Drivers of *Clostridioides difficile* hypervirulent ribotype 027 spore germination, vegetative cell growth and toxin production *in vitro*.

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ABSTRACT

Objectives *Clostridioides difficile* infection (CDI) is a considerable healthcare and economic burden worldwide. Faecal microbial transplant remains the most effective treatment for CDI, but is not at the present time the recommended standard of care. We hereby investigate which factors derived from a healthy gut microbiome might constitute the colonisation resistance barrier (CRB) in the gut, inhibiting CDI.

Method CRB drivers pH, short chain fatty acid (SCFA), and oxidation-reduction potential (ORP) were investigated *in vitro* using *C. difficile* NAP1/BI/027. Readouts for inhibitory mechanisms included germination, growth, toxin production and virulence gene expression. pH ranges (3 – 7.6), SCFA concentrations (25 – 200mM) and ORP (-300 - +200mV) were manipulated in brain heart infusion broth cultures under anaerobic conditions to assess the inhibitory action of these mechanisms.

Results <pH 5.3 completely inhibited *C. difficile* growth to OD of 0.019 vs. 1.19 for control pH 7.5. Toxin production was reduced to 25 units vs 3125 units for pH 7.6 (1 in 5 dilutions). Virulence gene expression reduced by 150 fold compared with pH 7.6 ($p<0.05$). Germination and proliferation of spores below pH 6.13 yielded an average OD of 0.006 vs. 0.99 for control. SCFA were potent regulators of toxin production at 25mM and above ($p<0.05$). Acetate significantly inhibited toxin production to 25 units independent of OD (0.8733) vs. control (OD 0.6 and toxin titer 3125) ($p<0.05$). ORP did not impact *C. difficile* growth.

Conclusion This study highlights the critical role that pH has in the CRB, regulating CDI *in vitro* and that SCFA can regulate *C. difficile* function independent of pH.

INTRODUCTION

Clostridioides difficile (*C. difficile*) remains a major cause of antibiotic associated diarrhoea and ulcerative colitis in the healthcare setting (1-3). The gut microbiota plays a vital role in protection against opportunistic pathogen infections and in many disease states an altered or dysbiotic gut microbiota is observed (4-7). A competent gut microbiota and environmental conditions created in the gut forms a barrier to infectious colonisation and is a main contributor to protect against enteric infection (7). A gut epithelial mucus layer forms a physical barrier between bacteria and host (8) moderating interaction of opportunistic pathogens with the gut epithelium and preventing bacterial translocation and infection (9). The gut microbiota/host interactions are also fundamentally important for training host innate immune responses to pathogen burden (10).

There is compelling evidence that oxidation-reduction potentials (ORP), pH and short chain fatty acids (SCFA) govern microbial colonisation in the gut (3, 4, 10-12). SCFA have also been shown to downregulate pathogenic virulence factors in enterohaemorrhagic *E. coli* O157:H7 by inhibiting shiga toxin synthesis (13). These protective mechanisms are collectively termed the colonisation resistance barrier (CRB).

Pinpointing an effective therapeutic for *C. difficile* infection (CDI) is challenging. Current treatment for CDI has changed from metronidazole and vancomycin to fidaxomycin and vancomycin (14-16). Fidaxomycin carries a lower risk of recurrence, but its cost-effectiveness is a topic of debate (17). The main risk factor to CDI is gut microbiota dysbiosis (12, 18), associated with age and/or administration of broad-spectrum antibiotics. Dysbiosis leads to an impairment in the CRB yielding opportunities for pathogenic bacteria to proliferate (3, 11, 12). Maintaining a diverse microbial ecosystem in the colon supports

adequate pH control, optimum ORP and increased SCFA production (19, 20), crucial elements of the CRB. In the present study, we sought to investigate which elements of the CRB act as drivers of virulence, germination and growth inhibition of *Clostrioides difficile* hypervirulent ribotype 027.

MATERIALS AND METHODS

Culture conditions for vegetative cells and spores

C. difficile NAP1/BI/027 (BAA-1803, Hall and O'Toole Prevot, ATCC, USA) was prepared alongside a third passage working cell bank and stored in 10% glycerol at -80°C. Unless otherwise stated, unsupplemented Brain Heart Infusion (BHI) (Oxoid, UK) broth with a final pH=7.5 was used for all growth experiments (19, 20). An anaerobic 1% inoculum of ~10^6 cells was used for each experiment (Don Whitley A45 anaerobic workstation; anaerobic gas mix (10% CO_2, 10% H_2, 80% N_2)) at 37°C for 48 hours.

