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Modulation of porcine intestinal epitheliocytes immunetranscriptome response by *Lactobacillus jensenii* TL2937

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RESEARCH ARTICLE

Abstract

In order to evaluate probiotic strains applicable for the beneficial immunomodulation of the porcine gut (immunobiotics), we previously developed a porcine intestinal epitheliocyte cell line (PIE cells). Here, transcriptomic studies using PIE cells were performed considering that this information would be valuable for understanding the mechanisms involved in the protective activity of the immunobiotic strain *Lactobacillus jensenii* TL2937 against intestinal inflammatory damage in pigs. In addition, those studies would provide criteria for selecting biomarkers for the screening of new immunobiotic strains. We performed microarray analysis to investigate the transcriptomic response of PIE cells to the challenge with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs) and, the changes induced by *L. jensenii* TL2937 in that response. The approach allowed us to obtain a global overview of the immune genes involved in the response of PIE cells to heat-stable ETEC PAMPs. We observed that *L. jensenii* TL2937 differently modulated gene expression in ETEC PAMPs-challenged PIE cells. Microarray and RT-PCR analysis indicated that the most remarkable changes in PIE cells transcriptomic profile after heat-stable ETEC PAMPs challenge were observed in chemokines, adhesion molecules, complement and coagulation cascades factors. In addition, an anti-inflammatory effect triggered by TL2937 strain in PIE cells was clearly demonstrated. The decrease in the expression of chemokines (*CCL8, CXCL5, CXCL9, CXCL10,* and *CXCL11*), complement (*C1R, C1S, C3,* and *CFB*), and coagulation factors (*F3*) by *L. jensenii* TL2937 supports our previous reports on the immunoregulatory effect of this strain. These results provided clues for the better understanding of the mechanism underlying host-immunobiotic interaction in the porcine host. The comprehensive transcriptomic profiles of PIE cells provided by our analyses successfully identified a group of genes, which could be used as prospective biomarkers for the screening and evaluation of new anti-inflammatory immunobiotics for the prevention of inflammatory intestinal disorders in pigs.

Keywords: porcine intestinal epitheliocytes, immunotranscriptomic response, ETEC, *Lactobacillus jensenii* TL2937, immunobiotic biomarkers

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infects humans and several species of animals. In farm animals like pigs, ETEC infection results in reduced growth rate, increased mortality and economic loss. ETEC interacts with intestinal epithelial cells (IECs), colonises the small intestine and secretes enterotoxins inducing intestinal acute diarrhoea and inflammation (Ondrackova *et al.,* 2012; Zhou *et al.,* 2012). In addition to its capacity to infect cells and induce damage through toxins, ETEC triggers inflammatory responses through other pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) that significantly contribute to intestinal tissue injury during infections (Geens and Niewold, 2010; Zhou *et al.,* 2012). Therefore, in order to protect pigs against ETEC without damaging host tissues it is important to generate both an adequate inflammatory response against the pathogen, and an efficient regulation of that response.

For several years, researchers have tested probiotics for decreasing pathogen load and ameliorating gastrointestinal disease symptoms in animals. The potential for positive manipulation of the porcine gut immune system through the introduction of beneficial microbes, described as immunobiotics, is currently a popular area of investigation (Villena and Kitazawa, 2014). In this regard, in order to identify potentially immunobiotic strains, we have developed and used several *in vitro* tests based in porcine cells including the clonal porcine intestinal epitheliocyte cell line (PIE cells) (Moue *et al.*, 2008).

Our previous studies on expression profiles of Toll-like receptors (TLRs) in PIE cells have shown that TLR4 is strongly expressed in this cell line. In fact, PIE cells are able to increase chemokine and cytokine expressions in response to LPS stimulation. We have also demonstrated that treatment of PIE cells with *Lactobacillus jensenii* TL2937 is able to down-regulate the levels of interleukin (IL)-6, IL-8, and MCP-1 produced in response to heat-stable ETEC PAMPs or LPS challenges (Shimazu *et al.,* 2012). Moreover, the effect of *L. jensenii* TL2937 on the expression of negative TLR regulators in PIE cells was also evaluated. The expression of SIGIRR, Tollip, A20, Bcl-3, MKP-1, and IRAK-M was studied, and it was found that MKP-1, A20, and Bcl-3 mRNA expression was up-regulated in PIE cells stimulated with this immunobiotic strain (Shimazu *et al.,* 2012). Our *in vivo* data concerning the immunoregulatory effect of *L. jensenii* TL2937 demonstrated that the administration of this immunobiotic bacterium improved immune health, growing performance, and productivity of piglets (Suda *et al.,* 2014).

The evidence of a positive impact of immunobiotics on animal health has increased in the last years, however the mechanisms through which these beneficial microbes influence the animal host remain largely undefined. Various immune pathways have been implicated in the beneficial effects of immunobiotics in the porcine host (Tsukida *et al.,* 2016; Villena and Kitazawa, 2014), but few comprehensive studies have been undertaken, primarily due to lack of available tools. The recent development of specialised expression microarrays based on porcine genome sequence provides one such tool that can evaluate multiple pathways simultaneously (Schroyen and Tuggle, 2015).

We hypothesised that transcriptomic analyses using PIE cells could provide valuable data to understand the mechanisms involved in the protective activity of immunobiotics against intestinal inflammatory damage in the porcine host. In addition, this approach would provide criteria for selecting new biomarkers for the screening and selection of immunobiotics strains in PIE cells that can be used for the development of new immunologically functional feeds, which may help to prevent inflammatory intestinal disorders in pigs. The aim of this study was therefore to investigate: (1) the transcriptomic response of PIE cells to the challenge with heat-stable ETEC PAMPs; and (2) the changes in the transcriptomic profiles induced by the immunobiotic strain *L. jensenii* TL2937 in ETEC PAMPs-challenged PIE cells. We also aimed to provide detailed interpretation of selected genes, which are prospective biomarkers for immunobiotics selection.

2. Materials and methods

Porcine intestinal epitheliocyte cells

PIE cells, which are non-transformed intestinal cultured cells originally derived from intestinal epithelia isolated from an unsuckled neonatal swine (Moue *et al.*, 2008), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% foetal calf serum, 100 mg/ml penicillin, and 100 U/ml streptomycin at 37 °C in an atmosphere of 5% CO_2. PIE cells grow rapidly and are well adapted to culture conditions even without transformation or immortalisation. However, the proliferative ability of PIE cells diminishes after 50 passages in culture. Therefore, we used PIE cells only between the 20th and 40th passages in these experiments (Shimazu *et al.*, 2012; Tomosada *et al.*, 2013).

Microorganisms

ETEC strain 987 (O9:H-:987 pilus+:heat-stable enterotoxin+) was kindly provided by M. Nakazawa at the National Institute of Animal Health (Tsukuba, Japan) (Shimazu *et al.,* 2012). ETEC cells were grown in tryptic soy broth (TSB; Becton, Dickinson and Company, San Jose, CA, USA) for 24 h at 37 °C with shaking. After overnight incubation, the subcultures of bacteria were centrifuged at 5,000×*g* for 10

min at 4 °C, washed with phosphate-buffered saline (PBS), and heat killed (100 °C, 30 min). Heat-stable ETEC PAMPs were suspended in DMEM for use.

L. jensenii TL2937 and *L. plantarum* TL2766 were grown in MRS medium (Difco, Detroit, MI, USA) for 16 h at 37 °C, washed with PBS, and heat killed (56 °C, 30 min). These bacterial samples were resuspended in DMEM, enumerated using a microscope and a Petroff-Hausser counting chamber, and stored at -80 °C until use (Shimazu *et al.*, 2012; Villena *et al.*, 2012).

