INTRODUCTION

Though the use of antibiotics is by far the most important iatrogenic causal factor (Peterfreund et al., 2012) for the development of *Clostridium difficile* infection (CDI), recently considerable attention has been focused on the role of proton pump inhibitors (PPIs) in promoting CDI (Yearsley et al., 2006; Kim et al., 2012). PPIs are a frequent outpatient medication, and they are near-ubiquitous in their use among hospitalized subjects (Fraeyman et al., 2013). With several PPIs now available as over-the-counter medications, and considering the general perception on the part of physicians and patients that this class of drugs is predominantly safe (McGinn et al., 2010), PPI use is currently at its greatest prevalence since the introduction of this drug class (Chuong et al., 2013). If direct evidence was forthcoming that these drugs might promote CDI, this discovery would result in a significant change in medical practice patterns towards limiting the scope of PPI use.

There is mounting evidence, almost solely from retrospective comparative studies, that the use of PPIs is associated with the development of both primary and recurrent CDI (Aseeri et al., 2008; Akhtar & Shaheen, 2007). Though recent publications are not unanimous in this conclusion (Rotramel et al., 2012), there exists a plurality of scientific evidence which strongly suggests that PPIs are an independent risk factor for the development of CDI among hospitalized patients. Several systematic reviews have further confirmed this relationship (Bavishi & Dupont, 2011; Leonard et al., 2007). However, there are myriad unanswered questions pertaining to the mechanism(s) by which PPIs promote CDI. Though previous publications on the topic have assumed that gastric acid suppression is a key gut-related factor to this phenomenon, there is no direct evidence thus far that the majority of CDIs in hospitalized human subjects begin with faecal–oral transmission, nor is there broad scientific affirmation that acid suppression allows ingested *C. difficile* organisms to survive the environment of the stomach would be more likely to create symptomatic CDI. Although the faecal–oral paradigm is conceptually appealing, it remains to be

Correlation between virulence gene expression and proton pump inhibitors and ambient pH in *Clostridium difficile*: results of an in vitro study

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Proton pump inhibitors (PPIs) are associated with the development of *Clostridium difficile* infection in humans. Though it is assumed that PPIs mediate this effect through gastric acid suppression, there has been little investigation into whether PPIs, or ambient pH, might directly affect the expression of *C. difficile* toxin genes. In the present study, *C. difficile* ribotypes 001, 027 and 078 obtained from human subjects were grown under anaerobic conditions prepared at pHs of 5, 7.3 and 9. Matched trios were exposed to 100 μM and 200 μM of omeprazole along with PPI untreated controls. Custom designed reverse transcription quantitative PCR hydrolysis probes were used to assess *C. difficile* gene expression for toxins A (tcdA), B (tcdB) and binary toxin (cdtB), as well as their positive regulators (tcdR and cdtR), using rrsA, which encodes 16S rRNA, as a constitutively expressed reference gene. tcdC and codY, negative regulators of toxin expression, were also assessed. Basic pH resulted in greater expression of tcdA, and with PPI exposure a 120-fold higher expression was noted with ribotype 001. tcdB and cdtB expressions were much less responsive to pH or PPIs, though a clear response to acidic pH and PPI exposure was observed in ribotype 027. tcdC and codY expressions were largely unaffected, except with ribotype 027; low pH and PPIs resulted in their greater expression, though to a lesser degree than with toxin genes and their positive regulators. Non-neutral pH and PPI exposure appear to have an effect on *C. difficile*, one that has a net effect towards toxin gene expression.

Abbreviations: BHIB, brain heart infusion broth; CDI, *Clostridium difficile* infection; IRB, Institutional Review Board; PPI, proton pump inhibitor; RT-qPCR, reverse transcription quantitative PCR.

Seven supplementary figures and two supplementary tables are available with the online version of this paper.
established on empirical grounds. This is a key issue, since the faecal-oral construct serves as the basis for proposing that PPIs promote CDI principally through their effects on gastric acid suppression.

A clinically relevant yet unanswered question relates to whether PPIs might have a direct effect on *C. difficile*, perhaps promoting toxin production or inducing other virulence behaviours apart from their effects on gastric pH. If such an influence was demonstrated, a further understanding of the potential dangers of PPIs would be provided, and a greater restriction on their seemingly promiscuous administration would be warranted. This would also introduce a broader concept that all drugs, including PPIs, may have an effect on *C. difficile* that is both different to the intended mechanism of action of the drug, and may create previously unrecognized consequences for *C. difficile* as well as other members of the gut microbiome.