Preparation of spore suspension

A spread of *C. difficile* ATCC 1803 was carried out on cycloserine/cefoxitin (250 mg/L and 8 mg/L respectively) supplemented BHI agar plates (to select for *C. difficile* and avoid contaminant organisms). The plates were incubated at 37°C anaerobically for approximately 7-10 days to encourage sporulation. Colonies were transferred to 1:1 sterile PBS (1%) and ethanol (100%) for at least 72 hours aerobically to purify the spore suspension. The spores were then centrifuged (4000 x g for 10 minutes), washed twice and resuspended in sterile 1% PBS before 1:10 serial dilutions were plated onto blood agar using the modified Miles and Misra drop plate technique (21) for enumeration.

pH experiments

pH ranges reflecting the gastrointestinal tract were examined (pH 3.3-7.6). Sterile BHI broth was prepared at appropriate pH with 0.1M citric acid and 0.2M disodium phosphate buffer in 10mL glass universals and pre-equilibrated anaerobically overnight. Vegetative cell and spore suspensions were used at a 1% inoculum of an overnight culture or stored spore suspension respectively (~10^6 CFU). Challenged vegetative cells were incubated for 48 hours anaerobically and spore suspensions for 72 hours anaerobically.

Continuous culture

A glass 500mL continuous culture vessel was sealed and filled with sterile pH buffered BHI with an anaerobic gas mix (10% CO_2, 10% H_2, 80% N_2) bubbled through the vessel. Vessel media (pH=7.5) was heated to 37°C on a hotplate/magnetic stirrer in a laminar flow hood and inoculum (5mL vegetative cells) added to media and continuously transferred to the vessel at 1 mL/minute. pH was adjusted by feeding appropriate pH buffered BHI (as described in pH experiments). pH of the vessel was continually monitored; samples were measured for OD (600nm) and purity streaks were conducted daily.

Manipulating ORP of BHI broth

100mL sterile, pH buffered BHI broth (pH 5.37, 6.65 and 7.07) was pre-equilibrated overnight anaerobically. An ORP probe (Cole Parmer, UK) was disinfected in 2% Virkon (v/v) for 15 minutes before being rinsed in sterile dH_20. The ORP probe was calibrated using pH 4 and pH 7 buffer saturated with quinhydrone. BHI broth ORP was continually measured (mV) as filter sterilised 4-5 mL of 2% (v/v) potassium ferricyanide was added to raise the ORP to + 150mV (19) or above until the level plateaued. The broth was then immediately inoculated with 1 mL of an overnight culture of *C. difficile*. Cultures were incubated for 48

hours anaerobically at 37°C. E_h readings were taken at 24 and 48 hours, followed by measurement of optical density (600nm) at 48 hours.

SCFA concentrations in BHI broth

BHI broth was buffered to desired pH ranges between pH 3.3-7.6 and sodium salts of SCFA (acetate, butyrate and propionate) were prepared in a 2M stock solution in H_2O and filter sterilised. Concentrations of SCFA used in experiments ranged from 25 – 300mM.

Vero cell culture conditions and assay design

African green monkey kidney vero cells (kindly provided by Dr Gillian Douce, University of Glasgow, UK) were cultured in 75cm^2 tissue culture flasks (Starlab, UK) using Eagle's Minimum Essential Medium (MEM) (Sigma Aldrich, UK) supplemented with 10% faecal calf serum and 1% penicillin/streptomycin (complete MEM) with 5% CO_2 at 37°C. 96 well (flat bottomed) microtiter plates were seeded with log 10^4 vero cells per well and incubated until a 95% confluent monolayer had formed on the base of the wells. Five-fold serial dilutions of the cell-free supernatants from pH and SCFA studies were carried out in complete MEM and exposed to the vero cell monolayer overnight. The monolayers were observed using a bright field inverted microscope (Olympus, UK) for cell rounding. The highest dilution of supernatant with less than 30% cell rounding indicated the toxin titer. Controls were clean BHI broth and MEM. Little to no variation was observed in the triplicate samples. There were carried out in duplicate to ensure reliability of results and confirmed by another researcher.

