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Original article

Quantitative analysis of the immune response upon *Salmonella typhimurium* infection along the porcine intestinal gut

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Abstract – *Salmonella enterica* serovar Typhimurium causes enteric disease and compromises food safety. In pigs, the molecular response of the intestine to *S. typhimurium* has been traditionally characterized by in vitro models that do not reflect the actual immunological competence of the intestinal mucosa. In this work, we performed an oral *S. typhimurium* infection study to obtain insight into the in vitro response in three different sections (jejunum, ileum and colon) of the porcine intestine. For this, samples from one-month-old infected piglets were collected during a time course comprising 1, 2, and 6 days post inoculation to evaluate the intestinal response by quantifying the mRNA expression of gene coding for 28 innate immune system molecules using quantitative real-time PCR assays. In addition, samples from non-infected control animals were also employed to establish differences in the steady state gene expression between intestinal sections. The panel of quantified molecules included an assortment of cytokines, chemokines, pattern-recognition receptors, intracellular signaling molecules, transcription factors and antimicrobial molecules. Changes in gene expression occurred in the three different parts of the intestine and during the course of the *S. typhimurium* infection. Moreover, the high variation observed in expression patterns of genes coding for inflammatory mediators could indicate that each intestinal section responds differently to the infection. Thus, on the contrary to findings in the jejunum and colon, a down-regulation and lack of induction of some proinflammatory cytokine transcripts was observed in the ileum. Nevertheless, all chemoattractant cytokines assayed were up-regulated in the ileum and jejunum whereas only interleukin-8 and MIP-1α mRNA were over expressed in the colon. In conclusion, our results reveal regional differences in gene expression profiles along the porcine intestinal gut as well as regional differences in the inflammatory response to *S. typhimurium* infection. Taken together, these data should provide a basis for a complete understanding of the porcine intestinal response to bacterial infection.

Salmonella **/ pig / intestinal gut / immune response / real-time PCR**

1. INTRODUCTION

Salmonellosis caused by the non-host-adapted bacteria *Salmonella enterica* subspecies *enterica* serovar Typhimurium is an important disease in animal safety and human health. In pigs, this threat is double, since it not only causes economical losses due to animal weakening and underproduction, but also due to the well-known public health risk of commercializing *Salmonella* infected pork products [3]. The symptomatology of salmonellosis by *S. typhimurium* is similar in humans and pigs, and it is characterized by enterocolitis,

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an infection of the lining of the small and large intestine [12].

Phagocytic cells, including macrophages, neutrophils and dendritic cells (DC) are critical components of the innate immune response to *S. typhimurium* [33]. In addition, although the intestinal epithelium has been traditionally identified only as a physical barrier against luminal bacteria, more recently, intestinal epithelial cells are considered as a source of chemokines, cytokines and other immunomodulators which lead to the recruitment of phagocytic cells to initiate both innate and adaptative immune responses [26]. The role as a sentinel of infection is developed by intestinal epithelial cells through the detection of pathogen-associated molecular patterns (PAMP) by the pattern-recognition receptors (PRR) expressed on its cellular membranes [8]. *Salmonella* contain several PAMP, including lipopolysaccharide (LPS), peptidoglycan, mannose and flagellin capable of activating an epithelial proinflammatory gene program in the gut [35].

In vitro studies have shown that *Salmonella* interaction with porcine epithelial cells results in a proinflammatory response characterized by the release of several cytokines and chemokines [2, 28, 30, 32]. However, although these in vitro models can provide valuable information, it is clear that a cell culture approach cannot define the physiological relevance of such cellular responses. In the intestine, different specialized cell types are present at the epithelial level, including enterocytes (absorptive cells), globet cells (mucus producing cells), M-cells (antigen sampling cells) and Paneth cells (secretory intestinal cells) [14, 15]. In addition, the mucosal immunity involves multiple cell types that reside at the site of infection or infiltrate from the circulation, establishing a complex communication network between them in the form of soluble and cell-bound molecules. Thus, DC form an extensive network in the lamina propia of the small as well as the large intestine. Mucosal DC constantly survey the luminal microenvironment which contains commensal microbiota and potentially harmful organisms regulating pathogen recognition and adaptative as well as innate defense activation [24]. On the contrary, resident and recruited macrophages, neutrophils and lymphocytes complete the plethora of cell types interacting in the intestinal mucosa. In this framework, it is clear that the response against luminal pathogens characterized by in vitro assays could not reflect the actual immunological competence of the intestinal mucosa.

In order to contribute to the knowledge of the porcine in vivo response to *Salmonella*, in this work, we pursued the characterization of the early immune response to *S. typhimurium* infection along the intestinal tract, focusing on mucosa response. To achieve this, an experimental infection covering from early times after infection (1 and 2 days) to middle times (6 days) was designed. Then, the mucosal immune response was evaluated by quantification of the relative mRNA expression of genes coding for molecules with a relevant function in innate immunity including the following: cytokines, chemokines, PRR, intracellular signaling molecules, transcription factors and antimicrobial molecules.

