Effect of dietary inorganic sulfur level on growth performance, fecal composition, and measures of inflammation and sulfate-reducing bacteria in the intestine of growing pigs[1]

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ABSTRACT: Two experiments investigated the impact of dietary inorganic S on growth performance, intestinal inflammation, fecal composition, and the presence of sulfate-reducing bacteria (SRB). In Exp. 1, individually housed pigs (n = 42; 13.8 kg) were fed diets containing 2,300 or 2,100 mg/kg of S for 24 d. Decreasing dietary S had no effect on ADG, ADFI, or G:F. In Exp. 2, pigs (n = 64; 13.3 kg) were fed diets containing 0, 0.625, 1.25, 2.5, or 5.0% $CaSO_4$, thereby increasing dietary S from 2,900 to 12,100 mg/kg. Two additional diets were fed to confirm the lack of an impact due to feeding low dietary S on pig performance and to determine if the increased Ca and P content in the diets containing $CaSO_4$ had an impact on growth performance. Pigs were fed for 35 d. Ileal tissue, ileal mucosa, and colon tissue were harvested from pigs fed the 0 and 5% $CaSO_4$ diets (low-S and high-S, respectively) to determine the impact of dietary S on inflammation-related mRNA, activity of mucosal alkaline phosphatase and sucrase, and pathways of inflammatory activation. Real-time PCR was used to quantify SRB in ileal and colon digesta samples and feces. Fecal pH, sulfide, and ammonia concentrations were also determined. There was no impact on growth performance in pigs fed the diet reduced in dietary S or by the increase of dietary Ca and P. Increasing dietary S from 2,900 to 12,100 mg/kg had a linear ($P < 0.01$) effect on ADG and a cubic effect ($P < 0.05$) on ADFI and G:F. Real-time reverse-transcription PCR analysis revealed that pigs fed high-S increased ($P < 0.05$) the relative abundance of intracellular adhesion molecule-1, tumor necrosis factor-α, and suppressor of cytokine signaling-3 mRNA, and tended ($P = 0.09$) to increase the relative abundance of IL-6 mRNA in ileal tissue. Likewise, pigs fed high-S had reduced ($P < 0.05$) abundance of nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor-α and increased ($P < 0.05$) phospho-p44/p42 mitogen-activated protein kinase in ileal tissue, but there was no effect of dietary S on mucosal alkaline phosphatase or sucrase activity. Pigs fed the high-S diet had decreased ($P < 0.05$) total bacteria in ileal digesta, but increased ($P < 0.05$) prevalence of SRB in colon contents. Fecal sulfide was increased ($P < 0.05$) and fecal pH was deceased ($P < 0.05$) in pigs fed high-S. The data indicate that growing pigs can tolerate relatively high amounts of dietary inorganic S, but high dietary S content alters inflammatory mediators and intestinal bacteria.

Key words: growing pig, inflammation, inorganic sulfur, sulfate-reducing bacteria

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INTRODUCTION

Investigating the chemistry and metabolism of S has a long history (Lewis, 1924; du Vigneaud, 1952; Dziewiatkowski, 1962). Sulfur is an important element essential for animal growth, being required for the formation of the many S containing compounds found in essentially all body cells. Unlike the extensive understanding of S-AA metabolism (Stipanuk, 2004; Shoveller et al., 2005; Baker, 2006; Burrin and Stoll, 2007), intake of inorganic S intake has received little attention (NRC, 1980), being required only under certain circumstances (Sasse and Baker, 1974; Lovett et al., 1986). Pigs forced to consume water with increased sulfates have been shown to scour initially, but can adapt to the increased sulfate such that performance may not be affected (Anderson and Stothers, 1978; Paterson et al., 1979; Veenhuizen et al., 1992). Even consumption of

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water containing up to 7,000 mg of sulfate/L has been shown not to affect pig performance (Anderson et al., 1994). Although addition of $CaSO_4$ has been shown to reduce urine and manure pH (Mroz et al., 2000), resulting in decreased ammonia emission (Canh et al., 1998), increased S excretion may have a variety of negative effects. Increased dietary S has been shown to increase S-containing odorants (Sutton et al., 1998; Whitney et al., 1999; Apgar et al., 2002), and there are abundant data indicating that increased H_2S causes gastrointestinal epithelium damage (Attene-Ramos et al., 2010), leading to the development of chronic diseases such as ulcerative colitis and colorectal cancer (Deplancke et al., 2000, 2003). Because various corn coproducts may have increased total S (Kerr et al., 2008), which could serve as a substrate for increased H_2S production by sulfate-reducing bacteria (**SRB**), thereby affecting gastrointestinal health and function, 2 experiments were conducted to investigate the impact of inorganic S on pig performance, markers of intestinal inflammation, manure composition, and the presence of SRB.

MATERIALS AND METHODS

All experimental protocols involving animals were approved by the Iowa State University Animal Care and Use Committee.

General

In Exp. 1, the control diet was formulated with dicalcium phosphate and sulfate-based trace minerals, whereas the low-S diet was formulated with defluorinated phosphate and nonsulfate-based trace minerals (Table 1). Each diet was formulated to contain 0.80% Ca, 0.42% available P, 17.6% CP, 3,250 kcal of ME/kg, and 1.12% standardized ileal digestible Lys, with other AA balanced relative to an ideal AA basis. The control and low-S diets were calculated to contain 2,135 and 1,755 mg/kg of total dietary S, respectively. In Exp. 2, the control diet was formulated with monoammonium phosphate and sulfate-based trace minerals, whereas the low-S diet was formulated with defluorinated phosphate and nonsulfate-based trace minerals (Table 2). The control and low-S diets contained 0.74% Ca and 0.49% available P. Because $CaSO_4$ was utilized as the source of supplemental S in the diets with increased S, dietary Ca content across the increased S diets were formulated equivalent to the Ca content at 5.0% $CaSO_4$, 1.54% Ca, with P balanced relative to the calculated Ca:P in the control and low-S diets, equating to 1.10% total P. Calcium sulfate was supplemented at 0, 0.625, 1.25, 2.5, and 5.0% to attain calculated total dietary S of 2,959, 4,014, 5,066, 7,183, and 11,399 mg/kg of diet, respectively. All diets with graded amounts of S were formulated with sulfate-based trace minerals. All diets in Exp. 2 were formulated to 21% CP, 3,325 kcal of ME/kg, and 1.15% standardized ileal Lys, with other AA balanced relative to an ideal AA basis.

