Deoxynivalenol induced inflammation and increased the adherence of entero-invasive *Escherichia coli* to intestinal epithelial cells via modulation of mucin and pro-inflammatory cytokine production

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Abstract

Deoxynivalenol (DON) is a mycotoxin that commonly occurs in crops. It was hypothesized that DON could trigger intestinal inflammation and increase the susceptibility of intestinal epithelial cells (IECs) to pathogen infection. Accordingly, the aim of this study was to investigate the effects of DON on intestinal susceptibility to pathogen infection. Semiconfluent Caco-2 cells were exposed to DON followed by acute entero-invasive *Escherichia coli* (EIEC) infection. The effects of DON and EIEC contamination on mucin, cytokines and related signal transduction pathways were examined as part of the local immune system. Caco-2 cells were able to generate a rapid immune response against DON with or without EIEC post-challenge. An increase in EIEC attachment to DON-exposed cells was observed, probably in part, mediated by modulation of secretory MUC5AC mucins and membrane bound MUC4 and MUC17 mucins. Cells were also able to express and produce important mediators of inflammation, such as cytokines as a result of activation of toll-like receptors signalling cascades, modulation of nuclear factor κ-light chain-enhancer of activated B cells (NK-κB) and/or mitogen-activated protein kinase (MAPK) pathways. These data indicate that DON may exert immunomodulatory effects on intestinal epithelial cells, which might thereby modify the susceptibility to bacterial infection.

Keywords: Bacterial infection; Cytokines; Deoxynivalenol; *Escherichia coli*; Mucins; Inflammatory responses

1. Introduction

The mycotoxin deoxynivalenol (DON) is secondary metabolite of *Fusarium* fungi that frequently contaminate both human food and animal feed. Human ingestion of DON contaminated grains can lead to different diseases, including nausea, vomiting, diarrhoea, abdominal pain, headache, dizziness, and fever (Sobrova et al. 2010).

DON has been known to be immunotoxic affecting cells of the immune system and the gastrointestinal tract. DON induces “ribotoxic stress response”, which results in the elevation of many pro-inflammatory genes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) as well as cytokines such as interleukin (IL)-6, IL-1β and tumour necrosis factor (TNF)-α (Moon et al. 2002, Moon et al. 2003, Pestka et al. 2004).

Foodborne pathogens can cause severe diseases including diarrhoea, which are of considerable public health concerns. The most common foodborne pathogens found to contaminate food include *Escherichia coli* (*E. coli*) spp., *Clostridium perfringens*, *Listeria spp.* and *Salmonella enterica* serotypes Enteritidis (Maciorowski et al. 2007). Among them, the most important foodborne pathogen is *E. coli*. Entero-invasive *E. coli* (EIEC) are intracellular pathogens that invade colonic epithelial cells by first penetrating the intestinal epithelium through microfold cells (M cells) to gain access to the submucosa, producing a rather localized infection with subsequent destruction of the underlying mucosa as a result of their active replication (Croxen et al. 2013).

The mucus lining the intestinal epithelium provides an important physicochemical barrier against ingested pathogens and toxins. It is

84 composed of a mixture of mucins, which are heavily glycosylated with O-
85 linked oligosaccharides and N-glycan chains linked to protein backbones.
86 There are two different structural and functional classes of mucins: secreted
87 gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC19), and
88 transmembrane (membrane-bound) mucins (MUC1, MUC3A, MUC3B, MUC4,
89 MUC12, and MUC17). To initiate infection processes, bacteria need to find
90 ways to ensure their survival and colonization by first adhering to the intestinal
91 epithelium (Torres et al. 2003). Mucins can effectively impede the ability of
92 bacteria and viruses to invade and colonize the cells, prevent their spread
93 along the mucosal surfaces, and limit the amount of microbial-produced toxins
94 reaching mucosal cells (Liévin-Le Moal et al. 2006).

95 Previous studies from our laboratory and others have shown that DON
96 affected mucus production (Wan et al. 2014, Antonissen et al. 2015, Pinton et
97 al. 2015) and also cytokine production (Van De Walle et al. 2008, Van De
98 Walle et al. 2010, Wan et al. 2013). Bacterial pathogens such as *E. coli* could
99 also trigger host inflammatory responses by enhancing mucin expression that
100 protect bacterial intrusion to gut epithelium (Vieira et al. 2010, Xue et al. 2014),
101 and eliciting cytokine production (Jung et al. 1995, Eaves-Pyles et al. 2008). *E.*
102 *coli* can also be recognized by toll-like receptors (TLRs) (mainly TLR4), which
103 transduce signals to the nucleus, activate the transcription factor nuclear
104 factor κ-light chain-enhancer of activated B cells (NF-κB) and initiate
105 inflammatory responses by producing inflammatory cytokines and
106 chemokines (Underhill et al. 2002, Dąbek et al. 2010). The intestinal tract is
107 the first barrier to ingested mycotoxins but also the first line of defence against
108 intestinal infection. Ingestion of some mycotoxins increases the susceptibility

to experimental or natural mucosal infections (Tai et al. 1988, Fukata et al. 1996, Stoev et al. 2000, Oswald et al. 2003, Vandenbroucke et al. 2011). So far, there is only one study reporting that DON rendered the intestinal epithelium more susceptible to *Salmonella Typhimurium* with a subsequent potentiation of the inflammatory responses in the gut (Vandenbroucke et al. 2011).

Accordingly, it was hypothesized that DON could also increase the susceptibility of intestinal epithelial cells (IECs) to an entero-invasive pathogen infection and trigger intestinal inflammation. With DON and EIEC infections being emerging issues with possible deleterious consequences for both animal and human health and with the gastrointestinal tract being the primary target, the aim of this study was to investigate the effects of low and relevant concentrations of DON on intestinal susceptibility to acute EIEC infection. Of the cell line models, the Caco-2 cell line, originally isolated from human colon adenocarcinoma, in spite of lack of mucus production, is among the most commonly used *in vitro* model of intestinal epithelium to study bacterial adherence and invasion (Resta-Lenert et al. 2003, Ganan et al. 2010, Resta-Lenert et al. 2011, Khodaii et al. 2017). Caco-2 cells were grown at semi-confluence stages (2 to 3 days of culture) and used in this study, as they were substantially more susceptible to infection than confluent cells.

2. Materials and Methods

2.1 Chemicals and reagents

Minimum Essential Medium (MEM), foetal bovine serum (FBS) and all other cell culture reagents were obtained from Gibco-Life Technology (Eggenstein,

Germany). DON was obtained from Sigma Chemical Company (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) purchased from Sigma and stored at -20 °C before use. In addition, phosphate buffered saline (PBS), sodium biocarbonate, diethylpyrocarbonate (DEPC) and chloroform are purchased from Sigma. RNAisoPlus was purchased from Takara (Otsu, Japan). HiScript^{TM} RT SuperMix for qPCR and AceQ qPCR SYBR Green Master Mix were obtained from Vazyme Biotech Co. (Piscataway, NJ, USA).

2.2 Cell line and culture conditions

Caco-2 cells (passages 22-31) obtained from the ATCC (HTB-37) were maintained at 37 °C, 5% CO_2, 90% relative humidity in MEM+20% FBS. Routinely, cells were sub-cultured once a week using trypsin-EDTA (0.25%, 0.53 mM) and seeded at a density of $2x10^6$ cells per 180 cm^2 flask. All cells were screened for mycoplasma contamination with a MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland) prior to use.

2.3 Bacteria preparation

Entero-invasive *Escherichia coli* (EIEC O29:NM) was from Prof Wei Chen from the State Key Laboratory of Food Science and Technology, Jiangnan University. EIEC were incubated overnight at 37 °C in Luria-Bertani (LB) broth until the stationary phase was reached. Subcultures of the overnight cultures in fresh medium were grown to a phase of exponential growth. Cells were centrifuged at 4,000 rpm (Beckman Coulter, Fullerton, CA; GS-6R centrifuge) for 5 min, wash with PBS twice and suspended in MEM+20% FBS to desired concentrations before adding to the epithelial cell layers.

159

160 2.4 Cell viability by CCK-8 assay

161 A CCK-8 colorimetric assay was performed to assess cell viability/cytotoxicity
162 in response to different concentrations of DON without or with EIEC post-
163 treatment. The CCK assay is a colorimetric assay based on the reduction of a
164 tetrazolium salt, WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenyl]-2H-
165 5-tetrazolio]-1,3-benzene disulfonate sodium salt), to a water-soluble
166 formazan by cellular NADH or NADPH (Ishiyama et al. 1997). The assay was
167 performed following manufacturer's instruction (Dojindo, Kunamoto, Japan). In
168 brief, cells were seeded at 2×10^4 cells/well in 96-well culture plates and
169 allowed to grow for 3 days. At 3 days, cells were treated with different
170 concentrations of DON (0, 2, 4, 8, 16 µM) for 24 h. After 24 h incubation,
171 Caco-2 cells were treated with medium containing exponentially grown EIEC
172 bacteria at a multiplicity of infection (MOI) of 250:1 to the cells for 1 h. After
173 that, cells were washed and incubated in medium with gentamicin (50 µg/ml)
174 for another 1 h. At the end of the incubation, CCK-8 solution (10 µl) was
175 added to each well, and the cells were incubated at 37 °C for 1 h. The colour
176 intensity (absorbance) was determined using a microplate reader (model 550,
177 BioRad) at 450 nm. Cell viability was expressed as the percentage of the
178 mean value normalized to the control (untreated cells). For each treatment,
179 the mean value was obtained from at least six wells.

