escherichia coli} (STEC) O157:H7 is a leading cause of gastrointestinal illness linked to food and waterborne outbreaks worldwide. Patients infected with STEC O157 often present with hemorrhagic colitis, although some can develop hemolytic uremic syndrome (HUS) [1] that can lead to kidney failure and death. In the U.S., an annual 46.2% hospitalization rate and 0.5% death rate were reportedly attributable to STEC O157 [2], while the incidence of all STEC infections was estimated to be 5.9 cases per 100,000 individuals in 2018 [3]. Considerable variation in the number of infections, however, has been documented across different regions in the U.S. [4].

STEC pathogenesis is mainly due to the production of one or more Shiga toxins (Stx) encoded by genes carried on lambda-like bacteriophages [5]. Other virulence factors are also important. For instance, STEC strains possessing genes like \textit{eae} (intimin) found on the locus of enterocyte effacement (LEE) pathogenicity island [6], contribute to attaching and effacing lesion formation on intestinal epithelial cells. \textit{E. coli} strains possessing \textit{stx} as well as the LEE island are classified as enterohemorrhagic \textit{E. coli} (EHEC), which were found to be more virulent than STEC strains lacking these factors [7]. Furthermore, EHEC strains producing the Stx2a and/or Stx2c variants were observed to be more virulent causing a higher frequency of HUS than strains producing the Stx1 variant alone or with...
Stx2a [5,8,9]. Differences have been reported across populations, however, indicating that strain specific factors are also important for clinical phenotypes. Indeed, we previously showed that a diverse set of O157 strains could be classified into nine distinct clades based on the phylogenetic analysis of 96 single nucleotide polymorphisms (SNPs) [10]. Most importantly, strains belonging to clade 8 were significantly more common among HUS cases and more frequently possessed genes encoding Stx2a and/or Stx2c compared to other clades. Follow up studies showed that clade 8 strains also had higher stx2 expression levels following exposure to epithelial cells [11] and more than the expected number of Stx-encoding bacteriophage insertion sites occupied by prophages lacking stx [12]. Notably, deletion of a non-Stx-encoding prophage in a high stx2a-expressing clade 8 strain (TW14313) drastically reduced stx2a expression levels in the mutant relative to the wild type [12]. Taken together, these data suggest that clade 8 strains possess unique traits that may enhance their ability to cause more severe disease.

A genome analysis of the clade 8 O157:H7 strain (TW14359), which was implicated in the 2006 North American spinach outbreak that caused a higher-than-average frequency of HUS [13], uncovered several traits that could promote virulence [14]. One candidate gene, norV, encodes a nitric oxide (NO) reductase (NorV) that detoxifies NO in oxygen limiting conditions [15]. Two versions of norV have been identified [14]. An intact functional norV was detected in TW14359 [14], while a non-functional copy containing a 204 bp deletion was found in the EDL933 (clade 3) [16] and the Sakai (clade 1) [17] O157:H7 outbreak strain genomes [14]. An initial screen of 100 O157:H7 strains recovered from multiple sources detected the 204 bp deletion in 58% of strains and a correlation with stx1 [14], which we found to be overrepresented among strains representing clades 1–3 [10]. These findings indicate that norV functionality and its impact on virulence may be restricted to strains with a specific genetic background.

A prior study also demonstrated that possession of an intact norV protected different EHEC O157:H7 strains from growth inhibition by NO under anaerobic conditions unlike strains possessing the non-functional version of norV [18]. Additionally, mutants lacking norV had decreased survival within macrophages. Since macrophage uptake and the subsequent production of NO are a critical part of the innate immune response to bacterial pathogens, these data suggest that possession of a functional norV can protect a bacterium from macrophage killing. Indeed, the same study found that insertion of a functional norV into the EDL933 chromosome subsequently increased stx2 expression in the presence of NO inside macrophages [18]. As these data were generated with a strain that was not classified as clade 8, the function of norV in a more virulent strain background is not known. Consequently, the goal of this study was to examine the effects of NO and norV mutagenesis on growth, stx2 expression, and macrophage survival using the clade 8 spinach outbreak strain, TW14359.

