Acta Scientiarum
http://periodicos.uem.br/ojs/acta
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v47i1.71652

Barrows and gilts respond differently as experimental models for *Escherichia coli* challenge during the nursery phase

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ABSTRACT. The objectives of this study were to evaluate the effect of *Escherichia coli* (*E. coli*) F4 challenge in nursery piglets, and to develop an experimental model to be used in research. Ninety-six weaned piglets were divided in a 2 x 2 factorial design consisting of *E. coli* challenge (challenged or not challenged) and sex (barrows or gilts). Pig growth performance, fecal score, blood count, intestinal morphometry, and cecal content (microbiological and short-chain fatty acid analysis) data were collected and analyzed. Pigs in the non-challenged treatment had 6% greater average daily gain during day 8-28 (the period in which the health challenge was administered) and 1.2% greater average daily feed intake in over the entire experiment, when compared to challenged pigs. Gilts in the non-challenged group had a higher villus: crypt ratio when compared to piglets in the challenged group. These findings indicate that the pathogen challenge using *Escherichia coli* F4 strains, especially in gilts, proved to be an effective method to reproduce commercial health challenges in the first two weeks postweaning and may be used in experimental models.

Keywords: health; disease challenge model; swine.

Received on March 15, 2024.
Accepted on March 28, 2025.

Introduction

Modernization of the swine industry has increased the sanitary and environmental challenges for piglets, especially during the post-weaning and nursery phases (Oliveira Jr. et al., 2013). During these periods, piglets undergo biological stress resulting from physiological, environmental, and social challenges, which contributes to the occurrence of diseases and impairs piglet health, welfare and performance (Campbell et al., 2013).

During the nursery phase, piglets are in constant contact with several pathogenic microorganisms, such as *Escherichia coli*, which combined with stressors, predispose the piglets to infections (Kummer et al., 2009). Enterotoxigenic *E. coli* (ETEC) is a major bacterial agent that affects the health of piglets in the post-weaning period and is one of the main microorganisms related to the occurrence of diarrhea in the nursery phase (Che et al., 2017). The impairments on gastrointestinal tract caused by the pathogen affects the animal's health, its growth and development throughout the production cycle (Wang et al., 2018; Che et al., 2017; Zhu et al., 2018).

The consequences of pathogenic *E. coli* in piglets are diverse such as damage intestinal integrity and morphology, trigger an infectious and inflammatory process, and result in intestinal dysfunction. Intestinal integrity is essential in defense against pathogens (Pi et al., 2014). When the small intestine is compromised, the digestion and absorption of nutrients, such as proteins and amino acids, are also diminished (Chen et al., 2014). This results in piglets becoming increasingly susceptible to other infections and enteric diseases, which affects pig growth performance and leads to economic losses.

Given the challenges the piglets face throughout the nursery phase, experimentally disease challenges that replicate conditions found in commercial productions are of the major importance. There are few studies that evaluate the effect of disease challenges in piglets. Thus, the objectives of the present study are to evaluate the effects of an *E. coli* F4 challenge on gilts and barrows during the nursery phase on parameters related to the intestinal health and productive outcome of piglets and to analyze the feasibility of using such a challenge under experimental conditions.

Material and methods

Animals, experimental design and procedures

The experiment was approved by the Ethics Committee on the Use of Animals of the School of Veterinary Medicine and Animal Science at the University of São Paulo, under protocol number: 3743220518. The study was carried out on at the Swine Research Laboratory of the University of São Paulo, located in the city of Pirassununga, São Paulo, Brazil (21° 59' 46'' S and 47° 25' 36'' W).

A total of ninety-six piglets (48 barrows and 48 gilts) of Choice Genetics terminal cross line, obtained from a commercial swine herd, were used. The piglets were weaned at an average age of 24 days and with an average weight of 6.7 ± 0.92 kg. The piglets were housed in a nursery unit equipped with 48 raised stalls, 50% slatted floor, with semi-automatic feeders and nipple drinkers. The temperature was controlled using side curtains and heating lamps.

The experimental design was carried out in randomized blocks (based on initial weight and sex) with four treatments and 12 repetitions, in a 2 x 2 factorial treatment design (challenged or not challenged with *E. coli*) X (barrow and gilt). The experimental unit was composed of the mean (BW 6.71 ±0.4 kg) of the two pigs present in each pen. The experimental period was 42 days, subdivided into pre-challenge (0 to 7 days), peri-challenge (8 to 28 days), and post-challenge (29 to 42 days). The diets were formulated to meet or exceed the nutritional requirements of the nursery phase according to the NRC (2012) and are presented the in supplementary materials.

The disease challenge with *Escherichia coli* F4 was performed on day 8, 9 and 17 of the experiment. The days were selected to mimic the most challenging days on a commercial farm. On the first and second challenge, piglets in the challenged group received 1 ml of a solution containing the bacteria (concentration of 106 CFU mL-1 of *E. coli*) and the piglets in the non-challenged group received 1 ml of saline solution. Another bacterial inoculation was performed on the 17th day of the experiment, in which piglets from the challenged group received 2 ml of the bacterial solution (concentration of 109 CFU mL-1 of *E. coli*) while piglets from the non-challenged group received 2 mL of saline solution. The challenges were performed with the aid of a nasogastric tube.

The bacterial inoculum was prepared from the field strain *Escherichia coli* F4 (LT+, Sta+ and STb+). The strain was cultivated in culture medium for 16 hours at 37ºC, and then washed sequentially in PBS (phosphate-buffered saline) until a concentration of 106 or 109 CFU mL-1 was reached according to the methodology of Rodrigues et al. (2020).

On days 11, 28, and 42 of the experiment, serial slaughters were performed to evaluate intestinal health in different times post-challenge. Nine piglets per treatment, piglets closest to the mean weight of each treatment, were selected for slaughter, totaling 54 animals during the experimental period (27 barrows and 27 gilts). The slaughter was carried out through electronarcosis followed by exsanguination, in the slaughterhouse of the University of São Paulo, located in Pirassununga, São Paulo, Brazil, using trained staff.

Performance, incidence of diarrhea, hemogram, microbiological and short chain fatty acids composition of the cecal content

To evaluate the productive performance, piglets were weighed on day 0, 7, 14, 21, 28, 35th, and 42 of experiment. The feed provided and leftovers were evaluated daily. Based on these data, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion (FC, ADFI:ADG) were calculated.