2. MATERIALS AND METHODS

2.1. Bacterial strain

The *Salmonella enterica* serovar Typhimurium phagetype DT104 was an isolate from a carrier pig [11]. This isolate was serotyped using slide agglutination with antisera purchased from Bio-Rad (Hercules, CA, USA). Phage typing was performed in accordance with the methods of the Spanish National Reference Laboratory, Algete, Madrid, Spain.

Bacteria were grown in Luria Bertani (LB) broth to log stationary phase at 250 rpm, 37 °C for 8 h to an $OD_{600\ nm}$ of 0.8. After harvesting by centrifugation at 8 000 *g*, the bacterial pellet was resuspended in phosphate-buffered saline (PBS), and adjusted to a final concentration of 10^8 cfu/mL in PBS.

2.2. Experiment design and disease progress

Sixteen male and female crossbred weaned piglets, approximately 4 weeks of age, were used. Before infection, faecal samples from each animal were analyzed to confirm that piglets were free of *Salmonella*. Pigs were housed in an environmentally controlled isolation facility at 25 °C and under

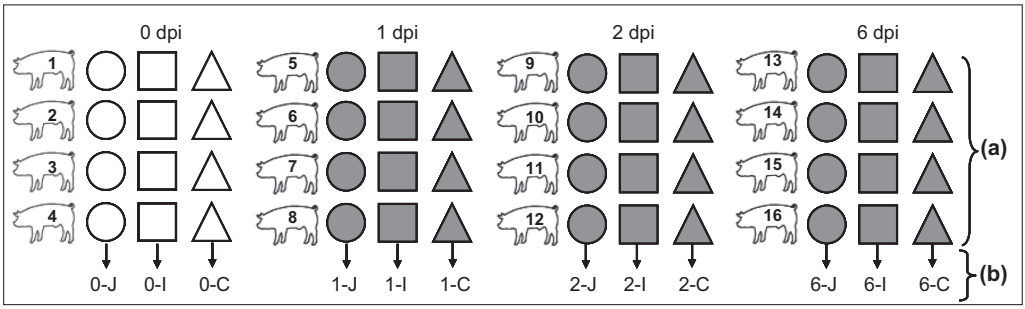

Figure 1. Schematic representation of the experimental design used in this study. Samples of the jejunum (○), ileum (□) and colon (△) were collected from each animal at different time points (0, 1, 2 and 6 dpi). Open symbols mean non-infected pigs; filled symbols mean *S. typhimurium* infected pigs (a). Total RNA was extracted from all 48 intestinal samples. RNA isolated from the same intestinal segment collected from four pigs in each time point was pooled for the subsequent quantitative PCR analysis (b).

constant light with ad libitum access to feed and water. After an acclimation period of 5 days, pigs were challenged orally with 10^8 cfu of *S. typhimurium* ($n = 12$), whereas the control group ($n = 4$) received sterile medium orally. The four non-infected control pigs were necropsied 2 h prior to experimental infection. Four randomly chosen infected pigs were necropsied at each time point of 1 day post-infection (dpi), 2 dpi and 6 dpi, respectively. Faecal swabs, blood and tissue samples were aseptically collected. The jejunum, ileum and colon were independently sectioned in pieces of around 10 cm and immediately frozen in liquid nitrogen for mucosa isolation and RNA purification (Fig. 1). Serum samples were frozen for use in enzyme-linked immunosorbent assay (ELISA) assays. All the infected animals were fecal-culture positive for *Salmonella* and developed similar clinical signs of gastrointestinal disease, including increased rectal temperature, diarrhea and lethargy.

All procedures involving animals respected European regulations regarding the protection of animals used for experimental and other scientific purposes.

2.3. Serum ELISA

Serum samples were harvested in polypropylene tubes and stored at −20 °C until use. ELISA was used for serum interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) analysis using Swine IL-8 and Swine TNF-α ELISA kit (Invitrogen, Carlsbad, CA, USA), respectively, according to the manufacturer's protocol. Respective standard curves were used to determine the amounts (pg/mL) of each cytokine in the porcine serum samples. IL-8 and TNF-α levels were measured both in control and infected pigs at each sampling time (Fig. 2).

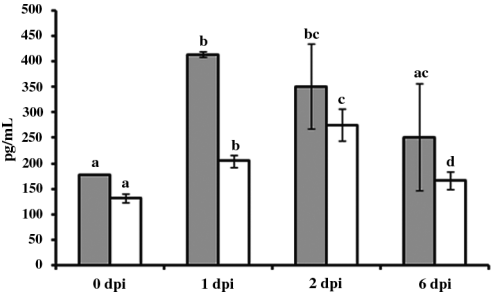

Figure 2. Serum IL-8 (filled bar) and TNF-α (open bar) levels in control and infected pigs with *S. typhimurium*. Means ± standard deviation of the values obtained from 16 (0 dpi), 12 (1 dpi), 8 (2 dpi) and 4 (6 dpi) animals are indicated. The same letters above the bars indicate no significant differences ($p < 0.05$).