149 *qPCR virulence/colonisation gene analysis*

150 Bacterial cell pellets were treated with RNAprotect (Qiagen, UK) upon conclusion of
151 experimentation according to the manufacturer's instructions. The pellets were stored at –
152 80°C until RNA extraction. RNA was extracted and purified using the RNAeasy kit (Qiagen,
153 UK) with an additional bead beating step upon addition of RNeasy lysis buffer (Qiagen, UK)
154 using 0.1mm glass beads (Sigma, UK). DNase treatment was conducted using the RNase free
155 DNase set following the manufacturer's instructions (Qiagen, UK). Reverse transcription was
156 carried out with the Quanti-Tect reverse transcription kit according to manufacturer's
157 instructions (Qiagen, UK). In order to quantify virulence gene expression, quantitative PCR
158 was carried out using PrecisionPLUS 2x qPCR MasterMix premixed with SYBR Green
159 (PrimerDesign, UK). The virulence/colonisation genes and primers utilised in this
160 experiment (22, 23) are described in supplementary file 1 (Life Technologies, UK). The
161 cycling protocol was 95°C hot start for 2 minutes, x40 cycles of 95°C for 15 seconds and
162 60°C annealing temperature for 1 minute. Cycle threshold (Ct) values were collected and
163 percentage expression calculated in comparison to a housekeeping gene (*rpoA*) by calculation
164 of ΔΔCT.

165

166 *Statistical Analysis*

167 Statistical analysis on data to determine statistical significance between groups was carried
168 out on Graphpad Prism 7 (GraphPad Software, USA). Shapiro-Wilkes normality test was
169 conducted to determine distribution of data. Statistical analysis between groups of parametric
170 data was carried out using a one-way analysis of variance (ANOVA) with Dunnet's post hoc
171 test or a Kruskal-Wallis test with Tukey's post hoc test for multiple comparisons. In cases
172 where treatment/dose was investigated, a two-way ANOVA with Dunn's post hoc analysis
173 was used. Statistical significance was achieved if $p < 0.05$.

174

RESULTS

pH strongly influences C. difficile germination, growth, toxin production and colonisation factor gene expression.

A narrow pH threshold of less than 0.5 pH units differentiated between inhibition of growth and full confluent growth of *C. difficile* vegetative cells and spores (**Figure 1**). Already revived vegetative cells successfully proliferated at pH=5.83 and above ($p<0.01$), whilst spores germinated successfully at pH=6.19 and above ($p<0.01$). Some of the variability in results was likely due to noticeable 'clumping' in all *C. difficile* cultures in which confluent growth had occurred. The OD of the higher pH cultures (pH=6.65, pH=7.07 and pH=7.67) were not statistically significant from pH=5.83 ($p>0.1$). Additional studies demonstrated (**Figure 2**) no germination for an inoculated spore suspension at pH=5.83 (**Fig 2A**), and a shift in lag phase from spore and vegetative cells at pH=6.65 (**Fig 2B**) and pH=7.76 (**Fig 2C**). Inoculation with a spore suspension versus inoculation with a live culture did not result in a change of maximum optical density (OD) (1.0-1.5). These effects of pH fluctuations on vegetative cell suspensions were replicated in a continuous culture model (**Fig 2D**). Lowering pH below 5.80 resulted in a drop in OD, which was successfully recovered when pH was raised to above pH=6 in three separate cycles.

Toxin production was significantly decreased due to the effect of pH=5.37 and 5.83 on *C. difficile* ($p<0.01$, **Figure 3**). Maximum toxin production was found at pH=7.67, with a step-wise increase in a vegetative cell/spore mixed culture (expressed as the dilution at which the toxin longer resulted in verocell rounding). Gene expression of TcdA appeared to increase above pH=6.65 although expression was only significantly higher compared with control at pH=7.67 ($p=0.03$, **Fig 4A**). TcdB expression was not increased when *C. difficile* was challenged over the range pH=5.8 to pH=6.19, but was significantly higher at pH=6.65

(p=0.002), pH=7.07 (p=0.007) and pH=7.67 (p=0.0015, **Fig 4B**). Cwp84 expression increased significanlty (ten-fold) at pH=7.67 compared with control (p=0.0368, **Fig 4C**). CWp84 expression was not significantly increased at any other pH condition in comparison with the control, which may be linked to fibrous filament morphology found at pH=7.67 (**Figure 1**). Flagellar protein FliD expression was not detectable at pH=5.83 and pH=6.19, and was significantly lower at pH=6.65 (p=0.0122) and pH=7.07 (p=0.0024) (**Fig 4D**).

C. difficile toxin production is regulated by acetate

Acetate, propionate and butyrate all had inhibitory effects on *C. difficile* toxin production at varying concentrations (**Figure 5**). Acetate (at all concentrations used) at pH=6.67 did not influence growth, but significantly inhibited toxin production at 50mM, 100mM and 200mM ($p<0.01$, **Figure 5**). Butyrate reduced *C. difficile* toxin titer at 25mM, 100mM and 200mM but did not affect growth ($p<0.01$). Propionate-treated cell supernatant resulted in high vero cell rounding at 200mM, with low bacterial OD, but 25mM, 50mM and 100mM significantly affected toxin burden on vero cell rounding ($p<0.01$, **Figure 5**). Experimental error was ruled out by challenging vero cells with supernatants of three biological replicates and conducting the assay with three technical replicates.