Druing the experimental period, feces in all pens were evaluated and classified twice a day to estimate the incidence of diarrhea. Fecal score was based on the methodology of Pedersen and Toft (2011), in which stool scores range from 1 to 4 and are associated to the level of diarrhea and presence of liquid or pasty feces. The feces were evaluated and scored according to the following characteristics: score 1, feces firm and molded; score 2, soft and molded feces; score 3, loose feces; score 4, watery feces. Fecal scores of 1 and 2 were considered healthy and scores of 3 and 4 associated with diarrhea.

At slaughter, blood samples were collected to assess red blood cell count, leukocyte count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Additionally, platelet count and a morphological analysis of leukocytes were conducted using the May-Grunwald-Giemsa blood smear technique.

Post-slaughter samples of jejunum (2.0 cm) were collected to evaluate the integrity of the intestinal mucosa. The samples were prepared in accordance with the method outlined by Zhaxi and colleagues (2020). A microscope equipped with a camera and ImageJ software was utilized to measure parameters such as villi height, crypt depth, and the villus: crypt ratio.

Cecal content samples were obtained to assess the concentrations of short-chain fatty acids (SCFA) – acetate, propionate, isobutyrate, butyrate, isovalerate and valerate and to analyze the populations of intestinal bacteria, including *Enterobacterium, Escherichia coli*, *Lactobacilli* and *Bifidobacterium*. The samples were diluted in phosphate-buffered saline (PBS), with *E. coli, Enterobacterium*, and *Bifidobacterium* samples being fractionated from 10-1 to 10-3g, and *Lactobacillus* samples from 10-1 to 10-5. Selective culture media were employed for the cultivation of each bacterium group. Prior to statistical analysis, all colony counts (CFU g-1) underwent logarithmic transformation (log10).

For statistical analysis, the Shapiro-Wilk test was used to assess data normality. When the data did not follow a normal distribution, they were transformed using the PROC RANK procedure of SAS Institute Inc (SAS, 2009). Data was anaylzed using analysis of variance (ANOVA). When there was a statistical difference by the F test ($p < 0.05$) for the interactions, Tukey test was used to compare means. Data were submitted to the statistical package of the SAS software (2009) through the MIXED procedure. For the data "incidence of diarrhea", SAS NPAR1WAY procedure was used. Dunn's test was applied as post-hoc for multiple paired comparisons, with $p < 0.05$ being considered significant, for variables that were rejected by the Kruskal-Wallis test at a 5% probability level.

Results and discussion

The performance of piglets was compared in periods related to experimental procedures: pre-challenge; peri-challenge and post-challenge (day 0-7; 8-28; and 29-42 respectively) and total experimental period (day 0-42). Results can be found in Table 1.

Table 1. Performance of animals challenged or not with *Escherichia coli* F4.

Variables	Sex	Challenge		Sex mean	MSE	P-value		
		Without	With			Sex	Challenge	Sex*Challenge
Initial weight, kg	Gilt	6.78	6.77	6.77	0.398	0.810	0.245	0.924
	Barrow	6.64	6.63	6.64				
	Challenge average	6.71	6.70					
Body weight day 7, kg	Gilt	8.77	8.56	8.66	0.506	0.902	0.136	0.536
	Barrow	8.62	8.53	8.58				
	Challenge average	8.70	8.54					
ADG day 0-7, kg	Gilt	0.285	0.256	0.270	0.019	0.782	0.163	0.521
	Barrow	0.283	0.272	0.277				
	Challenge average	0.284	0.264					
ADFI day 0-7, kg	Gilt	0.383	0.375	0.379	0.026	0.788	0.850	0.348
	Barrow	0.382	0.395	0.389				
	Challenge average	0.383	0.385					
FC day 0-7	Gilt	1.345	1.477	1.411	0.047	0.836	0.302	0.556
	Barrow	1.356	1.459	1.408				
	Challenge average	1.351	1.468					
Body weight day 28, kg	Gilt	18.48	17.80	18.14	0.96	0.876	0.055	0.895
	Barrow	18.74	17.96	18.35				
	Challenge average	18.61	17.88					
ADG day 8-28, kg	Gilt	0.462	0.435	0.449	0.024	0.643	0.029	0.970
	Barrow	0.478	0.450	0.464				
	Challenge average	0.470	0.443					
ADFI day 8-28, kg	Gilt	0.706	0.665	0.686	0.042	0.775	0.065	0.990
	Barrow	0.723	0.682	0.702				
	Challenge average	0.715	0.673					
FC day 8-28	Gilt	1.527	1.528	1.527	0.037	0.759	0.920	0.951
	Barrow	1.510	1.514	1.512				
	Challenge average	1.518	1.521					
Body weight day 42, kg	Gilt	25.79	25.43	25.61	1.10	0.737	0.590	0.895
	Barrow	26.23	26.01	26.12				
	Challenge average	26.01	2.72					
ADG day 29-42, kg	Gilt	0.521	0.496	0.509	0.027	0.136	0.834	0.282
	Barrow	0.535	0.572	0.553				
	Challenge average	0.528	0.534					
ADFI day 29-42, kg	Gilt	0.979 ab	0.913 ab	0.946	0.064	0.843	0.339	0.014
	Barrow	0.896 b	1.030 a	0.963				

	Challenge average	0.937	0.971					
	Gilt	1.877	1.829	1.853				
FC day 29-42	Barrow	1.693	1.804	1.749	0.094	0.330	0.715	0.368
	Challenge average	1.785	1.817					
	Gilt	0.452	0.426	0.439				
ADG day 0-42, kg	Barrow	0.464	0.461	0.463	0.019	0.354	0.267	0.388
	Challenge average	0.458	0.443					
	Gilt	0.744	0.699	0.721				
ADFI day 0-42, kg	Barrow	0.724	0.750	0.737	0.043	0.791	0.628	0.079
	Challenge average	0.734	0.725					
	Gilt	1.640	1.637	1.638				
FC day 0-42	Barrow	1.554	1.625	1.590	0.040	0.346	0.257	0.210
	Challenge average	1.597	1.631					

MSE: mean standard error; ADG: Average daily gain; ADFI: Average daily feed intake; FC: Feed Conversion; Means followed by distinct letters differs by the test of Tukey with $p < 0.05$.