2.4. Intestinal mucosa isolation and RNA purification

All 48 samples (intestinal sections and sampling times, Fig. 1) were independently processed for mucosa isolation and RNA purification. Two cm in length of each intestinal sample stored at −80 °C

was treated with RNA*later*®-ICE (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Thirty mg of intestinal mucosa were isolated by scraping the luminal surface with a razor and homogenization in 600 µL of RLT buffer (Qiagen, Valencia, CA, USA) using a rotor-stator homogenizer. Further RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Eluted RNA was treated with DNase using a TURBO DNA-*free* Kit (Ambion). Treated RNA was precipitated by adding 1/10 volume 3 M NaOAc, pH 5.2 and 2.5 volumes ethanol and incubating at −80 °C overnight. RNA was pelleted by centrifugation at 16 000 *g* and washed with 75% ethanol twice. RNA was resuspended in RNase free water, quantified using a NanoDrop™1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to obtain a RNA concentration of 1 µg/µL. RNA integrity was evaluated using denaturing agarose gels after each step of the purification procedure: after column elution, DNase treatment, and after sodium acetate precipitation. RNA pools were prepared by mixing equal amounts of RNA from the same intestinal section (jejunum, ileum or colon) collected from the four pigs at each time point (0, 1, 2 or 6 dpi), resulting in three control pools (0-J, 0-I and 0-C) and nine infected pools (1-J, 1-I, 1-C, 2-J, 2-I, 2-C, 6-J, 6-I and 6-C) (Fig. 1).

2.5. Primer design for quantitative real-time PCR (qPCR)

qPCR primers used in this work (Tab. I) were designed using Beacon Designer™ (Biosoft International, Palo Alto, CA, USA) based on the cDNA sequences obtained from the GeneBank database. Each primer was homology searched by a NCBI BLAST search to ensure that it was specific for the target mRNA transcript. To optimize the amplification procedure, all primer pairs were designed to be used at the same annealing temperature (57 °C). Melting curves followed by gel electrophoresis were used to confirm product specificity after PCR cycles.

2.6. qPCR assays

Pooled RNA (1.5 µg) was reverse transcribed to cDNA using the iScript™ cDNA Synthesis kit (Bio-Rad) in a total volume of 30 µL. cDNA solutions were diluted by adding 70 µL of UHQ water obtained from a Milli-Q Plus water system (Millipore, Bedford, MA, USA) and stored at −20 °C. Relative gene expression was determined by qPCR using an iQ5 Thermo Cycler (Bio-Rad). Each sample was amplified by duplication in the same 96 well PCR plate and plates were repeated at least twice. Twenty µL real-time PCR reactions were carried out using 2 µL of diluted cDNA as template and the iQ™ SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instructions. Final concentration of the primers in the PCR reaction was 0.4 µM. The qPCR conditions were 5 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 45 s at 72 °C. After amplification, a melting program was run to verify the presence of only one PCR product. To ensure the sensitivity and accuracy of the results obtained by qPCR, internal normalization of the gene expression analysis was carried out using beta-actin and cyclophilin-A as the internal control genes (reference genes) [29]. Previously, to accurately ascertain which reference genes would be the most reliable for use as normalisers in qPCR, we subjected ribosomal 18S RNA, beta-actin and cyclophilin-A expression data to analysis using the BestKeeper tool [27]. The results from the BestKeeper analysis indicate that 18S RNA was the least reliable reference gene in the context of our bacterial experimental infection.

2.7. Data analysis

Two different approaches were performed for mRNA quantification: (i) to establish the changes in gene expression that occur in an intestinal section during the experimental infection relative to uninfected controls and (ii) to compare the level of target gene expression among different segments of porcine intestine at the non-infected state relative to the expression in the jejunum. In both cases, the relative gene expression was assessed by the $2^{-\Delta\Delta Ct}$ method [19], in which the PCR cycle used for quantification (Ct, threshold cycle) has been replaced by the term Cq (quantification cycle), according to the recommendations of the RDML (real-time PCR Data Markup Language) data standard[1]. ΔΔCq values were calculated according to the formula $\Delta\Delta Cq = [(Cq_{TGX} - Cq_{RGX})_{Time\ Y}] - [(Cq_{TGX} - Cq_{RGX})_{Time\ 0}]$ for the first approach and $\Delta\Delta Cq = [(Cq_{TGJ} - Cq_{RGJ})_{Time\ 0}] - [(Cq_{TGX} - Cq_{RGX})_{Time\ 0}]$ for the second, where Cq_{TG} is the quantification cycle of the target gene, Cq_{RG} the geometric mean of the quantification cycle of the reference genes, X is any intestinal section, Y is any time point (dpi) and J is the jejunum. The mean and standard deviation of ratios ($2^{-\Delta\Delta Cq}$ values) obtained for a gene

[1] http://www.rdml.org

Table I. List of genes and sequences of the primers used for quantitative PCR analysis.

