JÉSSICA APARECIDA BARBOSA

PERFORMANCE AND INTESTINAL HEALTH OF PIGLETS WEANED AT 21 OR 25 DAYS OF AGE CHALLENGED WITH *ESCHERICHIA COLI K88* $^+$

LAVRAS-MG

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Master's dissertation presented to the University Federal of Lavras, as part of the requirements of the Graduate Program in Animal Science, area of concentration in Production and Nutrition of Non-Ruminants, to obtain the title of Master.

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Approved in May 28, 2019.

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2019

À minha família, em especial, à minha mãe,
Nely, pelo apoio, incentivo, compreensão e
por ser o meu maior exemplo de vida.

Dedico

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ABSTRACT

The objective of this study was to evaluate the effects of weaning at the 21 and 25 days old of piglets challenged or not by *Escherichia coli K88*$^{+}$ and its effects on the performance, diarrhea incidence, intestinal morphometry, microbiological counts and volatile fatty acids concentration on cecal content in the first weeks of nursery phase. A total of 84 barrows (DB90 × PIC337) were divided into two groups: 42 animals weaned at 21 days (5.2 ± 0.3 kg) and 42 animals weaned at 25 days (6.8 ± 0.3 kg). The experimental design was a 2x2 factorial, two weaning ages (21 x 25 days), and with and without challenge, totaling four treatments, seven replicates and three piglets per pen. The experimental period was 24 days. On days 4 and 5, the challenged group received orally 1 mL of the solution with the concentration of 10^6 CFU/ mL *E. coli K88*$^{+}$. Data were analyzed using SAS software (P ≤ 0.05 and trend 0.05 ≤ 0.10 and the Tukey test used for comparison of means. At four experimental days, the animals weaned at 25 days old had higher BW (P <.0001), ADG (P =0.0006) and ADFI (P<.0001) compared the animals weaned at 21 days old. Piglets weaned at 25 days had higher ADG and ADFI throughout the experimental period (P<0.0001). The challenged group presented lower ADFI (P=0.05) than the non-challenged group. From 0 to 4 experimental days, weaning at 21 days resulted in a higher incidence of diarrhea (P = 0.0001) compared to weaning at 25 days. From 4 to 12 days, higher incidence of diarrhea occurred in non-challenged animals (P=0.03) than challenged. Piglets weaned at 25 days had higher villus height in the jejunum (P= 0.0345) and ileum (P = 0.0421) than piglets of 21 days. There was a higher *E. coli* count in piglets weaned at 21 days compared to 25 days weaning (P = 0.039). Age and challenge did not affect the *Lactobacillus* count (P> 0.05). The production of acetic, propionic and butyric volatile fatty acids (AGV) was higher (P <0.05) in the inoculated groups compared to the not inoculated groups. In conclusion, weaning at 25 days of age improves performance, decrease diarrhea incidence and *E.coli* in the first days of nursery phase, suggesting greater maturity and adaptation to stressors.

Key words: intestinal morphometry, physiological maturity, piglet, weaning

RESUMO

O objetivo deste estudo foi avaliar os efeitos do desmame aos 21 e 25 dias de idade de leitões desafiados ou não por *Escherichia coli* K88+ e seus efeitos sobre o desempenho, incidência de diarreia, morfometria intestinal, contagem microbiológica e concentração de ácidos graxos voláteis no ceco nas primeiras semanas da fase de creche. Um total de 84 suínos machos (DB90 × PIC337) foram divididos em dois grupos: 42 animais desmamados aos 21 dias (5,2 ± 0,3 kg) e 42 animais desmamados aos 25 dias (6,8 ± 0,3 kg). O delineamento experimental utilizado foi o fatorial 2x2, com duas idades ao desmame (21 x 25 dias), com e sem desafio, totalizando quatro tratamentos, sete repetições e três leitões por baia. O período experimental foi de 24 dias. Nos dias 4 e 5, o grupo desafiado recebeu por via oral 1 mL da solução com a concentração de 106 UFC / mL de *E. coli K88* +. Os dados foram analisados por meio do software SAS ($P \leq 0,05$ e tendência $0,05 \leq 0,10$) e o teste de Tukey para comparação de médias. Aos quatro dias experimentais, os animais desmamados aos 25 dias apresentaram maior peso corporal ($P < 0,0001$), GPD ($P = 0,0006$) e CRMD ($P < 0,0001$) comparado aos animais desmamados aos 21 dias de vida. Leitões desmamados aos 25 dias apresentaram maior GPD e CRMD ao longo do período experimental ($P < 0,0001$). O grupo desafiado apresentou menor CRMD ($P = 0,05$) comparado ao não desafiado. De 0 a 4 dias experimentais, o desmame aos 21 dias resultou em maior incidência de diarreia ($P = 0,0001$) em relação ao desmame aos 25 dias, sendo que, entre 4 e 12 dias, ocorreu maior incidência de diarreia nos animais não desafiados ($P = 0,03$). Leitões desmamados aos 25 dias apresentaram maior altura das vilosidades no jejuno ($P = 0,0345$) e no íleo ($P = 0,0421$) e menor contagem de *E.coli* ($P = 0,039$) em comparação aos 25 dias de desmame. A idade e o desafio não afetaram a contagem de *Lactobacillus spp*.($P > 0,05$). A produção dos ácidos graxos voláteis acéticos, propiônicos e butíricos (AGV) foi maior ($P < 0,05$) nos grupos inoculados em comparação aos não inoculados. Em conclusão, o desmame aos 25 dias de idade melhora o desempenho, diminui a incidência de diarréia e a contagem de *E.coli* nos primeiros dias da fase de creche, sugerindo maior maturidade e adaptação aos estressores.

Palavras-chave: idade de desmame, leitão, maturidade fisiológica, morfometria intestinal

DESEMPENHO E SAÚDE INTESTINAL DE LEITÕES DESMAMADOS AOS 21 OU 25 DIAS DE IDADE E DESAFIADOS OU NÃO COM *ESCHERICHIA COLI* K88+

Elaborado por **Jéssica Aparecida Barbosa** e orientado por **Vinícius de Souza Cantarelli**

Na natureza, o desmame dos leitões acontece por volta das 12-17 semanas de idade. No entanto, na suinocultura moderna os leitões são separados de suas mães, misturados com outras leitegadas em um ambiente novo e deixam de se alimentar de leite, passando a receber uma ração seca em torno de três semanas de idade. Todas essas mudanças tornam o processo de desmame altamente estressante para os leitões, o qual resulta em aumento de diarreia e consequente perda de peso. A idade ao desmame é um fator que pode contribuir para minimizar este quadro, pois permite melhor desenvolvimento fisiológico e imunológico desses animais. Assim, avaliamos os efeitos do desmame aos 21 e 25 dias de idade sobre parâmetros de desempenho e saúde intestinal de leitões na creche. Um grupo de leitões foi desmamado aos 21 dias e outro aos 25 dias de idade, onde metade dos leitões de cada idade recebeu um inóculo de *E. coli* $k88^+$ via oral. Foi avaliado o desempenho, microbiologia, incidência de diarreia e integridade intestinal. Em geral, os resultados obtidos neste estudo demonstraram que o desmame aos 25 dias aumenta o consumo de ração, o ganho de peso, promove maior altura de vilosidades e como consequência reduz a incidência de diarreia nos primeiros dias em comparação com o desmame aos 21 dias. Portanto, desmamar os leitões com maior idade permite melhor desenvolvimento do sistema digestório, melhor aproveitamento de nutrientes e melhor desempenho nas primeiras semanas de creche.

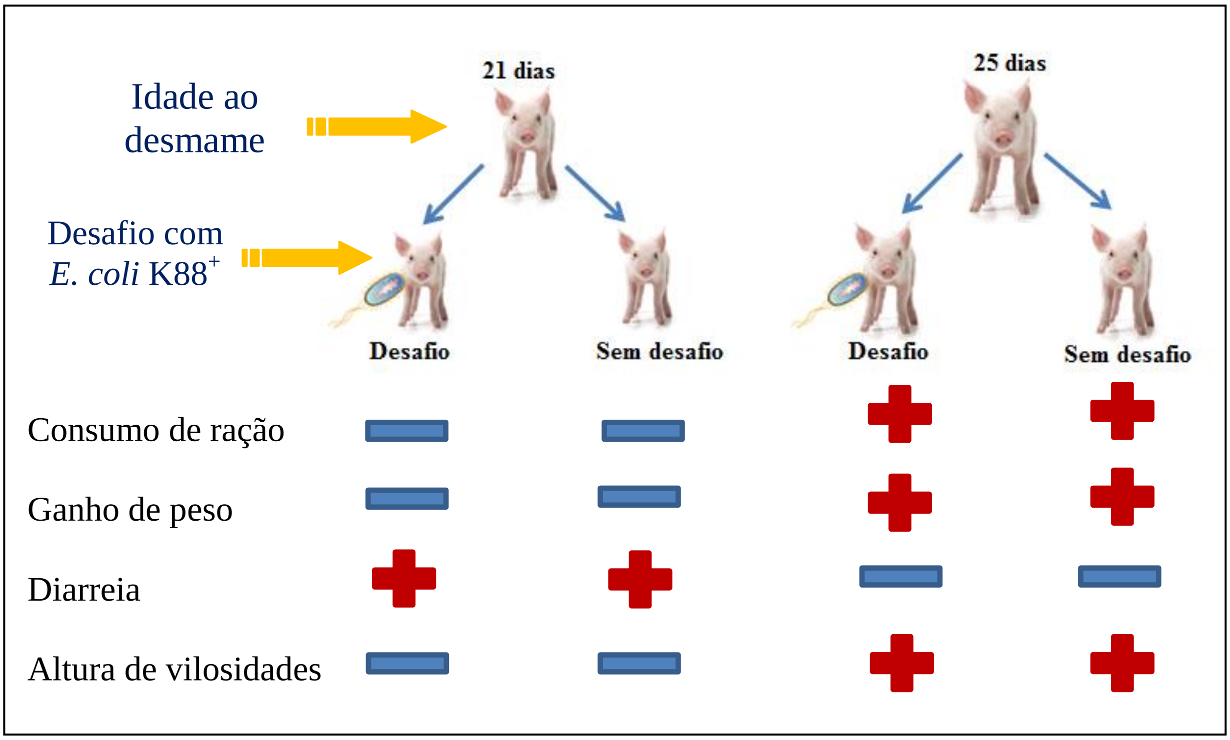

Figura1. Principais efeitos do desmame aos 21 ou 25 dias de idade de leitões desafiados ou não com *E.coli* K88+ nas primeiras semanas de creche.