Anti-inflammatory effect of lactobacilli in PIE cells

PIE cells were seeded at 3×10^4 cells per well in 12-well type I collagen-coated plates (Sumitomo Bakelite Co., Tokyo, Japan) and cultured for 3 days. After changing medium, lactobacilli (5×10^7 cells/ml) were added and 48 h later, each well was washed vigorously with medium at least three times to eliminate all stimulants. Then cells were stimulated with ETEC PAMPs (5×10^7 cells/ml) or LPS (1000 ng/ml; from *E. coli* O55:B5 prepared by phenol extraction followed by gel-filtration chromatography; Sigma, St. Louis, MO, USA) for 3, 6, 12, or 24 h for RT-PCR studies or for 12 h for microarray studies.

Microarray analysis

Total RNA was isolated from PIE cell sample using PureLink RNA Mini Kit (Life Technologies Inc. Gaithersburg MD, USA) and treated with DNase. RNA integrity of all samples were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), using the RNA 6000 Nano Kit. Complementary DNA synthesis, using 200 ng of RNA, and hybridisation with Porcine (V2) Gene Expression Microarray (Agilent Technologies) was performed at Hokkaido System Science Co. Scanning and digitisation of Microarray were done by Agilent Technologies Microarray Scanner and Agilent Feature Extraction 10.7.3.1, respectively. Data normalisation and expression analysis were performed with GeneSpring software version 13.1 (Agilent Technologies). Genes with significant changes in transcript abundance were selected on the basis of two criteria: a t-test *P*-value of less than 0.05, which was considered statistically significant, and a cut-off in transcript abundance of at least 2-fold. Statistical analysis was conducted with the Limma package from BioConductor in R software (version 3.2.5). Results were expressed as log2 scale (log2 ratio). Genes whose expressions were log2>1 and $P<0.05$ were annotated using 'Database for Annotation, Visualization, and integrated Discovery (DAVID), version 6.7 (https://david.ncifcrf.gov/), or GeneAnalytics (geneanalytics.genecards.org). Genes were further analysed according to Gene Ontology (GO) classification. Microarray data have been submitted to NCBI-GEO under the accession number GSE77095.

Quantitative expression analysis by RT-PCR

We performed two-step real-time quantitative PCR to characterise the expression of selected genes in PIE cells. Total RNA was isolated from each PIE cell sample using TRIzol reagent (Invitrogen). All cDNAs were synthesised using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, UK) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyl-X-rhodamine (ROX) (Invitrogen). The primers used in this study are listed in Supplementary Table S1. The PCR cycling conditions were 2 min at 50 °C, followed by 2 min at 95 °C, and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The reaction mixtures contained 5 µl of sample cDNA and 15 µl of master mix, which included the sense and antisense primers. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples.

Statistical analysis

Statistical analyses were performed using GLM and REG procedures available in the SAS computer program (SAS, Cary, NC, USA). Comparisons between mean values were carried out using one-way ANOVA and Fisher's least significant difference (LSD) test. For these analyses, *P*-values <0.05 were considered significant.

3. Results

Immunotranscriptomic changes in PIE cells after challenge with ETEC PAMPs

We first investigated the transcriptomic response of PIE cells to challenge with heat-stable ETEC PAMPs that is able to stimulate a potent inflammatory response in this cell line (Shimazu *et al.*, 2012; Tomosada *et al.*, 2013). Microarray analysis was performed using PIE cells 12 h after the stimulation with heat-stable ETEC PAMPs. When these cells were compared with unchallenged PIE cells we found that there were 3,095 transcripts (representing 715 unique genes) and 2,481 transcripts (representing 561 unique genes) up-regulated and down-regulated, respectively (Figure 1A, B). Out of these differentially regulated genes, several were assigned to immune related functions according to GO database (Figure 1C, Supplementary Table S2). Changes of immunotranscriptome response in PIE cells after heat-stable ETEC PAMPs challenge included genes in the following GO Biological Process pathways: 'innate immune response', 'inflammatory response', 'cytokine-mediated signalling pathway', 'cell adhesion', 'cell surface receptor signalling pathway', and 'blood coagulation' among others (Figure 1C). Of interest, immune related genes were found

in the group of up-regulated genes, while no immune genes were detected in down-regulated transcripts.

The most remarkable changes in PIE cells after heat-stable ETEC PAMPs challenge were observed in expression of chemokines. The highest fold changes (log2 ratio) were observed for *CXCL5* (9.2), *CXCL10* (9.2), *CXCL11* (9.1), *CXCL9* (6.9), *CSF2* (6.9), *CCL4* (6.8), and *CXCL8* (6.6) (Supplementary Table S2). Chemokines that are involved in neutrophils recruitment and activation such as *CXCL5*, *CXCL11* and *CXCL8* were up-regulated more than 6 folds (log2 ratio). In addition, we observed a significant up-regulation of chemokines that are chemotactic factors for monocytes and T lymphocytes including *CXCL10, CXCL9, CCL8, CCL20, CCL23, CX3CL1,* and *CXCL12* (Supplementary Table S2). Moreover, chemokines involved in monocyte migration such as *CCL2* were also increased.

Challenging PIE cells with ETEC PAMPs significantly increased the expression of inflammatory (*TNF, IL-1α, IL-6, IL-18,* and *IL-25*) and angiogenic (*VEGFA*) cytokines genes (Supplementary Table S2). Colony stimulating factor 2 genes (*CSF2*) encoding a cytokine that is able to stimulate the growth and differentiation of hematopoietic precursor of granulocytes and macrophages was also significantly up-regulated.

In addition, we found the up-regulation of genes for adhesion molecules in PIE cells after stimulation with ETEC PAMPs including *SELE, SELL*, *SELP,* and *ICAM-1* (Supplementary Table S2). There was also a 6-fold increase in the expression of *LGALS9* (galectin 9), which is involved in epithelial-lymphocytes interaction.

The microarray analysis revealed increases in the expression of several factors belonging to the complement system including *C1R, C1S, C3, C8, CFB* and *CD46* (Supplementary Table S2). Interestingly, up-regulation of genes related to the coagulation cascade was also observed. The most remarkable effect was detected in tissue factor (*F3*) that was increased more than 4 folds (Supplementary Table S2). The expression of thrombin (*F2*), and fibrinogen (*FGG*) was increased as well.