In the first period (day 0-7) and in total period (day 0-42) no significant interactions between sex and challenge were found ($p > 0.05$) and no significant differences were observed for any of the variables analyzed ($p > 0.05$). During the second period (day 8-28) no interaction was observed between sex and *E. coli* challenge ($p > 0.05$), but non-challenged piglets had a 6.0% higher ADG ($P = 0.029$) than challenged piglets. For the same phase, non-challenged piglets tended to have a 4.0% greater weight at 28 day ($P = 0.055$) and a 6.2% higher ADFI ($P = 0.065$) than piglets challenged with *Escherichia coli*. In the third phase (day 29-42), an interaction between sex and challenge was observed, in which challenged barrows had a greater ADFI (14.9% increase) when compared to non-challenged barrows, while gilts did not differ statistically ($P = 0.014$).

Unchallenged piglets had better growth performance when compared to piglets challenged by *E. coli* during the second experimental period (day 8-28), which corresponds to the period when the sanitary challenges were performed. The piglets that did not receive the bacterial inoculum had the greater ADG (day 8-28), tended to have a greater weight at 28 day, and ADFI (day 8-28) than those that were challenged. The systemic effects of inflammatory and infectious processes are known (Oliveira Jr. et al., 2013; Webel et al., 1997). Activation of the immune system triggers metabolic and neuroendocrine responses due to the action of inflammatory cytokines, which modulate immune-physiological interactions and modify nutritional requirements to allow reestablishment of homeostasis (Johnson, 1997; Oliveira Jr. et al., 2013). Among the cytokines involved in the process, there are Tumor Necrosis Factor α (TNF-α), Interleukin 1 (IL-1) and Interleukin 6 (IL-6), which lead to loss of muscle mass and proteolysis, increased secretion of hormones such as glucocorticoids that have a catabolic function in adipose and muscle tissues, promoting an anorexic effect and reducing the synthesis of anabolic hormones (Webel et al., 1997). Alterations observed in the present study coincide with effect of inflammatory processes associated with the experimental inoculation of *E. coli* F4. Other studies found similar results, such as Gao et al. (2013), who observed that animals of different breeds (Landrace and Jinhua) challenged with a solution containing *E. coli* (ETEC K88) had reduced growth and average daily feed intake during the study period.

Between day 29 and 42, challenged barrows had greater ADFI than unchallenged barrows. The increase in feed consumption by the barrows challenged with *E. coli* may have been due to their recovery after removal of the stimulus and source of infection since piglets were inoculated with the bacterial strains in the previous phase. Furthermore, the rapid recovery of barrows may be associated with the low concentration of *E. coli* inoculate used in the present study. Che et al. (2017) performed a disease challenge on piglets consisting of 100 mL doses with concentrations of 109 CFU mL-1 of *E. coli*. Owusu-Asiedu et al. (2003) used 6 mL doses of a bacterial solution containing 1010 CFU mL-1 of *E. coli* K88 (F4), while the present study used only 2 mL with 106 CFU mL-1 of *E. coli* (first and second challenge) and 2 mL with 109 CFU mL-1 of *E. coli* (third challenge). Additionally, the increased feed consumption observed in barrows (challenged with *E. coli*) when compared to gilts during the third phase of the experiment (between day 29-42) may be related to early life experiences (such castration), which influences and affects the way in which piglets cope with the weaning process and subsequently events, as suggested by Van Erp-Van der Kooji et al. (2000), not to mention hormonal status, since lower concentrations of testicular androgens and estrogens may have a suppressive effect on feed intake (Weiler et al., 1998; Santollo et al., 2021).

The percentages of each fecal score, mean fecal score and total fecal score, related to the treatment and sex of the animals, were compared in weeks of the experiment and periods of health challenges, as follows: week 1 (S1 – day 0 to 7), week 2 to week 4 (S2-S4 – day 8 to 28), week 5 to week 6 (S5-S6 – day 29 to 42).

Analyzes were also compared in relation to total period of experiment (Total score – day 0 to 42). All results are presented in Table 2.

Table 2. Incidence of diarrhea of piglets (barrow and gilt) challenged or not with *E. coli* F4.

Variables	Sex	Challenge		Sex mean	MSE	P value		
		Without	With			Sex	Challenge	Sex* Challenge
Score 1 S1, %	Gilt	85.22	78.05	81.63	3.02	0.250	0.058	0.818
	Barrow	80.82	75.08	77.95				
	Challenge average	83.02	76.56					
Score 2 S1, %	Gilt	12.40	17.91	15.15	2.41	0.237	0.019	0.706
	Barrow	14.70	21.97	18.34				
	Challenge average	13.55	19.94					
Score 3 S1, %	Gilt	1.94	3.84	2.89	1.19	0.869	0.660	0.280
	Barrow	3.51	2.68	3.09				
	Challenge average	2.72	3.26					
Score 4 S1, %	Gilt	0.44	0.20	0.32	0.41	0.485	0.278	0.581
	Barrow	0.97	0.26	0.62				
	Challenge average	0.71	0.23					
Fecal score S1	Gilt	1.18	1.26	1.22	0.04	0.322	0.190	0.568
	Barrow	1.25	1.28	1.26				
	Challenge average	1.21	1.27					
Score 1 S2-4, %	Gilt	85.39	83.06	84.23	2.64	0.503	0.034	0.159
	Barrow	87.20	77.36	82.28				
	Challenge average	86.30	80.21					
Score 2 S2-4, %	Gilt	13.01	15.44	14.22	2.34	0.473	0.036	0.219
	Barrow	11.99	20.15	16.07				
	Challenge average	12.50	17.79					
Score 3 S2-4, %	Gilt	1.60	1.21	1.40	0.81	0.822	0.362	0.148
	Barrow	0.81	2.43	1.62				
	Challenge average	1.21	1.82					
Score 4 S2-4, %	Gilt	0.00	0.29	0.15	0.11	0.307	0.136	0.307
	Barrow	0.00	0.06	0.03				
	Challenge average	0.00	0.18					
Fecal score S2-4	Gilt	1.16	1.19	1.17	0.03	0.580	0.034	0.146
	Barrow	1.14	1.25	1.19				
	Challenge average	1.15	1.22					
Score 1 S5-6, %	Gilt	27.02	21.61	24.32	3.38	0.457	0.070	0.675
	Barrow	31.09	22.76	26.93				
	Challenge average	29.05	22.19					
Score 2 S5-6, %	Gilt	54.99	58.18	56.58	2.62	0.401	0.252	0.748
	Barrow	58.37	60.20	59.29				
	Challenge average	56.68	59.19					
Score 3 S5-6, %	Gilt	16.63	14.88	15.75	1.73	0.062	0.357	0.077
	Barrow	9.57	14.66	12.12				
	Challenge average	13.10	14.77					
Score 4 S5-6, %	Gilt	1.37	5.33	3.35	1.17	0.181	0.044	0.300
	Barrow	0.96	2.37	1.67				
	Challenge average	1.17	3.85					
Fecal score S5-6	Gilt	1.92	2.04	1.98	0.06	0.116	0.032	0.685
	Barrow	1.80	1.97	1.89				
	Challenge average	1.86	2.00					
Score 1 total, %	Gilt	65.91	61.74	63.82	2.13	0.743	0.013	0.316
	Barrow	67.44	58.78	63.11				
	Challenge average	66.67	60.26					
Score 2 total, %	Gilt	26.90	30.10	28.50	1.70	0.217	0.018	0.418
	Barrow	27.90	33.80	30.85				
	Challenge average	27.40	31.95					
Score 3 total, %	Gilt	6.67	6.21	6.44	0.89	0.257	0.308	0.143
	Barrow	4.18	6.55	5.37				
	Challenge average	5.42	6.38					
Score 4 total, %	Gilt	0.53	1.95	1.24	0.40	0.187	0.048	0.222
	Barrow	0.48	0.86	0.67				
	Challenge average	0.51	1.41					
Total fecal score	Gilt	1.42	1.48	1.45	0.03	0.647	0.016	0.426

