

Intestinal expression of pro-inflammatory cytokines induced by oral intake of lipopolysaccharide (LPS) from *E. coli* in weaned pigs^a

*Expresión intestinal de citoquinas proinflamatorias inducidas mediante ingestión de lipopolisacáridos de *E. coli* en lechones recién destetados*

*Expressão intestinal de citocinas pró-inflamatórias induzida pela ingestão de lipopolissacarídeo de *E. coli* em leitões recém desmamados*

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Summary

Background: treatments with lipopolysaccharide (LPS) from *E. coli* are an accepted way of inducing inflammation in immunological studies since they have the ability to activate a coordinate series of signs through the synthesis of pro-inflammatory cytokines such as interleukin 8 (IL-8), IL-18 and tumor necrosis factor alpha (TNF- α), which can cause significant changes in intestinal structure and functionality. **Objective:** the aim of this study was to evaluate the effect of adding LPS of *E. coli* on pro-inflammatory cytokine gene expression IL-8, IL-18 and TNF- α in early-weaned pigs. **Methods:** fieldwork was conducted at Centro San Pablo, belonging to the Universidad Nacional de Colombia with 32 pigs at 21 days of age, with 6.5 ± 0.5 kg of weight. Animals were fed with a basal diet supplemented with two levels of inclusion of LPS of *E. coli* serotype 0111:B4 (0 to 0.3 $\mu\text{g}/\text{mg}$ of food). Pigs were slaughtered in stages on days 1, 5, 7 and 10 post-weaning and complete extraction of small intestine was made. Gene expression was evaluated by qPCR. Blocks at random in a factorial arrangement 2 x 4 were used as statistical design. **Results:** the basal diet (without addition of LPS) presented increase in mRNA expression ($p < 0.01$) of all the cytokines in jejunum

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for each post-weaning day, which suggests an inflammatory response and extensive tissue damage in pigs after early weaning. In diet 1 (with consumption of 0.3 μg LPS / mg diet) cytokines TNF- α , IL-8 and IL-18 showed a significant increase in their levels of expression ($p < 0.01$). All cytokines presented significant increase ($p < 0.01$) in jejunum for each post-weaning day. **Conclusion:** The increase observed in the expression of TNF- α can be involved in the development of post-weaning diarrhea.

Key words: gut immunology, intestinal villi, pig weaning, RT-PCR, TNF- α .

Resumen

Antecedentes: los tratamientos con lipopolisacáridos (LPS) de *E. coli* son una forma aceptada para inducción de inflamación en estudios inmunológicos ya que tienen la capacidad de activar una gran variedad de rutas de señalización a través de la síntesis de citoquinas proinflamatorias, tales como interleuquina 8 (IL-8), IL-18 y factor de necrosis tumoral alfa (TNF- α), las cuales pueden provocar cambios importantes en la estructura y funcionalidad intestinal. **Objetivo:** el presente estudio tuvo como objetivo evaluar el efecto de la adición de LPS de *E. coli* sobre la expresión génica de las citoquinas proinflamatorias IL-8, IL-18, y TNF- α en lechones recién destetados. **Métodos:** este trabajo se realizó con 32 lechones de 21 días de edad, con un peso de $6,5 \pm 0,5$ kg en el Centro San Pablo, perteneciente a la Universidad Nacional de Colombia. Los animales fueron alimentados con una dieta basal adicionada con dos niveles de inclusión de LPS de *E. coli* serotipo 0111:B4 (0 y 0,3 $\mu\text{g}/\text{mg}$ de alimento). Los animales se sacrificaron escalonadamente los días 1, 5, 7 y 10 posdestete, y se realizó extracción completa del intestino delgado. Se evaluó la expresión génica por qPCR. El diseño estadístico empleado fue de bloques al azar en un arreglo factorial de 2X4. **Resultados:** la dieta basal (sin adición de LPS) presentó incremento en la expresión de mRNA ($p < 0,01$) de todas las citoquinas en yeyuno para cada día posdestete, lo que sugiere una respuesta inflamatoria y grandes daños tisulares en el lechón luego del destete precoz. En la dieta 1 (con consumo de 0,3 μg LPS /mg dieta) las citoquinas TNF- α , IL-8, e IL-18 presentaron incrementos significativos en sus niveles de expresión ($p < 0,01$). Todas las citoquinas presentaron incremento significativo ($p < 0,01$) en yeyuno para cada uno de los días posdestete. **Conclusión:** el incremento observado en la expresión de TNF- α puede estar implicado en el desarrollo de diarreas posdestete.

