

***E. coli* Lipopolysaccharide Decreases the Expression of Proteins of Tight Junctions in the Jejunum of Weaning Piglets**

Lipopolisacárido de *E. coli* Disminuye la Expresión de Proteínas de Uniones Apretadas en Yeyuno de Lechones Posdestete

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Abstract. In order to assess the effect of the addition of *E. coli* lipopolysaccharide (LPS) on the expression of tight junction proteins in the jejunum of weaned piglets (the animals were weaned at 21-days-old), 64 piglets were slaughtered at 1, 5, 7 and 10 days postweaning and complete extraction of jejunum was carried out in order to evaluate the molecular expression of Claudine 3 (C3), Claudine 4 (C4), and Zonula Occludens-1 (ZO-1). To induce intestinal inflammation, animals were fed a basal diet supplemented with four levels of *E. coli* LPS (0, 0.3, 0.5, and 1.0 $\mu\text{g mg}^{-1}$ of feed). The statistical design consisted of randomized blocks in a 4X4 factorial arrangement (four LPS concentrations and four post-weaning periods). A decrease was observed ($P < 0.01$) in the expression of C3, C4, and ZO-1 in the animals that were fed the diet with the highest LPS-inclusion level. LPS contributes to the appearance of anatomical and intestinal functional disorders which are denoted by a decrease in the molecular expression of proteins from the intestinal barrier. This, in turn, is associated with an indiscriminate paracellular transport of molecules, microorganisms, and toxic compounds into the circulatory system. This results in post-weaning diarrhea and a decrease in the productive efficiency of piglets.

Key words: Diarrhea, fever, digestive functions, gastrointestinal pathophysiology.

Resumen. Con el objetivo de evaluar el efecto de la adición de lipopolisacárido LPS de *E. coli* sobre la expresión de proteínas de las uniones apretadas en yeyuno de lechones destetos, se sacrificaron 64 lechones escalonadamente los días 1 (21 días de edad, día del destete), 5, 7 y 10 posdestete, y se les extrajo completamente el yeyuno para la evaluación de la expresión molecular de Claudina 3 (C3), Claudina 4 (C4), y Zonula Occludens-1 (ZO-1). Para inducir la inflamación intestinal los animales fueron alimentados con una dieta basal, adicionada con cuatro niveles de LPS de *E. coli* (0; 0,3; 0,5 y 1,0 $\mu\text{g mg}^{-1}$ de alimento). El diseño estadístico utilizado fue de bloques al azar en arreglo factorial de 4X4 (cuatro concentraciones de LPS en cuatro tiempos posdestete). Se observó la disminución ($P < 0,01$) en la expresión de C3, C4 y ZO-1 en los animales que consumieron la dieta con mayor nivel de inclusión de LPS. El LPS contribuye a desordenes anatómicos y funcionales a nivel intestinal, representados por la disminución en la expresión molecular de proteínas de la barrera intestinal, lo que está asociado al transporte paracelular indiscriminado de moléculas, microorganismos y compuestos tóxicos hacia la circulación sistémica. Todo lo anterior conlleva la presentación de diarreas posdestete y a la disminución en la eficiencia productiva de los lechones.

Palabras clave: Diarreas, fiebre, funciones digestivas, fisiopatología gastrointestinal.

In the countries with the higher levels of pig production, piglets are weaned 14 to 28 days after being born (Marion *et al.*, 2002). As a result, piglets are lighter and the digestive system is less developed during weaning, particularly the gut. This renders these animals more susceptible to digestive problems in the post-weaning period (Reis *et al.*, 2007).

Besides its digestive functions, the gut of piglets forms a physical barrier that prevents toxic compounds and pathogens from entering the intestinal mucosa and the circulatory system. This barrier is mainly composed of tight junctions, which are multiprotein complexes that bind and seal the spaces between adjacent enterocytes and limit the paracellular movement of

molecules and luminal bacteria within the mucosa of the tissues (Lamb-Rosteki *et al.*, 2008). Junctions are formed by the proteins: Zonula Occludens 1 (ZO-1) and two Claudines (C3 and C4), which are connected to the epithelial cells (Tlaskalová-Hogenová *et al.*, 2004), act as a structure, and regulate the permeability of the tight junctions (McLaughlin *et al.*, 2004).

Similarly, tight junctions participate in the preservation of cellular polarity and are considered to play a key role in intestinal diffusion mechanisms (Shen and Turner, 2005). However, diffusion mechanisms may be dramatically altered by the physiological processes triggered by weaning and the presence of microbial infections (Fan, 2002).