Gene name	Foward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Accession number
β-Actin	CAGGTCATCACCATCGGCAACG	GACAGCACCGTGTTGGCGTAGAGGT	U07786
Cyclophilin-A	CCTGAACATACGGGTCCTG	AACTGGGAACCGTTTGTGTTG	AY266299
IL-8	TTCGATGCCAGTGCATAAATA	CTGTACAACCTTCTGCACCCA	M86923
MCP-1	ACCAGCAGCAAGTGTCCTAAAG	GTCAGGCTTCAAGGCTTCGG	NM_214214
MIP-1α	TCTCGCCATCCTCCTCTG	TGGCTGCTGGTCTCAAAATA	AY643423
MIP-1β	CAGCACCAATGGGCTCAGA	TTCCGCACGGTGTATGTGA	EF107667
TNF-α	CCTCTTCTCCTTCCTCCTG	CCTCGGCTTTGACATTGG	NM_214022
IL-1β	GGCCGCCAAGATATAACTGA	GGACCTCTGGGTATGGCTTTC	NM_214055
IFN-γ	CAAAGCCATCAGTGAACTCATGA	TCTCTGGCCTTGGAACATAGTCT	X53085
IL-6	TGGCTACTGCCTTCCCTACC	CAGAGATTTTGCCGAGGATG	NM_214399
IL-12p40	GGAGTATAAGAAGTACAGAGTGG	GATGTCCCTGATGAAGAAGC	U08317
IL-4	TTGCTGCCCCAGAGAAC	TGTCAAGTCCGCTCAGG	AY294020
IL-10	CAGATGGGCGACTTGTTG	ACAGGGCAGAAATTGATGAC	L20001
TLR-1	TGCTGGATGCTAACGGATGTC	AAGTGGTTTCAATGTTGTTCAAAGTC	AB219564
TLR-2	TCACTTGTCTAACTTATCATCCTCTTG	TCAGCGAAGGTGTCATTATTGC	AB085935
TLR-3	AGTAAATGAATCACCCTGCCTAGCA	GCCGTTGACAAAACACATAAGGACT	DQ266435
TLR-4	GCCATCGCTGCTAACATCATC	CTCATACTCAAAGATACACCATCGG	AB188301
TLR-5	CAGCGACCAAAACAGATTGA	TGCTCACCAGACAGACAACC	NM_001123202
TLR-6	AACCTACTGTCATAAGCCTTCATTC	GTCTACCACAAATTCACTTTCTTCAG	AB085936
TLR-7	TCAGTCAACCGCAAGTTCTG	GATGGATCTGTAGGGGAGCA	NM_001097434
TLR-8	AAGACCACCACCAACTTAGCC	GACCCTCAGATTCTCATCCATCC	AB092975
TLR-9	CACGACAGCCGAATAGCAC	GGGAACAGGGAGCAGAGC	AY859728
TLR-10	CCTGTCCAACTGCCTCATTTG	CTAAGTGTTCTAAGGATGTGTTTCTG	AB219565
NOD1	ACCGATCCAGTGAGCAGATA	AAGTCCACCAGCTCCATGAT	AB187219
NOD2	CCTTTTGAAGATGCTGCCTG	GATTCTCTGCCCCATCGTAG	NM_001105295
MyD88	TGGTGGTGGTTGTCTCTGATGA	TGGAGAGAGGCTGAGTGCAA	NM_002468
NF-κB	CTCGCACAAGGAGACATGAA	ACTCAGCCGGAAGGCATTAT	DQ834921
Caspase-1	CTCTCCACAGGTTCACAATC	GAAGACGCAGGCTTAACTGG	NM_214162
PBD-1	ACCGCCTCCTCCTTGTATTC	GGTGCCGATCTGTTTCATCT	NM_213838
PBD-2	CTGTCTGCCTCCTCTCTTCC	CAGGTCCCTTCAATCCTGTT	NM_214442

in different plates were calculated. When the ratio was lower than 1, which means down-regulation, the fold change was calculated as 1/ratio and a minus sign (−) was added to denote down-regulation (Tabs. II and III). With this method, a fold-change value of 1 or −1 represents no difference in gene expression. In both approaches, the differences in mRNA expression among groups were assessed by the Student's paired *t*-test previous determination of normal distribution of the data and variance homogeneity using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

3. RESULTS

3.1. Serum levels of IL-8 and TNF-α in swine infected with *S. typhimurium*

Serum levels of IL-8 and TNF-α (shown as arithmetic mean levels in pg/mL) were measured in order to evaluate the host response to the enteric pathogen (Fig. 2). Significantly elevated levels of TNF-α ($p < 0.05$) were noted in all infected animals versus healthy controls, whereas only serum IL-8 levels were significantly higher than in uninfected animals ($p < 0.05$) at 1 and 2 dpi. In general, detectable amounts of serum IL-8 were higher than TNF-α levels although these differences were only statistically significant at 1 dpi. In addition, a time-dependent effect was observed in the protein release of IL-8 and TNF-α with a maximal level of IL-8 production occurring early at 1 dpi when compared with the higher production of TNF-α at 2 dpi.