Palabras clave: destete, inmunología intestinal, RT-PCR, TNF- α , vellosidad intestinal.

Resumo

Antecedentes: os tratamentos com lipopolissacarídeos (LPS) de *E. coli* são uma forma aceita de indução de inflamação para estudos imunológicos quanto que têm a capacidade de ativar uma grande variedade de vias de sinalização através da síntese de citocinas pró-inflamatórias tais como a interleucina 8 (IL-8), IL-18 e fator de necrose tumoral alfa (TNF- α), o que pode causar alterações importantes na estrutura e funcionalidade do intestino. **Objetivo:** o presente estudo teve como objetivo a adição de LPS de *E. coli* sobre a expressão génica das citocinas pró-inflamatórias IL-8, IL-18 e TNF- α em leitões desmamados. **Métodos:** este trabalho foi feito com 32 leitões de 21 dias de idade, com um peso de $6,5 \pm 0,5$ kg no Centro San Pablo, pertencente à Universidade Nacional de Colômbia. Os animais foram alimentados com uma dieta basal adicionada com dois níveis de inclusão de LPS de *E. coli* serotipo 0111:B4 (0 y 0,3 $\mu\text{g}/\text{mg}$ de alimento). Os animais foram sacrificados nos dias 5, 7 e 10 após o desmame, e foi realizada a remoção completa do intestino delgado. A expressão do gene foi avaliada por qPCR. O desenho estatístico empregado foi de bloco ao acaso num fatorial de 2x4. **Resultados:** a dieta basal (sem LPS adicionado) mostrou aumento da expressão de mRNA ($p < 0,01$) de todas as citocinas no jejuno para cada dia após o desmame, sugerindo uma resposta inflamatória e grande danos teciduais no porco após o desmame precoce. Na dieta 1 (com um consumo de 0,3 mg de LPS / mg dieta) citocinas TNF- α , IL-8 e IL-18 mostraram aumentos significativos nos níveis de expressão ($p < 0,01$). Todas as citocinas mostraram aumento significativo ($p < 0,01$) no jejuno, para cada um dos dias após o desmame. **Conclusão:** o aumento observado na expressão de TNF- α pode estar envolvido no desenvolvimento de diarréia pós-desmame.

Palavras chave: desmame, imunologia intestinal, RT-PCR, TNF- α , vilosidades intestinais.

Introduction

The intestinal epithelium plays an active role in defending the body against infections. It serves as a dynamic barrier, regulating the uptake of nutrients and water, and preventing the absorption of bacteria and endotoxins normally found in the intestinal lumen (Pie *et al.*, 2004). Intestinal epithelial cells can activate immune and inflammatory responses by producing cytokines—peptides that are crucial for the recruitment and activation of neutrophils, macrophages, T and B cells and dendritic cells (Pie *et al.*, 2004). Some cytokines—such as interleukin 8 (IL-8), IL-18, and tumor necrosis factor (TNF- α)—are constitutively expressed by intestinal epithelial cells under normal physiological conditions. However, they are abundantly over-expressed in response to microbial infections (Stadnyk, 2002; Gopal *et al.*, 2008) and during various physiological processes triggered by the weaning (Pie *et al.*, 2004; Garriga *et al.*, 2005).

Early weaning is considered to have a negative impact on pig performance (Gomez *et al.*, 2008). Immediately after weaning, pigs enter a fasting period that leads to a reduction of gastrointestinal tract lactobacilli, thus increasing *E. coli* populations, which in turn release pro-inflammatory lipopolysaccharide (LPS) from their cell walls (Amador *et al.*, 2007). The LPS are recognized by specific receptors on the cell membrane and activate various cellular signaling pathways (Chow *et al.*, 1995).

