Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic Escherichia coli K88

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ABSTRACT: We investigated whether spray-dried plasma (SDP) improved growth and health of piglets challenged with enterotoxigenic Escherichia coli K88 (ETEC). Forty-eight pigs weaned at 21 d (BW = 4.88 ± 0.43 kg) received one of four diets containing 6% SDP or fish proteins (as-fed basis) either nonmedicated (SDP-NM and FP-NM diets) or medicated with 0 or 250 mg/kg of colistine + 500 mg/kg of amoxycycline (SDP-M and FP-M diets), for 15 d. On d 4, pigs were orally challenged with ETEC. On d 15, eight pigs per dietary group were killed, blood and saliva were collected for analysis of K88 fimbriae-specific immunoglobulin (Ig)-A, and jejunum was removed for villi preparation, histological analysis, and cytokine expression. The presence or absence of K88 receptors (K88+ and K88− pigs respectively) was determined by villous adhesion assay. Effects of protein source on ADG (P = 0.04) and ADFI (P < 0.01), as well of medication on ADFI (P < 0.02), of all pigs were observed. In sacrificed pigs, there was an effect of protein source on ADG (P = 0.03) and ADFI (P < 0.001), as well an interaction between medication and presence of K88 receptors (P = 0.02) for feed:gain ratio. Plasma K88 specific IgA were low in all K88− pigs and higher in K88+ pigs fed FP-NM compared with all other groups (P < 0.05), except SDP-M. An interaction was found among protein source, medication, and presence of K88 receptors (P = 0.04). Saliva IgA concentrations were high in all pigs fed FP-NM and low in all other pigs. Jejunum of pigs fed FP-NM showed some ulcerations, edema, and mild inflammatory cell infiltration (ICI). In pigs fed FP-M, edema was reduced. Conversely, only a mild ICI was observed in pigs fed SDP-NM and SDP-M. Crypt depth was increased in K88+ pigs fed SDP-NM and an interaction between protein source and presence of K88 receptors was observed (P < 0.05). Expressions of tumor necrosis factor-α and interleukin (IL)-8 were lower in pigs fed SDP-NM and SDP-M than in those fed FP-NM and FP-M, either K88− or K88+ (P < 0.01). In pigs fed FP diets, expression of IL-8 tended to increase (P = 0.08) in K88+ compared with K88− subjects. Expression of interferon-γ increased in K88− and K88+ pigs fed FP-M as compared with other pigs (P < 0.01). These results indicate that feeding with SDP improved growth performance and protected against E. coli-induced inflammatory status, and suggest that use of SDP-NM can be considered a valid antibiotic alternative.

Key Words: Cytokines, Immunoglobulin A, Intestine, Pigs, Spray-Dried Plasma

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Introduction

Weaning is frequently associated with infection, disease, and diarrhea in pigs, which are mainly caused by enterotoxigenic Escherichia coli (ETEC) K88 and which may lead to a high rate of mortality (Moon and Bunn, 1993; Osek, 1999). Feeding piglets with spray dried animal plasma (SDP) may improve feed intake and growth rate (Kats et al., 1994; Coffey and Cromwell, 2001) and decrease intestinal diseases (van der Peet-Schwering and Binnendijk 1995). Some authors did not find an effect of SDP on pig gut morphology, disaccharidase activities, or fecal bacterial counts (van Dijk et al., 2002a,b). However, recent studies have reported an improvement of growth performance and decreased ETEC shedding by feeding SDP (Owusu-Asiedu et al., 2003a). The mechanisms by which SDP might counteract pathogen infectivity may include improvement of immunocompetence or reduction of pathogen adhesion to

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the mucosa by the immunoglobulins and glycoproteins present in SDP (Sanchez et al., 1993; Van Dijk et al., 2001a). Spray dried animal plasm is also able to lower inflammatory cytokine expression in many tissues (Touchette et al., 2002). Whether SDP affects cytokine expression in intestine remains to be seen.

The effect of SDP on pigs challenged with lipopolysaccharide (LPS) has been investigated (Touchette et al., 2002); however, an infection by live bacteria, such as ETEC, could have more dramatic consequences in the intestine than LPS. Under such conditions, the effects of SDP may be different. Moreover, because the pathogenic action of ETEC is exerted by binding to specific intestinal receptors, the efficacy of SDP in preventing ETEC K88 infection may be related to the presence of K88 receptors in intestine.

In this study, we used weaned pigs challenged with ETEC K88 as a model of in vivo pig inflammatory conditions to investigate the following: 1) whether SDP improved growth performance and immune defenses and decreased intestinal inflammation; 2) whether any such effects might be related to the presence of ETEC receptors; and 3) whether SDP could replace antibiotic therapy.

