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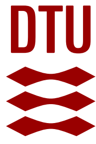

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Necrotizing Enterocolitis in Preterm Pigs Is Associated with Increased Density of Intestinal Mucosa-Associated Bacteria Including Clostridium perfringens

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1 **Necrotizing enterocolitis in preterm pigs is associated with increased density of**
2 **intestinal mucosa-associated bacteria including *C. perfringens***
3
4 Short title: Tissue-associated bacteria and NEC
5
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17
18 **Key words**: Animal model, Cell culture, Gene expression, Necrotizing enterocolitis, Microbiota,
19 Quantitative PCR, *Clostridium perfringens*
20
21 **Abbreviations**: NEC, necrotizing enterocolitis; FISH, fluorescence *in situ* hybridization; PBS,
22 phosphate buffered saline; MOI, multiplicity of infection; SEM, standard error of mean; RIN, RNA
23 integrity number
24

25 ## Abstract

26 *Background:* Necrotizing enterocolitis (NEC) is associated with changes in the luminal gut
27 microbiota. It is not known whether the mucosa-associated microbiota is affected by NEC and
28 stimulates inflammatory lesions. *Objective:* We hypothesized that the density of the mucosa-
29 associated microbiota correlates with NEC severity in preterm pigs and that *C. perfringens*, which
30 has been associated with NEC in preterm infants, is stimulating the expression of immune genes in
31 intestinal epithelial cells. *Methods:* First, we determined the density of total bacteria and *C.*
32 *perfringens* in the distal small intestinal mucosa of 58 NEC- and healthy preterm pigs using
33 quantitative PCR. Next, we analyzed in IPEC-J2 cells the effect of different infection densities of *C.*
34 *perfringens* type A on the expression of genes related to intestinal function and immune response.
35 *Results:* Total bacterial and *C. perfringens* densities were higher in NEC- versus healthy pigs, and
36 correlated positively with NEC severity. In IPEC-J2 cells expression levels of immune-related
37 genes (*CCL5, NFKBIA*, *IL8*, *IL1RN,* and *TNFAIP3*) increased, while the expression of the
38 sodium/glucose co-transporter (*SLC5A1)* decreased, with increasing density of *C. perfringens*.
39 *Conclusions:* The density of mucosa-associated bacteria, and specifically *C. perfringens*, may
40 stimulate the progression of NEC in preterm pigs. *C. perfringens* affects newborn porcine intestinal
41 epithelial cells by changing their immune gene expression patterns, which may enhance the
42 inflammation and development of lesions in the immature intestine.

43

44 ## Introduction

45 An unbalanced intestinal microbiota is a risk factor for necrotizing enterocolitis (NEC), but its exact
46 contribution remains unclear. In general, studies show that infants developing NEC have a different
47 gut microbiota than infants staying healthy [1]. Differences include lower bacterial diversity, higher
48 density of total bacteria and increased numbers of Proteobacteria like *E. coli* and *Klebsiella* [2–5],

49 but also of *Clostridium spp.* including *C. perfringens* [6,7; ?ny kilde/Sim et al 2015?], which may
50 lead to a more severe and often lethal disease progression than other NEC-related pathogens
51 [7;schlapbach?]. Thus, pathogens may at a certain threshold alone or together with other members
52 of the microbiota stimulate NEC development. Still, in other studies only minimal changes are
53 observed [8,9]. Therefore, cause and effect are difficult to separate.
54 The majority of intestinal microbiota analyses have relied on fecal samples to reflect the intestinal
55 microbiota, and only few studies have included intestinal tissue or contents [3,7,8]. This may add
56 further to the ambiguity regarding the composition of the microbiota due to differences in sample
57 material [10]. The bacteria that are most relevant to NEC and directly affects intestinal epithelial
58 cells may be those in close contact with the intestinal epithelial cells, the mucosa-associated
59 microbiota. Access to intestinal samples from preterm infants is obviously difficult. Hence, the
60 mucosa-associated microbiota is best investigated in appropriate animal models of NEC, coupled
61 with cell studies. To provide novel insight into the association between NEC and the density of the
62 mucosa-associated microbiota, and the transcriptional immune response of epithelial cells to *C.*
63 *perfringens*, we used a preterm pig model of NEC [11] and a porcine intestinal IPEC-J2 cell line
64 [12]. We hypothesized that NEC severity would correlate with the density of the distal small
65 intestinal mucosa-associated bacteria. Although the etiology of NEC is multifactorial and several
66 bacterial pathogens have been associated with the disease [2–7], we choose *C. perfringens* for more
67 in-depth analysis. *C. perfringens* has repeatedly been associated with NEC in preterm pigs
68 [reviewed in 13] and in preterm infants [6,7; ?ny kilde/Sim et al 2015?], where it often lead to a
69 fulminant disease course [7; schlapbach?]. Furthermore, it is know to produce a large number of
70 toxins and is a common cause of severe diseases including enteric disease in humans and animals
71 [reviewed in Hatheway 1990]. (Thomas et al 1984?). The tissue samples originated from previous
72 studies of diet-dependent differences in NEC-sensitivity and the mucosa-associated microbiota [14–