Once initiated, this cascade of reactions can lead to a septic state and to an unregulated systemic response that may progress to failure of multiple organs. A large number of cytokines have been implicated in such adverse effects, including TNF- α and interleukin (IL) (Asaduzzaman *et al.*, 2008). The release of these pro-inflammatory cytokines causes changes in the structure and functional capacity of the intestine (García-Herrera *et al.*, 2008).

The administration of LPS from *E. coli* is one of the most used models to study acute infection processes, because LPS response is highly reproducible, without the side effects commonly

associated with chronic infections (Albin *et al.*, 2007). This study aimed to evaluate the effect of oral administration of LPS from *E. coli* on gene expression of pro-inflammatory cytokines IL-8, IL-18 and TNF- α in weaned pigs.

Materials and Methods

Ethical considerations

All experimental procedures were conducted according to the guidelines suggested by the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985). This research was approved by the Ethics Committee for Animal Research of the Universidad Nacional de Colombia, Medellín (CEMED 001 Act of January 26, 2009).

Location

The animal work was conducted at the San Pablo Center of the Universidad Nacional de Colombia, Medellín, located in Rio Negro, Antioquia, at 2,100 meters of altitude, corresponding to tropical lower-montane wet forest (LM-wf) zone, with average annual temperatures between 12 and 18 °C.

Animals and diet

A total of 32 piglets were used; animals were a Duroc x Landrace crossbred and, each was weaned exactly at 21 days of age, weighing 6.5 ± 0.5 kg. The pigs were housed in collective cages (four animals per cage) located in a room with controlled temperature (26 ± 3 °C). The animals were supplied water *ad libitum* throughout the experiment. The basal diet (BD) offered contained milk and milk derivatives, and was enriched with vitamins, minerals, and lysine-HCL. The diet was balanced to meet all the minimum nutritional requirements proposed by NRC (1998) (Tables 1 and 2). Two experimental diets were evaluated. The control diet (basal) was compared against the same diet added with LPS of *E. coli* in concentrations of 0.3 $\mu\text{g}/\text{mg}$ of feed. Each pen was offered 300 g feed/day. However, extra feed was offered when pigs required it. The experimental diets were offered from days 1 to 10 after weaning. No solid food was offered to the piglets during lactation.

Table 1. Composition of the basal diet.

Ingredients	%
Powdered milk	59.0
Casein	6.05
Dairylac 80 (lactose) ^A	15.0
Proliant 1000 (whey) ^B	8.0
Hemoglobin	2.5
Corn starch	4.32
Palm oil	2.363
Sea salt	0.203
Mono-dicalcium phosphate	0.314
Common salt	0.4
Lysine	0.439
Methionine	0.326
Threonine	0.279
Tryptophan	0.061
Toxins adsorbent ^C	0.05
Vitamins ^D	0.36
Minerals ^E	0.12
Flavors ^F	0.217

^A Dairylac 80 (Pro-Ag Products Ltd., Winnipeg, Canada)

^B Proliant 1000 (Alitecno S.A.C., Lima, Peru)

^C Toxibond (Biomix, Medellín, Colombia)

^D Composition per kg of food: vitamin A 1020 IU; vitamin D 198 IU; vitamin E 6 IU; vitamin K 1.20 mg; riboflavin 7.20 mg; vitamin B₁₂ 0.04 mg; coline 968.58 mg; niacin 36 mg; pantothenic acid 16.55 mg; thiamine 30 mg; pyridoxine 31 mg; biotin 0.08 mg; folic acid 0.75 mg.

^E Composition per kg of food: copper 14.40 mg; iron 120 mg; manganese 36 mg; selenium 0.30 mg; iodine 0.96 mg; zinc 144 mg.

^F Sweet Vanilla, fruit essence (Prodia, Medellín, Colombia).

Table 2. Proximal analysis of the basal diet.

