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Effect of breast feeding time on physiological, immunological and microbial parameters of weaned piglets in an intensive breeding farm

G.R. García[a,c], C.A. Dogi[a,c,*], G.E. Ashworth[b], D. Berardo[b], G. Godoy[b], L.R. Cavaglieri[a,c], A. de Moreno de LeBlanc[c,d], C.R. Greco[a]

[a] Departamento de Microbiología e Inmunología, Argentina
[b] Departamento de Fisiología Animal, Universidad Nacional de Río Cuarto, Ruta 36 km, 601, 5800 Río Cuarto, Córdoba, Argentina
[c] Member of Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina
[d] Centro de Referencia para Lactobacilos (CERELA), Tucumán, Argentina

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ABSTRACT

The aim of this work was to study the long-lasting consequences of different weaning age on physiological, immunological and microbiological parameters of weaned piglets. Piglets were weaned at 14 days (14W) or 21 days (21W). Blood samples were taken for IgG and cortisol determination on preweaning day and at 4; 20 and 40 post-weaning days. Three animals of each group were sacrificed. Small intestines for morphometric studies and secretory-IgA determination in fluid were taken. The cecum was obtained for enterobacteria, lactobacilli and total anaerobes enumeration. A significant decrease in piglet's plasma IgG concentrations was observed immediately after weaning and no differences were found between 14W and 21W. An increase in intestinal S-IgA was observed according to piglet's age. This increase was significantly higher in piglets 14W compared to piglets 21W. Animals from 14W group showed a decrease in villus length and in the number of goblet cells and intraepithelial lymphocytes. Other parameters were not affected by the weaning age. A short-term increase in cortisol was observed after weaning in both experimental groups. Enterobacteria decreased significantly after weaning in both groups, reaching values of weaning after 40 days. Lactobacilli counts decreased in both groups after weaning; however their counts were always higher than those obtained for enterobacteria. No differences were observed between 14W and 21W with regards to counts of anaerobes. The shortening of breast feeding time would favor an early synthesis of intestinal S-IgA after weaning. The changes observed in the microbiota could decrease postweaning enteric infections. However, early weaning induced negative effects on the cells of gut innate immunity and villi atrophy. This work provides knowledge about advantages and disadvantages at different weaning and long-lasting consequences on pig health. It is critical that swine producers become aware of the biological impacts of weaning age, so as to be able to decide the appropriate management strategies according to their facilities and rearing environment.

1. Introduction

Weaning has been shown to be a stressful period, with potential effects on the development of the immune system (Johnson et al., 2006), the intestine, and the intestinal microbiome (Pluske, 2013). Weaning causes physiological changes in structure and function of the intestine during the transition from liquid to a solid diet, these changes affect the absorptive capacity of the small intestine which can likely influence growth and feed efficiency (Lallès et al., 2007). In addition, the immunoregulatory and immunoprotective components of maternal milk are removed; as a consequence, pigs at weaning are highly susceptible to pathogenic enteric conditions such as postweaning diarrhea that may be caused by serotypes of enterotoxigenic *Escherichia coli* (Pluske, 2013; Rist et al., 2013). The establishment of a diverse bacterial microbiota plays a key role in the maintenance of the gastrointestinal health by preventing colonization by pathogens (Van Kessel et al., 2004). Beneficial microbiota is especially important at periods such as weaning, when the animal still has an immature immune system and depends on certain compounds in the sow's milk to prevent the growth of opportunistic bacteria (Edwards and Parret, 2002).

* Corresponding author at: Departamento de Microbiología e Inmunología, Universidad Nacional de Río Cuarto, Ruta 36 km, 601, 5800 Río Cuarto, Córdoba, Argentina. Fax: +54 358 4676231.
E-mail address: cdogi@exa.unrc.edu.ar (C.A. Dogi).