ORP has no significant role in the regulation of C. difficile growth

At pH=6.65, increased ORP significantly increased growth (OD) compared with control (p=0.003) (**Figure 6**) but no other significant effects were observed when ORP was varied under experimental conditions.

DISCUSSION

Understanding what constitutes the CRB is important to preventing CDI in an ageing population. This study demonstrates the exquisite sensitivity of *C. difficile* to subtle changes around pH=5.67. Increased filamentous morphology was observed at higher pH (pH=6.7 and above for vegetative cells) which correlates with an increased expression of *Cwp84* at pH=7.67, perhaps due to shorter lag phase. Previous work has shown increased colonisation gene expression in response to a neutral/basic pH gut environment, observations noted in a dysbiotic gut (24, 25).

pH in the colon can reach as low as 5.37–5.83 when consuming a high fibre diet (26, 27). We demonstrate that manipulation of colonic pH may prevent the colonisation, germination, growth and toxin production of hypervirulent *C. difficile* 027. May *et al.,* (1994) highlight the influence of dietary fibre on the CRB, attributed to increased SCFA production and lowered pH around the inhibitory ranges we have identified (28). We demonstrate a prolonged lag phase, decrease in toxin production at pH range 5.37–7.07 and correlation with decreased expression of the virulence genes *tcdA* and *tcdB.* Decreased cytotoxicity and enhanced colonisation resistance was previously demonstrated in fermentation systems supplemented with oligosaccharides, which was not attributable *Bifidobacteria* spp. but may be related to pH=5.5 maintenance in this model (29). Wetzel and McBride (2019) described increased toxin A production at pH=5.5 with a solid media matrix. Our results in liquid media suggest a clear impact of narrow pH thresholds on *C. difficile* germination and growth. pH impact on toxin appears to be related to influence on lag phase length and not the impact on the cells toxin production capacity.

We demonstrate that *C. difficile* spore suspension growth was inhibited below pH=6.19 but proliferation of vegetative cell suspensions above pH=5.67 suggesting that at pH<6.19 germination and outgrowth of spores specifically is prevented. Maximal germination has previously been shown to occur above pH=6.5 (30). *C. difficile* can withstand oxidative environments like *S. enterica* serotype Typhimurium (31). *C. difficile* utilises TcdB-induced, NADPH oxidase (NOX) epithelial cell ROS upregulation to facilitate survival over a wide range of redox potentials, indicating specialised survival mechanisms in *C. difficile* at extreme E_h ranges (32). We observed that extreme positive E_h at the point of inoculation does not affect *C. difficile* growth, and that pH drives its ability to proliferate at extreme E_h. Manipulation of pH in the colon is a plausible mechanism for influencing *C. difficile* virulence and pathogenicity regardless ORP perturbations.

We found significant effects of SCFA on toxin gene expression but no effect on *C. difficile* growth. We observed that 100mM sodium butyrate and 100mM sodium propionate increased *tcdA* gene expression. Similarly, 100mM sodium butyrate and 50mM sodium acetate increased *tcdB* gene expression (Data not shown). However, this was not reflected with toxin titer in response to the SCFA challenge, which was downregulated. A similar occurrence was discussed by Dupuy and Sonenshein, (1998) who found that *C. difficile* toxin gene expression was increased as a stringent response to catabolism repression at stationary phase of growth (33). Increased virulence as a stringent response to stressors is one mechanism by which *C. difficile* can utilise a competitive environment to its advantage. This highlights the importance of identifying colonisation/virulence modifying therapeutics which may alter the CRB in the colon.

The limitations of this work are that it does not combine all explored factors and include complexities of the gut microbiome. Further work is needed to determine if these findings translate to humans. Future work into the combined effect which incorporates a competent microbiome, with a detailed understanding of the impact this has on *C. difficile* germination, growth and virulence would be a beneficial next step.

CONCLUSION

This work highlights colonic environmental mechanisms which can be exploited for developing CDI therapeutics (34). SCFA and pH, within physiological ranges, are important to prevent *C. difficile* colonisation, germination, growth and/or virulence *in vitro*. Studies which enhance the CRB in humans at risk of CDI are warranted, particularly if they can reduce the antibiotic burden, and they may be achievable relatively simply and inexpensively through dietary means.

