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

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Abstract

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

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

32 **Introduction.**

33 *Salmonella* infections are an important source of food-born 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

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

2.4 Adhesion and invasion of *Salmonella*.

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

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

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

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

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

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

2.7 Effect of viability of bacteria on defensin gene expression.

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

2.8 Quantitative PCR.

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

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

2.9 Statistical analyses.

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

3. Results

3.1 *Scanning Electron Microscopy.*

The apical surface of IPEC-J2 cells was visualized using scanning electron microscopy (Figure 1). The cell surface shows the presence of microvilli, characteristic of intestinal epithelial cells. The microvilli are spread all over the apical surface of the cells, but density and structure differs somewhat between separate cells (Fig. 1A). In Figure 1.B, a *S. Typhimurium* is shown that is adhered to the cell surface. The picture shows that the flagella are attached to the microvilli of the intestinal cell. In Figure 1C and an enlargement thereof in 1D, the start of the invasion process is captured. The intestinal cell microvilli in the area surrounding the bacterium are shorter and deformed. The bacterium is engulfed and seems to invade the IPEC-J2 cell. The flagella are still visible while the bacterium enters the IPEC-J2 cell. Some unidentified balloon- or elongated shaped structures were also visible, most clearly shown in Fig 1C. These are considered cell debris, likely due to the experimental handling of the cell mixtures, since they were also observed in non-infected IPEC cells.

3.2 *Adhesion and invasion.*

The capacity of the porcine pathogens *S. Typhimurium* and four *S. Choleraesuis* strains to adhere and invade IPEC-J2 cells was assessed. In these experiments, no difference in adhesion between these bacteria was observed although large variations in adhesion were detected for all bacteria between different experiments, resulting in relatively high standard deviations (Table 2). The invasion of IPEC-J2 cells was comparable for all strains as well, except for *S. Choleraesuis* 977, which showed a reduced capacity to invade the epithelial cells.

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

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

3.4 Effect of infection on defensin gene expression.

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

4. Discussion

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

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

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

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

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

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

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

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

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291 References

- 292 Barnea, M., Madar, Z., Froy, O., 2008. Glucose and insulin are needed for optimal defensin
293 expression in human cell lines. *Biochem. Biophys. Res. Commun.* 367, 452-456.
- 294 Beutler, B., 2004. Innate immunity: an overview. *Mol. Immunol.* 40, 845-859.
- 295 Bolton, A. J., Martin, G. D., Osborne, M. P., Wallis, T. S., Stephen, J., 1999. Invasiveness of
296 *Salmonella* serotypes Typhimurium, Choleraesuis and Dublin for rabbit terminal ileum in
297 vitro. *J. Med. Microbiol.* 48, 801-810.
- 298 Bolton, A. J., Osborne, M. P., Stephen, J., 2000. Comparative study of the invasiveness of
299 *Salmonella* serotypes Typhimurium, Choleraesuis and Dublin for Caco-2 cells, HEp-2 cells
300 and rabbit ileal epithelia. *J. Med. Microbiol.* 49, 503-511.
- 301 Chiu, C. H., Su, L. H., Chu, C., 2004. *Salmonella enterica* serotype Choleraesuis:
302 epidemiology, pathogenesis, clinical disease, and treatment. *Clin. Microbiol. Rev.* 17, 311-
303 322.
- 304 Delbridge, L. M., O'Riordan, M. X., 2007. Innate recognition of intracellular bacteria. *Curr.*
305 *Opin. Immunol.* 19, 10-16.
- 306 Fehlbaum, P., Rao, M., Zasloff, M., Anderson, G. M., 2000. An essential amino acid induces
307 epithelial beta -defensin expression. *Proc. Natl. Acad. Sci. U S A* 97, 12723-12728.
- 308 Fraser, J. N., Davis, B. L., Skjolaas, K. A., Burkey, T. E., Dritz, S. S., Johnson, B. J., Minton,
309 J. E., 2007. Effects of feeding *Salmonella enterica* serovar Typhimurium or serovar
310 Choleraesuis on growth performance and circulating insulin-like growth factor-I, tumor
311 necrosis factor-alpha, and interleukin-1beta in weaned pigs. *J. Anim. Sci.* 85, 1161-1167.
- 312 Hendriksen, S. W., Orsel, K., Wagenaar, J. A., Miko, A., van Duinkerken, E., 2004. Animal-to-
313 human transmission of *Salmonella* Typhimurium DT104A variant. *Emerg. Infect. Dis.* 10,
314 2225-2227.
- 315 Lehrer, R. I., 2004. Primate defensins. *Nat. Rev. Microbiol.* 2, 727-738.
- 316 Oswald, I. P., 2006. Role of intestinal epithelial cells in the innate immune defence of the pig
317 intestine. *Vet. Res.* 37, 359-368.