Materials and Methods

Animals and Housing

Forty-eight pigs (Large White), male and female, that were weaned at 21 d of age, were obtained from eight litters in a commercial piggery where ETEC K88 infection was reported. Animals were housed in pens with a mesh floor in groups of four for the first 2 d, and then individually. Pigs were kept at a controlled temperature (28°C at the beginning and 24°C at the end of the experiment, with a 1°C decrease every 3 d). Infrared lamps were located over piglets for the first 7 d.

Experimental Design and Diets

The experimental design was a factorial arrangement of dietary protein source and antimicrobial medication. The diets contained 6% SDP or herring fish proteins (as-fed basis), nonmedicated (SDP-NM and FP-NM diets, respectively) or medicated with 0 or 250 mg/kg of colistin + 500 mg/kg of amoxycycline (SDP-M and FP-M diets, respectively), and are reported in Table 1. Fish protein was supplied by Prodotti Gianni (Milan, Italy) and SDP was provided by APC Europe (Barcelona, Spain). The diets were formulated to contain about 3,700 Kcal of DE/kg and 1.5% Lys. Trace minerals and vitamins were provided according to NRC (1998). The diets contained 6% SDP or herring fish proteins (as-fed basis), nonmedicated (SDP-NM and FP-NM diets, respectively) or medicated with 0 or 250 mg/kg of colistin + 500 mg/kg of amoxycycline (SDP-M and FP-M diets, respectively), and are reported in Table 1. Fish protein was supplied by Prodotti Gianni (Milan, Italy) and SDP was provided by APC Europe (Barcelona, Spain). The diets were formulated to contain about 3,700 Kcal of DE/kg and 1.5% Lys. Trace minerals and vitamins were provided according to NRC (1998). The calcium and phosphorus levels of the SDP diet were lower than those of the FP diet. Despite this, the supplementation of calcium formate and monocalcium phosphate in the SDP diet was not increased above that in the FP diet, in order to avoid possible side effects on gastric secretion caused by high amounts of calcium and phosphorus salts (Bolduan et al., 1988; Puscas et al., 2001).

Pigs were divided in four groups of 12 animals and fed the SDP-NM, FP-NM, SDP-M, or FP-M diets for 15 d. Food and water were provided ad libitum. Gender of the animals was balanced across dietary treatments. Pig weights were recorded on d 1 and 15. On d 4 of dietary treatments, all pigs were orally challenged with ETEC (1 × 1010 bacteria/mL of PBS). On d 15, 8 pigs were randomly selected from each group and anaesthetized with sodium thiopental (10 mg/kg BW). Blood from the anterior vena cava and saliva were collected for immunoglobulin (Ig)-A detection. The jejunum was removed and portions processed for chemical composition, villi preparation, histological analysis, and cytokine expression. Euthanasia of the pigs was then performed by intracardiac injection of Tanax (0.5 mL/kg BW; Intervet Italia, Peschiera Borromeo, Italy).

<table>
<thead>
<tr>
<th>Table 1. Composition (% as-fed basis) of experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Item</strong></td>
</tr>
<tr>
<td><strong>Protein source</strong></td>
</tr>
<tr>
<td><strong>Ingredients</strong></td>
</tr>
<tr>
<td>Corn extruded</td>
</tr>
<tr>
<td>Barely flaked</td>
</tr>
<tr>
<td>Barley</td>
</tr>
<tr>
<td>Wheat middlings</td>
</tr>
<tr>
<td>Spray-dried milk whey</td>
</tr>
<tr>
<td>Spray-dried milk whey-lard</td>
</tr>
<tr>
<td>Soybean meal, 50% CP</td>
</tr>
<tr>
<td>FP</td>
</tr>
<tr>
<td>SDP</td>
</tr>
<tr>
<td>Spray-dried skim milk</td>
</tr>
<tr>
<td>Dextrose</td>
</tr>
<tr>
<td>Calcium formate</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
</tr>
<tr>
<td>Lysine-HCL</td>
</tr>
<tr>
<td>ni-Methionine</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Vitamin and mineral supplement</td>
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<tr>
<td><strong>Nutrient composition</strong></td>
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<tr>
<td>DE, kcal/kg</td>
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<tr>
<td>CP, %</td>
</tr>
<tr>
<td>Calcium, %</td>
</tr>
<tr>
<td>Phosphorus, %</td>
</tr>
<tr>
<td>Lysine, %</td>
</tr>
<tr>
<td>Threonine, %</td>
</tr>
<tr>
<td>Methionine, %</td>
</tr>
<tr>
<td>Tryptophan, %</td>
</tr>
</tbody>
</table>

