DANTE TEIXEIRA VALENTE JÚNIOR

CRUDE PROTEIN AND LACTOSE ON PERFORMANCE, INTESTINAL MORPHOLOGY AND EXPRESSION OF GENES RELATED TO INTESTINAL INTEGRITY AND IMMUNE SYSTEM OF PIGLETS

Dissertation presented to the Animal Science Graduate Program of Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Alysson Saraiva

VIÇOSA - MINAS GERAIS
2020

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

V154c
2020

Valente Júnior, Dante Teixeira, 1993-
Crude protein and lactose on performance, intestinal morphology and expression of genes related to intestinal integrity and immune system of piglets / Dante Teixeira Valente Júnior. - Viçosa, MG, 2020.
35 f. : il. ; 29 cm.

Inclui anexo.
Orientador: Alysson Saraiva.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f.21-27.

1. Suínos - Alimentação e ração. 2. Leitões. 3. Sistema imunológico. 4. Intestinos. 5. Lactose. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-Graduação em Zootecnia. II. Título.

CDD 22 ed. 636.40855

DANTE TEIXEIRA VALENTE JÚNIOR

CRUDE PROTEIN AND LACTOSE ON PERFORMANCE, INTESTINAL MORPHOLOGY AND EXPRESSION OF GENES RELATED TO INTESTINAL INTEGRITY AND IMMUNE SYSTEM OF PIGLETS

Dissertation presented to the Animal Science Graduate Program of Universidade Federal de Viçosa, in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

APPROVED: July 28, 2020.

Assent:

Dante Teixeira Valente Júnior
Author

Alysson Saraiva
Adviser

ACKNOWLEDGMENTS

To God for give me more than I deserve every day.

To Federal University of Viçosa and the Department of Animal Science for the opportunity to complete my Master of Science program.

To Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Instituto Nacional de Ciência e Tecnologia - Ciência Animal (INCT-CA) for financial support.

To my parents, Dante and Maria de Fátima, for their immense love, trust and immeasurable support.

To my grandparents, uncles and cousins, for their support and encouragement during this journey.

To my sister Marina for all the teaching, advices and affection.

To my girlfriend Maykelly for love, companionship and encouragement.

To my advisor, Prof. Alysson Saraiva for all the knowledge transmitted during those years, and for guidance in the elaboration and execution of this study. I also would like to thank Prof. Gabriel C. Rocha, Prof. Paulo H. R. F. Campos and Dr. Fabrício Santos, for their availability, attention and contributions to this study.

To my friends from Paula Cândido and to those I made in Viçosa, for the greast moments lived.

To the hard workers Arlindo, Fernando, Francisco, José Roberto, Leandro and Valdeir, from UEPE-Suinocultura (UFV), for the friendship and teachings transmitted over the years.

To my work team, Gustavo, Lívia, Marcos and Matheus, for their friendship and help during the execution of this study.

To the Grupo de Discussão em Suinocultura (GDSUI) for enriching my professional career.

ABSTRACT

VALENTE JÚNIOR, Dante Teixeira, M.Sc., Universidade Federal de Viçosa, July, 2020. **Crude protein and lactose on performance, intestinal morphology and expression of genes related to intestinal integrity and immune system of piglets**. Adviser: Alysson Saraiva.

The present study was carried out to evaluate the effects of crude protein (CP) and lactose (LAC) levels on performance, intestinal morphology and gene expression associated to epithelial integrity and activation of the immune system of piglets. A total of 120 commercial castrated male and female hybrid piglets, weaned at 21 days of age and 7.88 ± 0.9 kg initial body weight, were assigned to a randomized block design, in a 2 × 3 factorial arrangement, with 2 levels of crude protein (CP, 20.0 and 24.0%) and 3 levels of lactose (LAC, 8.0, 12.0 and 16.0%), with 10 replicates and two piglets per experimental unit. There was no interaction ($P > 0.05$) between CP and LAC levels for any performance variables and serum urea nitrogen concentration (SUN). Piglets fed diets with 24.0% CP had higher average daily feed intake (ADFI; $P = 0.048$) compared to those fed 20.0% CP. Diets containing 12.0 or 16.0% LAC resulted in higher ($P < 0,01$) ADFI and average daily gain (ADG) of piglets compared to the 8.0% LAC diet. Pigs fed 12.0% LAC diets had greater ($P = 0.018$) final body weight (final BW) than those fed 8.0 and 16.0% LAC diets. There was no effect ($P = 0.164$) of LAC concentration on feed:gain ratio (F:G). Piglets consuming 20.0% CP diets had lower ($P < 0.001$) SUN than piglets fed 24.0% CP diets. Regardless of diet CP levels, piglets fed 12.0 or 16.0% LAC diets had higher duodenum and ileum villous height ($P = 0.02$ and $P = 0.006$, respectively). Interactions ($P < 0.05$) were observed between CP and LAC for duodenum, jejunum and ileum crypt depth. Piglets fed 24.0% CP diet with 12.0 or 16.0% LAC had lower duodenum crypt depth, and piglets fed 24.0% CP diet with 12.0 LAC had lower ileum crypt depth than those fed with 8.0% LAC. There were interactions ($P < 0.05$) between the CP and LAC for duodenum and ileum villus height:crypt depth ratio. Piglets fed 24.0% CP diet with 12.0 or 16.0% LAC had higher duodenum and ileum villus height:crypt depth ratio compared to those fed 24.0% CP with 8.0% LAC. Interactions ($P < 0.05$) were observed between CP and LAC levels for gene expression of IL-6 and TNFα. However, there was no effect of LAC on the expression of these cytokines in the diets with 20.0 or 24.0% CP. Similarly, there was a tendency for interaction ($P = 0.07$) between CP and LAC in the IL-1β gene expression. Whilst, at the levels of 20.0 and 24.0% CP there was no effect of LAC on the expression of IL-1β. There was interaction ($P = 0.025$) between CP and LAC for haptoglobin (Hp) gene expression. At 24.0% CP piglets fed 16.0% LAC had lower expression of Hp. Gene expression of TGF β1 and occludin were not influenced ($P > 0.05$) by CP and LAC levels of the diets. There was interaction ($P = 0.012$) between CP and LAC in gene expression of the ZO-1, and the diet with 16.0% LAC was more efficient in decreasing gene expression of ZO-1 compared to 8.0 and 12.0% LAC. In conclusion, concentration of 12.0% LAC in the diet improves performance of piglets from 21 to 35 days old regardless of diet CP content. The inclusion of 12.0 and 16.0% LAC in the diet promotes improvements in intestinal morphology, being more evident in the diet with 24.0% CP compared to 20.0% CP. In addition, increasing inclusion of LAC in the diet with higher level of CP (24.0%) decreases the immune system activation and reduces the permeability of the intestinal epithelium.

Keywords: Immune system. Intestinal morphology. Lactose. Piglets. Prebiotic. Weaning.

RESUMO

VALENTE JÚNIOR, Dante Teixeira, M.Sc., Universidade Federal de Viçosa, julho de 2020. **Proteína bruta e lactose no desempenho, morfologia intestinal e expressão de genes relacionados à integridade intestinal e sistema imunológico de leitões**. Orientador: Alysson Saraiva.

O presente estudo foi realizado para avaliar os efeitos dos níveis de proteína bruta (PB) e lactose (LAC) sobre o desempenho, morfologia intestinal e expressão gênica associada à integridade epitelial e ativação do sistema imunológico de leitões. Um total de 120 leitões híbridos machos e fêmeas castrados comerciais, desmamados aos 21 dias de idade e 7,88 ± 0,9 kg de peso corporal inicial, foram distribuídos em um delineamento de blocos ao acaso, em um arranjo fatorial 2 × 3, com 2 níveis de proteína bruta (PB, 20,0 e 24,0%) e 3 níveis de lactose (LAC, 8,0, 12,0 e 16,0%), com 10 repetições e dois leitões por unidade experimental. Não houve interação (P> 0,05) entre os níveis de PB e LAC para nenhuma das variáveis de desempenho e concentração de uréia sérica. Leitões alimentados com dietas com 24,0% PB tiveram maior consumo de ração médio diário (CRD; P = 0,048) em comparação aqueles alimentados com 20,0% PB. Dietas contendo 12,0 ou 16,0% de LAC resultaram em maior (P <0,01) CRD e ganho de peso médio diário (GPD) dos leitões em comparação com a dieta de 8,0% de LAC. Leitões alimentados com dietas de 12,0% LAC tiveram maior (P = 0,018) peso médio final (PMF) do que aqueles alimentados com dietas de 8,0 e 16,0% LAC. Não houve efeito (P = 0,164) da concentração de LAC na conversão alimentar. Os leitões que consumiram dietas com 20,0% PB tiveram menor (P <0,001) concentração de ureia sérica do que os leitões alimentados com dietas com 24,0% PB. Independentemente dos níveis de PB da dieta, os leitões alimentados com 12,0 ou 16,0% de LAC apresentaram maior altura das vilosidades do duodeno e íleo (P = 0,02 e P = 0,006, respectivamente). Interações (P <0,05) foram observadas entre PB e LAC para profundidade de cripta do duodeno, jejuno e íleo. Leitões alimentados com dieta de 24,0% PB com 12,0 ou 16,0% LAC tiveram menor profundidade de cripta do duodeno, e leitões alimentados com dieta 24,0% PB com 12,0% LAC tiveram menor profundidade de cripta do íleo em relação aqueles alimentados com 8,0% LAC. Houve interações (P <0,05) entre o PB e LAC na relação altura das vilosidades:profundidade de cripta do duodeno e íleo. Leitões alimentados com dieta de 24,0% PB com 12,0 ou 16,0% LAC tiveram maior relação altura de vilosidades:profundidade de cripta no duodeno e íleo em comparação aqueles alimentados com 24,0% PB com 8,0% LAC. Foram observadas interações (P

<0,05) entre os níveis de PB e LAC na expressão gênica de IL-6 e TNFα. Porém, não houve efeito da LAC na expressão dessas citocinas nas dietas com 20,0 ou 24,0% PB. Da mesma forma, houve tendência de interação (P = 0,07) entre PB e LAC na expressão do gene IL-1β. Já nos níveis de 20,0 e 24,0% PB não houve efeito da LAC na expressão de IL-1β. Houve interação (P = 0,025) entre PB e LAC na expressão do gene da haptoglobina (Hp). Com 24,0% de PB, os leitões alimentados com 16,0% de LAC apresentaram menor expressão de Hp. A expressão gênica de TGF β1 e occludina não foi influenciada (P> 0,05) pelos níveis de PB e LAC das dietas. Houve interação (P = 0,012) entre PB e LAC na expressão gênica de ZO-1, e a dieta com 16,0% LAC diminuiu a expressão gênica de ZO-1 em comparação com 8,0 e 12,0% LAC. Em conclusão, a concentração de 12,0% de LAC na dieta melhora o desempenho de leitões de 21 a 35 dias de idade, independentemente do teor de PB da dieta. A inclusão de 12,0 e 16,0% de LAC na dieta promove melhorias na morfologia intestinal, sendo mais evidente na dieta com 24,0% de PB em comparação com 20,0% de PB. Além disso, o aumento da inclusão de LAC na dieta com maior nível de PB (24,0%) diminui a ativação do sistema imunológico e a permeabilidade do epitélio intestinal.