3.2. mRNA quantification of immune response genes by real-time PCR

To gain insight into the mechanisms of porcine intestinal defense to bacterial infection, we quantified the expression of genes coding for 28 immune related molecules including proinflammatory and chemoattractant cytokines, PRR, intracellular signaling molecules, transcription factors and antimicrobial molecules after an in vitro experimental infection with *S. typhimurium*. Statistically significant changes in gene expression relative to uninfected animals are shown in Table II. In addition, the expression of the genes was also quantified in non-infected animals (steady state) and the results are shown in Table III.

3.2.1. *Cytokine mRNA expression after* S. typhimurium *infection*

Among cytokines, mRNA expression of chemoattractant cytokines IL-8, MCP-1, MIP-1α and MIP-1β were significantly up-regulated in the jejunum and ileum. In the colon, only changes in IL-8 and MIP-1α gene expression were observed, with an overexpression of their respective mRNA occurring later than in the jejunum and ileum. Similarly, mRNA expression of proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-6 and IL-12p40 was also analyzed. As shown in Table II, TNF-α mRNA was up-regulated in the three porcine intestinal sections whereas IL-1β, IFN-γ and IL-6 mRNA were increased in the infected jejunum and colon but significantly down-regulated ($p < 0.01$) or not induced in the ileum. Interestingly, IL-12p40 was up-regulated in the colon but down-regulated in the jejunum, albeit not as markedly as in the ileum. Finally, no changes in gene expression of anti-inflammatory cytokines IL-4 and IL-10 were observed along the porcine intestine in response to *S. typhimurium* infection.

3.2.2. *Pattern recognition receptor mRNA expression after* S. typhimurium *infection*

Toll-like receptors (TLR) and NOD-like receptors (NLR) play an important role in the sensing of Gram-negative bacterial infections. Consequently, the mRNA expression of TLR (TLR-1 to TLR-10), NOD1 and NOD2 was analyzed in our model of experimental *S. typhimurium* infection. In TLR there were very few significant changes in gene expression, with the up-regulation of TLR-2 mRNA in the ileum being the most relevant result. TLR-4 and TLR-7 mRNA expression were not differentially regulated in the infected intestinal tracts whereas a general pattern of down-regulation was observed for TLR-1, TLR-3, TLR-5, TLR-9 and TLR-10 genes (Tab. II).

Table II. Changes in gene expression relative to uninfected controls within each intestinal section at 1, 2 and 6 dpi. Only genes with statistically significant changes are shown.