Crude Protein (%)	21.0
Ethereal Extract (%)	8.35
Ashes (%)	5.42
Humidity (%)	7.215
Gross energy (Kcal/kg)	3,708.0

Treatments

Two experimental diets were tested: a basal diet (BD) and the BD added with LPS of *E. coli* (serotype 0111: B4; Sigma-Aldrich, St. Louis, MO, USA) as follows:

1. BD: Basal Diet (BD); no addition of LPS from *E. coli*.
2. BD+LPS: BD added with 0.3 µg of LPS from *E. coli*/mg of feed.

Clinical evaluation

Before starting the experiment, all animals were clinically evaluated and laboratory monitored to avoid including previously sick or diarrheic pigs. Such monitoring was also conducted three times daily throughout the study, keeping records of any health alteration. Rectal temperature was measured daily at 8:00 am using a rectal mercury thermometer. The reference temperature was 38 °C. Consistency of the stools was daily assessed by direct observation, assigning them a number from 0 to 3 according with fecal appearance: A 0 value indicates normal feces, and therefore no diarrhea presence. A value of 1 describes a slightly pasty diarrhea, 2 value indicates a moderate, semi-liquid diarrhea, and 3 value severe, very liquid diarrhea. Daily grades during the experimental period were added together and an index of diarrhea severity was estimated (Reis de Souza *et al.*, 2010). This index is expressed by the following equation:

$$DSI = dFC/Ep$$

Where:

DSI= Diarrhea severity index.

dFC= Daily fecal consistency grade.

Ep= Experimental period (days).

Sampling of intestinal tissue

The pigs were slaughtered as follows: eight pigs were slaughtered on the weaning day to check the overall health status of the organs before LPS consumption. Two more pigs from each treatment were slaughtered on days 5, 7, and 10 after weaning. All piglets were slaughtered 2.5 hours after their last meal. Pigs were sedated via CO₂ inhalation for 3 minutes before being humanely killed by exsanguination through sectioning of the jugular vein. The small intestine was removed from the pyloric junction to the ileocecal valve. The mesentery was longitudinally cut with blunt scissors. Then, the intestine was weighed, measured and lined up without any strain over a table, and divided into three segments (duodenum, jejunum, and ileum) of equal size. 20 cm were cut from the center of each segment, and the digesta was removed by rinsing it with cold saline infusion (Makkink *et al.*, 1994; Reis *et*

al., 2005). Subsamples (1 cm) were then obtained from each segment and were prepared for total RNA extraction. Samples were preserved in liquid nitrogen and RNA was stored in a freezer at -70 °C until laboratory analyses.

RNA extraction

Total RNA was extracted from intestinal samples (25 mg) using a commercial kit (Tissue & Cells ULTRACLEAN™ RNA Isolation Kit, MO, USA; BIO Laboratories Inc., San Diego, CA, USA). The tissue was macerated in stone mortars using liquid nitrogen. Work surfaces were previously treated with diethylpyrocarbonate (DEPC) to remove possible RNases. The RNA samples were kept stored at -70 °C throughout the experiment and only aliquots on ice were taken for analysis. Integrity of the extracted material was evaluated running the samples on 1% agarose gel with ethidium bromide staining (100V for 50 min). Ribosomal RNA (rRNA) bands were observed, along with the presence of messenger RNA (mRNA). The extraction was repeated under identical conditions when enough amount of material was not observed, or not clear rRNA bands were detected.

RNA quantification

Purified samples of total RNA were quantified using 1 µL of the solution in a Nanodrop 1000 (Thermo Fisher Scientific Inc, Waltham, MA, USA). It was assessed at a rate of 260/230 in order to detect the presence of any potential pollutants. The solution in which RNA was stored during the last extraction step was used as a blank.

Complementary DNA (cDNA) synthesis

The cDNA was synthesized from total RNA using a commercial kit (QuantiTect® Reverse Transcription Kit; QIAGEN, Valencia, CA, USA). This kit uses random hexamer and poly-thymidine primers to maximize the retro-transcription reaction, obtaining both rRNA and mRNA. The same amount of sample was used for all reverse transcription reactions (1 µg of RNA in each reaction) based on the quantification results. The remaining genomic DNA was removed followed by the addition of enzyme and triphosphate nucleotides to synthesize cDNA according to manufacturer's recommendations. The obtained cDNA was stored at -20 °C.