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During the weaning period, there is a brief period of fasting resulting from the forced removal of milk and the subsequent provision of solid food. This may cause a decrease in nutrient absorption and an increase in the substrate available for enteropathogenic bacteria in the hindgut (Pluske *et al.*, 2003). Consequently, weaning is associated with a dramatic change in the composition of the intestinal microbiota (Chow *et al.*, 2010).

In modern production systems, early weaning causes the lactobacilli population to disappear from the gastrointestinal tract and favors an increase in the *E. coli* population, releasing proinflammatory substances such as lipopolysaccharides (LPS) from the cell walls (Amador *et al.*, 2007). LPS causes physical damage in the gut by activating a coordinated series of cell signaling pathways which favor an inflammatory response, which in turn produces important changes in the structure and functional capacity of the gut (García *et al.*, 2008). These intestinal changes are characterized by an alteration in the functions of the tight junctions as well as by an increase in the indiscriminate paracellular transport of molecules (Pitman and Blumberg, 2000; García *et al.*, 2003).

The administration of *E. coli* LPS is one of the most used models to study acute infection processes because the LPS response is highly reproducible, without the side effects commonly associated with chronic infections. Therefore, the aim of this study was to determine the effect of *E. coli* LPS administration on the molecular expression of tight junction proteins in the jejunum of piglets at different post-weaning periods.

MATERIALS AND METHODS

Ethical considerations. All experimental procedures were conducted according to guidelines suggested by "The International Guiding Principles for Biomedical Research Involving Animals" (CIOMS, 1985), and approved by the Ethics Committee on Animal Experimentation of the Universidad Nacional de Colombia (Medellín; CEMED 001 from January 26, 2009).

Location. The fieldwork was conducted at the San Pablo Center of the Universidad Nacional de Colombia, located in Rionegro, Antioquia, at an altitude of 2,100 m, corresponding to a tropical lower-montane wet forest (LM-wf) zone, with average annual temperatures between 12 and 18 °C.

Animals. A total of 52 pigs were used. The pigs were group-penned in nursery crates with trough-type feeders in a temperature-controlled room (26 ± 3 °C).

Diets. Four experimental diets were evaluated: a control diet (basal diet) and three others containing *E. coli* LPS, serotype O111: B4 (Sigma-Aldrich™, St Louis, MO, USA), as follows:

- Basal Diet (BD) 0.0 µg LPS mg⁻¹ feed.
- Diet 1 (D1): BD plus 0.3 µg LPS mg⁻¹ feed.
- Diet 2 (D2): BD plus 0.5 µg LPS mg⁻¹ feed.
- Diet 3 (D3): BD plus 1.0 µg LPS mg⁻¹ feed.

The basal diet consisted of powdered milk and milk byproducts and was supplemented with vitamins, minerals, and HCL-lysine, meeting all minimum nutritional requirements proposed by the NRC (2012) (Table 1). The pigs were offered a minimum of 3000 g feed/day per crate, additional feed was provided when required. The diets were administered from days 1 to 10 after weaning. Water was provided *ad-libitum* throughout the experiment. No solid feed was offered to the piglets during lactation.

Small intestine sampling. The pigs were sequentially slaughtered as follows:

Four pigs were slaughtered on day 1 (weaning day), representing the control group. This group was used to assess the overall health status and to evaluate the macroscopic appearance of the organs before the pigs received LPS. Four pigs of each LPS-inclusion level (0, 0.3, 0.5, and 1.0 µg LPS mg⁻¹ feed) were slaughtered on days five, seven, and 10 after weaning. All animals were slaughtered 2.5 hours after their last meal and were previously sedated by carbon dioxide inhalation for three minutes. The pigs were then slaughtered by exsanguination through a section of the jugular vein. After the slaughter, the small intestine was removed from the pyloric junction to the ileocecal valve (Segalés and Domingo, 2003). The intestine was lined up on a table, measured without any tension, divided into three equally sized sections (duodenum, jejunum, and ileum) and a 20 cm sample was taken from the center of the jejunum. The digesta contained in the samples was removed by washing it with cold saline infusion as previously described (Makkink *et al.*, 1994; Reis *et al.*, 2005). Subsamples (1 cm) were then obtained from each segment and were prepared for total RNA extraction. The samples were preserved in liquid nitrogen and the RNA was stored in a freezer at -70 °C.

Table 1. Composition of proximal analysis and the basal diet used in gastrointestinal pathophysiology study of post weaning pigs.