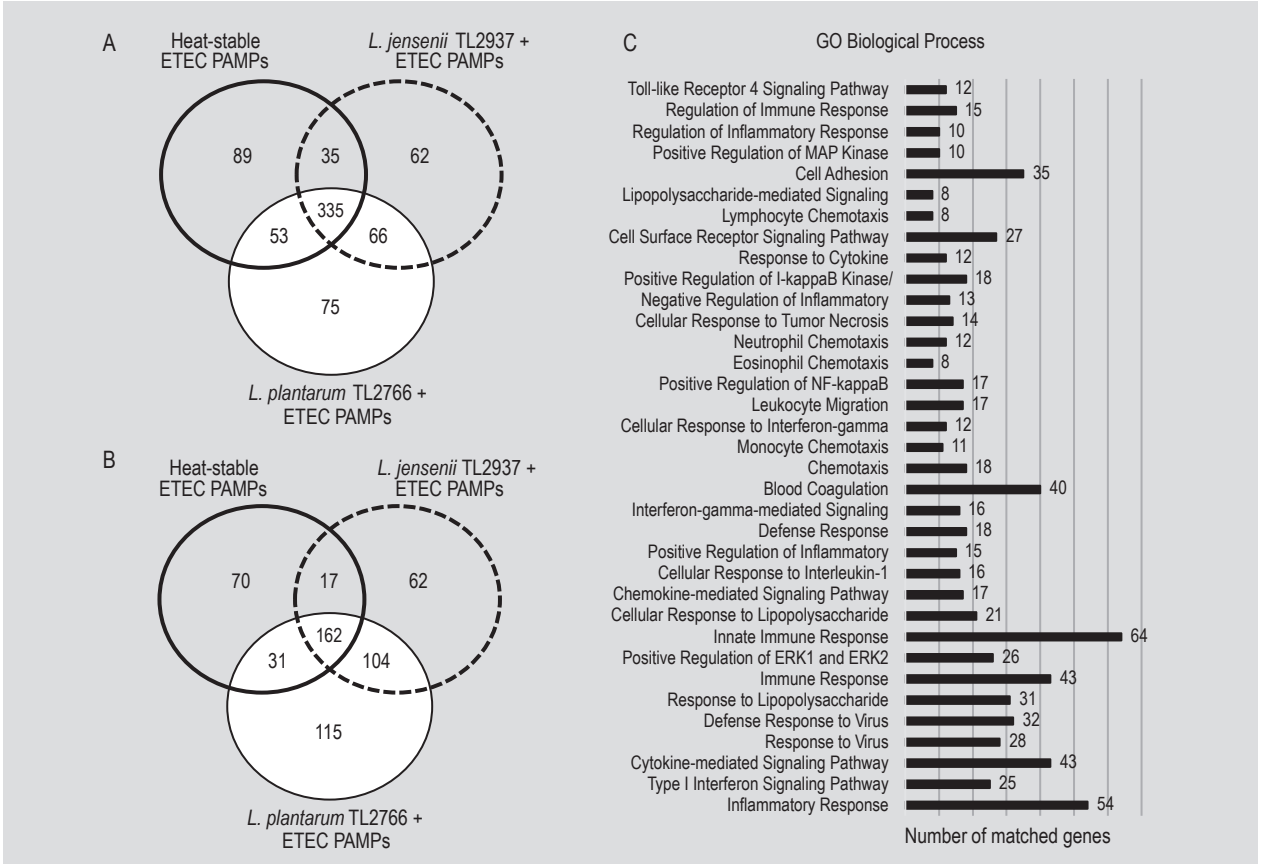

Figure 1. Differentially regulated genes in porcine intestinal epithelial (PIE) cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs). Untreated PIE cells challenged with heat-stable ETEC PAMPs were used as controls. Venn diagrams showing the number of differentially up-regulated (A) and down-regulated (B) genes for each experimental group. Number of matched genes categorised according to gene Ontology (GO) database (C).

Changes in the expression of two pattern recognition receptors were detected: *TLR2*, and *PGLYRP2* (Supplementary Table S2). *TLR4* expression was not modified in ETEC-challenged PIE cells. Gene expression of several factors binding to DNA and RNA from viruses involved in innate antiviral response was increased in PIE cells after heat-stable ETEC PAMPs challenge (Supplementary Table S2). In addition, expression of interferon *(IFN)-β* was increased 3-folds. Transcriptional regulators of type I IFN-dependent immune responses (*IRF5* and *IRF7*) and signal transducers and transcription activators that mediate cellular responses to IFNs (*STAT1* and *STAT2*) were up-regulated in PIE cells after ETEC PAMPs stimulation (Supplementary Table S2).

A significant increase in the expression of NFκ polypeptide genes (*NFKBIA, NFKB2, NFKB1*) was detected in ETEC PAMPs-challenged PIE cells (Supplementary Table S2). We also detected increases in the expression of serum amyloid A2 (*SAA2*), and inducible nitric oxide synthase 2 (*NOS2*).

RT-PCR analysis of genes in PIE cells after challenge with ETEC PAMPs

To further evaluate the changes in gene expressions induced by heat-stable ETEC PAMPs in PIE cells, RT-PCR was performed on the genes that showed significant up-regulation in the microarray profiles. From the 82 immune and immune-related genes up-regulated by ETEC PAMPs (Figure 2, Supplementary Table S2, Figure S4) we selected 26 belonging to cytokines, chemokines, adhesion molecules, complement and coagulation factors, and *SAA2* to be studied by RT-PCR. We confirmed that the direction of the changes in gene expression was in agreement with results obtained in the microarray analysis in all the studied genes. We detected a significant increase in expression of *CCL8, CXCL9* and *CXCL11* with a peak at 12 h after ETEC PAMPs challenge (Figure 3). Similarly, we observed increases in expression of *CCL4* and *CXCL5* with peaks at 3 h, and in *CCL2, CCL5* and *CSF2* with peaks at 24 h after the ETEC stimulation of PIE cells. In addition, expression of *CCL20, CXCL2* and *CXCL8* increased from 3 h and stayed in the same level during the complete studied period (Figure 3). Stimulation of PIE cells with LPS also induced increases in all the studied chemokines, and the kinetic changes were similar to those observed in ETEC PAMPs-challenged PIE cells but with lower values. The only exception was *CXCL8*, which expression started to decrease after 12 h in LPS-stimulated PIE cells while it increased in ETEC PAMPs-challenged PIE cells (Figure 3).

Both, ETEC PAMPs and LPS increased the expression of *IL-1α* and *IL-6*. However, levels of both cytokines were lower in LPS-stimulated PIE cells when compared to those stimulated with ETEC PAMPs (Supplementary Figure S1). *IFN-β* was slightly up-regulated from 3 h after the challenge

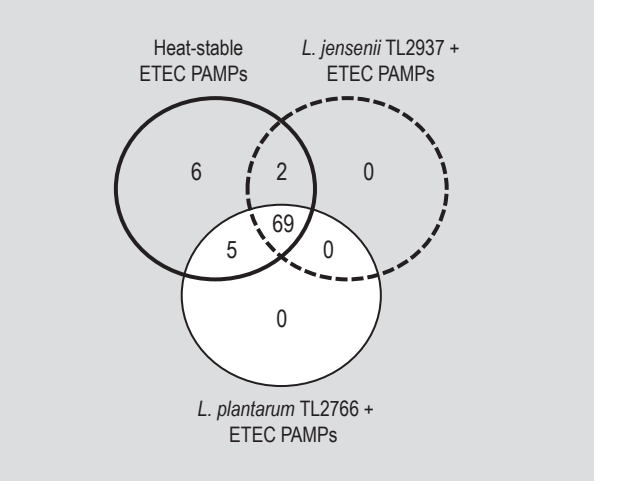

Figure 2. Differentially regulated genes that are known to have immune related functions in porcine intestinal epithelial (PIE) cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs). Untreated PIE cells challenged with heat-stable ETEC PAMPs were used as controls. Venn diagram showing the number of differentially up-regulated genes for each experimental group.

with ETEC or LPS with no differences between them (Supplementary Figure S1). Both, ETEC PAMPs and LPS increased the expression of *SAA2* (Supplementary Figure S1), *SELE, VCAM-1,* and *galectin 9* (Figure 4). However, expression levels of these transcripts were lower in LPS-stimulated PIE cells when compared to those stimulated with ETEC PAMPs.

Stimulation of PIE cells with ETEC PAMPs or LPS induced an increase in gene expression of all the studied complement factors (*C1R, C1S, C3* and *CFB*), which gradually increased until 24 h post-stimulation (Figure 4). In addition, coagulation factors *F2* (thrombin) and *F3* (tissue factor) were up-regulated after the challenges with ETEC PAMPs or LPS. *F2* levels were similar with both stimuli; however expression of *F3* was lower in LPS-treated PIE cells when compared to ETEC PAMPs-challenged cells (Figure 4).

Modulation of ETEC PAMPs-induced immunotranscriptome changes in PIE cells by *L. jensenii*

Next, we analysed microarray data to evaluate the effect of the immunobiotic *L. jensenii* TL2937 strain on the immunotranscriptomic response of PIE cells after the challenge with heat-stable ETEC PAMPs. For that purpose, PIE cells were stimulated with *L. jensenii* TL2937 or *L. plantarum* TL2766 (non-immunomodulatory strain), and then challenged with ETEC PAMPs. Comparative analysis of microarray profiles indicated that *L. jensenii* TL2937 differentially modulated the expression of several genes