Palavras-chave: Desmame. Lactose. Leitões. Morfologia intestinal. Prebiótico. Sistema imune.

SUMMARY

Crude protein and lactose on performance, intestinal morphology and expression of genes related to intestinal integrity and immune system of piglets 9

ACKNOWLEDGMENTS 9

ABSTRACT 9

1 INTRODUCTION 10

2 MATERIAL AND METHODS 11

2.1 Animals, experimental design, housing and diets 11

2.2 Slaughter procedures and intestinal samples collection 12

2.3 Sample collection for gene expression analysis 12

2.4 Blood analysis 13

2.5 Intestinal morphology assessment 13

2.6 Gene expression analysis 13

2.7 Statistical analysis 14

3 RESULTS 14

3.1 Performance and serum urea nitrogen concentration 14

3.2 Intestinal morphology 15

3.3 Gene expression 15

4 DISCUSSION 15

4.1 Performance and serum urea nitrogen concentration 15

4.2 Intestinal morphology 17

4.3 Gene expression 19

5 CONCLUSION 20

6 REFERENCES 21

ANEXO 35

Crude protein and lactose on performance, intestinal morphology and expression of genes related to intestinal integrity and immune system of piglets

Dante Valente Teixeira Júnior[1], Gustavo de Amorim Rodrigues[1], Marcos Henrique Soares[1], Gabriel Cipriano Rocha[1], Paulo Henrique Furtado Campos[1,2], Alysson Saraiva[1,2]

[1] Department of Animal Sciences, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil;

[2] Muscle Biology and Nutrigenomics Laboratory, Universidade Federal de Viçosa, Viçosa, MG, 36570-900, Brazil;

ACKNOWLEDGMENTS

The authors would like to thank the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)* and *Instituto Nacional de Ciência e Tecnologia de Ciência Animal (INCT-CA)* for financial support.

ABSTRACT

The present study was carried out to evaluate the effects of crude protein (CP) and lactose (LAC) levels on performance, intestinal morphology and gene expression associated to epithelial integrity and activation of the immune system of piglets. A total of 120 weaned piglets with initial weight 7.88 ± 0.9 kg were assigned to a randomized block design, in a 2 × 3 factorial arrangement (20.0 and 24.0% CP and 8.0%, 12.0%, and 16.0% LAC), with 10 replicates. Piglets fed 24.0% CP had higher average daily feed intake (ADFI). Diets containing 12.0 or 16.0% LAC resulted in higher ADFI and average daily gain of piglets compared to 8.0% LAC. Pigs fed 12.0% LAC diets had greater final body weight than those fed 8.0 and 16.0% LAC diets. Piglets consuming 20.0% CP diets had lower SUN. Piglets fed 12.0 or 16.0% LAC diets had higher duodenum and ileum villous height (VH). Interactions were observed for duodenum, jejunum and ileum crypt depth (CD) and duodenum and ileum VH:CD ratio. Piglets fed 24.0% CP diet with 12.0 or 16.0% LAC had lower duodenum and ileum CD, and higher duodenum and ileum VH:CD ratio than those fed 8.0% LAC. Interactions were observed between CP and LAC levels for gene expression of IL-6, TNFα, haptoglobin (Hp) and zonula occludens-1 (ZO-1), and a tendency for interaction of IL-1β gene expression was observed. However, there were no

effect of LAC on expression of IL-6, TNFα and IL-1β. At 24.0% CP, piglets fed 16.0% LAC had lower expression of Hp and ZO-1. Concentration of 12.0% LAC in the diet improves performance of piglets regardless of diet CP content. In addition, increasing inclusion of LAC in the diet promotes improvements in intestinal morphology and integrity, and decreases the immune system activation, being more evident in piglets fed higher CP diets.
Key words: inflammatory response, intestinal morphology, lactose, piglets, prebiotic, weaning.

1 INTRODUCTION

Weaning of piglets is the most critical phase in the whole pig production cycle. Piglets are subjected to several stressors such as separation of the sow and littermates, environmental changes, establishment of a new social hierarchy and adaptation to a solid and less digestible diet with the presence of anti-nutritional factors (Jiao et al., 2014).

The stressful events associated with weaning cause damage to the mucosa of the gastrointestinal tract (GIT), changing its morphology and permeability (Kim, Hansen, Mullan, & Pluske, 2012; Moeser, Pohl, & Rajput, 2017) which together with changes in the intestinal microbiota and activation of the immune system compromise piglet performance (Moeser et al., 2017; Pluske, Turpin, & Kim, 2018; Wen et al., 2018).

Thus, with lesser digestive and absorptive capacity of the GIT after weaning, diets with higher crude protein (CP) levels provide greater amount of fermentative substrate in the large intestine benefiting pathogenic bacteria species (Heo et al., 2009; McLamb, Gibson, Overman, Stahl, & Moeser, 2013).

Protein fermentation in the large intestine results in increased production of toxic compounds such as ammonia, biogenic amines, hydrogen sulfide and branched chain fatty acids which may cause damage to the intestinal epithelium and are directly correlated with the incidence of post-weaning diarrhea in piglets (Kröger et al., 2013; Pieper et al., 2016a; Wen et al., 2018).

In this way, lactose (LAC) serve as a fermentative substrate in the large intestine as lactase activity reduces after weaning (De Vos et al., 2014), resulting in reduced protein fermentation (Pieper, Vahjen, & Zentek, 2016b).

In addition, LAC beneficially modulates the microbiota by increasing lactic acid, with reduction of intestinal pH and promotes an increase in short-chain fatty acids (SCFAs) production, leading to decreased inflammatory response and increased proliferation of intestinal epithelial cells (Pierce, Callan, McCarthy, & O'Doherty, 2007; Soares et al., 2020; Venema, 2012), reflecting in greater integrity and functionality of the intestinal villi (Soares et al., 2020).

In our recent study, assessing LAC and CP levels for weaned piglets, supply of higher dietary concentrations of LAC improved the intestinal morphology and integrity without affecting piglet performance (Soares et al., 2020). Unlike the present study, Soares et al. (2020) used antibiotic and zinc oxide as growth promoters in the diets, as well as more digestible protein sources in the diet with higher CP content.

However, the ban/restriction of antibiotics and zinc oxide use as growth promoters in the diets for weaned piglets has been discussed around the world due antimicrobial resistance and excess zinc excretion, respectively (Pei et al., 2019; Pluske et al., 2018). Thus, higher inclusion of LAC in diets without these growth promoters may result in greater magnitude responses on intestinal health, immune response and piglet performance (Soares et al., 2020). In addition, the magnitude of LAC effect may also be dependent on the protein content of the diet (Pierce et al., 2007).

Thus, the hypothesis is that increasing the inclusion of LAC in diets with high CP concentration improves performance, morphology and epithelial integrity of the intestine, in addition to decreasing the activation of the immune system of piglets weaned at 21 days of age.

In this way, the present study was carried out to evaluate the effect of CP and LAC levels on performance, intestinal morphology and gene expression associated to epithelial integrity and activation of the immune system of piglets during the first two weeks after weaning.

2 MATERIAL AND METHODS

All methods involving the handling of pigs followed the ethical principles of animal research (CONCEA) and were approved by the Commission of Ethics in the Use of Production Animals (CEUAP) of the Universidade Federal de Viçosa (protocol 100/2018).

2.1 Animals, experimental design, housing and diets

A total of 120 castrated male and female piglets (Agroceres PIC) weaned at 21 days of age and 7.88 ± 0.86 kg of body weight were assigned to a randomized block design, in a 2 × 3 factorial arrangement, with two levels of crude protein (CP, 20.0 and 24.0%) and three lactose levels (LAC, 8.0, 12.0 and 16.0%), with 10 replicates and 2 piglets per experimental unit, represented by the cage.

Piglets were housed during the experimental period of 14 days in metal cages, with plastic slatted floors, equipped with semiautomatic feeders and nipple drinkers, located in climatic-controlled nursery rooms in which the temperature was maintained within the thermoneutral zone during the experimental period by means of air conditioners. Ambient temperature inside the rooms was recorded daily (5:00 p.m.), using maximum and minimum thermometers, kept in an empty cage in the center of the room, half height of the piglets' body.

Experimental diets (Tables 1) were formulated to meet the nutritional requirements of pigs in the pre-initial phase, as recommended by Rostagno et al. (2011). The dietary treatments consisted of: 20.0% CP + 8.0% LAC, 20.0% CP + 12.0% LAC, 20.0% CP + 16.0% LAC, 24.0% CP + 8.0% LAC, 24.0% CP + 12.0% LAC and 24.0% CP + 16.0% LAC.