Ingredients	%	Proximal composition	
Powdered Milk	59.0	Crude Protein (%)	21.0
Casein	6.05	Ethereal Extract	8.35
Dairylac 80 (lactose) ^A	15.0	Ash (%)	5.42
Proliant 1000 (whey) ^B	8.0	Humidity (%)	7.215
Hemoglobin	2.5	Gross energy (kcal kg ⁻¹)	3708.0
Corn starch	4.32		
Palm oil	2.363		
Sea salt	0.203		
Monodicalcic phosphate	0.314		
Common salt	0.4		
Lysine	0.439		
Methionin	0.326		
Threonine	0.279		
Tryptophan	0.061		
Toxins ^C adsorbent	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 UI, vitamin D 198 UI, vitamin E 6 UI, 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)

Total RNA extraction and material quality check.

Total RNA was extracted using an ULTRACLEAN™ Tissue and Cells RNA Isolation Kit (MO BIO Laboratories Inc., San Diego, CA, USA). Samples (25 mg) were broken down in a mortar using liquid nitrogen. The RNA was stored at -80 °C and only aliquoted on ice for analysis. The integrity of the extracted material was assessed by running the samples on agarose gel with 1% ethidium bromide (100V for 50 min) and observing the rRNA bands. When not enough of the material or bands was clearly observed, the extraction was repeated under identical conditions (Freeman *et al.*, 1999).

Synthesis of copy DNA (cDNA) from total RNA. A QuantiTect™ Reverse Transcription Kit (QIAGEN™) was used to synthesize the cDNA from the total RNA

extracted. 1 µg RNA samples were used for all reverse transcription reactions. Genomic DNA debris was removed and enzyme plus triphosphate nucleotides were added to synthesize the cDNA following the manufacturer's recommendations. The obtained cDNA was stored at -20 °C until it was used (Kubista *et al.*, 2006).

RT-PCR from cDNA for ZO-1, C3 and C4 and constitutive expression gene. The primers of the genes of interest and constitutive expression (Cyclophilin) are shown in Table 2. These primers were taken from the literature, verifying that their design had been performed on the intron/exon edges to avoid amplification of genomic DNA. Additionally, the primers were evaluated through bio-informatic analysis to verify their specificity

with the studied mRNA and rRNA fragment and the absence of secondary structures, such as lenses or dimer formations, between them or with the other

primers. The primers were chosen to amplify the less than 150 base pairs fragments, as recommended by some researchers (Bustin, 2000; Kubista *et al.*, 2006).

Table 2. Primer sequences and references used in the gastrointestinal physiopathology study of post weaning pigs.

Gene	Sequence	Alignment T°	Reference
CyclophilinF	5'-GCTCCACGGGAGGTTTCTG-3'	58 °C	Paulin <i>et al.</i> , 2007
Cyclophilin R	5'-GGTACACCTGTCAAACGGTAACG-3'		
*C3 F	5'-GATGCAGTGCAAAGTGACGA-3'	58 °C	Mariani <i>et al.</i> , 2009
C3 R	5'-GTCCTGCACGCAGTTGGT-3'		
*C4 F	5'-TATCATCCTGGCCGTGCTA-3'	55 °C	Mariani <i>et al.</i> , 2009
C4 R	5'-CATCATCCACGCAGTTGGT-3'		
**ZO-1 F	5'-ACCCACAAACCCACCAA -3'	56.8 °C	Xu <i>et al.</i> , 2011
ZO-1 R	5'-CCATCTCTTGCTGCCAAACTATC-3'		

*Claudine 3; Claudine 4; **Zonula Occludens-1

Standardization strategy, data processing, and relative quantification. To determine the expression level of each gene, a relative quantification method using a constitutively expressed gene (Cyclophilin) was used, following recommendations from several reports on gene choice and overall normalization strategy (Muller *et al.*, 2002; Vandesompele *et al.*, 2002; Huggett *et al.*, 2005). The amount of material from which the RNA was extracted, the RNA amount in the reverse transcription reaction, and the amount of cDNA for PCR amplification were identical for all samples. The chosen constitutively expressed gene was validated in previous trials under similar experimental conditions (Paulin *et al.*, 2007). The results were analyzed using the ImageJ software, version 1.43.

mRNA expression of intestinal ZO-1, C3 and C4 using RTPCR and quantification of PCR products. The mRNA amplification of C3, C4 and ZO-1 was conducted on individual samples of the middle intestine segment (Petersen *et al.*, 2001). The general protocol for PCR was: Cyclophilin: 95 °C x 1 min; 37 cycles 95 °C x 30 seg; 58 °C x 30 seg; 72 °C x 40 seg; 72 °C x 3 min; 10 °C x ∞; C3: 95 °C x 1 min; 39 cycles 95 °C x 30 seg; 58 °C x 30 seg; 72 °C x 40 seg; 72 °C x 3 min; 10 °C x ∞; C4: 95 °C x 1 min; 37 cycles 95 °C x 30 seg; 55 °C x 30 seg; 72 °C x 40 seg; 72 °C x 3 min; 10 °C x ∞; ZO-1: 95 °C x 1 min; 39 cycles 95 °C x 30 seg; 56.8 °C x 30 seg; 72 °C x 40 seg; 72 °C x 3 min; 10 °C x ∞.