2. Materials and Methods

2.1. Bacterial Strains, norV Detection, and Growth Conditions

The E. coli O157:H7 spinach outbreak strain, TW14359, was used for all experiments; it possesses stx2a and stx2c and was previously classified as clade 8 by SNP genotyping [10]. This strain was previously recovered by the Michigan Department of Health and Human Services (MDHHS) from a patient who had consumed spinach linked to the national 2006 outbreak [13]. An additional set of 303 previously characterized O157 strains with seven stx profiles representing the predominant clades 1–3 and 6–9 [10], was screened for the presence of an intact (functional) or deleted version of norV. These strains were recovered from clinical cases in Michigan between 2001 and 2006 as described [19]. Differences in the frequencies of stx profiles and norV type were detected using the likelihood chi-square test or Fisher’s exact test for sample sizes less than five.

After overnight growth in Luria–Bertani (LB) broth at 37 °C and DNA isolation, PCR was performed to detect the presence of a functional norV in all 303 strains. The primer set norV698_F and norV1373_R, which targets the region flanking the norV deletion (Table 1),
was used with the following PCR conditions: 10 min at 94 °C plus 30 cycles of 1 min at 92 °C, 30 s at 53 °C and 1 min at 72 °C followed by 20 min at 72 °C.

Table 1. Oligonucleotide primers developed for use in this study.

| Primer     | Sequence (5'-3')                              | Target     | Amplicon Size |
|------------|-----------------------------------------------|------------|---------------|
| norv698_F  | ATAACCCGGACGCAAATTGT                         | norV       | 675 bp (intact) |
| norv1373_R | TATCCGGGACTTCATCCA                           |            | 470 bp (deletion) |
| norV-del1  | GCAATTAGCAAGA                                 | Kanamycin marker | 1577 bp |
| norV-del2  | CAATTTTTGAGACCGCTGAA                         |            |               |
|            | AAAATTGAGTTGCTGTC                            |            |               |
|            | AGGCTGAGGACGCTGTC                            |            |               |
|            | CACCGTTGGCGGCGG                             |            |               |
|            | GCGGAAGCGCGAACCA                            |            |               |
| norV-del3  | GTATTCGCCACGACATCAA                          | ΔnorV      | 1868 bp (TW14359) |
| norV-del4  | AAATCTGGCACCACCAATCAC                       |            | 1905 bp (ΔnorV) |
| norvcompl_F| AGATATGTCATATCGACCATTGGA                     | norV + flanking region and norV promoter | 1620 bp |
| norvcompl_R| AATGATCAATACGCGGTGC                         |            |               |

2.2. Construction of a norV Mutant

norV was deleted from the chromosome of wild-type (WT) strain TW14359 using the red recombination procedure [20]. Briefly, recombinant PCR products containing a kanamycin (Km) resistance marker flanked by 50 bp sequences homologous to the upstream and downstream regions of norV were generated from plasmid pKD4 [21,22] using primers norv-del1/del2 (Table 1). PCR products were electroporated into the red recombinase-producing WT strain containing pKM208 as described previously [23]. Transformants were identified by growth on LB agar supplemented with 25 µg/mL Km following incubation at 37 °C. The deletion in norV (ΔnorV) was confirmed by PCR-based restriction fragment length polymorphism using the norv-del3/del4 primer set followed by digestion with PstI for 2 h at 37 °C.