Piglets had free access to feed and water throughout the experimental period (21 to 35 days of age). During the trial, feed was weighed before feeding and feed wastage and leftovers were collected and weighed daily to determine average daily feed intake (ADFI). At the end of the experimental period, piglets were individually weighed to determine final body weight (final BW), average daily gain (ADG), and feed:gain ratio (F:G).

2.2 Slaughter procedures and intestinal samples collection

At 35 days of age, one piglet from each experimental unit, with average body weight closest to the average weight of the piglets in the block was electrically stunned followed by exsanguination to collect histological samples.

Segments of 2.0 x 2.0 cm of the intestine were sampled corresponding to duodenum (10 cm from pylorus), jejunum (the middle portion) and ileum (5 cm proximal to the ileocecal junction), according to Yang, Jiang, Zheng, Wang, & Yang (2014). Histological sections were then washed in physiological solution and fixed in 4.0% paraformaldehyde solution for 24 hours at room temperature.

2.3 Sample collection for gene expression analysis

In order to collect jejunum samples for gene expression of cytokines and tight junction's proteins, the instruments that came into contact with the tissue were sanitized with alcohol 70%, dried with paper towel and sprayed with RNAse Exterminator (Protech Technology Enterprise CO., Ltd., Taipei, Nanking Dist., Taiwan) between each sampling.

The portion of the sampled jejunum was washed with sterile saline serum for removal of the digesta in contact with the tissue. From the tissue sample obtained, about ten 0.25 cm^3 subsamples were collected, washed individually in sterile serum, placed in cryotubes with a capacity of 1.8 mL, frozen instantaneously in liquid nitrogen, and stored at - 80°C for further analysis.

2.4 Blood analysis

At the end of the experimental period, the remaining piglet of each experimental unit was fasted for 14 hours and then blood was collected by puncture in the orbital sinus, using hypodermic needles (40 x 1.6 mm) and packed in 10 mL tubes without anticoagulants. Samples were immediately sent at room temperature to the Viçosa clinical laboratory for determination of serum urea nitrogen concentration (SUN) (Ureal Cobas c 311, Roche Diagnostics GmbH, Basel, Switzerland).

2.5 Intestinal morphology assessment

The histological samples were cross-sectionally cut and dried in ethyl crescent gradients, diaphanized in xylene and embedded in liquid paraffin at 60°C. Transverse cuts 5 µm thickness were stained with hematoxylin and eosin. Three cuts of 5 µm thickness each were placed per slide. The cuts were semi-serial using 1 in 10 cuts.

For morphological readings, an optical microscope (EVOS® XL Core) was used with 10x magnification, coupled to the image analyzer *ImageJ 1.50i; java1.6.0_20* (National Institutes of Health, USA). Heights of 20 villi and their 20 crypts were selected and measured.

2.6 Gene expression analysis

Total RNA extraction was performed using Trizol (Life Technologies) following the manufacturer instructions. The RNA concentration was estimated by NanoVueTM Plus spectrophotometer (GE Healthcare, Freiburg, Germany), and RNA integrity was evaluated through 1.0% agarose gel electrophoresis. Complementary DNA synthesis was performed according to Goscript Reverse Transcription System protocol (Promega, Madison, WI, USA; Applied Biosystems, Foster City, CA, USA). GenBank numbers to access the primers of the genes are shown in Table 2. Primers were used for reverse transcription quantitative PCR with GoTaq qPCR Master Mix (Promega) in ABI Prism 7300 Sequence Detection Systems thermocycler (Applied Biosystems). Geometric means of Ct values of GAPDH was used to normalize target genes expression. Gene of interest relative expression was calculated by $2^{-\Delta Ct}$ (Livak & Schmittgen, 2001).

2.7 Statistical analysis

The cage was considered the experimental unit for analysis of performance data (ADFI, ADG, F:G, and final BW). One piglet from each experimental unit, slaughtered at 35 days of age, was considered the experimental unit for analysis of serum urea nitrogen concentration, intestinal morphology and gene expression. The data were analyzed using the GLM procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC) licensed by Universidade Federal de Viçosa. The means were compared by Tukey test. When there was interaction LAC levels were analyzed within 20.0 and 24.0% CP diets. For all statistical analyzes probability values less than 0.05 were considered significant and probability values between 0.05 and 0.10 was considered as trend.

3 RESULTS

3.1 Performance and serum urea nitrogen concentration

During the experimental period the average temperature inside the nursery rooms was 27.35ºC ± 1.19ºC the minimum temperature was 26.48 ± 0.85ºC and the maximum temperature was 28.21 ± 0.79ºC. The thermoneutral zone for piglets in the first two weeks after weaning is established between 27 to 30°C (Kummer, Gonçalves, Lippke, Marques, & Mores, 2009). Therefore, in the present study the piglets were kept in thermoneutral environment.

There was no interaction (P>0.05) between the CP and LAC for any performance variables (Table 3). Piglets fed diets with 24.0% CP had higher ADFI (P = 0.048) compared to those fed 20.0% CP. Diets containing 12.0 or 16.0% LAC resulted in higher ADFI and ADG (P = 0.003 and P = 0.007, respectively) of piglets compared to the 8.0% LAC diet. Similarly, the diet with 12.0% LAC resulted in a higher final BW (P = 0.018) of piglets compared to 8.0 and 16.0% LAC diets. However there was no effect (P = 0.164) of LAC concentration on F:G.

There was also no interaction (P=0.699) between dietary CP and LAC for SUN. Piglets consuming 20.0% CP diets had lower (P<0.001) SUN than piglets fed 24.0% CP diets (Table 3).

3.2 Intestinal morphology

There were no interaction (P > 0.05) between the CP and LAC diets for duodenum, jejunum and ileum villous height, and for jejunum villous height:crypt depth ratio (Table 4). No effect (P > 0.05) of LAC and CP was observed in jejunum villous height:crypt depth ratio. However, piglets fed 12.0 or 16.0% LAC diets had higher (P < 0.05) duodenum and ileum villous height.

Interactions (P < 0.05) were observed for duodenum, jejunum and ileum crypt depth, and duodenum and ileum villous height:crypt depth ratio.

There were no differences (P > 0.05) in jejunum crypt depth of piglets fed 8.0, 12.0 or 16.0% LAC in diets with 20.0 or 24.0% CP. Nonetheless, piglets fed 24.0% CP diet with 12.0 or 16.0% LAC had lower (P < 0.05) duodenum and ileum crypt depth, and higher (P < 0.05) duodenum and ileum villous height:crypt depth ratio than those fed 8.0% LAC.

3.3 Gene expression

Interactions (P < 0.05) were observed between CP and LAC levels of the diets for gene expression of IL-6, TNFα, haptoglobin (Hp) and zonula occludens-1 (ZO-1), and a tendency (P = 0.07) for interaction of IL-1β gene expression was observed (Table 5). However, there were no effect of LAC on expression of IL-6, TNFα and IL-1β. At 24.0% CP, piglets fed 16.0% LAC had lower expression of Hp and ZO-1.

The gene expression of TGF β1 and occludin were not influenced (P> 0.05) by CP and LAC levels of the diets.

4 DISCUSSION

4.1 Performance and serum urea nitrogen concentration

The stress caused by weaning causes physiological changes that compromise the piglet's intestinal health (Moeser et al. 2017; Pluske et al., 2018; Wen et al., 2018). Thus, after weaning piglets have reduced digestive and absorptive capacity of GIT, resulting in greater amount of protein substrate for the proliferation of pathogenic bacteria in the large intestine (Heo et al., 2009; McLamb et al., 2013).

Toxic compounds such as ammonia, biogenic amines, hydrogen sulfide and branched-chain fatty acids generated from residual protein fermentation cause damage to the intestinal epithelium, impair energy metabolism of enterocytes, and are directly correlated with incidence of post-weaning diarrhea in piglets (Pieper et al., 2016a; Rist, Weiss, Eklund, & Mosenthin, 2013; Wen et al., 2018).

Although high levels of CP in the diet have been associated with incidence of diarrhea and decreased performance of weaned piglets, in the present study no effects were observed on ADG and F:G.

Interestingly, piglets fed 24.0% CP diet had a higher ADFI, despite the high concentration of soybean meal in the diet. In agreement with the present study, Nyachoti, Omogbenigun, Rademacher, and Blank (2006) and Yue and Qiao (2008) also observed a reduction in ADFI when the CP content in the diet was reduced from 23.0 to 19.0%.

According to Leray, Segain, Cherbut, and Galmiche (2003), the reduction of CP in the diet decreases the gastric emptying through modulation of cholecystokinin (CCK) release and gastric muscle sensitivity to CCK in rats. Likewise, Tian et al. (2016) observed an increase in CCK concentrations in the gastric antrum of weaned piglets fed 17% CP compared to those fed 20% CP. Thus, the reduction in ADFI of piglets fed 20.0% CP observed in the present study may be related to less gastric emptying, through the modulation of CKK release.

Nevertheless, Kim, Heo, Mullan, and Pluske (2011) and Wu et al. (2015) did not observe differences in ADFI of piglets fed 23.0 or 19.0% CP in the diets. The discrepancies between our study and those others may explained by the greater piglets weight at the beginning of the experimental period (7.88 vs ~5.7 kg), which may have mitigated post-weaning stress.

However, the increase of ADFI in piglets fed 24.0% CP did not result in better ADG and F:G, which is explained by greater amino acid catabolism, evidenced by higher SUN observed in piglets fed 24.0% CP.

LAC has been studied as an alternative to mitigate the effects of diets with high levels of CP. In our recent study, Soares et al. (2020) did not observe positive effects of dietary LAC levels on performance variables of weaned piglets, regardless of CP level in the diets.