The density of the RT-PCR products from each protein was expressed in units relative to the

band density of the constitutive expression gene (cyclophilin gene).

Statistical design: The experiment was performed as a randomized block design (2 blocks) in a 4x4 factorial arrangement (four experimental diets and four periods after weaning) (Steel and Torrie, 1980). The animals were blocked by initial weight. Each animal was assigned one of 16 treatments and each treatment had four repetitions. The General Linear Model procedure (GLM) of SASTM (2006) was used for the statistical analysis of the data. The Duncan test (P<0.05) was used to separate the treatment means.

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 had to be slaughtered or excluded from the experiment. No food orts were observed during the experiment.

Data on the molecular expression of each of the genes for the studied proteins were obtained from the ratio between the expression of each protein's gene and the expression of the constitutive gene (Cyclophilin). Initially, the values for the molecular expression of the tight junction proteins in the basal diet were compared through the various post-weaning periods in order to determine the exclusive

effect of weaning on the expression of these proteins. Table 3 shows the data obtained with the basal diet (BD) during the various experimental periods for the jejunum.

For C3, C4, and ZO-1, a statistically significant ($P < 0.01$) decrease was observed in the molecular

expression, starting from the first day after weaning (day 1). Similarly, on day 5 after weaning, the animals presented the lowest expression values in terms of cyclophilin (1.34, 1.18, and 0.90 respectively). All studied variables showed partial recovery from days five to ten. However, a statistical difference was observed between days 1 and 10 ($P < 0.01$).

Table 3. Mean molecular expression levels of tight junction proteins in the jejunum of weaned pigs not consuming *E. coli* LPS, as postweaning days increase (effect of weaning).

Variables	Post-weaning periods (days)				Standard mean error
	1	5	7	10	
C3	1.78 a	1.34 b	1.41 b	1.54 c	0.03
C4	1.44 a	1.18 b	1.21 b	1.34 c	0.02
ZO-1	1.37 a	0.90 b	0.99 b	1.19 c	0.05

The means with different superscripts in the same row are statistically significant ($P < 0.01$).

C3: Claudine 3; **C4:** Claudine 4; **ZO-1:** Zonula Ocludens 1.

For tight junction proteins C3, C4, and ZO-1, significant decreases ($P < 0.01$) were observed between the different diets (Table 4). The animals on the D3 diet had the lower molecular expression values (1.12, 0.92, and

0.66 respectively) with respect to BD (1.51, 1.29, and 1.11, respectively). However, it is worth mentioning that there were no statistically significant differences between D1 and D2 ($P > 0.01$).

Table 4. Changes in the molecular expression of tight junction proteins in the jejunum of weaned piglets that ingested various LPS levels after weaning (until the tenth day after weaning).

Variables	Diets				Standard mean error
	BD	D1	D2	D3	
C3	1.51 a	1.32 b	1.23 b	1.12 c	0.03
C4	1.29 a	1.11 b	1.08 b	0.92 c	0.04
ZO-1	1.11 a	0.87 b	0.84 b	0.66 c	0.03

BD: Basal diet without *E. coli* LPS; **D1:** BD plus 0.3 µg of *E. coli* LPS /mg of feed; **D2:** BD plus 0.5 µg of *E. coli* LPS /mg of feed; **D3:** BD plus 1.0 µg of *E. coli* LPS /mg of feed.

The means with different superscripts in the same row are statistically significant ($P < 0.01$).

C3: Claudine 3; **C4:** Claudine 4; **ZO-1:** Zonula Ocludens 1.

Tables 5 and 6 show the general average values of the molecular expression of the studied tight junction proteins for each treatment and for each exposure period. No statistical interaction was observed between the LPS concentrations and postweaning periods for any of the studied variables. Therefore, it was not necessary to analyze or break down said factors independently.

With regard to the molecular expression parameter for the C3, C4, and ZO-1 tight junctions during the LPS exposure periods, the overall evaluation of all the animals (DB, B1, D2, and D3; Table 5) showed a significant decrease ($P < 0.01$) from day one after weaning. Additionally, the animals presented the lowest values at day 10 after weaning (1.04, 0.87, and 0.52 respectively).

Table 5. Changes in the molecular expression of tight junction proteins in the jejunum of weaned piglets exposed to LPS during various periods at different post-weaning periods.