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During natural weaning, the piglet is progressively introduced to several external feed sources and its fermentative capacity and immune system is progressively developed for approximately 10 weeks, at which time the animal is completely weaned. The number of births per year is an important indicator of the sow's productivity and it is related to the duration of breastfeeding. Early weaning of pigs enhances growth and feed efficiency, reduces the potential for vertical transmission of pathogens (Butler et al., 2008) and therefore is being adopted widely by the swine industry. However, this early weaning result in postweaning diarrhea, mainly related to intestinal dysbiosis during this rapid adaptation to the new diet. There are several studies suggesting that early weaning causes substantial changes in the intestinal bacterial community (Franklin et al., 2002; Konstantinov et al., 2006). The weaning age has declined over time in order to increase sow productivity and the fact that weaning under modern-day commercial conditions inflicts stress (environmental, nutritional, psychological/social) on pigs and is associated with marked changes in gastrointestinal tract (GIT) physiology, microbiology and immunology. Considering this, the aim of the present work was to study the effect of weaning at 14 and 21 days on systemic and mucosal immunology and on the gut morphology and bacterial community of weaned piglets in an intensive breeding farm. The work was conducted to provide knowledge about advantages and disadvantages of weaning at different ages and long-lasting consequences in an intensive breeding farm, where the high production indexes could be in detriment of animal welfare.

2. Materials and methods

2.1. Animals and experimental design

All studies were approved by Subcommittee on Animal Bioethics of Universidad Nacional de Rio Cuarto (Córdoba, Argentina), as established by Resolution 253/10 of the Council.

A total of 30 (Large White x Landrace) pigs, obtained from an intensive indoor breeding farm, were randomly selected from different litters and divided into two groups: 1) 14 day weaning age (14W) and 2) 21 day weaning age (21W). Piglets were ear tagged for identification and reared in confinement with controlled light and temperature. Pigs had *ad libitum* access to water and feed. The general health status of pigs was evaluated by recording any changes in behaviour, activity, posture, presence of diarrhea, feed and water intake, possible illness and deaths. No clinical signs of disease were observed during health monitoring in the experimental period.

Three animals of each group were sacrificed one pre-weaning day (−1) and at 4, 20 and 40 post-weaning days.

2.2. Blood samples

Before sacrifice, peripheral blood was collected (10 ml) from 15 14W and 21W pigs by puncture of vena cava (at −1 and 4 days postweaning) and of external yugular vein (at 20 and 40 days postweaning), using heparin as anticoagulant. All blood samples were collected between 0630 and 0800 h, and blood collection typically lasted less than 1 min per pig. For cortisol determination, blood samples were also taken at the moment of the weaning and 4 and 24 h after weaning. The samples were transported on ice and then centrifuged at 400 *g* for 15 min. Plasma was removed, frozen and stored at −20 °C until IgG and cortisol determination.

2.3. Gut samples

Animals were anaesthetized with thiopentone sodium perfusion and infarction was induced by lidocaine hydrochloride 2% intrathecal. Intestinal fluid was collected from the small intestines; approximately 10 cm of ileum was taken and washed with 9 ml of 0.85% NaCl and centrifuged at 5000 *g* during 15 min at 4 °C. The supernatant was recovered and stored at −20 °C until IgA determination.

The supernatant was recovered and stored at −20 °C until IgA determination.

For histological study, three portions (2 cm length each) from the ileum were taken and fixed by immersion in 10% (v/v) buffered formalin immediately after slaughter.

For microbiological determinations, the cecum was aseptically removed, weighed and placed into sterile tubes containing 70 ml of peptone solution (0.1%) and kept on ice.

2.4. IgG and S-IgA analysis

IgG in plasma and IgA in intestinal fluid were measured by an indirect enzyme-linked immunosorbent assay (ELISA) with a commercial kit specific for swine IgG and IgA (Bethyl, Montgomery, USA) according to the manufacturer recommendations.

2.5. Cortisol analysis

Cortisol concentrations were determined in plasma samples using a radioimmunoassay kit (RIA, Cortisol DSL-2100, Diagnostic Systems Laboratories, Inc.) in accordance with the manufacturer's protocol. The tubes were counted on a Wizard Auto Gamma counter.