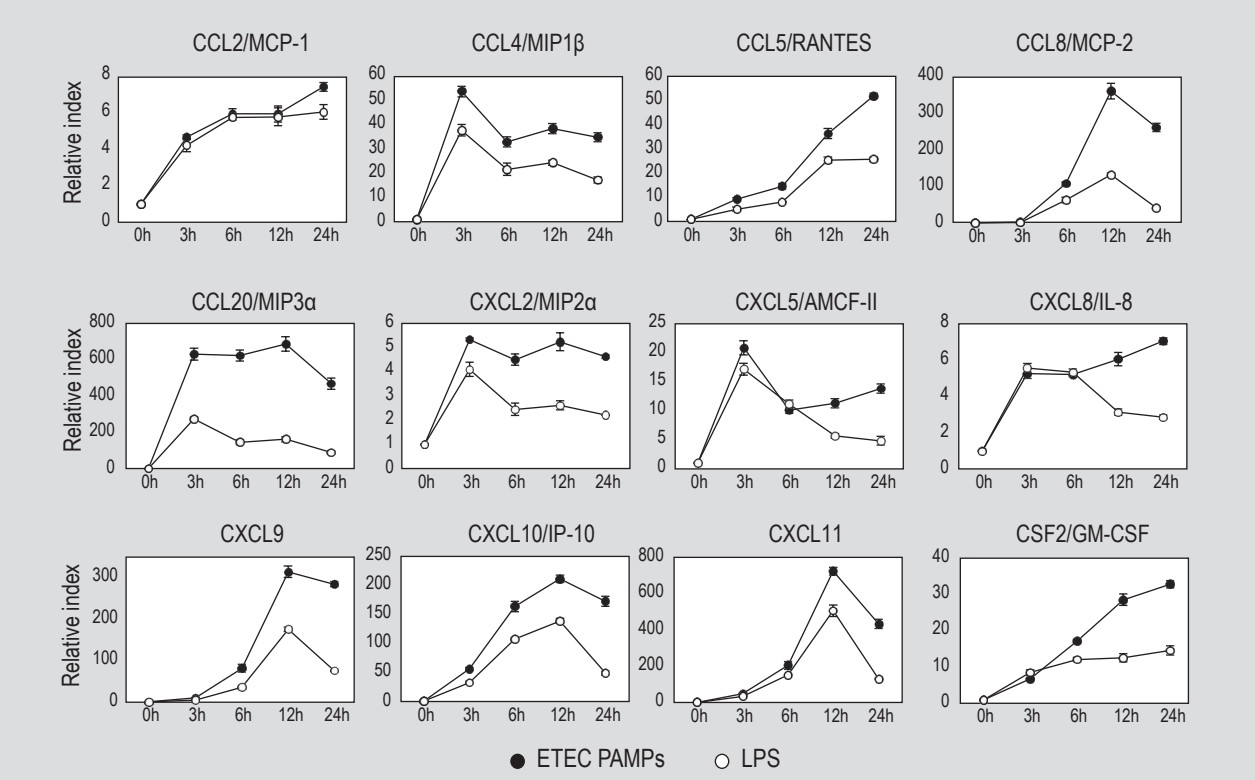

Figure 3. Expression of chemokines in porcine intestinal epithelial (PIE) cells after the challenge with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs) or lipopolysaccharide (LPS), analysed by RT-PCR. The results represent data from three independent experiments.

related to immune responses in PIE cells after the ETEC PAMPs stimulation (Supplementary Table S2).

The Venn diagram analysis was used to find genes that were uniquely and commonly modulated between lactobacilli-treated and control PIE cells (Figure 2). Of the 82 differentially expressed genes in the Venn diagram analysis, 6 were unique to the ETEC PAMPs challenge, while no unique genes were found in the TL2937 stimulation plus ETEC PAMPs challenge, or the TL2766 stimulation plus ETEC PAMPs challenge. In addition, 2 genes were common to TL2937 treatment plus ETEC PAMPs challenge and control, while 69 genes were common to all the three treatments. The cluster analysis in Supplementary Figure S4 depicts the transcriptomic patterns of differentially modulated genes between lactobacilli-treated and control PIE cells. The treatment with TL2766 plus ETEC PAMPs challenge clustered closer to the control than the treatment with TL2937 plus ETEC PAMPs.

Closer examination of the variations in gene expression induced by the immunobiotic strain TL2937 revealed differences in several up-regulated genes sheared with control PIE cells (Supplementary Table S2). Most remarkable differences were found in the genes belonging to cytokines and chemokines. Although expression of chemokines such as *CXCL5, CXCL10, CXCL11, CXCL9, CCL8, CCL11* and *CCL2* was up-regulated in TL2937-treated PIE cells after the challenge with ETEC PAMPs, the increases were significantly lower when compared to control PIE cells without lactobacilli treatment (Supplementary Table S2). Other chemokines such as *CCL4, CCL5, CCL20, CXCL2,* and *CXCL8* were not different between TL2937-tretated and control ETEC PAMPs-challenged PIE cells. In addition, we observed that gene expression of the pro-inflammatory cytokines and chemokines *TNF, CXCR6,* and *IL-25*, which were up-regulated in the ETEC PAMPs-challenged control PIE cells, was not increased in cells treated with TL2937.

We also observed an increased expression of selectins (*SELE, SELL,* and *SELP*) genes in TL2937-treated PIE cells after the challenge with ETEC PAMPs when compared to the control cells, while galectin 9 (*LGALS9*) was diminished in TL2937-tretated cells (Supplementary Table S2). No significant differences were found in expression of other adhesion molecules such as *VCAM-1* or *ICAM-1*.

Expression of *TLR2* was up-regulated in TL2937-treated PIE cells after the challenge with ETEC PAMPs, however the increase was lower when compared to the control PIE cells without lactobacilli treatment (Supplementary Table S2). In addition, we observed that *PGLYRP2* gene expression was