*Fish protein from herrings (Prodotti Gianni, Milan, Italy).
Spray-dried plasma, from APC Europe, Barcelona, Spain.
Lard was spray dried together with milk whey.
Vitamin and mineral mixture supplied per kilogram diet: vitamin A, 26,500 IU; vitamin D3, 2,400 IU; vitamin K3, 2 mg; vitamin E, 35 mg; vitamin B1, 10 mg; vitamin B2, 8 mg; vitamin B6, 6 mg; vitamin B12, 0.04 mg; niacin, 55 mg; biotin, 0.15 mg; d-pantothenic acid, 30 mg; folacin, 1 mg; iron (as FeSO4), 2 mg; zinc (as ZnSO4), 175 mg; copper (as CuSO4), 150 mg; manganese (as MnSO4), 50 mg; KI, 2.5 mg; cobalt (as CoCO3), 2 mg; selenium (as Na2SeO4), 0.2 mg.
Based on chemical composition.
**Bacterial Preparation**

The ETEC K88 strain O149 (provided by Istituto Zootecnico Sperimentale della Lombardia e dell’Emilia, Reggio Emilia, Italy) was grown in Luria broth (LB) medium containing 1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0. Tryptone and yeast extract were from Oxoid (Basingstoke, England). After overnight incubation at 37°C with shaking, bacteria were diluted 1:100 in fresh LB. Following incubation, the bacterial cells were harvested by centrifugation at 3,000 × g for 10 min at 4°C, washed in PBS, and resuspended in PBS. Bacteria grown to mid-log phase (about 0.3 OD600) was determined by densitometry and confirmed by serial dilution followed by viable plate counts on LB agar.

**Microbial Analysis**

On d 11, feces were individually collected, and on d 15, at slaughter, a segment of the mid-jejunum (about 50 cm) of each pig was excised and opened longitudinally. Mucosa was removed by gently scraping. For the quantification of ETEC K88, 1 g of each sample was diluted in 4 mL of Ringer’s solution (1:5 vol/vol), and 10-fold serial dilutions were made up to 10⁻¹⁰ dilution. Aliquots of 0.1 mL were inoculated onto a violet-red bile agar medium (containing 0.1 g/L of 4-methyl-umbelliferyl-β-glucoronide) and incubated at 43°C for 24 h. Colony-forming units were determined at the dilution, which showed 30 to 300. Specificity of colonies was confirmed by Wood’s lamp fluorescence, growth on Kligler iron agar media (Oxoid) and API 20 E (Bio-Mérieux, Rome, Italy). The presence of K88 antigen was assessed by slide agglutination test using rabbit immune sera against ETEC K88 antigens (bank of Istituto Zootecnico Sperimentale della Lombardia e dell’Emilia-Romagna).

**In Vitro Villous Adhesion Assay**

To determine the presence of K88 receptors, a villous adhesion assay was performed according to Van den Broeck et al. (1999).

*Collection of Villi.* A segment of the mid-jejunum (15 cm) was excised and the intestinal content removed by three washes with PBS at 4°C. The segment was opened and washed for 15 min in Krebs-Henseleit buffer (120 mM NaCl, 14 mM KCl, 25 mM NaHCO₃, 1 mM KH₂PO₄, pH 7.4) containing 1% (vol/vol) formaldehyde at 4°C. The villi were gently scraped from the mucosa with a glass slide and washed four times in Krebs-Henseleit buffer, for 1 h per wash. Villi were frozen in dimethyl sulfoxide (DMSO)-Hanks’ medium solution (Sigma, Milan, Italy), 650 mg/100 mL of BSA, 10 mL of fetal calf serum, 30 mL of DMSO, and 30 mL of glycerol. The villi suspension was then stored for 24 h at −20°C, followed by storage at −80°C.

*Villous Adhesion Assay.* Villi were thawed at 20°C and placed into Krebs buffer containing 1% formaldehyde for 1 h, washed twice with Krebs buffer without formaldehyde and finally transferred into 900 μL of PBS supplemented with 10 g/L of D-mannose (Sigma). Then, 100 μL of bacterial solution was added (4 × 10⁹ bacteria/mL of PBS) and incubated for 1 h with gentle shaking at room temperature. After incubation, villi were examined by phase-contrast microscopy at a magnification of 1,000×. The bacteria adhering along a 50-μm length of villous brush border was observed in 20 different places, and the bacterial adhesion was calculated by the number of bacteria (n) along the 250-μm length of villous brush border. Pigs were considered positive with n = 30.