Dissertação de mestrado em Zootecnia na UFLA, defendida em 28 de maio, 2019.

LIST OF FIGURES

LIST OF TABLES

-

SUMMARY

FIRST PART

1. INTRODUCTION

Weaning is a natural process that occurs around 12 to 17 weeks of age of the pig in the nature. However, in intensive production, the age of weaning was reduced to 3 weeks of life. This practice has brought benefits to the reproductive efficiency of sows by increasing the number of weaned piglets/sow/year and optimizing facilities.

Due to the hyperprolificity of the sows, the number of light-born piglets has progressively increased in the world pig husbandry; animals that present digestive, physiological and immunological immaturity than to the others of the litter. In addition, these prematurely weaned piglets are more difficult to adapt to the stressful effects of weaning when they are performed around 21 days and require a more complex pre-starter diet, including ingredients that increase the cost of the feed.

Weaning is an intrinsic stage in the pig farm production and one of the main challenges facing the piglet. Stressors such as maternal separation, changing facilities and ambient temperature, and changes in the type of diet, combined with the immaturity of the immune and digestive systems, result in negative impacts on piglets, such as changes in intestinal integrity and increased inflammatory response.

One of the main aggravating factors in this critical period is the low feed and water intake in the first nursery days, leading to a higher incidence of classic post-weaning diarrhea associated with *Escherichia coli K88*$^{+}$ (ETEC) proliferation which generates significant losses of performance. The use of nutritional strategies in this phase will not maximize the performance of the piglets if the intake pattern is not ideal in the first days of nursery phase.

Nutritional and management strategies as increasing weaning age (from 21 to 25 days, for example), can minimize the damages caused by the early management, since it allows more time for the physiological and immunological development. Better feed intake in the first days after weaning will promote greater intestinal health and consequently better performance in the first weeks of nursery.

The wean age is directly related to the duration and intensity of the factors mentioned above. Recent research has compared the performance and intestinal health of weaned piglets at 14, 18, 21, 28, 35 or 42 days, with positive results when it is performed over 21 days. There are still no studies assessing weaning at 25 days of age compared to weaning at 21 days in a challenging situation.

The objective of this study was to evaluate the effects of weaning at 21 and 25 days of age on performance and intestinal health parameters of piglets challenged or not with *Escherichia coli K88*$^{+}$ during the first weeks of the nursery phase.

2. BACKGROUND

2.1 Characteristics of the post-weaning period

The weaning process is considered the most challenging moment in pig life. Several environmental, psychosocial and nutritional stressors added to immature digestive and immune systems promotes gastrointestinal dysfunction and consequent break growth performance in piglets (MONTAGNE et al., 2007; EVERAERT et al., 2017).

Psychological stressors such as mother's separation, mixture of litter and establishment of a new social hierarchy with changes of environment and temperature, promotes damage to the intestinal architecture and decrease intestinal barrier functions (KIM et al.2012), favoring the translocation of pathogens in the gut mucosa (MOESER et al., 2007a, SMITH et al., 2010) leading to post-weaning diarrhea classical in the production system (SUN et al., 2017).

Among the stressors, diet change is one of the most important events that occur in this period. The transition of milk highly digestible by new diet formulated with cereal-based ingredients with lower digestibility and palatability results in low voluntary feed intake and starvation period. This transient anorexia lead to the low energy intake in the first week (VAN BEERSCHREURS and BRUININX, 2002), causing structural changes in the intestine, such as villus atrophy and cryptic hyperplasia which decrease in the secretion and activity enzymes present on the brush border of enterocytes (MARION et al., 2005), compromising the ability of digestion and absorption of nutrients (LALLÈS et al., 2004; BÄUMLER et al., 2016).

According to Le Dividich et al. (2000), between 3 and 4 weeks of age the voluntary metabolizable energy intake (ME) varies around 60-70% of the energy intake from milk in the pre-weaning period. Energy requirements for body maintenance are met after the third day of weaning and it would take approximately two weeks for piglets to fully recover their energy intake during the lactation period.

Brooks et al. (2003) reported that after weaning, 50% of the weaned piglets eat for the first time whitin 24 hours and 10% eat only after 48 hours. Water and feed fasting 24 hours after weaning leads to significant impacts on the performance and intestinal mucosal structure, for example, villous atrophy and crypts hyperplasia in the small intestine (HORN et al., 2014). These changes related to the low feed intake after weaning are characterized as acute and transient, in which shortly after the recovery in feed intake occurs a period of intestinal maturation (BOUDRY et al., 2004b).

Over the postnatal period, the gastrointestinal tract of piglets have important maturation processes with rapid changes in size, digestive barrier, fast rate of protein turnover, changes in microbiota composition and immunological functions (SMITH et al., 2010; KIM et al., 2012). These processes present a high degree of plasticity and are modified by environmental stimulus. Thus, the events that occur during weaning can shape the gastrointestinal function (MOESER et al., 2017), characterized by morphological, enzymatic and inflammatory alterations (BOUDRY et al., 2004b; WIJTTEN et al., 2011).

During lactation, intake of highly digestible and high lactose milk promotes the growth of lactic acid bacteria which naturally decrease stomach pH by the production of lactic acid as a result of bacterial fermentation (SURYANAYANA et al., 2012). At weaning, the reduction of lactose in the diet decreasing lactic acid associated with the physiological inability to secrete enough hydrochloric acid raises the stomach pH, favoring growth and proliferation of pathogenic bacteria such as *Escherichia coli* and also the decrease in digestion and absorption of nutrients (VIOLA & VIEIRA, 2003; CHAMONE et al., 2010).

The maintenance of low gastric pH (2.0 - 4.0) is ideal both to allow the conversion of pepsinogen to pepsin for adequate protein digestion and to prevent the proliferation of pathogenic bacteria (VIOLA & VIEIRA, 2003), which use the undigested protein substrates to develop and cause physiological disorders such as diarrhea in the first few weeks post weaning (LANGE et al., 2010).

In modern pig farms, piglets come to be weaned until three weeks of age which may amplify the physiological effects of weaning (MELOTTI et al., 2011; MCLAMB et al., 2013).

All of these changes are crucial for a nursery phase to be a major challenge for swine farming. Several nutritional and management strategies (JAYARAMAN et al. 2017) such as increase weaning age, are sought to minimize the physiological impacts caused in response to early weaning in order to improve intestinal health and performance of piglets in this period (CAMPBELL et al. 2013).

2.2 Weaning age

Under normal conditions weaning is a gradual process that occurs around 12 to 17 weeks, a period that coincides with the almost complete development of the GIT and the immune system (MOESER et al., 2017).

In technified pig farms the weaning age decreased over the years (YANG et al., 2016) in order to increase productivity and today this process takes place in about 3 to weeks. Early weaning around the 21 days, practiced on most farms, yielded gains in productivity by increasing litter/sow/year, optimizing facilities and reducing vertical transfer of pathogens (BUTLER et al., 2008).

However, early weaning in production systems occurs at the time the gastrointestinal tract barrier is still under development (POHL et al., 2015), resulting in delayed maturation and intestinal barrier dysfunction, reduction in weight gain and increase susceptibility to enteric diseases (MOESER et al., 2017). The impact of weaning stressors does not only provide physiological changes in the gut, but is also related to immunological changes and to microbiota composition in weaned piglets (PLUSKE, 2013).

The physical and functional gastrointestinal changes in early-weaned pigs have short- and long-term effects, and may persist into adulthood (MOESER et al., 2017). The stressors of weaning are intrinsic to this period and will affect all piglets at this stage, however, weaning piglets between 4 and 5 weeks of age may have a better development and function of GIT and immune system, which may have a beneficial impact on post-weaning piglets (MARSI et al., 2015; XUN et al., 2018).

2.2.1 Effects of weaning age on intestinal barrier morphology and function

The intestinal epithelium play primary functions in the digestion and absorption of nutrients and forms a selective barrier against pathogens and other antigens (YANG et al., 2013). Its development depends on genetic, nutritional, immunological and microbial composition (PLUSKE, 2013).

Intestinal development is a dynamic and continuous process that occurs through cell renewal, involving stages of cell proliferation and differentiation in the crypt, as well as apoptosis and peeling losses naturally at the apex of the villus (BOUDRY et al,. 2004b; DONG & PLUSKE, 2007). Dietary nutrients and nutritional status directly affect this process of epithelial renewal (EVERAERT et al., 2017).

