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Effects of dietary humic and butyric acid on growth performance and response to lipopolysaccharide in young pigs[1]

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ABSTRACT: Humic acid (MFG) and fat-protected butyric acid (BA) has been shown to modulate energy metabolism and inflammation. Therefore, the objectives of this study were to determine the effects of MFG and BA, alone and in combination, on growth performance and response to lipopolysaccharide (LPS)-induced inflammation in young pigs. An experiment was conducted using 448 crossbred weanling pigs, which were stratified by gender and BW and were randomly assigned to 1 of 4 dietary treatments in a 2 × 2 factorial arrangement consisting of control and MFG with or without BA. The pigs were housed at a density of 8 pigs/pen and with 14 pens/dietary treatment. Growth performance and feed intake were assessed for 35 d. To assess the inflammation-related properties of MFG and BA, on d 36 a subset of 48 pigs from each treatment was intramuscular injected with either sterile saline or *Escherichia coli* LPS (20 μg/kg BW; *E. coli* serotype O55:B5) for 4 h in a 2 × 2 × 2 factorial arrangement (±LPS, ±MFG and ±BA; $n = 6$ pigs/treatment group) to assess their febrile response as well as serum, liver, and muscle cytokine responses. Results from this study showed that neither BA nor MFG alone or in combination altered pig ADG, ADFI, and G:F. Moreover, in the presence of LPS, the combination of MFG and BA resulted in a 62% decrease ($P = 0.08$) in serum cortisol compared to when neither compound was added to the diet. In contrast, serum IGF-I was increased ($P < 0.01$) by 59% from the use of both MFG and BA, as opposed to when neither was added, with pigs subjected to LPS. However, both MFG and BA inclusion appear to have a complex role in modulating different aspects of the immune response to LPS, particularly when both are fed in combination. Humic acid also appeared to play a role in decreasing oxidative stress.

Key words: humic acid, inflammation, pig, protected butyric acid

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INTRODUCTION

Dietary humic acid has been shown to increase ADG and G:F in young pigs (Ji et al., 2006; Wang et al., 2008), but the mechanisms responsible for improved growth performance are unknown. It is possible that humic acid improves growth performance by decreasing the activation of inflammation-related pathways, which are generally catabolic in nature (Gabler and Spurlock, 2008). Indeed, studies in some species suggest that humic acid alters inflammatory processes. In broilers, humic acid has been shown to decrease blood heterophil counts and the heterophil:lymphocyte (Rath et al., 2006), while in rats, oral administration of potassium humate has been shown to decrease carrageenan-induced paw edema (Naude et al., 2010) and leonardite humate attenuates the magnitude of the delayed-type hypersensitivity response (Van Rensburg et al., 2007). Mechanistically, humic acid directly suppresses the activation of nuclear factor-kappa B (**NF-κB**) by *Escherichia coli* lipopolysaccharide (**LPS**) in human umbilical cord endothelial cells by preventing the degradation of its inhibitor, **IκBα** (Gau et al., 2000).

Another dietary additive that regulates performance and inflammation is the short chain fatty acid butyric

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acid (**BA**). Recently, Lu et al. (2012) reported that butyrate supplementation to gestating sows and piglets enhanced postweaning growth performance, which was suggested to be mediated by increased substrate oxidation. Additionally, dietary butyrate has been shown to reduce liver steatosis and inflammation in animals (Mattace Raso et al., 2013) and suppress inflammatory responses in numerous cell types (Weber and Kerr, 2006; Ohira et al., 2013). Therefore, the objectives of this study were to determine the effects of humic acid and BA, alone and in combination, on growth performance and response to LPS-induced inflammatory stimuli in young pigs.

MATERIALS AND METHODS

All animal care and handling procedures used in this study were reviewed and approved by the Iowa State University Animal Care and Use Committee.

Animals and Experimental Design

An experiment using 448 crossbred (Pig Improvement Company males and Newsham females) weanling (20 d of age) pigs was conducted to determine the effects of dietary humic acid (MFG; Kent Nutrition Group, Muscatine, IA) and fat-protected BA (Nutri-Ad, Inc., Elgin, IL) on growth performance and response to LPS. The MFG is derived from Menefee Humate. The MFG is a natural occurring mined mineral material derived exclusively from the Menefee geological formation in New Mexico. It consists of highly compressed and biodegraded fresh water carbon (subbituminous coal), trace minerals, silicon dioxide, humic acid (50%), and fulvic acid (4%). The fat-protected BA contained 21% BA. Pigs were stratified by gender and BW at weaning and were randomly assigned to 1 of 4 dietary treatments in a 2 × 2 factorial arrangement consisting of control and MFG with or without BA. The pigs were housed at a density of 8 pigs/pen with 14 pens/dietary treatments. Pigs were allowed ad libitum access to feed and water with pig performance evaluated for 35 d. The diets (Table 1) were fed in 3 phases and met or exceeded the nutritional requirements for young pigs (NRC, 1998). Pig BW and ADFI were determined when dietary phases were changed and at the completion of the study.