The present study is the first to investigate whether PPIs have a direct effect on the expression of *C. difficile* genes associated with virulence as assessed using reverse transcription quantitative PCR (RT-qPCR) techniques. The effect of ambient pH on bacterial gene expression is also investigated, which is another uncertain variable in *C. difficile* activity. The comparative effects of PPIs on gene expression, as well as the effects of escalating doses of PPIs and varying ambient pHs, are described. The present work provides the first evidence, seminal as it may be, that PPIs may, in fact, promulgate CDI through mechanisms unrelated to their effects on pH, and that the manner by which they promote CDI is much more complex and multifaceted than has been previously appreciated.

**METHODS**

This was an Institutional Review Board (IRB)-approved study performed using clinical *C. difficile* isolates obtained from *C. difficile*-infected patients at least 18 years of age who were treated at our institution and who consented to be enrolled in an IRB-approved *C. difficile* biobank belonging one of the authors (D.S.).

**C. difficile culture and omeprazole treatment.** Pre-reduced brain heart infusion broth (BHIB, pH 7.3) and pH-adjusted BHIB (pH 5.0 and pH 9.0) were prepared by addition of 1 N HCl and 1 N NaOH, respectively. Three *C. difficile* isolates represented by ribotypes 027 (toxinotype III), 078 (toxinotype V) and 001 (toxinotype 0) were grown under anaerobic conditions at 37 °C. Early stationary phase (3 McFarland units; OD600 = 0.5) cultures were split into matched trios and were treated with 100 μM or 200 μM omeprazole. These preparations were then incubated under strict anaerobic conditions with their matched, PPI untreated controls for either 4 or 12 h. Appropriate 1 ml cell aliquots for each ribotype, broth pH and omeprazole dose were collected at 4 and 12 h time points and mRNA transcripts were immediately stabilized by the addition of RNA later reagent (Life Technologies).

**RNA isolation.** Total RNA was extracted using aliquots from all cell treatments and controls. Cells were harvested by centrifugation at 16 000 g for 15 min, and cell walls were digested by resuspension in 500 μl freshly prepared lysozyme buffer (50 mM glucose, 100 mM NaCl, 10 mM EDTA, 10 mM Tris/HCl pH 8.0) containing 0.5 μl Ready-Lyse™ lysozyme (Epitrend Biotechnologies) for 20 min. Whole cell lysis was achieved using a hot-TRIZol (Life Technologies) extraction method. TRIZol reagent was added to cells followed by incubation at 65 °C for 15 min, with intermittent vortexing. Cell lysates were extracted with chloroform, followed by centrifugation at 12 000 g for 15 min. Upper aqueous phases were then mixed with an equal volume of RNase-free 70% EtOH, and crude RNA extracts were clarified by passage through RNeasy mini columns (Qiagen Sciences). Trace genomic DNA was removed by a 40 min on-column digestion with 0.5 Kunitz units RNase-free DNase I (Qiagen Sciences) and the columns were subsequently washed as per the manufacturer’s directions. Purified total RNA was eluted with RNase-free water and final total RNA yields were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). RNA samples with A260/A280 absorbance ratios between 1.80 and 2.10 were considered acceptable for cDNA synthesis. Final RNA concentrations were adjusted to 50 ng μl⁻¹ and were stored at −70 °C.

**cDNA synthesis.** Absence of contaminating genomic DNA was determined by a 35-cycle PCR using 16S rRNA gene primers and 50 ng RNA template. cDNA syntheses were performed on 450 ng total RNA using the Invitrogen SuperScript III First-Strand synthesis system (Life Technologies) with 50 ng μl⁻¹ random hexamer primers. Synthesis products were then digested with RNase-H for 20 min at 37 °C, and were stored at −20 °C.