Variables	Post-weaning periods (days)				Standard mean error
	1	5	7	10	
C3	1.78 ^a	1.22 ^b	1.18 ^b	1.04 ^c	0.03
C4	1.44 ^a	1.06 ^b	1.02 ^b	0.87 ^c	0.04
ZO-1	1.37 ^a	0.76 ^b	0.69 ^b	0.52 ^c	0.03

The means with different superscripts in the same row are statistically significant ($P < 0.01$).

C3: Claudine 3; **C4:** Claudine 4; **ZO-1:** Zonula Ocludens 1.

DISCUSSION

The intestinal barrier is formed by many tight junctions where the proteins ZO-1, C3, and C4 are responsible for restricting the paracellular passage of luminal bacteria and molecules within the mucosa of the tissues (McLaughlin *et al.*, 2004). When the intestinal microbiota has an optimal balance, it prevents pathogen or virulent microorganisms from multiplying and adhering to and invading the epithelial cells, as well as the circulatory system (Tlaskalová-Hogenová *et al.*, 2004), which favors the integrity of the intestinal wall. However, under pathophysiological situations or stress, the MLCK pathway is activated, which increases intestinal permeability, solute cotransport, and the presence of proinflammatory cytokines and pathogenic bacteria.

Many cytokines, including TNF- α , are capable of increasing intestinal permeability because they can directly activate the MLCK (myosin regulatory light chain) pathway, thus shortening the actin filaments in the enterocytes, which ultimately breaks the tight union (Chin *et al.*, 2002). A change in this conformation favors the development of pathogenic problems at the intestinal level (Saitou *et al.*, 2000). Since tight junctions are important for maintaining the structural integrity of the epithelial barrier, they are the target of multiple infectious agents (Shen and Turner, 2005). Furthermore, these cytokines increase intestinal permeability by reducing the expression of the genes for proteins, such as ZO-1 and Claudines in the tight junctions, thus causing deep alterations in the normal physiology of enterocytes (Tsukita and Furuse, 2002; Bruewer *et al.*, 2003). The results obtained in this study showed a reduction in the molecular expression of ZO-1, C3, and C4, which may be associated with the post-weaning physiological intestinal inflammation in the piglets fed with the basal diet.

The events in which the MLCK pathway is activated are associated with a decrease in transepithelial resistance (Berkes *et al.*, 2003), an increase in indiscriminate paracellular transport of molecules, and a bacterial invasion of tissues (Shifflett *et al.*, 2005). Thus, the modulation of MLCK activity represents a point of convergence of multiple signaling pathways regulating the function of the tight junctions, thus causing changes in their structure and, in turn, in the proteins that compose said junctions (Yu *et al.*, 2010).

As mentioned above, the decrease in the molecular expression of the tight junction proteins observed in the animals that consumed the BD might be associated with the inflammatory and immune responses produced by different stress manifestations during weaning. These include: abrupt separation from the mother, relocation to new social groups, and changing to solid food (Kojima *et al.*, 2007). In addition, physiological responses to the stress caused by weaning involve complex interactions and responses from the central nervous, endocrine, and immune systems, which influence the health and welfare of the animals in response to environmental and handling conditions (Yang and Glaser, 2000; Davis *et al.*, 2006).

In this study, a decrease in the molecular expression of tight junction proteins was observed in the pigs that were fed with diets containing increasing concentrations of LPS. In the studies conducted by Pié *et al.* (2004), Amador *et al.* (2007), and Parra *et al.* (2013), the addition of LPS to the intestinal medium increased the production of various proinflammatory cytokines, mainly TNF- α , which may activate a wide range of signaling pathways that affect cell turnover and growth by stimulating apoptosis (Bruewer *et al.*, 2003), thus favoring permeability to bacteria and compounds that might strengthen infectious and inflammatory processes (Berkes *et al.*, 2003). In addition,

McLaughlin *et al.* (2004) reported that TNF- α modifies the structure of claudines by weakening the multiprotein complex of the tight junctions, which is crucial for the maintenance of intestinal barrier properties. This is consistent with the results obtained in this study, which showed that LPS decreases the expression of ZO-1, C3, and C4, possibly due to an increase in the production of TNF- α , as reported by Parra *et al.* (2013).