To determine the dietary effects of MFG and BA on an inflammatory response, at the completion of the growth portion of the study a subset of 48 pigs from each treatment were either injected intramuscular with sterile saline or with *E. coli* LPS (20 μg/kg BW; *E. coli* serotype O55:B5; Sigma Chemical Co., St. Louis, MO) in a 2 × 2 × 2 factorial arrangement (±LPS, ±MFG and ±BA; n = 6 pigs/treatment group). Pigs did not have access to feed during the course of the 4-h LPS challenge. Four hours after LPS or saline injection, rectal temperatures were recorded, blood samples (10 mL) for serum were obtained, and pigs were sacrificed using a penetrating captive bolt gun and exsanguination. A sampling time of 4 h was chosen because previous studies have found that LPS decreased the relative abundance of IGF-I mRNA in LM at 4 h after LPS challenge (Spurlock et al., 1998; Weber and Kerr, 2008). Likewise, elevated levels of tissue (Brix-Christensen et al., 2005) and circulating (Wright et al., 2000) cytokines have been found in pigs at 4 h after LPS injection. Liver and longissimus dorsi

Table 1. Composition of experimental diets (as-fed basis)

Item	Phase 1 (d 0 to 10)	Phase 2 (d 10 to 20)	Phase 3 (d 20 to 35)
Ingredient, %			
Corn	40.29–40.76	52.82–53.18	60.66–60.96
Soybean meal	29.08	28.34	32.68
Whey	20.00	10.00	–
Fish meal	4.00	4.00	–
Soybean oil	1.75	1.25	2.00
Monocalcium phosphate	1.10	1.41	1.86
Spray-dried plasma	1.00	–	–
Calcium carbonate	0.57	0.73	1.15
Zinc oxide	0.43	–	–
Sodium chloride	0.32	0.42	0.50
Hydrolyzed brewers dried yeast, Y600[1]	0.30	–	–
Vitamin–trace mineral premix[2]	0.17	0.17	0.17
Humic acid[3]	0.00–0.25	0.00–0.25	0.00–0.25
Butyric acid[4]	0.00–0.22	0.00–0.11	0.00–0.06
L-Lys HCl	0.18	0.19	0.32
DL-Met	0.15	0.10	0.14
Copper sulfate	0.08	0.08	0.08
L-Thr	0.05	0.06	0.11
Flavoring agent[5]	0.05	0.05	0.03
Antioxidant[6]	0.02	0.02	0.02
Calculated composition, %			
CP	22.5	21.0	20.0
Crude fat	6.90	5.65	5.34
Lys	1.55	1.40	1.35
Thr	0.99	0.89	0.85
Trp	0.28	0.25	0.24
Ca	0.91	0.91	0.86
P	0.80	0.80	0.75

[1]Grain Processing Corporation, Muscatine, IA.

[2]Provided per kilogram in diet: retinyl acetate, 108 μg; cholecalciferol, 1.16 μg; DL-α-tocopheryl acetate, 2.0 mg; vitamin K, 5.3 mg; vitamin B_{12}, 0.044 mg; riboflavin, 13.6 mg; D-pantothenic acid, 40 mg; niacin, 80 mg; folic acid, 1.1 mg; biotin, 0.18 mg; pyridoxine, 3.4 mg; choline chloride, 5 mg; selenium (selenium yeast), 0.30 mg; iodine (calcium iodate), 1.77 mg; copper (copper sulfate), 16 mg; manganese (manganese sulfate), 34 mg; iron (ferrous sulfate), 134 mg; and zinc (zinc sulfate), 137 mg.

[3]Humic acid used was MFG (Kent Nutrition Group, Muscatine, IA).

[4]Butyric acid used was Ultramix C (Nutriad Inc., Elgin, IL).

[5]Kent PPE Flavor (Kent Nutrition Group, Muscatine, IA).

[6]Endox (Kemin Industries, Des Moines, IA).

samples were collected, rinsed with sterile PBS, snap-frozen in liquid nitrogen, and stored at –80°C pending analysis. Serum samples were stored at –80°C until analyzed for cytokines and metabolites.

Serum Cytokines, Inflammatory Measures, and Metabolites

Serum concentrations of IL-6 were measured by using a porcine-specific ELISA kit (R & D Systems, Minneapolis, MN), and measurements were conducted according to the manufacturer's recommendations. The limit of detection for the IL-6 ELISA was 10 pg/mL and the intra- and interassay CV were 2.9 and 8.5%, respectively. When the concentrations of IL-6 were below the limit of detection for the ELISA, the limit of detection was used as the concentration for data analysis. Serum IGF-I concentrations were determined in duplicates by using a commercially available kit (Active IGF-I ELISA; Diagnostic Systems Laboratories Inc., Webster, TX).

Serum samples were pretreated with the solutions provided with the kit to separate IGF-I from binding proteins. The samples were run in duplicate using the manufacturer's protocol. The assay was validated for porcine plasma by spiking a pooled porcine plasma sample with known quantities of standard and by serial dilution of the pooled plasma sample. Based on 2 assays, the intra- and interassay CV were less than 9%. The percentage recovery from pooled porcine serum was 99%, and the assay sensitivity was 0.03 ng/mL.

Serum cortisol was determined by using a commercially available kit (Active Cortisol EIA; Diagnostic Systems Laboratories Inc.) that had been validated previously for porcine serum (Weber and Spurlock, 2004). The cortisol ELISA had a limit of detection of 1 ng/mL and intra-and interassay CV of less than 12%. Serum triglycerides were measured using an enzymatic kit (T7531; Pointe Scientific, Lincoln Park, MI). The intra- and interassay CV for the triglyceride assay were 1.0 and 2.9%, respectively.

Serum glucose concentrations were determined by using a kit (GAHK20; Sigma Chemical Co.) based on hexokinase activity as describe by the manufacturer. Glutathione concentrations in serum were measured according to the protocol described by Hu (1994). Briefly, 100 μL of serum was deproteinated with 10% trichloroacetic acid. To 170 μL of 0.1 *M* sodium phosphate (5 m*M* EDTA, pH 8.0) in a black 96-well microplate (Greiner Bio-One, Santa Clara, CA), 20 μL of the deproteinated serum was added. Next, 10 μL of 1 mg/mL *o*-phthalaldehyde in absolute methanol was added to each well. After 15 min the fluorescence at 350 nm excitation and 420 nm emission was read using a fluorescent plate reader. The concentration of glutathione was determined using a standard curve of known concentrations of glutathione dissolved in sterile water.