	Jejunum fold-change[a]	Ileum fold-change[a]	Colon fold-change[a]		Jejunum fold-change[a]	Ileum fold-change[a]	Colon fold-change[a]
IL-8				***TLR-2***			
1 dpi	**3.54****	**5.29****	1.23	1 dpi	−1.44	**3.59****	−1.59
2 dpi	**8.43****	**5.59****	**3.53****	2 dpi	1.34	**2.56***	1.08
6 dpi	3.31	3.61	**3.30****	6 dpi	**−3.31***	**5.58****	−1.2
MCP-1				***TLR-3***			
1 dpi	2.27	**4.80****	−1.37	1 dpi	−1.44	**−2.35***	−2.37
2 dpi	**2.77***	**3.59***	1.25	2 dpi	1.2	−2.21	−1.60
6 dpi	−1.06	1.62	1.11	6 dpi	1.27	1.22	**−3.09***
MIP-1α				***TLR-5***			
1 dpi	2.19	1.6	−1.01	1 dpi	−1.12	−2.05	−1.97
2 dpi	**3.86***	**3.84****	1.64	2 dpi	−1.14	−2.22	−1.53
6 dpi	1.76	**3.58***	**4.52****	6 dpi	−1.51	−1.10	**−2.39***
MIP-1β				***TLR-9***			
1 dpi	1.73	1.88	−1.27	1 dpi	−1.02	−1.35	1.45
2 dpi	**2.65***	1.87	−1.12	2 dpi	1.67	−1.11	**3.06***
6 dpi	−1.26	**2.79***	2.1	6 dpi	1.3	**−2.93***	1.41
TNF-α				***TLR-10***			
1 dpi	2.59	**2.86***	1.02	1 dpi	−1.09	−2.24	1.09
2 dpi	**3.59****	1.95	**2.22***	2 dpi	−1.43	−1.79	1.34
6 dpi	1.65	1.48	1.48	6 dpi	1.21	**−2.73***	−1.65
IL-1β				***NOD1***			
1 dpi	1.72	1.46	−1.22	1 dpi	2.34	**2.85***	1.15
2 dpi	**15.38****	1.02	**3.09***	2 dpi	**5.86****	**2.97***	1.57
6 dpi	**−3.48****	−5.02**	**3.33****	6 dpi	1.77	1.75	1.43
IFN-γ				***NOD2***			
1 dpi	1.36	1.9	−1.21	1 dpi	1.96	1.17	−1.04
2 dpi	**2.87****	−1.35	1.31	2 dpi	**3.05***	1.17	1.18
6 dpi	−2.14	1.46	**3.61****	6 dpi	−1.31	−1.13	−1.31
IL-6				***NF-κB***			
1 dpi	2.09	2.1	1.07	1 dpi	1.03	1.97	1.07
2 dpi	**5.43****	−1.32	**2.89***	2 dpi	1.1	**3.43***	−1.41
6 dpi	−1.98	**-4.00****	2.23	6 dpi	−1.65	**2.28***	−1.44
IL-12p40				***Caspase-1***			
1 dpi	1.32	−1.49	−1.69	1 dpi	1.31	**−2.51***	−1.07
2 dpi	1.05	**−4.36****	1.58	2 dpi	1.42	**−2.60***	−1.14
6 dpi	**−3.03***	**−4.61****	**2.44***	6 dpi	1.26	1.09	−1.35
TLR-1				***PBD-2***			
1 dpi	−1.25	−2.12	−1.73	1 dpi	**−19.56****	**2.95***	1.02
2 dpi	**−2.23***	1.1	−1.08	2 dpi	**−17.21****	**6.97****	2.34
6 dpi	−1.1	−1.59	−1.67	6 dpi	**−18.30****	1.1	−1.22

[a] Differences were analyzed using the Student's paired *t*-test.
Statistically significant changes are in bold: *$p < 0.05$, **$p < 0.01$.

Table III. Changes in gene expression relative to the jejunum at the steady state (non-infected samples). Fold-change values with the same letters above are not significantly different ($p < 0.05$). Only genes with statistically significant changes are shown.

	Jejunum		Ileum		Colon	
	Fold-change	SD	Fold-change	SD	Fold-change	SD
IL-8	1^{a}	0.14	-2.80^{b}	0.37	-1.89^{ab}	0.45
MCP-1	1^{ab}	0.82	-1.15^{a}	0.12	2.69^{b}	0.03
MIP-1α	1^{a}	0.62	-1.98^{a}	0.98	-2.62^{b}	0.44
IL-1β	1^{a}	0.49	4.89^{b}	0.63	1.26^{a}	0.20
IFN-γ	1^{a}	1.39	-1.57^{a}	0.12	-6.21^{b}	0.34
IL-6	1^{a}	0.40	5.48^{b}	0.27	-1.00^{a}	0.69
IL-12p40	1^{a}	0.25	4.31^{b}	0.44	-2.50^{c}	0.12
IL-4	1^{a}	0.09	-1.86^{b}	0.04	-4.10^{b}	1.05
TLR-2	1^{a}	1.22	1.87^{a}	0.46	9.14^{b}	0.85
TLR-4	1^{a}	0.25	1.56^{ab}	0.63	2.07^{b}	0.12
TLR-7	1^{a}	0.85	3.83^{b}	0.52	5.30^{b}	0.59
TLR-8	1^{a}	0.52	2.34^{a}	0.34	5.63^{b}	1.15
TLR-9	1^{a}	0.10	17.39^{b}	0.07	1.75^{c}	0.11
TLR-10	1^{a}	0.25	11.80^{b}	0.48	1.69^{a}	0.16
NF-κB	1^{a}	1.70	-6.22^{b}	0.60	-2.59^{ab}	1.56
PBD-1	1^{a}	0.03	9.92^{b}	0.24	NA	
PBD-2	1^{a}	0.02	-3.65^{b}	0.43	3.50^{c}	0.33

NA, no amplification.

Regarding intracellular NOD receptors, NOD1 gene expression was up-regulated in the jejunum and ileum whereas NOD2 increased its expression only in the jejunum (Tab. II).

3.2.3. *Transcript expression of MyD88, NF-κB and caspase-1 in the porcine intestinal mucosa after* S. typhimurium *infection*

Myeloid differentiation primary response gene 88 (MyD88) is an adapter protein that is involved in TLR-induced activation of nuclear factor-kappa B (NF-κB) which subsequently leads to transcription of proinflammatory cytokines. Caspase-1 is responsible for maturation of certain inflammatory cytokines including IL-1β. In our study, no changes were observed in MyD88 mRNA expression in any of the intestinal sections analyzed. On the contrary, significant up-regulation of NF-κB1 gene expression ($p < 0.05$) and down-regulation of caspase-1 mRNA expression ($p < 0.05$) were detected in the ileum (Tab. II).