Weaning causes consequences throughout the GIT (PLUSKE et al., 2016). However, the small intestine has been reported to be the most affected portion during this process, responding with anatomical, physiological and immunological adjustments to adapt the changes occurred due to stress in this period (BOUDRY et al., 2004b; LALLÈS et al., 2004; BURKEY et al., 2009; WIJTTEN et al., 2012; PLUSKE, 2013).

The renewal of the intestinal epithelium in piglets occurs rapidly, every three or four days and this process requires a high energy requirement (DANIEL et al., 2014). As a consequence of the low feed intake after early weaning, the intestinal structure responds with changes in its morphology and barrier function (WIJTTEN et al., 2011).

Another important fact is that intestinal invasion by pathogens at this stage also causes damage to the intestine, and epithelial cells respond increasing their rate of cell turnover, impacting the villus and crypt architecture (HU et al., 2013). As a consequence of changes in villi and crypts, the villus: crypt ratio is significantly lower in weaned piglets than suckling pigs (PLUSKE et al., 2003).

Lallès et al. (2004) describe that during the first two days after weaning the small intestine lose 20% to 30% of its relative weight and that villous atrophy can range from 45 to 70% of the pre-weaning values. In contrast to villus height, no clear changes are observed in crypt in the first days after weaning (SPREEUWENBERG et al., 2001; MARION, 2002; HEDEMANN et al., 2006).

Several authors have reported that villous atrophy that occurs immediately after weaning is more pronounced in the proximal than in the distal portion of the small intestine (HAMPSOM, 1986; MARION et al., 2002; MONTAGNE et al., 2007) due to lack of luminal nutrient (STOLL et al., 2000).

According to Hampson et al. (1986) and Li et al. (1991), the damage that occurs in the intestinal epithelium is transient and can finish until to 12 days after weaning. The weaning age is an important factor related to the severity lesion in the intestinal barrier after weaning (MOESER et al., 2017), influencing the recovery period of villous atrophy (PLUSKE et al., 2003).

Molly (2001) reported that three to seven days after weaning in piglets weaned at 24 days of age there was a reduction about 59% in villus height and about 144% increase in crypt depth. Hu et al. (2013) found that between 3 and 7 days after weaning, shorter villi and deeper crypts in the jejunum can be observed in weaned piglets at 21 days and that recovery to normal morphometry occurs from 14 days after weaning.

Comparing weaned piglets at 7 days of age with suckling piglets, Marion et al. (2002) observed at 3 days after weaning, the villus height was on average reduced to 59% of the pre-weaning values in the proximal region compared to the distal region of small intestine and the values remained reduced at 14 days after weaning.

In results of several studies, Marsi et al. (2015) found that the impacts on intestinal architecture are very visible in weaned piglets aged less than 28 days, while late weaning has little or no effect on the intestinal mucosa seen at physiological maturity in this period. However, it is economically unfeasible to wean over five weeks in the farm cycle.

Evaluating the influence of weaning at 17, 21, 28 and 35 days of age on changes in intestinal development, Gu et al. (2002) observed that at 5 days after weaning villous atrophy was higher in piglets weaned at 17 days and took 11 days to recover the architecture compared other ages. However, in animals weaned at 28 days there was no reduction in villus height and 15 days after weaning there was a 111% increase in villus height.

In this context, piglets weaned at 14 days compared to 21 or 28 presented no expression of disaccharidases and greater villous atrophy at 7 and 14 days after weaning, as well as slow recovery in villus architecture, while weaning at 21 and 28 days had increased villi from seven to 14 nursery days (TSUKAHARA et al., 2016).

In some cases, weaning age may influence only functional changes in intestinal permeability without causing damage to gut architecture as described by Smith et al. (2010). According to the weaning age increased from 15 to 28 days, improvements were observed in the permeability function indicated by the higher TER and lower mannitol flux, even though no differences in intestinal morphology were observed between the ages.

Weaning age also plays a critical role in intestinal permeability, which may compromise the barrier function and favor the translocation of pathogens. Evaluating weaning at 19 and 28 days of age on intestinal function 24 hours after weaning, Moeser et al. (2007b) observed that weaning at 19 days induced acute disturbances in intestinal permeability in the jejunum and colon marked by the reduction in transepithelial electrical resistance (TER) and increased mannitol flux among tight junctions compared to piglets weaned at 28 days.

In response to the challenge with $2x10^9$ CFU of *E. coli* F18, piglets weaned early at 16 and 18 days of age had a fast onset and greater severity of clinical diarrhea compared to piglets weaned at 20 days, in addition to a marked reduction in weight gain (McLAMB et al., 2013). The severity of diarrhea in early weaning can be explained by reduction significant in ileal villus height and the increase in permeability due to the reduction of TER, compared to weaning at 20 days, which did not present any of these changes.

In four weeks of evaluation, Pohl et al. (2017) concluded that piglets weaned early at 15 days had diarrhea most of the time (43.6%) compared to wean at 28 days (4.80%). For ileal permeability, younger weaning showed an increase in mannitol flux rate compared to older piglets at seven weeks of age and the decrease in TER occurred both at 49 days and at 140 days of age, showing a lasting effect of change in the development of the intestinal barrier.

Xun et al. (2018) investigated the morphology, intestinal permeability and gene expression of tight junctions and D-lactate proteins in the jejunum and ileum at 56 days of piglets weaned at 21, 28, 35 and 42 days. It was observed that piglets weaned at 21 days of age had a lasting impairment in intestinal barrier functions characterized by decreased gene expression of occludin and claudin and D-lactate compared to weaned at 35 and 42 days. D-lactate is a marker that assesses the extent of damage in the intestinal mucosa and may indicate presence of bacterial infection (BURTIS et al., 2016). The higher their values, the greater the intestinal damage (XIAO et al., 2014).

2.2.2 Effect of weaning age on piglet immunity

Even as the birth, in which the immune system needs to adapt to microbial colonization and milk antigens, the weaning also represents a period of great challenge for the development of the immune system (MOESER et al., 2017), being characterized by exposure of the immune system of piglets to a wide range of new dietary antigens and new pathogens in the environment (LALLÈS et al., 2007).

At birth, piglets acquire the elements of adaptive immunity via colostrum rich in immunoglobulins, antimicrobial peptides and growth factors which provides systemic immunity and also via milk, which contains a greater amount of IgA, in addition to other protective factors of the intestinal mucosa (STOKES et al., 2001). However, early weaning occurs at a time when there is a decline in passive immunity and active immunity is not yet developed, making weaning even more challenging because high vulnerability of piglets to pathogens (PIÉ et al., 2004).

GIT is an important interface between the host and the environment and due to this interaction, the gastrointestinal environment has an immune mucosal system composed of dendritic cells, macrophages, cytokines and lymphocytes (PLUSKE et al., 2018), in which they are responsible for recognizing and responding adequately to the wide range of luminal antigens (STOKES et al., 2017).

Several endogenous (microbiota) and exogenous factors (diet, aerosols) constantly challenge the mucosal immune system (REN et al., 2015). To maintain intestinal integrity, the mucosal immune system must respond quickly and robustly to mobilize innate and adaptive responses to an antigenic challenge, without excessive activation of the inflammatory response and damage to the intestinal epithelium of the piglets (MOESER et al., 2017; PLUSKE et al., 2018).

The combined performance of stressors associated with weaning, especially the low feed intake immediately after weaning (PIÉ et al., 2004), are responsible for causing an inflammatory response that triggers intestinal health disorders, compromising intestinal epithelial barrier function (MOESER et al., 2007b; POHL et al., 2017).

García et al. (2016) demonstrated that early weaning at 14 days induced negative effects on mucosal immunity and intestinal morphology compared to 21-day weaning. Although they had increased expression of secretory IgA, they had lower villus length and less number of goblet cells producing mucin and intraepithelial lymphocytes than those of 21 days.

According to some authors, early weaning can stimulate the maturation adaptive system in recently weaned piglets by inducing the early synthesis of IgA in the gut due to the greater impacts of weaning at early ages (KRAMER et al., 1995; HU et al., 2013). Already

Xun et al. (2018) found a significant increase in secreted IgA expression in the jejunum and ileum in piglets weaned at 21 days compared to weaning at 28, 35 and 42 days.

The post-weaning classical epithelial barrier rupture provides increased intestinal permeability, promoting an inflammatory response exacerbated by the translocation of endotoxins and other antigenic agents (MOESER et al., 2007b; POHL et al., 2017). Pié et al. (2004) reported increased expression of inflammatory cytokines in GIT (IL-1β, IL-6, TNF-α), which remain at elevated levels in the early days until they return to baseline levels.

When challenged with *E. coli* ETEC, weaned piglets at 16 and 18 days presented reduced IL-6, IL-8 and neutrophil responses compared to weaned at 20 days (McLAMB et al., 2013). The impaired immune response in piglets younger contributed to the diarrhea and increased permeability in response to the challenge with ETEC, since they presented significantly a fast and severe onset of post-weaning diarrhea.

Smith et al. (2010) investigated the immune response in the jejunum of weaned piglets at 15 and 28 days of age and demonstrated that the jejunal mucosa of early animals had a higher density of cellular infiltrates (lymphocytes, macrophages and neutrophils) in the lamina propria in relation to late weaning, suggesting an increase in inflammation and antigenic stimulation in the mucosa due to the barrier function impaired by increased intestinal permeability.

In addition, Moeser et al. (2007b), Smith et al. (2010), McLamb et al. (2013) and Pohl et al. (2017) evaluated the activation of mast cells in the intestinal mucosa through weaning stress and concluded that as the weaning age decreases, the number of degranulated mast cells increases.