2.6. Morphometric analysis

Ileum samples for histological study were dehydrated and embedded in paraffin wax, sectioned at 4 μm and stained with haematoxylin and eosin. The morphometric measurements taken from the intestinal histological sections included crypt depth, villus length and width. The same villi were used to determine the number of goblet cells. Measurements were performed in 10 well oriented villi from each animal. The number intraepithelial lymphocytes (IEL) per 100 epithelial cells in the complete villous was counted. Digital images were captured with an Axiophot microscope (Carl Zeiss, Thornwood, NY) fitted with high resolution Powershot G6 7.1 megapixels digital camera (Canon Inc., Japan). Digital image analysis and morphometric measurements were performed with Axiovision AxioVs40 V4.6.3.0. Software (Carl Zeiss, Göttingen, Germany).

2.7. Microbiological analysis

Samples were immediately homogenized, serial dilutions were obtained and aliquots (0.1 ml) of the appropriate dilution were spread onto the surface of the agarized media: Reinforced Clostridial (RCA, Britania, Buenos Aires, Argentina) for total anaerobic bacteria; Mann–Rogosa–Sharp (MRS Britania, Buenos Aires, Argentina) for total lactobacilli and MacConkey (Britania, Buenos Aires, Argentina) for Enterobacteriaceae enumeration. Colony counts were expressed as log10 numbers of bacteria per gram of cecum.

2.8. Statistical analysis

Results were expressed as the mean values of independent results ± the standard deviation (DS). Three pigs of each group were sacrificed in each trial and samples were collected. The experimental protocols were repeated 2 independent times and the samples obtained for the analysis of different data correspond to 6 pigs for each group (from both trials). The results were analyzed by analysis of variance (ANOVA). Means were compared using Fisher's

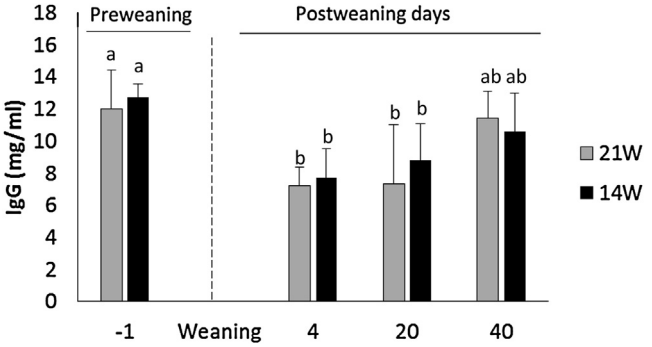

Fig. 1. Total plasma IgG concentration (mg/ml) in piglets weaned at 21 days (21W) or 14 days of age (14W). IgG levels were determined using indirect ELISA at one pre-weaning day and at 4, 7, 20 and 40 post-weaning days. Each point represents the mean of $n = 15 \pm$ SD pigs from each group. Different letters show significantly differences ($P < 0.001$).

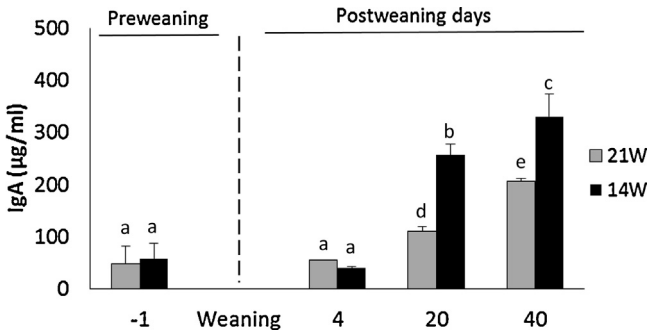

Fig. 3. Total S-IgA concentration (µg/ml) in the small intestine fluid from pigs of different experimental groups were determined using indirect ELISA at one pre-weaning day and at 4, 20 and 40 post-weaning days. Each point represents the mean of $n = 3 \pm$ SD pigs from each group. Different letters show significantly differences ($P < 0.001$).

protected LSD test, and $p < 0.05$ was considered significant. The analysis was conducted using PROC GLM in SAS (SAS Institute, Cary, NC,USA).