Serum glutathione peroxidase activity and thiobarbituric acid reactive substances (**TBARS**) were determined using kits (Cayman Chemical, Ann Arbor, MI). For the glutathione peroxidase assay, the serum samples were diluted 1:20 in the sample buffer provided with the kit (Toepfer-Berg et al., 2004). Serum haptoglobin was determined using a colorimetric assay kit (Phase Haptoglobin Assay; Tridelta, Kildare, UK) previously used in pigs (LeFloc'h et al., 2008).

Liver and Muscle 4-Hydroxynonenal Protein Adducts

Liver and longissimus dorsi muscle proteins were extracted using T-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with protease inhibitor tablets (Roche, Indianapolis, IN) and 12 m*M* butylated hydroxytoluol. Following homogenization, the lysates were centrifuged at 10,000 × *g* for 5 min at 4°C and the resulting supernatant was assayed for protein concentration using the bicinchoninic acid assay (Pierce) method, and this was then stored at –80°C until used for slot blot analysis. For the determination of tissue and serum 4-hydroxynonenal (**4-HNE**) adducts, slot blot analysis was performed according to previously published procedures used to evaluate the effect of inflammation on tissue 4-HNE adduct levels (Yin et al., 2009). The protein lysates (15 μg) were blotted onto nitrocellulose membranes in duplicate using a slot blot apparatus. The blots were stained with Ponceau S to visualize protein transfer, and the membranes were incubated overnight at 4°C with the 4-HNE goat anti-rabbit antibody (AB5605; Millipore, Temecula, CA) at a dilution of 1:5,000. A goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce) and a chemiluminescent detection kit was used along with a Kodak Image Pro 4000 mm imaging system and software (Molecular Imaging Systems, Rochester, NY) to visualize and quantify the reaction complexes.

Ribonucleic Acid Isolation and Real-Time-PCR

Total RNA was isolated from liver and LM samples using Trizol (Invitrogen, Inc., Carlsbad, CA) reagent according to the manufacturer's protocol, and the RNA was resuspended in nuclease-free water. To eliminate any genomic DNA contamination, the RNA samples were treated with a deoxyribonuclease I kit (DNA-free; Ambion, Inc., Austin, TX) per the manufacturer's instructions. Total RNA was quantified by measuring the absorbance at 260 nm using a spectrophotometer (ND-100; NanoDrop Technologies, Rockland, DE) and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm (NanoDrop Technologies). All samples had 260:280 nm above 1.8. Additionally, the integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal

Table 2. Primers used for real-time PCR

Transcript[1]	Primer sequences (5′ → 3′)[2]	Amplicon size, bp	Accession number
GAPDH	(S) GTCTGGAGAAACCTGCCAAA (AS) CCCTGTTGCTGTAGCCAAAT	228	AF017079
IGF-I	(S) TTCGCATCTCTTCTACTTGGCCCT (AS) CGTACCCTGTGGGCTTGTTGAAAT	152	NM_214256
IL-1β	(S) CCTCCTCCCAGGCCTTCTGT (AS) GGGCCAGCCAGCACTAGAGA	178	M86725
IL-6	(S) GCCACCTCAGACAAAATGCT (AS) TCTGCCAGTACCTCCTTGCT	143	NM_214399
LPS-BP	(S) ATGAGTTCCACAGCCTGGAC (AS) GAGTCGGAGATGGCCAAATA	97	NM_001128435
SOCS3	(S) AGATCCCTCTGGTGTTGAGC (AS) CGTTGACTGTTTTCCGACAG	115	AY785557
TNF-α	(S) CCCAAGGACTCAGATCATCG (AS) ATACCCACTCTGCCATTGGA	101	x57321

[1]GAPDH = glyceraldehyde phosphate dehydrogenase; LPS-BP = lipopolysaccharide binding protein; SOCS3 = suppressor of cytokine signaling 3; TNF-α = tumor necrosis factor α.

[2]S = sense primer; AS = antisense primer.

bands stained with ethidium bromide after electrophoresis on 1.2% agarose gels (E-gel; Invitrogen, Inc.). A good preparation was indicated by the presence of 28S and 18S bands that were not smeared and by the 28S band stained with a greater intensity than the 18S band. Total RNA (1 μg) was reverse transcribed using a commercially available cDNA synthesis kit (iScript; BioRad Laboratories, Hercules, CA).

Real-time PCR detection of inflammation-related mRNA was conducted using the primers presented in Table 2. Amplification was performed in a total volume of 25 μL containing 1x 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 parameters: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Optical detection was performed at 72°C. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. 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. The PCR products were also sequenced to confirm the identity of each gene. The mRNA abundance values for each sample were normalized to glyceraldehyde phosphate dehydrogenase according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The mRNA expression of glyceraldehyde phosphate dehydrogenase was not affected ($P > 0.10$) in liver or LM by dietary treatment or injection with LPS.

Statistical Analysis

Data were analyzed by ANOVA as a randomized complete block design using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). For the growth performance portion of the experiment, a 2 × 2 factorial arrangement consisting of dietary MFG and BA treatments was used. Data from the LPS challenge study conducted at the completion of the experiment were analyzed as a 2 × 2 × 2 factorial arrangement of treatments that included LPS, MFG, and BA treatments. The pen was the experimental unit for the growth performance data. For the data collected on the subset of pigs used in the LPS challenge portion of the study, individual pig was deemed the experimental unit. The residual mean square error term was used to test all main effects and interactions. Means were evaluated using the PDIFF and STDERR options of GLM. Differences were considered significant at $P \leq 0.05$ and a tendency at $P \leq 0.10$.