**Preparation of Fimbriae**

Fimbriae for the K88-specific IgA analysis were prepared from *E. coli* K88 with the method described by Van den Broeck et al. (1999). Bacteria were homogenized (Carlo Erba Reagenti, Milan, Italy) for 15 min at 24,000 rpm. Fimbriae present in the supernatant was precipitated with 40% (wt/vol) ammonium sulfate, and then resuspended and dialyzed overnight against ultrapure water to remove ammonium sulfate. The solution was then centrifuged and the final pellet containing the fimbriae was lyophilized.

**Detection of Fimbriae-Specific IgA**

The amount of K88-specific IgA antibody in plasma and saliva was assayed by ELISA, as reported by Van den Broeck et al. (1999). Wells of a 96-well microtiter plate (NUNC, Life Technologies, Milan, Italy) were coated with K88 fimbrial adenalin, Mab (CVL, Addlestone, U.K.), at a concentration of 1 μg/mL in 50 mM carbonate–bicarbonate buffer, pH 9.4 (Sigma). After incubation at 37°C for 3 h, and then at 4°C overnight, PBS and 0.2% (vol/vol) Tween 20 (Merck, Milan, Italy) were added for 30 min at room temperature. The ETEC K88 fimbrial antigen was added at a concentration of 50 μg/mL in ELISA dilution buffer. The plate was then incubated at 37°C for 1 h. In the next step, pooled sera were added in series of twofold dilutions in ELISA dilution buffer. After incubation at 37°C for 1 h, the wells were treated with goat anti-pig IgA, conjugated horseradish peroxidase (Bethyl Laboratories, Montgomery, TX), and 20% fetal calf serum, at 37°C for 1 h. After addition of 2,2-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid substrate) (Roche Diagnostics, Milan, Italy) and incubation at 37°C for 15 min, the OD₄⁰⁵ were recorded with a microplate reader (Sunrise Microplate Reader, TECAN, Milan, Italy). The concentration values, expressed as arbitrary units per milliliter, were calculated using a four-parameter analysis with the formula:

\[ y = D + (A - D)(1 + (x/C)^B) \]

where \( A = y \), if \( x = 0 \); \( B = \) increase (slope) factor; \( C = 50\% \) intercept; and \( D = y \), if \( x \geq \) infinity. Pooled sera
obtained from 5 pigs positive for *E. coli* K88 adhesion were used as standard, and 100 arbitrary units were attributed to each milliliter.

**Histological Analysis**

Pieces of jejunum (3 cm) were dissected, fixed in Bouin’s solution for 12 h, and embedded in paraffin at 58°C. Sections (7 μm) were stained with Mallory stain and then examined by light microscopy. Histopathological observations were blindly carried out by two observers on at least four sections of the jejunum of each animal. The variables selected to establish the extent of the most evident tissue modifications were ulcerations, edema, inflammatory cell infiltration (ICI), and dilatation of blood vessels. A range of scores from 0 to 3, defined as 0 = normal; 1 = mild alterations; 2 = moderate alterations; and 3 = severe alterations, was considered to evaluate the severity of morphological damages. Crypt length was measured with a micrometer.

**Analysis of Cytokine mRNA Level**

Constitutive level of interleukin (IL)-8, tumor necrosis factor (TNF)-α and interferon (IFN)-γ mRNA in jejunum was analyzed by reverse transcription-PCR. Total RNA was extracted from 50 mg of tissue homogenized in 1 mL of TRIzol reagent (Life Technologies, Gibco BRL, Milan, Italy). One microgram of RNA was reverse-transcribed to cDNA in 20 μL of reaction mixture containing 50 mM Tris(HCl) pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM DTT, 0.5 mM deoxynucleoside triphosphates (dNTPs; Pharmacia Biotech, Milan, Italy), 2.5 mM oligo (dT)$_{18}$, 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies), and 40 U of RNase inhibitor (Promega, Florence, Italy). The mixture was incubated at 37°C for 60 min and heat-inactivated at 95°C for 5 min. The reverse transcriptase product (4 μL) was amplified in a 25-μL PCR reaction mixture containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 2.5 U of Taq DNA polymerase, 0.3 μM each of sense and antisense primers. Samples were heat denaturated at 95°C and then subjected to 35 cycles of PCR, followed by a last cycle at 72°C for 7 min on a DNA thermal cycler (Applied Biosystem, Rome, Italy). The PCR cycle conditions were 30 s at 95°C and 2.5 min at 62°C. The relative intensity of the bands was analyzed by Scion image software (Scion Corp., Frederick, MD). Each sample was also amplified with actin as the internal control. The ratio of cytokine:actin mRNA intensities was used to evaluate the relative levels. The sequences of pig primers were the following: IL-8 sense 5′-TTT CTT CAG CTC TCT TCT GTG AGG-3′ and antisense 5′-CTG CTG TTG TTG CTT CTC-3′ (expected fragment size of 269 bp); TNF-α sense 5′-ATC GGC CCC CAG AAG GAA GAG-3′ and antisense 5′-GAT GGC AGA GAG GAG GTT CTT-3′ (expected fragment size of 557 bp); IFN-γ sense 5′-GTT TTT CTT CAG CTC TCT GTG AGG-3′ and antisense 5′-CTG CTG TTG TTG CTT CTC-3′ (expected fragment size of 410 bp); actin sense 5′-GGA CTT CGA GCA GGA GAG GAT-3′ and antisense 5′-GCA CGG TGT TTT AGG CTT AGA GG-3′ (expected fragment size of 233 bp). The primers were provided by M-Medical (Genenco, Florence, Italy).