For complementation of ΔnorV, a 1620 bp region containing the norV coding region plus additional flanking regions including the norV promoter, were amplified by PCR from strain TW14539 genomic DNA using Takara LA Taq polymerase (Takara Bio; Madison, WI, USA) with the norv-compl_F/norvcompl_R primer set. The resulting PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen; Carlsbad, CA, USA) to construct the pCR2.1-TOPO norV plasmid. This plasmid was transformed into the ΔnorV strain, creating the ΔnorV::norV WT strain. Next, we electroporated the pCR2.1-TOPO empty vector into the ΔnorV strain, which resulted in a ΔnorV::vector strain for use as a control. Transformants were grown overnight in LB supplemented with 100 µg/mL ampicillin at 37 °C.

2.3. Bacterial Gene Expression

Prior to RNA extraction, the WT and mutant strains were grown for 6 and 24 h in LB broth at 37 °C in the presence or absence of 200 µM of the nitric oxide donor (NOR4) (Sigma-Aldrich; Burlington, MA, USA). The concentration of NO was determined using the Nitrate/nitrite Colorimetric Assay Kit (Sigma-Aldrich). Strains were cultivated at 37 °C with shaking (aerobic) or without shaking in the presence of 5% CO₂ (anaerobic). cDNA was synthesized from the extracted RNA by reverse transcription as described previously [24]; three independent RNA populations were extracted per sample. Quantitative real-time PCR (qRT-PCR) was performed utilizing amplification conditions and primers specific for the B subunit gene of stx2, that differentiates between stx2a and stx2c as was carried out previously [11,24]. Expression data are reported for “stx2” for simplicity. For normalization,
published conditions and primers were used to amplify the 16S rRNA gene, rrsH [24]. Transcription differences were quantified as described [11,22], and expression levels were presented for strain TW14359 relative to ΔnorV or the same strain grown in different conditions (e.g., aerobic vs. anaerobic). Fold change in stx2 expression was calculated using the ddCt method [25]; a fold change of ≥2 was considered biologically significant.

2.4. Cell Culture and EHEC Infection

THP-1 monocyte-like cells (ATCC TIB-202) were cultured in Roswell Park Memorial Institute 1640 (RPMI) growth medium (Gibco; Amarillo, TX, USA) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (FBS; Atlanta Biologicals; Minneapolis, MN, USA), and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO2. Cells were differentiated into macrophages by incubation with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) in RPMI medium with 2% FBS for 24 h as was described [26]. Using a published protocol [27] that was modified for EHEC O157:H7, the cells were seeded at a density of 1 × 10⁶ cells per well in 24 well plates. Bacteria were added to the cell monolayer at a multiplicity of infection (MOI) of 10 and incubated at 37°C with 5% CO2 for 1 h. The cells were washed with phosphate-buffered saline (PBS) and fresh medium containing 100 mg/mL of gentamicin was added for 1 h to kill extracellular bacteria. The infected monolayers were lysed by adding 0.1% triton X-100 (Sigma-Aldrich) in PBS. Lysates were diluted and plated onto LB agar and incubated at 37°C overnight to quantify colony forming units (CFUs). The number of intracellular (phagocytosed) bacteria was determined by normalizing to the total number of bacteria left in the well after the 1 h infection period. The percent survival was calculated by dividing the number of intracellular bacteria at 24 h by the total number of intracellular bacteria 1 h after adding antibiotics and multiplying by 100. A Student’s t-test was used to determine if there were significant differences between strains as measured using the mean; a p-value ≤ 0.05 was considered significant.