However, in the present study, 12.0 or 16.0% LAC in diet promoted an increase in ADG of piglets, which is consistent with higher ADFI and final BW observed. Nevertheless, the increase in weight gain had no influence in F:G. Unlike Soares et al. (2020), we did not use antibiotics and zinc oxide as growth promoters in diets, which may have evidenced the positive responses of increased LAC in diets on piglet' performance.

Similar results on growth performance were also reported by Kim et al. (2010), Lynch, Callan, and O'Doherty (2009) and O'Doherty, Dillon, Figat, Callan, and Sweeney (2010) supplementing LAC in the diet for weaned piglets.

LAC beneficially modulates the microbiota by increasing lactic acid, with reduction of intestinal pH and promotes an increase in short-chain fatty acids (SCFAs) production, leading to decreased inflammatory response and increased proliferation of intestinal epithelial cells (Jeong et al., 2019; Pierce et al., 2007; Soares et al., 2020). Which may explain the improved performance of piglets that consumed diets with higher LAC content.

The highest ADFI of piglets fed 12.0 or 16.0% LAC diets can be attributed at least to two factors. Firstly, diets containing LAC are more palatable (Lynch et al., 2009) and thus are more attractive to piglets, stimulating consumption. Secondly, there is an association between feed digestibility and feed intake by piglets, and LAC supplementation in the diet of weaned piglets improves nutrient digestibility coefficients (Jeong et al., 2019; Kim et al., 2010; O'doherty et al., 2010).

The SUN is commonly used as an indicator of the efficiency of use of dietary nitrogen for protein synthesis (Heo et al., 2009; Morales et al., 2016). As observed in the present study, at higher CP diets SUN concentration increases, indicating greater catabolism of amino acids as piglets are subjected to a greater supply of nitrogen (Fang et al., 2019; Wu et al., 2015).

4.2 Intestinal morphology

Villous height is positively correlated to enzyme production and surface area for digestion and absorption of nutrients, being a good indicator of intestinal health (Soares et al., 2020). In the present study, the inclusion of 12.0 or 16.0% LAC in the diets promoted an increase in duodenum and ileum villous height regardless of CP level in the diet, which is consistent with the results of ADFI and ADG.

The renewal rate and migration of enterocytes from crypts to villi is a normal process in the intestine, however, the speed at which this process occurs changes the intestinal morphology (Parker et al., 2017). At high rate of cell renewal that can be caused by pathogenic bacteria colonization in the intestine, the crypts become deeper resulting in greater energy losses to support cell renewal (Baurhoo, Letellier, Zhao, & Ruiz-Feria, 2007). In this sense, the performance of piglets is associated with the villous height:crypt depth ratio (Lu, Zou, & Wang, 2008). The higher villous height and lower the crypt depth the better the digestion and absorption of nutrients (Li et al., 1991).

In this study, the addition of 12.0 or 16.0% LAC in the diet with 24.0% CP decreased duodenum and ileum crypt depth and increased duodenum and ileum villous height:crypt depth ratio (Figure 1), improving the performance of piglets.

LAC in the intestine acts as an inhibitor of protein fermentation. Carbohydrate fermenting bacteria incorporate amino acids as microbial protein, limiting the availability of substrate for protein fermentation (Pierce et al., 2006a; 2006b), decreasing the concentration of toxic compounds such as ammonia and hydrogen sulfide in the intestinal lumen. These metabolites interfere the energy metabolism of intestinal mucosal cells, inducing cellular energy deficiency (Blachier, Mariotti, Huneau, & Tomé, 2007; Leschelle et al., 2002), that negatively influences the intestinal morphology of piglets.

In this sense, supplementation of 12.0 or 16.0% LAC in the diet may have reduced protein fermentation and the formation of ammonia and hydrogen sulfide in the intestinal lumen, increasing the longevity of enterocytes, resulting in increased villous height.

Another hypothesis is that the use of LAC as a fermentative substrate for acidophilic bacteria such as *Lactobacillus* spp., resulting in reduction of intestinal pH through the production of lactic acid and control of the population of pathogenic bacteria (Daly et al., 2014; Jeong et al., 2019; O'doherty et al., 2010). With the reduction of intestine colonization by pathogens, the turnover of intestinal epithelial cells decreases, resulting in greater villous height and reduction of crypt depth.

Studies with LAC supplementation in the diet of weaned piglets reported increased production of volatile fatty acids in different segments of the intestine (Pierce et al., 2007; Lynch et al., 2009; Jeong et al., 2019). Among volatile fatty acids, butyrate is associated with increased secretion of GLP-2, which plays an important role in proliferation, differentiation, and reduction of enterocytes apoptosis (Liu, Ipharraguerre, & Pettigrew, 2013; Sigalet et al., 2015). Therefore, one can infer the supply of the higher levels of LAC (12.0 or 16.0%) in the present study may have contributed to a higher production of volatile fatty acids and this may be associated with increased villous height and villous height:crypt depth ratio of the intestinal segments.

4.3 Gene expression

Weaning is associated with an increased inflammatory response, contributing to disorders of the intestinal mucosa through the positive regulation of pro-inflammatory cytokines such as IL-6, TNFα and IL-1β (Pié et al., 2004). In addition to causing damage to the intestinal epithelium, proinflammatory cytokines divert nutrients used from growth in response to infection to control the immune challenge, causing deficiency in the use of energy and nutrients, thus impairing the piglets' performance (Elsasser, Caperna, Li, Kahl, & Sartin, 2008).

Although LAC did not alter the gene expression of IL-6, TNFα and IL-1β in the present study, the pattern of response between the CP levels with the increase in LAC levels in the diet was different (Figure 2). Piglets fed 24.0% CP had a numerical reduction of 32.0, 42.6 and 45.3% in the expression of IL-6, TNFα and IL-1β, respectively, by increasing LAC in the diets, suggesting that increasing LAC inclusion in diets with higher levels of CP may attenuate the inflammatory response, which is consistent with the results of intestinal performance and morphometry in this study.

Haptoglobin (Hp) is an acute-phase protein which has its expression increased when there is a high degree of infection and inflammation (Soler, Gutiérrez, Müllebner, Cerón, & Duvigneau, 2013). As a result, Hp has been widely used as a marker of infection in pigs (Benga et al., 2009).

In this study the inclusion of LAC did not influence the expression of Hp in the jejunum of piglets fed the 20.0% CP diet. However, as the levels of LAC increased in the diet with 24.0% CP the expression of Hp in the jejunum was reduced (Figure 2), evidencing the inclusion of LAC in the diet with 24.0% CP attenuated intestinal inflammation, resulting in the improvement of intestinal morphology and performance of piglets.

The inflammatory response can be regulated by the expression of anti-inflammatory cytokines such as TGF β1 through the inactivation of NF-κB (Monteleone, Pallone, & MacDonald, 2004). However, in the present study there was no effect of the diets on the expression of TGF β1 in jejunum, indicating the attenuation of the inflammatory response by LAC could be mainly linked to other two factors.

First, LAC may attenuate the inflammatory response by increasing volatile fatty acids (Jeong et al., 2019), such as acetate, propionate and butyrate, which bind to GPR41, GPR43 and GPR109a receptors in the intestinal epithelium, leading to inactivation of NF-κB and suppression of pro-inflammatory cytokines (Levy, Blacher, & Elinav, 2017). Second, LAC favors the growth of acidophilic bacteria decreasing the population of pathogenic bacteria (Kim et al., 2010; Jeong et al., 2019), which reduces pathogen bonds in the intestinal epithelium and consequently the activation of the immune system.

Occlusive junction proteins such as occludin and ZO-1 are responsible for decreasing the permeability of the intestinal epithelium, preventing the passage of microorganisms and toxins into the intercellular space, maintaining the integrity and functionality of the intestinal epithelium (He et al., 2017).

In the present study, the levels of CP and LAC did not influence the expression of occludin. However, increasing LAC in the 24.0% CP diet resulted in a reduction in the expression of ZO-1 in the piglets' jejunum.

A possible explanation for this result is related to the regulation of ZO-1 on cell proliferation. It has been shown in tissues with greater cell proliferation ZO-1 expression can be decreased (Balda, Garrett, & Matter, 2003; De Longh, Wederell, Lovicu, & McAvoy, 2005; Unger et al., 2002).

Balda and Matter (2009) and González-Mariscal et al. (2014) reported that ZO-1 directs the transcription factor ZONAB away from the nucleus, decreasing the induction of genes such as CD1, CDK4 and PCNA involved in cell proliferation. Thus, the reduced expression of ZO-1 correlates with the increased proliferation of epithelial cells (González-Mariscal et al., 2014), which is consistent with the results of intestinal morphology of the present study.

Although at the same time that the increase in LAC levels in the diet promoted a reduction in inflammatory response and an improvement in intestinal morphology and consequently in performance of the piglets, interestingly, a reduction in ZO-1 expression occlusive junction protein was observed. In this sense, one can infer the improvement of intestinal health promoted by LAC may have reduced the need for ZO-1 synthesis in the intestinal barrier.

5 CONCLUSION

In conclusion, 12.0% LAC in the diet improves performance of piglets from 21 to 35 days of age regardless CP content. The inclusion of 12.0 or 16.0% LAC in the diet promotes improvements in intestinal morphology, which are more evident in the diet with 24.0% CP. In addition, increasing LAC content in the diet with 24.0% CP decreases the activation of the immune system and reduces the permeability of the intestinal epithelium.