Another alternate mechanism involved in the intestinal alterations resulting from the TNF- α production and from the inflammation caused by LPS (Hataya *et al.*, 2003; Waseem *et al.*, 2008, Parra *et al.*, 2013) is the shortening of the actin filaments in the enterocytes, which makes the structure of the tight junctions permeable to bacteria and compounds that might strengthen infections and inflammations (Berkes *et al.*, 2003). This favors the infiltration of leukocytes, the expression of the gene for proinflammatory cytokines, the overexpression of heat shock proteins, the modification of tissues caused by proteases, and, finally, the appearance of epithelial functional disorders related to absorption, mineral secretion, and intestinal permeability (Lallès *et al.*, 2004).

Studies conducted by Lamb-Rosteki (2008) demonstrated that the integrity and function of the epithelial barrier is interrupted after one hour of exposure to *C. jejuni* in broilers. The interruption of such barrier is represented by the paracellular breaking and the granular accumulation of Claudines. This is consistent with this study's findings since, as the time of exposure to *E. coli* LPS increased, a decrease in the molecular expression of C3, C4, and ZO-1 was observed, as well as a reduction in the integrity and renewal rate of the epithelial barrier's multiprotein complex. On the other hand, exposure to some toxic secondary metabolites produced by fungi (deoxynivalenol) decreases C3 in caco-2 cells, as well as C3 and C4 in IPEC-1 cells, leading to the alteration of the structure of the tight junctions, which are responsible for the paracellular passage of molecules (McLaughlin *et al.*, 2004; Pinton *et al.*, 2009). Therefore, the decrease in the expression of proteins C3, C4, and ZO-1 observed in this study might cause the paracellular movement of compounds and a decrease in cell polarity, which would ultimately favor the appearance of diarrhea (Mush *et al.*, 2006).

CONCLUSIONS

Weaning is associated with multiple environmental and nutritional factors that produce different manifestations of stress, which in turn cause the protein

complexes forming tight junctions to deteriorate, thus decreasing intestinal barrier functioning.

E. coli LPS decreases the expression of tight junction proteins, which might contribute to the development of anatomical and functional disorders in the intestine. Such disorders are characterized by indiscriminate paracellular transport of molecules, microorganisms and toxic compounds into the circulatory system. This results in post-weaning diarrhea and a decrease in the productive efficiency of piglets.

Due to the large anatomical, physiological, and immunological similarities between human beings and pigs, the latter might be an excellent animal model for developing therapeutic strategies in human medicine. Likewise, these results could be a starting point in the search for therapies for Crohn's disease in human beings.

BIBLIOGRAPHY

- Amador, P., J. García, M.C. Marca, J. de la Osada, S. Acín, M.A. Navarro, M.T. Salvador, M.P. Lostao and M.J. Rodríguez-Yoldi. 2007. Intestinal D-galactose transport in an endotoxemia model in the rabbit. *The Journal of Membrane Biology* 215(2-3): 125-133.
- Berkes, J., V.K. Viswanathan, S.D. Savkovic and G. Hecht. 2003. Intestinal epithelial responses to enteric pathogens: effects on tight junction barrier, ion transport, and inflammation. *Gut* 52(3): 439-451.
- Brewer, M., A. Luegering, T. Kucharzik, C.A. Parkos, J.L. Madara, A.M. Hopkins and A. Nusrat. 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *Journal of Immunology* 171(11):6164-72.
- Bustin, S.A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 25(2): 169-193.
- Chin, A.C., D.A. Teoh, K.G. Scott, J.B. Meddings, W.K. Macnaughton and A.G. Buret. 2002. Strain-dependent induction of enterocyte apoptosis by *Giardia lamblia* disrupts epithelial barrier function in a caspase-3-dependent manner. *Infection and Immunity* 70(7): 3673-3680.
- Council for International Organizations of Medical Sciences. (CIOMS). 1985. *International Guiding Principles*