73 16]. Therefore, the present study included 58 preterm pigs to investigate the association between the
74 microbiota and NEC across diets. Furthermore, we investigated the effect of increasing numbers of
75 *C. perfringens* on immune gene expression pattern in IPEC-J2 cells. This cell line is derived from
76 the jejunum of a neonatal, unsuckled pig [12], and is a suitable *in vitro* model for newborn epithelial
77 cells [17]. Our choice of genes was based on our previous diet studies in preterm pigs [17,18] and
78 studies on the effects of pathogens on IPEC-J2 cells [12].
79

80 Materials and methods

81 Intestinal microbiota analyses

82 Fifty-eight preterm pigs were delivered by caesarean section, and surgical procedures, rearing, diet
83 intervention, euthanasia and tissue evaluation and collection have been described previously [14–
84 16]. The pigs were fed the following enteral diets: porcine colostrum (n = 5), formula (n = 9),
85 formula + probiotics (n = 13) [14], formula containing lactose (n = 11) or maltodextrin (n = 11) as
86 the principal carbohydrate source [15], or formula with a casein:whey-ratio of 60:40 (n = 9) [16].
87 Information on the composition of the diets can be found in table 1. After euthanasia, NEC was
88 blinded evaluated in the stomach, proximal-, middle-, and distal small intestine, and colon using a
89 score ranging from 1 (no or minimal focal hyperaemic gastroenterocolitis) to 6 (severe extensive
90 hemorrhagic and necrotic gastroenterocolitis). A score of minimum three in one intestinal region
91 was defined as a case of NEC. All animal protocols and procedures were approved by the Danish
92 National Committee on Animal Experimentation.
93 To get an approximation of the mucosa-associated microbiota for both FISH and quantitative PCR,
94 the luminal content was gently removed by squeezing along the length of the intestine and the
95 remaining tissue used for analysis. To visualize the association between the microbiota and the
96 intestinal tissue, fluorescence *in situ* hybridization (FISH) was performed on distal small intestinal