Barrow	1.38	1.50	1.44
Challenge average	1.40	1.49	

MSE: mean standard error. Means followed by distinct letters differs by the test of Tukey with $p < 0.05$.
*Fecal consistency categories: score 1 = firm and moldy, score 2 = firm and soft, score 3 = soft and score 4 = watery; scores 1 and 2 represents normal feces and scores 23 and 4 represents diarrhea (Pedersen & Toft, 2011).

No significant interaction on fecal score analysis was observed between sex (barrow or gilt) and challenge with *E. coli* during all experiment ($p > 0.05$). In the first period (day 0 to 7), challenged animals had increased score 2 (32.0%; P = 0.019), while the control group tended to have a higher score 1 (8.4%; P = 0.058) and no interactions between sex and treatment were found. On the second period 4 (day 8 to 28), which corresponds to the period in which the health challenges were performed, non-challenge piglets had an increase of 7.0% in the score 1 (P = 0.034) and an increase of 42.3% in score 2 when compared to *E. coli* challenged group (P = 0.036). Challenged piglets had a mean fecal score 6.0% higher than the animals in the non-challenged group (P = 0.034). During the last period (S5-S6 – day 29 to 42), the *E. coli* challenged group had a higher score 4 (229.0%; P = 0.044) and a higher mean fecal score (7.5%; P = 0.032) when compared to the unchallenged group. Unchallenged piglets tended to have a 30.9% increase in score 1 (P = 0.070). In relation to the total experimental period (day 0 to 42), there was a 10.6% increase in Score 1 in the group not challenged by *E. coli* (P = 0.013). Piglets that received the *E. coli* challenge had higher scores 2 (16.6%; P = 0.018) and 4 (176.4%; P = 0.048), as well as a 6.4% increase in mean fecal score for the entire experimental period (P = 0.016).

When considering the total experimental period, the challenged group had a higher frequency of score of 4 (considered diarrhea) and total fecal score and a lower frequency of score 1 (considered normal feces). Furthermore, average fecal score for the period in which the sanitary challenges occurred (S2-S4) was greater for animals challenged with *E. coli*; in the subsequent period (S5-S6), challenged piglets also showed higher frequency of Score 4 (diarrhea). These results may demonstrate the effects of the bacteria *Escherichia coli* F4 (enterotoxigenic), which is associated with diarrheal conditions in the nursery phase. This enteric pathogen is considered as one of the main causes of diarrhea in weaned piglets due to colonization in the gastrointestinal tract (Zhu et al., 2018). Bacteria recognizes and adheres to specific receptors present in the gastrointestinal tract of piglets, with the help of fimbriae and adhesins, to finally start the production of enterotoxins, causing diarrhea (Moonens et al., 2015; Zhu et al., 2018). Piglets at this stage are also more susceptible to diseases due to the various stressors to which they are exposed in the post-weaning period (Campbell et al., 2013). The fecal score results obtained in the study demonstrate the effectiveness of the *Escherichia coli* F4 challenge.

Other studies corroborate with our findings. Che et al. (2017) observed that piglets challenged with solutions containing 109 CFU mL-1 of ETEC (*E. coli* O149) had a higher fecal score (diarrhea) during the first 24 hours after the challenge. SØrensen et al. (2009) observed variable effects on fecal characteristics and consistencies after a E. coli O149 challenge, demonstrating that controlled models of challenge with *E. coli* can be used to induce conditions like those present in commercial swine facilities that lead to diarrhea.

Data referring to the hemogram are presented in Table 3 and were divided by slaughter date due to the collection of samples. Slaughter 1 occurred during the first phase of nursery period (day 11), Slaughter 2 during the second phase of nursery period (day 28), and Slaughter 3 during the third phase of nursery period (day 42).

Table 3. Hemogram of piglets during nursery phase challenged or not with *E. coli* F4.

Slaughter 1								
Variables	Sex	Challenge		Sex mean	MSE	P value		
		Without	With			Sex	Challenge	Sex* Challenge
Red cells ($x10^6 \mu L^{-1}$)	Gilt	6.30	5.87	6.09	0.267	0.559	0.317	0.401
	Barrow	6.27	6.23	6.25				
	Challenge average	6.28	6.05					
Hemoglobin ($g\ dL^{-1}$)	Gilt	11.78	10.65	11.22	0.520	0.709	0.375	0.105
	Barrow	10.82	11.20	11.01				
	Challenge average	11.30	10.93					
Hematocrit (%)	Gilt	36.50	33.70	35.10	1.654	0.855	0.284	0.262
	Barrow	34.73	34.80	34.76				
	Challenge average	35.61	34.25					
Mean corpuscular volume (fL)	Gilt	58.02	57.46	57.74	1.329	0.227	0.978	0.560
	Barrow	55.49	56.00	55.74				
	Challenge average	56.75	56.73					

