Effects of dietary glycyl-glutamine on growth performance, small intestinal integrity, and immune responses of weaning piglets challenged with lipopolysaccharide


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Effects of dietary glycyl-glutamine on growth performance, small intestinal integrity, and immune responses of weaning piglets challenged with lipopolysaccharide


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ABSTRACT: The objectives of this study were to determine the effects of dietary glycyl-glutamine (Gly-Gln) on postweaning growth, small intestinal morphology, and immune response of stressed or nonstressed piglets. Pigs (n = 144; initially 4.49 kg and 14 d of age) were randomly allocated to 24 pens (6 pigs/pen) in an environmentally controlled nursery and assigned to *Escherichia coli* lipopolysaccharide (LPS) challenge (PBS vs. LPS) and Gly-Gln supplementation (0 vs. 0.15%) in a 2 × 2 factorial arrangement of treatments with 6 pens/treatment. The LPS was the stress-inducing agent, and it was injected on d 7 and 14 of the 21-d experiment. Inflammatory challenge with LPS reduced ADG (P < 0.05) and tended to reduce ADFI (P = 0.06) of piglets from d 7 to 21 of the experiment. Supplementation of Gly-Gln increased ADG and G:F from d 0 to 21 (P < 0.05). On d 21 (1 wk after the second LPS injection), there was an LPS challenge × diet Gly-Gln interaction for ADFI (P < 0.05), but it was difficult to ascertain whether Gly-Gln increased ADFI. A trend for an LPS challenge × diet Gly-Gln interaction was observed for ADG (P = 0.07). There were no differences in lymphocyte proliferation among treatments. The LPS challenge increased crypt depth (CD) of the duodenum and decreased the ratio of villus height (VH) to CD of the ileum (P < 0.05) on d 14 (1 wk after the first LPS injection), whereas dietary supplementation of Gly-Gln increased VH of the ileum and VH:CD of the duodenum (P < 0.05). The concentration of peripheral blood IL-1β was increased by injection of LPS (P < 0.05) and was decreased by dietary Gly-Gln supplementation during the experimental period (P < 0.05); however, there was no interaction of LPS challenge × Gly-Gln addition for IL-1β concentration. Concentrations of peripheral blood IL-2 tended to increase at d 14 (P = 0.09) and soluble IL-2 receptor tended to decrease at d 7 (P = 0.06) in piglets supplemented with Gly-Gln; therefore, the peripheral blood IL-2/soluble IL-2 receptor system tended to favor the secretion of IL-2 during the first 2 wk of the experiment. In conclusion, considerable suppression of growth and immune function occurred in early weaning piglets challenged with LPS, and such depression could be alleviated by dietary Gly-Gln supplementation independent of the LPS challenge.

Key words: glycyl-glutamine, growth, immunity, lipopolysaccharide, weaning piglet

INTRODUCTION

Early weaning of piglets leads to a sudden cessation of passive immunity from the milk of the sow, which, along with the immature immune system of the piglets, makes them vulnerable to diseases (Deprez et al., 1986). In addition, the reduction in feed intake associated with weaning affects intestinal integrity and causes pathological disorders (Spreeuwenberg et al., 2001). Dietary supplementation with some nutrients or immunomodulators to rectify the intestinal impairment and modulate the immune function of animals can be considered a potential means to improve their performance and health status (Li et al., 2007). Studies have indicated that an immunological challenge can result in some physiological changes (van...
Effects of glycyl-glutamine on piglets

Heugten et al., 1996; Johnson, 1997; Yi et al., 2005), through the release of proinflammatory cytokines (Johnson, 1997; Webel et al., 1997). Peripheral concentrations of IL-13, IL-2, and soluble IL-2 receptor (sIL-2R), which competes with the membrane IL-2 receptor to suppress the immune response, can reflect the immune function (Rubin and Nelson, 1990; Bailey et al., 1992; Taniguchi and Minami, 1993; Sadeghi et al., 1999). These cytokines can adversely affect growth and feed efficiency; thus, the modulation of these cytokines may have beneficial effects in alleviating the negative effects induced by an immunological stress (Carroll et al., 2003).

Previous studies have established that dietary glutamine supplementation improves intestinal morphology in weanling piglets with compromised immune function (Wu et al., 1996; Wang et al., 2008). Additionally, immunomodulation by glycyl-glutamine (Gly-Gln) is associated with inhibitory effects on the overproduction of inflammatory cytokines (Yi et al., 2005). However, few studies can be found on the changes in serum cytokines (Yi et al., 2005). How
tever, these cytokines may have beneficial effects in alleviating the negative effects induced by an immunological stress (Carroll et al., 2003).