6 REFERENCES

Balda, M. S., & Matter, K. (2009). Tight junctions and the regulation of gene expression. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1788(4), 761-767. *doi*:10.1016/j.bbamem.2008.11.024

Balda, M. S., Garrett, M. D., & Matter, K. (2003). The ZO-1–associated Y-box factor ZONAB regulates epithelial cell proliferation and cell density. *The Journal of Cell Biology*, 160(3), 423-432. *doi*:10.1083/jcb.200210020

Baurhoo, B., Letellier, A., Zhao, X., & Ruiz-Feria, C. A. (2007). Cecal Populations of Lactobacilli and Bifidobacteria and Escherichia coli Populations After In Vivo Escherichia coli Challenge in Birds Fed Diets with Purified Lignin or Mannanoligosaccharides. *Poultry Science*, 86(12), 2509-2516. *doi*:10.3382/ps.2007-00136

Benga, L., Hoeltig, D., Rehm, T., Rothkoetter, H.-J., Pabst, R., Valentin-Weigand, P., & Iras, F. (2009). Expression levels of immune markers in Actinobacillus pleuropneumoniae infected pigs and their relation to breed and clinical symptoms. *BMC Veterinary Research*, 5(1), 1-12. *doi*:10.1186/1746-6148-5-13

Blachier, F., Mariotti, F., Huneau, J. F., & Tomé, D. (2007). Effects of amino acid-derived luminal metabolites on the colonic epithelium and physiopathological consequences. *Amino Acids*, 33(4), 547-562. *doi*:10.1007/s00726-006-0477-9

Daly, K., Darby, A. C., Hall, N., Nau, A., Bravo, D., & Shirazi-Beechey, S. P. (2014). Dietary supplementation with lactose or artificial sweetener enhances swine gut Lactobacillus population abundance. *British Journal of Nutrition*, 111(S1), S30-S35. *doi*:10.1017/s0007114513002274

De Longh, R. U., Wederell, E., Lovicu, F. J., & McAvoy, J. W. (2005). Transforming Growth Factor-β-Induced Epithelial-Mesenchymal Transition in the Lens: A Model for Cataract Formation. *Cells Tissues Organs*, 179(1-2), 43-55. *doi*:10.1159/000084508

De Vos, M., Huygelen, V., Willemen, S., Fransen, E., Casteleyn, C., Van Cruchten, S., … Van Ginneken, C. (2014). Artificial rearing of piglets: Effects on small intestinal morphology and digestion capacity. *Livestock Science*, 159, 165-173. *doi*:10.1016/j.livsci.2013.11.012

Elsasser, T. H., Caperna, T. J., Li, C. J., Kahl, S., & Sartin, J. L. (2008). Critical control points in the impact of the proinflammatory immune response on growth and metabolism. *Journal of Animal Science*, 86, E105-E125. *doi*:10.2527/jas.2007-0634

Fang, L. H., Jin, Y. H., Do, S. H., Hong, J. S., Kim, B. O., Han, T. H., & Kim, Y. Y. (2019). Effects of dietary energy and crude protein levels on growth performance, blood profiles, and nutrient digestibility in weaning pigs. *Asian-Australasian journal of animal sciences*, 32(4), 556-563. *doi*:10.5713/ajas.18.0294

González-Mariscal, L., Domínguez-Calderón, A., Raya-Sandino, A., Ortega-Olvera, J. M., Vargas-Sierra, O., & Martínez-Revollar, G. (2014). Tight junctions and the regulation of gene expression. *Seminars in Cell & Developmental Biology*, 36, 213-223. *doi*:10.1016/j.semcdb.2014.08.009

He, L., Zhou, X., Huang, N., Li, H., Cui, Z., Tian, J., … Yin, Y. (2017). Administration of alpha-ketoglutarate improves epithelial restitution under stress injury in early-weaning piglets. *Oncotarget*, 8(54), 91965-91978. *doi*:10.18632/oncotarget.20555

Heo, J. M., Kim, J. C., Hansen, C. F., Mullan, B. P., Hampson, D. J., & Pluske, J. R. (2009). Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of Escherichia coli1. *Journal of Animal Science*, 87(9), 2833-2843. *doi*:10.2527/jas.2008-1274

Jeong, Y. D., Ko, H. S., Hosseindoust, A., Choi, Y. H., Chae, B. J., Yu, D. J., … Kim, J. S. (2019). Lactobacillus-based fermentation product and Lactose Level in the Feed for Weanling Pigs: Effects on Intestinal Morphology, Microbiota, Gas Emission, and Targeted Intestinal Coliforms. *Livestock Science*, 227, 90-96. *doi*:10.1016/j.livsci.2019.06.018

Jiao, L. F., Song, Z. H., Ke, Y. L., Xiao, K., Hu, C. H., & Shi, B. (2014). Cello-oligosaccharide influences intestinal microflora, mucosal architecture and nutrient transport in weaned pigs. *Animal Feed Science and Technology*, 195, 85-91. *doi*:10.1016/j.anifeedsci.2014.05.014

Kim, J. C., Hansen, C. F., Mullan, B. P., & Pluske, J. R. (2012). Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Animal Feed Science and Technology*, 173(1-2), 3-16. *doi*:10.1016/j.anifeedsci.2011.12.022

Kim, J. C., Heo, J. M., Mullan, B. P., & Pluske, J. R. (2011). Efficacy of a reduced protein diet on clinical expression of post-weaning diarrhoea and life-time performance after experimental challenge with an enterotoxigenic strain of Escherichia coli. *Animal Feed Science and Technology*, 170(3-4), 222-230. *doi*:10.1016/j.anifeedsci.2011.08.012

Kim, J. S., Shinde, P. L., Yang, Y. X., Yun, K., Choi, J. Y., Lohakare, J. D., & Chae, B. J. (2010). Effects of dietary lactose levels during different starter phases on the performance of weaning pigs. *Livestock Science*, 131(2-3), 175-182. *doi*:10.1016/j.livsci.2010.03.016

Kröger, S., Pieper, R., Schwelberger, H. G., Wang, J., Villodre Tudela, C., Aschenbach, J. R., … Zentek, J. (2013). Diets high in heat treated soybean meal reduce the histamine induced epithelial response in the colon of weaned piglets and increase epithelial catabolism of histamine. *PLOS One*, 8, e80612. *doi*:10.1371/journal.pone.0080612

Kummer, R., Gonçalves, M. A. D., Lippke, R. T., Marques, B. M. F. P. P., & Mores, T. J. (2009). Factors associated with nursery pig performance. *Acta Scientiae Veterinariae*, 37, s195-s209.

Leray, V., Segain, J.-P., Cherbut, C., & Galmiche, J.-P. (2003). Adaptation to low-protein diet increases inhibition of gastric emptying by CCK. *Peptides*, 24(12), 1929-1934. *doi*:10.1016/j.peptides.2003.10.015

Leschelle, X., Robert, V., Delpal, S., Mouillé, B., Mayeur, C., Martel, P., & Blachier, F. (2002). Isolation of pig colonic crypts for cytotoxic assay of luminal compounds: effects of hydrogen sulfide, ammonia, and deoxycholic acid. *Cell Biology and Toxicology*, 18(3), 193-203. *doi*:10.1023/a:1015515821390

Levy, M., Blacher, E., & Elinav, E. (2017). Microbiome, metabolites and host immunity. *Current Opinion in Microbiology*, 35, 8-15. *doi*:10.1016/j.mib.2016.10.003

Li, D. F., Nelssen, J. L., Reddy, P. G., Blecha, F., Klemm, R., & Goodband, R. D. (1991). Interrelationship between hypersensitivity to soybean proteins and growth performance in early-weaned pigs. *Journal of Animal Science*, 69(10), 4062-4069. *doi*:10.2527/1991.69104062x

Liu, Y., Ipharraguerre, I. R., & Pettigrew, J. E. (2013). DIGESTIVE PHYSIOLOGY OF THE PIG SYMPOSIUM: Potential applications of knowledge of gut chemosensing in pig production1. *Journal of Animal Science*, 91(5), 1982-1990. *doi*:10.2527/jas.2012-6193

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, 25(4), 402-408.

Lu, J. J., Zou, X. T., & Wang, Y. M. (2008). Effects of sodium butyrate on the growth performance, intestinal microflora and morphology of weanling pigs. *Journal of Animal and Feed Sciences*, 17, 568-578. *doi*: 10.22358/jafs/66685/2008

Lynch, B., Callan, J. J., & O'Doherty, J. V. (2009). The interaction between dietary crude protein and fermentable carbohydrate source on piglet post weaning performance, diet digestibility and selected faecal microbial populations and volatile fatty acid concentration. *Livestock Science*, 124(1-3), 93-100. *doi*:10.1016/j.livsci.2009.01.003

McLamb, B. L., Gibson, A. J., Overman, E. L., Stahl, C., & Moeser, A. J. (2013). Early Weaning Stress in Pigs Impairs Innate Mucosal Immune Responses to Enterotoxigenic E. coli Challenge and Exacerbates Intestinal Injury and Clinical Disease. *PLoS ONE*, 8(4), e59838. *doi*:10.1371/journal.pone.0059838

Moeser, A. J., Pohl, C. S., & Rajput, M. (2017). Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition*, 3(4), 313-321. *doi*:10.1016/j.aninu.2017.06.003

Monteleone, G., Pallone, F., & MacDonald, T. T. (2004). Smad7 in TGF-β-mediated negative regulation of gut inflammation. *Trends in Immunology*, 25(10), 513-517. *doi*:10.1016/j.it.2004.07.008

Morales, A., Buenabad, L., Castillo, G., Vázquez, L., Espinoza, S., Htoo, J. K., & Cervantes, M. (2016). Dietary levels of protein and free amino acids affect pancreatic proteases activities, amino acids transporters expression and serum amino acid concentrations in starter pigs. *Journal of Animal Physiology and Animal Nutrition*, 101(4), 723-732. *doi*:10.1111/jpn.12515

Nyachoti, C. M., Omogbenigun, F. O., Rademacher, M., & Blank, G. (2006). Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets1. *Journal of Animal Science*, 84(1), 125-134. *doi*:10.2527/2006.841125x