Mean corpuscular hemoglobin (pg)	Gilt	18.68	17.94	18.31				
	Barrow	17.26	17.93	17.59	0.500	0.285	0.893	0.061
	Challenge average	17.97	17.93					
Mean corpuscular hemoglobin concentration (%)	Gilt	32.24	31.46	31.85				
	Barrow	31.17	32.15	31.66	0.430	0.702	0.711	0.209
	Challenge average	31.70	31.81					
Erythroblasts/ 100 Leukocytes	Gilt	2.60	2.75	2.68				
	Barrow	0.33	1.25	0.79	1.306	0.146	0.294	0.883
	Challenge average	1.47	2.00					
Corrected Total Leukocytes (/µL)	Gilt	16495	15164.0	15829.5				
	Barrow	14995	17951.0	16473.0	2954.12	0.838	0.725	0.371
	Challenge average	15745	16557.5					
Rod neutrophils (/µL)	Gilt	0.40	0.85	0.63				
	Barrow	0.58	1.00	0.79	0.676	0.674	0.476	0.796
	Challenge average	0.49	0.93					
Segmented neutrophils (/µL)	Gilt	44.20	46.00	45.10				
	Barrow	48.00	39.75	43.88	9.10	0.885	0.704	0.558
	Challenge average	46.10	42.88					
Lymphocytes (/µL)	Gilt	52.20	50.00	51.10				
	Barrow	47.33	56.75	52.04	8.87	0.859	0.731	0.464
	Challenge average	49.77	53.38					
Monocytes (/µL)	Gilt	2.00	1.89	1.94				
	Barrow	2.74	1.50	2.12	0.64	0.974	0.180	0.416
	Challenge average	2.37	1.69					
Eosinophiles (/µL)	Gilt	0.40	1.00	0.70				
	Barrow	0.33	0.25	0.29	0.64	0.737	0.908	0.892
	Challenge average	0.37	0.63					
Basophiles (/µL)	Gilt	0.80	0.25	0.53				
	Barrow	1.00	0.75	0.88	0.48	0.458	0.383	0.736
	Challenge average	0.90	0.50					
Platelets ($x10^3$ μL^{-1})	Gilt	297.40	174.50	235.95				
	Barrow	446.33	260.50	353.42	90.11	0.355	0.152	0.781
	Challenge average	371.87	217.50					
Plasmatic proteins (g dL^{-1})	Gilt	5.28	4.89	5.08				
	Barrow	5.00	5.05	5.02	0.23	0.987	0.427	0.259
	Challenge average	5.14	4.97					
Neutrophil/lymphocyte ratio	Gilt	1.01	1.18	1.09				
	Barrow	1.11	0.78	0.94	0.38	0.877	0.594	0.586
	Challenge average	1.06	0.98					
Slaughter 2								
Red cells ($x10^6$ μL^{-1})	Gilt	7.19	7.29	7.24				
	Barrow	5.71	6.64	6.17	1.02	0.118	0.953	0.864
	Challenge average	6.45	6.96					
Hemoglobin (g dL^{-1})	Gilt	11.60	11.26	11.43				
	Barrow	9.61	10.68	10.15	1.65	0.320	0.270	0.872
	Challenge average	10.60	10.97					
Hematocrit (%)	Gilt	37.59	37.19	37.39				
	Barrow	31.67	35.08	33.37	5.47	0.480	0.403	0.373
	Challenge average	34.63	36.13					
Mean corpuscular volume (fL)	Gilt	52.61	51.37	51.99				
	Barrow	46.43	52.92	49.67	8.09	0.591	0.678	0.918
	Challenge average	49.52	52.14					
Mean corpuscular hemoglobin (pg)	Gilt	16.18	15.48	15.83				
	Barrow	14.03	16.05	15.04	2.49	0.680	0.628	0.806
	Challenge average	15.11	15.77					
Mean corpuscular hemoglobin concentration (%)	Gilt	30.84	30.29	30.57				
	Barrow	25.22	30.37	27.80	4.28	0.853	0.724	0.094
	Challenge average	28.03	30.33					
Erythroblasts/ 100 Leukocytes	Gilt	2.00	2.00	2.00				
	Barrow	0.83	3.40	2.12	2.12	0.925	0.460	0.688
	Challenge average	1.42	2.70					
Corrected Total Leukocytes (/µL)	Gilt	13940	14652	14296				
	Barrow	13540	17000	15270	3057.56	0.497	0.699	0.384
	Challenge average	13740	15826					
Rod neutrophils (/µL)	Gilt	0.33	0.00	0.17				
	Barrow	0.50	0.40	0.45	0.36	0.405	0.379	0.788
	Challenge average	0.42	0.20					