RESULTS

Growth Performance

Over the course of the 35-d study, dietary MFG or BA had no impact on ADG, ADFI, or GF (Table 3). The overall health of the experimental pigs remained excellent, with no mortalities observed, and only 1 pig required therapeutic treatment with injectable antibiotics.

Inflammatory Response

As expected, pigs challenged with LPS had increased ($P < 0.05$) rectal body temperatures indicative of a febrile response (Table 4). There was a complex relationship between dietary MFG and BA for the febrile response in pigs injected with LPS as indicated by the 3-way interaction ($P = 0.07$). In pigs fed no MFG, dietary BA led to a greater ($P < 0.05$) body temperatures when injected with

Table 3. Growth performance of pigs fed diets supplemented with humic acid (MFG; Kent Nutrition Group, Muscatine, IA) or butyric acid (BA)[1]

	Control		MFG		
Item	Control	BA	Control	BA	SE
ADG, g/d					
d 0 to 10	272	264	274	258	9.75
d 10 to 20	427	448	444	437	9.93
d 20 to 35	687	667	674	674	9.66
d 0 to 35	494	489	494	487	7.62
ADFI, g/d					
d 0 to 10	253	253	265	245	7.80
d 10 to 20	592	604	610	596	11.57
d 20 to 35	976	948	973	953	14.33
d 0 to 35	660	651	667	649	10.39
G:F					
d 0 to 10	1.07	1.04	1.03	1.05	0.011
d 10 to 20	0.72	0.74	0.73	0.73	0.007
d 20 to 35	0.71	0.71	0.69	0.71	0.006
d 0 to 35	0.75	0.75	0.74	0.75	0.005
BW, kg					
Initial	6.5	6.5	6.5	6.4	0.03
d 35	23.8	23.8	23.6	23.5	0.27

[1]Data represent 14 replicate pens/treatment with 8 pigs/pen.

Table 4. Effect of humic acid (MFG; Kent Nutrition Group, Muscatine, IA) and butyric acid (BA) on body temperature and serum cytokines and metabolites in pigs injected with *Escherichia coli* lipopolysaccharide (LPS) or pigs in the basal state

	Injection	Control		MFG		
Item	solution	Control	BA	Control	BA	SE
Body temperature, °C[1]	Saline	39.4[a]	39.6[a]	39.4[a]	39.4[a]	0.1
	LPS	40.6[b]	41.1[c]	41.2[c]	40.8[b]	
Cortisol, ng/mL[2]	Saline	6.7[a]	5.0[a]	9.6[a]	6.6[a]	8.0
	LPS	56.2[b]	55.6[b]	53.4[b]	21.0[a]	
Glucose, mg/dL[3]	Saline	117.2[a]	122.4[a]	107.8[a]	127.7[a]	8.8
	LPS	96.1[b]	91.1[b]	113.3[a,b]	110.1[a,b]	
Glutathione, μ*M*[4]	Saline	6.3[a]	6.5[a]	9.1[b]	8.6[a,b]	1.1
	LPS	8.4[a,b]	7.3[a,b]	7.1[a,b]	7.4[a,b]	
GPX activity, nmol·min/mL[5]	Saline	1,358	1,799	1,112	1,146	268
	LPS	1,596	1,477	1,418	1,146	
Haptoglobin, mg/mL[6]	Saline	0.212[a,b]	0.379[b]	0.053[a]	0.368[b]	0.064
	LPS	0.292[b]	0.235[a,b]	0.193[a]	0.236[a,b]	
IGF-I, ng/mL[7]	Saline	209.8[a]	227.2[a]	213.7[a]	177.8[a,b]	19.9
	LPS	120.2[b]	106.8[b]	114.4[b]	191.2[a]	
IL-6, pg/mL[8]	Saline	10.0	10.0	10.0	10.0	39.5
	LPS	57.9	191.4	61.0	77.5	
TBARS, μ*M*[9]	Saline	6.7[a]	9.4[a]	6.5[a]	10.1[a]	2.4
	LPS	13.0[b]	7.6[a]	10.3[a,b]	6.0[a]	
Triglycerides, mg/dL[10]	Saline	24.8[a,b]	27.9[a,b]	35.9[a,b]	41.6[a]	6.1
	LPS	42.1[a]	38.5[a]	34.4[a,b]	19.1[b]	

[a–c]Means within a variable with different letters are different at $P < 0.05$.

[1]Response to LPS ($P < 0.0001$) and LPS × MFG × BA ($P = 0.07$). $n = 6$ pigs/treatment.

[2]Response to LPS ($P < 0.0001$) and LPS × MFG × BA ($P = 0.08$).

[3]Response to LPS ($P < 0.01$) and LPS × MFG response ($P = 0.11$).

[4]Lipopolysaccharide × MFG response ($P = 0.04$).

[5]GPX = glutathione peroxidate activity. Response to MFG ($P = 0.07$).

[6]Dietary BA response ($P = 0.02$) and LPS × BA response ($P < 0.01$).

[7]Response to LPS ($P < 0.0001$) and LPS × MFG × BA ($P < 0.01$).

[8]Response to LPS ($P < 0.003$).

[9]TBARS = thiobarbituric acid reactive substances. Dietary BA × LPS ($P = 0.03$).

[10]Dietary MFG × LPS response ($P = 0.005$).

LPS, whereas in pigs fed MFG, feeding BA led to reduced ($P < 0.05$) body temperatures after LPS injection.