**Toxin-related gene expression.** Expression levels of toxin-related genes (Table 1) were measured using custom qPCR hydrolysis probe assays (TaqMan Gene Expression Assays, Applied Biosystems) for the following seven target genes of interest: toxin A (*tdaA*), toxin B (*tdbB*) and binary (*cdtB*), as well as the toxin-regulatory genes *tdIR* (an RNA-polymerase bacterial sigma factor for the expression of the genes for toxins A and B), *tdcC* (a putative negative regulator of toxin and A and B expression whose gene product sequesters sigma factors, thus preventing formation of an RNA holoenzyme), *cdtR* (a response regulator that controls *cdtB* expression) and *cchV* (a global negative regulator of toxin gene expression found in many low G+C content Gram-positive bacteria). Primer pairs were validated by dissociation curves, and amplification efficiencies were determined using serially diluted genomic DNA. Each qPCR assay primer and probe sequence is listed in Table 1. TaqMan assays (10 μl) were designed and conducted in triplicate following Minimum Information for the Publication of qPCR experiments guidelines where applicable (Bustin et al., 2009) and using Applied Biosystems Universal Assay Buffer with 100 ng cDNA (except for 16S, at 1 ng) per reaction. Following 15 min activation at 95 °C, reactions were amplified for 45 cycles in an ABI Prism 7900HT Sequence Detection System (SDS software v2.4). Co-amplification of the constitutively expressed rrsA gene, which encodes 16S rRNA, served as an endogenous reference control, where relative quantity of target gene expression was calculated from exponential-phase mean threshold cycle values normalized to reference gene expression. The threshold cycle (Ct) was defined as the fractional cycle number at which fluorescence exceeded the fixed threshold of 0.1 with automatic baseline measurements using Applied Biosystems RQ Manager software v. 1.2 (Applied Biosystems). Gene expression levels (fold change) relative to the comparison group (pH 7.3 and PPI untreated) were calculated using the comparative ΔΔCt method (Schmittgen & Livak, 2008). Expression results were log-transformed to ensure normality of relative quantities of gene expression.

As this study dealt with technical replicates, as opposed to biological replicates, measurements of fold change for gene expression were utilized, and statistical comparisons between technical replicates were not performed as this was not felt to be mathematically or methodologically appropriate.

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RESULTS

Table S1 (available in JMM Online) provides a list of RT-qPCR results for each *C. difficile* gene of interest. These results are summarized below. Figures pertaining to tcdA, tcdB and cdtB, which graphically represent fold change in gene expression, are provided herein, while figures for the remaining genes of interest are provided as supplementary materials. These graphs are presented as bars indicating the geometric mean of relative fold-change gene expression normalized to the levels of 16S rRNA, with error bars representing ±95% confidence intervals. As these are technical replicates they are not intended as a means of comparison between different groups, but rather they demonstrate variation in a single group under technical replication.

### Results for toxin genes

For *tcdA* expression (Fig. 1), at 4 h, without PPI treatment, an acidic pH promoted a 3.4-fold increased expression of *tcdA* in ribotype 027; ribotypes 001 (2.3-fold) and 078 (1.8-fold) each evidenced increased expression, but to lesser degrees than ribotype 027. At a basic pH, all ribotypes manifested a significantly higher expression of the toxin A gene as compared to other pHs, with ribotype 001 having the largest measured increase expression at 35-fold.

With PPI treatment, similar trends were observed. At a neutral pH, PPI exposure resulted in no measurable change in the expression of *tcdA*, with the exception of a twofold increase in expression for ribotype 027 with exposure to the highest dose of PPI. For both basic and acidic pHs, a higher degree of expression with PPI exposure was measured; with a basic pH, the greatest increase in expression was observed in ribotype 001 with exposure to 200 μM of PPI (120-fold increase).

At the 12 h time point, similar trends between the treatment groups were noted as above, though with smaller differences in measured fold-changes.

For *tcdB* expression (Fig. 2), at the 4 h time point, without PPI treatment, there was almost no difference in gene expression measured across varying pHs, with the