3.2.4. *mRNA expression for the antimicrobial peptide β-defensins following experimental* S. typhimurium *infection*

Mammalian antimicrobial peptides, including β-defensins, represent an ancient arm of innate immunity designed to directly neutralize invading microbes. In this work, we examined the effect of *S. typhimurium* infection over porcine β-defensin 1 (PBD-1) and 2 (PBD-2) expression along the porcine intestinal gut. The results from Table II indicate that PBD-2 mRNA expression was highly down-regulated in the jejunum but over-expressed in the ileum. In contrast, PBD-1 mRNA expression was not altered in our experimental infection model.

3.2.5. *Quantification of steady state expression of immune genes along the porcine intestinal gut*

The aim of this study was to compare the expression of selected innate immune genes (Tab. I) in three porcine intestinal sections at

the steady state. The results are given in Table III, in which statistically significant changes in gene expression relative to the jejunum are shown. Overall, diverse features of variation in gene expression patterns were seen among the jejunum, ileum and colon, particularly due to changes in the expression of proinflammatory cytokines and TLR. Thus, with the exception of TNF-α and IFN-γ, we found that all the proinflammatory cytokines analyzed were significantly more expressed in the ileum than in the jejunum and colon. In addition, ileal mucosa also showed the highest levels of mRNA expression of BDF-1, TLR-9 and TLR-10. On the contrary, mRNA expression of most TLR including TLR-2, TLR-4, TLR-7 and TLR-8 was higher in the colon than in the jejunum and ileum. Finally, some cytokines such as IL-8, MIP-1α and IL-4, as well as the transcription factor NF-κB, were more expressed in the jejunum than in the ileum and colon.

4. DISCUSSION

In this work we aimed to describe the state of the innate immune machinery in the porcine intestinal mucosa after infection with the enteropathogenic bacteria *S. typhimurium*. To achieve this, we quantified the expression of genes coding for selected immune-defense molecules in three different sections of the porcine intestinal tract (jejunum, ileum and colon) at steady state and at various times post-infection (1, 2 and 6 dpi). Other approaches mimicking in vivo *Salmonella* infections have been reported in pigs, i.e. the small intestinal segment perfusion model [25, 31] and the gut loop in the ileum or jejunum [20, 21]. Although highly valuable for minimizing individual variation among animals, these methods do not totally reflect the natural intestinal transit or the possible bacterial preference for infection of certain intestinal sections. Indeed, none of these procedures have been used to characterize the large intestine response to *Salmonella* infection. Therefore, to our knowledge, our study is the first attempt to simultaneously analyze the response of the porcine small and large intestine to an in vivo-induced bacterial infection.

Our results regarding TNF-α and IL-8 gene expression suggest that all porcine intestinal sections studied were able to sense bacterial presence. This was in agreement with the increased levels of IL-8 and TNF-α secretion in the serum of the experimentally infected animals and taken together, data of IL-8 and TNF-α gene and protein expression could indicate that an inflammatory process had been triggered along the porcine intestine after infection with *S. typhimurium*. Nevertheless, on the basis of changes in cytokine expression obtained in the present study, the extent of the inflammatory response could be varied greatly from the jejunum to the ileum and colon. Thus, some proinflammatory cytokines with an important role in the control of *Salmonella* infection such as IL-1β, IFN-γ, IL-6 and IL-12p40 [7, 17] differed in its mRNA expression among the three intestinal sections, showed a lack of induction and even down regulation in the ileum compared to the jejunum and colon (Tab. II). These results indicate that the ileum mucosa is not prompting the proinflammatory burst needed to face up to pathogens, neither by a lower intrinsic capacity of this intestinal section to mount an immune response against the bacteria; nor as the consequence of a pathogen-induced down-immune-modulation to obtain a successful invasion. The first assumption seems unlikely in the light of our finding that the higher basal expression of IL-1β, IL-6 and IL-12p40 mRNA was found in the ileum at steady state (Tab. III). However, some studies in agreement with the second interpretation, have demonstrated the ability of *S. typhimurium* to interfere with the host's inflammatory response to promote bacterial pathogenesis [4, 10].