**Statistical Analysis**

Data were analysed by analysis of variance using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). To analyze growth performance, a three-factor treatment analysis (protein source, medication, and sex) was performed. Because sex was not significant for any responses, it was removed from the model. For sacrificed animals, the model was a three-factor design and included protein source, medication, sensitivity of intestinal villous to ETEC adhesion, and first-level interactions. For the factor sensitivity of intestinal villous to ETEC adhesion, we grouped Level 0 with Level 1, and Level 2 with Level 3. For growth performance of sacrificed pigs, initial BW was added as covariate. An alpha level of $P < 0.05$ was used for determination of statistical significance, with statistical tendencies reported when $P < 0.10$.

**Results**

The effects of protein source and medication on growth performance of ETEC-challenged piglets are shown in Table 2. Two of the pigs fed FP-NM died after bacterial infection. Pigs fed SDP-NM and SDP-M had higher ADG ($P < 0.05$) and ADFI ($P < 0.01$) than did pigs fed FP-NM and FP-M. The addition of antibiotics induced a further increase in ADFI. There were no differences in feed:gain ratio among treatments. No interaction between the main factors was found.

The effects of protein source, medication, and presence of K88 receptors on growth performance of ETEC-challenged piglets that were killed are shown in Table 3. Considering the initial BW as covariate, the final BW of K88$^-$ pigs fed SDP-NM and SDP-M tended ($P = 0.09$) to be higher than that of K88$^+$ pigs fed FP-NM and FP-M. Compared with pigs fed FP-NM and FP-M, pigs fed SDP-NM and SDP-M had higher ADG ($P = 0.07$) and ADFI ($P < 0.05$). For feed:gain ratio, there were no effects of diet and an interaction between medication and presence of K88 receptor was observed.

The microbial analysis of ETEC K88 indicated that only piglets fed diets containing FP-NM excreted detectable amounts of bacteria (7.81 ± 0.78 cfu, data not shown), and no bacteria were found in the central jejunum of all groups.

Figure 1 shows the effect of protein source, medication, and presence of K88 receptors on K88$^-$ specific IgA in plasma and saliva. The titer of IgA in plasma (Figure 1A) was very low in all K88$^+$ piglets. Conversely, the amount of IgA was elevated ($P < 0.01$) only in K88$^+$ piglets fed FP-NM as compared to all the other groups,
Table 2. Effect of protein source and medication on growth performance of *Escherichia coli* K88 (ETEC K88)-challenged piglets

<table>
<thead>
<tr>
<th>Item</th>
<th>Medication (M):</th>
<th>Protein source (PS):</th>
<th>P-values</th>
<th>Main effect</th>
<th>Interactions</th>
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<td></td>
<td>MN</td>
<td>FP</td>
<td>SDP</td>
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<td>12</td>
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<tr>
<td>Initial BW, kg</td>
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<td>4.88</td>
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<tr>
<td>Final BW, kg</td>
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<td>7.13</td>
<td>7.34</td>
<td>7.68</td>
<td>0.34</td>
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<tr>
<td>ADG, g</td>
<td>128</td>
<td>148</td>
<td>164</td>
<td>186</td>
<td>18</td>
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<tr>
<td>ADFI, g</td>
<td>175</td>
<td>186</td>
<td>197</td>
<td>214</td>
<td>5.8</td>
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<tr>
<td>Feed:gain</td>
<td>1.81</td>
<td>1.48</td>
<td>1.30</td>
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</table>

*Fish protein (FP) and spray-dried plasma (SDP) diets.