Serum cortisol concentrations were increased ($P < 0.05$) in pigs treated with LPS injection, and there was a 3-way interaction ($P = 0.08$) such that serum cortisol concentrations were lower ($P < 0.05$) in pigs fed diets containing both MFG and BA in LPS-injected pigs. Serum glucose concentrations were lower ($P < 0.05$) in LPS-injected pigs. A trend ($P = 0.11$) occurred between dietary MFG and LPS. Serum glucose concentrations were decreased ($P < 0.05$) in pigs challenged with LPS in pigs fed diets devoid of MFG, whereas serum glucose concentrations in pigs fed MFG and injected with LPS did not differ from those pigs fed MFG and injected with saline. For serum glutathione there was an interaction ($P = 0.04$) between LPS and MFG such that serum glutathione concentrations were increased ($P < 0.05$) in saline-injected pigs fed MFG, but serum glutathione concentrations were not different between dietary treatments in LPS-injected pigs. Serum glutathione peroxidase activity was decreased ($P < 0.05$) in pigs fed MFG. For serum haptoglobin, there was an interaction ($P < 0.01$) between dietary BA and LPS because in saline-injected pigs, serum haptoglobin concentrations were greater ($P < 0.05$) in pigs fed BA. However, in LPS-challenged pigs there was no effect of dietary BA on serum haptoglobin. There was a 3-way interaction ($P < 0.01$) for serum IGF-I. Serum IGF-I concentrations were reduced ($P < 0.05$) in LPS-challenged pigs as compared to saline-injected pigs except for the group fed the combination of MFG and BA. As expected, serum IL-6 concentrations were greater ($P < 0.003$) in pigs challenged with LPS, but there was no effect of dietary treatment. For serum TBARS, there was an interaction ($P = 0.03$) between LPS and dietary BA because LPS-challenged pigs fed BA had reduced ($P < 0.05$) serum TBARS. However, dietary BA had no effect on serum TBARS in saline-injected pigs. There was an interaction ($P = 0.005$) between MFG and LPS for serum triglyceride concentrations. Pigs fed MFG and challenged with LPS had reduced ($P < 0.05$) serum triglycerides, whereas serum triglycerides were not different between saline or LPS-challenged pigs fed diets containing no MFG.

Liver IGF-I mRNA levels were not affected by LPS or dietary treatments (Table 5). As expected, greater mRNA abundance of IL-1β ($P = 0.003$) and IL-6 ($P = 0.10$)

Table 5. Effect of dietary humic acid (MFG; Kent Nutrition Group, Muscatine, IA) and butyric acid (BA) on inflammation-related mRNA in liver and LM in pigs injected with *Escherichia coli* lipopolysaccharide (LPS)

Item	Injection solution	Control		MFG		SE
		Control	BA	Control	BA	
Liver						
IGF-I	Saline	0.052	0.030	0.025	0.049	0.027
	LPS	0.026	0.024	0.047	0.041	
IL-1β[1]	Saline	0.0015	0.0002	0.0013	0.0003	0.0172
	LPS	0.0635	0.0619	0.0558	0.0262	
IL-1Ra[2]	Saline	0.0051	0.0019	0.0021	0.0032	0.0312
	LPS	0.0884	0.0243	0.0900	0.0073	
IL-6[3]	Saline	0.0048	0.0046	0.0021	0.0021	0.0091
	LPS	0.0109	0.0136	0.0064	0.0269	
LPS-BP[4]	Saline	0.368[a]	0.239[a]	0.164[a]	0.283[a]	0.230
	LPS	0.832[a]	0.156[b]	0.902[a]	0.228[b]	
SOCS3[5]	Saline	0.005	0.002	0.002	0.003	0.032
	LPS	0.085	0.090	0.024	0.007	
TNF-α[6]	Saline	0.008	0.005	0.025	0.015	0.016
	LPS	0.016	0.007	0.053	0.013	
LM						
IGF-I[7]	Saline	0.011	0.028	0.034	0.018	0.006
	LPS	0.007	0.007	0.011	0.007	
IL-1β[8]	Saline	0.0018	0.0008	0.0017	0.0007	0.0009
	LPS	0.0041	0.0023	0.0047	0.0021	
IL-6	Saline	0.0003	0.0013	0.0014	0.0007	0.0029
	LPS	0.0094	0.0025	0.0025	0.0009	
TNF-α	Basal	0.0006	0.0009	0.0012	0.0006	0.0008
	LPS	0.0019	0.0005	0.0027	0.0005	

[a–c]Means within a variable with different letters are different at $P < 0.05$.

[1]Response to LPS ($P = 0.003$). $n = 6$ pigs/treatment.

[2]Lipopolysaccharide response ($P = 0.01$), dietary BA ($P = 0.09$), and LPS × BA response ($P = 0.09$).

[3]Response to LPS ($P = 0.10$).

[4]LPS-BP = lipopolysaccharide binding protein. Dietary BA response ($P = 0.03$) and LPS × BA response ($P = 0.08$).

[5]SOCS3 = suppressor of cytokine signaling 3. Response to LPS ($P = 0.05$).

[6]TNF-α = tumor necrosis factor α.

[7]Response to LPS ($P < 0.001$).