180

181 2.5 Invasion and adhesion assay

182 The number of cells in each of the 24-well plate following DON treatment was
183 first determined by trypan blue (Gibco) exclusion assay for calculation of MOI.

Invasion assay were performed using the gentamicin protection assay as described previously (Boudeau et al. 1999). Briefly, Caoc-2 cells were seeded in 24-well plates ($1x10^5$ cells) and cultured for 2 days. After 2 days, cells were treated with different concentrations of DON (0, 8, 16 µM) for 24 h. After 24 h incubation, cells were treated with medium containing exponentially grown EIEC at a MOI of 250:1 to the cells. After 1 h of incubation at 37°C, cells were washed and incubated in medium with gentamicin (50 µg/ml) (for cells infected with invasive bacteria or uninfected controls) for another 1 h at 37°C. The cells were then lysed with 0.1% Triton X-100 (Sigma) in deionized water. Samples were diluted and plated onto LB agar plates to determine the colony forming unit (CFU) recovered from the lysed cells. In control experiments, gentamicin has no significant effect on any of the parameters measured. Furthermore, no significant bacterial overgrowth was observed over the duration of the experiment under all conditions tested. The percentage of invading bacteria was expressed as $CFU_{(invading\ bacteria)}$ of infected cells divided by $CFU_{(total\ bacteria)}$, normalized to the number of cells in each of the 24-well plate following different concentrations of DON treatment.

To determine the total number of cell-associated bacteria corresponding to adherent and intracellular bacteria, cells were lysed after 1 h infection period, and the bacteria were quantified as described above. The number of adhering bacteria was determined by subtracting the number of invading bacteria from the total number of cell-associated bacteria. The percentage of adhering bacteria was expressed as $CFU_{(adhering\ bacteria)}$ of infected cells divided by $CFU_{(total\ bacteria)}$, normalized to the number of cells in each of the 24-well plate following different concentrations of DON treatment.

209

210 2.6 Quantification of MUC protein expression

211 Expression of MUC protein by Caco-2 cells was studied using biotinylated
212 wheat germ agglutinin (WGA) (Vector Labs; Burlingame, CA, USA). Briefly,
213 Caco-2 cells were seeded onto 96-well plates and treated or not with DON
214 with or without EIEC infection as described above and were fixed with 4%
215 paraformaldehyde (PFA; Sigma) during 30 min at room temperature. After
216 fixation, cells were washed two times with 0.05% PBS-Tween 20 (PBS-Tw).
217 Cells were blocked for 1 h at room temperature in PBS supplemented with 2%
218 BSA and 0.1% Triton-X100. Biotinylated-conjugated WGA (1:10 000 dilution)
219 was then added to the wells for 1 h at room temperature followed by
220 incubation with avidin peroxidase (1:10 000 dilution) for another hour. Cells
221 were washed two times with PBS-Tw and 3,3',5,5'-tetramethylbenzidine
222 peroxidase (TMB) substrate (BioLegend; San Diego, CA, USA) was added.
223 Reaction was stopped with 2N sulphuric acid (Merck; Darmstadt, Germany)
224 and the optical density was read at 490 nm using the Multiskan microplate
225 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Relative
226 MUC protein expression was normalized to the number of cells in the well
227 following each treatment.

228

229 2.7 Quantitative polymerase chain reaction (qPCR) analysis

230 Cells were seeded and treated as above. After 1 h infection with EIEC, total
231 RNA was extracted using RNAiso^{TM} Plus according to manufacturer's
232 instructions. The concentration of RNA was measured by using NanoDrop
233 ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE) with

234 purity ascertained by (A260/A280) of >1.8. RNA integrity was checked by
235 running the RNA sample on ~1% agarose gel. Total RNA (500 ng) from each
236 sample was converted into cDNA using HiScript™ RT SuperMix for qPCR
237 according to the manufacturer's instructions. qPCR was performed to quantify
238 the products of interest, cytokines and chemokines (IL-1β,–IL-6, IL-8, TNF-α,
239 MCP-1), mucins (MUC1, MUC3A, MUC4, MUC5AC, MUC5B, MUC17), Toll-
240 like receptors (TLR-1, -2, -4, -5, -6) and related signalling molecules (MyD88,
241 NF-κB). Assessment of glyceraldehyde-3-phosphate dehydrogenase
242 (GAPDH) levels was also performed which served as an internal control for
243 RNA integrity and loading. Human specific primers were described in Table 1.
244 For analyses on a StepOnePlus™ Real-Time PCR system (Applied
245 Biosystems, Foster City, CA, USA), 2 µl of 5X diluted cDNA was added to
246 AceQ qPCR SYBR Green Master Mix, to obtain final primer concentrations of
247 500 nM/primer in final volume of 10 ul. The sample was centrifuged briefly
248 and run on the PCR machine using the default fast program (45 cycles of 95
249 °C for 3 s, 60 °C for 30 s). To ensure the reliability of qPCR data, the
250 amplicons are kept short (<250 bp) (Nolan et al. 2006). All PCR reactions
251 were performed in duplicate. Negative controls, consisting in PCR mix
252 components without cDNA was used for all primers. The relative product
253 levels were quantified using the $2^{-\Delta\Delta CT}$ method as described previously (Livak
254 et al. 2001).
255
256 2.8 Protein extraction, SDS-PAGE and immunoblotting
257 The cells, undergoing the same treatment as described for qPCR, were
258 washed with PBS and extracted for total proteins (whole cell extracts). In brief,

100 µl of RIPA lysis buffer, supplemented with protease inhibitor cocktail (Sigma) and phosphatase inhibitors (Cell Signaling, Beverly, MA, USA) was used to extract total proteins from the cells. Protein concentration was determined by the DC protein assay (BioRad).

Proteins (10-50 µg) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel (BioRad), separated by electrophoresis (SDS-PAGE), and then blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Darmstadt, Germany). The membrane was blocked with 5% BSA in Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 (TBST) buffer. Proteins were probed by immunoblotting with diluted rabbit primary antibodies from Cell Signaling (1:1000) for NF-κB p65 (#8242), p44/42 MAPK (Erk1/2) (#4695), phospho-p44/42 MAPK (Erk1/2) (#4370), JNK2 (#9258), phospho-SAPK/JNK (#4668), p38 MAPK (#8690), phospho-p38 MAPK (#4511) and GAPDH (ab181602; Abcam), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#170-6515, BioRad) secondary antibodies. The blots were developed using Clarity Western ECL blotting kit (BioRad) and chemiluminescence was detected with a digital imaging system (ChemiDoc XRS+ system with image lab software, BioRad). Quantification was performed by Image Lab software version 6.0 (Bio-Rad) by densitometric analysis (Schneider et al. 2012).

2.9 Statistical analyses

All assays were expressed as mean ± standard error of mean (SEM) for the number of separate experiments indicated. Data analyses were performed using the GraphPad PRISM 9.0 software (Graphpad Software Inc., San

Diego, CA). All data were first evaluated for normality with the Shapiro–Wilk and Levene's variance homogeneity test. For parametric data, one-way analysis of variance (ANOVA) followed by Dunnet's test against a control group; for non-parametric data, one-way ANOVA with the Kruskal-Wallis test, followed by the Dunn's multiple comparisons test was used to identify significant differences against a control group. Data are significantly different at $p<0.05$, according to the post hoc ANOVA statistical analysis.

3. Results

3.1 DON and EIEC lowered cell viability

CCK assay was performed to investigate the effect of DON pre-treatment on cell viability and to determine the concentrations of DON used in subsequent experiments (Fig. 1A). DON resulted in a significant concentration-dependent reduction in cell viability at 4, 8 and 16 µM ($p<0.05$). DON at the concentrations of 8 and 16 µM were selected for the subsequent experimental assays. The combined effect of DON and EIEC on cell viability was also assessed. Addition of EIEC to cells pre-treated with 8 and 16 µM DON significantly reduced cell viability compared to control (PBS) ($p<0.0001$), but the reduction was not significantly different from cells treated with EIEC or DON alone (Fig. 1B).

3.2 DON increased EIEC adhesion but reduced invasion

Since adhesion and invasion are both important processes in bacterial pathogenesis, the effects of DON on EIEC adhesion and invasion were assessed by a modified bacterial adherence assay and gentamicin protection

assay (Fig. 2). A preliminary bacterial invasion experiment was conducted in our laboratory by adding EIEC at MOI of 1:1, 2.5:1, 10:1, 25:1, 100:1, 250:1, 500:1, 1000:1 to the cells for 1 or 2 h. Results showed that EIEC at MOI of 250:1 for 1 h showed the highest invasion number to the cells from the colony counts (data not shown). Therefore, the MOI of 250:1 and 1 h of EIEC incubation were chosen for all the subsequent experiments. Pre-treatment with 8 and 16 µM DON caused a significant increase in EIEC adherence (p<0.05) (Fig. 2A). However, cells pre-treated with DON reduced EIEC invasion significantly at (p<0.05) (Fig. 2B).

3.3 DON and EIEC contamination altered mucin gene expression and protein production

Mucin production is important in forming intestinal mucus, and acts as a physical barrier against bacterial infection. The effect of DON and EIEC exposure on mucin mRNA expression was determined by qPCR (Fig. 3) and protein production using the lectin wheat germ agglutinin (WGA) assay (Fig. 4).