**Materials and Methods**

The animal protocol for this research was approved by the South China Agricultural University Animal Care and Use Committee.

**Animal Care and Management**

A total of 144 piglets [Duroc × (Landrace × Yorkshire)] weaned at 14 d of age (initial BW = 4.49 kg) were randomly allotted to 4 treatments in a 2 × 2 factorial arrangement based on initial BW and sex. Piglets were housed in 36 pens, with 6 pigs per pen and 6 pens per treatment. The main effects were LPS challenge (0 vs. 200 units) and Gly-Gln supplementation (0 vs. 0.15%; as-fed basis). Piglets had ad libitum access to feed, and troughs were weighed back and feed was added daily to determine pen feed intake. The initial room temperature was 26 to 28°C, which was reduced by 1.2°C each week during the experiment. Body weight and feed intake were determined before each LPS injection and weekly thereafter.

**Diets and Experimental Design**

The experimental diets (Table 1) were formulated using primarily corn and soybean meal to meet or exceed NRC (1998) requirements for all nutrients. A basal diet was formulated, and the experimental diet was created by supplementation with 0.15% Gly-Gln (purity, 99.3%). The Gly-Gln (Tian Cheng Drug Co., Tianjin, China), concanavalin A (ConA) and Escherichia coli LPS (Sigma Chemical Co., St. Louis, MO), RPMI 1640 (Gibco BRL, Grand Island, NY), and swine IL-2, sIL-2R, and IL-1β ELISA kits (Rapid-bio International Inc., Beijing, China) were obtained from commercial companies. The LPS was injected intramuscularly (200 µg/kg of BW) on d 7 and 14 after blood sampling. The LPS dose used was selected based on the results of previous studies (Johnson and Borell, 1994; van Heugten et al., 1994; Webel et al., 1997). The concentration of LPS (500 µg/mL) was made by dilution with PBS (0.01 M and pH 7.4). The control piglets were injected with an equal volume of PBS solution.

**Blood Sampling and Lymphocyte Proliferation Response**

On d 7, 14, and 21, six piglets were selected from each treatment (1 piglet per pen) based on the litter of origin, BW gain, and health and were killed for blood and tissue analyses. A blood sample from each pig was collected into a 10-mL heparinized vacuum tube (sodium heparin, spray coated, 150 USP units, Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) by vena cava puncture before LPS injection and was centrifuged at 1,000 × g at 4°C for 30 min, and the serum sample was stored at −20°C until analysis. A second blood sample (approximately 5 mL) was taken from the same piglet for the lymphocyte proliferation analysis. All blood-related measurements were analyzed in duplicate.

In vitro cellular immune response was measured using a lymphocyte blastogenesis assay according to the

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**Table 1. Ingredient composition of the control diet (as-fed basis)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn, 7.8% CP</td>
<td>43.30</td>
</tr>
<tr>
<td>Soybean meal, 44% CP</td>
<td>25.40</td>
</tr>
<tr>
<td>Soy protein isolate, 65% CP</td>
<td>3.00</td>
</tr>
<tr>
<td>Fish meal, 62.5% CP</td>
<td>6.00</td>
</tr>
<tr>
<td>Whey powder, 2.5% CP</td>
<td>7.50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.50</td>
</tr>
<tr>
<td>Lactose</td>
<td>10.00</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.75</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.05</td>
</tr>
<tr>
<td>Chlorine chloride, 50%</td>
<td>0.10</td>
</tr>
<tr>
<td>l-Lysine-HCl</td>
<td>0.22</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.10</td>
</tr>
<tr>
<td>l-Threonine</td>
<td>0.08</td>
</tr>
<tr>
<td>Premix²</td>
<td>1.00</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
</tr>
</tbody>
</table>