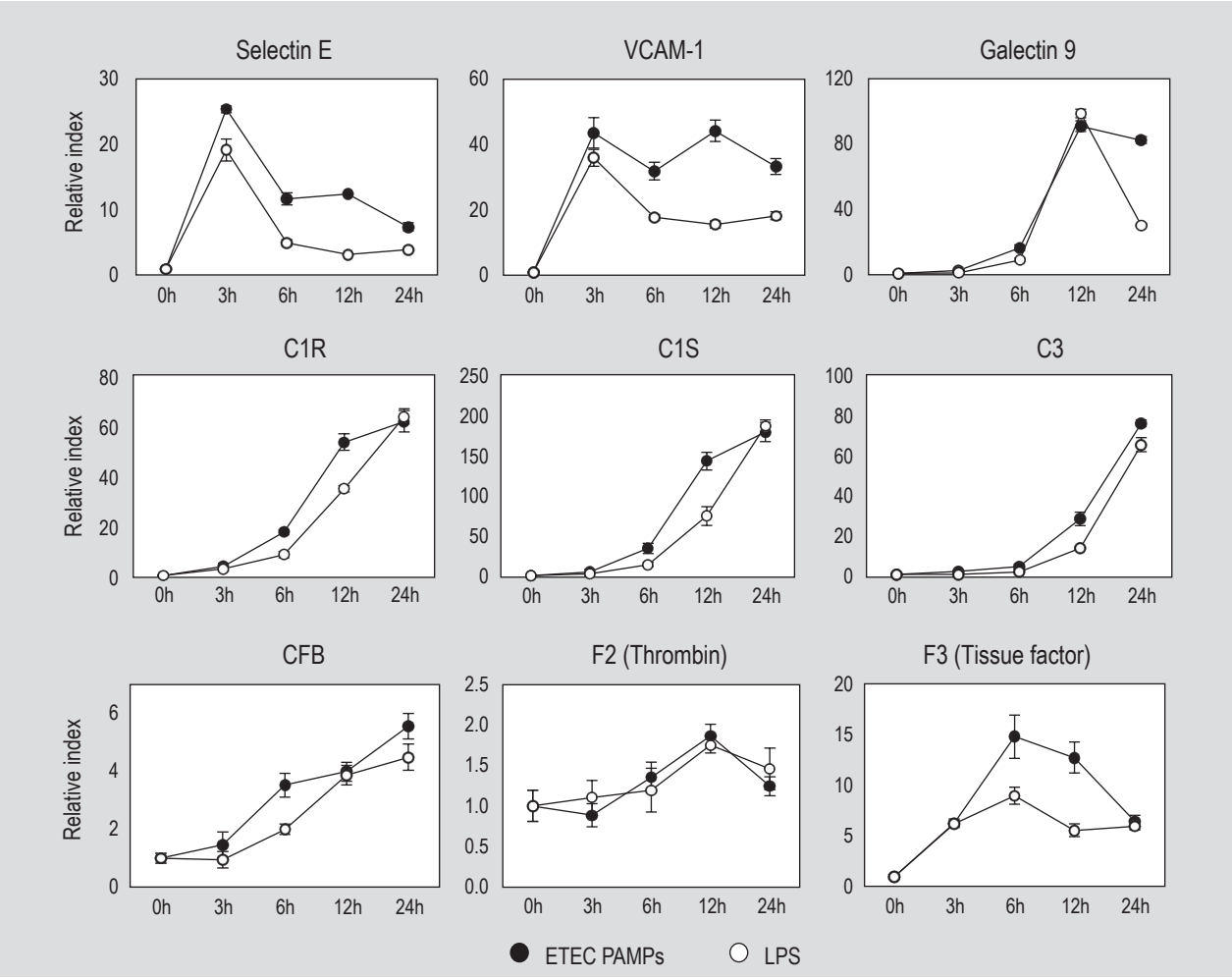

Figure 4. Expression of adhesion molecules and, complement and coagulation factors in porcine intestinal epithelial (PIE) cells after the challenge with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs) or lipopolysaccharide (LPS), analysed by RT-PCR. The results represent data from three independent experiments.

not affected in cells treated with TL2937 when compared to controls.

Although expression *C1R, C1S, CFB, C3* and *F3* (tissue factor) were up-regulated in TL2937-treated PIE cells after the challenge with ETEC PAMPs, the increase was significantly lower when compared to the control PIE cells without lactobacilli treatment (Supplementary Table S2). Interestingly, expression of *F2* (thrombomodulin) was higher in immunobiotic treated cells than in ETEC PAMPs-challenged control PIE cells.

Finally, we also detected that expression of *SAA2* in TL2937-treated PIE cells after the challenge with ETEC PAMPs was lower when compared to control PIE cells without lactobacilli treatment (Supplementary Table S2).

It should be noted that immunotranscriptomic response of PIE cells treated with the non-immunomodulatory strain *L. plantarum* TL2677 was almost identical to the response of ETEC PAMPs-challenged control PIE cells (Figure 2, Supplementary Table S2). The microarray data revealed differences only in the expression of *SELE, SELP, SELL,* and *F2* genes, which were higher in TL2677-treated PIE cells than in control cells. In addition, the expression level of *C5AR1, C8A, CD2, EGF, FGG, IL-25, TNF, IL-18RA,* and *LGALS9* genes in TL2766-treated PIE cells was lower than those observed in ETEC PAMPs-challenged control PIE cells.

RT-PCR analysis of genes in PIE cells challenged by ETEC PAMPs and stimulated by *L. jensenii*

In order to confirm the changes induced by TL2937 strain in the immunotranscriptome response of heat-stable ETEC PAMPs-challenged PIE cells, RT-PCR was performed on selected genes. Genes with or without significant differences between TL2937-tretated and non-treated PIE cells were chosen. The transcriptional changes evaluated by RT-PCR indicated a similar overall trend in the transcription.

Expression of *CCL4, CCL5, CCL20, CXCL2, CXCL8* and *CSF2* in TL2937-treated PIE cells was not different from the control PIE cells after the challenge with heat-stable ETEC PAMPs (Supplementary Table S2). In contrast, levels of *CCL2, CCL8, CXCL5, CXCL9, CXCL10* and *CXCL11* were significantly lower in *L. jensenii* TL2937-tretaed PIE cells when compared to the controls (Figure 5). No differences in the expression profiles of chemokines were found between *L. plantarum* TL2677-treated PIE cells and control PIE cells after the challenge with ETEC PAMPs (Supplementary Figure S2, Figure 5).

In agreement with the results from our microarray analysis, both lactobacilli strains were able to increase the expression of selectin E (*SELE*) and reduce the expression of galectin 9 (*LGALS9*), with no significant differences between them (Supplementary Figure S3). Moreover, no differences in *VCAM-1* were found between lactobacilli-treated and control PIE cells.

Expression of *IL-1α* in TL2937- or TL2766-treated PIE cells was not different from the control PIE cells after the challenge with heat-stable ETEC PAMPs. In contrast, expression levels of *IL-6* (Supplementary Figure S3) and *SAA2* (Figure 6) were significantly lower in *L. jensenii* TL2937-tretaed PIE cells when compared to the controls.

We also observed that complement factors (*C1R, C1S, C3* and *CFB*) were significantly down-regulated in *L. jensenii* TL2937-tretaed PIE cells when compared to the controls (Figure 6). *L. jensenii* TL2937 significantly reduced the level of *F3* in ETEC PAMPs-challenged PIE cells. Again, no differences in expression profiles of complement factors or *F3* were found between *L. plantarum* TL2677-treated PIE cells and the control PIE cells after the challenge with ETEC PAMPs (Figure 6). Microarray data indicated a significant up-regulation of *F2* by lactobacilli treatment, however RT-PCR indicated no modification of the expression of *F2* after bacterial treatments (Supplementary Figure S3). This was the only discrepancy between microarray and RT-PCR data.

4. Discussion

In the last decades, pig farms have significantly increased in size due to the growing food demand of human population. This fact has unfortunately increased the risk of diseases in those animals. Therefore, a better understanding of the immune system in the porcine host is essential, because susceptibility to infectious diseases has great influence on pig performance (Boddicker *et al.*, 2013). Studies of transcriptomic pig immune response using whole porcine genome microarrays have enriched our knowledge of the pig immune system (Gao *et al.*, 2010). In this regard,

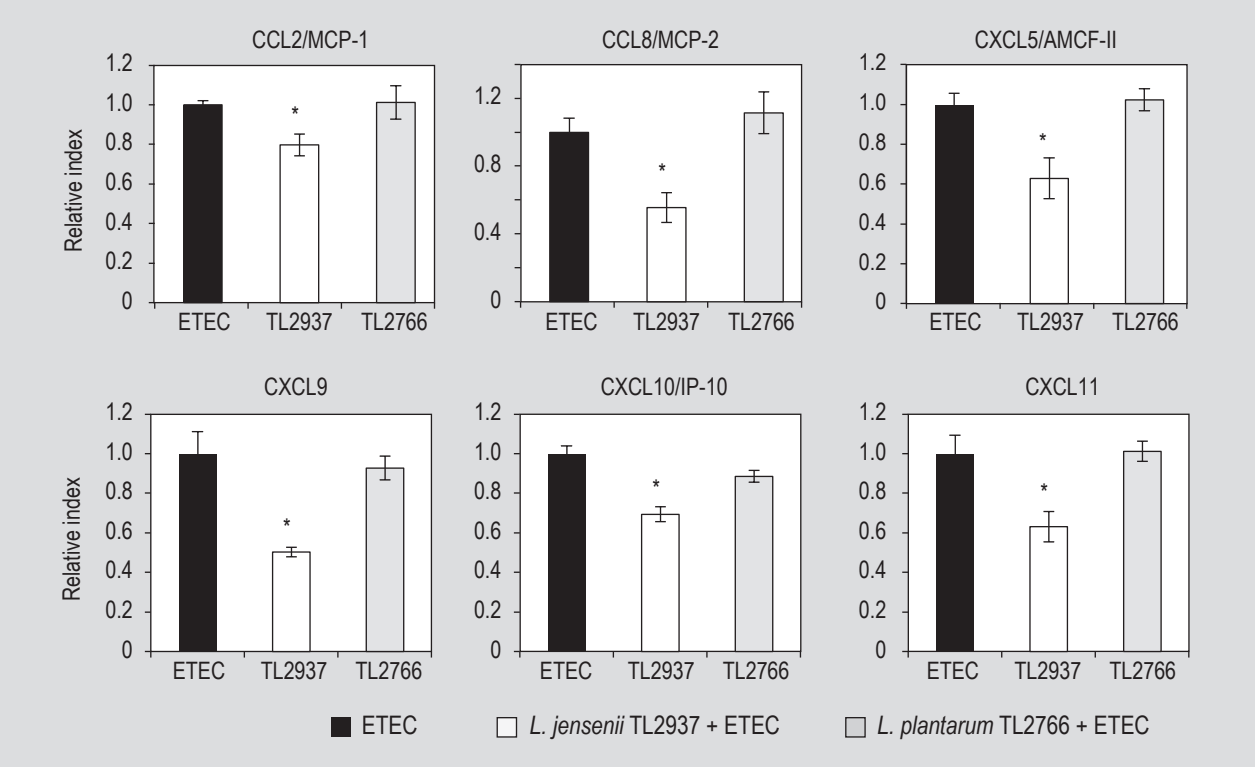

Figure 5. Expression of chemokines in porcine intestinal epithelial (PIE) cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs), analysed by RT-PCR. Untreated PIE cells challenged with heat-stable ETEC PAMPs were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences (*P*<0.05).