- for Biomedical Research Involving Animals. Geneva. 28 p.
- Chow, J., S.M. Lee, Y. Shen, A. Khosravi and S.K. Mazmanian. 2010. Host-bacterial symbiosis in health and disease. *Advances in Immunology* 107: 243-274.
- Davis, M.E., S.C. Sears, J.K. Apple, C.V. Maxwell and Z.B. Johnson. 2006. Effect of weaning age and commingling after the nursery phase of pigs in a wean-to-finish facility on growth, and humoral and behavioral indicators of well-being. *Journal of Animal Science* 84: 743-756.
- Fan, M.Z. 2002. Growth and ontogeny of the gastrointestinal tract. pp. 31-60. In: Xu, R.J. and P. Cranwell (eds.). *The neonatal pig: gastrointestinal physiology and nutrition*. Nottingham University Press, Nottingham. 360 p.
- Freeman, W.M., S.J. Walker and K.E. Vrana. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 26(1): 112-22.
- García, J., B. Abad and M.J. Rodríguez-Yoldi. 2003. Effect of lipopolysaccharide on D-fructose transport across rabbit jejunum. *Inflammation Research* 52(4): 177-184.
- García, J, M.C. Marca, E. Brot-Laroche, N. Guillén, S. Acin, M.A. Navarro, J. Osada and M.J. Rodríguez-Yoldi. 2008. Protein kinases, TNF- α , and proteasome contribute in the inhibition of fructose intestinal transport by sepsis in vivo. *American Journal of Physiology. Gastrointestinal and Liver Physiology* 294(1): G155-G164.
- Hataya, Y., T. Akamizu, H. Hosoda, N. Kanamoto, K. Moriyama, K. Kangawa, K. Takaya and K. Nakao. 2003. Alterations of plasma ghrelin levels in rats with lipopolysaccharide-induced wasting syndrome and effects of ghrelin treatment on the syndrome. *Endocrinology* 144: 5365-5371.
- Huggett, J., K. Dheda, S. Bustin and A. Zumla. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity* 6(4): 279-84.
- Kojima, C.J., J.A. Carroll, R.L. Matteri, K.J. Touchette and G.L. Allee. 2007. Effects of weaning and weaning weight on neuroendocrine regulators of feed intake in pigs. *Journal of Animal Science* 85(9): 2133-2139.
- Kubista, M., J.M. Andrade, M. Bengtsson, A. Forootan, J. Jonák, K. Lind, R. Sindelka, R. Sjöback, B. Sjögreen, L. Strömbom, A. Ståhlberg and N. Zoric. 2006. The real-time polymerase chain reaction. *Molecular Aspects of Medicine* 27(2-3): 95-125.
- Lallès, J.P., G. Boudry, C. Favier, N. Le Floch, S. Pié, C. Piel and B. Sève, 2004. Gut function and dysfunction in young pigs: physiology. *Animal Research* 53: 301-316.
- Lamb-Rosteki, J., L. Kalischuk, G. Inglis and A. Buret. 2008. Epidermal growth factor inhibits *Campylobacter jejuni*-induced Claudin-4 disruption, loss of epithelial barrier function, and *Escherichia coli* translocation. *Infection and Immunity* 76(8): 3390-3398.
- Makkink, C.A., P.J.M. Berntsen, B.M.L. op den Kamp, B. Kemp and W.A. Verstegen. 1994. Gastric protein breakdown and pancreatic enzyme activities in response to two different dietary protein sources in newly weaned pigs. *Journal of Animal Science* 72(11): 2843-2850.
- Mariani, V., S. Palermo, S. Fiorentini, A. Lanubile and E. Giuffra. 2009. Gene expression study of two widely used pig intestinal epithelial cell lines: IPEC-J2 and IPI-2I. *Veterinary Immunology and Immunopathology* 131(3-4): 278-284.
- Marion, J., M. Biernat, F. Thomas, G. Savary, Y. Le Breton, R. Zabielski, I. Le Huërou-Luron and J. Le Dividich, 2002. Small intestine growth and morphometry in piglets weaned at 7 days of age. Effects of level of energy intake. *Reproduction, Nutrition, Development* 42(4): 339-354.
- McLaughlin, J., P. Padfield, J. Burt and C. O'Neil. 2004. Ochratoxin a increases permeability through tight junctions by removal of specific claudin isoforms. *American Journal of Physiology. Cell physiology* 287(5): C1412-C1417.
- Muller, P.Y., H. Janovjak, A.R. Miserez and Z. Dobbie. 2002. Processing of gene expression data generated by quantitative real time RT-PCR. *Biotechniques* 32(6): 1372-1374, 1376, 1378-1379.
- Musch, M.W., M.M. Walsh-Reitz and E.B. Chang. 2006. Roles of ZO-1, occludin, and actin in oxidant-induced barrier disruption. *American Journal of Physiology and Gastrointestinal Liver Physiology* 290: G222-G23.

