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1 *Salmonella* serovar specific upregulation of porcine defensins 1 and 2 in a
2 jejunal epithelial cell line.

3

4 Edwin J.A. Veldhuizen^{a*}, Ingrid Koomen^a, Ton Ultee^b, Albert van Dijk^a, Henk P. Haagsman^a

5

6 ^aDepartment of Infectious Diseases and Immunology & ^b Center for Cell Imaging, Department of
7 Biochemistry & Cell Biology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The
8 Netherlands.

9

10 * Corresponding author: Department of Infectious Diseases & Immunology, Faculty of Veterinary
11 Medicine, Utrecht University, P.O. Box 80.165, 3508 TD, Utrecht, The Netherlands. Tel +31 30
12 2535361, Fax: + 31 30 2532333, E.J.A.Veldhuizen@uu.nl

13 **Abstract**

14 Defensins are antimicrobial important effector peptides of the innate immune system, which provides
15 protection against bacterial infections in the intestine. *Salmonella* Choleraesuis and *Salmonella*
16 Typhimurium are the most commonly isolated serovars in pig, but disease outcome is dependent on
17 the *Salmonella* serovar. These infections are a serious problem for the swine industry and are also
18 posing a major threat to public health because of *Salmonella*-related food-borne illnesses in human.
19 To understand the innate immune response of pigs upon *Salmonella* infections, we studied the effect
20 of these *Salmonella* serovars in the porcine ileal epithelial cell line IPEC-J2 on defensin gene
21 expression. With the use of scanning electron microscopy, we first visualized the surface
22 characteristics of this cell line, and captured the invasion of *Salmonella* into the epithelial cell. Gene
23 expression levels of porcine β -defensin 1 and 2 were both induced upon *Salmonella* Typhimurium
24 infection but *Salmonella* Choleraesuis had no effect. Invasion, adhesion and defensin susceptibility of
25 both serovars were similar, which could not explain the observed difference in host response to these
26 *Salmonellae*. In addition, induction of defensins was dependent on viability of *Salmonella*
27 Typhimurium, since *Salmonella* cell- or secreted components had no effect on defensin gene
28 expression. These results provide further insight into the porcine innate immune response towards
29 *Salmonella* infections, and could partially explain the different epidemiology of *Salmonella* infections
30 in pig.

31 Keywords: Innate immunity, gut, antimicrobial peptide, pig, Salmonella

32

32 **Introduction.**

33 *Salmonella* infections are an important source of food-borne illnesses and therefore a major public
34 health concern. In addition, Salmonellosis is a major cause of disease in pigs and a serious problem
35 for the swine industry. In pig, *Salmonella enterica* serovar Choleraesuis (*S. Choleraesuis*) and
36 *Salmonella enterica* serovar Typhimurium DT104 (*S. Typhimurium*) are the most frequently isolated
37 serovars (Chiu et al., 2004, Schwartz, 1999). In contrast to *S. Typhimurium*, which is able to infect a
38 very broad range of hosts including humans, *S. Choleraesuis* appears to be specifically adapted to pig
39 (Chiu et al., 2004). Interestingly, these serovars produce different patterns of disease in growing pigs:
40 *S. Typhimurium* usually causes mild enteritis and self-limiting diarrhea, while *S. Choleraesuis* leads to
41 systemic disease such as septicaemia (Fraser et al., 2007, Schwartz, 1999).

42 In the intestine, the first line of defense against invading pathogens, such as *Salmonella*, is provided
43 by the innate immune system (Beutler, 2004). Part of this immune response is the release of
44 antimicrobial peptides (AMPs) into the lumen of the intestinal tract. These peptides are capable of
45 directly killing a wide variety of bacterial and viral pathogens. One major subclass of AMPs is the
46 group of β -defensins (Lehrer, 2004). Up to date, 12 porcine β -defensins (pBDs) have been identified
47 and the expression of pBD-1 and pBD-2 in the small intestine of the pig has recently been described
48 (Sang et al., 2006, Veldhuizen et al., 2007).