Segmented neutrophils (/µL)	Gilt	39.10		34.06		36.58				
	Barrow	34.85		46.70		40.77	8.21	0.586	0.626	0.259
	Challenge average	36.98		40.38						
Lymphocytes (/µL)	Gilt	58.83		63.72		61.28				
	Barrow	45.45		51.61		48.53	9.63	0.220	0.374	0.976
	Challenge average	52.14		57.66						
Monocytes (/µL)	Gilt	1.00		1.00		1.00				
	Barrow	1.50		1.60		1.55	0.63	0.429	0.941	0.981
	Challenge average	1.25		1.30						
Eosinophiles (/µL)	Gilt	1.14	a	0.24	b	0.69				
	Barrow	0.63	ab	0.80	ab	0.71	0.42	0.742	0.031	0.011
	Challenge average	0.89		0.52						
Basophiles (/µL)	Gilt	0.00		0.00		0.00				
	Barrow	0.17		0.00		0.08	0.141	0.515	0.515	0.515
	Challenge average	0.08		0.00						
Platelets (x10^3 µL^{-1})	Gilt	579.00		443.25		511.13				
	Barrow	508.33		426.00		467.17	168.1	0.663	0.329	0.693
	Challenge average	543.67		434.63						
Plasmatic proteins (g dL^{-1})	Gilt	5.73		5.49		5.61				
	Barrow	4.64		5.30		4.97	0.80	0.273	0.272	0.898
	Challenge average	5.19		5.39						
Neutrophil/lymphocyte ratio	Gilt	0.67		0.57		0.62				
	Barrow	0.84		1.02		0.93	0.21	0.247	0.908	0.492
	Challenge average	0.75		0.80						
Slaughter 3										
Red cells (x10^6 µL^{-1})	Gilt	7.70	ab	7.56	ab	7.63				
	Barrow	7.15	b	7.91	a	7.53	0.27	0.708	0.078	0.024
	Challenge average	7.42		7.74						
Hemoglobin (g dL^{-1})	Gilt	12.74		12.33		12.53				
	Barrow	12.18		12.33		12.25	0.48	0.568	0.787	0.568
	Challenge average	12.46		12.33						
Hematocrit (%)	Gilt	41.64		41.10		41.37				
	Barrow	39.75		40.65		40.20	1.60	0.478	0.911	0.658
	Challenge average	40.70		40.88						
CHCH (%)	Gilt	30.56		29.95		30.26				
	Barrow	30.60		30.28		30.44	0.27	0.515	0.122	0.609
	Challenge average	30.58		30.11						
Erythroblasts/ 100 Leukocytes	Gilt	3.00		5.25		4.13				
	Barrow	1.75		6.00		3.88	1.48	0.786	0.058	0.275
	Challenge average	2.38		5.63						
Corrected Total Leukocytes (/µL)	Gilt	19987.0		23580.0		21783.5				
	Barrow	20889.0		19231.0		20060.0	3284.40	0.640	0.820	0.593
	Challenge average	20438.0		21405.5						
Rod neutrophils (/µL)	Gilt	0.3094		0.7275		0.52				
	Barrow	0.03354		0.09311		0.06	0.295	0.226	0.306	0.467
	Challenge average	0.17		0.41						
Segmented neutrophils (/µL)	Gilt	49.49		53.46		51.48				
	Barrow	58.58		44.30		51.44	6.97	0.997	0.429	0.185
	Challenge average	54.04		48.88						
Lymphocytes (/µL)	Gilt	41.44		42.67		42.06				
	Barrow	36.95		47.51		42.23	7.08	0.972	0.285	0.390
	Challenge average	39.19		45.09						
Monocytes (/µL)	Gilt	7.35	a	1.21	b	4.28				
	Barrow	3.58	ab	6.03	a	4.80	1.31	0.412	0.120	0.006
	Challenge average	5.46		3.62						
Eosinophiles (/µL)	Gilt	0.69		2.03		1.36				
	Barrow	0.68		1.26		0.97	0.65	0.501	0.195	0.309
	Challenge average	0.68		1.64						
Basophiles (/µL)	Gilt	0.20		0.00		0.10				
	Barrow	0.25		0.75		0.50	0.29	0.207	0.723	0.274
	Challenge average	0.23		0.38						
Platelets (x10^3 µL^{-1})	Gilt	391.00		604.82		497.91				
	Barrow	502.23		306.09		404.16	120.48	0.472	0.935	0.094
	Challenge average	446.62		455.46						
Plasmatic proteins (g dL^{-1})	Gilt	5.94		6.03		5.99				
	Barrow	5.91		5.93		5.92	0.22	0.788	0.717	0.825
	Challenge average	5.92		5.98						

Neutrophil/lymphocyte ratio	Gilt	1.20	1.64	1.42				
	Barrow	2.38	0.98	1.68	0.66	0.981	0.292	0.302
	Challenge average	1.79	1.31					

MSE: mean standard error. Means followed by distinct letters differs by the test of Tukey with $p < 0.05$.

For the first nursery period (Slaughter 1), no significant effects were found for the variables analyzed ($p > 0.05$) and no interaction between sex and challenge was observed ($p > 0.05$). Samples collected during Slaughter 2 indicated a significant interaction between sex and treatment for the variable "Eosinophils count", in which unchallenged gilts had 375.0% greater eosinophil numbers compared to challenged gilts, while the barrows did not differ statistically ($P = 0.011$). As for the third phase of the nursery period (Slaughter 3), there was a significant interaction between sex and treatment for variables: "Red Cells", in which challenged barrows had a 10.6% higher value compared to unchallenged barrows ($P = 0.024$) and "Monocytes", in which unchallenged gilts and challenged barrows showed an increase of 507.0 and 398.0%, respectively, when compared to challenged gilts ($P = 0.006$). Challenged piglets tended to have 136.5% greater value for "Erythroblasts/100 Leukocytes" in relation to the non-challenged group ($P = 0.058$).

In the present study, it has been noted that the responses of some blood parameters to *E. coli* challenge were variable, including interactions with sex. Modifications on red cell count, eosinophil count and monocyte count can be related to *E. coli* challenge and a weaning-induced systemic inflammatory response in piglets (Sugiharto et al., 2014). It is also known that some differences and modifications on the total blood count can be influenced by other factors, such as piglet age (Davis et al., 2006). Davis et al. (2006) demonstrated that weaned piglets had increased total white blood cell count following weaning and increased age, consequently. Despite the significant effects found in some variables of the blood tests, the values observed in this study remained within the physiological intervals considered normal for the swine species and for the age group of the animals in the study, according to Kaneko, Harvey, and Bruss (1997) and Friendship and Henry (1992).

The results regarding intestinal morphometry are shown in Table 4 and dived by slaughter age. For the Slaughter 1 period, there was no interaction between sex and challenge ($p > 0.05$), but the non-challenged group tended have a 30.2% greater value for the variable "Crypt Depth" in relation to the challenged group ($P = 0.062$). Furthermore, gilts tended to have a 29.6% increase in Crypt Depth when compared to barrows ($P = 0.058$). During the period covered by Slaughter 2, there was a significant interaction between sex and treatment for "Villus height" ($P = 0.004$) and "Villus: Crypt Ratio" ($P = 0.044$). Unchallenged gilts had an increase of 107.6% on villus height when compared to challenged gilts, while barrows had similar statistical behavior. Furthermore, unchallenged gilts showed an 85% higher villus: crypt ratio compared to not challenged barrows, while piglets in the challenged group, both barrows and gilts, were statistically similar. In the third slaughter period, there were no significant effects between groups for all parameters analyzed ($p > 0.05$) and no interaction between the factors analyzed (sex and challenge with *E. coli*).

Table 4. Intestinal morphometry of piglets during nursery phase challenged or not with *E. coli* F4.