3. Results

3.1. norV Distribution by Clade and Association with Stx Gene Profiles

To detect the presence of the intact (functional) and deleted norV variants, all 303 O157 clinical isolates were examined. A 470 bp product representing the presence of a non-functional norV containing a 204 bp deletion was found in 197 (65.0%) strains. By contrast, a 675 bp product representing the intact and functional version of norV was found in the remaining 106 (35.0%) strains. Differences in the distribution of the intact norV were observed across the O157 clades previously classified using SNP genotyping. [10]. Among the predominant O157 clades examined, the non-functional norV containing the deletion was detected only in strains belonging to clades 1–3, while the intact norV was detected in all strains representing clades 6–9 (Figure 1). Among those 197 strains containing the 204 bp deletion, most (83.2%) belonged to clade 2, the predominant lineage examined in this subset of clinical strains. By contrast, strains with the deleted version of norV were significantly more likely to possess both stx1 and stx2 relative to all other strains (Fisher’s exact test p ≤ 0.0001). Among the 186 strains with stx1 and stx2, most belonged to clades 2 (n = 152) and 3 (n = 32).
Table 2. Distribution of stx profiles by clade and norV type among 303 Shiga toxin-producing Escherichia coli O157 clinical isolates.

| Clade | norV† | stx1 | stx1, stx2 | stx1, stx2c | stx1 | stx2c | stx2, stx2c | stx2 | stx2c | Total * |
|-------|-------|------|------------|------------|------|-------|------------|------|-------|--------|
| 2     | Deletion | 2 (1.2) | 152 (92.7) | – | – | 10 (6.1) | – | – | 164 |
| 3     | Deletion | 1 (3.0) | 32 (97.0) | – | – | – | – | – | 33 |
| 6     | Intact | – | – | – | – | 1 (12.5) | 1 (12.5) | 6 (75.0) | 8 |
| 7     | Intact | 1 (3.5) | 1 (3.5) | 7 (24.1) | – | 6 (20.7) | 14 (48.3) | 29 |
| 8     | Intact | – | 1 (1.5) | 1 (1.5) | – | 30 (45.5) | 33 (50.0) | 1 (1.5) | 66 |
| 9     | Intact | – | 1 (33.3) | 2 (66.7) | – | – | – | 3 |

* Percentages were calculated using the total number of isolates per clade as the denominator. † Differences in the frequencies of stx profiles by norV type were detected using the likelihood chi-square test; the Fisher’s exact test was used for sample sizes less than five.

3.2. Impact of NO on stx2 Expression in the Clade 8 Outbreak Strain

To determine whether strain TW14359 had higher stx2 expression in response to NO, the strain was grown in LB with and without the NOR4 nitric oxide donor. In aerobic conditions, the stx2 expression levels were not significantly altered in the presence or absence of NO after 6 h of growth (data not shown). The same was true in anaerobic conditions after 6 h. Increasing the incubation time, however, was associated with an increase in stx2 expression but only in anaerobic conditions. Specifically, after 24 h of anaerobic growth, stx2 expression was 2.2-fold higher in the presence of NO relative to the absence of NO (Figure 2). No significant difference was observed for aerobic conditions after 24 h of growth, indicating that NO activates stx2 expression in the clade 8 spinach outbreak strain only in anaerobic conditions.
3.2. Impact of NO on stx2 Expression in the Clade 8 Outbreak Strain across Growth Conditions

Since norV contributes to NO detoxification and an intact norV was present in all 66 clade 8 strains examined, we constructed an isogenic non-polar null norV mutant (ΔnorV) in the wild-type (WT) spinach outbreak strain TW14359. stx2 expression was quantified in ΔnorV relative to the WT strain in the presence and absence of NO in both aerobic and anaerobic conditions following 24 h of growth. Under aerobic conditions, stx2 transcription levels were similar between the WT and ΔnorV in the presence and absence of NO (Figure 3A). As compared to the WT strain, stx2 transcription levels were 1.0 ± 0.3 in the absence of NO and 1.7 ± 0.6 with NO. This finding suggests that norV does not affect stx2 expression in aerobic conditions regardless of NO availability. By contrast, growth in anaerobic conditions for 24 h showed a difference in stx2 expression in the presence of NO (Figure 3B). Specifically, the mutant ΔnorV strain had a 3.2-fold increase in stx2 expression as compared to the WT strain when NO was present. The WT and ΔnorV revealed similar transcript levels of stx2 in the absence of NO, suggesting that norV inhibits stx2 expression solely in the presence of NO.