49 In order to study the effect of *Salmonella* infection on defensin regulation in the porcine GI-tract we
50 used the porcine cell line IPEC-J2. These cells are derived from porcine jejunum and have
51 demonstrated to possess many of the characteristics attributed to intestinal epithelial cells *in vivo*
52 (Schierack et al., 2006). Infection of intestinal cells with *S. Typhimurium* resulted in a clear
53 upregulation of both pBD-1 and pBD-2 mRNA levels after 24h of infection. Furthermore, this
54 upregulation required viable bacteria and was independent of *S. Typhimurium* secreted components.
55 In contrast, infection of IPEC-J2 cells with *S. Choleraesuis* had no or little detectable effect on
56 defensin regulation.

57 **2. Materials and methods**

58 *2.1 Bacterial strains and culture conditions*

59 Overnight cultures of *Salmonella enterica* serovar Typhimurium DT104 (Hendriksen et al., 2004),
60 and 4 field strains of *Salmonella Choleraesuis* (denoted as *S. Choleraesuis* 994-997) were prepared
61 freshly for every experiment by cultivation from a frozen stock at 37 °C in Trypticase Soy Broth
62 (TSB, Oxoid Limited). Before use in adhesion, invasion and infection experiments, an overnight
63 culture was subcultured 1:100 and incubated for 2 h at 37 °C. Bacteria were then collected in the
64 exponential phase, spun down and resuspended in IPEC-J2 cell culture medium at 1×10^8 colony
65 forming units (CFU) /ml.

66 *2.2 Cell culture*

67 The IPEC-J2 cell line (Schierack et al., 2006) was maintained in 50% Dulbecco's Modified Eagle
68 Medium and 50% Nutrient Mixture F12 (Ham) (1:1 DMEM/F12) (GIBCO, Invitrogen life
69 Technologies, Carlsbad, CA), supplemented with (10 U/ml) penicillin, (10 mg/ml) streptomycin, (2
70 mM) L-glutamine and 5% fetal bovine serum (vol/vol) (all provided by GIBCO, Invitrogen life
71 Technologies, Carlsbad, CA). Cells were grown in monolayers at 37 °C in 5% CO₂ atmosphere.
72 Medium was changed every other day and cells were passed once a week. For infection experiments,
73 cells were cultured in 12-well cell culture plates (surface area= 4.75 cm² per well; Costar, Corning
74 Incorporated, Corning, NY) and grown to confluent monolayers in 7 days. In all experiments IPEC-J2
75 cells were used between passages 80 and 100.

76 *2.3 Scanning electron microscopy (SEM).*

77 IPEC-J2 cells were seeded on plastic slides in 12-well tissue-culture plates. After the cells reached
78 confluency, cells were incubated with *S. Typhimurium* or *S. Choleraesuis* for 1 h. The cells were then
79 washed three times with plain DMEM/F12 and incubated with Karnovskys glutaraldehyde fixative for
80 a week. Subsequently, the following fixation, wash and rehydration steps were performed: washing
81 with 0.1 ml of cacodylate buffer (pH 7.4); post-fixation with 2% OsO₄ in 0.1 ml cacodylate buffer

82 (pH 7.4) for 2 h. Rinsing in 0.1 M cacodylate for 10 min and additionally 6 times in distilled water for
83 30 min; incubation in 2% tannic acid for 1 h; washing 6 times 1 h in distilled water; fixation in 2%
84 OsO₄ buffered in 0.1 M cacodylate buffer pH 7.4 for 1 h; dehydration using serial solutions of 50%,
85 70%, 80%, 96%, and 100% acetone, and critical-point-dried using a Bal-Tec CPD 030 system.
86 Finally, the samples were coated with platinum to a thickness of 10 nm and examined using a Philips
87 XL 30 SFEG scanning electron microscope.

88 *2.4 Adhesion and invasion of Salmonella.*

89 IPEC-J2 cells were grown to confluency in twelve-well tissue-culture plates, washed three times with
90 antibiotic free medium, and incubated with 1 ml plain DMEM/F12 medium (without serum, L-
91 Glutamine or antibiotics) containing 1×10^8 CFU/ml bacteria for 1 h at 37 °C. The monolayers were
92 washed three times with plain media and lysed in 1% Triton-X100 in PBS at room temperature for 5
93 min in order to release the bacteria. The suspensions were serially diluted and 100 µl of each dilution
94 was plated on Trypticase Soy Agar (TSA, Oxoid Limited). The plates were incubated for 24 h at 37
95 °C. Numbers of cell-associated bacteria were calculated as total (adhering and intracellular) bacterial
96 CFU. In the invasion assay, the IPEC-J2 monolayers in the wells were washed once with 1 ml warm
97 plain medium after 1 h incubation with bacteria and then incubated for 2 h with 1 ml of 300 µg/ml
98 colistin in warm plain DMEM/F12 to kill extracellular bacteria. Cells were washed three times with
99 plain DMEM/F12, and finally lysed in 1% Triton X-100. The number of intracellular bacteria was
100 determined by colony-plating as described above.