Cytokine mRNA quantification

The primers for the genes of interest and constitutive expression of rRNA are shown in table 3. These primers were taken from the literature, verifying that its design had been carried out at the edges intron/exon to avoid amplification from genomic DNA. Additionally, they underwent several bioinformatic analyses to verify their specificity with the mRNA or rRNA fragment studied and the absence of secondary structures, such as the formation of lenses or dimers with themselves or with the other primer. Additionally, primers with T_m above 60 °C were used to amplify fragments with less than 150 base pairs, as several researchers have recommended (Bustin, 2000; Kubista et al., 2006).

Table 3. Primers for Real-time PCR.

Target gene		Sequence	T _m Primer	T _m amplicon	Source
28s rRNA	Forward	GCTCCACGGGAGGTTTCTG	63 °C	82.3 °C*	[1]
	Reverse	GGTACACCTGTCAAACGGTAACG	63 °C		
TNF- α	Forward	AAGGACTCAGATCATCGTCTCAAAC	62 °C	81.4 °C*	[1]
	Reverse	CGGCTTTGACATTGGCTACA	62 °C		
IL-8	Forward	TGGACCAGAGCCAGAGAGACTAG	62 °C	80.8 °C*	[2]
	Reverse	AAACTGCCAAGAAGGCAACAG	62 °C		
IL-18	Forward	TCCTTTTCATTAACCAGGGACATC	62 °C	79.2 °C*	[1]
	Reverse	GGTCTGAGGTGCATTATCTGAACA	62 °C		

*Obtained in the experiment. [1]Paulin et al., 2007; [2]Allen et al., 2001.

A commercial kit was used for the qPCR (QuantiFast™ SYBR® Green PCR kit, QIAGEN, Valencia, CA, USA) in a Smart Cycler® (Cepheid Inc., Sunnyvale, CA, USA). All qPCR reactions were run with 0.1 µM of each primer and 1 µL of cDNA, starting with a five-minute activation of the enzyme and then 40 cycles with two stages. The first stage was held at 95 °C for 15 seconds. The second stage was held at 60 °C for 30 seconds (62 °C for 28s rRNA). A denaturation curve was generated at the conclusion of the run to find the T_m of the amplified fragment and confirm its identity.

Standardization strategy, data management and relative quantification

A relative quantification method was used to determine the level of expression of each gene. The method included the use of a constitutively expressed gene (28s ribosomal RNA) following the recommendations of several researchers regarding the choice of gene and overall standardization strategy (Dheda *et al.*, 2005; Huggett *et al.*, 2005; Muller *et al.*, 2002; Vandesompele *et al.*, 2002). The amounts of material from which RNA was extracted, the amount of RNA in reverse transcription reaction, and the amount of cDNA for PCR amplification were identical for all samples. The chosen constitutive expression gene was validated in previous studies under similar experimental conditions (Paulin *et al.*, 2007). The results were analyzed with the assumption-free method, which employs fluorescence data for each qPCR reaction to establish the reaction zone in which there was exponential kinetics (Ramakers *et al.*, 2003). Based on this region, a linear regression was established that fit the data thereby obtaining the reaction efficiency from the slope of the line and the intercept (N_0) as an estimate of the initial concentration in terms of fluorescence of SYBR Green. The software recommended by the equipment manufacturer was used for these calculations (LinRegPCR 11.0). The N_0 values of each cytokine gene were expressed in arbitrary fluorescence units and were normalized using the corresponding N_0 value for the constitutive expression gene in each animal.

Statistical design

The experiment followed a randomized block design (two blocks) in a 2 x 4 factorial arrangement (two experimental diets by four periods after weaning; Steel and Torrie, 1985). Animals were blocked by initial weight. Each animal was assigned one of eight treatments and each treatment had four replicates. Statistical analysis was conducted using the General Linear Model procedure (GLM) of SAS (2006). Differences between treatment means were determined by least squares and analyzed by ANOVA. The Duncan test was used to compare treatment means ($p < 0.05$).