3. Results

3.1. Plasma IgG quantification

The results for plasma IgG determination in piglets are shown in Fig. 1. A significant decrease in IgG concentrations was observed after weaning and then the values remain without significant differences until 40 days postweaning, when IgG concentrations reach the values obtained before weaning. No differences were found between 14W and 21W.

3.2. Cortisol determination

The higher cortisol level was obtained at the moment of the weaning (10.40 ± 0.80 µg/dl) and then the levels diminished until restored to the basal concentrations found at the preweaning day (2.20 ± 0.90 µg/dl for 14W group and 2.10 ± 0.80 µg/dl for 21W). A decrease in cortisol concentrations was observed 4 days after weaning, due to a negative retro alimentation. On 20 and 40 postweaning days the levels were similar to those obtained at the preweaning. No differences were observed in serum cortisol concentration between the two experimental groups (Fig. 2).

3.3. Intestinal S-IgA determination

The results for intestinal S-IgA determination in piglets are shown in Fig. 3. From day 20 postweaning an increase in intestinal IgA was observed according to pigletís age. This increases was significantly higher ($p < 0.0001$) in animals weaned at 14 days (14W) compared to animals weaned at 21 days (21W).

3.4. Intestinal morphology

Results for morphometric analysis are shown in Table 1. There were significant differences ($P < 0.05$) in crypt depth and villus width associated with age at the last point evaluated. However, there were no significant differences between treatments in these endpoints. Animals from 14W group showed a significant decrease ($P < 0.05$) in villus length 4 days after weaning and then similar values to those obtained at the preweaning were observed. On the contrary animals from 21W group showed an increase in villus length across the time. A significant decrease ($P < 0.05$) in the number of goblet cells and IEL was found after weaning, compared between treatments. In 14W group, IEL decreased 4 days after weaning and then reached values similar to those of the preweaning, however, the number of goblet cells remained diminished throughout the experience.

3.5. Microbial determination in cecum samples

Microbial determination in cecum is shown in Fig. 4. No significant differences were observed between the two experimental groups. The number of enterobateria decreased significantly after weaning in both groups, reaching values of preweaning at 40 days. A decrease in lactobacilli counts was observed postweaning and the numbers of this bacterial group were restored at 20 days in both experimental groups. Despite this postweaning lactobacilli decrease, lactobacilli counts were always higher than those obtained for enterobacteria. The results obtained from total anaerobes counts showed that this bacterial population remained constant over time and no differences between weanling groups were observed. It is important to remark that diarrhea was not observed in any of the two experimental groups.

4. Discussion

The weaning is a critical moment for piglets because it produces an important stress due to feeding change, removing from sow and regrouping in postweaning phase. Many authors have reported the negative impact of weaning on the health and growth of the piglets. However, little information has been provided about the long-lasting impact of the weaning age on several aspects of the pigs health. The present work is a longitudinal study aimed at assessing how early weaning affects physiological, microbiological and immunological parameters of the pig. Pigs have an epitheliochorial placenta which is impermeable to immunoglobulins, therefore the colostrum is the principal source of immunoglubulins for the neonates and in porcine is characterized by very high levels of IgG. Maternal immunoglobulins are absorbed from the intestine into the systemic circulation from colostrum ingested within the first 36 h after birth (Salmon et al., 2009; Butler et al., 2008). According to that, in the present work, the highest levels of IgG in pigletís plasma were detected the day before weaning, and then the values decreased with the age of the pigs. The weaning age has no effect on plasma IgG levels.

The IgA, in the form of secretory IgA (S-IgA) is the major antibody isotype present in mucosal secretions and has many functional attributes (Lamm, 1976). Similarly to the IgG, during the suckling phase of development, luminal intestinal secretory IgA is provided predominantly by the colostrum and breast milk, whereas in post-weaned animals, secretory IgA is synthesized by the weanlings on

Table 1
Histological studies on ileum of piglets weaned at 14 and 21 days of age (Media ± SD).