Table 1. Composition of experimental diets, Exp. 1 (as-is basis)[1]

Item	Low sulfur	Control
Ingredient, %		
Corn	68.94	68.39
Soybean meal	22.28	22.35
Whey, dried	5.00	5.00
Limestone	0.17	0.80
Defluorinated P[2]	1.99	—
Dicalcium P[3]	—	1.84
Sodium chloride	0.35	0.35
Vitamin mix[4]	0.30	0.30
Trace mineral, low-S[5]	0.15	—
Trace mineral, high-S[6]	—	0.15
Choline chloride, 60%	0.07	0.07
L-Lys·HCl	0.42	0.42
L-Thr	0.13	0.13
L-Trp	0.02	0.02
DL-Met	0.13	0.13
L-Val	0.05	0.05
Total	100.00	100.00
Composition		
Calculated sulfur, mg/kg	1,755	2,135
Analyzed sulfur,[7] mg/kg	2,100	2,300

[1]All diets were formulated to 0.80% Ca, 0.42% available P, 3,250 kcal of ME/kg, 17.6% CP, 1.12% standardized ileal digestible Lys, and balanced for other AA on an ideal AA basis. Diets were analyzed to contain an average of 16.7% CP.

[2]Contains approximately 32% Ca, 18% P (90% available), and 566 mg/kg of total sulfur.

[3]Contains approximately 22% Ca, 18% P (95% available), and 11,050 mg/kg of total sulfur.

[4]Provided the following per kilogram of diet: 6,614 IU of vitamin A, 1,653 IU of vitamin D_3, 33 IU of vitamin E, 0.03 mg of vitamin B_{12}, 10 mg of riboflavin, 50 mg of niacin, and 26 mg of D-pantothenic acid.

[5]Provided the following per kilogram of diet: 10 mg of Cu (sulfate), 150 mg of Fe (sulfate), 0.36 mg of I (ethylenediamine dihydroiodide), 5 mg of Mn (sulfate), 150 mg of Zn (sulfate), and 0.30 mg of Se (Na selenite).

[6]Provided the following per kilogram of diet: 10 mg of Cu (carbonate), 150 mg of Fe (carbonate), 0.36 mg of I (ethylenediamine dihydroiodide), 5 mg of Mn (oxide), 150 mg of Zn (oxide), and 0.30 mg of Se (Na selenite).

[7]Analyzed by thermocombustion to the nearest 100 mg/kg.

Dietary S was measured by thermal combustion (Elementar Analysensysteme GmbH, Hanau, Germany) as described previously (Kerr et al., 2008).

Animal Management

Pigs (Exp. 1: 42 pigs, 13.8 kg initial BW; Exp. 2: 63 pigs, 13.4 kg initial BW) were randomly allotted to individual stainless-steel pens (0.46 × 1.22 m) and allowed free access to feed and water. Pigs and feeders were weighed at the beginning and end of each experiment (Exp. 1, 24 d; Exp. 2, 35 d) to obtain BW gain and feed disappearance, respectively. At the termination of Exp. 2, fresh fecal samples from pigs fed the diets containing 0 or 5% $CaSO_4$ (**low-S** or **high-S**, respectively) were collected, placed on ice, and analyzed within 2 h for pH, reduced S (S^{-2}), and ammonia. An additional sample (5 g) was frozen and processed at a later date for SRB enumeration.

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Table 2. Composition of experimental diets, Exp. 2 (as-is basis)

			Calcium sulfate addition, %				
Item	Low-S	Control	0	0.625	1.25	2.5	5.0
Ingredient, %							
Corn	57.49	56.96	50.04	49.49	48.96	47.91	45.69
Soybean meal	24.50	24.56	25.05	25.10	25.14	25.20	25.39
Whey, dried	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fish meal, select	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Plasma, porcine	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Soy oil	0.15	0.33	2.95	3.15	3.35	3.75	4.60
Limestone	0.14	0.63	2.61	2.28	1.95	1.29	—
Defluorinated P[1]	0.61	—	—	—	—	—	—
Monoammonium P[2]	—	0.41	2.22	2.22	2.22	2.22	2.22
Sodium chloride	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin mix[3]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Trace mineral, low-S[4]	0.15	—	—	—	—	—	—
Trace mineral, high-S[5]	—	0.15	0.15	0.15	0.15	0.15	0.15
Se premix, 0.06% Se	0.05	0.05	0.05	0.05	0.05	0.05	0.05
DL-Methionine	0.01	0.01	0.03	0.03	0.03	0.03	0.03
$CaSO_4$	—	—	—	0.63	1.25	2.50	5.00
Calculated composition[6]							
Ca, %	0.74	0.74	1.54	1.54	1.54	1.54	1.54
P_{total}, %	0.69	0.68	1.10	1.10	1.10	1.10	1.10
$P_{available}$, %	0.40	0.40	0.83	0.83	0.83	0.83	0.83
S, mg/kg	2,440	2,688	2,959	4,014	5,066	7,183	11,399
Analyzed S,[7] mg/kg	2,500	2,500	2,900	3,600	5,000	8,200	12,100

[1]Contains approximately 32% Ca, 18% P (90% available), and 566 mg/kg of total S.

[2]Contains approximately 35% Ca, 24% P (99% available), and 15,000 mg/kg of total S.

[3]Provided the following per kilogram of diet: 6,614 IU of vitamin A, 1,653 IU of vitamin D_3, 33 IU of vitamin E, 0.03 mg of vitamin B_{12}, 10 mg of riboflavin, 50 mg of niacin, and 26 mg of D-pantothenic acid.

[4]Provided the following per kilogram of diet: 10 mg of Cu (sulfate), 150 mg of Fe (sulfate), 0.36 mg of I (ethylenediamine dihydroiodide), 5 mg of Mn (sulfate), and 150 mg of Zn (sulfate).

[5]Provided the following per kilogram of diet: 10 mg of Cu (carbonate), 150 mg of Fe (carbonate), 0.36 mg of I (ethylenediamine dihydroiodide), 5 mg of Mn (oxide), and 150 mg of Zn (oxide).

[6]All diets were formulated to 21% CP, 3,325 kcal of ME/kg, 1.15% standardized ileal digestible Lys, and balanced for other AA on an ideal AA basis. Diets were analyzed to contain an average of 21.4% CP.

[7]Analyzed by thermocombustion to the nearest 100 mg/kg.