Significant up-regulation of *MUC5AC* mRNA was found when cells were treated with all the treatment groups (Fig. 3A). Significant up-regulation of *MUC5B* mRNA was also observed when cells were treated with 16 µM DON alone and with EIEC post-infection (Fig. 3B).

MUC1, *MUC4* and *MUC17* mRNA expression showed similar responses when treated with DON, where they experienced a gradual increase with increasing DON concentrations (Fig. 3C, E, F). For *MUC1*, in general, the mRNA expression level in cells with DON treatment alone

334 remained unchanged when compared to PBS control. DON pre-treatment
335 lowered *MUC3* mRNA expression level significantly (p<0.05). Addition of
336 EIEC did not affect *MUC3* mRNA expressions (Fig. 3D). When Caco-2 was
337 treated 16 µM DON, *MUC4* and *MUC17* mRNA expression increased
338 significantly (p<0.05) compared to the PBS control. Addition of EIEC further
339 increased *MUC4* and *MUC17* mRNA expression (p<0.01) (Fig. 3E-F).
340 To investigate the effects of DON and EIEC treatment on the mucus
341 secretion, mucin-like glycoprotein secretion in cell lysates and those secreted
342 into culture supernatants were measured and analysed. Exposure to 8 and 16
343 µM DON alone increased mucin protein production significantly (p<0.05).
344 Addition of EIEC, however, did not cause any significant change to the
345 amount of mucin protein produced compared to their pathogen-free
346 counterparts (Fig. 4).
347
348 3.4 DON and EIEC modulated pro-inflammatory cytokine and chemokine
349 gene expression
350 The effects of treating Caco-2 monoculture with DON and EIEC on pro-
351 inflammatory cytokine and chemokine mRNA expression were examined by
352 qPCR (Fig. 5). DON with and without EIEC post-treatment significantly
353 downregulated *IL1B* gene expression (p<0.0001) (Fig. 5A). Pre-treatment with
354 16 µM DON upregulated *IL6* mRNA expression significantly (p<0.05); addition
355 of EIEC further increased the *IL6* mRNA (p<0.001) (Fig. 5B). In the absence
356 of DON, EIEC contamination alone significantly increased *IL8* and *TNFA*
357 mRNA expressions (p<0.001 and 0.05, respectively) (Fig. 5C and D). Pre-
358 treatment with all concentrations of DON lowered the *IL8* expression

359 significantly (p<0.0001), but no significant change in *TNFA* was observed.
360 Treatment with DON and EIEC both significantly down-regulated *CCL2* mRNA
361 expression (p<0.001), though it appeared DON's ability to modulate *CCL2*
362 gene expression was greater than that of EIEC (p<0.0001) (Fig. 5E).
363
364 3.5 DON and EIEC regulated TLRs and MyD88 gene expression
365 Pathogen associated molecular patterns (PAMPs) can be recognized
366 by TLRs, which along with MyD88, can stimulate cytokines and chemokines
367 production through NF-κB activation. TLRs and MyD88 gene expression in
368 response to DON and EIEC were assessed by qPCR (Fig 6). DON showed
369 modest upregulation of *TLR1* gene at all concentrations tested; addition of
370 EIEC to 16 µM DON pre-treated cells, however, showed a remarkable
371 increase in *TLR1* mRNA (p<0.001) (Fig. 6A). For *TLR5*, the gene expression
372 was downregulated significantly by DON at all concentrations in the absence
373 and presence of EIEC (p<0.0001) but no change was observed when cells
374 were infected with EIEC alone (Fig. 6C). Due to the low expression of *TLR4* in
375 Caco-2 cell line, its expression data for uninfected control and treatment
376 groups were not shown. For *TLR2*, *TLR6* and *MYD88*, their gene expressions
377 were not affected by the treatments significantly (Fig. 6B, D and E).
378
379 3.6 DON and EIEC tended to activate the NF-κB p65 signalling pathway
380 p65 (RELA) and p50 (NF-κB1) is the most commonly found
381 heterodimer complex of NF-κB, which participate in nuclear translocation and
382 activation of NF-κB to regulate gene expression and thus major cellular
383 functions (Garg et al. 2013). Post-translational modifications of NF-κB,

384 especially of the RELA subunit, further enhance the NF-κB function as a
385 transcription factor (Huang et al. 2010). Therefore, in our study, qPCR and
386 western blot analyses were used to quantify *RELA* (or NF-κB p65) levels as
387 an indicator for NF-κB activation in response to DON and EIEC contamination
388 (Fig. 7). Treatment with DON alone resulted in an ascending trend in *RELA*
389 mRNA expression. Addition of EIEC further increased the mRNA expression
390 (p<0.01) (Fig. 7A). Results obtained from western blot also revealed similar
391 patterns for NF-κB protein expression. Up-regulation of NF-κB p65 protein
392 expression was observed in cells infected with EIEC without and with DON
393 pre-treatment (Fig. 7B).
394
395 3.7 EIEC inhibited DON-induced MAPK signalling pathway activation
396 Previous studies have shown the involvement of MAPK signalling pathways in
397 DON-induced inflammation in intestinal cells (Van De Walle et al. 2008, Van
398 De Walle et al. 2010). In this study the three classical MAPK signalling
399 pathways, JNK, p38 MAPK and extracellular signal-regulated kinase (ERK)
400 were investigated in DON treated cells with and without EIEC post-infection
401 (Fig. 7C). Western blot data showed that DON treatment alone induced
402 significantly the phosphorylation of ERK and JNK, but not p38. However,
403 addition of EIEC to DON treated cells inhibited these MAPK signalling
404 pathways activation. EIEC alone also significantly inhibited p38 signalling
405 pathways but no change in p38 phosphorylation protein level was observed in
406 cells pre-treated with DON.
407
408 **4. Discussion**

The present study was the first to investigate the effects of low and relevant concentrations of DON on intestinal susceptibility to acute (1-2 h) EIEC infection. The effects of DON and EIEC contamination on mucin, cytokines and related signal transduction pathways were examined in the intestinal epithelial cells as part of the local immune system. The concentrations of DON are in accordance with the levels probably encountered in the gastrointestinal tract of animals or human tissues after consumption of food or feed contaminated with DON (Sergent et al. 2006). Assuming that DON ingested in one meal is diluted in 1 litre of gastrointestinal fluid and is totally bio-accessible, the *in vitro* concentrations to be used in this study correspond to food contamination ranging from 1.18 mg/kg to 4.72 mg/kg of DON (Van De Walle et al. 2008). The infection protocol (MOI and EIEC treatment duration) was established based on a preliminary experiment in our laboratory. Similar infection protocols were also adopted by other investigators (Resta-Lenert et al. 2003, Ganan et al. 2010, Resta-Lenert et al. 2011).

Bacterial adherence to host cells is the initial crucial step towards colonization and establishment of infection within the host (Torres et al. 2003). In the present study, DON increased the adhesion of EIEC on intestinal epithelial cells (IECs) but caused a reduced invasion into IECs. This may be attributed to the induction of mucin gene and protein expression in IECs as demonstrated in our study. Indeed, numerous studies have shown altered mucin expression in chronic intestinal inflammatory diseases and cancer, both in animal models and patient cohorts (Ho et al. 1993, Reis et al. 1999, Ho et al. 2006, Longman et al. 2006, Moehle et al. 2006, Heazlewood et al. 2008,

Larsson et al. 2011). Both secretory and membrane-bound mucins are important constituents of the physicochemical barrier for the protection of the epithelial cell surface against undesirable harmful pathogens (Liévin-Le Moal et al. 2006). Over-expression and hyper-secretion of the secretory mucins, in particular, MUC5AC and MUC5B are two of the important characteristics of the inflammatory process in mucosa. Previous studies conducted by our laboratory indicate the modulation of biosynthesis of MUC5AC and MUC5B following exposure to DON in differentiated Caco-2 cells (Wan et al. 2014). However, no data are available concerning the effects of DON and EIEC on mucin production. Our finding demonstrated that MUC5AC and MUC5B mRNA was significantly increased upon DON and EIEC treatment. The rapid elevation of secretory mucin in responses to xenobiotics and bacterial infection is crucial for protecting intestine against pathogens and its metabolites (Snyder et al. 1987).

DON is known for its ability to activate signalling pathway and gene expression in goblet cells. Several studies indicated the potential involvement of mitogen-activated protein kinases (MAPKs). The initial binding of DON to ribosome leads to the activation of protein kinase R (PKR) that in turn causes the activation of the MAPKs and subsequently up-regulates human *MUC5AC* gene transcription. Less is known about the regulation of MUC5B expression. However, based on the possible common regulatory mechanism between MUC5AC and MUC5B (Moniaux et al. 2001), it is possible that *MUC5B* mRNA expression are regulated by MAPKs activation as well.

There is increasing evidence for the role of membrane-bound mucins in maintaining intestinal mucosal integrity. Among all identified membrane-bound

mucins, MUC3 and MUC17 are the membrane-bound mucins that are moderately expressed in the colon (Hattrup et al. 2008) and abundantly in both goblet cells and enterocytes of the small intestine (Ho et al. 1993, Kim et al. 2010). On the other hand, MUC1 and MUC4 are also expressed in normal intestinal tissues, but they are markedly upregulated in response to bacterial infection (McAuley et al. 2007, Lindén et al. 2008). In this study, we have shown a significant induction in *MUC1*, *MUC4* and *MUC17* but not *MUC3* mRNA in cells with DON and EIEC co-exposure. This was in agreement with a previous study that also demonstrated the protective role of MUC17 in protection of the intestinal mucosa against an EIEC strain (Resta-Lenert et al. 2011). MUC17 contributes significantly to maintaining cell homeostasis and modulating chronic inflammatory responses by activating signalling pathways associated with inflammation and cancer. It was postulated that NF-κB contributes, at least partly to the mucin regulation because all intestinally expressed mucin genes contain a potential or experimentally proven binding site for NF-κB (Moehle et al. 2006). The NF-κB regulatory pathway plays an important role in cell activation and production of diverse inflammatory mediators, including a variety of cytokines and chemokines (Hayden et al. 2004). But of course, NF-κB is not the only transcriptional regulator influencing mucin expression. Further studies are necessary to understand the mechanisms controlling the expression of mucin.