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FIGURE LEGENDS

Figure 1: a) C. difficile vegetative cell and spore growth when challenged with pH ranging from pH 3.36 to pH 7.67 (n = 9). Letters highlight statistically significant groups ($p<0.05$)

Figure 2: Vegetative cell vs spore lag phase at A) pH 5.83 B) pH 6.65 and C) pH 7.67 (n=3). D) pH Effect of pH on C. difficile growth study conducted in chemostat continuous flow culture showing fluctuations in pH leads to decrease and recovery in OD. Conducted over 35 days with continuous 1 mL/min flow rate of clean, pH buffered BHI broth.

Figure 3: Toxin titer of C. difficile 027 challenged with pH 5.37 – 7.67 (n=3). Toxin titer was enumerated as the lowest serial dilutions that resulted in 30 % cell rounding on vero cell monolayers (expressed as dilution – 1 in 5 increments). Letters highlight statistically significant groups ($p<0.05$)

Figure 4: a) C. difficile tcdA expression at varied pH ranges pH (5.6 - 7.6). b) C. difficile tcdB expression at varied pH ranges (pH 5.6 – 7.6), c) C. difficile Cwp84 expression at varied pH ranges (pH 5.6 – 7.6). d) C. difficile FliD expression at varied pH ranges (pH 5.6 – 7.6). e) C. difficile toxin titer at varied pH (pH 5.3 - 7.6) with control (culture in BHI at pH 7.5). The experiment was conducted from biological replicates of the effect of pH on C. difficile growth work (n = 3). This work was conducted from biological replicates of the effect of pH on C. difficile growth work (n = 3). Each biological replicate was tested in duplicate. Letters highlight statistically significant groups ($p<0.05$).

Figure 5: Quantification of toxin titer and OD (600 nm) of C. difficile 027 in response to challenge of varied concentrations of SCFA sodium salts of acetate, butyrate and propionate

316 from 25 – 200 mM (n = 3). Left axis displays toxin titer, enumerated as the lowest serial
317 dilutions that resulted in 30 % cell rounding on vero cell monolayers (expressed as dilution –
318 1 in 5 increments). Data is shown in bar chart form. Right axis displays OD (600 nm). Data is
319 shown in scatterplot form. Letters highlight statistically significant groups ($p<0.0001$)
320
321 **Figure 6**: Effect of perturbations in ORP at point of inoculation on C. difficile 027 growth
322 (n=3). ORP is expressed as ΔEh and results are shown as OD (600 nm). Asterisk highlights
323 statistical significance from control group. ** $p<0.01$.
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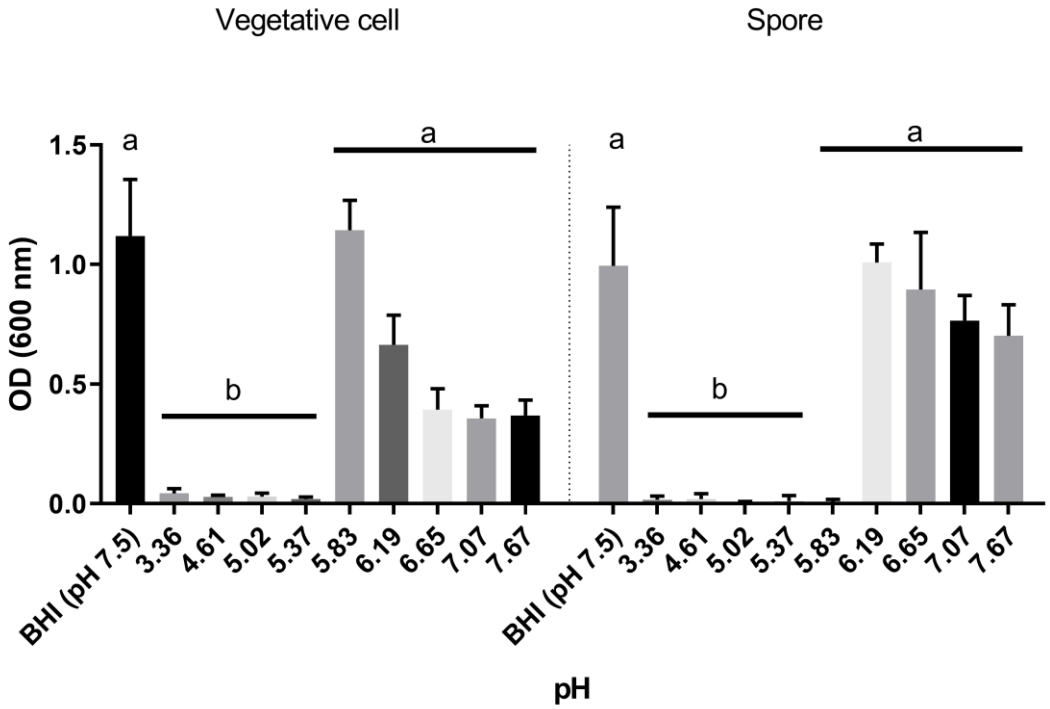
425 Figure 1