	Pre and postweaning Days	Weaning at 14 days	Weaning at 21 days
Cryp depth (μm)	−1	125.20 ± 56 a	104.45 ± 11 a
	4	113.41 ± 30 a	158.18 ± 0.06 b
	20	119.44 ± 22 a	256.63 ± 42 c
	40	177.48 ± 33 b	304.70 ± 68 c
Villus length (μm)	−1	379.88 ± 137 b	290.52 ± 58 a
	4	290.41 ± 72 a	341.83 ± 90 a
	20	333.60 ± 73 ab	421.04 ± 98 b
	40	329.48 ± 73 ab	348.49 ± 62 ab
Villus width (μm)	−1	125.20 ± 25 a	120.14 ± 18 a
	4	113.41 ± 21 a	111.36 ± 19 a
	20	119.44 ± 26 a	129.18 ± 28 a
	40	177.48 ± 61 b	170.10 ± 29 b
Goblet cells (Number/10 villi)	−1	17.60 ± 4.22 c	16.40 ± 4.33 a
	4	14.90 ± 3.48 bc	19.10 ± 4.89 a
	20	6.50 ± 1.35 a *	18.90 ± 1.45 a *
	40	13.40 ± 3.10 b *	20.30 ± 3,59 a *
IEL (IELs/100 epithelial cells)	−1	21.25 ± 4.03 b	36.25 ± 12.53 a
	4	14.25 ± 6.70 a *	44.50 ± 7.14 a *
	20	34.50 ± 4.85 c	36.00 ± 4.94 a
	40	47.00 ± 10.32 c	49.83 ± 12.53 a

Each point represents the mean of $n = 3 \pm$ SD. Different letters show significant differences ($P < 0.001$) within each column.
* means significant differences between treatments (14W and 21W).

their own adaptive immune system. Despite the beneficial effect of breast-feeding in newborns, it is believed that maternal antibodies may have a suppressive effect on the development of mucosal immune response in their offspring, leading to a partially developed immune system at weaning (Kramer and Cebra, 1995; Wagstrom et al., 2000; de Moreno de LeBlanc et al., 2008). According to that, in the present work, piglets increased IgA concentrations in the intestinal contents from 20 days post-weaning, which would be related to the lack of maternal antibodies and the maturation of the own immune system. It is also important to note that piglets weaned at 14 days showed a significant increase of intestinal S-IgA compared to animals weaned at 21 days. This result suggests that the early weaning stimulated the maturation of the own adaptive immune system in the newborn piglets. Therefore, the shortening of breast feeding period could induce an earlier active synthesis of gut IgA in the pigs.

The process of weaning is one of the most stressful events in the pig's life. Cortisol is a traditional measure of stress in pigs, being elevated by acute stress (Ruis et al., 2001). Cortisol is a steroid hormone that regulates the metabolism and the body's reaction to stress and inflammation. To determine whether weaning age influenced baseline activity of the hypothalamic–pituitary–adrenal (HPA) axis, serum cortisol was measured in 14W and 21W animals. According to the marked increases in cortisol concentrations at the weaning day compared to the preweaning, weaning indeed became a stressful event for the piglets. However, the stress associated with weaning was transient, with a short-term increase in cortisol after weaning. These results are similar to those obtained by Kick et al. (2012). A significant lower cortisol value was found at day 4 post weaning, compared to 20 and 40 post weaning days. This fact could be explained by the negative feedback effect of cortisol on the hypothalamus and pituitary. Cortisol secretion is controlled by the hypothalamic pituitary adrenal axis (HPA), which is a self-regulated dynamic feedback neuroendocrine system (Faghih et al., 2011). The values obtained in the postweaning period were around 3 and 3.5 μg/dl and, according to previously reported are not indicative of stress (Franklin et al., 2002; Sutherland et al., 2006; Jarvis et al., 2008). The results demonstrated that the cortisol levels was not influenced by the weaning age.

Regarding intestinal morphology, Pluske (2013) reported that weaning induces structural and functional changes in the small intestine including shortening of the villi (villous atrophy) and an increase in crypt depth. In the present work, a shortening of villi was observed in animals early weaned (14W) immediately after weaning and this fact did not occur in animals from 21W group.