101 *2.5 Susceptibility of Salmonella towards porcine β -defensin 2.*

102 The antimicrobial activity of a synthetic peptide (Genosphere Biotechnologies, Paris, France)
103 corresponding to the mature 37 form of pBD-2
104 (DHYICAKKGGTCNFSPCPLFNRIEGTCYSGKAKCCIR) was assessed against *S. Typhimurium*
105 and *S. Choleraesuis* 994, as described before (Veldhuizen et al., 2008). In short, 25 µl 2.10^6 CFU/ml
106 mid-logarithmic bacteria were incubated in 5 mM phosphate, pH 7.0, 1/200 TSB with 25 µl pBD2 (0-

107 256 µg) for 3 h at 37 °C. Subsequently, 200 µl TSB was added, further diluted 10-1000 fold in TSB
108 and transferred onto TSA plates. After 24 h incubation at 37 °C colonies were counted.

109 *2.6 Infection of IPEC-J2 cells with Salmonella.*

110 IPEC-J2 cells were grown to confluency in complete IPEC-J2 medium as described above and
111 infected with 1 ml of 1×10^8 CFU/ml bacteria in plain DMEM/F12 for 3 h. After incubation, cells
112 were washed twice and incubated with fresh complete medium containing 300 µg/ml colistin. After 6
113 and 24 h (and in initial experiments also after 12 h) post infection, IPEC-J2 cells were dissolved in 1
114 ml TRIzol ® (Invitrogen life technologies, Carlsbad, CA) for quantitative PCR analysis.

115 *2.7 Effect of viability of bacteria on defensin gene expression.*

116 *S. Typhimurium* were grown to log phase and collected as described above and resuspended in IPEC-
117 J2 medium. The bacteria were then subjected to one of the following treatments: 1) incubation at 80
118 °C for 60 min. 2) Incubation in IPEC-J2 medium containing 1 mg/ml colistin, or 3) incubation in
119 IPEC-J2 medium (without antibiotics) for 1h, followed by filtration (0.45µm pore size) to remove
120 bacteria. Subsequently, the medium containing the killed bacteria (treatment 1 and 2), or the
121 *Salmonella* secreted proteins were used in the IPEC-J2 infection experiments. A small portion of the
122 solution containing the treated bacteria was serially diluted and colony-plated on TSA to detect the
123 presence of viable *S. Typhimurium*.

124 *2.8 Quantitative PCR.*

125 Total cellular RNA was isolated from cell cultures using TRIzol as recommended by the suppliers. An
126 extra wash step with ice cold 70% ethanol was performed to remove the final traces of organic
127 solvents. Purity and quality of the RNA extracts was checked on 1% agarose gels and using UV
128 absorption at 260/280 nm. Approximately 500 ng RNA was used to produce cDNA, using iScript
129 (Bio-rad Laboratories BV, Veenendaal, The Netherlands) according to the manufacturer's
130 recommendations. Quantitative PCR (qPCR) was performed on cDNA samples using a qPCR
131 mastermix for Primer-Probe reactions (RT-QP2X-03, Eurogentec Nederland b.v., The Netherlands).

132 All qPCR reactions were performed according to the manufacturer's recommendation, using gene
133 specific primers and probes at concentrations depicted in table 1. Probes were labeled with 5' FAM
134 (pBD-1 and HPRT) or 5' Yakima Yellow (pBD-2) and 3' Deep Dark Quencher 1 (all probes). All
135 qPCR reactions were performed in 25 μ l on an ABI Prism 7000 sequence detection system (Applied
136 Biosystems, Weiterstadt, Germany), using the following cycling protocol: 10 min 95 °C
137 (denaturation); 40 cycles: 15s at 95 °C, 20s 60 °C, 40s 72°C. Relative gene expression to the
138 housekeeping gene HPRT, expressed as $\Delta C_t = C_t(\text{pBD}) - C_t(\text{HPRT})$, was performed in order to
139 correct for the variance in amounts of cDNA input in the reactions.