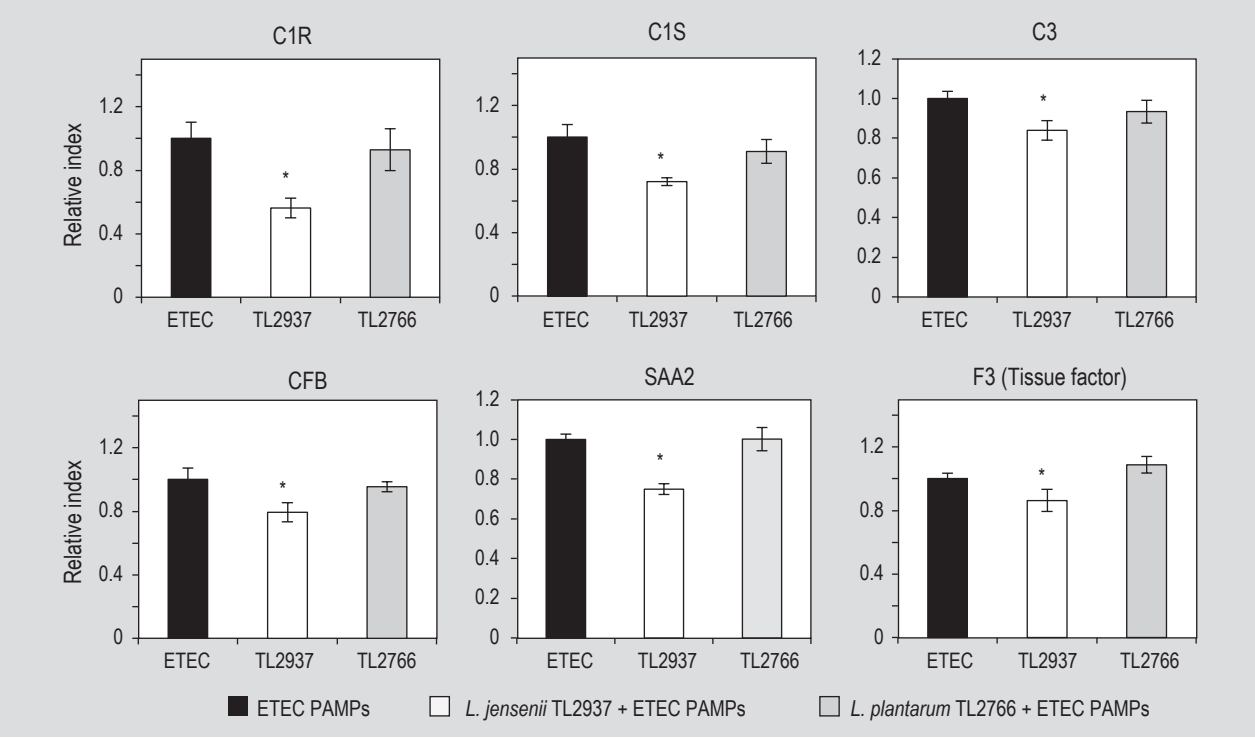

Figure 6. Expression of serum amyloid A2 (SAA2), complement and tissue factors in porcine intestinal epithelial (PIE) cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs), analysed by RT-PCR. Untreated PIE cells challenged with heat-stable ETEC PAMPs were used as controls. The results represent data from three independent experiments. Asterisks indicate significant differences (*P*<0.05).

commercially available microarrays have been used to study pig immune responses against pathogens considering that gastrointestinal pathogens-associated diarrhoea results in morbidity and mortality in neonatal and weaned piglets, and is one of the economically most important diseases in swine husbandry.

High-throughput microarray technology has been employed for screening genes involved in disease processes or immune responses to pathogenic ETEC. Zhou *et al.* (2012) used the porcine IPEC-J2 cell line, originally derived from jejunal epithelia, for studying ETEC-IECs interactions and evaluating porcine transcriptional changes upon infection with three ETEC strains expressing different fimbriae (F4ab, F4ac and F18ac). The work found that 2,443, 3,493 and 867 genes were differentially expressed in IPEC-J2 cells that after infection with F4ab, F4ac and F18ac ETEC, respectively. These results are in line with the knowledge that F4ac is the most common antigenic variant of F4 fimbriae causing piglet diarrhoea (Ji *et al.*, 2010). In addition, the small intestinal segment perfusion technique and microarray analysis have been used to evaluate porcine early immune responses induced by different ETEC strains expressing heat-stable or heat-labile enterotoxins (Loos *et al.*, 2012). Interestingly, most transcripts down-regulated by ETEC identified in that study were not associated with immune responses, while the immunomodulatory genes were abundant in the up-regulated transcripts.

Similarly, we demonstrated here that stimulation of PIE cells with heat-stable ETEC PAMPs significantly alters gene expression profiles. Of the transcripts differentially modulated by ETEC PAMPs challenge, 715 and 561 unique genes were up-regulated and down-regulated, respectively. Moreover, the majority of genes associated with immune responses were present in the group of genes up-regulated after heat-stable ETEC PAMPs challenge. In fact, we found significant increases in the expression levels of cytokines, chemokines, and adhesion molecules genes in ETEC-stimulated PIE cells (Figure 7). Our findings are in agreement with previous studies. Geens and Niewold (2010) found genes differentially expressed between unchallenged IPEC-J2 cells and those stimulated with LPS or ETEC, including increases in *CXCL2, IL-8, AREG* and *CYP1A1*. In addition, the immune genes *IL-8, LT-β, CXCL2, CCL20,* and *AMCF-II* were highly up-regulated in IPEC-J2 cells upon F4ab, F4ac, or F18ac ETECs strains challenges (Zhou *et al.*, 2012). Collectively, these results show that ETEC stimulates a typical inflammatory response in porcine IECs, the extent of which is different according to the different ETEC strains, bacterial viability, and infection time.