### Table 1. Sequences for hydrolysis probes used in qPCRs

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Oligonucleotide sequence (5′–3′)</th>
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<tbody>
<tr>
<td>tcdA</td>
<td>Forward primer GGTAATAATTCAAAACGGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse primer AGCATCGTATAGGCAGATG</td>
</tr>
<tr>
<td></td>
<td>Reporter probe AGGCTAATACAGCTATGGGTGAA</td>
</tr>
<tr>
<td>tcdB</td>
<td>Forward primer GTAATTAGTTAGTGACAGCAATCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer CCAATTATACCTATCTTAGCTTCTTAC</td>
</tr>
<tr>
<td></td>
<td>Reporter probe ATGGGTCACTCGTTAC</td>
</tr>
<tr>
<td>tcdC</td>
<td>Forward primer ACCAACATCATGTATAGTCAAAACAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer CAAATAGCTTCTTCTTGAACAGCTACT</td>
</tr>
<tr>
<td></td>
<td>Reporter probe CAGCATCGAAGAAACAG</td>
</tr>
<tr>
<td>codY</td>
<td>Forward primer TCTTTTCTGAAAGATAATGATAACAA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer TGAATTTGAAATACCTTGAAGAAGACAG</td>
</tr>
<tr>
<td></td>
<td>Reporter probe GAGCACTATTACATG</td>
</tr>
<tr>
<td>cdtB</td>
<td>Forward primer TCAAAATGGATTCACTACATGTAACTACA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer CAGATTTCCATATCCTTTCACTATCTT</td>
</tr>
<tr>
<td></td>
<td>Reporter probe ACGGCATTTGAATAT</td>
</tr>
<tr>
<td>cdtR</td>
<td>Forward primer GGATTTGATTAGTTAGTAAAGTTAATTGAC</td>
</tr>
<tr>
<td></td>
<td>Reverse primer GTATCTTTTGTGACCTTTATACAGCTTT</td>
</tr>
<tr>
<td></td>
<td>Reporter probe ACTTTGACATTTCA</td>
</tr>
<tr>
<td>16s</td>
<td>Forward primer TTAGCGATTTACTTCCGTTAAGA</td>
</tr>
<tr>
<td></td>
<td>Forward primer CCATCTGTACTGGTCACCT</td>
</tr>
<tr>
<td></td>
<td>Reporter probe CCGGGACGGGTGACTAA</td>
</tr>
</tbody>
</table>

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http://jmm.sgmjournals.org
exception of an 8.4-fold increase in *tcdB* expression in ribotype 027 at pH 5. As opposed to the trends observed with *tcdA*, *tcdB* expression did not appear to respond uniformly to PPI treatment, and there was also no clear response to gene expression with escalating doses of omeprazole. The greatest fold change measured with PPI exposure was observed in ribotype 027 at pH 5 with 100 μM PPI (21-fold increase). The addition of 200 μM PPI actually resulted in a slight decrease in *tcdB* expression in this ribotype (19-fold increase compared to reference gene).

Similar findings were observed for each group at the 12 h time point.

For *cdtB* expression (Fig. 3), ribotype 001 is *cdtB* negative, and therefore is not included in the results related to binary toxin and its regulator. Without PPI exposure, there was little change in *cdtB* expression based on varying pH alone, with the exception of a 5.9-fold increased expression in ribotype 027 at pH 5. Gene expression in ribotype 027 increased with PPI exposure, though without a clear response to escalating PPI doses, with the highest fold increase observed with 100 μM PPI at pH 5 (18-fold).

Similar trends were observed at the 12 h time point, with almost identical results compared to those measures at the 4 h time point.

**Regulator genes for toxin expression**

For *tcdR* expression (Fig. S1), with no omeprazole treatment ribotype 027 demonstrated a significantly increased expression of *tcdR* (34-fold higher) at pH 5; no change to the expression of this gene was noted for this ribotype at other pHs. Ribotypes 078 and 001 manifested no changes in *tcdR* expression without PPI exposure.

With PPI exposure, ribotype 027 evidenced the greatest increase in gene expression at an acidic pH, with an even higher degree of gene expression measured with escalating doses of PPI (138-fold at 100 μM omeprazole; 152-fold at 200 μM omeprazole). Ribotype 078 was found to have a 10-fold higher *tcdR* expression with 100 μM omeprazole at pH 5, but otherwise demonstrated no change to gene expression with PPI exposure. Ribotype 001 produced a small 1.9-fold change with 100 μM of PPI at pH 7.3, and a 6.1-fold increase in gene expression at pH 9 with 200 μM PPI, but was otherwise unaffected by omeprazole dosage or pH changes.
Similar trends were noted with measurements for the three ribotypes at the 12 h time point.