Fecal pH was measured by mixing 1 g of feces with 10 mL of water before insertion of a calibrated probe (model 530, probe #476436, Corning Inc., Corning, NY). The S^{-2} probe (model 290A+, probe #9616, Thermo Scientific, Beverly, MA) was calibrated using a standard curve, 5 levels, of sodium sulfide dissolved in water. Because of the instability of the sodium sulfide crystals, the concentrated solution was titrated immediately before use to determine its exact concentration. A control solution and blank were analyzed at the end of each assay to check validity of probe operation. To analyze fecal samples for S^{-2}, 3 g of feces were mixed with 30 mL of water and then 2 g of this was weighed into a 100-mL beaker, after which 38 mL of deaerated nanopure water, 40 mL of SAOB solution (Thermo Scientific), and a stir bar were added. The pH of the mixture was maintained at pH = 12 for the probe to accurately measure S^{-2}. The calibrated probe was inserted into each beaker and the concentration recorded. The NH_3 probe (model 290A+, probe #9512, Thermo Scientific) was calibrated using a standard curve, 3 levels, of ammonium chloride dissolved in water. A control solution and blank were analyzed at the end of each day to check validity of probe operation. To analyze fecal samples, 3 g of feces were mixed with 30 mL of water and then 1 g was weighed into a 100-mL beaker, after which 99 mL of nanopure water and a stir bar were added. During mixing, 2 mL of ISA (Thermo Scientific) was added. The pH of the mixture was maintained at $\geq$11 for the probe to accurately measure ammonia. The probe was inserted into the beaker, and the concentration was recorded.

Tissue Sample Collection

At the end of Exp. 2, after feeding the experimental diets for 35 d, pigs fed the low-S or high-S diets (n = 8/treatment) were killed for the collection of ileum and colon tissue samples, ileum mucosal scrapings, and intestinal contents. Pigs were killed for intestinal tissue, mucosal, and digesta sample collection by penetrating captive bolt followed by exsanguination. Tissue samples were collected from the ileum 15 cm cranial to the ileal-cecal junction and from the colon at the location 15 cm distal to the cecum. After dissection, the tissue samples were rinsed with ice-cold PBS and immediately placed into cryogenic vials and snap-frozen in liquid N. For determination of ileum mucosal enzyme activity,

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the remaining portion of the ileum was opened longitudinally, and the mucosa was scraped with a microscope slide and snap-frozen. All tissue and mucosal samples were stored at −80°C pending analysis.

Ileum and Colon Tissue Protein Extraction and DNA Determination

Snap-frozen samples of ileum and colon tissue were homogenized in a tissue protein extraction reagent (Pierce, Rockford, IL) supplemented with protease (Roche, Indianapolis, IN) and phosphatase (Sigma Chemical Co., St. Louis, MO) inhibitors. The tissue homogenates were centrifuged at 10,000 × g for 5 min at room temperature, and the supernatants were used for Western blot analysis. Intestinal tissue homogenate protein concentrations were determined (Bicinchoninic Acid Assay, Pierce) using BSA as a standard. The DNA concentrations of the intestinal tissue homogenates were determined (Hoechst 33258 dye, Sigma Chemical Co.; Labarca and Paigen, 1980). Calf thymus DNA (Sigma Chemical Co.) was used for the standards for DNA determinations.

Ileal Mucosal Sucrase and Alkaline Phosphatase Activity

Mucosal sucrase and alkaline phosphatase activities were determined using previously detailed methods (Tang et al., 1999) with minor modifications (Weber et al., 2010). Briefly, ileal mucosal samples (0.2 g) were homogenized in 1 mL of ice-cold deionized water and centrifuged at 2,200 × g for 30 min at 4°C. The supernatant was diluted in deionized water 1:25 for sucrase activity and 1:6 for alkaline phosphatase activity. Protein concentrations of the diluted mucosal homogenates were determined (Bicinchoninic Acid Assay, Pierce) using BSA as a standard. Sucrase activity was determined using the sucrose concentration and incubation conditions described by Kidder and Manners (1980). The glucose liberated from sucrose was measured using a hexokinase-based glucose assay kit (GAHK20, Sigma Chemical Co.). Alkaline phosphatase activity was determined using a commercially available kit (A7505, Pointe Scientific, Canton, MI) per the manufacturer's instructions.

Isolation of Total RNA and Real-Time Reverse-Transcription PCR

Total RNA was isolated from ileum and colon tissue samples using Trizol (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's protocol, and the RNA pellets were resuspended in nuclease-free water. To eliminate any genomic DNA contamination, the RNA samples were treated with a DNase I kit (DNA-free, Ambion Inc., Foster City, CA). Total RNA was quantified by measuring the absorbance at 260 nm (NanoDrop ND-100 spectrophotometer, NanoDrop Technologies, Rockland, DE), and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm. All samples had 260:280 nm ratios above 1.8:1. Additionally, the integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 1.2% agarose gels (E-gel, Invitrogen). Total RNA (1 μg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript, BioRad Laboratories, Hercules, CA). The iScript kit used a blend of oligo (dT) and random hexamer primers for cDNA synthesis, and the reverse transcriptase was RNase H^+ to ensure removal of the RNA template.

Real-time PCR detection of the mRNA was conducted utilizing SYBR Green and the primers presented in Table 3. Amplification was carried out in a total volume of 25 μL containing 1× iQ SYBR Green Supermix (BioRad Laboratories), forward and reverse primers (0.1 μg/μL), and 1 μL of the 20-μL cDNA reaction. After an initial 5 min denaturation step at 95°C, the reactions were cycled 40 times under the following conditions: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Optical detection was carried out at 72°C. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. Thermal cycling conditions and real-time detection were conducted using an iQ5 multi-color real-time PCR detection system (Bio-Rad Laboratories). A nontemplate control was run with every assay, and all determinations were performed in duplicate. The presence of a single PCR product of the correct size for each primer set was verified by visualizing the PCR products via electrophoresis on 1% agarose gels stained with ethidium bromide. External cDNA standards were constructed by cloning the corresponding reverse-transcription PCR product into a PCR 4-TOPO vector (Invitrogen), and the resultant plasmids were sequenced at the Iowa State University DNA facility for verification. Serial dilutions of a known amount of plasmid containing the cDNA of interest were included on each 96-well plate. The abundance of each gene product was calculated by regressing against the standard curve generated in the same reaction with their respective plasmids. Expression of the genes of interest was normalized to the mRNA expression of cyclophilin A. The mRNA expression of cyclophilin A was not affected by dietary S.