Mast cells activate the release of mediators such as histamine and proteases that can induce defects in the intestinal barrier and activate enteric secretory neurons that trigger the pathophysiological mechanisms of diarrhea (OVERMAN et al., 2012). Mast cells also interact with receptor 1 of *Corticotropin Releasing Factor* (CRF) shown to have increased expression in the intestinal mucosa of prematurely weaned piglets and trigger immediate and long-term disorders of intestinal barrier rupture (McLAMB et al., 2013; MEDLAND et al., 2016).

Thus, stress events occurring at early weaning significantly interfere with the absorptive and secretory function of the gastrointestinal tract as weaning age decreases and these impacts may extend into subsequent stages where the animals may exhibit a development below that genetic potential (HU et al., 2013).

2.2.3 Relationship between weaning age and intestinal microbiota of piglets

Post-weaning disorders do not only result from changes in the architecture and function of GIT, but also result in changes in the intestinal microbiota (KONSTANTINOV et al., 2004) and, the rupture that occurs in the microbiota is one keys that leads to post-weaning dysbiosis and diarrhea.

Has been estimated that intestine contain approximately 500 to 1000 bacterial species (SONNENBURG et al., 2004; KIM et al., 2015). The gastrointestinal tract piglets are composed of a complex and dynamic microbial ecosystem whose composition varies over time between individuals and along the GIT (KONSTANTINOV et al., 2004; ISAACSON et al., 2012). Several factors influence composition including maternal microbiota, medium pH, substrate availability, peristalsis, mucus secretion, milk intake, dietary composition, age and antimicrobial agents (HAO et al., 2004; KIM et al., 2012).

In equilibrium, the microbiota acts in digestion and fermentation of carbohydrates, production of volatile fatty acids and vitamins, and plays an important role in the maintenance of normal functions in intestine, as protection against pathogens until regulation of the immune response of the host (KAMADA et al., 2013; FRESE et al., 2015). Any disturbance in the microbial ecosystem creates an opportunity for pathogenic organisms to colonize and cause diseases (FOUHSE 2016).

The microbial composition in suckling pigs is molded soon after birth with exposure to a maternal microbiota and beginning of colostrum and milk intake, which provide nutritional benefits favoring the establishment of *Lactobacillus spp.* in the stomach due to low pH and anaerobic bacteria in the large intestine such as *Eubacterium*, *Streptococcus spp*., *Bifidobacterium* and *Clostridium spp*. (PETRI et al., 2010).

Significant differences occur in the composition of the fecal microbiota in nursing compared to weaned piglets. Overall, Firmicutes and Bacteroidetes were the most abundant phylum in swine fecal samples representing 79% to 90% of the fecal bacterial community regardless of age (PAJARILLO et al., 2014; NIU et al., 2015).

Comparing relative abundances of the intestinal microbiota in faecal samples of nursing and weaned piglets through the 16S rRNA gene sequencing technique, Guevarra et al. (2018), found significant differences occur in the composition of the fecal microbiota in nursing compared to weaned piglets.

Suckling piglets present greater abundance phylum Bacteroidetes (44.4%), Firmicutes (41.07%), Spirochaetes (9.87%) and Proteobacteria (2.94%), while weaned piglets present greater predominance in Bacteroidetes (63.14%), Firmicutes (34.27%) and Proteobactérias (1.79%) (GUEVARRA et al.,2018). These differences show the direct effect of the change in dietary composition, environment and among other factors on the intestinal microbiota (KIM et al., 2012).

At weaning, several factors contribute to the loss of intestinal microbiota diversity, especially during the first week (TAO et al., 2016; HOLMAN et al., 2017). Dietary changes and low feed intake in the early days lead to the reduction of some beneficial microorganisms, such as Lactobacillus spp. (LALLÈS et al., 2007).

Associated with this, the inflamed bowel in the weaning transition exposes the mucus layer that protects the intestinal epithelium, where the glycans that make up this layer become more available for attachment of pathogenic microorganisms such as Clostridium spp. and E. coli (BÄULMER et al., 2016; GRESSE et al., 2017).

2.2.4 Impacts of weaning age on nursery phase growth performance

One of the main challenges arising from early weaning and the physiological immaturity is the low feed intake in the first nursery days, which intensifies the disorders in the GIT promoting an exacerbated inflammatory response. The immunological challenge to weaning can lead to reduced animal growth, where the nutrients are redirected to the immune response rather than muscle growth (LEDUR et al., 2012).

The weaning age correlates directly with the physiological maturity of the piglets, and the low feed intake observed after weaning can be minimized as the age at weaning increases even in a few days (COLSON et al., 2006), and promotes a better adaptation to stress in the initial nursery phase (FACCIN et al., 2018).

Gain with late weaning is observed during the first days after weaning and this effect may persist until the growing and finishing phase (SMITH et al., 2008). However, some studies comparing two or three ages report that this difference is not always observed until the end, in which, even increasing the age and consequently the weight at weaning, the difference in weight between the ages disappears around the seventh week and remains the same until slaughter (LEIBBRANDT et al., 1975; DUNSHEA et al., 2003a; COLLINS et al., 2010).

Smith et al. (2008) evaluated the effect of weaning with a mean age of 15 and 20 days on performance and feed cost of 2467 piglets. Piglets weaned at 20 days had a 6% increase in weaning weight for each additional day in lactation and 3.3% of the exit weight in nursery phase, which resulted in a 7% reduction in feed cost per kg of weight gain, compared to the younger piglets.

Leliveld et al. (2013) studied the effect of weaning at 21, 28 and 35 days on performance in the post weaning phase from to 10 weeks of age and observed that ADG (363, 402 and 476 g / d), ADFI (560, 620 and 680 g / d mean) increased linearly according to age respectively and dietary FC was also better with increasing age (1.57, 1.55, 1.43).

According to Colson et al. (2006), weaning at 21 days presents more negative consequences on growth rate and endocrine responses to stressors than weaning at 28 days. Compared to suckling piglets, both ages had lower ADG in the seven days post weaning. However, for endocrine changes, cortisol increases in weaning regardless of age, but the effects were more intense and lasting at weaning at 21 days.

Another important factor that influences the nursery phase is weaning weight (DUNSHEA et al., 2003a; HE et al., 2016), which also be correlated with age. According to data found by Main et al. (2004b), piglets weaned at 21.5 days of mean were 3.13 kg heavier at weaning than those at 15 days average, which reflected positively throughout the experimental period.

However, in some situations, younger and heavier piglets may not perform better than lighter and older weaned piglets, as observed by Montsho et al. (2016). Investigating the performance of weaned animals at 21, 28 and 35 days with initial weights (4.50 kg, 4.30 kg, 4.30 kg respectively), the authors found that even with a higher weight, weaning performance at 21 days was worse (lower final body weight, ADG, ADFI and higher FC) compared to animals at 28 and 35 days. Older piglets exhibit GIT maturity and immune system, better ingestion capacity and feed demand.

2.3 Post-weaning diarrhea

Intestinal dysbiosis induced by stressful weaning factors are one of the main causes of post-weaning diarrhea that results in a significant difference in weight among piglets in nursery phase, being the proliferation of enterotoxigenic *Escherichia coli* (ETEC) is the main cause of diarrhea in recently weaned piglets (MADEC et al., 2000; HEO et al., 2013).

This negative gram bacterium presents high resistance to antimicrobial agents (SILVA et al., 2015), morbidity from 30 to 40%, reaching up to 80% in some litters and mortality around 10% (FAIRBROTHER, & GYLES, 2012).

ETEC is characterized by inducing aqueous diarrhea in first two nursery weeks and results in significant economic loss due to dehydration, reduction in average daily weight gain, increase light animals and may lead death of animals in some cases (SOBESTIANSKY et al., 2012; GRESSE et al., 2017).

Infection occurs via the faecal-oral route and the main virulence factors are F4 (K88), F5 (K99), F6 (P987), F7 (F41) and F18 fimbriae and heat-labile (LT) and heat-stable (ST) enterotoxins (KIM et al., 2010). ETEC F4 (K88) has the ability to colonize all jejunal and ileal epithelium (SUN et al., 2017) and soon after the oral intake, in 2 to 3 hours the bacteria that resist the acidic environment stomach reach the small intestine (NGUYEN et al., 2015).

The damage occurs without destroying the intestinal villi (ZHANG et al., 2007), through the interaction and adhesion of fimbriae (adhesins) with specific receptors of mannose, galactose or glycoproteins present in enterocyte epithelial cells (RHOUMA et al., 2015).

After fixation and multiplication, they release enterotoxins that act increasing the secretion of water and electrolytes (Na+ and CL-) and decreasing absorption liquids, increasing permeability and induce fluid excretion in intestinal lumen (VANNUCCI, et al., 2009). Although there is no direct damage to the intestinal epithelium by ETEC enterotoxins, the barrier injury and villus structure occurs through excessive fluids loss resulting in hypovolemia and intestinal ischemia (McLAMB et al., 2013).

Due impact of post-weaning diarrhea on productive system, ETEC is one of the main concerns related to intestinal health piglets in nursery phase and the oral challenge with ETEC K88 has been constantly used in experimental conditions to approximate the response of some additive or husbandry, for example, age of weaning to the reality of a commercial environment (BOSI et al., 2004; KIARIE et al., 2011).

Volumes between 1 and 6 ml and concentrations ranging from 1 x $10^{6,}$ 10^7, 10^8, 10^9; 6.3×10^9, 10^{10} and 10^{12} CFU / ml for oral challenge with ETEC are found in literature and present satisfactory or not results in performance reduction, changes in intestinal morphometry and immune response and in inducing mild to severe diarrhea (SILVEIRA et al., 2018; ADEWOLE et al., 2016).