[8]Dietary BA response ($P = 0.02$) and LPS response ($P = 0.005$).

mRNA were observed in LPS-challenged pigs. There were interactions ($P < 0.09$) between LPS and dietary BA for liver IL-1Ra and LPS-binding protein mRNA abundance. Levels of IL-1Ra and LPS-binding protein mRNA were greater ($P < 0.05$) in pigs challenged with LPS and fed diets containing no BA, but liver IL-1Ra did not differ between saline- or LPS-injected figs fed BA. Liver suppressor of cytokine signaling 3 (**SOCS3**) mRNA expression levels were increased ($P < 0.05$) in LPS-injected pigs but were not affected by dietary treatments. The expression level of tumor necrosis factor α (**TNF-α**) mRNA was not affected by LPS or dietary treatment in liver or muscle; however, muscle IGF-I mRNA was decreased in pigs injected with LPS. Dietary BA led to a decrease ($P = 0.02$) in muscle IL-1β mRNA regardless of LPS challenge status, and muscle IL-1β mRNA levels were increased ($P = 0.005$) in LPS-challenged pigs.

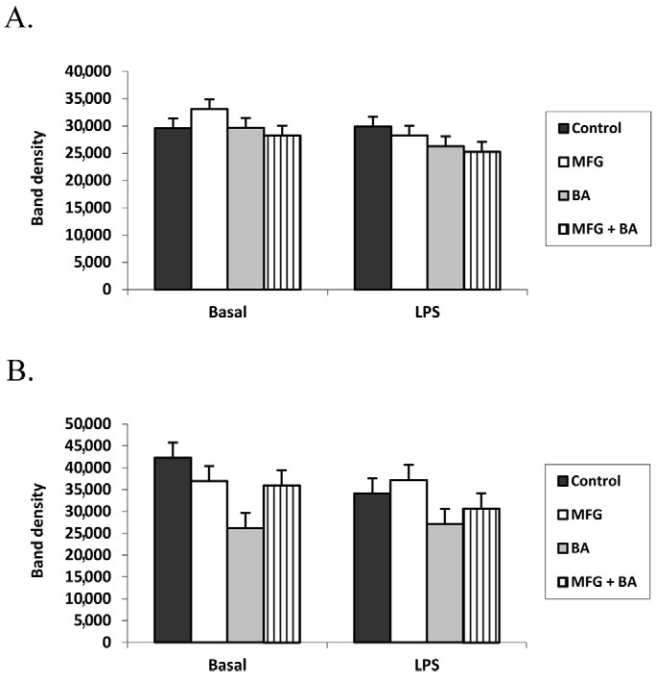

Figure 1. Effect of humic acid (MFG; Kent Nutrition Group, Muscatine, IA) and butyric acid (BA) on 4-hydroxynonenal adducts in (A) liver and (B) longissimus dorsi tissue of pigs injected with lipopolysaccharide (LPS) or pigs in the basal state. Each bar in the graph represents 6 pigs/treatment. Data presented are densitometric units. There was a significant effect of BA in liver ($P < 0.01$) and longissimus dorsi tissue ($P < 0.003$).

As a further indicator of oxidative stress, 4-HNE protein adducts were measured in liver and muscle tissue. It was observed that 4-HNE protein adducts were lower ($P < 0.05$) in both liver and muscle when pigs were fed BA (Fig. 1).

DISCUSSION

The main objective of this study was to determine the effects of a specific humic acid (MFG) and a fat-protected BA, alone and in combination, on growth performance and their ability to attenuate inflammatory stimulation in young pigs. Overall, inclusion of MFG (0.25% in all phases) and BA (0.06 to 0.22% across phases) alone or in combination in the diets of weanling pigs at these levels had no effect on growth performance parameters in relatively healthy pigs. These results are similar to other nursery pig research that has previously reported sodium butyrate failed to alter growth performance in clinically healthy pigs (Weber and Kerr, 2008).

Regarding dietary humic acid, Ji et al. (2006) reported an increase in pig ADG at 5 wk postweaning at 0.5% humic substance inclusion but not at 1.0% inclusion. Furthermore, at 14 wk postweaning, both levels showed significant decreases in ADFI and increases in G:F. Additional work by

Ji et al. (2006) showed a significant increase in ADG, decrease in ADFI, and increase in G:F during later phase diets with 0.5% humic substances. However, it is important to note that within these studies, different combinations of humic substances were used and likely composed of different concentrations of humic and fulvic acids.

Constant inflammatory challenges have been shown to be detrimental to pig health and growth performance (Williams et al., 1997; Rakhshandeh et al., 2012). Even though we observed no growth performance benefits from BA and or MFG inclusion in the diet of clinically healthy nursery pigs, we hypothesized that these 2 compounds would provide anti-inflammatory benefits to an LPS challenge. As expected, when pigs were challenged with the inflammatory stimuli LPS and monitored for a period of 4 h, they responded significantly with increased body temperature, serum cortisol, and IL-6 concentrations compared to saline-injected pigs. This LPS-induced inflammatory response also decreased blood glucose and IGF-I concentrations compared to the saline-injected control pigs. These parameters follow the similar classic proinflammatory cytokines profile induction as previously reported in LPS challenges pigs (Webel et al., 1997; Carroll et al., 2005; Gabler et al., 2008). Additionally, compared to the saline control, LPS induced increased mRNA levels of IL-1β, IL-1 receptor antagonist, IL-6, and SOCS3 in liver and increased IL-6 mRNA levels in muscle and decreased muscle IGF-I mRNA levels.