- 318 Paulin, S. M., Jagannathan, A., Campbell, J., Wallis, T. S., Stevens, M. P., 2007. Net
319 replication of *Salmonella enterica* serovars Typhimurium and Choleraesuis in porcine
320 intestinal mucosa and nodes is associated with their differential virulence. *Infect. Immun.* 75,
321 3950-3960.
- 322 Reed, W. M., Olander, H. J., Thacker, H. L., 1986. Studies on the pathogenesis of *Salmonella*
323 typhimurium and *Salmonella choleraesuis* var kunzendorf infection in weanling pigs. *Am J*
324 *Vet. Res.* 47, 75-83.
- 325 Sang, Y., Patil, A. A., Zhang, G., Ross, C. R., Blecha, F., 2006. Bioinformatic and expression
326 analysis of novel porcine beta-defensins. *Mamm. Genome* 17, 332-339.
- 327 Sang, Y., Ramanathan, B., Ross, C. R., Blecha, F., 2005. Gene silencing and overexpression
328 of porcine peptidoglycan recognition protein long isoforms: involvement in beta-defensin-1
329 expression. *Infect. Immun.* 73, 7133-7141.
- 330 Schierack, P., Nordhoff, M., Pollmann, M., Weyrauch, K. D., Amasheh, S., Lodemann, U.,
331 Jores, J., Tachu, B., Kleta, S., Blikslager, A., Tedin, K., Wieler, L. H., 2006. Characterization
332 of a porcine intestinal epithelial cell line for in vitro studies of microbial pathogenesis in
333 swine. *Histochem. Cell. Biol.* 125, 293-305.
- 334 Schmidt, L. D., Kohrt, L. J., Brown, D. R., 2008. Comparison of growth phase on *Salmonella*
335 enterica serovar Typhimurium invasion in an epithelial cell line (IPEC J2) and mucosal
336 explants from porcine small intestine. *Comp. Immunol. Microbiol. Infect. Dis.* 31, 63-69.
- 337 Schwartz, K., J., 1999. *Salmonellosis*. 8th ed.. Iowa State Univ Ames, Iowa,
- 338 Skjolaas, K. A., Burkey, T. E., Dritz, S. S., Minton, J. E., 2006. Effects of *Salmonella enterica*
339 serovars Typhimurium (ST) and Choleraesuis (SC) on chemokine and cytokine expression in
340 swine ileum and jejunal epithelial cells. *Vet. Immunol. Immunopathol.* 111, 199-209.
- 341 Veldhuizen, E. J.A., Hendriks, H. G., Hogenkamp, A., van Dijk, A., Gastra, W., Tooten, P.
342 C., Haagsman, H. P., 2006. Differential regulation of porcine beta-defensins 1 and 2 upon
343 *Salmonella* infection in the intestinal epithelial cell line IPI-2I. *Vet. Immunol. Immunopathol.*
344 114, 94-102.

Veldhuizen, E. J.A., Rijnders, M., Claassen, E. A., van Dijk, A., Haagsman, H. P., 2008. Porcine beta-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria. *Mol. Immunol.* 45, 386-394.

Veldhuizen, E. J. A., van Dijk, A., Tersteeg, M. H.G., Kalkhove, S. I., van der Meulen, J., Niewold, T. A., Haagsman, H. P., 2007. Expression of beta-defensins pBD-1 and pBD-2 along the small intestinal tract of the pig: Lack of upregulation in vivo upon *Salmonella* typhimurium infection. *Mol. Immunol.* 44, 276-283.

Wang, T. T., Nestel, F. P., Bourdeau, V., Nagai, Y., Wang, Q., Liao, J., Tavera-Mendoza, L., Lin, R., Hanrahan, J. W., Mader, S., White, J. H., 2004. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* 173, 2909-2912.

Wehkamp, J., Harder, J., Wehkamp, K., Wehkamp-von Meissner, B., Schlee, M., Enders, C., Sonnenborn, U., Nuding, S., Bengmark, S., Fellermann, K., Schröder, J. M., Stange, E. F., 2004. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. *Infect. Immun.* 72, 5750-5758.

Figure legends.

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

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

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

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

Table 1. Primer and probe sequences used for quantitative PCR

Gene	Sequence	Concentration
HPRT	Forw. tggaaagaatgtcttgattgtgaag	300 nM
	Rev. atctttggattatgctgcttgacc	300 nM
	Probe acactggcaaaacaatgcaaacctgct	200 nM
pBD-1	Forw. tgccacaggtgccgatct	400 nM
	Rev. ctgtagctgcttaaggaataaaggc	400 nM
	Probe ttggagcacacttgcccggcata	200 nM
pBD-2	Forw. ccagaggccgaccactaca	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

Figure 1