1 Provided the following amounts of vitamins and trace minerals per kilogram of diet: vitamin A, 18,000 IU; vitamin D₃, 4,000 IU; vitamin E, 50 mg; thiamine, 4 mg; riboflavin, 4 mg; niacin, 25 mg; d-pantothenic acid, 30 mg; pyridoxine, 1.5 mg; choline chloride, 500 mg; vitamin B₁₂, 30 µg; folic acid, 2 mg; biotin, 0.16 mg; cobalt, 0.15 mg (as CoSO₄·7H₂O); copper, 225 µg (as CuSO₄·5H₂O); manganese, 48 mg (as MnO₂); iron, 150 mg (as FeSO₄·7H₂O); zinc, 150 mg (as ZnO); iodine, 1.5 mg (as KI); and selenium, 0.3 mg (as Na₂SeO₃).
method of Blecha et al. (1983). Peripheral blood was added into a Ficoll-Hypaque solution (density: 1.077 ± 0.001 g/mL; Jingyang Co., Tianjin, China). The lymphocytes were collected by density-gradient centrifugation (3,000 × g, 15 min, 4°C), washed 3 times with PBS, and resuspended in 5 mL of RPMI 1640 complete culture medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, 100 units of penicillin/mL, 100 µg of streptomycin/mL, and 25 µg/mL HEPES. Cell viability was assessed by trypan blue dye exclusion (Wu et al., 1991). Cell concentrations were adjusted to 1 × 10^7 cells/mL, and 100 µL of cell suspension and the T-lymphocyte mitogen (ConA: 40 µg/mL) were added to a 96-well microtiter plate. Cells were incubated at 37°C with 5% CO2 in an incubator. After 48 h of incubation, 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.; 5 mg/mL) was added to each well, and the plates were incubated for another 4 h. Subsequently, 100 µL of 10% SDS dissolved in 0.04 M HCl solution was added to the plates, which were incubated for 2 h to lyse the cells and solubilize the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.; 5 mg/mL). Finally, the plates were placed in an automated ELISA reader (Bio-Rad 550, Bio-Rad Laboratories, Hercules, CA) for measurement of absorbance at 570 nm. The lymphocyte proliferation value was expressed as a stimulation index, which was calculated as the absorbance of wells incubated without ConA divided by the absorbance of wells incubated without ConA.

**Small Intestinal Morphology**

The samples of small intestine tissues (approximately 2 cm) were again collected on d 7, 14, and 21 (1 piglet per pen) for determination of intestinal morphology and integrity. The segment approximately 15 cm away from the pyloric junction was considered the duodenum, that 55 cm away from the pyloric junction was considered the jejunum, and a distal segment approximately 15 cm proximal to the ileocecal junction was considered the ileum (Tang et al., 1999; Brown et al., 2006). Intestinal morphological measurements included the following 3 indices: villus height (VH), crypt depth (CD), and VH:CD. These indexes were quantified according to Nabuurs et al. (1993). Mean values of VH, CD, and their ratio within each segment were calculated.

**Analysis of Serum Cytokines**

Serum concentrations of cytokines (IL-1β, IL-2, and sIL-2R) were determined using a commercially available porcine ELISA kit (Rapid-bio International Inc., Beijing, China). All samples were analyzed in duplicate. The detectable ranges of assays were 31.25 to 250 pg/mL for IL-1β analysis, 7.8 to 250 pg/mL for IL-2 analysis, and 125 to 500 pg/mL for sIL-2R analysis. The detection limit of all 3 ELISA kits was 10 pg/mL. All analyses were conducted as described by the manufacturer.

**Statistical Analyses**

Data were analyzed by ANOVA using the GLM procedure (SAS Inst. Inc., Cary, NC) appropriate for a factorial arrangement of treatments in a randomized complete block design. The statistical model included the effects of challenge (PBS or LPS), dietary Gly-Gln (0 or 0.15%), and their interactions. The pen was used as the experimental unit for analysis. An α level of P < 0.05 was used to indicate statistical significance, and P < 0.10 was used to indicate a statistically significant trend.

**RESULTS**

**Growth Performance**

Before LPS challenge (from d 0 to 7), piglet growth was not different among treatments (Table 2). During the first challenge period (from d 7 to 14), the LPS challenge tended to decrease ADFI (P = 0.06) compared with that of PBS-treated piglets. During the second challenge period (from d 14 to 21), Gly-Gln supplementation appeared to have no effect on ADG and ADFI in piglets challenged with LPS, but it increased ADG and ADFI in piglets not challenged with LPS (LPS challenge × Gly-Gln, P = 0.07 and P < 0.05 for ADG and ADFI, respectively). The interaction for ADG (P < 0.05) and a trend for an interaction for ADFI (P = 0.06) observed during the 21-d experimental period appeared to be caused by the interactions observed during the second LPS challenge period. Supplementation of Gly-Gln increased G:F from d 14 to 21 and also during the 21-d experimental period (P < 0.05).

**Morphology of Small Intestine**

In all 3 segments of the small intestine, there were no LPS challenge × Gly-Gln interactions for VH, CD, or VH:CD at d 7, 14, or 21 (Figures 1 and 2). Before the LPS challenge (d 7), there were no differences among treatments in small intestinal morphology. The administration of LPS increased CD of the duodenum and decreased VH:CD of the ileum on d 14 (P < 0.05), whereas Gly-Gln supplementation increased VH:CD (P < 0.05) and tended to increase VH (P = 0.09) of the duodenum on d 14 and VH (P < 0.05) of the ileum on d 21. There were no differences in the morphology of the jejunum among treatments.