111q	Sex	Challenge		Sex average	MSE	P value		
		Without	With			Sex	Challenge	Sex*Challenge
			Slaughter 1					
Villus height (µm)	Gilt	290.32	265.45	277.89				
	Barrow	276.81	294.84	285.83	51.85	0.933	0.846	0.605
	Challenge average	283.57	280.15					
Crypt depth (µm)	Gilt	319.40	201.82	260.61				
	Barrow	202.98	199.15	201.07	33.06	0.058	0.062	0.070
	Challenge average	261.19	200.49					
Villi height: crypt depth ratio	Gilt	0.91	1.33	1.12				
	Barrow	1.35	1.51	1.43	0.277	0.278	0.163	0.381
	Challenge average	1.13	1.42					
			Slaughter 2					
Villus height (µm)	Gilt	494.15 a	238.01 b	366.08				
	Barrow	293.53 ab	357.14 ab	325.34	42.12	0.027	0.246	0.004
	Challenge average	393.84	297.58					
Crypt depth (µm)	Gilt	307.71	261.65	284.68				
	Barrow	351.53	269.72	310.63	56.10	0.182	0.557	0.683
	Challenge average	329.62	265.69					

	Gilt	1.61	a	0.91	ab	1.26				
Villi height: crypt depth ratio	Barrow	0.87	b	1.39	ab	1.13	0.31	0.711	0.603	0.044
	Challenge average	1.24		1.15						
Slaughter 3										
	Gilt	250.52		268.96		259.74				
Villus height (µm)	Barrow	327.38		322.66		325.02	39.97	0.993	0.119	0.775
	Challenge average	288.95		295.81						
	Gilt	242.00		257.55		249.78				
Crypt depth (µm)	Barrow	278.64		283.87		281.26	41.74	0.782	0.413	0.891
	Challenge average	260.32		270.71						
	Gilt	1.09		1.05		1.07				
Villi height: crypt depth ratio	Barrow	1.26		1.16		1.21	0.27	0.772	0.543	0.904
	Challenge average	1.17		1.11						

MSE: mean standard error. Means followed by distinct letters differs by the test of Tukey with $p < 0.05$.

Our findings suggest compromised intestinal integrity and morphology, which are associated with the activation of innate immunity and the local inflammatory process (Che et al., 2017). Such alterations in organism's homeostasis may be related to the presence of *Escherichia coli* F4, which is an enterotoxigenic bacterium. It is known that the maintenance of intestinal integrity is extremely important in the post-weaning period, due to the vital function of this organ, which is responsible for the final digestion and absorption of nutrients (Chen et al., 2014). Thus, both the length and width of the villi and the depth of the crypt are related to the maintenance of intestinal integrity and health of the animals, and the greater the villus: crypt ratio, the better the intestinal digestive and absorptive capacity (Bontempo et al., 2006). Gao et al. (2013) observed that pigs challenged with *E. coli* K88 had greater villus atrophy (i.e., lower villus height) and reduced crypt depth when compared to unchallenged pigs and Che et al. (2017) observed that piglets challenged by *E. coli* had lower villus height, evaluating jejunum and ileum samples, compared to unchallenged pigs, which corroborates with our findings. The compromised intestinal integrity, demonstrated by the results regarding intestinal morphometry, may be correlated to the impairments observed on the piglet's growth performance and incidence of diarrhea. Furthermore, unchallenged gilts had superior villus: crypt ratio compared to barrows not challenged by *E. coli*.

The results of the of the populations of intestinal bacteria and concentrations of short-chain fatty acids (SCFA) are presented in Tables 5 and 6, respectively. The results were divided accordingly the Slaughter dates.

Table 5. Microbiological composition of the cecal content of nursery piglets challenged or not with *E. coli* F4.

Variables	Sex	Challenge		Sex mean	MSE	P value		
		Without	With			Sex	Challenge	Sex*Challenge
Slaughter 1								
	Gilt	3.006	2.846	2.926				
E. coli	Barrow	2.768	3.185	2.977	0.523	0.773	0.610	0.183
	Challenge average	2.887	3.016					
	Gilt	2.745	2.901	2.823				
Enterobacteria	Barrow	3.163	3.818	3.491	0.709	0.508	0.557	0.873
	Challenge average	2.954	3.360					
	Gilt	7.799	8.104	7.952				
Lactobacillus	Barrow	7.529	7.864	7.696	0.279	0.433	0.359	0.859
	Challenge average	7.664	7.984					
	Gilt	5.107	5.262	5.184				
Bifidobacterium	Barrow	4.748	5.189	4.968	0.380	0.643	0.416	0.501
	Challenge average	4.928	5.225					
Slaughter 2								
	Gilt	4.654	4.791	4.722				
E. coli	Barrow	4.090	5.399	4.744	0.430	0.795	0.163	0.224
	Challenge average	4.372	5.095					
	Gilt	4.075	4.208	4.142				
Enterobacteria	Barrow	4.295	4.805	4.550	0.49	0.517	0.367	0.781
	Challenge average	4.185	4.507					
	Gilt	7.343	7.358	7.351				
Lactobacillus	Barrow	6.747	7.708	7.227	0.361	0.934	0.089	0.149
	Challenge average	7.045	7.533					
	Gilt	5.401	5.873	5.637				
Bifidobacterium	Barrow	5.356	5.850	5.603	0.27	0.596	0.039	0.805
	Challenge average	5.378	5.862					

Slaughter 3									
	Gilt	4.382		3.610		3.996			
E. coli	Barrow	5.246		4.465		4.855	1.02	0.313	0.281	0.882
	Challenge average	4.814		4.038					
	Gilt	8.105		7.690		7.897			
Lactobacillus	Barrow	8.003		7.667		7.835	0.420	0.822	0.314	0.682
	Challenge average	8.054		7.678					
	Gilt	4.788	a	4.272	ab	4.530			
Bifidobacterium	Barrow	3.847	b	4.619	ab	4.233	0.309	0.457	0.774	0.029
	Challenge average	4.317		4.445					

MSE: mean standard error. Means followed by distinct letters differs by the test of Tukey with $p < 0.05$.

In the microbiological analysis, no significant differences and interactions were observed between the variables analyzed at the first slaughter. At Slaughter 2, challenged piglets had an 8.9% higher Bifidobacterium value (P = 0.039) and tended to have a Lactobacilli value 6.9% greater (P = 0.089) when compared non-challenged piglets. No interactions between sex and challenge were found at the second slaughter ($p > 0.05$). In the third nursery phase (Slaughter 3), there was a significant interaction between sex and treatment for the Bifidobacterium variable (P = 0.029), in which unchallenged gilts showed an 24.4% increase in relation to unchallenged barrows, while piglets in the challenged group were statistically similar.