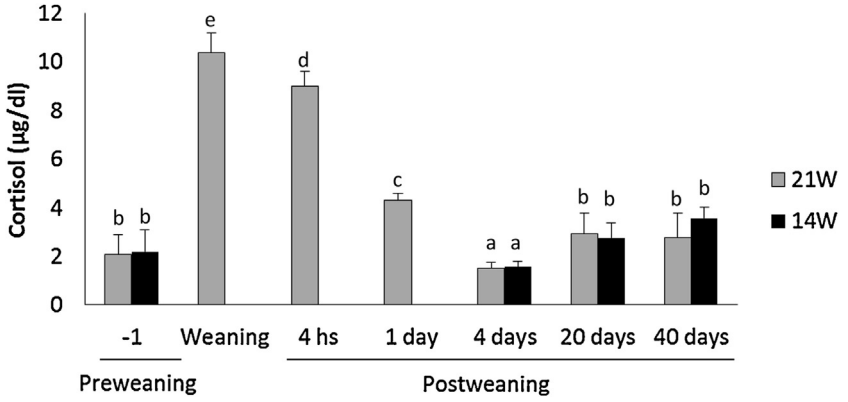

Fig. 2. Cortisol (μg/dl) in plasma samples from pigs of different experimental groups were determined using radioimmunoassay kit at one pre-weaning day, weaning, 4 h postweaning and at 1, 4, 20 and 40 post-weaning days. Each point represents the mean of $n = 3 \pm$ SD pigs from each group. Different letters show significantly differences ($P < 0.05$).

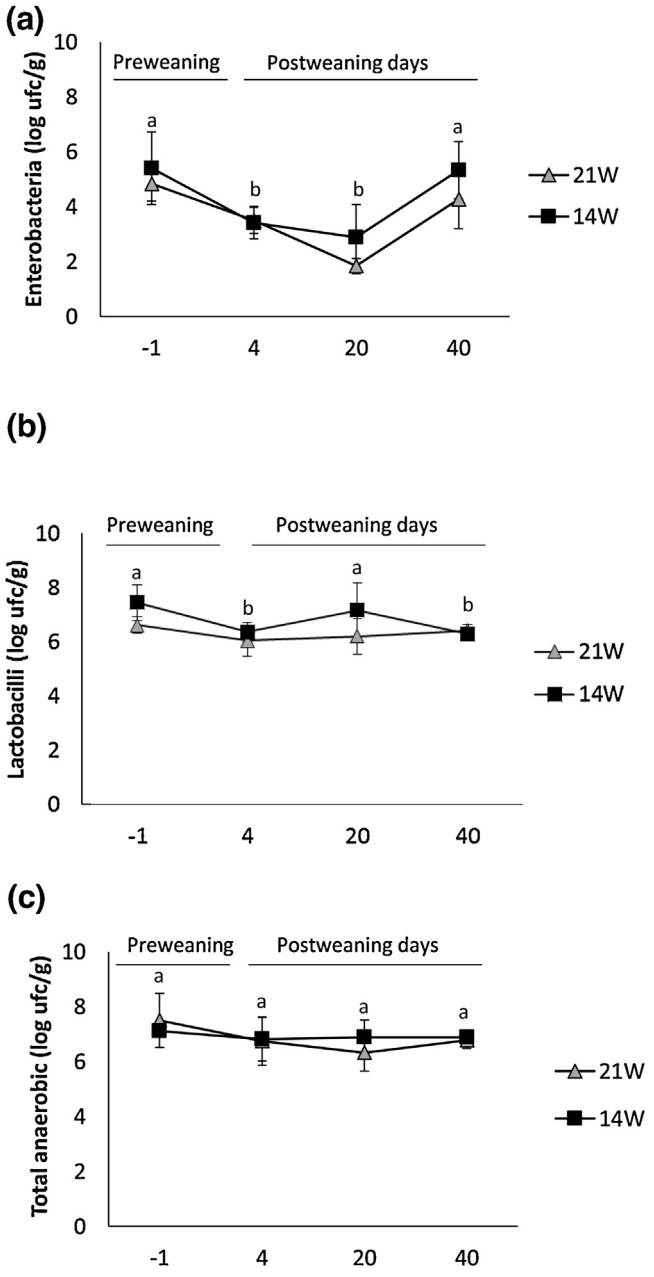

Fig. 4. Intestinal enterobacteria (a), lactobacilli (b) and total anaerobic bacteria (c) populations of weaned pigs. Each point represents the mean of $n = 3 \pm$ SD. Different letters show significant differences ($P < 0.001$).