**Immune Function**

There were no differences among the treatments in lymphocyte proliferation in response to ConA stimulation. As indicated in Table 3, LPS challenge × Gly-Gln interactions were not detected in concentrations of se-
rum IL-1β throughout the 21-d experiment. Administration of LPS increased serum IL-1β concentration 1 wk after the first challenge (d 14, \( P < 0.01 \)), whereas Gly-Gln supplementation decreased serum IL-1β concentration of the piglets on d 7, 14, and 21 (\( P < 0.01 \)).

Supplementation with Gly-Gln decreased sIL-2R concentration before the LPS challenge (d 7, \( P = 0.06 \)) and increased IL-2 concentration 1 wk after the first challenge (d 14, \( P = 0.09 \)). Serum IL-2 and sIL-2R concentrations were not affected by LPS treatment, and there were no interactions between LPS challenge and Gly-Gln.

**DISCUSSION**

Early weaning and an inflammation challenge result in a severe reduction of ADFI, which can consequently cause intestinal atrophy (Wu et al., 1996; Wang et al., 2008) and a maladjustment of immune function in piglets (Johnson, 1997). Previous studies have indicated that these changes can be mediated by some inflammatory cytokines, which amplify the cellular immune response and inhibit growth (Johnson, 1997; Johnson et al., 2006). Regulation of cytokine secretion would be greatly beneficial to the growth and immune system of early weaned piglets challenged with an immunological stress (Yi et al., 2005). Glutamine plays a key role in maintaining intestinal integrity and gut barrier function (Bai et al., 1996; Wu et al., 1996; Li et al., 2007). The dipeptide Gly-Gln is more stable than glutamine; in addition, it can be hydrolyzed to Gln (Furst et al., 1997). Therefore, the present study was designed to examine the effect of Gly-Gln on the performance, intestinal integrity, and immune function of piglets challenged with LPS.

In our study, the *E. coli* LPS challenge decreased the performance of weaning pigs, which is consistent with the result of Liu et al. (2003). Dietary Gly-Gln had no effect on piglet performance during the first week of the experiment (before the LPS injection), as reported by Liu et al. (2003) and Yi et al. (2005). The ADFI of piglets challenged with LPS was reduced after the first and second LPS challenges, which is similar to the results of van Heugten et al. (1996, 1997). We showed that during the second week of the experiment (i.e., 1 wk after the first challenge, on d 7 to 14), supplementation of Gly-Gln still had no effect on piglet performance, perhaps because piglets were unable to compensate for the reduction in ADFI because of the LPS challenge. In contrast, Lackeyram et al. (2001) observed that supplementation of corn- and soybean-based diets with 0.8% glutamine was effective in enhancing BW gain of early weaned, 10-d-old piglets in a 12-d study. During the third week (i.e., 1 wk after the second challenge, d 14 to 21), ADG and G:F improved by feeding 0.15% Gly-Gln, which is consistent with a previous report in piglets (Johnson et al., 2006).

In the present study, we showed that supplementation of Gly-Gln had no effect on enteric integrity in the first week of feeding without the LPS challenge, but had a significant effect with the LPS challenge. Similarly, Yi et al. (2002) reported no improvement in the small intestinal morphology of piglets supplemented with 1.2% glutamine at d 7 postweaning. Shorter duodenum VH and decreased ileum VH:CD were observed on d 14, which implies that LPS challenge might indirectly have affected small intestinal morphology by inducing the reduction of ADFI. It is important that feeding the diet containing 0.15% Gly-Gln effectively prevented duodenum and ileum atrophy during the second week.
after weaning. During the entire experimental period, there was no effect of the diet on ADFI between control and Gly-Gln-fed piglets. However, Gly-Gln supplementation resulted in an improvement in ADG and G:F, indicating that Gly-Gln may play an important role in maintaining intestinal integrity, which consequently enhances digestion and absorption, thereby alleviating the performance depression caused by an LPS challenge and weaning stress.

Consistent with the report of Liu et al. (2003), we observed a decrease in serum IL-1β concentration by the supplementation of Gly-Gln and an activation of the acute inflammatory response (indicated by the increased IL-1β secretion) in piglets challenged with LPS. This inflammatory response could result in partitioning nutrients toward the immune system rather than toward growth (Higashiguchi et al., 1995). The data

Figure 1. Villus height (A), crypt depth (B), and villus height-to-crypt depth ratio (C) in the duodenum of piglets on d 7, 14, and 21 of the experiment. Control/−LPS and control/+LPS represent the control without and with lipopolysaccharide (LPS) challenge, respectively. Gly-Gln/−LPS and Gly-Gln/+LPS represent glycyl-glutamine (Gly-Gln)-supplemented groups without and with LPS challenge, respectively.