3. GENERAL CONSIDERATIONS

In the current scenario of the large number of smaller born piglets and decrease in the use of growth promoting antimicrobials, it is necessary to search for management strategies that minimize the physiological impacts caused by weaning.

Reduction of weaning age to around 21 days of age brought productivity benefits, however, when weaned at this age, these animals show more pronounced digestive, physiological and immunological immaturity when compared to late weaned piglets, have lower feed intake in the early days, are more susceptible to farm pathogens and require a diet that increases feed costs.

Thus, the earlier weaning, the more severe the TGI dysfunctions and the slower recovery of the intestinal barrier after weaning, resulting in lower growth rates compared to late weaning.

Thus, weaning over 21 days, such as at 25 days of age, can bring current and future benefits to the production system due to reduced use of antimicrobials and increased hyperprolificity. Providing improvements in intestinal health and consequently better performance in the first weeks of nursery phase.

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SECOND PART

RESEARCH ARTICLE

(Journal of Animal Science norms)

Performance and intestinal health of piglets weaned at 21 or 25 days of age challenged with *Escherichia coli k88* $^{+}$

1. INTRODUCTION

The hyperprolificity of sows increased in the number of piglets born, also the weight variability at birth and higher incidence of light piglets. The nursery phase represents a critical period in the current pig production system, where early weaning has a major contribution to the stress caused to piglets (Moeser et al, 2017).

One of the factors that amplify the physiological effects of stress at this stage is the age of weaning, which is currently occurring around of three weeks of age (Melotti et al., 2011; Mclamb et al., 2013,). Gastrointestinal tract of weaned piglets before or around 21 days of age is still under development, and low feed intake and high diarrhea incidence leading to performance loss are common when weaning occurs at this age (Pohl et al., 2015 ; Pluske et al., 2016; Medland et al., 2016).

The most widely used practice to minimize damage at nursery phase and improve better performance in recent decades has been the use of antibiotics in animal feed (Morés, 2014; Cheng et al., 2014). However, in the context of decrease the use of performance enhancing antimicrobials in animal production, producers and researchers have sought management solutions that can assist post-weaning piglets and limit the use of antimicrobials (Who, 2002).

Weaning stressors are intrinsic to this period and will affect all piglets at this stage; however increasing weaning age to around four 25 days may minimize the damage caused by early management and be an effective alternative to reduce negative effects the removal of growth-promoting antimicrobials. Weaning above 21 days allows more time for the physiological and immunological development of these animals (Dong & Pluske, 2007), which may have a beneficial impact on piglets in the first weeks of nursery (Marsi et al., 2015; Xun et al., 2018).

Thus, the aim of this study was to evaluate the effects of weaning at 21 and 25 days of age on the performance and intestinal health parameters of piglets challenged or not with enterotoxigenic *Escherichia coli K88*$^{+}$ (E.coli K88^{+} ETEC) during the first weeks of nursery phase.

2. MATERIALS AND METHODS

All experimental procedures for the present study were approved by the Ethics Committee on Animal use of the Federal University of Lavras (UFLA) under the Protocol 015-18. The experiment was performed in the weaning facilities of the Swine Experimental Center at the Department of Animal Science of the Federal University of Lavras, Lavras, MG, Brazil.

2.1 Animals, housing, and feeding

A total of 84 barrows (DB90×PIC337) were divided into two groups: 42 piglets weaned at 21 days of age (5.2 ± 0.3 kg) and 42 piglets weaned at 25 days (6.8± 0.3 kg).

To reduce the potential confounding effects of parity and maternal immunity in this study, all animals were obtained from the same batch farrowing group from sows from third to eighth parity order, and were individually identified at birth with different colored ear tags.

The pigs were housed in raised decks pens (120 cm × 114 cm) with fully plastic slatted floor. Each pen had an adjustable nipple drinker and a galvanized steel trough. Previously housing, the room was cleaned and disinfected program prior to the arrival of pigs. For environmental control, ventilation and temperature of rooms were controlled by infrared bulbs and adjustment of windows.

All animals received the same commercial diet, which was formulated to meet the nutritional requirements of each stage, divided into two phases (phase 1, 0 to 7 days; and phase 2, 8 to 24 days). Ingredients and nutritional composition of the diets are presented in Table1. The pigs had *ad libitum* access to feed and water throughout the experimental period.

2.2 Experimental design and Treatments

The four treatments were arranged in a 2 × 2 factorial with main effects of weaning age (21 vs. 25 days) and challenged or not by *E. coli K88* $^{+}$ inoculation, with seven replicates, and three pigs per pen. The duration of the experimental period was 24 days.

Table 1. Basal diets of phase1, d1-7days and phase 2, 8 to 24 days

Ingredients (kg)	Starter 1	Starter 2
Corn	27.87	37.10
Soybean meal (46 %, CP)	9.80	19.80
Gelatine	5.00	
Biscuit flour	6.00	6.00
Degummed soybean oil	1.00	1.50
Sugar	2.00	3.00
Performa Rapid1 [1]	36.00	
Performa Rapid2 [2]		26.00
Colistin Sulfate 50%	0.04	0.04
Doxycycline 50%	0.04	0.04
Bacitracinzinc	0.06	0.06
Sodium chloride	0.03	0.30
Limestone	0.35	0.34
Dicalcium phosphate	1.69	1.70
Whey	9.00	4.00
Spray-dried animal plasma	1.00	
Mycofix Focus [3]	0.10	0.10
Bacsol[4]	0.03	0.02
Calculated values	**Starter 1**	**Starter 2**
Metabolizable Energy (kcal / kg)	3462	3419
Crude protein (%)	18.84	18.91
Digestible lysine (%)	1.407	1.299
Digestible methionine (%)	0,302	
Met + Cis digestible (%)	0.604	0.599
Digestible threonine (%)	0.677	0.652
Digestible tryptophan (%)	0.179	0.180
Lactose (%)	13.03	
Calcium (%)	0.733	0.702
Match available (%)	0.666	0.650
Sodium (%)	0.375	

*Starter 1: 21 to 28 days; Starter 2: 29 to 42 days. [1] Concentrate containing per kg of product: Protein Crude: 238g; Benzoic Acid: 13.69g; Folic acid: 2.29mg; Pantothenic acid: 49.97mg; *Bacillus lincheniformis*: 1.76x10E9 UFC; *Bacillus Subtilis*: 1.76x10E9 UFC; Biotin: 0.37mg; Calcium (min): 2000mg, Calcium (max): 3000 mg; Chlorohydroxyquinoline: 333.33mg; Copper: 555.55mg; Ethoxyquin 416.48mg; Ethereal extract: 60mg; Iron: 270.49mg; Gross Fiber: 45g; Phosphorus: 2800mg; Iodine: 3.40mg; Lactose: 103.80g; Lysine: 25mg; Manganese: 169.42mg; Mineral Matter 80g; Methionine: 11g; Niacin: 104.10mg; Selenium: 0.97mg; Threonine: 17g; Tryptophan:400mg; Humidity: 120g; Vitamin A: 31924.00UI; Vitamin B1: 5mg; Vitamin B12: 65.24mg; Vitamin B2:

14.16mg; Vitamin B6: 9.72mg; Vitamin D3: 5829.60UI; Vitamin E: 94.38 UI; Vitamin K3: 8.33mg; Zinc: 8333.33mg. [2] Commercial concentrate containing per kg of product: Crude Protein: 190g; Benzoic Acid:19.04mg; Folic acid: 3.17mg; Pantothenic acid: 69.23mg; *Bacillus lincheniformis*: 2.46x10E9 UFC; *Bacillus Subtllis*: 2.46x10E9 UFC; Biotin: 0.52mg; Calcium (min): 1600mg, Calcium (max):3500mg; Chlorohydroxyquinoline: 461.54mg; Copper: 729.23mg; Ethoxyquin 576.92mg; Ethereal extract: 40mg; Iron: 367.61mg; Gross Fiber: 45mg; Phosphorus: 500mg; Iodine: 4.62mg; Lysine: 24.mg; Manganese: 230.25mg; Mineral Matter: 80mg; Methionine: 11.50g; Niacin: 144.23mg; Selenium: 1.35mg; Threonine:14.50g;Tryptophan:3000mg; Humidity: 120g; Vitamin A:44230.80UI; Vitamin B1: 6.92mg; Vitamin B12: 90.38mg; Vitamin B2: 19.62mg; Vitamin B6: 13.46mg; Vitamin D3: 8076.90UI; Vitamin E: 130.77 UI; Vitamin K3: 11.54mg; Zinc: 11.54g. [3]Adsorvent.[4]Commercial product containing per kg of product *Saccharomyces cerevisiae* 2.5 x 106 CFU / g; *Bacillus Subtilis* 2,0 x 107 CFU / g.

2.3 Experimental procedure

Piglets were weighed on the first, 4, 12, and 24 days of the experiment to measuring average daily gain (ADG). Feed and the leftovers were daily quantified in order to calculate average feed daily intake (AFDI) and feed conversion ratio (FCR). The feed was weighed and given twice a day in the morning and in the afternoon by amount of feed identification available in feeders.

Fecal consistency was visually examined at the same time, by the same person. Following the methodology of Casey et al. (2007), the absence of diarrhea was determined by observation normal feces and the presence of diarrhea was determined by the observation of liquid and pasty stools. The diarrhea incidence (%) was calculated as the sum of the total number of pens where diarrhea was observed, per treatment, over the total number of pens for each period, and the quotient multiplied by 100.