97 samples without luminal content from two of the studies [15,16] as previously described [14].
98 Quantitative PCR was performed on distal small intestinal samples without luminal content that was
99 collected as full thickness tissue samples of 3 cm, snap frozen in liquid nitrogen and stored at -80°C
100 until analysis. DNA from the tissue samples was extracted using the QIAamp DNA mini kit
101 (Qiagen, West Sussex, UK) according to the manufacturer's instructions. Quantification of total
102 bacteria [19] and *C. perfringens* [20] was performed according to previous studies, but with minor
103 modifications. Briefly, the reaction mixture (25 µl) for total bacteria included 25 ng extracted DNA,
104 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Nærum, Denmark), 0.9 µM of each
105 primer (0.09 µM of primer 8FB) and 0.2 µM TaqMan probe. For *C. perfringens*, 20 µl reaction
106 mixture included 20 ng extracted DNA, 2× Taqman Universal PCR Master Mix (Applied
107 Biosystems), 0.25 mg/ml bovine serum albumin (Applied Biosystems), 1 µM of each primer and
108 0.1 µM TaqMan probe. Primers and probes (table 2) were synthesized at DNA Technology, Aarhus,
109 Denmark. Quantitative PCR was performed on a RotorGene 3000 Detection System (Corbett Life
110 Science, Sydney, Australia) under the following conditions: 95°C for 10 min, followed by 40 cycles
111 at 95°C for 30 s, 55°C for 30 s, 60°C for 45 s, 65°C for 15 s, and 72°C for 15 s for total bacteria,
112 and 95°C for 10 min, 45 cycles at 94°C for 10 s, 55°C for 20 s, and 70°C for 10 s for *C.*
113 *perfringens*. Standard curves were generated using *C. perfringens* DNA (NCTC 10240, National
114 Veterinary Institute, Frederiksberg C, Denmark) ranging over five (total bacteria) and six (*C.*
115 *perfringens*) tenfold dilutions from the limit of detection (total bacteria, 0.1 pg DNA/µl and *C.*
116 *perfringens*, 0.0125 pg DNA/µl). Cycle threshold was determined using the Rotor-Gene 3000 data
117 analysis software (Corbett Life Science) using the Auto-Find Threshold function. Standards and
118 samples were run in triplicates, and every reaction plate included one non-template control in
119 triplicate. Due to lack of sample material, samples from 49 pigs were included in the total bacteria
120 assay, while samples from all 58 pigs were included in the *C. perfringens* assay. The results were

121 calculated as relative quantities measured as pg DNA/25 ng of extracted total DNA (total bacteria)
122 and pg DNA/20 ng of extracted total DNA (*C. perfringens*).
123
124 **Gene expression in IPEC-J2 cells during *C. perfringens* infection**
125 IPEC-J2 cells [12] were maintained as previously described [17]. *C. perfringens* type A (NCTC
126 10240, National Veterinary Institute) was cultured (16 h; 37°C; anaerobic; brain heart infusion
127 broth, SSI diagnostics, Hillerød, Denmark), washed twice in Dulbecco's phosphate buffered saline
128 (PBS, Sigma-Aldrich, Brøndby, Denmark) and resuspended in growth medium that was prepared
129 according to [17], but without antibiotics. For the experiment, IPEC-J2 cells were grown in 6-well
130 plates (Corning Costar cell culture plates, Sigma-Aldrich) until near-confluence was achieved and
131 incubated in growth medium without antibiotics for 24 h. They were infected for 2 h with *C.*
132 *perfringens* at multiplicity of infection (MOI; n = 5) = 0, 10, 20 and 50 determined by OD600
133 measurement. Hereafter, the supernatant was collected, and the cells washed (PBS) and harvested
134 (1× trypsin:EDTA, Sigma-Aldrich). The cells and supernatant were centrifuged (10 min; 1000 rpm;
135 4°C), and the cell pellet stored at -80°C. The gene expression analysis was done as previously
136 described using reverse transcription quantitative real-time PCR [17,21]. Genes are denoted by their
137 gene symbol and information on primers for reference genes and genes showing significant
138 differences can be found in table 3, while information on the 48 primer pairs analyzed have been
139 published previously [17]. *RPL13A* and *ACTB* were the most stably expressed reference genes of 5
140 candidate genes, and used to normalize all samples in GenEx5 (MultiD Analyses AB, Göteborg,
141 Sweden). After normalization, quantification cycle was converted to relative quantities. Relative
142 expression of the sample with the lowest level of expression was scaled to 1 for each primer assay.
143
144 **Statistical analyses**

145 The density of total bacteria and *C. perfringens* was analyzed using a Mann-Whitney test with a
146 Dunn's multiple comparison post-hoc test in GraphPad Prism (Version 5.02, La Jolla, CA, USA)
147 and the results considered significant when $p < 0.05$. Correlation analysis was performed using
148 Spearman correlation analysis in GraphPad Prism, and correlations were considered significant if ρ
149 < -0.5/> 0.5, equal to $p < 0.001$. Analysis of the gene expression in IPEC-J2 cells was performed as
150 previously described [17]. Data was $\log_2$ transformed and tested with a one-way ANOVA with a
151 Tukey-Kramer's post-hoc test (GenEx5, MultiD Analyses AB). Gene expression was considered
152 significant if $p < 0.05$ and relative gene expression differences were > 2.0-fold between the groups.
153
154 **Results**