Results

Pigs fed the basal diet maintained normal body temperature, while animals that received LPS had rectal temperatures above 38 °C throughout the experiment. However, pigs fed LPS did not show symptoms of disease, so none required slaughter or exclusion from the experiment. No leftovers were observed during the experiment.

Both treatments had increasing numbers of diarrhea cases during the first 5 days after weaning (Figure 1). Beginning on day 5, diarrhea incidence started to decline in BD, reaching its lowest level on day 10 after weaning. The opposite occurred in pigs exposed to LPS (BD+LPS), in which diarrhea continued to increase from the fifth day, rapidly reaching its peak at day 7, and remaining high until the end of the test (day 10). The incidence of diarrhea was much higher in BD+LPS compared with BD, particularly from day 5 until day 10 after weaning (Figure 1).

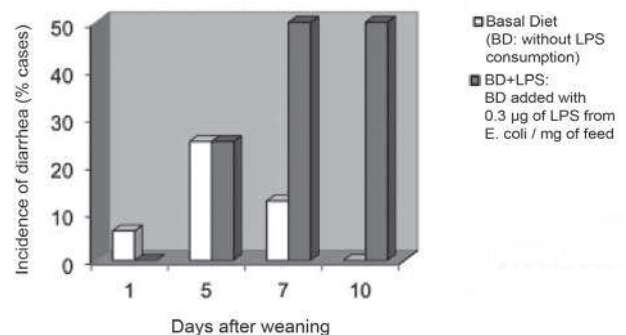


Figure 1. Diarrhea incidence in weaned pigs during 10 days ingesting LPS compared with pigs not consuming LPS.

An increase in mRNA expression of cytokines TNF- α , IL-8, and IL-18 was observed from days 1 to 5 after weaning ($p < 0.01$; Table 4). All variables under study showed a partial recovery from days 5 to 10. However, a statistical difference was observed between days 1 and 10 ($p < 0.01$).

Table 4. Mean expression levels of cytokine mRNA in intestine of weaned pigs not consuming LPS from *E. coli*, as post weaning days increase (effect of weaning on BD).

Cytokine	Day after weaning				SEM
	1	5	7	10	
TNF	275,258 ^a	948,802 ^b	652,465 ^c	353,665 ^d	28,341
IL-8	3,380 ^a	25,554 ^b	12,009 ^c	10,458 ^c	1,075
IL-18	6,180 ^a	33,600 ^b	14,551 ^c	12,185 ^c	2,014

^{abcd} Means with a different superscript within the same row are statistically different ($p < 0.01$). SEM: Standard error of the mean.

The expression of these cytokines in different parts of the small intestine of pigs not consuming LPS was also assessed. Significant differences ($p < 0.01$) were found in transcription levels of cytokines for selected intestinal segments. The middle part of the intestine (jejunum) showed the highest gene expression for all cytokines (Table 5).

Table 5. Expression levels of cytokine mRNA in intestinal segments of weaned pigs not consuming LPS from *E. coli* (effect of weaning on BD).

Cytokine	Cytokine expression			SEM
	Duodenum	Jejunum	Ileum	
TNF- α	1,114,148 ^a	1,473,336 ^b	1,301,145 ^c	28,341
IL-8	10,212 ^a	39,829 ^b	19,424 ^c	1,075
IL-18	30,199 ^a	68,782 ^b	56,926 ^c	2,014

^{abc} Means with a different superscript within the same row are statistically different ($p < 0.01$). SEM: Standard error of the mean.

No statistical interaction was observed between LPS concentration and post-weaning period of slaughtering for any of the variables under the present study, therefore it was unnecessary to analyze those two factors independently.

Tables 6, 7 and 8 show the overall mRNA cytokine expression in both treatments, cytokine expression according to the length of exposure to LPS, and the expression in the intestinal segments of pigs consuming LPS, respectively. The expression of cytokines TNF- α , IL-8, and IL-18 was significantly higher for BD+LPS ($p < 0.01$; Table 6).

Intestinal production of TNF- α , IL-8 and IL-18, as measured by mRNA expression, significantly raised with increasing exposure to LPS ($p < 0.01$), reaching its peak on day 10 post-weaning (Table 7).