Figure 2. Villus height (A), crypt depth (B), and villus height-to-crypt depth ratio (C) in the ileum in piglets at d 7, 14, and 21 of the experiment. Control/−LPS and control/+LPS represent the control without and with lipopolysaccharide (LPS) challenge, respectively. Gly-Gln/−LPS and Gly-Gln/+LPS represent glycyl-glutamine (Gly-Gln)-supplemented groups without and with LPS challenge, respectively.
showed that the adverse effects of LPS on intestinal integrity could be mediated by a decline in ADFI, but also could be affected directly as a result of a systemic inflammatory response (IL-1β secretion). In addition, there were no LPS × Gly-Gln interactions for serum cytokines, and supplementation of Gly-Gln improved ADG of LPS-challenged weanling piglets without affecting serum IL-2/sIL-2R. However, complex mechanisms concerning the effects of Gly-Gln on inflammatory response may include the intestinal mucosal immune function. Recently, Li et al. (2004) reported that feeding glutamine decreased the inflammatory response induced by LPS in the intestine of young rats. In the current study, we showed that Gly-Gln promoted IL-2 secretion during the early postweaning period. Accordingly, the increased secretion of IL-2 in peripheral blood may help explain the increased lymphocyte proliferation (Li et al., 2007). In the current study, however, we did not find significant lymphocyte proliferation.

In summary, supplementing the diet with Gly-Gln for 2 wk improved the growth and intestinal integrity of weanling piglets. The adverse effects of LPS on intestinal integrity could be mediated by the decline in ADFI, but also could be affected directly as a result of a systemic inflammatory response (IL-1β secretion), whereas Gly-Gln may be able to limit this proinflammatory response in LPS-challenged piglets. In addition, there were no LPS × Gly-Gln interactions for serum cytokines, and supplementation of Gly-Gln improved the ADG of LPS-challenged weanling piglets in the absence of an effect on serum IL-2/sIL-2R.

LITERATURE CITED


Table 3. Effects of dietary glycyl-glutamine (Gly-Gln) supplementation on serum IL-1β, IL-2, and soluble IL-2 receptor (sIL-2R) concentration in piglets challenged with lipopolysaccharide (LPS)1,2

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Gly-Gln</th>
<th>Control</th>
<th>Gly-Gln</th>
<th>SEM</th>
<th>LPS</th>
<th>Gly-Gln</th>
<th>LPS × Gly-Gln</th>
<th>P-value</th>
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</thead>
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<tr>
<td>IL-1β, pg/mL</td>
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<td></td>
<td></td>
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<tr>
<td>d 7</td>
<td>115.68</td>
<td>69.09</td>
<td>109.04</td>
<td>71.69</td>
<td>7.85</td>
<td>NS</td>
<td>&lt;0.01</td>
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<td>NS</td>
</tr>
<tr>
<td>d 14</td>
<td>114.04</td>
<td>70.80</td>
<td>138.80</td>
<td>102.12</td>
<td>7.57</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>d 21</td>
<td>94.55</td>
<td>44.88</td>
<td>124.05</td>
<td>90.40</td>
<td>9.54</td>
<td>NS</td>
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<tr>
<td>IL-2, pg/mL</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>d 7</td>
<td>68.22</td>
<td>71.59</td>
<td>69.58</td>
<td>70.09</td>
<td>1.11</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>d 14</td>
<td>69.00</td>
<td>84.69</td>
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</tr>
<tr>
<td>d 7</td>
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<td>71.59</td>
<td>69.58</td>
<td>70.09</td>
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<td>NS</td>
<td>3</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>IL-1β, pg/mL</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>d 7</td>
<td>200.39</td>
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<td>189.76</td>
<td>150.6</td>
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<tr>
<td>d 14</td>
<td>152.72</td>
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<td>175.51</td>
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<td>12.61</td>
<td>NS</td>
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<tr>
<td>d 21</td>
<td>138.64</td>
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<td>8.35</td>
<td>NS</td>
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</tr>
</tbody>
</table>

1Values are means for 6 piglets (1 piglet per pen).

2LPS (Sigma Chemical Co., St. Louis, MO) was injected on d 14 and d 21. −LPS = without challenge with LPS; +LPS = with challenge with LPS.

3NS = not statistically significant (P > 0.10).