155 **Intestinal microbiota analyses**

156 Visual inspection of FISH images showed a higher number of bacterial micro-colonies associated
157 with the mucosa in NEC- than healthy pigs. *C. perfringens* was part of the micro-colonies, which
158 were found along the length of the villi and down to the crypts in NEC- and healthy pigs.
159 Quantitatively, total bacterial density was higher in NEC- (n = 34) compared with healthy pigs (n =
160 15), and the same was observed for the density of *C. perfringens* when comparing NEC- (n = 38)
161 with healthy pigs (n = 20, figure 1A,B). The correlation between the density of total bacteria and
162 the distal small intestinal NEC score was $\rho = 0.440$, $p < 0.01$ (figure 1C) mainly driven by the low
163 NEC score pigs. An even more positive correlation was found between the distal small intestinal
164 NEC score and the density of *C. perfringens* ($\rho = 0.687$, $p < 0.001$, figure 1D). Finally, a positive
165 correlation between the densities of total bacteria and *C. perfringens* was observed ($\rho = 0.585$, p <
166 0.001).
167

168 **Gene expression in IPEC-J2 cells during *C. perfringens* infection**

169 The *in vitro* experiment showed that the cellular response of IPEC-J2 cells changed with increasing
170 number of *C. perfringens* type A. A small effect was observed on the RNA integrity, measured by
171 RNA integrity number (RIN), as this decreased from MOI = 0 (mean RIN = 9.98 ± 0.02) to MOI =
172 20 (mean RIN = 9.38 ± 0.25). The largest effect was seen at MOI = 50 since a RIN for only one
173 replicate was obtainable, which indicated a high degree of RNA degradation resulting from cell
174 lysis (figure 2A). To assure the most accurate results, the MOI = 50 group and one MOI = 10
175 replicate were excluded in the statistical analysis as large differences in RIN may affect the results.
176 Of the 22 genes, passing the data evaluation (table 3), six were differentially expressed between the
177 MOI groups (figure 2B). The expression of *CCL5, NFKBIA, IL8* and *TNFAIP3*, encoding proteins
178 involved in inflammation, was up-regulated in MOI = 10 compared with MOI = 0. Furthermore,
179 *NFKBIA* and *TNFAIP3* expression was higher at MOI = 20 compared with MOI = 0. The
180 expression of *CCL5* and *IL8* decreased at MOI = 20 to levels between MOI = 0 and MOI = 10,
181 while the decrease in *IL1RN* resulted in a difference between MOI = 10 and MOI = 20. The
182 expression of *SLC5A1* (sodium/glucose co-transporter) decreased with increasing MOI.
183

184 **Discussion**

185 In this study, we observed an association between NEC severity and the density of total bacteria,
186 including *C. perfringens* Type A, in the distal small intestinal mucosa of preterm pigs. The results
187 indicate that the mucosal bacterial density is a factor associated with the progression of NEC. Even
188 though this study does not clarify the exact contribution of the microbiota to the pathogenesis, we
189 speculate that reaching a certain threshold of bacterial contact stresses the immature intestinal
190 epithelium and set off the inflammatory process towards NEC. Although 14 of the 38 pigs with
191 NEC did not have lesions in the distal small intestine, but in another intestinal region, the density of
192 total bacteria in the distal small intestine was similar to pigs with NEC in this region (data not