Western Blot Analysis of Ileum and Colon Tissue

Intestinal tissue protein extracts (50 μg/lane) were fractionated by SDS-PAGE using 4 to 12% bis/Tris gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature with 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (Sigma Chemical Co.). Then the membranes were incubated overnight at 4°C with rabbit antibodies directed

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Table 3. Primers used for real-time reverse-transcription PCR analysis

Gene[1]	Primer sequences (5′ → 3′)[2]	Amplicon length, bp
ICAM-1 (*ICAM1*)	(Sense) AGGAGGCTCCATGAAGGTGA (AS) GAGTGCCATCGTTTTCCACA	149
IGF-I (*IGF1*)	(Sense) GCCTCAGGGCTCAATTCATA (AS) GGTGCCTGACAAGGACGTAT	137
IL-6 (*IL6*)	(Sense) GCCACCTCAGACAAAATGCT (AS) TCTGCCAGTACCTCCTTGCT	143
IL-8 (*IL8*)	(Sense)AACTGAGAGTGATTGAGAGTGGA (AS)GCTGTTGTTGTTGCTTCTCAGTT	136
IL-10 (*IL10*)	(Sense) GCATCCACTTCCCAACCA (AS) CTTCCTCATCTTCATCGTCAT	445
PCNA (*PCNA*)	(Sense) TACGCTAAGGGCAGAAGATAATG (AS) CTGAGATCTCGGCATATACGTG	191
SOCS3 (*SOCS3*)	(Sense) AGATCCCTCTGGTGTTGAGC (AS) CGTTGACTGTTTTCCGACAG	77
TNFα (*TNFA*)	(Sense) CCCAAGGACTCAGATCATCG (AS) ATACCCACTCTGCCATTGGA	101
Cyclophilin A (*PPIA*)	(Sense) GCGTCTCCTTCGAGCTGTT (AS) CCATTATGGCGTGTGAAGTC	160

[1]ICAM-1 = intracellular adhesion molecule 1; PCNA = proliferating cell nuclear antigen; SOCS3 = suppressor of cytokine signaling 3; TNFα = tumor necrosis factor α.
[2]AS = antisense primer.

against nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (**IκBα**; 4812, Cell Signaling Technology, Danvers, MA) or phospho-p44/p42 mitogen-activated protein kinase (**MAPK**) antibody (9101, Cell Signaling Technology) diluted 1:1,000 in blocking solution. After washing with Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with a horseradish peroxidase labeled secondary antibody (Zymed, Invitrogen) diluted 1:5,000 in blocking solution. The reaction complexes were visualized using a chemiluminescent detection kit (Pierce). The membranes were exposed to x-ray film, and the films were scanned and densitometry analysis was conducted (Fluor-S MultiImager, BioRad Laboratories). For normalization purposes, the blots were stripped (Restore Western Blot Stripping Buffer, Pierce) for 15 min at 37°C. Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), which was used to normalize ileum tissue IKBα content, was detected using a mouse anti-GAPDH monoclonal antibody (Y3322, BioChain Institute Inc., Hayward, CA) diluted at 1:2,000 in blocking solution. A rabbit anti-p44/p42 MAPK antibody (9102, Cell Signaling Technology) was used for normalizing ileum tissue phospho-p44/p42 MAPK. The GAPDH and MAPK reaction complexes were visualized as described before.

Microbial Analysis Quantitative PCR

Microbial analysis was performed on digesta samples obtained from the small and colon, and fecal samples obtained from pigs fed the low-S and high-S diets. A 1-mL sample of digesta or feces was mixed with an equal volume of Tris-saturated phenol and 10% (vol/vol) zirconium beads (0.1 mm). Samples were homogenized for 30 s at a speed of 4 m/s in an instrument (FastPrep, Q-BIOgene, Irvine, CA) followed by centrifugation for 10 min at 14,000 × *g* at room temperature. A 600-μL aliquot of the supernatant was then added to 500-μL binding matrix (Fastprep Binding Matrix, Q-BIOgene) and extracted using a kit (FastDNA Spin kit, Q-BIOgene) according to the manufacturer's instructions.

Real-time PCR assays were performed (Rotorgene 6000, Corbett Robotics Inc., San Francisco, CA) using the kit (QuantiTect SYBR Green PCR kit, Qiagen, Valencia, CA). Template DNA (5 μL) was used in a reaction mixture containing 10 μL of mixture (2× Quantitect SYBR Green PCR Mastermix, Qiagen), 500 n*M* each forward and reverse *dsrA* primer: group 1 (*Desulfobulbus*-like) SRB, Grp1fd/Grp1rd; group 2 (*Desulfovibrio*-like) SRB, Grp2fd/Grp2rd; group 3 (*Desulfovibrio*-like) SRB, Grp3fw/Grp3rd (Spence et al., 2008); and H_20 to a final volume of 20 μL. Reaction conditions were 95°C for 15 min (1×), then 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s (40×). A final melt curve analysis was performed to determine the presence or absence of nonspecific amplification products. For quantification of 16S rDNA, 5 μL of template DNA was used in a reaction mixture containing 10 μL of mixture (2× Quantitect Probe PCR Mastermix, Qiagen), 500 n*M* each forward (1055F) and reverse (1392R) primer, 250 n*M* probe (16STaq1115-BHQ), and H_2O to a final volume of 20 μL (Harms et al., 2003). Reaction conditions for quantification of 16S rDNA were 95°C for 15 min (1×), then 95°C for 15 s, and 58°C for 60 s (40×). All samples were run in triplicate, and H_2O replaced template in control reactions. Standard curves for SRB group assays and 16S rDNA were determined as described previously (Spence et al., 2008).

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Table 4. Performance of pigs fed reduced dietary S, Exp. 1[1]

Item	Low S	Control	SEM	P-value[2]
S,[3] mg/kg	1,755	2,135	—	—
S,[4] mg/kg	2,100	2,300	—	—
Observations, n	21	21	—	—
ADG, kg	0.782	0.789	0.024	0.82
ADFI, kg	1.974	1.951	0.028	0.56
G:F	0.395	0.402	0.008	0.54

[1]Average initial and final BW of 13.8 and 32.7 kg, respectively, over the 24-d trial.
[2]Initial BW used as a covariate.
[3]Calculated.
[4]Analyzed.

Statistical Analysis

Data in each experiment were subjected to ANOVA (SAS Inst. Inc., Cary, NC) with means reported as LS-MEANS. In Exp. 2, preplanned contrasts between pigs fed the control and low-S diet, and between pigs fed the control and 0% $CaSO_4$ were tested to determine if low-S or increased Ca and P affected pig performance relative to pigs fed the control diet, respectively. In addition linear, quadratic, and cubic effects on pig performance relative to dietary S were tested using contrast statements. The data obtained from tissue, mucosal, digesta, and fecal samples collected from the animals fed the low-S and high-S diets were analyzed as a single factor ANOVA using Proc GLM of SAS. The pig was considered the experimental unit in all experiments. Differences were considered statistically significant when $P < 0.05$, and treatment trends were discussed when $0.05 < P < 0.10$.