For the control of respiratory diseases, the pigs received on the first experiment day an intramuscular dose (100 mg/mL, 0.15 mL per animal; 2.5 mg/kg) of antibiotic based on tulathromycin (Draxxin®, Zoetis). This antimicrobial acts mainly in the lungs to prevent factors that are not associated with enteric challenge but could impair the performance of the animals and confound the study results.

The challenged group was orally inoculated with 1 mL of a solution containing 10^6 concentration of CFU/ml of *E. coli k88*$^+$ on days 4 and 5. The not challenged group received 1 mL of saline solution in the same period to standardize the management of all animals. The inoculum was placed at the entrance of the esophagus with the aid of a syringe coupled to a scalp to quantify the dosage per animal. The bacterial inoculum used was prepared from the bacterial strain *E. coli* K88+ (LT+, STa+ and STb+), which was validated by the Swine Health Laboratory of São Paulo University – VPS, Faculty of Veterinary Medicine and Animal Science - USP. The bacterial inoculum was prepared in the Laboratory of Microbiology in Department of Animal Science of UFLA. To reach concentration of 10^6 CFU/ml bacteria, the strain was cultured in culture medium (Luria Bertani Broth, Kasvi) for 16 hr., at 37 ° C followed by serial dilution in PBS (Phosphate Buffered Saline) to the final concentration of 10^6 CFU/ml.

2.4 Sample collection and chemical analyses

On the 12th of the experiment, one pig with (body weight) BW closest to the pen average weight was euthanized by electronarcosis (> 300v, 1.25A, for 4 seconds) followed by exsanguination, totaling 28 piglets. The procedure was performed in the Department of Veterinary Pathology of the University of Lavras.

After euthanasia, samples of jejunum (2.0 cm) and ileum (2.0 cm) were collected at 2.0 m and 10.0 cm respectively before íleo-cecal junction to perform histological analysis (villus height, crypt depth and villus: crypt ratio). After careful removal of the luminal content and washing with saline solution, the jejunum and ileum samples were fixed in 10% formaldehyde for 48 h and transferred to 70% alcohol solution until the slides were prepared.

The intestinal samples were dehydrated, included in paraffin blocks, sectioned in a microtome in the thickness of 4μm and stained by Hematoxylin and Eosin, based on Luna (1968). The slides were photographed through the trinocular microscope (CX31, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil) and digital image capture camera (SC30, Olympus Optical do Brasil Ltda., São Paulo, SP, Brazil). The villus height and crypt depth were measured using ten well-oriented villus and their respective crypts in each tissue to obtain the average per animal, through the ImageJ® 1.41 image software. The villus: crypt ratio was calculated and all analyses were performed by a single person.

Microbiological analysis was performed by sampling of cecal content of piglets. The cecal content was homogenized and the collected samples were diluted in 20% glycerol and frozen until analysis was performed. Bacterial counting was performed using the plate counting technique. The total content collected was homogenized and an aliquot of one ml was diluted in PBS (Phosphate Buffered Saline) for the serial dilution from 10^{-1} to 10^{-9}. After dilution, duplicate plating was performed and after incubation the colony count of *Escherichia coli* (MacConkey agar, Kasvi), *Lactobacillus spp* (MRS agar, Kasvi).

All plates were incubated for 24 to 48 hours at 37 ° C. Colony counts (CFU/g) were subjected to log transformation ($\log_{10}$) before statistical analysis. The analyzes were performed at the Microbiology Laboratory of the Department of Animal Science of the Federal University of Lavras, based on Wang et al (2011).

For the determination of acetic, propionic and butyric acid, 1g of the sampled cecal content was weighed in microtube and 1ml of distilled water was added immediately after slaughter. Subsequently, the solution was homogenized on a tube shaker and centrifuged at 15,000 g for 60 minutes at 4 ° C. Subsequently, the supernatant extract (± 0.4 mL) of each sample was transferred to chromatographic vials, in which were added 100 μL of 3: 1 solution of 25% metaphosphoric acid with 98-100% formic acid and 50 μL of 100 mM 2-ethyl-butyric acid (internal standard). From this extract, it was injected automatically by the injector system, 1 μL in gas chromatograph (HP HP 7890A; HP 7683B Injector, Agilent Technologies) equipped with HP-FFAP capillary column (1909F-112, 25 m, 0.32 mm, 0.5 μW; JeW Agilent Technologies). The drag gas used was H2, maintained in a flow rate of 31.35 mL / min. The temperature of the injector and detector was 260 ° C. The total time of the chromatographic analysis was 16.5 minutes, divided into three heating ramps: 80 ° C (1min), 120 ° C (20 ° C / min; 3min) and 205 ° C (10 ° C / min; 2 min). The concentration of VFA (mM) was determined based on external calibration curve made with chromatographic standards (Ferreira et al. 2016). The analyzes were performed at the Breeding Animal Nutrition Laboratory of the Escola Superior de Agricultura "Luiz de Queiroz" (ESALQ) of the Federal University of São Paulo (USP).

2.5 Statistical analysis

Data were analyzed as a completely randomized design with pen as the experimental unit. The Shapiro-Wilk Test was used to evaluate normality, when this assumption was not met, data were transformed using PROC RANK (SAS INSTITUTE, 2009). Then, the data were submitted to analysis of variance, using the statistical package SAS through the test F. Differences between means were considered significant at $p \leq 0.05$ and trend $0.05 \leq$ P-value <0.10., and compared by Tukey test. For the statistical analysis of the diarrhea incidence, the

Kruskall Wallis nonparametric test at 5% of significance were used (SAS INSTITUTE, 2009).

3. RESULTS AND DISCUSSION

Results of growth performance during the four days after weaned piglets at 21 and 25 days old are presented in Table 2. The piglets wean at 25 days presented higher initial weigth (P <.0001) than piglets wean at 21 days. At four experimental days, the animals weaned at 25 days old had higher BW (P <.0001), ADG (P =0.0006) and ADFI (P<.0001) compared the animals weaned at 21 days old. In the same period, there was a tendency for weaned piglets at 251 days to have better FCR (P=0.0817) compared to weaning at 25 days.

Table 2. Effect of weaning age on growth performance of piglets during the four experimental days.

Item	**Treatments**		CV %	SEM	P- value
	21	25			
Initial BW, kg	5.040	6.856	19.527	0.202	<.0001
Days, 4 days					
BW, kg	5.005	7.155	21.327	0.245	<.0001
ADG, kg	0.001	0.058	158.774	0.009	0.0006
ADFI, kg	0.072	0.130	39.164	0.007	<.0001
FCR kg/ kg	0.582	1.788	149.232	0.419	0.0817

*Notes.*ADFI: average daily feed intake; ADG: average daily gain; BW: body weight; FCR: feed conversion rate; SEM: standard error of the mean.

The low or none feed intake in the first days after weaning leads to insufficient energy and nutrients intake, compromising weight gain and the development of GIT (Burrin & Stoll, 2003). According to Le Dividich et al. (2000), the metabolizable energy intake (ME) by feed intake varies around 60-70% of the energy intake from milk in the pre-weaning period and energy requirements for maintenance are met after the third day after weaning.

According to Martinez-Ramirez et al. (2009), the protein and energy restriction in the first days reduce the amount of circulating T3 (triiodothyronine) and IGF-1 (insulin-like growth factor 1), damaging growth performance. This corroborates the results of this study, where the piglets with 21 days of weaning did not feed intake in the first four days of weaning when compared with weaning piglets at 25 days, where added to their physiological immaturity reflected the lower weight gain during this period, which was not observed in weaned at 25 days (Main et al. (2004b; Smith et al., 2008).

Growth performance results during the first three weeks post weaning of the piglets weaned in different wean age challenge or not are presented in Table 3.

Table 3. Effect of weaning age on the growth performance of piglets challenged or not with ETEC k88+

Item	Treatments				CV %	SEM	P –Value		
	Challenge		No challenge				C	WA	C*WA
	21	25	21	25					
Initial BW, kg	5.040	6.856	5.018	6.908	19.527	0.202	0.957	<.0001	0.899
Days, 4 to 12									
BW, kg	6.652	9.370	6.574	9.654	21.444	0.322	0.76	<.0001	0.600
ADG, kg	0.196	0.264	0.181	0.289	24.990	0.011	0.758	<.0001	0.176
ADFI, kg	0.221	0.313	0.236	0.364	25.094	0.013	0.051	<.0001	0.282
FCR kg/ kg	1.134	1.188	1.317	1.268	12.617	0.029	0.024	0.958	0.355
Days, 12 to 24									
BW, kg	11.811	15.314	11.707	15.636	17.067	0.439	0.842	<.0001	0.696
ADG, kg	0.430	0.495	0.427	0.498	14.974	0.012	0.829	0.019	0.697
ADFI, kg	0.697	0.823	0.709	0.911	17.655	0.026	0.254	0.0008	0.347
FCR kg/ kg	1.558	1.712	1.667	1.834	8.280	0.038	0.038	0.001	0.441
Days, 0 to 24									
BW, kg	11.811	15.314	11.707	15.636	17.067	0.439	0.842	<.0001	0.696
ADG, kg	0.282	0.352	0.279	0.364	18.111	0.009	0.821	0.0001	0.663
ADFI, kg	0.352	0.448	0.361	0.494	18.742	0.013	0.171	<.0001	0.358
FCR kg/ kg	1.254	1.274	1.298	1.364	7.093	0.009	0.053	0.207	0.490

*Notes.*ADFI: average daily feed intake; ADG: average daily gain; BW: body weight; FCR: feed conversion rate; C: challenge with ETEC; WA: weaning age; C x WA: interaction between challenge and weaning age. SEM: standard error of the mean.