140 2.9 Statistical analyses.

141 Statistical analyses were performed using SPSS version 15.0 for windows. All data were analyzed for
142 normality using the Shapiro-Wilk test. Differences between treated and control groups were
143 determined using the Mann-Whitney test. Significant differences were defined as $p < 0.05$

144 **3. Results**

145

146 *3.1 Scanning Electron Microscopy.*

147

148 The apical surface of IPEC-J2 cells was visualized using scanning electron microscopy (Figure 1).

149 The cell surface shows the presence of microvilli, characteristic of intestinal epithelial cells. The

150 microvilli are spread all over the apical surface of the cells, but density and structure differs somewhat

151 between separate cells (Fig. 1A). In Figure 1.B, a *S. Typhimurium* is shown that is adhered to the cell

152 surface. The picture shows that the flagella are attached to the microvilli of the intestinal cell. In

153 Figure 1C and an enlargement thereof in 1D, the start of the invasion process is captured. The

154 intestinal cell microvilli in the area surrounding the bacterium are shorter and deformed. The

155 bacterium is engulfed and seems to invade the IPEC-J2 cell. The flagella are still visible while the

156 bacterium enters the IPEC-J2 cell. Some unidentified balloon- or elongated shaped structures were

157 also visible, most clearly shown in Fig 1C. These are considered cell debris, likely due to the

158 experimental handling of the cell mixtures, since they were also observed in non-infected IPEC cells.

159

160 *3.2 Adhesion and invasion.*

161

162 The capacity of the porcine pathogens *S. Typhimurium* and four *S. Choleraesuis* strains to adhere and

163 invade IPEC-J2 cells was assessed. In these experiments, no difference in adhesion between these

164 bacteria was observed although large variations in adhesion were detected for all bacteria between

165 different experiments, resulting in relatively high standard deviations (Table 2). The invasion of

166 IPEC-J2 cells was comparable for all strains as well, except for *S. Choleraesuis* 977, which showed a

167 reduced capacity to invade the epithelial cells.

168

169 *3.3 Susceptibility of Salmonella towards porcine β -defensin 2.*

170 The antimicrobial effect of pBD2 was assessed against *S. Typhimurium* and *S. Choleraesuis* 994.
171 Bacteria were incubated with pBD-2 and plated on TSA plates for colony counting. No significant
172 difference in susceptibility was observed between the two *Salmonella* strains (Figure 2). At 4 $\mu\text{g/ml}$
173 pBD-2 the number of viable bacteria decreases dramatically and at 8-16 $\mu\text{g/ml}$ all *Salmonella* were
174 effectively killed by pBD-2. These minimal bactericidal concentration (MBC) values correspond to
175 approximately 4-8 μM pBD-2, which is in the expected activity range of defensins.

176

177 *3.4 Effect of infection on defensin gene expression.*

178

179 In order to determine the effect of *Salmonella* infection on defensin regulation, IPEC-J2 cells were
180 infected with *S. Typhimurium* and *S. Choleraesuis* strains 994 and 997, which have comparable (944)
181 and reduced (997) invasion characteristics to *S. Typhimurium*. The effect of infection on porcine β -
182 defensin gene expression is shown in Figure 3. A highly significant upregulation of both pBD-1
183 ($p < 0.01$ at $t = 24$ h) and especially pBD-2 ($p < 0.01$ at $t = 24$ h) was observed when IPEC-J2 cells were
184 infected with *S. Typhimurium*. The observed increase of 5 units in the $40 - \Delta C_t$ value (Fig. 3B)
185 corresponds to a 32-fold increase in gene expression for pBD-2. Induction of pBD-1 gene expression
186 was approximately 8-fold (three $40 - \Delta C_t$ units) after 24 h. In contrast, *S. Choleraesuis* (both strains) or
187 sham treatment of IPEC-J2 cells did not lead to a significant increase in pBD-1 expression, indicating
188 that this is a *S. Typhimurium* serovar specific process. The higher gene expression of pBD-2 for *S.*
189 *Choleraesuis* 997 infected cells compared to the control cells did actually reach statistical significance
190 ($p = 0.024$ at $t = 24$ h), but the extent of induction and time dependency was completely different from
191 that observed for *S. Typhimurium*. To further study the requirements for defensin upregulation, *S.*
192 *Typhimurium* were killed before IPEC-J2 infection by either heat treatment or colistin treatment. Both
193 methods resulted in complete killing of bacteria (results not shown). In addition, to detect whether *S.*
194 *Typhimurium* secreted virulence factors or other secreted proteins were responsible for the observed
195 β -defensin upregulating effect, a *S. Typhimurium* sample was allowed to grow in antibiotic-free
196 IPEC-J2 cell medium for 3 h after which the bacteria were discarded through filtration, resulting in a
197 soluble *S. Typhimurium* secretome fraction. The filtration step successfully removed viable bacteria