O'Doherty, J. V., Dillon, S., Figat, S., Callan, J. J., & Sweeney, T. (2010). The effects of lactose inclusion and seaweed extract derived from Laminaria spp. on performance, digestibility of diet components and microbial populations in newly weaned pigs. *Animal Feed Science and Technology*, 157(3-4), 173-180. *doi*:10.1016/j.anifeedsci.2010.03.004

Parker, A., Maclaren, O. J., Fletcher, A. G., Muraro, D., Kreuzaler, P. A., Byrne, H. M., … Pin, C. (2017). Cell proliferation within small intestinal crypts is the principal driving force for cell migration on villi. *The FASEB Journal*, 31(2), 636-649. *doi*:10.1096/fj.201601002

Pei, X., Xiao, Z., Liu, L., Wang, G., Tao, W., Wang, M., … Leng, D. (2019). Effects of Dietary Zinc Oxide Nanoparticles Supplementation on Growth Performance, Zinc Status, Intestinal Morphology, Microflora Population, and Immune Response in Weaned Pigs. *Journal of the Science of Food and Agriculture*, 99(3), 1366-1374. *doi*:10.1002/jsfa.9312

Pié, S., Lallès, J. P., Blazy, F., Laffitte, J., Sève, B., & Oswald, I. P. (2004). Weaning Is Associated with an Upregulation of Expression of Inflammatory Cytokines in the Intestine of Piglets. *The Journal of Nutrition*, 134(3), 641-647. *doi*:10.1093/jn/134.3.641

Pieper, R., Vahjen, W., & Zentek, J. (2016b). Intestinal lactose and mineral concentration affect the microbial ecophysiology along the gastrointestinal tract of formula-fed neonatal piglets1. *Journal of Animal Science*, 94(9), 3786-3795. *doi*:10.2527/jas2016-0459

Pieper, R., Villodre Tudela, C., Taciak, M., Bindelle, J., Pérez, J. F., & Zentek, J. (2016a). Health relevance of intestinal protein fermentation in young pigs. *Animal Health Research Reviews*, 17(2), 1-11. *doi*:10.1017/S1466252316000141

Pierce, K. M., Callan, J. J., McCarthy, P., & O'Doherty, J. V. (2007). The interaction between lactose level and crude protein concentration on piglet post-weaning performance, nitrogen metabolism, selected faecal microbial populations and faecal volatile fatty acid concentrations. *Animal Feed Science and Technology*, 132(3-4), 267-282. *doi*:10.1016/j.anifeedsci.2006.02.010

Pierce, K. M., Sweeney, T., Brophy, P. O., Callan, J. J., Fitzpatrick, E., McCarthy, P. & O'Doherty, J. V. (2006a). The effect of lactose and inulin on intestinal morphology, selected microbial populations and volatile fatty acid concentrations in the gastro-intestinal tract of the weanling pig. *Animal Science*, 82(3), 311-318. *doi*:10.1079/ASC200634

Pierce, K. M., Sweeney, T., Callan J. J., Byrne, C., McCarthy P. & O'Doherty, J. V. (2006b). The effect of inclusion of a high lactose supplement in finishing diets on nutrient digestibility, nitrogen excretion, volatile fatty acid concentrations and ammonia emission from boars. *Animal Feed Science and Technology*, 125(1–2), 45-60. *doi*:10.1016/j.anifeedsci.2005.05.016

Pluske, J. R., Turpin, D. L., & Kim, J. C. (2018). Gastrointestinal tract (gut) health in the young pig. *Animal Nutrition*, 4(2), 187-196. *doi*:10.1016/j.aninu.2017.12.004

Rist, V. T. S., Weiss, E., Eklund, M., & Mosenthin, R. (2013). Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: a review. *Animal*, 7(7), 1067–1078. *doi*:10.1017/S1751731113000062

Rostagno, H. S., Albino, L. F. T., Donzele, J. L., Gomes, P. C., Oliveira, R. F., Lopes, D. C., ... Euclides, R. F. (2011). *Brazilian tables for poultry and swine: Composition of feedstuffs and nutritional requirementes* (3rd ed.). Viçosa, MG, Brasil: Editora UFV.

Sigalet, D. L., Brindle, M., Boctor, D., Casey, L., Dicken, B., Butterworth, S., … Holst, J. (2015). A Safety and Dosing Study of Glucagon-Like Peptide 2 in Children With Intestinal Failure. *Journal of Parenteral and Enteral Nutrition*, 41(5), 844-852. *doi*:10.1177/0148607115609566

Soares, M. H., Rodrigues, G. A., Barbosa, L. M. R., Valente Júnior, D. T., Santos, F. C., Rocha, G. C., ... Saraiva, A. (2020). Effects of crude protein and lactose levels in diets on growth performance , intestinal morphology , and expression of genes related to intestinal integrity and immune system in weaned piglets. *Animal Science Journal*, 91, 1-11. *doi*:10.1111/asj.13429

Soler, L., Gutiérrez, A., Müllebner, A., Cerón, J. J., & Duvigneau, J. C. (2013). Towards a better understanding of salivary and meat juice acute phase proteins determination in pigs: An expression study. *Veterinary Immunology and Immunopathology*, 156(1-2), 91-98. *doi*:10.1016/j.vetimm.2013.09.018

Tian, Z., Ma, X., Yang, X., Fan, Q., Xiong, Y., Qiu, Y., … Jiang, Z. (2016). Influence of low protein diets on gene expression of digestive enzymes and hormone secretion in the gastrointestinal tract of young weaned piglets. *Journal of Zhejiang University-SCIENCE B*, 17(10), 742-751. *doi*:10.1631/jzus.b1600229

Unger, R. E., Oltrogge, J. B., Briesen, H. V., Engelhardt, B., Woelki, U., Schlote, W., … Kirkpatrick, C. J. (2002). Isolation and molecular characterization of brain microvascular endothelial cells from human brain tumors. *In Vitro Cellular & Developmental Biology - Animal*, 38(5), 273-281. *doi*:10.1290/1071-2690(2002)038<0273:iamcob>2.0.co;2

Venema, K. (2012). Intestinal fermentation of lactose and prebiotic lactose derivatives, including human milk oligosaccharides. *International Dairy Journal*, 22(2), 123-140. *doi*:10.1016/j.idairyj.2011.10.011

Wen, X., Wang, L., Zheng, C., Yang, X., Ma, X., Wu, Y., … Jiang, Z. (2018). Fecal scores and microbial metabolites in weaned piglets fed different protein sources and levels. *Animal Nutrition*, 4(1), 31-36. *doi*:10.1016/j.aninu.2017.10.006

Wu, Y., Jiang, Z., Zheng, C., Wang, L., Zhu, C., Yang, X., … Ma, X. (2015). Effects of protein sources and levels in antibiotic-free diets on diarrhea, intestinal morphology, and expression of tight junctions in weaned piglets. *Animal Nutrition*, 1(3), 170-176. *doi*:10.1016/j.aninu.2015.08.013

Yang, K. M., Jiang, Z. Y., Zheng, C. T., Wang, L., & Yang, X. F. (2014). Effect of Lactobacillus plantarum on diarrhea and intestinal barrier function of young piglets challenged with enterotoxigenic Escherichia coli K88. *Journal of Animal Science*, 92(4), 1496-1503. *doi*:10.2527/jas.2013-6619

Yue, L. Y., & Qiao, S. Y. (2008). Effects of low-protein diets supplemented with crystalline amino acids on performance and intestinal development in piglets over the first 2 weeks after weaning. *Livestock Science*, 115(2-3), 144-152. *doi*:10.1016/j.livsci.2007.06.018

Table 1. Composition of experimental diets

Ingredient, %	Crude protein, %					
	20.0			24.0		
	Lactose, %					
	8.0	12.0	16.0	8.0	12.0	16.0
Corn	42.79	42.79	42.79	31.80	31.80	31.80
Soybean meal	18.50	18.50	18.50	30.00	30.00	30.00
Starch	9.00	4.92	0.85	9.00	4.92	0.85
Lactose	8.17	12.25	16.32	8.17	12.25	16.32
Micronized soybean	12.00	12.00	12.00	12.00	12.00	12.00
Blood plasma	4.00	4.00	4.00	4.00	4.00	4.00
Soybean oil	1.10	1.10	1.10	1.40	1.40	1.40
Dicalcium phosphate	1.70	1.70	1.70	1.60	1.60	1.60
Limestone	0.92	0.92	0.92	0.92	0.92	0.92
Salt	0.45	0.45	0.45	0.45	0.45	0.45
L-lysine, 78.0%	0.40	0.40	0.40	0.05	0.05	0.05
Vitamin premix[a]	0.30	0.30	0.30	0.30	0.30	0.30
DL-methionine, 99.0%	0.21	0.21	0.21	0.11	0.11	0.11
Mineral premix[b]	0.20	0.20	0.20	0.20	0.20	0.20
Antioxidant	0.01	0.01	0.01	0.01	0.01	0.01
L-threonine, 98.5%	0.175	0.175	0.175	-	-	-
L-valine, 96.5%	0.065	0.065	0.065	-	-	-
L-tryptophan, 99.0%	0.020	0.020	0.020	-	-	-
Calculated nutritional composition[c]						
Calcium, %	0.850	0.850	0.850	0.850	0.850	0.850
ME, kcal/kg	3400	3400	3400	3400	3400	3400
Available phosphorus, %	0.450	0.450	0.450	0.450	0.450	0.450
Lactose, %	8.00	12.00	16.00	8.0	12.000	16.00
SID Lysine, %	1.350	1.350	1.350	1.350	1.350	1.350
SID Met+Cis, %	0.756	0.756	0.756	0.756	0.756	0.756
Crude protein, %	20.00	20.00	20.00	24.00	24.00	24.00
Sodium, %	0.280	0.280	0.280	0.280	0.280	0.280
SID threonine, %	0.850	0.850	0.850	0.850	0.850	0.850
SID tryptophan, %	0.243	0.243	0.243	0.285	0.285	0.285
SID valine, %	0.931	0.931	0.931	1.071	1.071	1.071
SID isoleucine, %	0.747	0.747	0.747	0.941	0.941	0.941

[a]Content per kilogram of product: folic acid (125.00 mg), calcium pantothenate (4,000.00 mg), biotin (12.50 mg), niacin (825.00 mg), vitamin B6 (250.00 mg), vitamin B2 (1,350.00 mg), selenium (75.00 mg), vitamin B1 (250.00 mg), vitamin A (2,100,000 U.I.), vitamin B12 (6,000.00 mcg), vitamin D3 (350.000 U.I.), vitamin E (5,000 U.I.), vitamin K3 (850.00 mg).