RESULTS AND DISCUSSION

Dietary S Impact on Pig Performance

Reducing dietary S by approximately 200 mg/kg had no effect on pig performance in Exp. 1 (Table 4) or Exp. 2 (Table 5). This was expected because the lack of an effect of decreasing dietary S on pig performance has been noted by others (Whitney et al., 1999; Apgar et al., 2002). Because $CaSO_4$ was used to increase dietary S in Exp. 2, we could not maintain the same amount of dietary Ca as in the control or low-S diets. Consequently, Ca equivalent to the that calculated in the diet containing the 5% $CaSO_4$, 1.54% Ca, was maintained across all increased S diets, with total P maintained according to the same Ca:P ratio as in the control and low-S diets. Although we had concern on the impact of increasing Ca and P on pig performance, the increase in Ca and P had no effect on ADG, ADFI, or G:F (control vs. 0% $CaSO_4$), indicating that responses to dietary S were not confounded by dietary Ca or P. Dietary S amounts were chosen based on expected impacts on pig performance and fecal consistency. Using an estimated 2.5 g of water intake/g of feed intake, dietary content of 0.625, 1.25, 2.5, and 5.0% $CaSO_4$ would have corresponded to water sulfate concentrations of 1,250, 2,500, 5,000, and 10,000 mg/L, respectively. Based on the literature (CAST, 1974; NAS, 1977; NRC, 1998), we would have expected no effect on pig performance with 0.625 or 1.25% $CaSO_4$, a slight depression of pig performance at 2.5% $CaSO_4$, and an additional reduction in pig performance at 5.0% $CaSO_4$. Although there was a linear ($P < 0.01$) decrease in ADG as dietary S increased in Exp. 2, the largest depression was noted in pigs fed the 2.5 and 5.0% $CaSO_4$ (Table 5). We cannot explain the cubic ($P < 0.01$) effect of $CaSO_4$ on ADFI where pigs fed the 0.625 and 5.0% $CaSO_4$ had the least feed intake. Likewise, the cubic ($P < 0.01$) effect of $CaSO_4$ on G:F cannot be explained, where apparently pigs fed the 0.625% $CaSO_4$ had improved G:F and pigs fed the 2.5% $CaSO_4$ had reduced G:F, whereas the G:F of pigs fed 1.25 or 5.0% $CaSO_4$ were unaffected relative to pigs fed the control diet. Most studies have shown no effect of moderate amounts of water sulfates on pig performance (Anderson and Stothers, 1978; Paterson et al., 1979; DeWit et al., 1987; Anderson et al., 1994; Patience et al., 2004). Likewise, Gomez et al. (1995) reported that 2,200 mg of inorganic sulfate/L water, relating to approximately 1.2% $CaSO_4$ in the feed, did not affect growth rate, even though nonpathogenic diarrhea was persistent. Although Canh et al. (1998) reported that dietary addition of 1.2 or 2.4% $CaSO_4$ reduced urinary and manure pH, they did not report animal performance changes. In contrast, Maenz et al. (1994) reported that 2,560 mg of sulfate/L water resulted in improved growth rates in 6.6-kg pigs fed for 10 d. The fact that pig performance was not largely affected by up to 10,000 mg/kg of diet in Exp. 2 is remarkable given that 4,000 mg/kg is considered toxic in ruminants (Kandylis, 1984).

It has been suggested that sulfate is poorly absorbed in the intestine leading to increased concentrations in the lower bowel and, consequently, diarrhea (McLeese et al., 1992). Others, however, have reported a parallel increase in urinary sulfate excretion with increased S consumption, indicating near complete absorption of S (Berry et al., 1969; Krijgsheld et al., 1979; Florin et al., 1991; Cole and Evrovski, 2000). Although we did not measure urinary sulfate or total S balance, we did find an increase ($P < 0.01$) in fecal sulfide content from pigs fed the high-S relative to pigs fed the low-S diet (Table 6), and even though we did not objectively

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Table 5. Performance of pigs fed reduced and increased dietary sulfur, Exp. 2[1]

Item	Low-S[2]	Control[2]	Calcium sulfate,[3] % 0	0.625	1.25	2.5	5.0	SEM	*P*-value[4]
S,[5] mg/kg	2,440	2,688	2,959	4,014	5,066	7,182	11,399	—	—
S,[6] mg/kg	2,500	2,500	2,900	3,600	5,000	8,200	12,100	—	—
Observations, n	6	9	8	9	8	7	8	—	—
ADG,[7,8] kg	0.729	0.738	0.765	0.723	0.736	0.697	0.697	0.019	0.18
ADFI,[7,9] kg	1.228	1.225	1.252	1.164	1.230	1.231	1.146	0.032	0.19
G:F[7,10]	0.595	0.604	0.611	0.626	0.609	0.567	0.608	0.010	0.01

[1]Average initial and final BW of 13.4 and 38.8 kg, respectively, over the 35-d trial.
[2]Diets formulated to 0.74% Ca and 0.40% P.
[3]Diets formulated to 1.54% Ca and 0.83% P.
[4]*P*-value of diet effect when all diets included in the model and initial BW used as a covariate.
[5]Calculated.
[6]Analyzed.
[7]Preplanned comparisons between low-S, control, and 0% $CaSO_4$ diets were not statistically significant ($P \geq 0.22$, 0.53, and 0.29 for ADG, ADFI, and G:F, respectively).
[8]Linear, quadratic, and cubic effects of graded levels of $CaSO_4$ on ADG were $P = 0.01$, 0.65, and 0.72, respectively.
[9]Linear, quadratic, and cubic effects of graded amounts of $CaSO_4$ on ADFI were $P = 0.08$, 0.80, and 0.01, respectively.
[10]Linear, quadratic, and cubic effects of graded amounts of $CaSO_4$ on G:F were $P = 0.05$, 0.52, and 0.01, respectively.

determine stool consistency, pigs fed the 5% $CaSO_4$ appeared to have stools that were less firm than pigs fed the 0% $CaSO_4$. Based on the data of Berry et al. (1969), Krijgsheld et al. (1979), Florin et al. (1991), and Cole and Evrovski (2000), the observed increase in intestinal S is likely not due to the lack of absorption, but due to the greater sulfate concentration irritating the intestinal mucosal causing an accelerated transit of intestinal contents, resulting in stool looseness or diarrhea (Krijgsheld et al., 1979). In contrast, McLeese et al. (1992) and Maenz et al. (1994) reported no evidence of intestinal mucosal damage in pigs consuming drinking water containing 2,650 mg of sulfate/L of water for 10 d. Furthermore, sulfate supplementation of drinking water has been shown not to increase intestinal sulfate or H_2S concentrations, indicating that inorganic S is not an important modulator of intestinal H_2S concentrations, although it did alter the bacterial profiles of the stomach and distal colon of 1-yr-old mice (Deplancke et al., 2003). Pigs fed the high-S diet also had feces with lower pH ($P < 0.01$) and a tendency for decreased ammonia compared with pigs fed the low-S diet ($P = 0.08$, Table 6). This is supported by Mroz et al. (2000) who reported that addition of $CaSO_4$ reduced urine and manure pH in addition to decreased ammonia emission (Canh et al., 1998).