For \( tcdC \) expression (Fig. S2), without exposure to omeprazole ribotype 027 demonstrated the largest increase in gene expression (8.6-fold at pH 5). At neutral and basic pHs, no difference in \( tcdC \) expression was measurable for this ribotype. Ribotypes 078 (2.3-fold increase at pH 7.3) and 001 (4.8-fold increase at pH 9) demonstrated lesser degrees of increased gene expression with variations in ambient pH. PPI exposure evoked the greatest increase in gene expression in ribotype 027 at pH 5, though without evidence of a PPI dose-dependent effect. At pH 7.3 and 9, exposure to the 200 \( \mu M \) PPI dose elicited a slightly higher degree of \( tcdC \) expression in ribotype 027 compared to the 100 \( \mu M \) dose, though the relative difference was small.

Similar trends were observed with ribotypes 078 and 001, with the exception that at pH 9, the addition of 200 \( \mu M \) PPI resulted in a 17-fold increase in gene expression. Similar trends were noted at the 12 h time point for all ribotypes.

For \( cdtR \) expression (Fig. S3), without PPI exposure neither ribotype 027 nor 078 demonstrated changes in expression at pH 7.3 compared to reference gene expression. Both ribotypes increased gene expression at extremes of pH, although with trends that were in mirror image. Ribotype 027 produced a 9.2-fold increased expression at pH 5, with a 2.3-fold increased expression at pH 9. For ribotype 078, gene expression was only 1.5-fold higher at pH 5, but rose to a 6.8-fold higher expression at pH 9. With exposure to PPIs, both ribotypes increased gene expression, especially at non-neutral pHs. Ribotype 027 was the most responsive to PPI exposure, though there was no obvious dose-dependent change in gene expression for either ribotype 027 or 078. No significant difference in these trends were noted at the 12 h time point, with the exception of a 37-fold increase in gene expression for ribotype 027 at pH 5 with a 200 \( \mu M \) PPI dose, as compared to a 30-fold increase at just 4 h.

For \( codY \) expression (Fig. S4), ribotype 027 demonstrated the greatest response in gene expression among all three ribotypes. Without PPI exposure, pH 5 elicited a sixfold increase in \( codY \) expression, while at pH 9 only a 1.7-fold increase was measured. The other ribotypes were largely unresponsive to changes in pH, and none of the ribotypes demonstrated a difference in \( codY \) expression compared to the reference gene at pH 7.3. With the addition of PPI, no consistent dose-dependent change in gene expression was observed. Ribotype 027 manifested the greatest increase in gene expression at pH 5 (16-fold with 100 \( \mu M \) omeprazole), with 1.7–4.5-fold increases at neutral and basic pHs. The other ribotypes were largely unresponsive to PPI therapy, and no clear differences were noted between the 4 and 12 h time points.

**DISCUSSION**

To the authors’ knowledge, this is the first study to demonstrate that PPI therapy has a direct effect on the expression of \( C. \) difficile genes, both related to toxin production and toxin gene regulation. This introduces a completely new perspective for how the risk of developing CDI should be viewed from the standpoint of PPIs in particular, and for medications in general. The findings in the present work provide a basis for viewing pharmaceuticals in a bi-faceted manner, one of which focuses on the intended mechanism of action of the drug, and the other takes account of the potential unintended direct effects that drugs may have on organisms of the gut, apart from their intended instrumentality.

The present study provides information describing how ambient pH affects \( C. \) difficile gene expression, which has been underappreciated in the currently published literature. One of the more interesting observations from this study pertains to how extremes of pH had the tendency to promote the greatest degree of increased gene expression. The pHs chosen in this study would, in some respects, fall outside those commonly encountered in the human alimentary tract (pHs of 9, for example). These parameters were chosen in order to help describe the extent to which pH might affect \( C. \) difficile, even if beyond a clinical
context. When adding considerations regarding the influence of PPI therapy to those regarding pH, our data suggest that the success of PPI therapy in humans may at times have a synergistic effect on promoting CDI. For example, tcdR expression was increased 34-fold in ribotype 027 at pH 5 without PPI exposure. With PPI treatment, tcdR expression increased 138–152-fold depending on the dose of PPI chosen. This would suggest that administering PPIs without achieving the desired alkalization of the stomach may actually promote the expression of toxin genes in the human gut, and to a greater degree than either pH or PPIs could individually induce. Alternatively, the gene for toxin A was expressed to a much higher degree in alkaline environments, and this effect was exaggerated when PPIs were added. This would suggest that there are also circumstances in which elevations of gut pH, the goal of PPI therapy, may actually promote expression of some of the toxin genes in C. difficile. The manner in which all of these data fit together in a clinically relevant, cohesive archetype is not entirely clear from these seminal data, and will require further study with in vivo models. Our data on the effect of pH suggests that even transient exposure to an alkalinalized stomach may enable C. difficile to be more able to colonize effectively the colon and to promote CDI by stimulating toxin gene expression.