This physiological change could affect the absorptive capacity of the small intestine which can likely influence feed efficiency.

Concerning the intestinal barrier, both cells producing the mucous layer and immune cells were studied because they are the first line of host defense against noxious agents and infections (Cheroutre, 2004). A decrease in the number of goblet cells was found in the 14W compared to 21W group. Similar results were reported by Castillo et al. (2007). Goblet cells reside throughout the length of the small and large intestine and are responsible for the production and maintenance of the protective mucus blanket by synthesizing and secreting high-molecular-weight glycoproteins known as mucins. The number of goblet cells is regulated by both microbial and host related factors (Deplancke and Gaskins, 2001). However, little information is available on the effect of weaning on mucin secretion. On the other hand, intraepithelial lymphocytes were affected transiently by early weaning. Taking into account the results obtained for goblet cells and IEL counts, early weaning seems to have negative impacts on the gut innate immunity.

The porcine gastrointestinal tract harbors a complex and dynamic microbial ecosystem. Microbiota development is strongly affected by the host genotype as well as environmental factors, including maternal gastrointestinal tract and milk-associated microbiota, and diet after weaning (Bauer et al., 2006; Mach et al., 2015). Postweaning disorders in pigs result in gastrointestinal alterations, from architecture, function and also from major changes in the adapting enteric microbiota: while populations of *Lactobacillus* remained stable and abundant before weaning, their numbers dropped significantly after weaning in conjunction with a marked increase in enterobacteria counts (Konstantinov et al., 2004). Lactobacilli and enterobacteria have been traditionally selected as microbial groups with a particular significance for gut health. The presence of lactobacilli in the gastrointestinal tract of pigs is believed to be beneficial for the animal, whereas *Escherichia/Shigella* are pathobiont species, awaiting potential stressors to become pathogenic (Mach et al., 2015). Unexpectedly, in the present work, the decrease of lactobacilli after weaning was accompanied by a significant decrease in the number of enterobateria in both groups, reaching values of weaning at 40 days postweaning. This fact could be due to the hygienic–sanitary conditions of the farm and the addition of a dairy supplement in feed. Despite the decrease in lactobacillus counts observed postweaning, they counts were always higher than those obtained for enterobacteria. In contrast to our results, an inverse correlation between lactobacilli and enterobacteria during the first week postweaning has been previously reported (Jensen-Waern et al., 1998; Franklin et al., 2002; Castillo et al., 2007). The counts of anaerobes remained relatively constant along the whole experience and this could be considered as indicator of gastrointestinal stability (Franklin et al., 2002).

5. Conclusion

The significance and impact of this work lie in the study of the long-lasting effects of weaning age in pigs using a combination of immunological, microbiological and physiological parameters. The weaning age did not affect the systemic immunity of the piglets. However, differences were observed at mucosal level: the shortening of breast feeding would favor the early increases of intestinal S-IgA after weaning, leading to a faster developed adaptive immunity. Nevertheless, negative effects on the innate immunity were observed by early weaning. Weaning age had no influence on serum cortisol levels and on intestinal bacterial community; though some gut morphometric parameters seem to be affected by early weaning and could affect feed efficiency.

Weaning age has declined over time in order to increase sow productivity. Biological alterations in immune system and gut physiology could occur after weaning that may have both short and long-term effects on subsequent pig growth and health. It is critical that swine producers become aware of the biological impacts of weaning age and they can decide the appropriate management strategies to minimize the adverse effects of weaning and to improve swine production, according to their facilities and rearing environment.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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