One of the concerns with distillers dried grains with solubles (**DDGS**) is that DDGS contain relatively high content of S (Kerr et al., 2008). Regardless of whether the dietary S from sulfate is not absorbed, or if absorbed it is not utilized, increased S will ultimately end up in manure. With S-containing compounds being key odorants in livestock manure (Le et al., 2005), the minimization of manure S could be important in odor arising from animal production facilities. In Exp. 2, fecal sulfide concentrations were greatly increased by feeding the high-S diet, indicating that increased amounts of dietary S ended up in the manure. In swine (Le et al., 2007) and poultry (Chavez et al., 2004), increasing dietary methionine (as a source of dietary S) above the requirement has been shown to affect odorous emissions. Interestingly, however, Gralapp et al. (2002) and Spiehs (2001) noted no impact of feeding DDGS on odor dilution threshold. This lack of an effect in their data, however, may have been due to the limited storage length of manure, which has been shown to have an impact on manure composition and odor intensity (Trabue et al., 2008; Ziemer et al., 2009)

Table 6. Fecal composition of pigs fed normal and high dietary sulfur, Exp. 2[1]

Item	Calcium sulfate, % 0	5	SEM	*P*-value
S,[2] mg/kg	2,959	11,399	—	—
S,[3] mg/kg	2,900	12,100	—	—
Observations, n	9	7	—	—
pH	7.04	6.38	0.15	0.01
S, μmol/g	0.96	2.86	0.32	0.01
NH_4, μmol/g	81.9	45.9	13.3	0.08

[1]Fecal samples obtained after pigs consumed their respective diet for 35 d, average final BW of 38.8 kg.
[2]Calculated.
[3]Analyzed.

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Table 7. Effect of increased dietary inorganic sulfate on the relative abundance of cytokine, intracellular adhesion molecule 1 (ICAM-1), proliferating cell nuclear antigen (PCNA), suppressor of cytokine signaling-3 (SOCS3) mRNA in ileum and colon tissue

	Calcium sulfate,[1] %			
Item	0	5	SE	*P*-value
Ileum				
ICAM-1	0.025	0.065	0.011	0.03
IL-6	0.065	0.126	0.023	0.09
IL-8	0.315	0.893	0.329	0.24
IL-10	0.081	0.114	0.038	0.55
TNFα[2]	0.054	0.188	0.033	0.01
PCNA	0.620	0.495	0.231	0.69
SOCS3	0.216	1.263	0.331	0.04
Colon				
ICAM-1	0.031	0.016	0.006	0.11
IL-6	0.074	0.044	0.012	0.10
IL-8	0.324	0.197	0.107	0.43
TNFα	0.089	0.032	0.020	0.17
PCNA	0.224	0.257	0.047	0.63

[1]Pigs (n = 8/treatment) were fed diets containing 0 or 5% added inorganic sulfate in the form of $CaSO_4$. The mRNA abundance is normalized to the mRNA expression of cyclophilin A.

[2]TNFα = tumor necrosis factor α.

or the smaller amount of DDGS supplemented in the diet. Feather meal, which contains increased content of S-AA, and consequently S (NRC, 1998; Kerr et al., 2008), has been shown to affect odorous compounds in fresh feces (van Heugten and van Kempen, 2002), but these authors failed to measure S-containing odorants, intestinal health variables, or microbial ecology. With the relationship between urinary and fecal N excretion and ammonia emissions being well established (Latimer et al., 1993; von Pfeiffer, 1993), one could speculate that a reduction in S excretion would also decrease S-containing odorants.

Intestinal Inflammatory Measures and Enzyme Activities

In ileum tissue, the mRNA expression of intracellular adhesion molecule 1 (**ICAM-1**) and tumor necrosis factor α (**TNFα**) were increased ($P < 0.05$), and the expression of IL-6 mRNA tended ($P = 0.09$) to be increased in pigs fed the high-S diet as compared with pigs fed the low-S diet (Table 7). Feeding the high-S diet led to a marked increase (approximately 5-fold; $P < 0.05$) in ileal suppressor of cytokine signaling 3 (**SOCS3**) mRNA expression, but had no effect on the expression of IL-8 or IL-10 mRNA. In colon tissue, expression of ICAM-1, IL-8, or TNFα mRNA did not differ between pigs fed the high-S or the low-S diet, but there was a trend for decreased ($P < 0.10$) IL-6 mRNA in colon tissue from pigs fed the high-S diet. In ileum tissue from pigs fed the high-S diet, the abundance of IkBα protein was decreased ($P < 0.05$) and the activation of p44/42 MAPK was increased ($P < 0.05$) as compared with pigs fed the low-S diet (Figure 1). The increased ileal mRNA abundance of ICAM-1 and TNFα in conjunction with decreased IkBα and increased MAPK activation may be due to increased local H_2S concentrations in the ileum in pigs fed the high-S diet. Increasing dietary sulfate provides increased substrate for H_2S production by SRB that reside in the ileum (Table 8). Hydrogen sulfide directly induces the expression of inflammatory cytokines and activates MAPK in human U937 monocytes (Zhi et al., 2007). Likewise, the pharmacological inhibition of endogenous H_2S synthesis decreased the production of inflammatory cytokines in liver and lung tissue in a mouse cecal ligation and puncture sepsis model (Zhang et al., 2007). Recently, it was observed that exposing a human small intestinal cell line, FHs 74, to elevated H_2S increases the expression of the inflammation-related gene, cyclooxygenase-2 (Attene-Ramos et al., 2010). Our data further support the notion that increased H_2S in the small intestine, in response to increased dietary inorganic S levels, may upregulate genes involved in inflammation.