Besides acting as a physical barrier, IECs are able to express and produce important mediators of inflammation such as cytokines and chemokines and other signal molecules like TLRs that are important for host defence and bacterial recognition (e.g. lipopolysaccharides (LPS) from gram-

484 negative bacteria) (Arce et al. 2010). TLRs are expressed not only on immune
485 cells but also non-immune cells such as epithelial cells. They can be rapidly
486 induced in response to pathogens, cytokines and environmental stresses.
487 TLR-1, -2, -4, -5 and -6 are expressed on the cell surface, which are
488 implicated in the recognition of microbial membrane components for
489 antimicrobial host defence (Akira et al. 2006). In this study, we showed that
490 upon the treatment with DON and EIEC, the mRNA expression of TLRs was
491 differentially modulated. DON without or with EIEC post-treatment induced
492 *TLR1* gene expression but suppressed *TLR5* expression. *TLR2* and *TLR6*,
493 however showed no significant up-regulation in our present study. This
494 indicates that TLR-1, but not TLR-2 and -6 signalling, was involved in the
495 induction of the early inflammatory responses by EIEC/DON in Caco-2 cells.
496 In contrast, the downregulation of *TLR5* mRNA may function to attenuate
497 excessive inflammatory responses due to DON and EIEC co-exposure.
498 Surprisingly, very low or no expression of TLR-4 was present in any of
499 treatments in Caco-2, and thus respond minimally to EIEC or DON. This is in
500 contrast to other studies which showed induction of TLR expression in
501 response to bacteria toxin (LPS from *Salmonella typhimurium*) in swine
502 intestinal epithelial cell lines (IPEC-J2 and IPI-2I) (Arce et al. 2010), in bovine
503 intestinal epithelial cells following *E. coli* 987P infection (Takanashi et al.
504 2013), as well as in IPEC-1 cells following enterotoxigenic *E. coli* (ETEC-
505 O149) strain K88 treatment (Taranu et al. 2015). The decrease of TLR-4 and
506 other TLRs (-2, -3, -6) were observed in porcine epithelial cells, macrophages,
507 mesenteric lymph nodes and spleen of mice under the effects of mycotoxins
508 (e.g., T-2 toxins, DON and ZEA) (Seeboth et al. 2012, Islam et al. 2013,

509 Taranu et al. 2015). All these results indicate that differential regulation of TLR
510 gene expression may contribute to inflammatory immune response against
511 bacterial infection in intestinal epithelial cells. However, EIEC treatment for 1 h
512 after DON treatment did not result in more changes in most of the TLRs
513 expression in the present study, implying DON is the major contributing factor
514 for the immune responses.

515 MyD88 is one of the most important adaptor molecules for
516 inflammatory signalling pathways. MyD88 mediates the activation of TLRs
517 and IL-1R and leads to the production of proinflammatory cytokines through
518 the activation of NF-κB and MAPK signalling pathways (Akira et al. 2006).
519 Here we showed that *RELA* but not *MYD88* mRNA was significantly increased
520 in cells after DON with or without post-challenge with EIEC, in comparison to
521 the unchallenged cells, indicating that NF-κB instead of MyD88 played a more
522 important role in regulating the inflammatory responses induced by DON and
523 EIEC. NF-κB transcription factor plays a critical role in regulation of immune,
524 inflammatory and acute phase responses and is also implicated in the control
525 of cell proliferation and programmed cell death (Aggarwal et al. 2004). NF-κB
526 is harmful to the host when excessively or improperly activated. The ability of
527 DON to influence NF-κB activation has been extensively reported (Van De
528 Walle et al. 2008, Krishnaswamy et al. 2010, Kalaiselvi et al. 2013, Del Regno
529 et al. 2015, Adesso et al. 2017). In this study, we reported that DON
530 increased NF-κB activation during inflammation. EIEC treatment for 1 h after
531 DON treatment caused a higher up-regulation of *RELA* mRNA and NF-κB
532 protein. It is evident that activation of NF-κB is followed by a series of events,
533 leading to the activation of signalling pathways, including MAPKs that are

crucial for regulating inflammation and producing inflammatory factors (Van De Walle et al. 2008, Van De Walle et al. 2010). Activation of these signalling cascades could lead to the production of pro-inflammatory cytokines. In the present work, we found that EIEC alone induced the mRNA expression of *IL8* and *TNFA*. Derangement of cytokine production by bacterial infection can lead to chronic inflammatory conditions (Karin et al. 2006). However, it is surprising to show that DON treatment significantly downregulated the mRNA expression of *IL1B IL8* and *TNFA*. This result is in agreement with a previous report by Ghareeb *et al*., which found that in broiler chickens, chronic administration of DON for 5 weeks resulted in significant down-regulation of certain cytokines, such as *IFNG* and *IL1B* mRNA in jejunal tissues (Ghareeb et al. 2013). Similar suppression of splenic *IFNG* and *IL1B* mRNA was also observed in another study in pigs following DON exposure (Cheng et al. 2006). DON is known to be either suppress or stimulate immunological responses, depending on the dose, time and duration of exposure (Ghareeb et al. 2013). In this context, it becomes evident that DON has a modulating effect on the innate immune response. DON could modify the gene expression of cytokines, and thus may affect the susceptibility of human and animals to disease. In spite of the lack of quantifying the levels of proteins that are actually translated from the observed mRNA transcripts, this study is the first to present significant modulation of different pro-inflammatory cytokine mRNA expression in IECs and this might merit further investigation of the mechanisms in relation to the functional relevance of mRNA expression such as by determining the protein levels of the selected pro-inflammatory

558 cytokines by utilizing the quantitative sandwich enzyme immunoassay
559 (ELISA).

560 Moreover, to determine whether MAPK signalling pathways were
561 involved in the immune responses in cells upon DON and EIEC treatment, the
562 three MAPKs (JNK, p38 MAPK and ERK) were investigated. Consistent with
563 other previous studies, DON induced phosphorylation of JNK and ERK
564 proteins. Addition of EIEC to DON-pre-treated cells, however, suppressed
565 DON-induced phosphorylation of JNK and ERK. DON alone did not induce
566 p38 MAPK phosphorylation but EIEC alone inhibited the p38 MAPK signalling
567 pathway. Although it is evident that MAPK plays an important role in immune
568 responses to *E. coli* infection (Wang et al. 2007, Zhuang et al. 2017), its role
569 in the adherence and internalization of bacteria into epithelial cells was
570 unclear. It is postulated that such deactivation of MAPK pathways may
571 counteract the adhesion and invasion of bacteria into the cells, which are the
572 major contributing factors to intestinal infection and inflammation (Liu et al.
573 2012).

574 In conclusion, the above observations provide a context for the present
575 study, suggesting that exposure to DON could be a predisposing factor to
576 infectious disease. IECs are able to generate a rapid immune response
577 against DON and EIEC contaminants when they act alone or in combination.
578 The results also suggested the potential involvement in secretory MUC5AC
579 mucins and membrane bound MUC4 and MUC17 mucins in modifying the
580 attachment and invasion of EIEC and thus affecting the susceptibility to EIEC
581 infection. IECs are able to express and produce important mediators of
582 inflammation such as cytokines and other signal molecules like TLRs that are

583 important for host defence and bacterial recognition. The augmented mucin
584 production and inflammatory stimulation might be a consequence of activation
585 of NF-κB signalling pathway. DON exposure also activated the MAPK
586 signalling molecules, including ERK and JNK through phosphorylation.
587 However, addition of EIEC to DON pre-treated cells inhibited MAPK signalling
588 pathway which might help protecting intestinal epithelial cells from further
589 damages caused by bacterial infection. A summary of the mechanisms of host
590 defence responses against DON and EIEC co-exposure was depicted in Fig.
591 8. Nevertheless, further studies are necessary to examine different bacterial
592 infection scenarios and to identify the complex mechanism(s) by which this
593 mycotoxin acts on the intestinal tract to modulate invasion and colonization by
594 opportunistic pathogens by using molecular approaches, such as high-
595 throughput mRNA sequencing and proteomics. Epidemiological studies are
596 also needed to assess the extent to which DON are involved in the
597 development of infectious diseases in humans.
598