An interaction ($P < 0.05$) was found among protein source, medication, and presence of K88 receptors. In saliva (Figure 2B), a strong IgA titer was observed in all K88− and K88+ pigs fed FP-NM. A low titer was found in all other groups. Medication decreased salivary IgA content, and a trend toward a reduction was also observed for protein sources. An interaction ($P < 0.05$) between the two factors was observed. No effect of sensitivity of intestinal villous to ETEC K88 adhesion was observed.

Morphology of the jejunum of pigs fed FP-NM and SDP-NM is illustrated in Figure 2, and the histological scores of pigs treated with the different diets are reported in Table 4. The jejenum of pigs fed FP-NM showed little ulceration, moderate dilatation of blood vessels and edema, and mild ICI. The morphology of pigs fed FP-M (not shown) showed that edema was reduced, as indicated by the histological score. Conversely, the jejunum of pigs fed SDP-NM did not show any ulceration, vasodilation, or edema, and only a mild ICI (not shown). The assigned histological scores were 0. The addition of antibiotics did not result in further improvements (not shown), and the assigned scores were 0. The effect of dietary treatments on crypt depth of jejunum is shown in Table 4. Crypt depth was higher in the K88+ pigs fed SDP-NM compared to others. A significant interaction between protein source and presence of K88 receptors was observed. No effect was observed when medication was added to the diets.

The expression of proinflammatory cytokines in the intestine of the K88− and K88+ pigs fed the different dietary treatments are given in Figure 3. Expressions of TNF-α (Figure 3A) and IL-8 (Figure 3B) were lower in pigs fed SDP-NM and SDP-M than in those fed FP-NM and FP-M, for both K88− and K88+. No effect of medication or the presence of K88 receptors was found. A trend ($P = 0.10$) for an interaction for IL-8 was observed between protein source and the presence of K88 receptors. In pigs fed FP-NM and FP-M, a trend for an increase of IL-8 was observed in K88+ vs. K88− pigs.

Table 3. Effect of protein source, medication, and susceptibility to *Escherichia coli* K88 (ETEC K88) intestinal adhesion on growth performance of piglets challenged with ETEC K88 that were killed

<table>
<thead>
<tr>
<th>Item</th>
<th>Susceptibility (S):</th>
<th>Protein source (PS):</th>
<th>Medication (M):</th>
<th>P-values</th>
<th>Main effects</th>
<th>Interactions</th>
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<tr>
<td></td>
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<td>K88+</td>
<td>FP</td>
<td>SDP</td>
<td>SEM</td>
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<td>3</td>
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<tr>
<td>Initial BW, kg</td>
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<td>4.98</td>
<td>4.72</td>
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<td>5.06</td>
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<td>ADG, g</td>
<td>106</td>
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<td>106</td>
<td>126</td>
<td>175</td>
<td>138</td>
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<tr>
<td>ADFI, g</td>
<td>187</td>
<td>168</td>
<td>187</td>
<td>168</td>
<td>193</td>
<td>188</td>
</tr>
<tr>
<td>Feed:gain</td>
<td>1.96</td>
<td>1.36</td>
<td>1.96</td>
<td>1.36</td>
<td>1.21</td>
<td>1.39</td>
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*Fish protein (FP) and spray-dried plasma (SDP) diets.

The FP and SDP diets were either nonmedicated (NM) or medicated (M; 250 mg/kg of colistine + 500 mg/kg of amoxycycline).

The lowest $P$-value for the PS × M and PS × S terms was $P = 0.30$.

Effect of initial BW (covariate; $P < 0.01$).

As-fed basis.
Spray-dried plasma improves piglet health

Figure 1. Effect of protein source, medication, and susceptibility to *Escherichia coli* K88 intestinal adhesion on K88 specific IgA in plasma. Pigs were fed diets containing 6% (as-fed basis) spray dried plasma (SDP), or nonmedicated (SDP-NM and FP-NM), or medicated *Escherichia coli* with 0 or 250 mg/kg of colistin + 500 mg/kg of amoxycycline (SDP-M and FP-M). K88+ = susceptible pigs; K88− = unsusceptible pigs. Data are the least squares means ± SEM. n = 4 for FP-NM and SDM K88− or K88+; n = 5 for K88− FP-M and SDP-NM; n = 3 for K88+ FP-M and SDP-NM. Panel A: Plasma response; protein source × medication × susceptibility interaction (P < 0.05). Panel B: Salivary response; protein source × protein source interaction (P < 0.05).

The expression of IFN-γ (Figure 3C) increased (P < 0.05) in K88− and K88+ pigs fed FP-M compared with all other groups. The expression of this cytokine was affected by an interaction (P < 0.01) between protein source and medication. No effect of the presence of K88 receptors was found.