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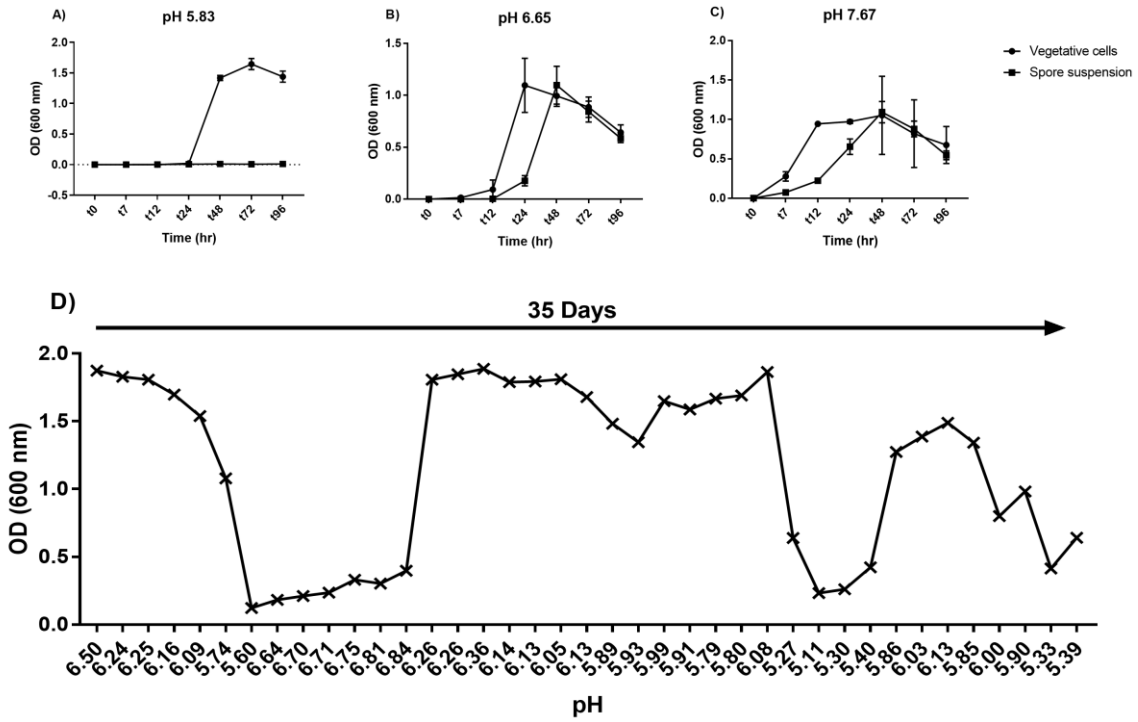
428 Figure 2



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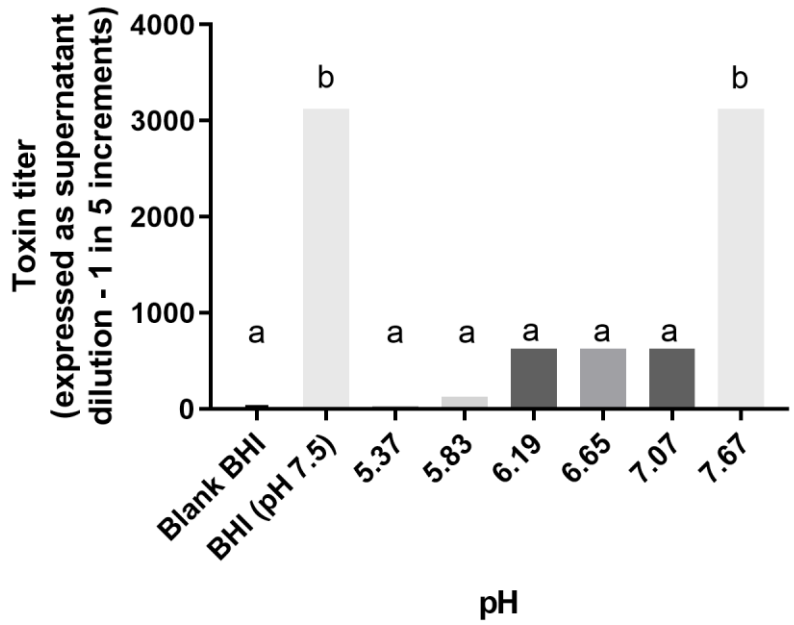