198 from this sample as detected by plating on TSA plates (results not shown). As is shown in Figure 4,
199 heat or colistin treated bacteria were not capable of significantly inducing the expression of pBD-1 or
200 pBD-2, although a tendency ($p=0.063$) towards pBD-1 upregulation is observed for colistin treated
201 bacteria after 6h. Similarly, the secretome of viable *S. Typhimurium* did also not have an effect on
202 defensin gene expression. Overall, these results show that viable *S. Typhimurium*, and not a cell- or
203 secreted component of *S. Typhimurium*, are required for upregulation of porcine β -defensins.

204 **4. Discussion**

205 From an immunological point of view, the epithelial lining of the intestine has long been considered
206 solely a physical barrier to protect the underlining mucosa. Intestinal epithelial cells form a tightly
207 packed monolayer covered by a mucus layer that is difficult to penetrate for pathogenic bacteria.
208 Although this is still an important function of epithelial cells, their role in the innate immune response
209 upon infection, and their role in homeostasis of the gut microbiota is now generally being recognized
210 (Oswald, 2006). Intestinal epithelial cells produce cytokines and chemokines, which are crucial for
211 the recruitment and activation of immune cells. In addition, they produce and secrete effector
212 molecules such as β -defensins that can directly affect bacterial populations in the gut. Many of the
213 characteristics of intestinal epithelial cells are maintained within the IPEC-J2 cell line, including the
214 presence of microvilli and the expression of porcine beta defensins, described in this report. The
215 increasing number of reports using IPEC-J2 cells also shows that the cell line has established itself as
216 a useful model for intestinal research.

217 In this report we used the IPEC-J2 cells as a model to determine the effect of *Salmonella* infection on
218 porcine β -defensin regulation of intestinal epithelial cells. First, adhesion and invasion characteristics
219 towards IPEC-J2 cells (Table 1) were tested for 4 *Salmonella* Choleraesuis strains and one *Salmonella*
220 Typhimurium. All tested *Salmonella* strains showed similar adhesion except for strain *S. Choleraesuis*
221 997, which had a reduced invasive activity (Table 2). This corresponds well to observations in other
222 cell lines where similar invasion of *S. Choleraesuis* and *S. Typhimurium* were detected in the human
223 colonic carcinoma cell line Caco-2, the human epithelial-like tumor cell line HEP-2, and rabbit ileal
224 epithelia (Bolton et al., 1999, Bolton et al., 2000). However, in our studies, there was a relatively large
225 variation observed in adhesion and invasion between experiments, which might have hampered
226 detection of relatively small differences between *Salmonella* serovars. For *S. Typhimurium*, Schmidt
227 et al (Schmidt et al., 2008) have described an effect of growth phase of the bacterium on invasiveness
228 of cell lines showing that log phase *S. Typhimurium* have higher invasive activity than stationary
229 phase bacteria. Although only log phase bacteria were used in this study, small day-to-day

230 experimental differences could have affected the outcome. The actual growth phase of *Salmonella*
231 could therefore, at least partially account for the high variance in the observed results.