**Discussion**

The results reported here indicate that SDP feeding to early-weaned piglets challenged with ETEC K88 improved growth and reduced inflammation in animals, as indicated by enhanced growth performance, reduced IgA secretion, decreased intestinal mucosal damage, and reduced proinflammatory cytokine expression in the gut. These findings are in agreement with previous data showing an improvement of growth and digestibility in piglets fed different sources of SDP (Gatnau and Zimmermann, 1994; Bosi et al., 2001; Coffey and Cromwell, 2001; Van Dijk et al., 2001a). We report original data on inflammatory cytokine expression in the intes-

Figure 2. Effect of protein source, medication and susceptibility to *Escherichia coli* K88 (ETEC K88) intestinal adhesion on cytokine expression in jejunum, assayed by reverse-transcription PCR. Pigs were fed diets containing 6% (as-fed basis) spray dried plasma (SDP) or fish proteins nonmedicated (SDP-NM and FP-NM) or medicated with 0 or 250 mg/kg of colistin + 500 mg/kg of amoxycycline (SDP-M and FP-M). K88+ = susceptible pigs; K88− = unsusceptible pigs. The figure represents the densitometric values of cytokine mRNA normalized to actin mRNA. Data are the least squares means ± SEM. n = 4 for either K88− or K88+ FP-NM and SDM; n = 5 for K88− FP-M and SDP-NM; n = 3 for K88− FP-M and SDP-NM. Panel A: TNFα response; effect of protein source (P < 0.01). Panel B: IL-8 response; effect of protein source (P < 0.01); protein source × susceptibility interaction (P = 0.10). Panel C: INFγ response; protein source × medication interaction (P < 0.01).
Histological scores of the depth of jejunum and histological scores in K88+ animals (Van den Broek et al., 1999). In pigs because serum IgG and IgA were detected only in K88+ animals, suggesting a prerequisite for the induction of an immune response specific for K88 ETEC on enterocyte brush borders is a prerequisite.

In a further improvement in pig health, supporting again the strong efficacy of SDP in preventing ETEC pathogenesis.

The SDP can be a viable alternative to antibiotics because it is as effective as medication against ETEC K88 infection and even more efficient than antibiotics in reducing the expression of proinflammatory cytokines. In addition, the results indicate that inclusion of antibiotics in the SDP-containing diet did not induce a further improvement in pig health, supporting again the potential role of SDP as an efficient antibiotic alternative. Our results are in agreement with those reported by Coffey and Cromwell (1995), who found that the performance-enhancing properties of SDP were independent of the growth-promoting properties of antimicrobials added to the diet. The activity of SDP and antibiotics appeared to be regulated by different mechanisms. Indeed, if medication and SDP acted through similar mechanisms to inhibit the pathogenic activity, an interaction between the two factors would have been observed. However, this was not the case, suggesting that SDP acted through other mechanisms.

It has been shown that the presence of receptors specific for K88 ETEC on enterocyte brush borders is a prerequisite for the induction of an immune response in pigs because serum IgG and IgA were detected only in K88+ animals (Van den Broek et al., 1999). In agreement with these findings, we have found a high IgA content in plasma of receptor-positive pigs fed the FP-NM diet and a low IgA titer in phenotypically adhesive pigs fed the SDP-NM diet. Decreased IgA production can be the consequence of an inhibition of ETEC K88 colonization or binding to intestinal receptors exerted by Ig and glycoproteins contained in SDP. Indeed, it has been shown that several glycoproteins obtained from plasma can inhibit adhesion of _Escherichia coli_ to small intestine (Sanchez et al., 1993). Different variants of K88 adhesin, designated K88ab and K88ac, which predominate in jejunum, and K88ad, which belongs to the ileum, have been identified in the small intestine (Chandler et al., 1994; Jeyasingham et al., 1999; Grange et al., 2002). Glycoproteins isolated from intestinal mucus and brush border membranes were reported to bind specifically to K88ab and K88ac fimbriae (Jin and Zhao, 2000), although only three have fulfilled the criteria as phenotype-specific K88 fimbrial receptors (Erickson et al., 1992; Grange et al., 2002). Thus, it is possible that SDP glycoproteins may have inhibited ETEC adhesion by competing with intestinal glycoprotein receptors present in the intestine. Relating to the Ig, these proteins may have acted by decreasing the number of bacteria available for gut colonization. In fact, our results on fecal excretion of ETEC K88 showed the presence of bacteria in feces of piglets fed FP-NM but not SDP-NM. These results do not agree with those reported by some authors that SDP was not able to reduce the number of ETEC in different sites of intestine (Van Dijk et al., 2002a). However, recent studies have shown a reduction in ETEC shedding after SDP was fed to pigs (Owusu-Asiedu et al., 2003a, b).