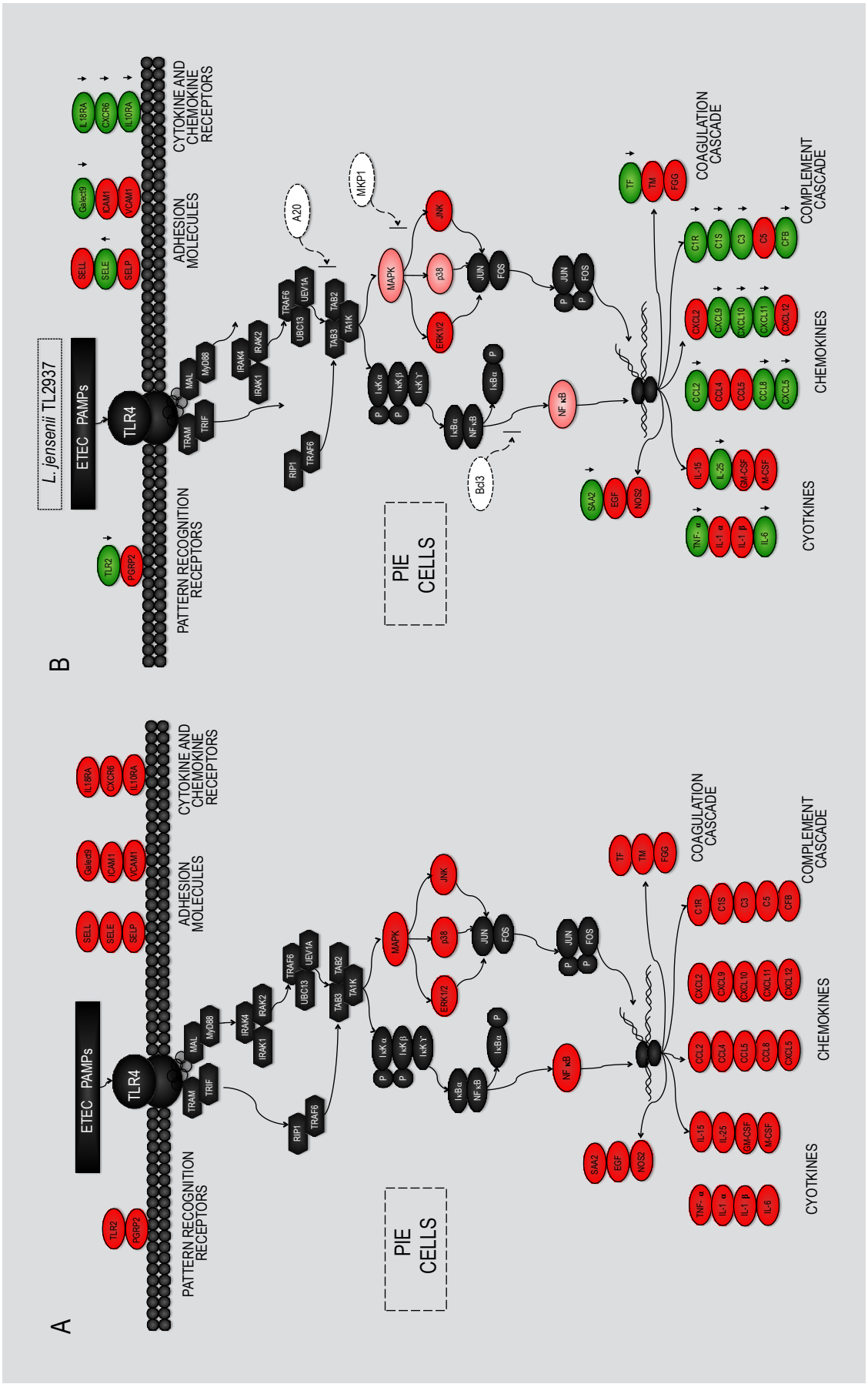

Figure 7. Global overview of the signalling pathways and immune genes differentially regulated in porcine intestinal epithelial (PIE) cells after the challenge with heat-stable enterotoxigenic *Escherichia coli* (ETEC) pathogen-associated molecular patterns (PAMPs) (A). Global overview of the signalling pathways and immune genes differentially regulated in PIE cells treated with *Lactobacillus jensenii* TL2937 and challenged with heat-stable ETEC PAMPs. Genes differentially regulated by TL2937 strain are shown in green (B).

ETEC adherence to IECs leads to NF-kB and MAPK pathway activation (Wang *et al.*, 2012), and it has been shown *in vitro* that ETEC infection modulates the pro-inflammatory responses through the regulation of NF-kB and MAPK signalling pathways (Zanello *et al.*, 2011). Moreover, *in vivo* studies in mice have found that the ETEC infection promotes intestinal expression of a set of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, IL-17, and IL-18, increases the expression of Rel and induces the phosphorylation of p38 and ERK1/2 proteins (Ren *et al.*, 2014). We have previously demonstrated that stimulation of PIE cells with heat-stable ETEC PAMPs activates NF-kB and induces the phosphorylation of ERK, p38, and JNK leading to the production of CCL2 (MCP-1), CXCL8 (IL-8) and IL-6 (Shimazu *et al.*, 2012). In the present work, we corroborated these findings by demonstrating that activation of NF-kB and MAPK pathways in PIE cells also increased the expression of several cytokines and chemokines including *CCL4, CCL5, CCL8, CCL20, CXCL2, CXCL5, CXCL9, CXCL10, CXCL11, CSF2,* and *IL-1α* by microarray analysis and RT-PCR. Moreover, some adhesion molecules were also significantly up-regulated in PIE cells after ETEC PAMPs stimulation including *SELE, SELP, SELL, ICAM-1,* and *VCAM-1*. These results indicate that PIE cells are able to mount a complex immune response involving changes needed to induce and promote the recruitment of inflammatory cells to the intestinal mucosa, which are intended to eliminate the pathogen. These features exhibit that PIE cells are an excellent laboratory tool to study treatments able to favourably modulate the inflammatory response.

In addition to cytokines and adhesion molecules, we also observed variations in other immune related genes in PIE cells after the challenge with ETEC PAMPs including PRRs, and complement and coagulation factors (Figure 7).

Challenge of PIE cells with ETEC PAMPs did not change the expression of *TLR4*. Loos *et al.* (2012) showed no differential regulation of TLR4 gene in porcine intestinal segments infected with ETEC strain. This last report was in agreement with a microarray study in IPEC-J2 cells showing no induction of TLR4 after co-incubation with ETEC compared to mock-infected cells (Geens and Niewold, 2010). Therefore, it seems that ETEC does not induce significant changes in TLR4 expression in porcine IECs. In contrast, ETEC PAMPs significantly up-regulated the expression of two PRRs in PIE cells: *TLR2* and *PGLYRP2* genes. This is in agreement with previous reports that have demonstrated that *PGYRP2* gene was highly up-regulated in IPEC-J2 cells after the challenge with ETEC strains (Zhou *et al.*, 2012).

The humoral innate immune system is comprised of soluble plasma components that include complement, acute phase proteins, and antimicrobial peptides (Shishido *et al.*, 2012). Acute phase proteins are blood proteins that are rapidly induced in response to inflammation caused by tissue injury, infections or stress. The major acute phase proteins in pigs are C-reactive protein, pig-MAP, haptoglobin, and serum amyloid A (SAA2) (Mair *et al.*, 2014). On the other hand, the complement cascade is activated through three independent pathways using several membrane-anchored, and soluble proteins that together constitute a proteolytic cascade (Shishido *et al.*, 2012). These processes result in the formation of the membrane attack complex that lyses host or pathogenic cells by formation of pores in the cellular membrane. Downstream of the multiple routes to complement activation, the generation of C3 and C5 convertases is a key reaction leading to the production of C3a and C5a, which are potent inflammatory mediators. It has been demonstrated that three IECs lines (T84, Caco2, and HT29) express mRNA for complement glycoproteins (Cao *et al.*, 2012). Those studies were consistent with reports of C4 expression at the protein level in T84 and Caco2, and factor B detected in Caco2 cell culture supernatants, confirming that IECs are a source of complement in the gut (Bernet-Camard *et al.*, 1996). Moreover, it was found that IECs express C5aR apically and directly respond to C5a through ERK signalling pathway with responses that promote inflammation (Cao *et al.*, 2012). Recent studies on the role of complement proteins during enteric infections have showed that this innate defence mechanism is important for the protection of mice against *Citrobacter rodentium,* which is an attaching and effacing mouse pathogen that models enteropathogenic and enterohemorrhagic *E. coli* in humans (Jain *et al.*, 2015). The authors showed that properdin knockout mice had increased diarrhoea and exacerbated inflammation combined with defective epithelial cell-derived IL-6 and greater numbers of colonising bacteria. Those effects were related to a failure to increase local C5a levels. In the current study, we detected significant increases in the expression of *SAA2* and several components of the complement system including *C1R, C1S, C3, CFB,* and *C5AR1* in PIE cells after the challenge with ETEC PAMPs, indicating that these factors may have an important role in the inflammatory response induced by ETEC infection in the porcine host.