599 **Competing interests**

600 The authors declare no competing interests.
601

602 **Figure Legends**

603 Fig. 1. Effects of DON without or with EIEC post-infection on cell viability. (A)
604 Preliminary screening of DON concentrations for subsequent experiments.
605 Cell viability data of caco-2 cells treated with different concentrations of DON
606 (0, 2, 4, 8 and 16 µM) for 24 hours. (B) Cell viability data of Caco-2 cells
607 treated with DON (8 and 16 µM) for 24 hours without or with EIEC bacteria
608 post-treatment at a multiplicity of infection (MOI) of 250:1 to the cells for 1 h.
609 Control received appropriate carriers. Results were shown as mean ± SEM,
610 which are from four independent experiments performed in six replicates. *, **,
611 ***, *** p<0.05, 0.01, 0.001 and 0.0001 compared to PBS control. One-way
612 ANOVA post Dunnet's test.
613
614 Fig. 2. Effects of 24 h of DON incubation on Caco-2 cells without or with EIEC
615 post-infection (1 h) on bacterial adhesion and invasion. The percentage of (A)
616 adhering and (B) invaded bacteria were calculated as described in Materials
617 and Methods. Results were shown as mean of ± SEM, which are from four
618 separate experiments performed in duplicates. *, ** p<0.05 and 0.01
619 compared to PBS control. One-way ANOVA post Dunn's test.
620
621 Fig. 3. Effects of 24 h DON incubation without or with EIEC post-infection (1
622 h) on mucin (*MUC*) gene expression. (A-B) Secretory *MUC5AC* and *MUC5B*,
623 and (C-F) membrane bound *MUC1, MUC3, MUC4* and *MUC17* mRNA
624 expression was measured by qPCR, with *GAPDH* as the internal control.
625 Results were shown as mean of ± SEM from five independent experiments. *,

626 **, *** p<0.05, 0.01 and 0.001 compared to PBS control. One-way ANOVA
627 post Dunnet's test.
628
629
630 Fig. 4. Effects of 24 h DON incubation without or with EIEC post-infection (1
631 h) on mucin (MUC) protein production as measured by WGA assay. Results
632 were shown as mean of ± SEM from six independent experiments. * p<0.05
633 compared to PBS control. One-way ANOVA post Dunn's test.
634
635 Fig. 5. Effects of 24 h DON incubation without or with EIEC post-infection (1
636 h) on cytokine and chemokine gene expression. (A-E) *IL1B*, *IL6, IL8*, *TNFA*
637 and *CCL2* mRNA expression was measured by qPCR, with *GAPDH* as the
638 internal control. Results were shown as mean of ± SEM from five independent
639 experiments. *, ***, **** p<0.05, 0.001 and 0.0001 compared to PBS control.
640 One-way ANOVA post Dunn's test.
641
642 Fig. 6. Effects of 24 h DON incubation without or with EIEC post-infection (1
643 h) on toll-like receptor (TLR) and *MYD88* gene expression. (A-E) *TLR1, TLR2,*
644 *TLR5, TLR6* and *MYD88* mRNA expression was measured by qPCR, with
645 *GAPDH* as the internal control. Results were shown as mean of ± SEM from
646 five independent experiments. *, ** p<0.05, 0.01 compared to PBS control.
647 One-way ANOVA post Dunn's test.
648
649 Fig. 7. Effects of 24 h DON incubation without or with EIEC post-infection (1
650 h) on NF-κB and MAPK signalling pathways. (A) *RELA* mRNA expression was

651 measured by qPCR, with *GAPDH* as the internal control. Results were shown
652 as mean of ± SEM from six independent experiments. (B) NF-κB p65 protein
653 expression was measured by Western blotting, with GAPDH as the internal
654 control. Representative photos of western blotting of NF-κB p65 and GAPDH.
655 Quantification of Western blot compared to PBS control from three
656 independent experiments. Inset shows group means. (C) Protein samples
657 were also analysed by Western blot with phospho-p38, JNK and ERK
658 antibodies. The total MAPK levels were used as an internal control.
659 Representative photos of western blotting of MAPKs and GAPDH. Results
660 were shown as mean of ± SEM from four independent experiments. *, ***, ****
661 p<0.05, 0.001 and 0.0001 compared to PBS control. One-way ANOVA post
662 Dunn's test.
663
664 Fig. 8. A summary of the proposed mechanisms of host defence responses
665 against DON and EIEC co-exposures. Upon the exposure to DON and EIEC,
666 intestinal epithelial cells are able to express and produce important mediators
667 of inflammation such as cytokines and modulate other signal molecules like
668 TLRs that are important for host defence and bacterial recognition. The
669 augmented mucin production and inflammatory stimulation might be a
670 consequence of activation of NF-κB signalling pathway. Infection of DON pre-
671 treated cells with EIEC inhibited MAPK signalling pathways which might help
672 protecting intestinal epithelial cells from further damages caused by bacterial
673 infection.
674
675 Table 1. Human specific primer sequences for qPCR.