It is interesting that upregulation of inflammation related genes was only observed in ileal tissue of pigs fed the high-S diet, whereas there was only a trend ($P = 0.10$) toward decreased IL-6 mRNA abundance in colon tissue. Differences in cellular populations between ileum and colon tissue may account for the differential responses in terms of inflammatory markers. It should be noted that in vitro studies evaluating the impact of H_2S on expression of inflammation-related genes utilized cell lines derived from the small intestine (Deplancke and Gaskins, 2003; Attene-Ramos et al., 2010). Therefore, direct effects of H_2S on inflammation-related genes and pathways in colonocytes remains unknown. In certain circumstances, it has been observed that H_2S has anti-inflammatory properties (Wallace, 2007). This differential response may be due to differences in H_2S

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concentrations between the ileum and colon because physiological concentrations of H_2S produce anti-inflammatory effects and greater concentrations induce inflammation (Wallace, 2007). One may speculate that H_2S concentrations are greater in the colon than ileum based on the increased estimates of SRB in the colon contents as compared with ileal contents (Table 8). However, intestinal H_2S was not measured in the present study; therefore, further research is needed to validate this assumption. Also, further studies are needed to characterize differences in H_2S production in various intestinal segments in response to dietary factors.

An alternative explanation for alterations in ileal inflammatory measures could be changes in the intestinal microbiota induced by the high-S diet. From the bacterial PCR analysis (Table 8), it was observed that feeding the high-S diet decreased estimated total bacteria in the ileum. It is becoming apparent that the composition of the intestinal microflora regulates intestinal inflammatory pathways. For example, certain commensal bacteria in the intestine antagonize NF-κB signaling (Kelly et al., 2004). Likewise, the production of polysaccharide A by the commensal bacteria, *Bacteriodes fragilis*, is associated with a downregulation of inflammatory pathways (Mazmanian et al., 2008). Therefore, it is possible that the high-S diet decreased the abundance of commensal bacteria with anti-inflammatory characteristics.

As an indicator of cellular proliferation, the abundance of proliferating cell nuclear antigen (**PCNA**) mRNA in ileum and colon tissue was also measured (Willing and Van Kessel, 2007). There was no difference in PCNA mRNA in ileum or colon tissue in pigs fed different amounts of S, but the DNA content in ileum tissue of pigs fed the high-S diet tended ($P = 0.10$) to be greater than ileum tissue DNA content of pigs fed the low-S diet(Table 9). Colon tissue DNA concentration or the protein content of ileum or colon tissue was affected by the high-S diet. Ileal mucosal sucrase or alkaline phosphatase activities were not affected by the high dietary S. Intestinal inflammation has been shown to increase intestinal crypt cell hyperplasia (MacDonald, 1992) and would presumably lead to an increase in intestinal tissue DNA content. This along with the lack of an effect on ileal enzyme activities is interesting given the increased mRNA abundance of ICAM-1 and TNFα and activation of inflammatory pathways. One explanation for the lack of an effect of the high-S diet on PCNA mRNA abundance, intestinal enzyme activity, or overt effects on animal growth may be the increased SOCS3 mRNA abundance in ileal tissue of pigs fed the high-S diet. Some studies have shown that SOCS3 works to restrain cytokine activation of inflammatory pathways in the intestine. For example, overexpression of SOCS3 in intestinal epithelial cells blocked the IL-6-induced activation of NF-κB (Wang et al., 2003). In addition, SOCS3 overexpression inhibited proliferation and IL-6 and TNFα induction of STAT3 and NF-κB, respectively, in nontransformed intestinal epithelial cells (Rigby et al., 2007). Therefore, an increase in SOCS3 mRNA in the ileum of pigs fed high-S likely counteracts the inflammatory activity of increased cytokines and minimizes cellular proliferation.

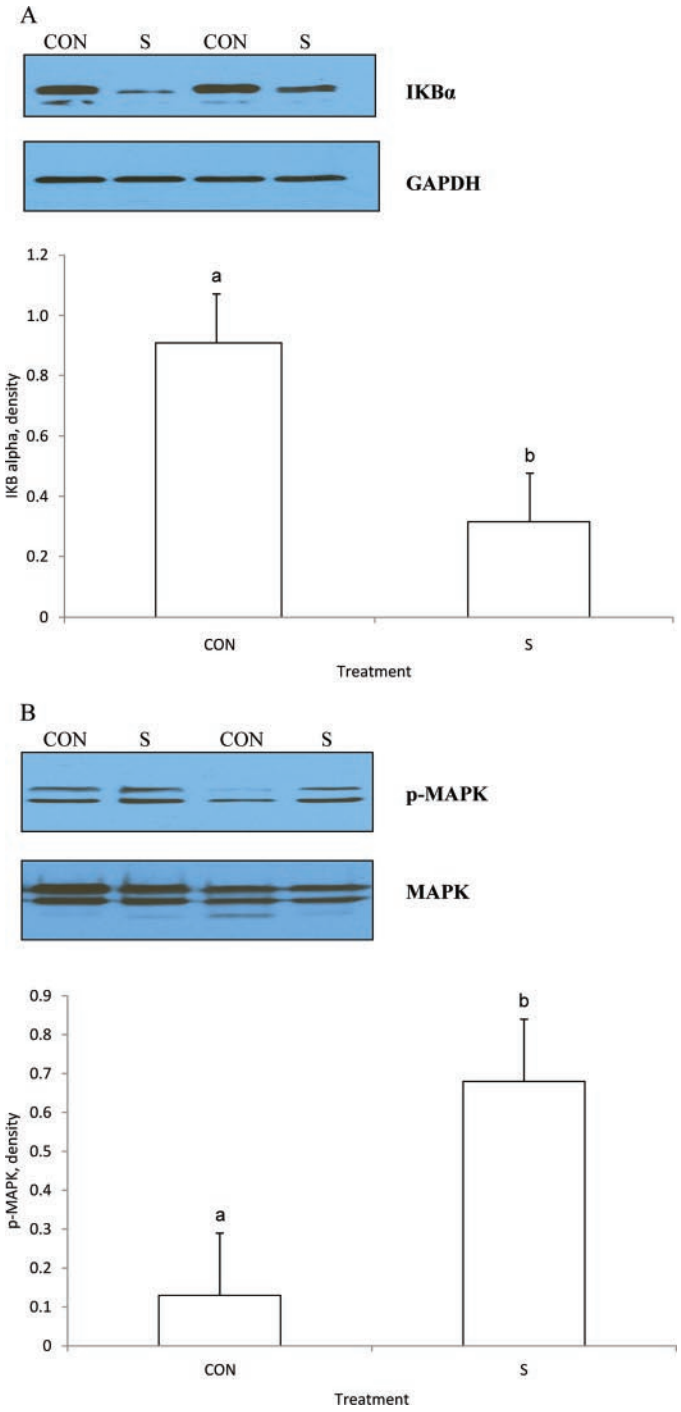

Figure 1. Representative Western blots of the effect of high dietary inorganic sulfate on (A) the abundance of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (IKBα) and (B) activation of p44/42 mitogen-activated protein kinase (MAPK) in ileum tissue from pigs (n = 8 pigs/treatment group) fed the control diet (CON) or 5% $CaSO_4$ (S). For density analysis, tissue IKBα content was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phospho-MAPK was normalized to total MAPK. [a,b]Means with different letters are different ($P < 0.05$). Color version available in the online PDF.