Next, we complemented the norV deletion in trans by expressing the WT norV on the pCR2.1-TOPO plasmid in the ΔnorV strain (ΔnorV::norVWT). norV expression in the complemented ΔnorV::norVWT strain was confirmed by qPCR, though the level of norV transcription was higher in the complemented strain than the WT. We then compared stx2 expression levels in the WT, ΔnorV, and ΔnorV::norVWT strains as well as the ΔnorV strain containing the empty vector (ΔnorV::vector) using the same conditions. Increased expression in stx2 was observed in the ΔnorV strain relative to the WT (data not shown), yet a similar increase was observed in both the ΔnorV::norVWT and ΔnorV::vector strains.

3.4. Role of norV in Uptake and Survival of the Clade 8 Outbreak Strain within Macrophages

To determine how norV impacts phagocytic uptake and intracellular survival of TW14359, we infected human THP-1-derived macrophages with the WT and isogenic ΔnorV strains. At 1 h post infection, no difference in phagocytic uptake was observed between the WT and ΔnorV strains (Figure 4A). Specifically, 0.31% ± 0.04 of the total WT cells per well and 0.34% ± 0.06 of ΔnorV cells were taken up by the THP-1 macrophages after 1 h. Enhanced survival within THP-1 macrophages, however, was observed for the
ΔnorV strain 24 h post infection relative to the WT (Figure 4B). Nonetheless, this difference was not statistically significant (Student’s t-test p = 0.10). After normalizing to initial uptake levels, the percent survival of the WT and ΔnorV strains within macrophages was 2.9% ± 0.7 and 4.1% ± 0.7, respectively.

Figure 4. (A) Phagocytic uptake and (B) intracellular survival of the E. coli O157:H7 wild-type (WT) TW14359 outbreak strain relative to the isogenic ΔnorV strain in THP-1 macrophages. Uptake was examined 1 h post-infection and survival was determined after 24 h. Boxes represent the means of three independent replicates and the bars show the standard deviation of the means. No significant differences were observed between strains for either phagocytic uptake or intracellular survival using a Student’s t-test.
4. Discussion

EHEC O157 strains belonging to the clade 8 lineage were shown to be more commonly isolated from HUS cases than other lineages [10] and have an enhanced ability to adhere to epithelial cells and express stx2 and other key virulence genes [11]. These findings suggest that clade 8 strains may be more virulent than strains representing other O157 lineages. Indeed, a prior study demonstrated enhanced virulence of the clade 8 spinach outbreak strain, K3995, relative to other O157:H7 strains in both rabbits and mice, which was due to increased Stx2 production [28]. This 2006 multistate spinach outbreak also contributed to a higher rate of HUS than has been observed for other O157:H7 outbreaks [13]. Additionally, a genome sequence comparison between the clade 8 spinach outbreak strain, TW14359, and two O157:H7 outbreak strains that caused low HUS case rates, found norV to be unique to the clade 8 strain [14]. Our evaluation of 303 O157 isolates, which represented the major phylogenic lineages previously defined by SNP genotyping [10], showed that 65% possess a 204 bp deletion in norV and that all of these strains belong to clades 2 and 3. The genome sequence of the EDL933 outbreak strain representing clade 3 also shows a deletion in norV [16] as does the clade 1 strain, RIMD 050995, linked to a 1996 outbreak in Sakai Japan [17]. Comparatively, the intact, functional version of norV was found in 35% of the 303 O157 strains evaluated herein and was restricted to clades 6–9.