Analysis of the febrile response and blood and tissue parameters to the interaction of LPS, BA, and MFG indicated a complex and varied response to dietary treatments. For example, serum haptoglobin was not altered due to LPS in the 4-h challenge period. However, BA treatment increased serum haptoglobin concentrations. Serum concentrations of IL-6 were not different due to BA supplementation, which is opposite to data previously published (Weber and Kerr, 2008). Furthermore, MFG appeared to have no effect on the LPS-induced IL-6 response. Serum cortisol concentration was similar to data published by Norimatsu et al. (1995). Interestingly, MFG + BA + LPS pigs had a 50% reduction in their LPS-induced cortisol response compared to all other LPS challenge treatments. Serum glucose concentrations of MFG pigs had a lower reduction after LPS challenge compared to the control pigs. This suggests that MFG may help to mitigate some of the metabolic stress induced by LPS challenge. The reduction in IGF-I concentrations were similar to data from Spurlock et al. (1998), but the ability of BA to modulate the LPS response differed in comparison to data from Weber and Kerr (2008). The interaction between BA and MFG treatments appeared to mitigate and preserve IGF-I concentration. Interestingly, the control diet pigs increased blood triglycerides whereas MFG pigs saw a reduction irrespective of LPS challenge. This is opposite to data published by Weber and Kerr (2008) that showed decreased triglyceride concentrations in BA treated pigs and a reduction in concentration due LPS challenge. These data may be explained by the fact that MFG contains a complex acid mixture of humic acid that can function as a lipid carrier within blood, enabling higher basal triglyceride levels.

Previous work by Weber and Kerr (2008) showed significant LPS effect on immune parameters and when pigs were supplemented with 0.2% sodium butyrate. However, we did not observe these differences in the current study due to BA supplementation. However, humic acid can directly suppress LPS-induced activation of NF-κB in human umbilical cord endothelial cells by preventing the degradation of its inhibitor, IκBα, and the subsequent proinflammatory cascade (Gau et al., 2000). Surprisingly, muscle and liver gene transcript abundance increased for TNF-α was not altered due to LPS challenge or diet. However, work by Webel et al. (1997) and Gabler et al. (2008) showed peak TNF-α protein levels at 2 h after challenge. As these samples were collected at 4 h after challenge, mRNA for TNF-α and other cytokines may be lower, even though translated protein concentrations are elevated. Liver IL-1β mRNA abundance was decreased in BA fed pigs in the basal state and not changed in MFG pigs. Furthermore, LPS-challenged pig muscle IL-1β abundance followed the same trend. Liver IL-1β and IL-6 mRNA abundance was not altered by diet. An important acute phase protein in LPS signaling and clearance is LPS binding protein (Mani et al., 2012). Interestingly, gene abundance for LSP binding protein was decreased due to BA treatments and increased due to LPS challenge in the liver.

Oxidative stress and reactive oxygen species also play a major role in cellular damage and dysfunction (Bottje and Carstens, 2009; Grubbs et al., 2013a,b). Overall, MFG and LPS resulted in no differences in 4-HNE adducts and TBARS in liver and muscle samples, but this may have been due to the short duration of the LPS challenge. Interestingly, BA significantly reduced liver and muscle 4-HNE adducts. Pigs fed MFG had higher glutathione concentrations and lower glutathione peroxidase activities compared to BA and control pigs. Although there was no change in TBARS, these data suggests the potential for MFG to mitigate some oxidative stress within the body.

In conclusion, this study showed that BA and MFG-derived humic acid, fed separately or in combination, had no effect on growth performance in healthy pigs at concentrations 0.06 to 0.22 and 0.25%, respectively. However, both MFG and BA inclusion appear to play a role in modulating different aspects of the immune response to LPS. Humic acid may play a role in negating the effects of oxidative stress, and further work is needed to explore this further. Additionally, MFG may play a role in LPS clearance. A time course with a longer

inflammatory challenge is warranted to further test the potential performance benefits of BA and MFG.

LITERATURE CITED

Bottje, W. G., and G. E. Carstens. 2009. Association of mitochondrial function and feed efficiency in poultry and livestock species. J. Anim. Sci. 87:E48–E63.

Brix-Christensen, V., J. Gjedsted, S. K. Andersen, C. Vestergaard, J. Nielsen, T. Rix, R. Nyboe, N. T. Andersen, A. Larsson, O. Schmitz, and E. Tonnesen. 2005. Inflammatory response during hyperglycemia and hyperinsulinemia in a porcine endotoxemic: The contribution of essential organs. Acta Anaesthesiol. Scand. 49:991–998.

Carroll, J. A., D. B. Carter, S. W. Korte, and R. S. Prather. 2005. Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge. Domest. Anim. Endocrinol. 29:564–572.

Gabler, N. K., J. D. Spencer, D. M. Webel, and M. E. Spurlock. 2008. *n*-3 PUFA attenuate lipopolysaccharide-induced down-regulation of toll-like receptor 4 expression in porcine adipose tissue but does not alter the expression of other immune modulators. J. Nutr. Biochem. 19:8–15.

Gabler, N. K., and M. E. Spurlock. 2008. Integrating the immune system with the regulation of growth and efficiency. J. Anim. Sci. 86:E64–E74.

Gau, R. J., H. L. Yang, S. N. Chowm, J. L. Suen, and F. J. Lu. 2000. Humic acid suppresses the LPS-induced expression of cell-surface adhesion proteins through the inhibition of NF-κB activation. Toxicol. Appl. Pharmacol. 166:59–67.

Grubbs, J. K., A. N. Fritchen, E. Huff-Lonergan, J. C. Dekkers, N. K. Gabler, and S. M. Lonergan. 2013a. Divergent genetic selection for residual feed intake impacts mitochondria reactive oxygen species production in pigs. J. Anim. Sci. 91:2133–2140.

Grubbs, J. K., A. N. Fritchen, E. Huff-Lonergan, N. K. Gabler, and S. M. Lonergan. 2013b. Selection for residual feed intake alters the mitochondria protein profile in pigs. J. Proteomics 80:334–345.

Hu, M. L. 1994. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol. 233:380–385.