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Table 8. Microbial evaluation of pigs fed normal and high dietary sulfur, Exp. 2[1]

Item[2]	Calcium sulfate, % 0	5.0	SEM	*P*-value
S,[3] mg/kg	2,959	11,399	—	—
S,[4] mg/kg	2,900	12,100	—	—
Small intestine				
SRB group 1	1,506	985	1,273	0.78
SRB group 2	7,811	3,476	2,706	0.28
SRB group 3	319	70	116	0.16
Total bacteria[5]	9.6×10^9	4.2×10^7	3.0×10^9	0.05
Colon				
SRB group 1	10,295	15,149	3,897	0.40
SRB group 2	50,228	200,482	34,626	0.01
SRB group 3	24,461	46,660	12,303	0.23
Total bacteria[5]	24.3×10^9	17.1×10^9	5.1×10^9	0.34
Feces				
SRB group 1	2,201	2,327	440	0.84
SRB group 2	340	805	219	0.16
SRB group 3	390,817	1,781,397	783,805	0.24
Total bacteria[5]	57.0×10^9	37.5×10^9	12.8×10^9	0.30

[1]Samples obtained after pigs consumed their respective diet for 35 d, average final BW of 38.8 kg. There were 6 samples obtained per dietary treatment from individually fed pigs. Data were expressed as copies per gram of material.

[2]Sulfate-reducing bacteria (SRB): group 1, *Desulfobulbus*-like SRB; group 2, *Desulfovibrio*-like SRB; group 3, *Desulfovibrio*-like SRB.

[3]Calculated.

[4]Analyzed.

[5]Total bacteria, copies of 16S rRNA genes.

Microbial Analysis

The predominant SRB found in the gastrointestinal tract belong to the genera *Desulfovibrio*, *Desulfobacter*, *Desulfomonas*, *Desulfobulbus*, and *Desulfotomaculum* (Gibson, 1990), with the *Desulfovibrio* and *Desulfobulbus* accounting for the majority of the colonic SRB (Gibson et al., 1993). Changes in the abundance of SRB and total bacteria were investigated using quantitative real-time PCR for enumeration of their *dsrA* and 16S rRNA genes, respectively. In a previous study designed to identify specific microbial groups responsible for odor production, the conserved *dsr* gene of SRB was cloned and sequenced from stored swine manure (Cook et al., 2008). Phylogenetic analysis of cloned *dsr* genes identified 3 major groups of SRB in swine manure with similarity to *Desulfobulbus* and *Desulfovibrio*-like species. Group 1 (*Desulfobulbus*-like) *dsr* sequences grouped most closely to *Desulfobulbus propionicus* (AF218452) and *Desulfobulbus elongatus* (AF418202). Group 2 (*Desulfovibrio*-like) *dsr* sequences did not group closely with known sequences, but did fall within the *Desulfovibrio* lineage. Group 3 (*Desulfovibrio*-like) *dsr* sequences were obtained from enrichment culture isolates and grouped most closely to *Desulfovibrio desulfuricans* (AF273034) and *Desulfovibrio intestinalis* (AF418183; Cook et al., 2008). Based on sequence alignments of cloned *dsrA* genes, degenerate PCR primer sets were designed for use in SYBR Green quantitative real-time PCR assays to specifically target and enumerate the 3 major groups of SRB identified in stored swine manure (Spence et al., 2008). In the current study, these group-specific primer

Table 9. Effect of increased dietary inorganic sulfate on intestinal tissue DNA and protein content, ileal sucrase, and alkaline phosphatase (ALP) activity

Item	Calcium sulfate,[1] % 0	5	SE	*P*-value
Ileum				
DNA, mg/g	0.37	0.46	0.04	0.10
Protein, mg/g	47.1	54.2	3.4	0.17
Sucrase activity, $\mu mol \cdot min^{-1} \cdot g$ of protein^{-1}	149.8	132.6	22.1	0.59
ALP activity, $mmol \cdot min^{-1} \cdot mg$ of protein^{-1}	25.1	24.1	2.8	0.80
Colon				
DNA, mg/g	0.70	0.63	0.11	0.64
Protein, mg/g	31.8	35.5	2.7	0.35

[1]Dietary inorganic S was increased by the addition of 5% $CaSO_4$ (n = 8 pigs/treatment).

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sets were used to enumerate the SRB population of intestinal and fecal samples in response to increased dietary S (Table 8). It was assumed that the SRB contained only 1 copy of *dsrA* (Klein et al., 2001).

In the small intestine, group 2 SRB were the dominant population of sulfate reducers, composing 81 and 77% in pigs fed the low-S or high-S diet, respectively. In the colon of pigs fed the low-S diet, the group 2 SRB accounted for 59% of the total SRB population, increasing to 77% of the total SRB population in pigs fed the high-S diet. In contrast, analysis of feces indicated the group 3 SRB were by far the dominant group of sulfate reducers in these samples, accounting for 99% of the total SRB population in feces of pigs fed both diets. Numerically, pigs fed greater amounts of dietary S had decreased SRB populations in the small intestine, but the opposite was noted in the colon and feces. Total bacterial population of the colon and fecal samples did not seem to be markedly affected by a change in dietary S. With the large variation in SRB and total bacterial populations noted between pigs, the only statistically significant changes were in a reduction in total bacteria in the small intestine ($P < 0.05$) and an increase in SRB group 2 in the colon ($P < 0.01$) in pigs fed the high-S diet (Table 8). As expected, there was an increase in bacteria concentrations (both SRB and total) in the progression from the small intestine, to the colon, and ultimately the feces. Taken together, the data indicate that growing pigs are quite tolerant of increased dietary inorganic S relative to growth performance, even though extremely high dietary S can alter inflammatory mediators and intestinal bacteria.

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