232

233 *S. Typhimurium* reproducibly induced gene expression of both pBD-1 and pBD-2, while no or little
234 effect was observed for the two *S. Choleraesuis* strains 994 and 997. These two *S. Choleraesuis* strains
235 were chosen because of the similar (strain 994) and differing (strain 997) adhesion and invasion
236 characteristic compared to *S. Typhimurium*, to possibly determine an effect of invasiveness on
237 defensin regulation upon infection. The effect of *S. Typhimurium* on defensin expression in IPEC-J2
238 cells corresponds well to the effect observed in the ileal cell line IPI-2I, (Veldhuizen et al., 2006). The
239 effect is apparently specific for *S. Typhimurium*, since *Arcobacter Cryaerophilus* and *Salmonella*
240 Enteritidis (in the IPI-2I cell line) or *S. Choleraesuis* (IPEC-J2) did not show the induced defensin
241 upregulation. However, studies by Sang *et al* also showed an increased pBD-1 expression in epithelial
242 cells upon infection with *Escherichia coli* and *Listeria Monocytogenes* (Sang et al., 2005). The time
243 course of pBD-1 expression was slightly different for ileal and jejunal cells. In the IPI-2I cell line
244 maximum induction was observed after 6 h while the jejunal cell line IPEC-J2 showed the highest
245 upregulation after 24 h. Whether this reflects a true difference between jejunal and ileal cells or
246 whether this is a cell line specific effect is not clear at this stage.

247

248 The fact that two related serovars such as *S. Typhimurium* and *S. Choleraesuis* have such a different
249 effect on defensin expression of epithelial cells is remarkable. Skjolaas *et al* described a comparable
250 difference in chemokine expression upon infection with these two *Salmonella* serovars (Skjolaas et
251 al., 2006). In these studies, IL-8 and MIF (Macrophage migration inhibitory factor) expression was
252 comparably regulated upon *Salmonella* infection but other innate immune genes such as CCL20 (CC
253 chemokine ligand 20) and osteopontin expression was *S. Typhimurium* specific. It is known that *in*
254 *vivo* the outcome of clinical disease is dependent on the *Salmonella* serovar, with *S. Choleraesuis*
255 being more likely to cause systemic disease than *S. Typhimurium* (Reed et al., 1986). In a recent study
256 this difference was at least partially contributed to a different localization and replication rate of these
257 serovars in the porcine intestine *in vivo*. *S. Choleraesuis* persisted better in the mesenteric lymph

258 nodes and showed a lower replication rate than *S. Typhimurium* in the epithelium layer (Paulin et al.,
259 2007). The described different response in terms of cytokine and defensin production of epithelial
260 cells to invasion of both *Salmonella* serovars could add to the pathogenic differences upon *Salmonella*
261 infection.

262 The experiments in this report indicated that viable *S. Typhimurium* are required to trigger the
263 induction of defensin gene expression, since bacterial or secreted components did not result in any
264 effect (Figure 4). This could mean that *Salmonella* has to invade the epithelial cell first before an
265 immune reaction is triggered, and would imply that cytosolic immunosensors, such as NOD or NOD-
266 like receptors could be involved (Delbridge and O'Riordan, 2007). However, that cannot easily
267 explain why *S. Choleraesuis*, which has a comparable invasion activity, does not elicit such a
268 response, since these receptors recognize ligands present in both *Salmonellae*. Elucidation of the
269 initial receptor and signal transduction pathways leading to defensin upregulation upon *S.*
270 *Typhimurium* infection could possibly resolve this issue. Besides providing insight into infection and
271 disease development in pigs, this could also lead to important clues on how to prevent *Salmonella*
272 infections. If the pathway leading to defensin production can be stimulated through simple feed
273 additives, the pig intestine would be well prepared for incoming pathogens, thereby preventing
274 infection. The potential of this approach is supported by recent studies that have shown that
275 probiotics, but also non-microbial compounds such as amino acids, vitamins and glucose, can induce
276 defensin expression (Barnea et al., 2008, Fehlbaum et al., 2000, Wang et al., 2004, Wehkamp et al.,
277 2004). The IPEC-J2 cell line provides a good and easy to use porcine model to test new feed additives
278 that could stimulate defensin production, or in a broader view, induce innate immunity in the porcine
279 intestinal tract.