The production of IgA antibodies in the saliva of pigs fed FP-NM was strong, although the titer was lower than that found in plasma. The SDP feeding was able to reduce the amount of salivary IgA. However, no effect of the phenotype for susceptibility to ETEC K88 adhesion and no interaction of this factor with the diet were observed. Thus, our results suggest that the susceptible phenotype is not a determinant for the production of salivary IgA.

The integrity of the intestinal barrier is fundamental for proper functioning of epithelial cells and to prevent the entry of pathogenic bacteria that might otherwise lead to inflammatory processes. Enterocytes are conti-
uously exfoliated from the tips of the villi and replaced
with new cells migrating out of the crypts. Our results of
dereper crypts after SDP feeding suggest that SDP
may increase enterocyte renewal and thus better main-
tain barrier integrity. This hypothesis is supported by
the data of mucosal morphology showing the presence of
ulcerations and inflammation in mucosa of pigs fed
FP-NM and not in pigs fed SDP-NM. Some authors
were unable to report an improvement of crypt cell
proliferation, crypt depth, or villus height by SDP feed-
ing (Jiang et al., 2000; Van Djik et al., 2001b). However,
these authors did not analyze the morphology of the
mucosa to study whether it was damaged before SDP
treatment and whether SDP could improve possible
mucosal alterations. It is possible that SDP may influ-
ence the villus-crypt structure only when the integrity
of intestinal barrier is compromised, as in our study.

It is well known that inflammation is mediated by
increased production of pro-inflammatory cytokines.
Among these, IL-8, TNF-α and IFN-γ are markedly
expressed in inflamed gut mucosa. Interleukin-8 is ex-
pressed in enterocytes and macrophages to recruit other
inflammatory cells and is highly selective for neutrophil
movement to inflammatory sites. Neutrophils are a rele-
vant class of inflammatory cells in infectious disease.
Tumor necrosis factor-α is involved in tissue damage
and may increase the ability of epithelial cells and mac-
rophages to secrete IL-8. Interferon-γ is produced by
macrophages and other cells and is an activating factor
in cell-mediated response. Our results showed a high
expression of IL-8 and TNF-α in intestine of pigs fed
FP-NM and FP-M, which tended to be higher in suscepti-
tible vs. unsusceptible pigs, whereas IFN-γ expression
did not change. Feeding SDP induced a marked de-
crease on expression of IL-8 and TNF-α, which was
higher in susceptible vs. unsusceptible pigs. Interest-
ingly, SDP was also able to induce a decrease of these
cytokines independent of the ETEC susceptibility.
Thus, all these data are in favor of a role of SDP in
decreasing intestinal inflammation by downregulating
inflammatory cytokine expression. Surprisingly, we
have found a strong IFN-γ expression in pigs fed FP-
M vs. the other groups. This finding can be ascribed to
the presence of antibiotics because previous studies
have reported that antimicrobial agents and antibiotics
can induce a high expression of IFN-γ (Tufano et al.,
1992; Noma et al., 2001). Some authors have recently
studied the expression of pro-inflammatory cytokines
in several pig tissues such as spleen, thymus, adrenal
and pituitary glands, hypothalamus and liver. They
have found that the expression of IL-1β,TNF-α and IL-
6 was reduced after SDP feeding, but not when pigs
were challenged with an injection of LPS (Touchette et
al., 2002). In our study, we have used an oral challenge
with ETEC. This bacterium can attach to intestinal
cells and then release toxins (Jin and Zhao, 2000). Pro-
tection at mucosal surfaces, against either invasive or
noninvasive bacteria, involves antiinflammatory cyto-
kine production by the cells to prevent both pathogen
attachment and development of inflammation in re-
sponse to bacterial infection. Indeed, we have found
an upregulation of proinflammatory cytokines following
ETEC infection. The intestinal immunological response
to oral challenge with ETEC can be different from that
elicited by LPS injection and more closely mimic the in-
vivo pig response to pathogen infection. Thus, by using
our experimental model, we can provide evidence that
feeding SDP to piglets infected with ETEC is able to
decrease inflammatory cytokine expression in intesti-
nal mucosa.

Implications

The results of the present study show that feeding
piglets a diet containing spray-dried plasma improves
growth and protects against enterotoxigenic Esche-
richia coli K88 infection by maintaining mucosal in-
tegrity, enhancing specific antibody defense, and de-
creasing inflammatory cytokine expression in intestine.
These data provide further support that spray-dried
plasma is a better source of protein than fish for wean-
ling pigs. In addition, this study suggests that the use
of spray-dried plasma can be considered a valid alterna-
tive to antibiotic treatment.

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