[b]Content per kilogram of product: Fe (20.0g), Cu (56.0g), I (400.0 mg), Zn (32.0 g), Mn (14.0 g).

[c]Values calculated according to Rostagno et al. (2011).

Abbreviation: SID, standardized ileal digestible.

Table 2. Oligonucleotides used on gene expression analysis of cytokines and tight junctions' proteins

Genes	GenBank number	Sequence	Size, bp
GAPDH	NM_001206359.1	F:´CAAAGTGGACATTGTCGCCATCA´ R:´AGCTTCCCATTCTCAGCCTTGACT´	124
TNF-α	NM_214022.1	F:5′CATCGCCGTCTCCTACCA3′ R:5′CCCAGATTCAGCAAAGTCCA3′	199
IL-1β	NM_214055.1	F:5′TCTGCCCTGTACCCCAACTG3′ R:5′CCCAGGAAGACGGGCTTT3′	132
IL-6	NM_001252429.1	F:5′CCTGTCCACTGGGCACATAAC3′ R:5′CAAGAAACACCTGGCTCTGAAAC3′	253
Hp	NM_214000.2	F:5'GCTAAGAATCTCCGCTTGG3' R:5'CAATCTCCACCTCCTGTTTC3'	100
TGF β1	NM_214015.1	F:5′GGACCTTATCCTGAATGCCTT3′ R:5′TAGGTTACCACTGAGCCACAAT3′	133
Occludin	NM_001163647.1	F:5′ TCCTGGGTGTGATGGTGTTC3′ R:5′ CGTAGAGTCCAGTCACCGCA3′	145
ZO-1	XM_003353439.2	F:5′AAGCCCTAAGTTCAATCACAATCT3 ′ R:5′ ATCAAACTCAGGAGGCGGC3′	130

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehyidrogenase; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; Hp, haptoglobin; TGF-β1, transforming growth factor beta 1; ZO-1, zonula occludens-1.

Table 3. Effects of crude protein (CP) and lactose (LAC) on performance and serum urea nitrogen concentration (SUN) of piglets from 21 to 35 days of age

CP (%)	LAC (%)			Mean	P-value			SEM
	8.0	12.0	16.0		CP	LAC	CP x LAC	(n=10)
				Initial body weight, kg				
20.0	7.88	7.86	7.90	7.88				
24,0	7.87	7.88	7.87	7.87	0.914	0.957	0.933	0.061
Mean	7.87	7.87	7.88					
				Final body weight, kg				
20.0	10.98	11.41	11.44	11.28				
24.0	11.06	11.82	11.47	11.45	0,305	0,018	0,603	0,208
Mean	11.02b	11.61a	11.46ab					
				Average daily feed intake, kg				
20.0	0.285	0.330	0.335	0.317 B				
24.0	0.313	0.355	0.351	0.340 A	0.048	0.003	0.899	0.014
Mean	0.299b	0.343a	0.343a					
				Average daily gain, kg				
20.0	0.222	0.254	0.254	0.243				
24.0	0.229	0.282	0.270	0.260	0.142	0.007	0.739	0.014
Mean	0.225b	0.268a	0.262a					
				Feed:gain ratio				
20.0	1.32	1.31	1.33	1.32				
24.0	1.39	1.27	1.33	1.33	0.741	0.226	0.268	0.037
Mean	1.35	1.29	1.33					
				SUN (mg/dl)				
20.0	19.15	18.53	17.63	18.44B				
24.0	27.63	25.02	25.57	26.07A	<0.001	0.274	0.699	1.177
Mean	23.39	21.78	21.60					

[A,B]Means followed by different uppercase letters in the lines differ ($p < .05$) from each other by Tukey test.
[a,b]Means followed by different lowercase letters in the lines differ ($p < .05$) from each other by Tukey test.
Abbreviations: CP, crude protein; LAC, lactose; SEM, standard error of the mean.

Table 4. Intestinal morphology of piglets 14 days post-weaning fed diets with different levels of lactose (LAC) and crude protein (CP)

CP (%)	LAC (%) 8.0	12.0	16.0	Média	P-value CP	LAC	CP x LAC	SEM (n=10)
			Duodenum villous height, µm					
20.0	241.346	248.399	252.511	247.418				
24.0	227.185	281.317	287.866	265.456	0.105	0.020	0.125	13.411
Média	234.265 b	264.858ab	270.188 a					
			Duodenum crypt depth, µm					
20.0	132.256a	141.732a	150.448a	141.478				
24.0	171.255a	135.180b	142.750b	149.728	0.064	0.051	<0.001	5.345
Média	151.755	138.456	146.599					
			Duodenum villous height:crypt depth					
20.0	1.876a	1.819a	1.711a	1.802				
24.0	1.366b	2.138a	2.076a	1.860	0.274	<0.001	<0.001	0.064
Média	1.621	1.978	1.893					
			Jejunum villous height, µm					
20.0	221.071	237.560	226.008	228.213				
24.0	208.201	225.373	223.127	218.901	0.329	0.351	0.890	11.577
Média	214.636	231.467	224.568					
			Jejunum crypt depth, µm					
20.0	109.792	119.440	112.890	114.041				
24.0	124.122	109.931	117.572	117.208	0.349	0.847	0.019	4.110
Média	116.957	114.685	115.231					
			Jejunum villous height:crypt depth					
20.0	2.061	2.043	2.029	2.044				
24.0	1.726	2.068	1.939	1.911	0.090	0.237	0.162	0.095
Média	1.893	2.056	1.984					
			Ileum villous height, µm					
20.0	172.518	190.873	184.764	182.718				
24.0	156.420	175.759	187.918	173.366	0.109	0.006	0.313	7.038
Média	164.469b	183.316a	186.341a					
			Ileum crypt depth, µm					
20.0	102.779a	113.101a	108.006a	107.962				
24.0	118.183a	102.287b	107.513ab	109.328	0.686	0.743	0.009	4,116
Média	110.481	107.691	107.759					
			Ileum villous heigth:crypt depth					
20.0	1.739a	1.738a	1.786a	1.754				
24.0	1.348b	1.765a	1.781a	1.631	0.013	<0.001	0.001	0.059
Média	1.543	1.752	1.783					

[A,B]Means followed by different uppercase letters in the lines differ (p < .05) from each other by Tukey test.

[a.b]Means followed by different lowercase letters in the lines differ (p < .05) from each other by Tukey test.

Abbreviations: CP, crude protein; LAC, lactose; SEM, standard error of the mean.

Table 5. Gene expression of cytokines and tight junction proteins in the jejunum of piglets 14 days post-weaning fed diets with different levels of lactose (LAC) and crude protein (CP)

CP (%)	LAC (%)			Mean	P-value			SEM (n=8)
	8.0	12.0	16.0		CP	LAC	CP x LAC	
				IL-6				
20.0	2.502	2.342	3.012	2.619				
24.0	3.732	2.909	2.537	3.059	0.103	0.307	0.035	0.317
Mean	3.117	2.626	2.775					
				TNFα				
20.0	3.233	3.480	3.985	3.566				
24.0	4.345	3.397	2.493	3.412	0.684	0.484	0.026	0.458
Mean	3.789	3.438	3.239					
				IL-1β				
20.0	2.242	2.743	2.873	2.619				
24.0	3.412	2.915	1.867	2.731	0.765	0.514	0.070	0.454
Mean	2.827	2.829	2.370					
				Haptoglobin				
20.0	1.721a	1.759a	1.944a	1.808				
24.0	2.403a	1.984ab	1.641b	2.009	0.168	0.286	0.025	0.172
Mean	2.062	1.872	1.792					
				TGF β1				
20.0	2.283	2.573	2.041	2.299				
24.0	2.638	2.525	2.259	2.474	0.381	0.236	0.701	0.225
Média	2.460	2.549	2.150					
				Occludin				
20.0	4.691	5.057	5.045	4.931				
24.0	5.546	5.384	3.829	4.920	0.978	0.309	0.156	0.529
Mean	5.119	5.221	4.437					
				ZO-1				
20.0	2.618a	3.048a	3.164a	2.944				
24.0	3.646a	3.111ab	2.369b	3.042	0.687	0.404	0.012	0.288
Mean	3.132	3.080	2.766					

[A,B]Means followed by different uppercase letters in the lines differ ($p < .05$) from each other by Tukey test.

[a,b]Means followed by different lowercase letters in the lines differ ($p < .05$) from each other by Tukey test.

Abbreviations: CP, crude protein; LAC, lactose; SEM, standard error of the mean; TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; TGF-β1, transforming growth factor beta 1; ZO-1, zonula occludens-1.