According to the chemoattractant cytokine expression, our results show that the immune response elapses from the small to the large intestine. Thus, the IL-8 and MIP-1α mRNA pattern of expression showed an earlier immune response in the jejunum and ileum than in the colon. Also, while in the ileum the expression of the genes remained at a relatively high level until 6 dpi in the jejunum the induction of the gene expression was not observed beyond 2 dpi. This evidence could suggest that the recruitment of phagocytes for clearance of

pathogen infection was maintained for a longer time in the ileum than in the jejunum, in agreement with previous observations showing that *Salmonella* colonizes the ileum more efficiently than the jejunum, possibly due to the existence of physiological and morphological differences between the two sites [3, 6]. In agreement with this, Hyland et al. (2006) [13] demonstrated that ileal Peyer's patches undergo a greater inflammatory cytokine response 6 h after *Salmonella* infection than the jejunal Peyer's patches.

Several *Salmonella* PAMP can act as activators of the TLR-2, -4, -5 and -9 signaling pathways [1, 16]. In a recent study, changes in TLR-2, TLR-4 and TLR-5 mRNA expression were observed in porcine jejunal mucosa upon *S. typhimurium* infection [20]. In our model of *S. typhimurium* challenge, those changes were not observed in the jejunum although TLR-2 mRNA expression was significantly increased in the ileum. We found that most TLR were not differentially regulated along the time course of the infection, suggesting that the expression of these receptors was constitutive in this infection model. Nevertheless, all TLR assayed in this study (except TLR-5) were regulated in a previous in vitro assay based on the stimulation of porcine jejunal or ileal cell lines with LPS from *S. typhimurium* [2], indicating that any extrapolation of such in vitro experiments to an in vivo situation should only be made with considerable caution. On the contrary, the unequal distribution of TLR-7, -8, -9 and -10 at the basal state identified in the present work along the intestinal tract (Tab. III), could reflect different susceptibilities to infection in each intestinal section, although we cannot exclude that the morphological differences among the jejunum, ileum and colon might be the cause of such a variation.

It has been reported that the intracellular NLR are able to sense the intracellular pathogen *S. typhimurium* and induce a proinflammatory response through NF-κB activation [7, 22]. In this work, NOD1 mRNA expression was up-regulated in the jejunum and ileum, while NOD2 mRNA up-regulation was only found in the jejunum. How the changes of these NLR will have any impact on the response to intestinal infection has not been well defined. However, recently published data indicates that TLR-2 and NOD1 are involved in chemokine production of murine epithelial cell lines in response to *Helycobacter muridarum* challenge [5]. Interestingly, in our conditions these two genes were the only PRR found up-regulated in the porcine ileum. This up-regulation could be pointing towards a role of both molecules also in the response against *S. typhimurium*.

NOD1 and NOD2 are able to activate caspase-1, which is responsible for the processing and maturation of the proinflammatory cytokines IL-1β and IL-18 [9]. The down-regulation of caspase-1 mRNA observed in the ileum in the present work (Tab. II) is difficult to interpret because, although some authors have reported that a caspase-1 deficiency confers resistance to oral infection with *S. typhimurium* [23], others demonstrated that the absence of caspase-1 does not result in resistance to oral infection by *S. typhimurium*, but rather, leads to increased susceptibility to infection [18].

In pigs, expression analysis of defensins has been assessed in in vitro and in vivo studies with contradictory results. Thus, Veldhuizen et al. [32] showed that PBD-1 and PBD-2 mRNA expression were up-regulated after in vitro infection of porcine intestinal epithelial cell lines with *S. typhimurium*. The same research group reported a lack of up-regulation of PBD-1 and PBD-2 in vivo in the small intestine upon *S. typhimurium* infection [31] whereas in a more recent work, Meurens et al. [20] observed up-regulation of PBD-2 in jejunal Peyer's patches. According to these in vivo observations, no changes in PBD-1 mRNA were observed in the present work. However, regarding PBD-2 mRNA expression, in our conditions we detected up-regulation in the ileum and, surprisingly, a high down-regulation in the jejunum that has not been previously reported (Tab. II). The biological significance of these large differences in the expression of both defensins along the pig intestine is difficult to establish. PBD-1 and PBD-2 seem to have a potent antimicrobial function but the differential regulation observed in our study might be related to a non-antimicrobial activity of defensins, such as chemotaxis of immature DC or

memory T-cells [34]. Therefore, further experiments are required to elucidate the role of PBD-1 and PBD-2 in the intestinal response to *S. typhimurium*.

In summary, using an oral *S. typhimurium* infection model, we identified genes differentially expressed in anatomical different parts of the porcine gut that should contribute to a better understanding of the porcine intestinal response to bacterial infection. Our results reveal that the jejunum, ileum and colon respond differently to infection with *S. typhimurium*, and showed ileum mucosa as unable to up-regulate some proinflammatory cytokines, which could help to a more successful colonization of this site by the infecting bacteria. In addition, differences in the pattern of gene expression among the three intestinal sections at the steady state have been established. We conclude that there are regional differences in the inflammatory response to *S. typhimurium* within the porcine gastrointestinal tract, and consequently, insight into the immunity mechanisms and pathways that contribute to the intestinal host defense development in this economically important species must be sought in this context.

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