Additional support for the distribution of the intact and deleted versions of norV was previously observed in different O157 strain collections. The initial screen of 100 O157 strains recovered from multiple sources detected the 204 bp deletion in 58% of strains and a correlation with stx1 [14]. In our analysis, however, the deletion was most common among strains possessing both stx1 and stx2 representing clades 2 and 3, which indicates variation across the strain collections. This variation is not surprising given that Stx-encoding bacteriophages can integrate within multiple sites in different E. coli genomes [29]; hence, geographic variation could be due to the prevalence of circulating bacteriophages in certain locations. Consistent with our data, another screen of 107 distinct O157 strains confirmed the norV deletion to be restricted to clades 1–3, while the intact norV was correlated with clades 6–8 [30]. These findings indicate that norV functionality is linked to the genetic background of a given O157 strain. Additionally, the intact norV was detected in a subset of 34 EHEC strains representing 10 non-O157 serotypes [31], suggesting a more widespread distribution. The association observed between clade 8 strains, which all contained an intact norV, and the presence of stx2 alone or with stx2c illustrates the importance of the O157 genetic background on virulence. Indeed, other strains with both an intact or deleted version of norV possess these stx subtypes either alone or in combination with stx1, suggesting that other factors also contribute to enhanced virulence in clade 8 strains. Examining norV function and its relationship with virulence is therefore warranted in a larger collection of clade 8 strains.

In anaerobic conditions, we demonstrated that NO induces stx2 expression in the clade 8 spinach outbreak strain, TW14359, resulting in a > 2-fold increase of stx2 transcription compared to growth in the absence of NO. In aerobic conditions, however, similar stx2 transcription levels were observed in the presence and absence of NO. As the primary habitat for EHEC during infection is in the oxygen restricted intestine, it can encounter stressful conditions that may include NO produced by macrophages or endothelial and epithelial cells [32]. Our data showing increased stx2 expression in TW14359 in anaerobic conditions, which mimic the gut environment, differ from responses observed in other O157 strains. For instance, in the clade 3 O157:H7 EDL933 outbreak strain, NO contributed to a decrease in Stx2 production by repressing the SOS response via the nitrite-sensitive repressor (NsrR) that senses NO [33]. NO was also shown to reduce expression of LEE-associated virulence genes, resulting in decreased adherence to epithelial cells in vitro [34]. These results, however, differ from those generated in another study using the same strain, EDL933, which found low NO concentrations induce production of Stx2 in anaerobic conditions [35]. The same study showed that a higher concentration of NO was needed...
for Stx1 production as well as Stx2 production in aerobic conditions, highlighting the complexity of NO signaling in EHEC O157.

Since NorV was previously shown to reduce NO, we sought to investigate the relationship between NorV and stx2 expression in the clade 8 TW14359 strain. Notably, the ΔnorV null mutant showed increased expression of stx2 relative to the wild-type (WT) strain, indicating that NorV inhibits stx2 expression in this strain. Expression of norV in trans on a plasmid in the ΔnorV strain, however, failed to decrease stx2 expression to levels similar to those observed for the WT strain. This failure to complement the WT phenotype is likely due to the use of ampicillin during the growth of the complemented strain since the pCR2.1-TOPO plasmid has an ampicillin resistance gene for selection. Multiple antibiotics, including ampicillin, have previously been shown to induce stx expression [36]. Contrary to our findings, Shimizu et al. [18] showed that by replacing the deleted norV copy found in the clade 3 EDL933 strain with an intact norV copy, stx2 expression was increased in the presence of NO. This finding suggests that NorV induces the expression of stx2 in EDL933. Moreover, another study showed that deleting norVV in the O157:H7 strain 620 was linked to a reduction in Stx production and persistence in the murine gut during infection; however, deletion of norVV in an O91:H21 strain (B2F1) had no impact on Stx production or persistence [31]. The reasons behind these phenotypic differences are not completely understood, although they are likely due to a variation in gene regulation and expression across O157 strains.