280 In summary, we further validated IPEC-J2 epithelial cells as a useful model for intestinal research, by
281 visualizing microvilli on the surface of the cells and describing β -defensin gene expression. The
282 expression of β -defensins was induced upon *S. Typhimurium* but not *S. Choleraesuis* infection. This
283 induction is dependent on viable *S. Typhimurium* since bacterial or secreted components did not
284 affect gene expression of these β -defensins.

285

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366 **Figure legends.**

367 Fig. 1. Scanning electron micrographs of IPEC-J2 cells. (A) Non-infected IPEC-J2 cells. (B)
368 *Salmonella* Typhimurium attached to the cell surface of an IPEC-J2 cell. (C) Invasion of *Salmonella*,
369 the bacterium is engulfed by the IPEC-J2 cell apical surface. (D) Enlargement of the invasion process
370 shown in 1C.

371 Fig. 2. Concentration dependent killing of (A) *Salmonella* Typhimurium and (B) *Salmonella*
372 Choleraesuis 994 by pBD-2. *Salmonella* were incubated with pBD-2 for 3 h, after which remaining
373 viable bacteria were detected using colony counting.

374

375 Fig 3. Effect of *Salmonella* infection of IPEC-J2 cells on the gene expression of pBD-1 and pBD-2.
376 IPEC-J2 cells were Sham treated (C) or infected with *Salmonella* Typhimurium (ST), *Salmonella*
377 Choleraesuis 994 (SC994) or *Salmonella* Choleraesuis 997 (SC997). Shown are mean expression
378 values ($n \geq 4$) expressed as 40 minus the corrected threshold Ct value, plus SEM; 6, 12 and 24 h after
379 infection. Experiments were (minimally) performed in duplicate and repeated on 2 separate days. ‘*’
380 indicates significant differences ($p < 0.05$) compared to the control levels at the same time point. ‘***’
381 indicates $p < 0.01$.

382

383 Fig. 4. Effect of viability of *S. Typhimurium* on the expression of pBD-1 and pBD-2 upon infection of
384 IPEC-J2 cells. *S. Typhimurium* were killed by heat inactivation or colistin treatment. Secreted
385 proteins of *S. Typhimurium* were collected by filtration after 1h incubation of *S. Typhimurium* in
386 IPEC-J2 cell medium. ‘C’ = sham treated cells, ‘ST’ = *S. Typhimurium* infected cells. ‘Heat’ = heat-
387 killed *S. Typhimurium*, ‘Col’ = Colistin killed *S. Typhimurium*, ‘Sec’ = secreted protein fraction of *S.*
388 *Typhimurium*. Shown are mean expression values ($n \geq 4$), expressed as 40 minus the corrected
389 threshold Ct value, plus SEM; 6 and 24 h after infection. Experiments were (minimally) performed in
390 duplicate and repeated on 2 separate days. ‘*’ indicates significant differences ($p < 0.05$) compared to
391 the control levels at the same time point. ‘***’ indicates $p < 0.01$.

392

Table 1. Primer and probe sequences used for quantitative PCR

Gene	Sequence	Concentration
HPRT	Forw. tggaaagaatgtcttgattgtgaag	300 nM
	Rev. atctttggattatgctgcttgacc	300 nM
	Probe aactggcaaaacaatgcaaacctgct	200 nM
pBD-1	Forw. tgccacagtgccgatct	400 nM
	Rev. ctgtagctgcttaaggaataaaggc	400 nM
	Probe ttggagcacactgcccggcata	200 nM
pBD-2	Forw. ccagaggtccgaccactaca	300 nM
	Rev. ggtcccttcaatcctgttgaa	300 nM
	Probe ctgcaacttctccccctgcccg	200 nM

Table 2. Adhesion and invasion of *Salmonella* Typhimurium and Choleraesuis in IPEC-J2

Cells. Data are expressed as the number of bacteria per IPEC-J2 cell.

	<i>S. Choleraesuis</i> 994	<i>S. Choleraesuis</i> 995	<i>S. Choleraesuis</i> 996	<i>S. Choleraesuis</i> 997	<i>S. Typhimurium</i>
Adhesion	2.35 ± 1.68	1.83 ± 0.47	1.49 ± 0.62	1.49 ± 0.25	1.94 ± 1.04
Invasion	0.98 ± 0.60	0.70 ± 0.37	0.65 ± 0.31	0.14 ± 0.07	0.94 ± 0.57