Table 6. Expression levels of cytokine mRNA in small intestine of weaned pigs with and without consumption of LPS from *E. coli*.

Cytokine	Treatment		
	BD	BD+LPS	SEM
TNF- α	557,547 ^a	5,138,613 ^b	328,796
IL-8	10,600 ^a	182,651 ^b	6,359
IL-18	16,629 ^a	55,683 ^b	2,974

BD: Basal diet (BD); BD+LPS: BD added with 0.3 μ g LPS from *E. coli* mg feed. ^{ab} Means with a different superscript within the same row are statistically different ($p < 0.01$). SEM: Standard error of the mean.

Table 7. Expression levels of cytokine mRNA in small intestine of weaned pigs consuming LPS (0.3 μ g LPS from *E. coli*/mg feed).

Cytokine	Post weaning period				SEM
	1	5	7	10	
TNF- α	275,258 ^a	1,548,803 ^b	5,536,653 ^c	9,524,654 ^d	328,796
IL-8	3,380 ^a	31,554 ^b	78,009 ^c	258,944 ^d	6,359
IL-18	6,180 ^a	46,180 ^b	53,600 ^b	140,551 ^c	2,974

^{abcd} Means with a different superscript within the same row are statistically different ($p < 0.01$). SEM: Standard error of the mean.

The middle intestine (jejunum) of piglets consuming LPS showed higher cytokines production, particularly TNF- α , whereas duodenum had the lowest production (Table 8). However, there were no statistically significant differences between jejunum and ileum for the different variables studied ($p > 0.01$).

Table 8. Expression levels of cytokine mRNA in the three sections of small intestine of weaned pigs consuming LPS (0.3 μ g LPS from *E. coli* /mg feed).

Cytokine	Intestinal Section			SEM
	Duodenum	Jejunum	Ileum	
TNF- α	1,897,744 ^a	5,093,345 ^b	4,924,750 ^b	328,796
IL-8	35,523 ^a	179,956 ^b	167,473 ^b	6,359
IL-18	62,536 ^a	126,158 ^b	118,355 ^b	2,974

^{abc} Means with a different superscript within the same row are statistically different ($p < 0.01$). SEM: Standard error of the mean.

Discussion

Literature is scarce on reports evaluating gene expression of proinflammatory cytokines in weaned piglets. However, *in vivo* and *in vitro* studies have

shown that after a bacterial infection in pigs, LPS has the ability to activate a variety of signaling pathways through the uncontrolled synthesis of proinflammatory cytokines such as IL-8, IL-18 and TNF- α (Johnson *et al.*, 2005), which can influence the integrity and functional capacity of intestinal cells, permeability to macromolecules and transport of ions and nutrients (Bauer *et al.*, 2006; Liu *et al.*, 2008). Total, efficient, and proper intestinal recovery may take several weeks (Yoo *et al.*, 2000; García-Herrera *et al.*, 2008).

Proinflammatory cytokines play an important role in intestinal physiology and may act on the intestinal barrier increasing epithelial ion secretion, specifically chloride (Cl⁻). The increased secretion of Cl⁻ in the crypts and decreased absorption of sodium (Na⁺) by the intestinal villi promote fluid loss in the small intestine. Both processes depend on the Na⁺ / K⁺-ATPase, located in the basolateral membrane and whose functional activity is affected by TNF- α (McKay and Baird, 1999; Pie *et al.*, 2004). Gene expression of TNF- α decreases the Na⁺ / K⁺-ATPase activity (Yu and Perdue, 2000) causing fluid accumulation in the small intestine. These factors combined appear to trigger bacterial endotoxin induced diarrhea in rabbits and pigs (García-Herrera *et al.*, 2003, 2008).

The increased expression of TNF- α observed in this study may contribute to the development of post-weaning diarrhea; this factor stimulates Cl⁻ secretion in the ileum (Musch *et al.*, 2002; Bertelsen *et al.*, 2004) and colon (Schmitz *et al.*, 1996).