193 shown). We speculate that the increased density of bacteria in the distal small intestine might reflect
194 the bacterial density in other regions of the intestine, and in the initial phase of NEC, another region
195 was stressed before the distal small intestinal region and the inflammatory process towards NEC
196 initiated here. The NEC-inducing effect of mucosal bacteria may interact with diet factors, because
197 a high intake of poorly digestible milk diets would likely increase bacterial proliferation and
198 metabolism. The results are in accordance with studies in preterm infants and pigs showing an
199 association between NEC and the density of both total bacteria [3,4] and *C. perfringens* [6,7,13].
200 The association between specific bacteria and NEC lesions could be due to increased density of
201 total bacteria, which was observed by the positive correlation between the density of total bacteria
202 and *C. perfringens*. However, the induction of intestinal injury by specific pathogens may still be
203 significant. Bjørnvad *et al*. [22] found that *C. perfringens* invaded the tissue more deeply in NEC-
204 compared with healthy pigs. In the present study, FISH observations showed bacteria closely
205 associated with the surface of the intestinal epithelium, and invasive pathogens could therefore
206 potentially affect the epithelial cells directly.
207 Gene expression changes in IPEC-J2 cells exposed to *C. perfringens* were observed by an increased
208 expression of inflammatory factors concomitant with a decrease in *SLC5A1*. In contrast, other genes
209 like *CD14*, *IL6*, *IL18* and *TLR4,* involved in the innate immune response, were not affected. Genes
210 encoding pro-inflammatory factors, *IL8* and *CCL5*, and anti-inflammatory factors, *IL1RN*, *NFKBIA*
211 and *TNPAIP3*, were affected by the increased numbers of *C. perfringens*, and indicate that
212 increased bacterial level initiates an inflammatory response. A concomitant up-regulation of IL-8
213 and IL-10 in plasma was also observed in preterm infants in response to severe NEC [23]. Likewise,
214 *CCL5*, *IL8* and *NFKBIA* were up-regulated with increasing NEC in preterm pigs [24, unpublished
215 data] generating further evidence for the applicability of IPEC-J2 cells as a low cost model for the
216 neonatal intestine. In preterm pigs given oral antibiotics NEC is prevented and *IL8* and other

217 immune-related genes are down-regulated [25]. The decrease in *SLC5A1* expression indicates that
218 absorptive functions are compromised during *C. perfringens* infection. This could cause
219 accumulation of nutrients available for bacterial fermentation and lead to bacterial overgrowth and
220 cytotoxic levels of metabolites. Conversely, providing milk diets that easily exceed the immature
221 digestive capacity of preterm newborns may provide substrates for bacterial fermentation. In quails,
222 excessive luminal fermentation by *Clostridium* species leads to NEC-like lesions [26]. Bacterial
223 toxins may also have a cytotoxic effect, and a rapid up-regulation of toxins by *C. perfringens* type C
224 when in close contact with Caco-2 cells was observed [27]. However, in preterm infants no positive
225 correlation was found between *C. perfringens* α-toxins and NEC severity [7]. Further studies should
226 investigate if the observed changes in gene expression of the epithelial cells are unique to *C.*
227 *perfringens* and if differences, for example production of metabolites, between pathogens involved
228 in NEC are associated with different disease courses.

229 In conclusion, mucosa-associated bacterial density is associated with NEC severity in preterm pigs.
230 Furthermore, specific pathogens may play a role in NEC development that was shown in this study
231 by the association between NEC severity and the density of *C. perfringens*, and by the ability of this
232 pathogen to induce changes in immune response genes of intestinal epithelial cells *in vitro*. It is
233 highly likely, that increased bacterial density in close association with the intestinal epithelium also
234 plays an important role for NEC development in preterm infants, both in its initiation and
235 progression to severe mucosal lesions. Our results suggest that preventive measures against NEC
236 might be directed towards stimulating the mucosal barrier against bacterial attachment, coupled
237 with provision of diets that minimize maldigestion and substrates available for fermentation. This
238 could include stimulation of intestinal mucus production by highly digestible and immune-
239 modulating milk diets [28] or by decreasing bacterial density using oral antibiotics [25], as shown in
240 preterm pig studies.

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336 ## Figure legends

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338 **Figure 1:** (A) Association between NEC and the density of total bacteria and (B) the density of *C.*
339 *perfringens* in NEC- (NEC-pigs) and healthy pigs (HEAL-pigs) determined by quantitative PCR.
340 Significant differences are indicated as * for $p < 0.05$ and *** for $p < 0.001$. Since two different
341 quantitative PCR assays have been used for quantification of total bacteria and *C. perfringens* figure
342 (A) and (B) cannot be directly compared. Spearman correlations between distal small intestinal
343 NEC score and the density of (C) total bacteria and (D) *C. perfringens*.