At Slaughter 2, challenged animals had higher Bifidobacterium content and tended to have higher Lactobacilli content when compared to animals not challenged with *E. coli*. These microorganisms (Lactobacilli and Bifidobacterium) are considered one of the main components of the intestinal microbiota of swine (Tsuchida et al., 2017), and thus, increases in their counts in pitlets challenged with *E. coli* F4 may be associated with host response to the presence of pathogenic bacteria in the intestinal environment. Intestinal microbiota is an important defense mechanism against pathogenic bacteria since commensal bacteria increase host resistance to the pathogen colonization in turn promoting host's health. (Shen et al., 2009). Also, I 1n humans, Bifidobacterium have beneficial effects on the physiology and pathology of some diseases, such as enterohemorrhagic caused by *Escherichia coli* - EHEC. Bifidobacterium produce acetate, increasing its concentration in the intestines and helping with the response against infectious agents by acting in vivo to promote defensive functions of host epithelial cells (Fukuda et al., 2011). In the present study, an increase in acetate levels was also observed in the same period (Slaughter 2), corroborating the results presented by Fukuda et al. (2011).

The fatty acids evaluated were acetate, propionate, isobutyrate, butyrate, isovalerate and valerate. No interactions were observed between sex and challenge for the first and last periods (Slaughter 1 and 3, respectively). For the Slaughter 2, there was a significant interaction between sex and treatment of animals for the variable acetate (P = 0.048), in which challenged gilts had a 113.5% higher acetate value when compared to unchallenged gilts, while barrows of both treatments were statistically similar.

Table 6. Composition of short chain fatty acids of cecal content of nursery piglets challenged or not with *E. coli* F4.

'12	Sex	Challenge		Sex mean	MSE	Valor de P		
		Without	With			Sex	Challenge	Sex*Challenge
Slaughter 1								
	Gilt	21.06	17.87	19.47				
Acetate	Barrow	17.99	18.51	18.25	2.92	0.686	0.655	0.539
	Challenge average	19.53	18.19					
	Gilt	8.67	8.50	8.59				
Propionate	Barrow	5.63	8.38	7.00	1.01	0.159	0.239	0.189
	Challenge average	7.15	8.44					
	Gilt	0.14	0.10	0.12				
Isobutyrate	Barrow	0.08	0.15	0.12	0.05	0.994	0.754	0.229
	Challenge average	0.11	0.12					
	Gilt	3.05	2.29	2.67				
Butyrate	Barrow	3.25	2.56	2.91	0.61	0.269	0.701	0.958
	Challenge average	3.15	2.42					
	Gilt	0.13	0.11	0.12				
Isovalerate	Barrow	0.09	0.13	0.11	0.05	0.846	0.806	0.454
	Challenge average	0.11	0.12					
	Gilt	0.34	0.68	0.51				
Valerate	Barrow	0.23	0.43	0.33	0.14	0.330	0.100	0.525
	Challenge average	0.29	0.56					

Slaughter 2										
	Gilt	8.24	b	17.60	a	12.92				
Acetate	Barrow	17.38	ab	14.25	ab	15.82	2.46	0.281	0.249	0.048
	Challenge average	12.81		15.93						
	Gilt	5.76		6.90		6.33				
Propionate	Barrow	6.81		6.79		6.80	1.03	0.608	0.544	0.530
	Challenge average	6.28		6.85						
	Gilt	0.15		0.16		0.15				
Isobutyrate	Barrow	0.17		0.25		0.21	0.06	0.305	0.330	0.444
	Challenge average	0.16		0.21						
	Gilt	1.63		2.89		2.26				
Butyrate	Barrow	3.02		2.80		2.91	0.61	0.428	0.315	0.258
	Challenge average	2.33		2.84						
	Gilt	0.17		0.19		0.18				
Isovalerate	Barrow	0.20		0.32		0.26	0.09	0.360	0.335	0.489
	Challenge average	0.18		0.26						
	Gilt	0.34		0.47		0.40				
Valerate	Barrow	0.49		0.65		0.57	0.13	0.150	0.123	0.259
	Challenge average	0.41		0.56						
Slaughter 3										
	Gilt	21.99		23.31		22.65				
Acetate	Barrow	20.91		22.79		21.85	1.88	0.657	0.380	0.873
	Challenge average	21.45		23.05						
	Gilt	10.82		12.58		11.70				
Propionate	Barrow	14.02		13.72		13.87	1.10	0.058	0.179	0.221
	Challenge average	12.42		13.15						
	Gilt	0.15		0.10		0.13				
Isobutyrate	Barrow	0.05		0.06		0.05	0.08	0.421	0.466	0.993
	Challenge average	0.10		0.08						
	Gilt	4.35		6.16		5.26				
Butyrate	Barrow	5.89		6.95		6.42	1.29	0.355	0.259	0.758
	Challenge average	5.12		6.56						
	Gilt	0.21		0.15		0.18				
Isovalerate	Barrow	0.06		0.07		0.06	0.12	0.335	0.242	0.997
	Challenge average	0.13		0.11						
	Gilt	1.04		1.61		1.32				
Valerate	Barrow	1.29		1.66		1.48	0.39	0.435	0.217	0.621
	Challenge average	1.16		1.63						

MSE: mean standard error. Means followed by distinct letters differs by the test of Tukey with $p < 0,05$.

In the period of the second slaughter, challenged gilts had higher acetate value when compared to unchallenged gilts. This result suggests that challenged gilts could be reacting to the infection and the presence of the *Escherichia coli* inoculated in the sanitary challenge. In humans, it is known that increases in the production and concentration of acetate modulates the gut's defensive response and improves defense mediated by epithelial cells, which leads to protection against infectious agents (Fukuda et al., 2011). In addition, short-chain fatty acids are responsible for providing energy to colonocytes, having essential roles in the reabsorption of water and sodium in the body and in the individual's intestinal health (Bergam, 1990; Zlotowski et al., 2008).

Conclusion

The *Escherichia coli* F4 challenge was effective mainly in the first two weeks post-challenge, as indicated by changes in the incidence of diarrhea, growth performance and intestinal morphometry. The effect of the challenge differed between the sexes, with gilts showing a greater response than the barrows, indicated by the differences observed mainly on the analysis of intestinal morphometry. Therefore, gilts may be recommended for use in experimental conditions with *E. coli* O149 challenge, as demonstrated in this study.

Data availability

In accordance with the principles of Open Science, the data generated and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Acknowledgements

Thanks to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (FCPES) – Finance code 001, and Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) – protocol numbers: 2019/19720-0; 2019/17453-4; 2020/05069-2 for partially funding this study.

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