In addition to its role in epithelial ion transport, proinflammatory cytokines are also involved in the integrity of the intestinal tissues (Williams, 2001), altering intestinal permeability through its effect on the structure of tight junctions between epithelial cells (McKay and Baird, 1999; Bruewer *et al.*, 2003; Ma *et al.*, 2004; Wang *et al.*, 2005), mainly at the jejunum (Spreeuwenberg *et al.*, 2001; Pie *et al.*, 2004; Lallès *et al.*, 2004). The LPS released into the gut cause the shortening of actin filaments in the enterocyte, disrupting the structure of tight junctions and causing the internalization of occludin. Under these circumstances, the intestinal

barrier may be affected, decreasing transepithelial resistance (Berkes *et al.*, 2003) and thus increasing indiscriminate paracellular transport of molecules, allowing gastrointestinal pathogens to access the systemic circulation (García-Herrera *et al.*, 2003; Pitman and Blumberg, 2000).

At the time of weaning, over-expressed mRNA of proinflammatory cytokines, especially TNF- α , may influence the integrity of intestinal epithelium, favoring the emergence of bacterial infections and diarrhea and increasing the transepithelial transport of microorganisms. In the presence of inflammatory processes, including those caused by bacterial infections, proinflammatory cytokines have been implicated in increasing apoptosis rates in intestinal epithelium (Ramachandran *et al.*, 2000), eliminating and controlling the number of cells in the proliferation area of intestinal crypts (Potten, 1992). Furthermore, TNF- α and IL-8 have mitotic effects on intestinal mucosa (Zachrisson *et al.*, 2001; Schaeffer *et al.*, 2006), causing the conversion of intestinal stem cells into secreting cells, thus decreasing the number of absorptive cells in the intestinal mucosa (Van *et al.*, 2005). This can lead to high malnutrition and mortality of pigs at this stage.

The immune response in the gastrointestinal tract is a constant balance between proinflammatory cytokine (IL-6, IL-8, IL-18, IL-12, TNF- α) and inflammatory cytokine release (IL-1RA, IL-4, IL-10). Under chronic inflammatory conditions, T helper type 1 lymphocytes (LTh1) induce the release of significant levels of proinflammatory cytokines, such as IL-12, IL-18 and TNF- α (Pizarro *et al.*, 1999; Reuter and Pizarro, 2004; Cavani *et al.*, 2000) and, in turn, suppress IL-10 by LTh2 production. Because of this, the activation of antigen-presenting cells, dendritic cell maturation, and intolerance to specific antigens are all increased, and the proliferation of T cells is limited (Enk *et al.*, 1993; Caux *et al.*, 1994). Inhibition of T cells leads to increased chloride secretion, and therefore to the onset of diarrhea (Xu and Verstraete, 2001). The IL-10 is a key molecule for the induction of T cells and the prevention of intestinal inflammation (León *et al.*, 2006).

The differential expression of proinflammatory cytokines in different parts of the intestinal tract of pigs occurs in response to intestinal disorders caused by stress and intestinal infections associated with the weaning (Oswald *et al.*, 2001; Fournout *et al.*, 2000). These inflammatory responses may reflect, at least in part, anatomical and functional differences between sections of the intestine (Beagley *et al.*, 1995). However, the anatomical changes that occur at weaning can be observed regardless of the weaning age, though its size may vary (Cera *et al.*, 1988; Pluske *et al.*, 2003).

The data from this study suggest that weaning is associated with increased intestinal inflammation, mainly caused by the expression of proinflammatory cytokines. An increase in proinflammatory cytokine expression was observed until day 5 after weaning. However, a decrease in the expression of these cytokines was noted after the fifth post-weaning day, without returning to the values found on day 1 after weaning. Therefore, we found a clear relationship between the expression of proinflammatory cytokines (TNF- α) and the incidence of diarrhea during the weaning stage.

Animals consuming BD+LPS presented an excessive inflammatory response due to the over-expression of proinflammatory cytokines. The mRNA expression of TNF- α , IL-8, and IL-18 increased by 922%, 1723%, and 335%, respectively, compared to BD, which coincides with the high rates of diarrhea observed during the post-weaning period. Considering the advantages of this inflammation model, it is necessary to conduct more studies in order to confirm the validity of the results observed.

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