One observation from the present work is that C. difficile response to both pH and PPI therapy may differ based on ribotype, and the response of the organism to pH or PPI therapy is not uniform. As a corollary to PPI effects, any discussion of C. difficile in general may be too broad and non-specific to reach valid conclusions without some further subcategorization of the type of C. difficile being discussed. The use of PCR ribotyping is slowly falling out of favour (Walk et al., 2012; Cloud et al., 2009; Morgan et al., 2008), since it has failed to correlate with clinical CDI outcomes, as had been initially anticipated. What constitutes the proper schema for genetic characterization of C. difficile along epidemiologically relevant lines continues to be debated, and in the absence of a newer, widely accepted taxonomy, ribotype descriptions remain a useful index for describing C. difficile between investigators. It is clear, however, that parsing the organism based on ribotype may not provide for a classification system that correlates with the behaviour of the organism, as suggested by the data in this study. For example, ribotype 001 produced the highest fold increase in tcdA expression in the present study (at pH 9 with 200 μM omeprazole), yet this ribotype is not known to be particularly virulent. It is quite likely that certain ribotypes harbour genetic and epigenetic factors that, when combined with environmental cues such as pH and various medications, lead to more virulent disease behaviour under certain conditions. Ribotype may simply be loosely associated with these yet undiscovered, but more clinically valuable, factors.

The present study has several limitations. Firstly, as with any in vitro study, the conditions defined within a research lab may be far removed from those of the human gut, and since the organism responds to its environment, the artificiality of the test environment can lead to limitations in the clinical application of the study results. However, to this the authors would point out that the purpose of the present study was largely directed towards ‘proof of concept’ that PPIs can affect C. difficile apart from pH, and it is readily acknowledged that more detailed studies will be required before a clinically relevant model of PPI influence is forthcoming. Secondly, the present study used RT-qPCR measurements, which are not always identical to a corresponding concentration of fully functional gene product. However, although still complex in their genetic controls, prokaryotes such as C. difficile have much less post-translational modification and fewer post-translational control mechanisms than do eukaryotes, and again, as a proof of concept study, the authors believe that the present work makes a strong case that the changes in gene expression measured herein would not have been dissipated by some post-transcriptional element(s) not accounted for in the use of PCR techniques. Thirdly, the present study deals with technical replicates, and in keeping with the need for in vivo studies with rodents, and eventually with humans, biological replicates will be needed. Fourthly, the study did not attempt to model the nutrient environment of the human colon, though since all of our cultures were identically treated sans pH and PPI influences, the net effect of this factor is anticipated to be nullified. Fifthly, there remains a question as to how PPIs administered to humans interact with C. difficile specifically in the alimentary tract. This has to do with issues of relevant dosages of PPIs in humans, as well as luminal exposure of PPIs in the gut. There is a precedent in the literature (Cederberg et al., 1992; Shin & Kim, 2013; Mirshahi et al., 1998; Suzuki et al., 1996) for the dosages of PPI used in the current study, and the authors are currently working on further evaluation of PPI effects in the human host, including PPI effects on gene expression and tight junction regulation in colonocytes, which along with the present data would help to guide PPI studies on C. difficile in a rodent model so that PPI effects on C. difficile and other gut organisms can be assessed in vivo.

PPIs exert an effect, independent of ambient pH, on C. difficile as measured by the expression of toxin genes and their regulators. This influence towards promoting gene expression has a synergistic effect with alkaline and acidic pHs, depending on the gene in question. The present findings indicate that PPIs promote C. difficile virulence behaviours in a manner independent of their effect on pH, but which can be augmented by changes in pH. Further studies towards detailing their mechanism of action are required.

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