There was not interaction between age and challenge (P <0.05) for growth performance. At the 4 to 12 days, weaned piglets at 25 days showed higher BW, ADG and ADFI (P <.0001) compared to conventional weaning (21d). In the same period, challenged piglets presented lower FCR (P = 0.024), with a tendency to lower ADFI (P= 0.051) than non-challenged piglets. From 12 to 24 days of experiment, the group weaned at 25 days presented higher BW (P<.0001), ADG (P = 0.019), ADFI (P=0.0008) and higher FCR (P=0.001) than to 21-days.The challenged piglets presented lower FCR (P=0.038) than non-challenged piglets.

Considering the total experimental period (days 0 to 24), piglets weaned at 25 days had higher BW (P<.0001), ADG (P=0.0001) and ADFI (P <0.0001) compared to 21 days. Moreover, there was a tendency for challenged animals to present lower FCR (P=0.053), compared to not challenged.

In the present study, weaning at 25 days reflected higher ADFI, ADG and BW from 4 to 12, 12 to 14 and throughout the experimental period. This is directly correlated with the physiological and digestive maturity of the piglets when they are weaned later (Smith et al., 2008; Tsukahara et al., 2016), which was probably a reflex of the creep feeding intake in the farrowing (Cabrera et al., 2013).

Leliveld et al. (2013) studied the effect of weaning at 21, 28 and 35 days growth performance and observed linearly increased on ADG (363, 402 and 476 g / d) and ADFI (560, 620 and 680 g/d mean) according to age respectively.

Another factor that influences the nursery phase which can also be correlated with age is the weaning weight (Dunshea et al., 2003a; He et al., 2016), According to data found by Main et al. (2004b), piglets weaned at 21.5 days of mean were 3.13 kg heavier at weaning than those at 15 days average, which reflected positively throughout the experimental period.

However, in some situations, younger and heavier piglets may not perform better than lighter and older weaned piglets, as observed by Montsho et al. (2016). Investigating the performance of weaned animals at 21, 28 and 35 days with initial weights (4.50 kg, 4.30 kg, 4.30 kg respectively), the authors found that even with a higher weight, weaning growth performance at 21 days was worse (lower final BW, ADG, ADFI and higher FCR) compared to animals at 28 and 35 days.

Thus, although the initial body weight difference between ages at weaning may have contributed to growth performance in this experiment, the digestive and immunological maturity the older piglets reflects in the greater capacity of feed intake in the first weeks of nursery phase.

In response to an infectious challenge to weaning, the immunological response can lead to reduced animal growth, redirecting nutrients to the synthesis of inflammatory response components than muscle growth (Han et al., 2018). According to Abbas et al., 2012 (2012), pro inflammatory cytokines reach receptors in the hypothalamus inducing hyperthermia and reduced appetite to save energy for immune response.

McLamb et al. (2013) reported that challenged weaned piglets at 16 and 18 days with *E. coli* ETEC F18, presented reduced growth rate in four days of challenge, however, growth was not affected in pigs weaned at 20d.

In this study, the challenge tended negatively affected the feed intake of the animals from 4 to 12 days. However, throughout the experimental period, the animals received a complex commercial diet, with inclusion of antibiotics and additives where it probably mitigated the effects of the challenge on the growth rate. On the other hand, the scenario could be different when using a less complex feed, without the inclusion of these agents.

In relation to diarrhea from 0 to 4 days, as shown in Table 4, no interaction was found between age and challenge ($P < 0.05$) for diarrhea incidence. There was a difference for age, where the incidence was higher for the weaning group at 21 days ($P = 0.0001$) compared to the 25 d group. In 4 to 12 days the diarrhea incidence was higher in the not challenged animals ($P = 0.03$) than those challenged. In the final period (12 to 21 days), the group weaned at 25d had a higher diarrhea incidence than the weaned at 21d ($P = 0.002$).

Table 4. Effect of weaning age on the diarrhea incidence in the piglets challenged or not with ETEC k88^{+}

Diarrhea incidence (%)	Treatments				CV %	SEM	P –Value		
	Challenge		No challenge				C	WA	C*WA
	21	25	21	25					
0 a 4, days	46.429	14.286	53.571	17.857	1.641	0.432	0.542	0.000	0.982
4 a 12, days	10.714	16.071	30.357	19.643	1.891	0.363	0.030	0.877	0.142
12 a 21, days	0.000	3.175	0.000	7.937	0.036	0.001	0.236	0.002	1.000

Notes. C: challenge with ETEC; WA: weaning age; C x WA: interaction between challenge and weaning age. SEM: standard error of the mean.

The period immediately weaning is characterized by a high incidence of intestinal disturbances and decrease of growth performance in piglets (Heo et al., 2013), and it is well established that symptoms are multifactorial, caused by low and variable feed intake in the first days after weaning (Lallès et al., 2004).

All of these changes are intrinsic to this period; however, the intensity of endocrine responses to weaning stressors correlates with the age of this management (Pluske et al., 2003; Smith et al., 2010). Colson et al. (2006) concluded that weaning at 21 days presents more negative consequences on growth rate due to higher level of cortisol excreted than weaning at 28 days, that is related to increased intestinal permeability and consequent diarrhea (McLamb et al. 2013).

The results of diarrhea incidence from 0 to 4 days after weaning found in the present study corroborate that the intensity of endocrine responses to weaning stressors is more pronounced in wean at 21 days compared to weaning at 25 days, which correlates with the low feed intake on these days and the morphometric characteristics.

It is important to highlight the association of these results with feed intake in this period. Weaned piglets at 25 days of age showed higher feed intake and lower diarrhea incidence in the first days after weaning compared to conventional 21-day weaning, which contributes positively to the better development and performance of piglets.

The proliferation of *E. coli* ETEC pathotype is the main cause of post weaning diarrhea in weaned piglets (Heo et al., 2013), being responsible for causing significant economic losses in the nursery phase (Ewing & Cole 1994; Barcellos et al., 2011).The oral infection model by *E.coli* ETEC K88+ has been constantly used in experimental conditions for post-weaning diarrhea (PWD) to approximate the response of some additive or husbandry in reduce infection (Adewole et al., 2016).

In the present study, challenged animals had a lower diarrhea incidence compared to those not challenged from 4 to 12 days. Several factors interpose with the efficacy of the challenge, such as volume and concentration of the strain (Adewole et al., 2016), loss of virulence factor, genotypic susceptibility of piglets (Petra et al., 2017), the presence or absence of F4 receptors on the brush border (Rhouma et al. 2017), environment, health status and diet (Gresse et al. 2017). They may interfere with the microbial community, causing intestinal balance to rupture (Faibrother et al., 2015).

As previously explained, all animals received a highly complex commercial diet with the inclusion of antimicrobials and other additives, which may modulate the pathogenic intestinal microbiota and be related to low incidence of diarrhea in *E. coli* challenged animals.

As a syndrome, are the causes of diarrhea in piglets, among them, the presence of nutrients in the intestine play an important role in its occurrence, mainly due to changes in osmotic pressure in the intestinal lumen (Sato et al., 2016).The excess of feed intake and the arrival of undigested and unabsorbed nutrients in the large intestine is the key point for the occurrence of nutritional diarrhea (Zlotowski et al., 2008; Barcellos et al., 2011).

The highest feed intake by weaning at 25 days compared to wean at 21 days may have influenced the persistence of diarrhea in these animals at the end of the experiment, which is also correlated with the worse feed conversion of these animals in this period.

The results of villi height, crypt depth and villus: crypt ratio in jejunum and ileum are presented in table 5. No interaction was found between age and challenge (P < 0.05) for intestinal morphometry. Piglets weaned at 25 days had greater villous height in jejunum (P=0.0345; Figure 2) and ileum (P=0.0421) than 21 days piglets. For the villus: crypt ratio of the ileum, higher ratio was observed for the challenged animals compared to the non-challenged animals (P = 0.001).

Table 5. Effect of weaning age on the villus height, crypt depth and villus/crypt ratio in the jejunum and ileum of piglets challenged or not with ETEC k88$^+$

Item	**Treatments**				SEM	**P –Value**		
	Challenge		No challenge			C	WA	C*WA
	21	25	21	25				
Jejunum								
Villus (µm)	450.25	474.52	433.24	467.31	12.396	0.357	0.034	0.707
Crypt (µm)	246.64	252.39	247.67	258.89	10.279	0.707	0.400	0.784
Ratio villus/crypt	1.86	1.97	1.78	1.90	0.077	0.277	0.231	0.962
Ileum								
Villus (µm)	371.56	398.49	374.68	404.49	12.763	0.733	0.042	0.914
Crypt (µm)	234.23	242.34	246.06	262.34	10.279	0.148	0.231	0.693
Ratio villus/crypt	1.79	1.66	1.54	1.57	0.077	0.001	0.307	0.102

Notes. C:challenge with ETEC;WA: weaning age; C x WA: interaction between challenge and weaning age. SEM: standard error of the mean.