Ji, F., J. J. McGlone, and S. W. Kim. 2006. Effects of dietary humic substances on pig growth performance, carcass characteristics, and ammonia emission. J. Anim. Sci. 84:2482–2490.

LeFloc'h, N., D. Melchior, and B. Seve. 2008. Dietary tryptophan helps to preserve tryptophan homeostasis in pigs suffering from lung inflammation. J. Anim. Sci. 86:3473–3479.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408.

Lu, H., S. Su, and K. M. Ajuwon. 2012. Butyrate supplementation to gestating sows and piglets induces muscle and adipose tissue oxidative genes and improves growth performance. J. Anim. Sci. 90(Suppl. 4):430–432.

Mani, V., T. E. Weber, L. H. Baumgard, and N. K. Gabler. 2012. Growth and development symposium: Endotoxin, inflammation, and intestinal function in livestock. J. Anim. Sci. 90:1452–1465.

Mattace Raso, G., R. Simeoli, R. Russo, A. Iacono, A. Santoro, O. Paciello, M. C. Ferrante, R. B. Canani, A. Calignano, and R. Melli. 2013. Effects of sodium butyrate and its synthetic amide derivative on liver inflammation and glucose tolerance in an animal model of steatosis induced by high fat diet. PLoS ONE 8:e68626.

Naude, P. J., A. D. Cromarty, and C. E. J. V. Rensburg. 2010. Potassium humate inhibits carrageenan-induced paw oedema and a graft-versus-host reaction in rats. Inflammopharmacology 18:33–39.

Norimatsu, M., T. Ono, A. Aoki, K. Ohishi, T. Takahashi, G. Watanabe, K. Taya, S. Sasamoto, and Y. Tamura. 1995. Lipopolysaccharide-induced apoptosis in swine lymphocytes in vivo. Infect. Immun. 63:1122–1126.

NRC. 1998. Nutrient requirements of swine. 10th ed. National Academy Press, Washington, DC.

Ohira, H., Y. Fujioka, C. Katagiri, R. Mamoto, M. Aoyama-Ishikawa, K. Amoko, Y. Izumi, S. Nishium, M. Yoshida, M. Usami, and M. Ikeda. 2013. Butyrate attenuates inflammation and lipolysis generated by the interaction of adipocytes and macrophages. J. Atheroscler. Thromb. 20:425–442.

Rakhshandeh, A., J. Dekkers, B. Kerr, T. Weber, J. English, and N. Gabler. 2012. Effect of immune system stimulation and divergent selection for residual feed intake on digestive capacity of the small intestine in growing pigs. J. Anim. Sci. 90:233–235.

Rath, N. C., W. E. Huff, and G. R. Huff. 2006. Effects of humic acid on broiler chickens. Poult. Sci. 85:410–414.

Spurlock, M. E., M. A. Ranalletta, S. G. Cornelius, G. R. Frank, G. M. Willis, S. Ji, A. L. Grant, and C. A. Bidwell. 1998. Leptin expression in porcine adipose tissue is not increased by endotoxin but is reduced by growth hormone. J. Interferon Cytokine Res. 18:1051–1058.

Toepfer-Berg, T. L., J. Escobar, W. G. V. Alstine, D. H. Baker, J. Salak-Johnson, and R. W. Johnson. 2004. Vitamin E supplementation does not mitigate the acute morbidity effects of porcine reproductive and respiratory syndrome virus in nursery pigs. J. Anim. Sci. 82:1942–1951.

Van Rensburg, C. E. J., J. R. Snyman, T. Mokoele, and A. D. Cromarty. 2007. Brown coal derived humate inhibits contact hypersensitivity: An efficacy, toxicity and teratogenicity study in rats. Inflammation 30:148–152.

Wang, Q., Y. J. Chen, J. S. Yoo, H. J. Kim, J. H. Cho, and I. H. Kim. 2008. Effects of supplemental humic substances on growth performance, blood characteristics and meat quality in finishing pigs. Livest. Sci. 117:270–274.

Webel, D. M., B. N. Finck, D. H. Baker, and R. W. Johnson. 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. J. Anim. Sci. 75:1514–1520.

Weber, T. E., and B. J. Kerr. 2006. Butyrate differentially regulates cytokines and proliferation in porcine peripheral blood mononuclear cells. Vet. Immunol. Immunopathol. 113:139–147.

Weber, T. E., and B. J. Kerr. 2008. Effect of sodium butyrate on growth performance and response to lipopolysaccharide in weanling pigs. J. Anim. Sci. 86:442–450.

Weber, T. E., and M. E. Spurlock. 2004. Leptin alters antibody isotype in the pig in vivo, but does not regulate cytokine expression or stimulate STAT3 signaling in peripheral blood monocytes in vitro. J. Anim. Sci. 82:1630–1640.

Williams, N. H., T. S. Stahly, and D. R. Zimmerman. 1997. Effect of chronic immune system activation on body nitrogen retention, partial efficiency of lysine utilization, and lysine needs of pigs. J. Anim. Sci. 75:2472–2480.

Wright, K. J., R. Balaji, C. M. Hill, S. S. Dritz, E. L. Knoppel, and J. E. Minton. 2000. Integrated adrenal, somatotropic, and immune responses of growing pigs to treatment with lipopolysaccharide. J. Anim. Sci. 78:1892–1899.

Yin, C., A. Pettigrew, J. P. Loftus, S. J. Black, and J. K. Belknap. 2009. Tissue concentrations of 4-HNE in the black walnut extract model of laminitis: Indication of oxidant stress in affected laminae. Vet. Immunol. Immunopathol. 129:211–215.