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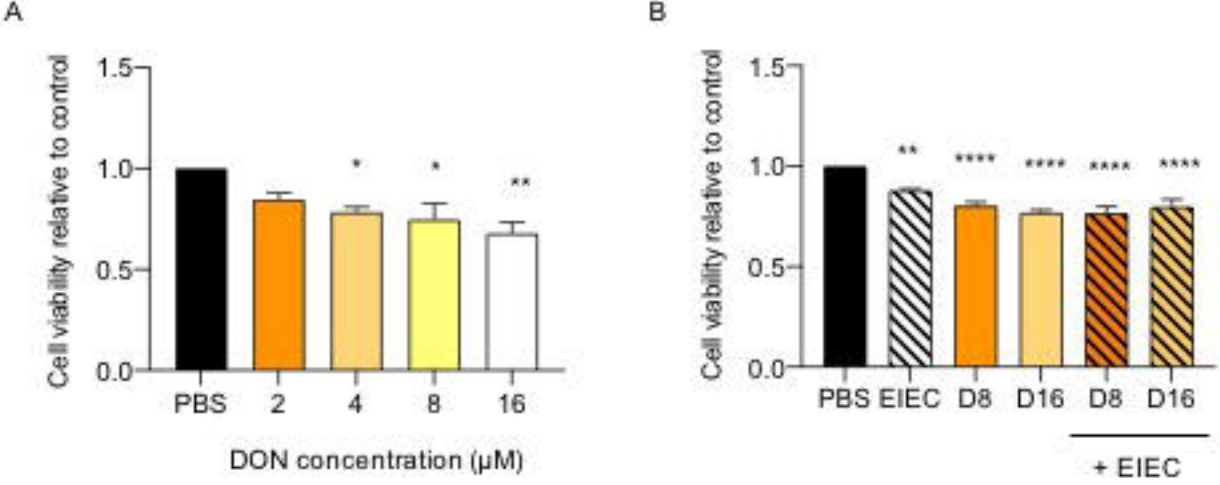
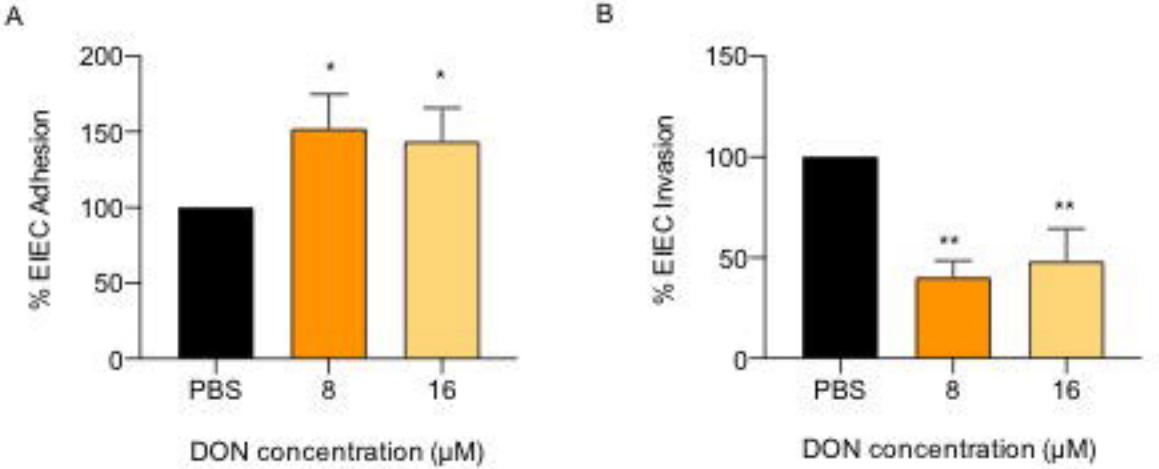
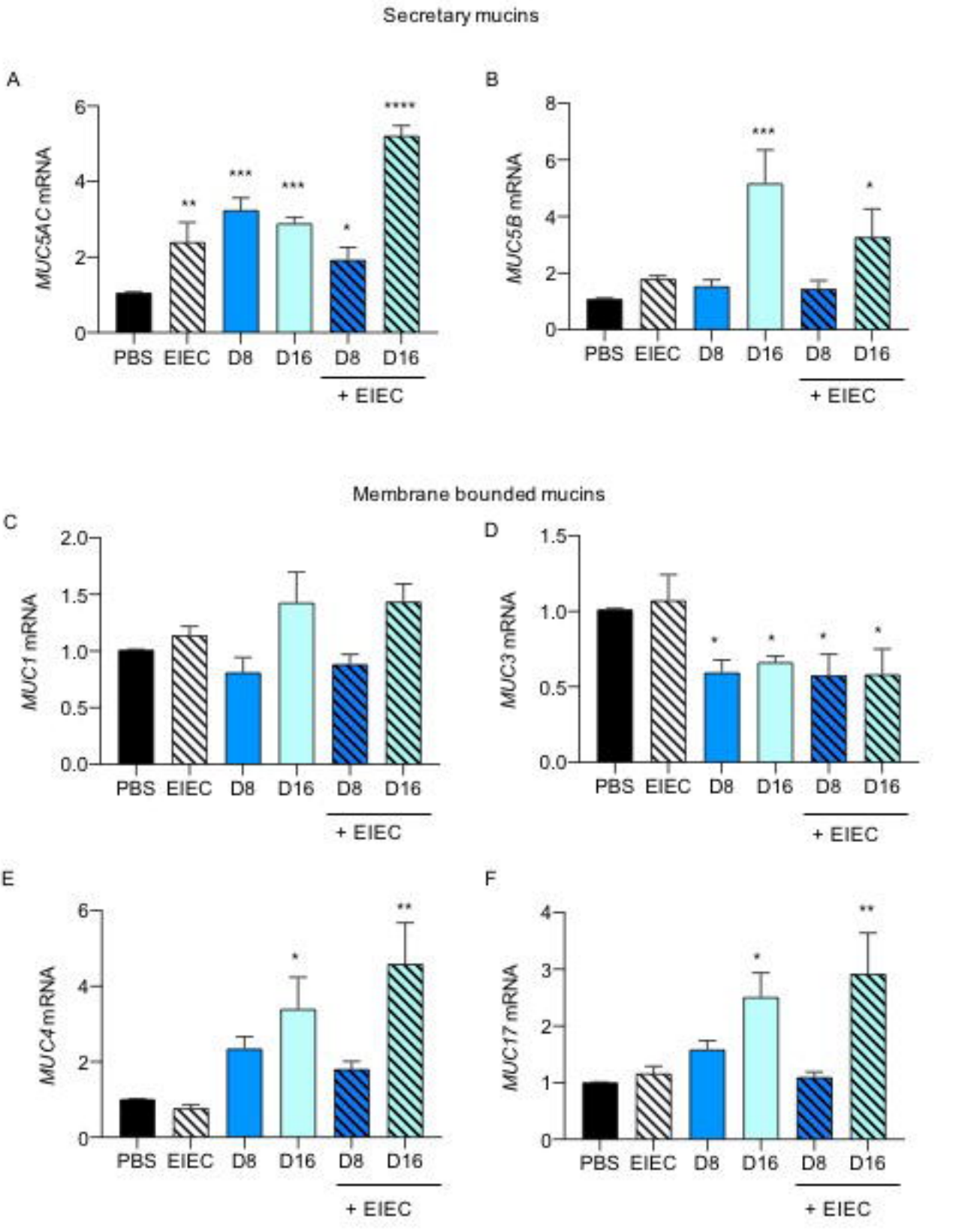
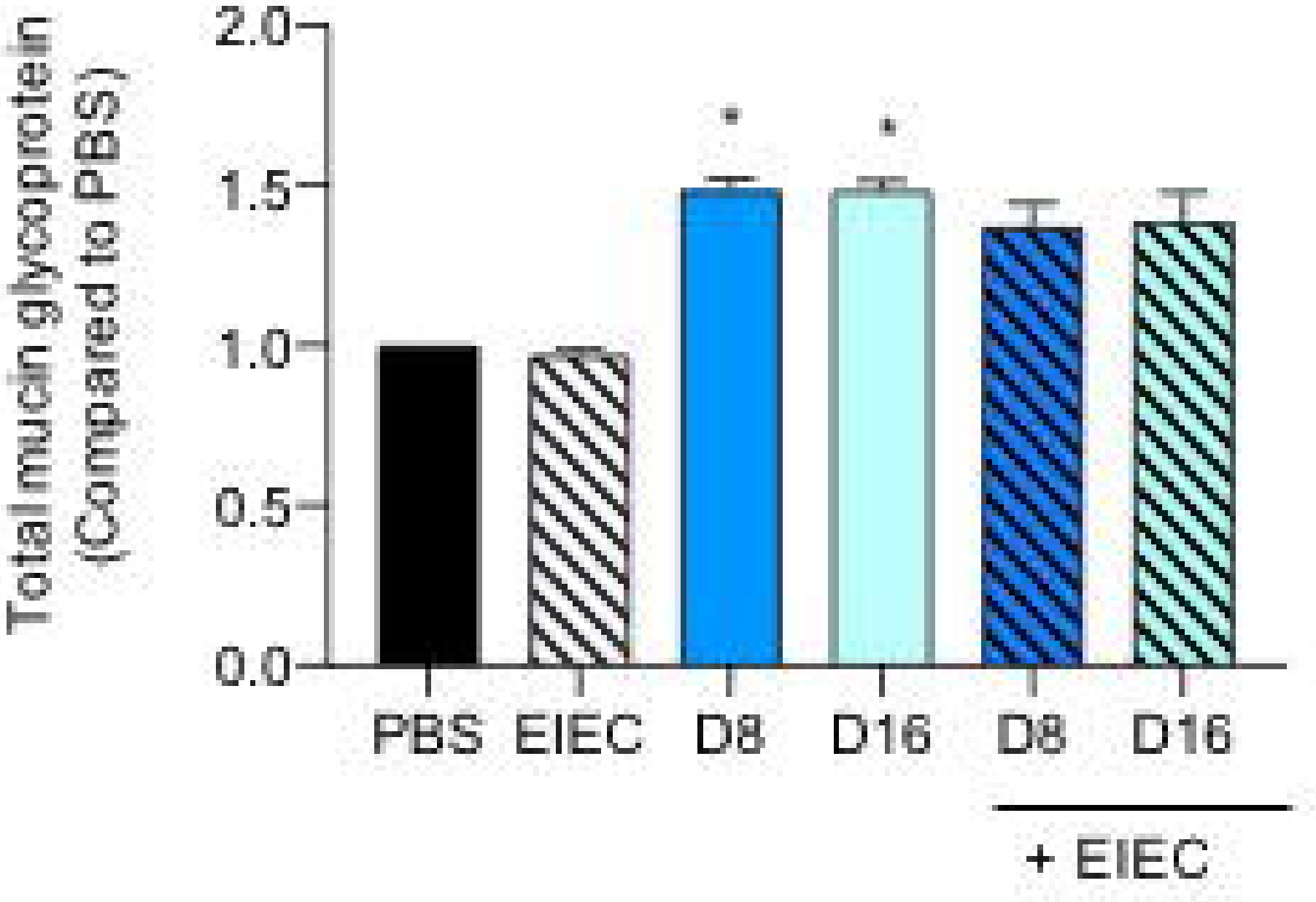
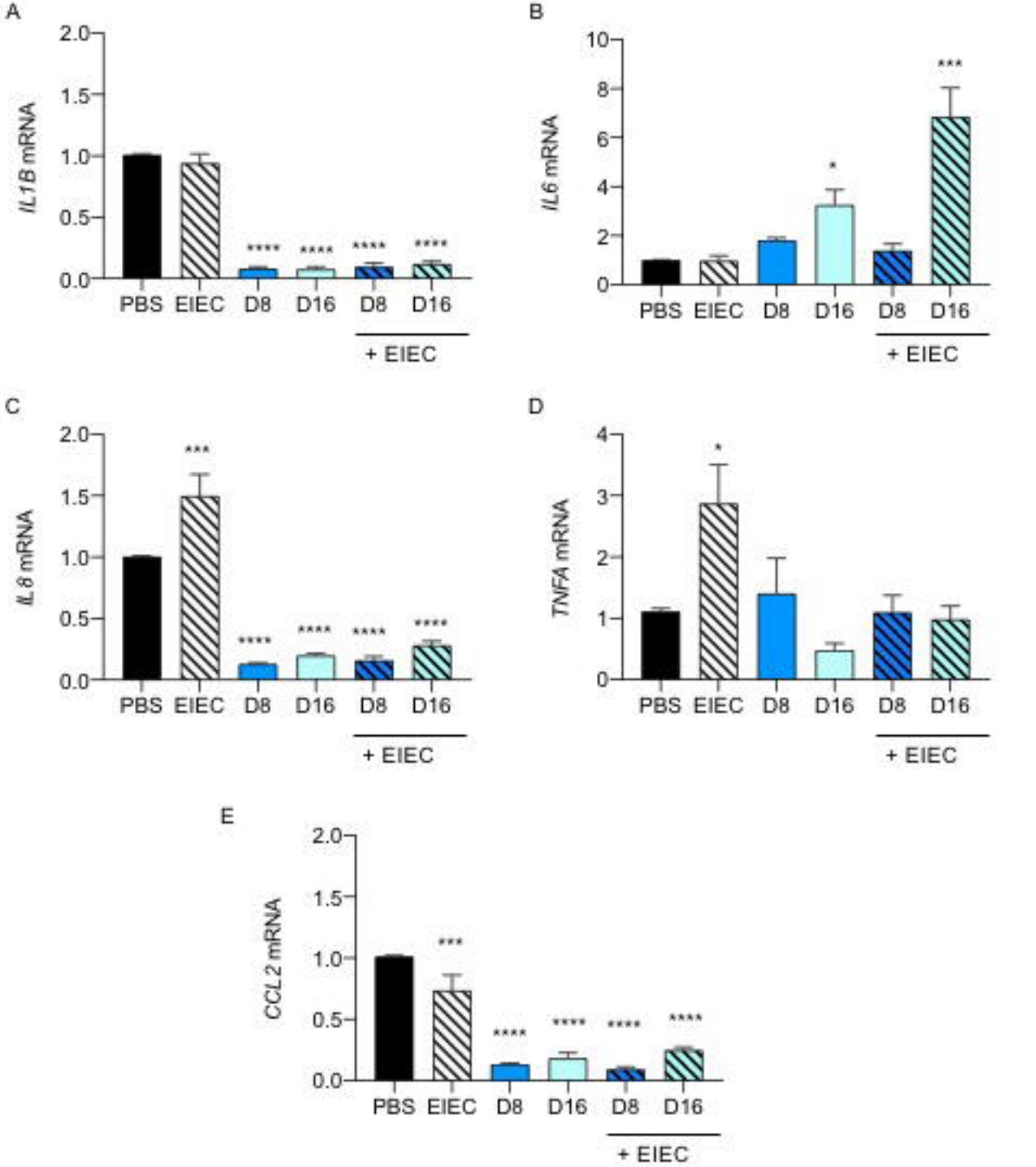
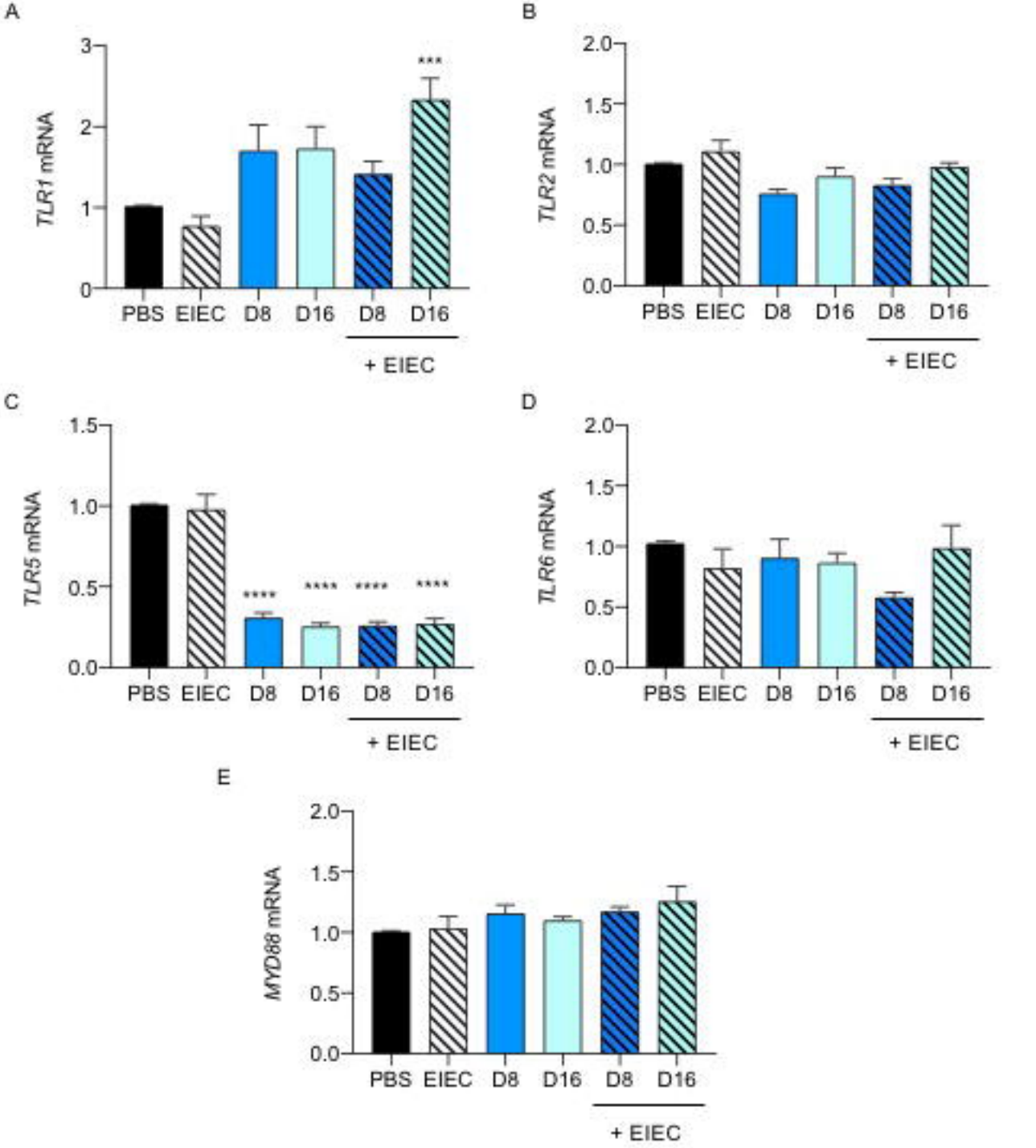