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345 **Figure 2:** (A) RNA degradation in IPEC-J2 cells measured by the RNA integrity number (RIN) and
346 (B) relative fold changes (mean + standard error of mean) of genes significantly different expressed
347 in IPEC-J2 cells stimulated with *C. perfringens* at multiplicity of infection (MOI) = 0 (white bars),
348 10 (light grey bars) and 20 (black bars). Different superscript letters (a,b) indicate significant
349 differences, $p < 0.05$ and fold change > 2.

Tables

Table 1: Diet composition in macronutrient content pr litre. All diets were fed as a bolus (15ml/kg body weight) every 3 h.

Diet	Energy, kJ	Protein, g		Carbohydrates, g			Fat, g
		Whey	Casein	Maltodextrin	Lactose	Other	
Sow colostrum#							
Formula##	4151	67	0	45		9	61
Formula with probiotics##	4151	67	0	45		9	61
Formula with lactose###	4648	63	0	8	48	2	70
Formula with maltodextrin###	4634	62	0	55	0	6	70
Formula Casein:whey ratio of 60:40###	4620	25	37	55	0	6	70

Porcine colostrum was collected manually from sows (Large White x Landrace, Research Station Sjælland II, Denmark) within 6 h of completed farrowing and stored at -20°C until used. Values for the composition of colostrum, see Sangild & Xu, 2004. Sangild PT & Xu RJ (2004) Colostrum. In Encyclopedia of Animal Science, pp. 1–3 [WG Pond and AW Bell, editors]. New York: Marcel Dekker.

Ingredients used: Pepdite, Maxipro and Liquigen-MCT, all products kindly donated by SHS International, Liverpool, UK. The probiotic mixture (kindly donated by Chr. Hansen, A/S) consisted of Bifidobacterium animalis (DSM15954) and 4 Lactobacillus species: acidophilus (DSM13241), casei (ATCC55544), pentosus (DSM14025), and plantarum (DSM13367). Probiotics were reconstituted in 1 % peptone-water and each strain was included at 10^9 colony-forming units (CFU)/g of viable lyophilized bacteria for a total concentration of 5 x 10^9 CFU/3 mL peptone-water. Boluses of probiotics or peptone-water placebo were administered (2 mL/ kg BW) every 6 h during the TPN period and every 3 h during the enteral phase. Boluses of probiotics were reconstituted fresh prior to every bolus administration.

Ingredients used: Seravit, Liquigen medium-chain triglyceride, and Calogen long-chain triglyceride (Nutricia, Allerød, Denmark); Variolac and Lacprodan alpha-15 and Miprodan (ARLA Foods Ingredient, Viby, Denmark); and Polycose (Abbott Nutrition, Columbus, OH).

Table 2. Oligonucleotide sequences of primers and probes used for quantification PCR and visualization (fluorescence *in situ* hybridization) of total bacteria and *C. perfringens* in distal small intestinal tissue of preterm pigs.

Primer/probe name	Oligonucleotide sequence (5'-3')	Reference
Quantitative PCR		
Total bacteria		
Forward primers	8FM: AGAGTTTGATCMTGGCTCAG	[19]
	8FB: AGGGTTCGATTCTGGCTCAG	
Reverse primer	Bact515R: TTACCGCGGCKGCTGGCAC	
TaqMan probe	Bact338K: [FAM]CCAKACTCCTACGGGAGGCAGCAG[TAMRA]	
C. perfringens		[20]
Forward primer	CPerf165F: CGCATAACGTTGAAAGATGG	
Reverse primer	CPerf269R: CCTTGGTAGGCCGTTACCC	
TaqMan probe	CPerf187F: [FAM]TCATCATTCAACCAAAGGAGCAATCC[TAMRA]	
Fluorescence *in situ* hybridization		
Total bacteria	S-D-bact-0338-a-A-18: [FITC]GCTGCCTCCCGTAGGAGT	[29]
C. perfringens	S-S-Cl.perf.-185-a-A-18: [Cy3]TGGTTGAATGATGATGCC	[11]

Table 3. Gene symbol, protein name, forward (F) and reverse (R) primer sequences, amplicon length and primer efficiency for reference genes and genes of interest in IPEC-J2 cells during *C. perfringens* infection.