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434 Figure 4

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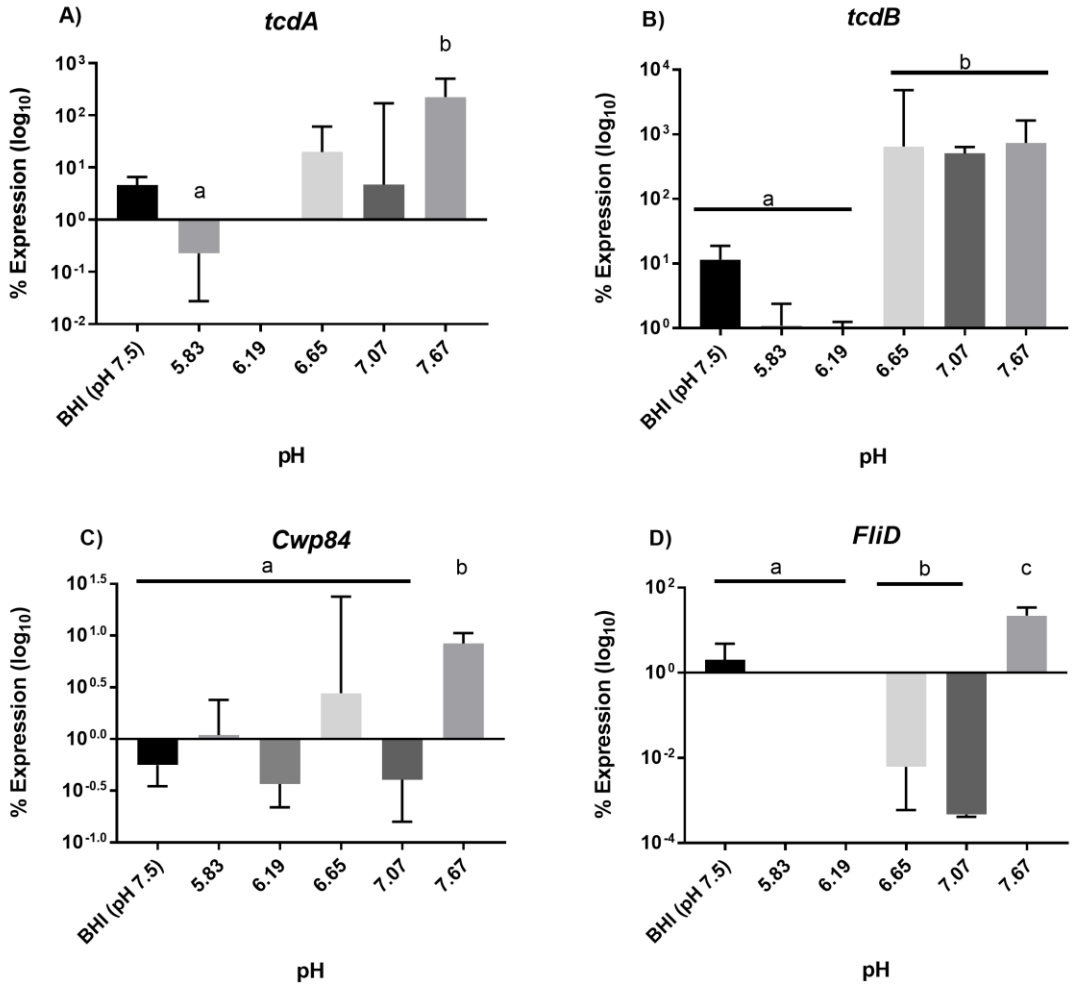