A
Cell viability relative to control
1.5
1.0
0.5
0.0
*
*
**
PBS
2
4
8
16
DON concentration (μM)
B
Cell viability relative to control
1.5
1.0
0.5
0.0
**
****
****
****
****
PBS
EIEC
D8
D16
D8
D16
+ EIEC

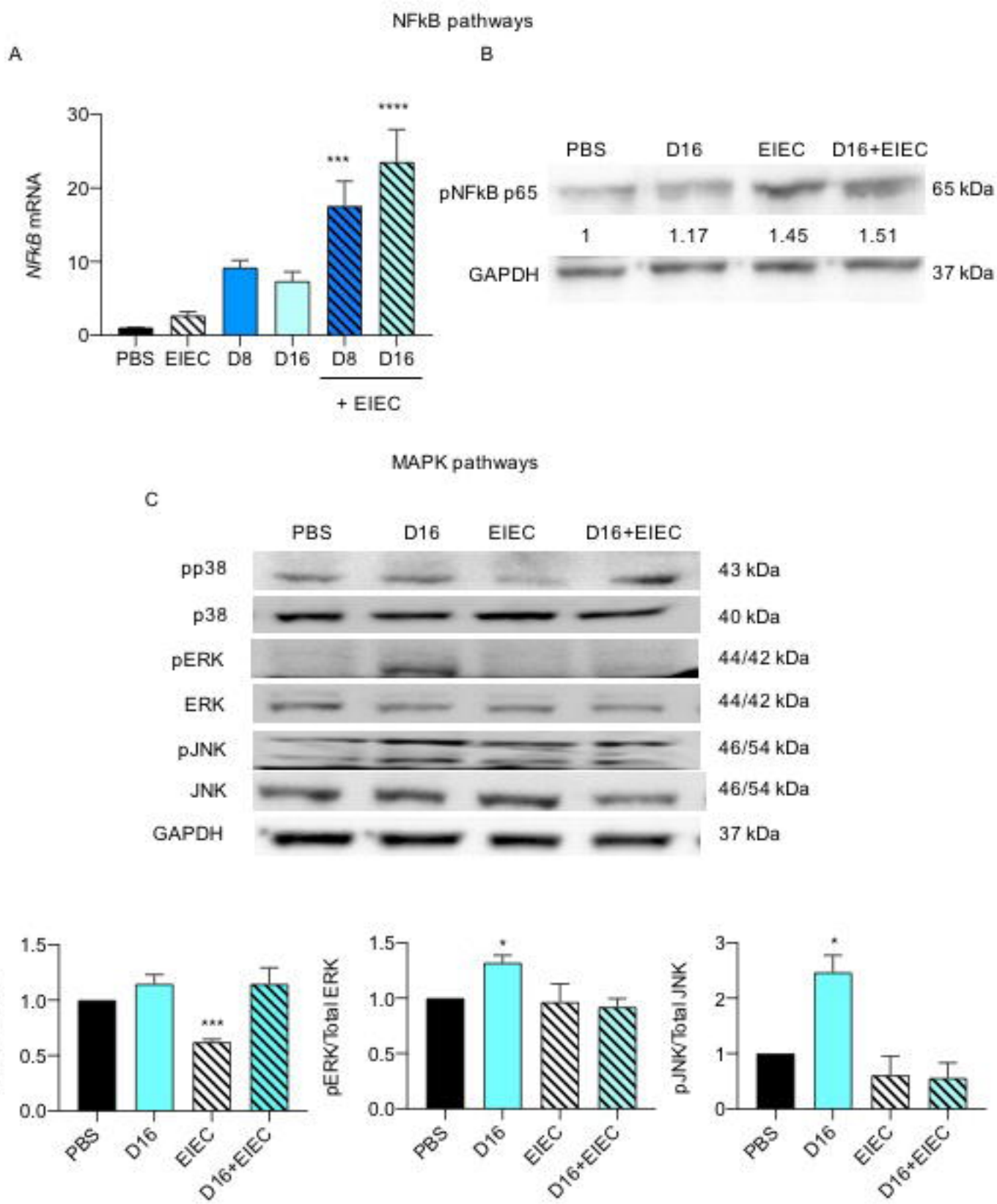

A
200
150
100
50
0
% EIEC Adhesion
*
*
PBS
8
16
DON concentration (μM)
B
150
100
50
0
% EIEC Invasion
**
**
PBS
8
16
DON concentration (μM)


Secretary mucins
A
MUC5AC mRNA
PBS
EIEC
D8
D16
D8
D16
+ EIEC
B
MUC5B mRNA
Membrane bounded mucins
C
MUC1 mRNA
D
MUC3 mRNA
E
MUC4 mRNA
F
MUC17 mRNA


Total mucin glycoprotein
(Compared to PBS)
2.0
1.5
1.0
0.5
0.0
*
*
PBS
EIEC
D8
D16
D8
D16
+ EIEC