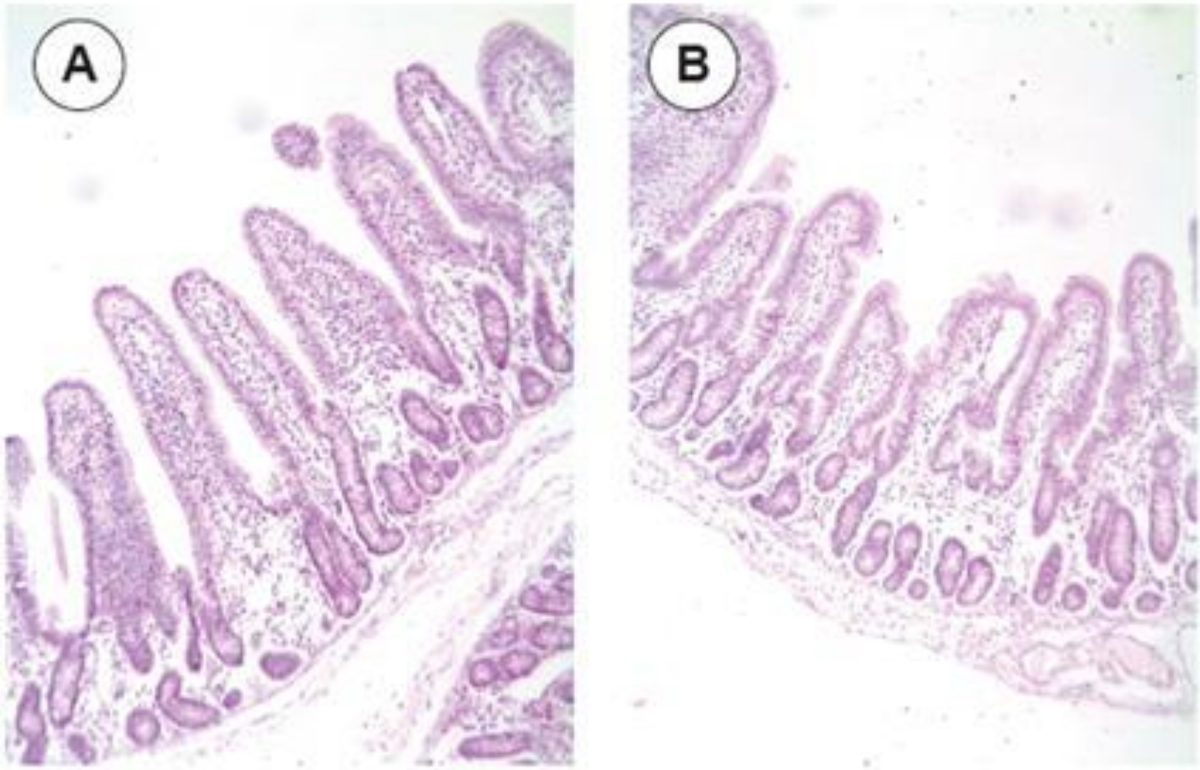

Figure.2. Jejunal sections from weaned piglets at different ages and challenged or not with *E.coli K88+*. A: weaning at 25d old; B: weaning at 21d old.

The morphological results obtained in this study demonstrated that there are differences in villus height in jejunum and ileum of wean age that corroborates with other studies conducted comparing ages at weaning in piglets (Marsi et al., 2015).

As an adaptive response to the weaning process, the intestinal structure responds with transient modifications in its morphology and barrier function (Wijtten et al., 2011), with characteristic atrophy of villi and cryptic hyperplasia, which reduces the capacity of digestion and absorption of nutrients (Pluske et al., 2016). The damage that occurs in the intestinal epithelium is transient and the weaning age reflects on the recovery time of villous atrophy (Hampson et al., 1986; Li et al., 1991) immediately after weaning (Lallès et al., 2007; Moeser et al., 2007a).

According to Hu et al. (2013), piglets weaned at 21 days have shorter villi between 3 and 7 days post-weaning, and take up to 14 days post weaning to recover those pre-weaning values. Weaned at 24 days it takes around seven days for recovery villous atrophy (Molly et al., 2001). Evaluating the villous of jejunum in piglets weaned at 14 and 28 days of age, Pluske et al. (2003) found that early weaning induced severe atrophy compared to late weaning. In relation to the recovery of villous atrophy, no recovery was observed in piglets weaned at 14 days of age, where at 14 days after weaning it was still shorter than those weaned at 28 days.

Low feed energy intake results in absence of nutrients in the intestinal lumen, which results in lower protein synthesis and rate of cell proliferation, contributing to villus atrophy (Burrin & Stoll, 2003) and, the longer villi suggest an increase in surface area favoring the absorption of available nutrients (Tsukahara et al., 2016).

Thus, it was hypothesized that weaning at 25 days of age resulted in a higher villus height in the jejunum and ileum compared to weaning at 21 days. It is important to highlight the association of these results with feed intake and the incidence of diarrhea. These older piglets showed higher feed intake and lower incidence of diarrhea in the first days after weaning, which contributed to intestinal maturation.

Regarding the microbiological count, the results are described in Table 6. No interaction was found between age and challenge ($P < 0.05$) for microbiological count. Between treatments, there was a significant effect of weaning age of the pigs for the *E. coli* count. Piglets weaned at 21 days presented higher *E. coli* counts in relation to 25 day weaning ($P = 0.039$). Age and challenge did not affect the *Lactobacillus* count ($P > 0.05$).

Table 6. Effect of weaning age on the *Escherichia coli* and *Lactobacillus* count in cecal content of piglets challenged or not with ETEC k88$^+$

Cecal content (log10 cfu/g)	**Treatments**				CV %	SEM	**P –Value**		
	Challenge		No challenge				C	WA	C*WA
	21	25	21	25					
E.coli	4.638	4.002	4.590	4.230	6.711	0.2899	0.200	0.039	0.515
Lacto	6.171	6.620	6.454	6.590	6.490	0.4157	0.747	0.445	0.678

Notes. C: challenge with ETEC; WA: weaning age; C x WA: interaction between challenge and weaning age. SEM: standard error of the mean.

The gastrointestinal tract piglets are composed of a complex and dynamic microbial ecosystem whose composition varies over time between individuals and along the GIT (Konstantinov et al., 2004; Isaacson et al., 2012). At weaning, several factors contribute for loss intestinal microbiota diversity, especially during the first week after weaning (Tao et al., 2015; Holman et al., 2017).

The higher *E. coli* count found in piglets weaned at 21 days compared to 25 days, were in agreement with previous studies. Franklin et al. (2002) found that in 10 days after weaning, piglets weaned at 17 days of age had a higher *E.coli* count than those weaned at 24 days. Leliveld et al. (2013) studied the effect of weaning age at 21, 28 and 35 days on counting faecal sample of *E. coli*. They found that piglets weaned earlier (3 weeks) presented highest score counts of this organism compared to weaned piglets later (4 weeks).

These findings are consistent with the physiological immaturity of the prematurely weaned piglet. With the immaturity of the digestive system and the reduction of gastric motility, larger amounts of undigested food are present in GIT favor the fermentation and proliferation of pathogenic bacteria such as *E. coli* (Suryanayana et al., 2012).

The results of the concentration of volatile fatty acids (acetic, propionic and butyric) in cecal content of weaned piglets at 21 and 25 days and challenged or not with *E.coli* are presented in table 6.

No interaction was found between age and challenge ($P < 0.05$) for VFA concentration. There was statistical difference only for the challenge, where the concentration of all acids was higher ($P < 0.05$) in the no inoculated groups compared to the inoculated groups (Table 7).

Table 7. Effect of weaning age on the concentration of volatile fatty acids in the cecal content of piglets challenged or not with ETEC k88^{+}

VFA (mmol/g)	Treatments				CV (%)	SEM	P- Value		
	Challenge		No challenge				C	WA	C*WA
	21	25	21	25					
Acetic	29.944	29.533	56.974	49.285	45.984	3.601	0.001	0.496	0.540
Propionic	12.555	12.123	21.645	19.000	44.362	1.369	0.003	0.523	0.645
Butyric	3.180	3.887	6.567	4.320	49.914	0.432	0.019	0.319	0.063
TOTAL	45.680	45.543	85.931	74.070	43.717	5.189	0.000	0.477	0.487

Notes. C: challenge with ETEC; WA: weaning age; C x WA: interaction between challenge and weaning age. SEM: standard error of the mean.

The altered balance of the intestinal microbiota is known as dysbiosis (Zhang et al., 2015). Loss of beneficial microorganisms, pathogen expansion and/or loss of diversity are events involving in this process (Petersen & Round, 2014) and are important in development intestinal disorders such as diarrhea (Minamoto et al., 2014).

In eubiosis, the microbiota produces of volatile fatty acids and vitamins. These acids, such as acetic, propionic and butyric, are the main end products derived from the microbial fermentation coming from the fermentation of carbohydrates in the large intestine (Cummings & Macfarlane,1991), and influence the protective function of the epithelium (Sicard et al., 2017).

Intestinal microbiota and VFA production are closely associated. VFA production may also be affected under dysbiosis conditions as proposed by Forslund et al. (2015), Rosario et al. (2018) and found by Borges et al. (2018). As found by these authors, external factors such as the environment may cause intestinal imbalance and favor the proliferation of pathogenic bacteria also produce VFA in the gut, which may also have occurred in the present study, since the highest production of VFA was found in the non-challenged group comparing to the challenged group.

4. CONCLUSIONS

These results suggest that weaned piglets at 25 days of age showed higher feed intake in the first four days of nursery, reflecting the lower incidence after weaning compared to conventional weaning at 21 days. In addition, weaning at 25 days of age promotes greater villus height and lower *Escherichia coli* count compared to weaning at 21 days of age.

In the period from 4 to 12 experimental days, the incidence of diarrhea was higher in non-challenged animals than in challenged animals. Even without changes in the diarrhea incidence in the challenged animals, the feed intake at four days was influenced by the challenge.

More studies are needed to evaluate the interaction of challenge with age in piglets consuming less complex diets.

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