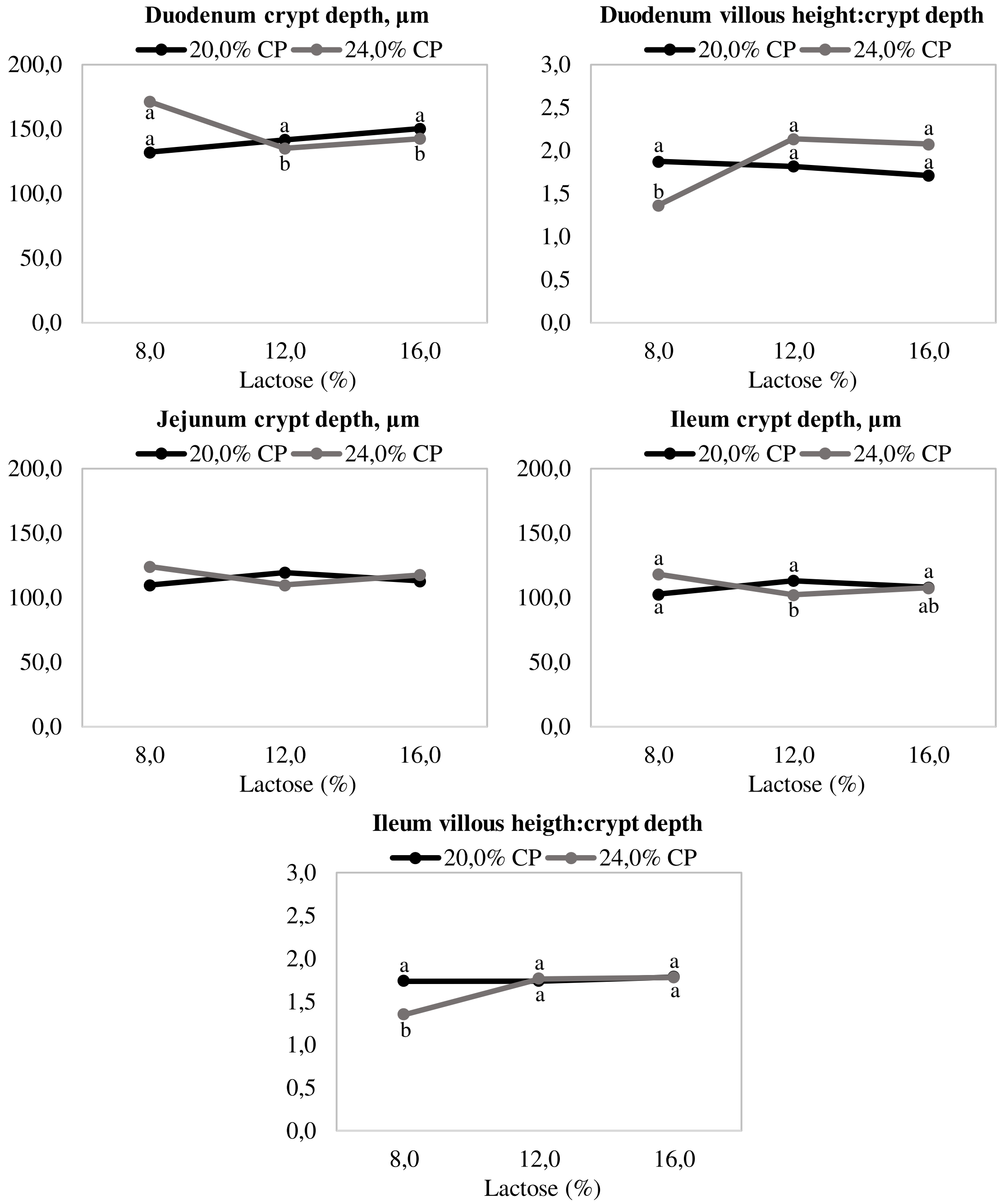

Figure 1. Intestinal morphology of piglets at 35 days of age according to crude protein (CP) and lactose in the diets. Means followed by distinct lowercase letters differ from each other by Tukey test (P<0.05).

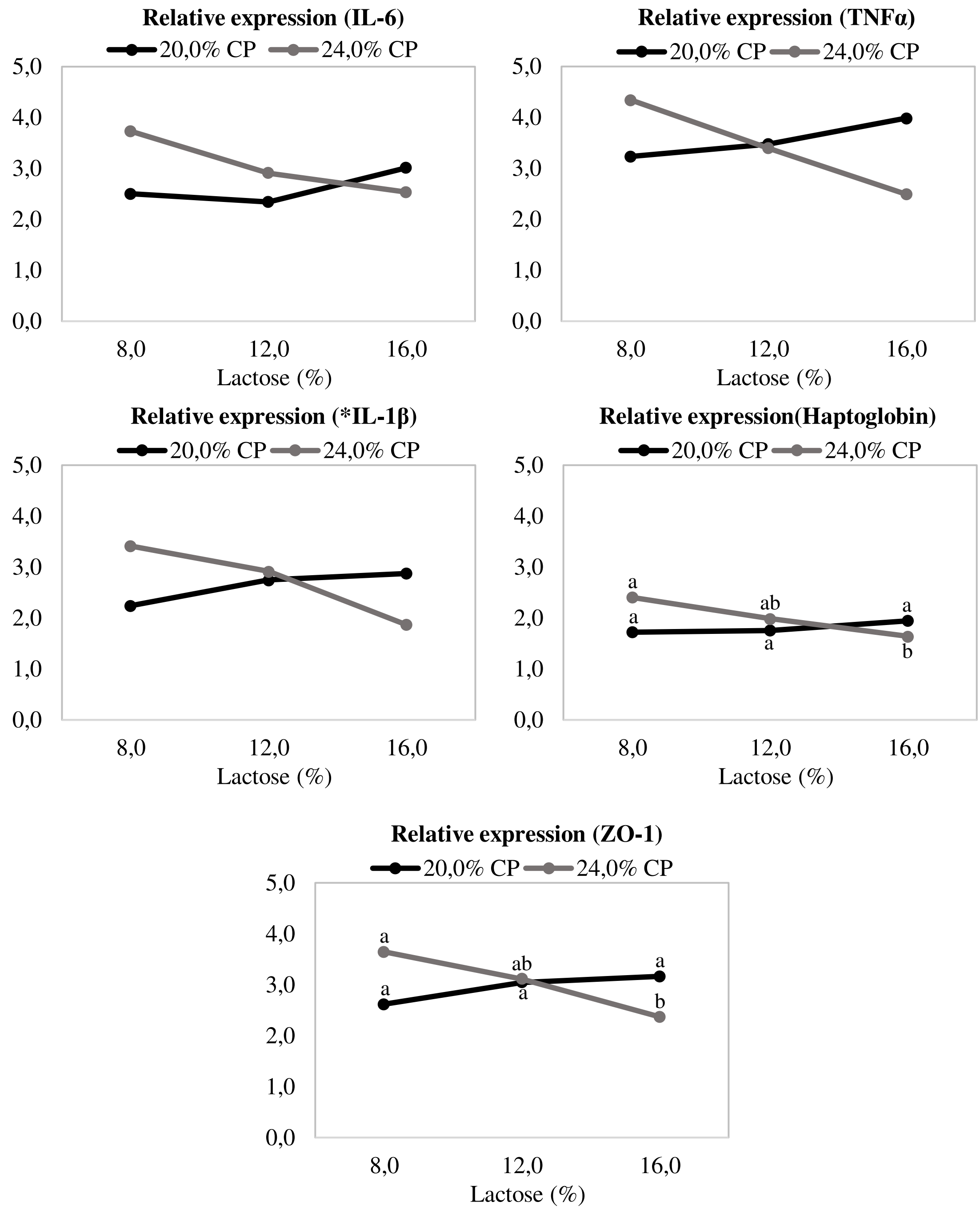

Figure 2. Intestinal gene expression of piglets at 35 days of age according to crude protein (CP) and lactose in the diets. Means followed by distinct lowercase letters differ from each other by Tukey test (P<0.05). Abbreviations: TNFα, tumor necrosis factor alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; ZO-1, zonula occludens-1.

ANEXO

UNIVERSIDADE FEDERAL DE VIÇOSA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS DE PRODUÇÃO
CEUAP/UFV

Campus Universitário – Viçosa, MG – 36570-900 – Telefone:(31) 3899.3275 – e-mail: ceuap@ufv.br – site: www.ceuap.ufv.br

Viçosa, 12 de Dez. de 2018

CERTIFICADO

Certificamos que o projeto intitulado "**Níveis de proteína bruta e de lactose em rações para leitões desmamados**", protocolo **nº 0100/2018**, sob a responsabilidade de **Alysson Saraiva** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo chordata, subfilo vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 8 de outubro de 2008, do decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo conselho nacional de controle da experimentação animal (concea), e foi apreciado pela comissão de ética no uso de animais de produção da universidade federal de viçosa (ceuap-ufv) em reunião de **30 de Nov. de 2018**.

Finalidade: (**X**)**Pesquisa** ( )**Ensino**

Vigência do Projeto: de **12 de Dez. de 2018** a **31 de Dez. de 2018**

Espécie/linhagem: **Suínos** (***Sus scrofa domesticus***) Nº de animais: **144**

Peso: +/- **6 kg** Idade: **21 dias** Sexo: **Macho/Femea** Origem: **Setor de Suinocultura/DZO/UFV - CNPJ/CPF: 25.944.455/0001-96**

CERTIFICATE

We certify that the project entitled "**Lactose and crude protein levels in diets for weaned piglets**", protocol **nº 0100/2018**, under the responsibility of **Alysson Saraiva** - which involves the production, maintenance and/or use of animals belonging to the phylum chordata, subphylum vertebrata (except man), for scientific research purposes (or education) - is in accordance with the law nº. 11.794, of October 8, 2008, Decree nº. 6899 of July 15, 2009, and the rules issued by the Brazilian National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on the use of farm animals of Universidade Federal de Viçosa (CEUAP-UFV) in its meeting on **Nov, 30th, 2018**.

Finality: (**X**)**Research** ( )**Education**

Duration of the Project: from **Dec, 12th, 2018** to **Dec, 31th, 2018**.

Species / strain: **Swine** (***Sus scrofa domesticus***) Nº of animals: **144**

Weight: +/- **6 kg** Age: **21 days** Sex: **Male/ Female** Source: **Setor de Suinocultura/DZO/UFV - CNPJ/CPF: 25.944.455/0001-96**

Luciana Navajas Rennó
Coordenadora da CEUAP/UFV