A
IL1B mRNA
2.0
1.5
1.0
0.5
0.0
****
****
****
****
PBS
EIEC
D8
D16
D8
D16
+ EIEC
B
IL6 mRNA
10
8
6
4
2
0
*
***
PBS
EIEC
D8
D16
D8
D16
+ EIEC
C
IL 8 mRNA
2.0
1.5
1.0
0.5
0.0
***
****
****
****
PBS
EIEC
D8
D16
D8
D16
+ EIEC
D
TNFA mRNA
4
3
2
1
0
*
PBS
EIEC
D8
D16
D8
D16
+ EIEC
E
CCL2 mRNA
2.0
1.5
1.0
0.5
0.0
***
****
****
****
PBS
EIEC
D8
D16
D8
D16
+ EIEC


A
TLR1 mRNA
3
2
1
0
***
PBS EIEC D8 D16 D8 D16
+ EIEC
B
TLR2 mRNA
2.0
1.5
1.0
0.5
0.0
PBS EIEC D8 D16 D8 D16
+ EIEC
C
TLR5 mRNA
1.5
1.0
0.5
0.0
****
****
****
****
PBS EIEC D8 D16 D8 D16
+ EIEC
D
TLR6 mRNA
2.0
1.5
1.0
0.5
0.0
PBS EIEC D8 D16 D8 D16
+ EIEC
E
MYD88 mRNA
2.0
1.5
1.0
0.5
0.0
PBS EIEC D8 D16 D8 D16
+ EIEC


NFkB pathways
A
B
30
20
10
0
NFkB mRNA
***
****
PBS
EIEC
D8
D16
D8
D16
+ EIEC
PBS
D16
EIEC
D16+EIEC
pNFkB p65
65 kDa
1
1.17
1.45
1.51
GAPDH
37 kDa
MAPK pathways
C
PBS
D16
EIEC
D16+EIEC
pp38
43 kDa
p38
40 kDa
pERK
44/42 kDa
ERK
44/42 kDa
pJNK
46/54 kDa
JNK
46/54 kDa
GAPDH
37 kDa
D
pp38/Total p38
1.5
1.0
0.5
0.0
***
PBS
D16
EIEC
D16+EIEC
pERK/Total ERK
1.5
1.0
0.5
0.0
*
PBS
D16
EIEC
D16+EIEC
pJNK/Total JNK
3
2
1
0
*
PBS
D16
EIEC
D16+EIEC

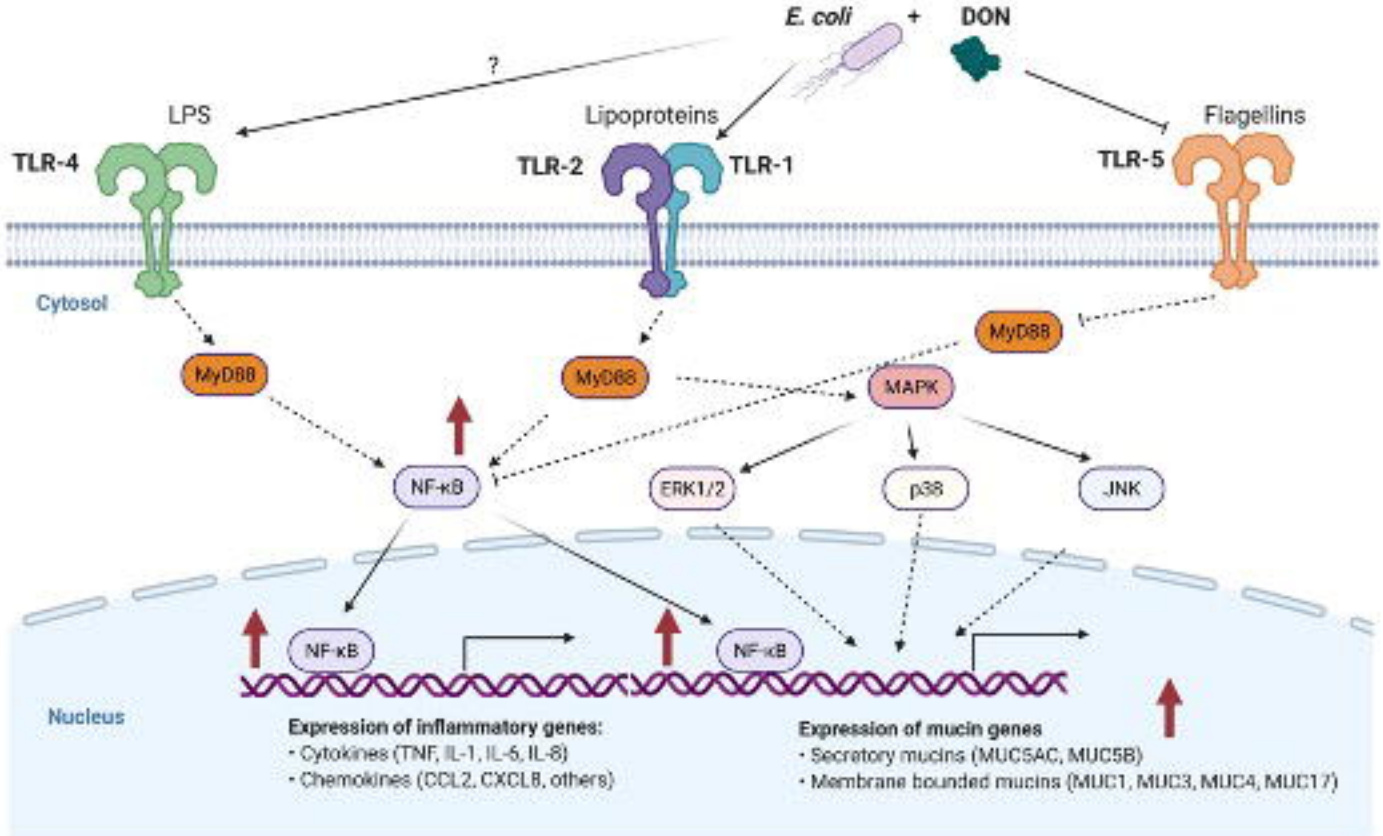


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Table 1. Human specific primer sequences for qPCR

Gene name	Abbreviated name	Product length (bp)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Accession Number	Reference
Mucin 1	MUC1	105	GTGGTGGTACAATTGACTCTGG	GTTATATCGAGAGGCTGCTTCC	NM_001204294.1	(Sperandio et al., 2013)
Mucin 3	MUC3	113	CTGCAACTACCAGCACTTCTTC	TATAGTTCCTGGACAGGGTGTG	NM_005960.2	(Sperandio et al., 2013)
Mucin 4	MUC4	113	AGGCTACCTCAAGACTCACCTC	TCATTCTCCTTGAAGAATCCTG	NM_018406.7	(Sperandio et al., 2013)
Mucin 5AC	MUC5AC	131	CTCCTACCAATGCTCTGTA	GTTGCAGAAGCAGGTTTG	NM_001304359.2	(Wan et al., 2014)
Mucin 5B	MUC5B	154	GACAGAGACGACAATGAG	CCTGATGTTTTCAAAAGTTTC	NM_002458.3	(Wan et al., 2014)
Mucin 17	MUC17	122	GTTTCAACACCACTGGCACC	CTGGTCCCGGTACTCCACTA	NM_001040105.1	NM_001040105.1
Interleukin-1β	IL1B	138	TGGAGCAACAAGTGGTGTTC	GCTGTAGAGTGGGCTTATCATC	NM_000576.2	(Tian et al., 2016)
Interleukin-6	IL6	100	TGAAAGCAGCAAAGAGGCACT	GCAAGTCTCCTCATTGAATCCAG	NM_000600.5	(Tian et al., 2016)
Interleukin-8	IL8	98	CTGATTTCTGCAGCTCTGTG	GGGTGGAAAGGTTTGGAGTATG	NM_000584.4	(Kina et al., 2009)
Tumor necrosis factor-α	TNFA	93	CTGCTGCACTTTGGAGTGAT	AGATGATCTGACTGCCTGGG	NM_000594.4	(Chanput et al., 2010)
Monocyte chemoattractant protein 1	CCL3	171	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC	NM_002982.4	NM_002982.3
Toll-like receptor-1	TLR1	135	GCCCAAGGAAAAGAGCAAAC	AAGCAGCAATATCAACAGGAG	NM_003263.4	(Lee et al., 2014)

Toll-like receptor-2	TLR2	125	TCTCCCATTTCCGTCTTTTT	GGTCTTGGTGTTCATTATCTTC	NM_001318796.1	(Lee et al., 2014)
Toll-like receptor-4	TLR4	213	GAAGCTGGTGGCTGTGGA	GATGTAGAACCCGCAAG	NM_138554.5	(Lee et al., 2014)
Toll-like receptor-5	TLR5	149	TTGCTCAAACACCTGGACAC	CTGCTCACAAGACAAACGAT	NM_003268.6	(Lee et al., 2014)
Toll-like receptor-6	TLR6	109	GTGCCATTACGAACTCTA	TTGTTGGGAATGCTGTT	NM_006068.4	(Lee et al., 2014)
Myeloid differentiation primary response 88	MYD88	143	GCAGAGCAAGGAATGTGACTTC	AGTCGCAGACAGTGATGAACCT	NM_001365877.1	(Tian et al., 2016)
RELA proto-oncogene, NF-kB subunit	RELA	112	TCTGCTTCCAGGTGACAGTG	ATCTTGAGCTCGGCAGTGTT	NM_021975.4	(Garg, Potter, and Abrahams, 2013)
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	159	CATGTTCGTCATGGGGTGAACCA	AGTGATGGCATGGACTGTGGTCAT	NM_002046.7	(Luo et al., 2017)