Gene symbol	Protein	Sequence (5´- 3´)	Amplicon length	Efficiency
Reference genes				
ACTB	β-actin	F: CTACGTCGCCCTGGACTTC R: GCAGCTCGTAGCTCTTCTCC	76	0.93
RPL13A	Ribosomal protein L13a	F: ATTGTGGCCAAGCAGGTACT R: AATTGCCAGAAATGTTGATGC	76	0.85
Genes of interest showing significant difference between groups				
CCL5	Chemokine (C-C motif) ligand 5	F: CTCCATGGCAGCAGTCGT R: AAGGCTTCCTCCATCCTAGC	121	0.92
IL1RN	Interleukin 1 receptor antagonist	F: TGCCTGTCCTGTGTCAAGTC R: GTCCTGCTCGCTGTTCTTTC	90	0.98
IL8	Interleukin 8	F: TTGCCAGAGAAATCACAGGA R: TGCATGGGACACTGGAAATA	78	0.80
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	F: GAGGATGAGCTGCCCTATGAC R: CCATGGTCTTTTAGACACTTTCC	85	0.88
SLC5A1	Sodium/glucose co-transporter	F: CTGCAAGAGAGTCAATGAGGAG R: CCGGTTCCATAGGCAAACT	99	0.95
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	F: CCCAGCTTTCTCTCATGGAC R: TTGGTTCTTCTGCCGTCTCT	113	0.90
Genes of interest not showing significant difference between groups				
APOA1	Apolipoprotein A-I	F: GTTCTGGGACAACCTGGAAA R: GCTGCACCTTCTTCTTCACC	86	0.81
C3	Complement component 3	F: ATCAAATCAGGCTCCGATGA R: GGGCTTCTCTGCATTTGATG	76	0.87
CD14	CD14 molecule	F:GGGTTCCTGCTCAGATTCTG R: CCCACGACACATTACGGAGT	164	0.83
CLDN3	Claudin 3	F: ATCGGCAGCAGCATTATCAC R: ACACTTTGCACTGCATCTGG	94	0.87
CXCL10	Chemokine (C-X-C motif) ligand 10	F: CCCACATGTTGAGATCATTGC R: GCTTCTCTCTGTGTTCGAGGA	141	0.82
DEFB1	Defensin, beta 1	F: ACCTGTGCCAGGTCTACTAAAAA R: GGTGCCGATCTGTTTCATCT	109	0.90
DEFB4A (*DEFB2*)	Defensin, beta 4A	F: CAGGATTGAAGGGACCTGTT R: CTTCACTTGGCCTGTGTGTC	99	0.83
HPRT1	Hypoxanthine phosphoribosyl-transferase 1	F: ACACTGGCAAAACAATGCAA R: TGCAACCTTGACCATCTTTG	71	0.88
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	F: CTCGCACAAGGAGACATGAA R: GGGTAGCCCAGTTTTTGTCA	97	0.88
IL18	Interleukin 18	F: CTGCTGAACCGGAAGACAAT R: TCCGATTCCAGGTCTTCATC	100	0.86
IL6	Interleukin 6	F: TGGGTTCAATCAGGAGACCT R: CAGCCTCGACATTTCCCTTA	116	085
MUC1	Mucin 1	F: GGATTTCTGAATTGTTTTTGCAG R: ACTGTCTTGGAAGGCCAGAA	116	0.81
OCLN	Occludin	F: CGGTGAGAAGATTGGCTGAT R: TTTCAAAAGGCCTGGATGAC	100	0.85
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b, regulatory subunit 1	F: GCAAACTGGCTACTGTGTGAAG R: GCACAGTCTGGTCATTGGAA	113	0.83
TGFB1	Transforming growth factor, beta 1	F: GCAAGGTCCTGGCTCTGTA R: TAGTACACGATGGGCAGTGG	97	0.81
TLR4	Toll-like receptor 4	F: TTTCCACAAAAGTCGGAAGG R: CAACTTCTGCAGGACGATGA	145	0.81