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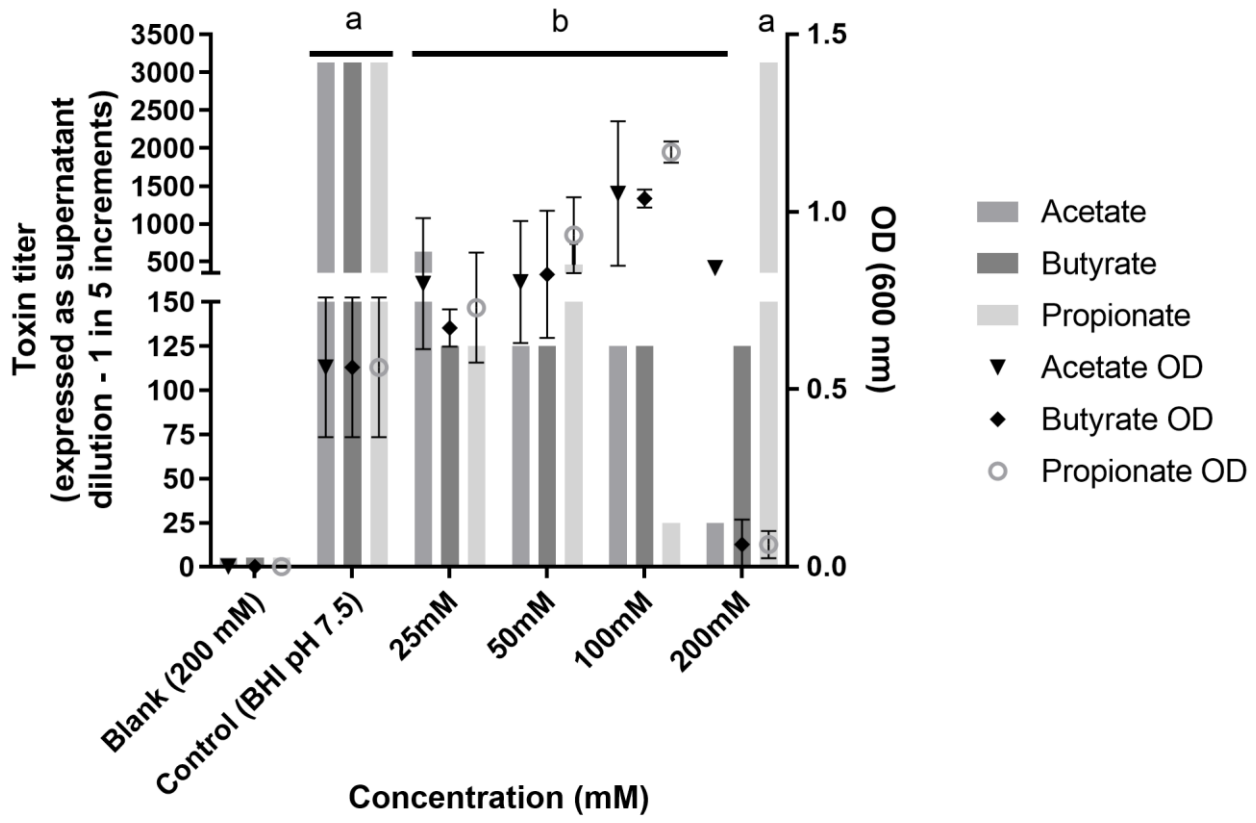
438 Figure 5



439

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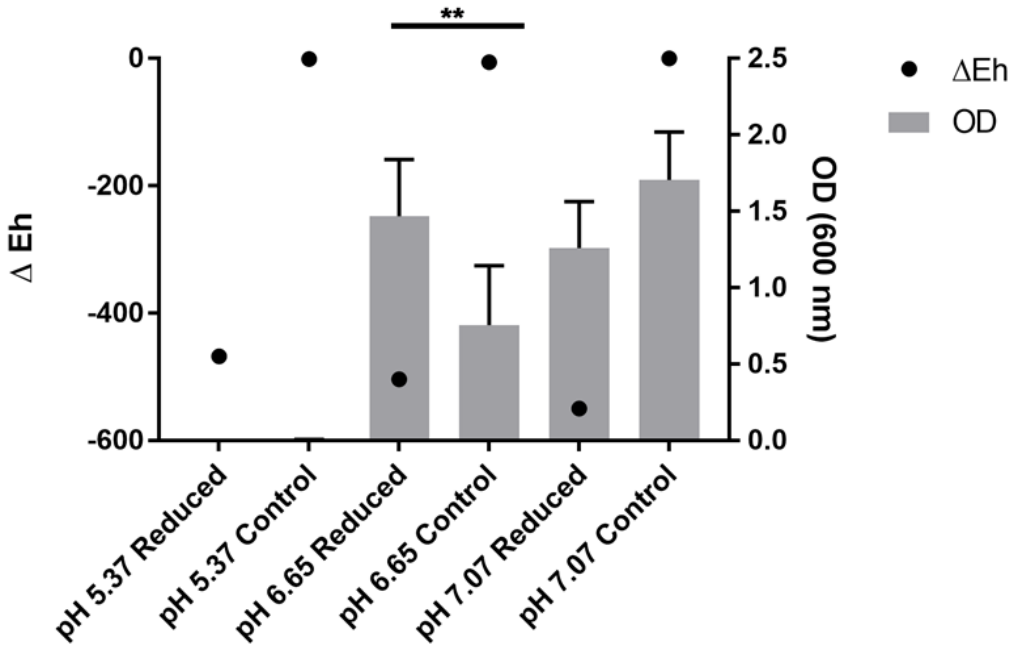
441 Figure 6



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