We observed a significant increase in the expression of some factors of the coagulation system in PIE cells treated with ETEC PAMPs, especially in tissue factor (TF, *F3* gene) expression. TF is the membrane receptor that initiates the extrinsic coagulation pathway. TF also plays an important role in inflammatory processes. Queiroz *et al.* (2011) evaluated whether ablation of TF reduced experimental colitis by using wild-type and TF-deficient mice treated with dextran sulfate sodium. The work demonstrated that the severity of colitis was diminished in TF-deficient mice compared with controls. Most notably, neutrophils infiltration at the site of inflammation, and cytokine levels were reduced in TF-deficient mice. It was shown that TF

induced high levels of the granulocyte-chemoattractant chemokine KC in the colon, thereby stimulating neutrophils influx with subsequent inflammatory-induced organ damage. Based on these findings, the increase in the TF expression observed in our current study allows us to speculate that coagulation system activation would also contribute to the activation of the inflammatory response induced by ETEC infection in pigs.

Several studies have shown that immunobiotics are able to beneficially modulate PRRs-mediated inflammatory response in the gut by modulating the functions of IECs (Villena and Kitazawa, 2014). In this regard, we selected lactobacilli strains with the capacity to regulate the inflammatory response induced by ETEC and LPS in PIE cells by evaluating the levels of IL-1α, IL-6, IL-8, and MCP-1 (Shimazu *et al.*, 2012). We found that IL-6 and IL-8 levels in PIE cells stimulated with some lactobacilli strains, especially *L. jensenii* TL2937, were significantly lower than those in control cells. Moreover, *L. jensenii* TL2937 have been shown to inhibit NF-κB and MAPK signalling pathways in ETEC- and LPS-challenged PIE cells through an up-regulation of the expression of the negative TLR regulators MKP-1, A20, and Bcl-3 (Shimazu *et al.*, 2012). In the present work, the treatment of PIE cells with *L. jensenii* TL2937 also resulted in differential expression of several pro-inflammatory mediators in response to the ETEC PAMPs challenge. Most notable changes were found in chemokines, since expression levels of *CCL2, CCL8, CXCL5, CXCL9, CXCL10,* and *CXCL11* were significantly lower in TL2937-treated PIE cells when compared to the controls (Figure 7). This finding is of interest because it not only confirms our previous *in vitro* (Shimazu *et al.*, 2012; Tsukida *et al.*, 2016; Villena *et al.*, 2012) and *in vivo* (Suda *et al.*, 2014) studies demonstrating the anti-inflammatory capacity of *L. jensenii* TL2937, but also provides a potential set of biomarkers for the screening and selection of new immunoregulatory bacterial strains.

Of note, *L. jensenii* TL2937 also induced a differential expression of adhesion molecules in ETEC PAMPs-challenged PIE cells; however, the same effect was obtained with the negative control strain *L. plantarum* TL2677. Therefore, adhesion molecules such as *SELE* or *LGALS9* would not have an application as biomarkers for immunobiotics' selection.

Interestingly, *L. jensenii* TL2937 was able to modify the expression of acute phase proteins and factors from the complement and coagulation system in PIE cells after the challenge with ETEC PAMPs (Figure 7). This is in line with our previous *in vivo* experiments using piglets of 3 weeks of age, which demonstrated that feeding with *L. jensenii* TL2937 significantly reduced blood complement activity and C reactive protein concentrations (Suda *et al.*, 2014). Moreover, using gene expression profiling microarrays, Bao *et al.* (2011) have analysed and screened porcine genes conferring resistance against ETEC infection by comparing responses of ETEC-resistant and ETEC-sensitive pig populations. The authors showed that transcriptomic differences between sensitive and resistant pigs included genes of complement and coagulation factors, although these genes have not been studied in detail or validated by RT-PCR. Then, similarly to cytokines and chemokines, down-regulation of acute phase proteins, complement, and coagulation factors by the immunobiotic TL2937 strain would be beneficial to modulate the inflammatory response and protect against the infection-induced intestinal damage. This is in agreement with the knowledge that complement is essential for the induction of TF expression upon *E. coli* stimulation, having both systems an important role in inflammation and sepsis (Brekke *et al.*, 2013). It has been reported that the inhibition of complement inhibits the *E. coli*-induced inflammatory response in blood cells by reducing the production of pro-inflammatory factors including cytokines (TNF-α, IL-6, INF-γ, and IL-1β), chemokines (IL-8, MCP-1, MIP-1α, MIP-1β, eotaxin, and IP-10), growth factors (VEGF, G-CSF, and GM-CSF) (Brekke *et al.*, 2008) and TF up-regulation and coagulation activation (Landsem *et al.*, 2013).

It was also reported that gut commensal microorganisms promote TF glycosylation, coagulation proteases activation, and phosphorylation of the cytoplasmic domain of TF in the small intestine (Reinhardt *et al.*, 2012). Moreover, the work demonstrated that the microbiota-induced extravascular TF-PAR1 signalling, and influenced vascular remodelling in the small intestine. These findings indicate that intestinal microorganisms are able to modulate the function of coagulation factors in the intestine. Some works have also demonstrated the capacity of immunobiotic bacteria to beneficially modulate the interaction between inflammation and coagulation during infections. Our previous studies performed with the immunobiotic strain *Lactobacillus rhamnosus* CRL1505 clearly demonstrated that the probiotic bacterium is able to influence lung immune-coagulative reaction triggered by TLR3 activation, by modulating the production of pro-inflammatory and anti-inflammatory cytokines as well as expression of TF and thrombomodulin in the lung (Zelaya *et al.*, 2014, 2015). This beneficial effect induced by the CRL1505 strain allows the reduction of lung tissue damage induced by respiratory syncytial virus (Zelaya *et al.*, 2014) or influenza virus (Zelaya *et al.*, 2015). In addition, the effect of probiotic bacteria on complement system gene expression in IECs has been described before. Studies in the cell line HT29 showed that *Bifidobacterium breve* IPLA20004 significantly up-regulated the expression of the complement component C3 and suggested that this effect could favor the recruitment of innate immune cells to the mucosa reinforcing the intestinal barrier (Sánchez *et al.*, 2015). To our knowledge, no study has described a beneficial effect of an immunobiotic bacterium on

complement and coagulation factors expression during ETEC-induced inflammation. Future investigation is necessary for understanding the beneficial effect of immunobiotic bacterium on these factors during ETEC infection in the porcine host.

In conclusion, the transcriptomic study performed in this work allowed us to obtain a global overview of the expression patterns of immune and immune-related genes involved in response of PIE cells to heat-stable ETEC PAMPs. The present study also confirmed that *L. jensenii* TL2937 differently modulates gene expression in ETEC PAMPs-challenged PIE cells. The microarray transcriptomic profiles and gene expression patterns studied by RT-PCR simultaneously demonstrated that an anti-inflammatory effect was triggered by the immunobiotic strain in PIE cells. The main outcome from the study was the differential regulation of chemokines, complement, and coagulation factors expression by *L. jensenii* TL2937. These results provided clues for the better understanding the mechanism underlying host-immunobiotic interaction in the porcine host. Moreover, our transcriptomic analysis successfully identified a group of genes (*CCL8, CXCL5, CXCL9, CXCL10, CXCL11, C1R, C1S, C3, CFB, F3, and SAA2*), which can be used as prospective biomarkers for the screening of new anti-inflammatory immunobiotics in PIE cells.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/BM2016.0095.

Figure S1. Expression of cytokines and serum amyloid A2 in porcine intestinal epithelial cells after the challenge with heat-stable enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns or lipopolysaccharide.

Figure S2. Expression of chemokines in porcine intestinal epithelial cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns.

Figure S3. Expression of cytokines, thrombin, and adhesion molecules in porcine intestinal epithelial cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns.

Figure S4. Heat map analysis of differentially regulated genes that are known to have immune related functions in porcine intestinal epithelial cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns.

Table S1. Primers sequences used in RT-PCR studies.

Table S2. Transcripts in porcine intestinal epithelial cells treated with *Lactobacillus jensenii* TL2937 or *Lactobacillus plantarum* TL2766 and challenged with heat-stable enterotoxigenic *Escherichia coli* pathogen-associated molecular patterns.

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