As part of the innate immune response to eliminate bacterial pathogens, NO is produced by the inducible NO synthase (iNOS) pathway within macrophages [37], which subsequently induces the bacterial SOS response. Therefore, we examined the role of norV in the survival of the O157:H7 strain TW14359 within THP-1 derived macrophages. Compared to the WT strain, the ΔnorV mutant was not impaired in its ability to survive within macrophages. This finding suggests that NorV is not required for NO detoxification by strain TW14359 to enhance survival inside macrophages and that other factors may be necessary. Our preliminary finding that norV expression did not differ in TW14359 in the presence or absence of NO (n = 2; data not shown) provides further support for this hypothesis. This finding, however, requires confirmation and an additional set of clade 8 strains should be evaluated to better define strain-specific responses.

In addition to NorV, EHEC was shown to possess hmp and hcp encoding a flavohemoglobin (Hmp) [38] and high affinity NO reductase (Hcp) [39], respectively, which act independently to lower the toxicity of NO in different conditions [40]. Therefore, it is likely that either Hmp or Hcp assist with NO detoxification in TW14359 to promote survival inside macrophages and that one or both products could compensate for the loss of NorV activity. Indeed, NorV and Hmp were previously found to work synergistically during anaerobic growth [40]. Our results are consistent with those from another study demonstrating that survival of a norV mutant in the non-pathogenic E. coli strain MG1655 was not impaired in J774.2 mouse derived macrophages compared to the wild-type strain [41]. Similarly, no significant differences were observed in the survival of a Salmonella enterica Typhimurium strain within murine macrophages relative to the isogenic mutant lacking norV [42]. Contrary to our results, however, the number of surviving bacterial cells within murine macrophages following infection with the EHEC O157 K15 strain containing an intact norV was greater than the number following infection with a norV deficient mutant [18]. While this finding suggests that norV confers a protective advantage in resisting killing within macrophages for strain K15, the discrepant results are most likely due to the use of different O157 strains. Unlike TW14359, which belongs to clade 8 and contains an intact norV, strain K15 was found to represent clade 7 [30], the lineage linked to less severe symptoms in our prior study [10], despite having an intact norV. There is also the possibility that the RAW264.7 mouse derived macrophage cells, which were used to examine the survival of the EHEC K15 strain after 10 h [18], may differ from the human derived THP-1 cells used herein. Indeed, a prior study observed key differences in protein expression profiles between mouse-derived RAW264.7 macrophages and THP-1 cells [43].
In summary, our study shows that NO induces the expression of stx2 in anaerobic conditions in the clade 8 spinach outbreak strain, TW14359, thereby increasing its pathogenicity. With that in mind, selected inhibition of NO production in the host could become the focus of extensive pharmaceutical research in the future. We also demonstrated that NorV inhibits stx2 expression in TW14359, which is the opposite of what we might expect when considering the enhanced virulence of clade 8 strains. While inhibiting Stx production is important for ensuring bacterial survival, a prior study showed that varying NO concentrations can induce different Stx production pathways [35], further highlighting the complex relationship between NO signaling and Stx regulation. These data are relevant since the level of Stx inhibition likely varies across conditions and environments encountered in the host. As we have shown that O157 strains can have variable levels of stx expression [11,44,45], it is clear that strain-specific factors can also impact transcriptional regulation. Given the importance of strain-specific differences, a more comprehensive investigation into how norV impacts stx expression in other clade 8 strain backgrounds is therefore warranted. Lastly, in contrary to data generated in another study [18], we showed that norV is not required for the survival of TW14359 within THP-1 macrophages. This finding further suggests that the mechanisms for surviving in the presence of nitrosative stress, particularly following phagocytic uptake, vary across strains and that these mechanisms are complex and may require multiple factors that differ depending on the environment.

5. Conclusions

Collectively, our investigation of NorV in a clade 8 strain from a 2006 multistate outbreak linked to spinach consumption suggests that it plays a role in the inhibition of Stx2 production. The specific mechanism of inhibition, however, remains unclear and requires further study.

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Informed Consent Statement: Patient consent was waived due to the use of specimens and data collected for public health purposes.

Data Availability Statement: All data supporting the results can be found within the manuscript.

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