- National Research Council (NRC). 2012. The nutrient requirements of swine. Eleventh Revised Edition. The National Academy Press, Washington. 400 p.
- Parra, S.J.E., T.J. Agudelo, P.D. Sanín, DJ. Forero, L.C. Muskus and H.A. López. 2013. Intestinal expression of pro-inflammatory cytokines induced by oral intake of lipopolysaccharide (LPS) from *E. coli* in weaned pigs. *Revista Colombiana de Ciencias Pecuarias* 26: 108-118.
- Paulin, S.M., A. Jagannathan, J. Campbell, T.S. Wallis and M.P. Stevens. 2007. Net replication of *Salmonella enterica* serovars Typhimurium and Choleraesuis in porcine intestinal mucosa and nodes is associated with their differential virulence. *Infection and Immunity* 75(8): 3950-3960.
- Petersen, Y.M., D.G. Burrin and P.T. Sangild. 2001. GLP-2 has differential effects on small intestine growth and function in fetal and neonatal pigs. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* 281(6): 1986-1993.
- Pié, S., S.P. Lallès, F. Blazy, J. Laffitte, B. Sève and I.P. Oswald. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *Journal of Nutrition* 134: 641-647.
- Pinton, P., J. Nougayréde, J. Del Rio, C. Moreno, D. Marin, L. Ferrier, A. Bracarense, M. Kolf-Clauw and I. Oswald. 2009. The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. *Toxicology and Applied Pharmacology* 237(1): 41-48.
- Pitman, R.S. and R.S. Blumberg. 2000. First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. *Journal of Gastroenterology* 35(11): 805-814.
- Pluske, J.R., D.E. Hopwood y D.J. Hampson. 2003. Relación entre la microbiótica intestinal, el pienso y la incidencia de diarreas, y su influencia sobre la salud del lechón tras el destete. pp. 94-108. En: XIX Curso de Especialización FEDNA, Madrid.
- Reis, S.T.C., C.M.J. Guerrero, B.A. Aguilera y L.G. Mariscal. 2005. Efecto de diferentes cereales sobre la morfología intestinal de lechones recién destetados. *Técnica Pecuaria en México* 43(3): 309-321.
- Reis, S.T.C., L.G. Mariscal, B.A. Aguilera y J.G.H. Cervantes. 2007. Digestibilidad de la proteína y energía en dietas para lechones complementadas con tres diferentes tipos de suero de leche deshidratado. *Revista Veterinaria México* 38(2): 141-151.
- SASTM. 2006. SAS/STAT User's Guide. Institute Inc. Statistical Analysis Systems Institute. Version 9.1th edition. Cary, NC: SAS Institute Inc.
- Saitou, M., M. Furuse, H. Sasaki, J.D. Schulzke, M. Fromm, H. Takano, T. Noda and S. Tsukita. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular Biology of the Cell* 11(12): 4131-4142.
- Segalés, J. y M. Domingo. 2003. La necropsia en el ganado porcino, diagnóstico anatomopatológico y toma de muestras. Boehringer Ingelheim, Madrid. 43 p.
- Shen, L. and J.R. Turner. 2005. Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. *Molecular Biology of the Cell* 16(9): 3919-3936.
- Shifflett, D.E., D.R. Clayburgh, A. Koutsouris, J.R. Turner and G.A. Hecht. 2005. Enteropathogenic *E. coli* disrupts tight junction barrier function and structure *in vivo*. *Laboratory Investigation; a Journal of Technical Methods and Pathology* 85(10): 1308-1324.
- Steel, R.G. and J.H. Torrie. 1980. Principles and procedures of statistics: a biometrical approach. Second edition. McGraw-Hill, New York. 633 p.
- Tlaskalová-Hogenová, H., R. Stepánková, T. Hudcovic, L. Tucková, B. Cukrowska, R. Lodinová-Zádníková, H. Kozáková, P. Rossmann, J. Bártová, D. Sokol, D.P. Funda, D. Borovská, Z. Reháková, J. Sinkora, J. Hofman, P. Drastich and A. Kokesová. 2004. Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. *Immunology Letters* 93(2-3): 97-108.
- Tsukita, S. and M. Furuse. 2002. Claudin-based barrier in simple and stratified cellular sheets. *Current Opinion in Cell Biology* 14(5):531-536
- Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data

by geometric averaging of multiple internal control genes. *Genome Biology* 3(7): Research 0034.

Waseem, T., M. Duxbury, H. Ito and M. Robinson. 2008. Exogenous ghrelin modulates release of pro- and anti-inflammatory cytokines in LPS-stimulated macrophages through distinct signaling pathways. *Surgery* 143: 334-342.

Xu, S., J. Lee and M. Miyake. 2011. Expression of ZO-1 and occludin at mRNA and protein level during preimplantation development of the pig parthenogenetic diploids. *Zygote* 20(2): 147-158.

Yang, E.V., and R. Glaser. 2000. Stress-induced immunomodulation: Impact on immune defenses against infectious disease. *Biomedicine and Pharmacotherapy* 54: 245-250.

Yu, D., A.M. Marchiando, C.R. Weber, D.R. Raleigh, Y. Wang, L. Shen and J.R. Turner. 2010. MLCK-dependent exchange and actin binding region-dependent anchoring of ZO-1 regulate tight junction barrier function. *Proceedings of the National Academy of Sciences of the United States of America* 107(18): 8237-8241.