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Effect of Low Crude Protein Formulation Strategies With and Without Pharmacological Zn for Nursery Pigs Challenged With Enterotoxigenic *Escherichia coli* F18

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Summary

A total of 90 weanling pigs (DNA 241 × 600; initially 13.2 ± 0.80 lb BW) were used in a 21-d study using two groups (45 pigs/group) to evaluate the effects of low crude protein (CP) formulation strategies with and without pharmacological zinc from zinc oxide (ZnO) on growth performance, fecal dry matter, and health parameters in enterotoxigenic *Escherichia coli* F18 (ETEC)-challenged nursery pigs. Within group, pigs were balanced for sex, stratified by weight, and allotted to one of five dietary treatments in a generalized randomized block design with 9 pigs per treatment (total of 18 replications per treatment). Diets were corn-soybean meal-based and formulated as: high CP (20.7% CP, 1.35% SID Lys), low CP (18.0% CP, 1.17% SID Lys), low CP + AA (18.0% CP, 1.35% SID Lys, similar SID AA:Lys ratios to high and low CP treatments by increasing synthetic AA inclusion), low CP + ZnO (18.0% CP, 1.17% SID Lys, 3,000 ppm Zn from ZnO), and low CP + AA + ZnO (18.0% CP, 1.35% SID Lys, 3,000 ppm Zn from ZnO). Following weaning (~19 d of age) pigs were group housed by treatment (4 or 5 pigs/pen) and provided their respective treatments during the pre-challenge period. Pigs were then moved to individual pens and continued their treatment diet on d 7. Inoculation occurred on d 7 with 6 mL of 107 CFU/mL ETEC F18 administered via orogastric gavage and followed with 6 mL of phosphate-buffered saline. Performance measures were collected on d 0, 7, 14, and 21 to calculate ADG, ADFI, and G:F. On d 7, 10, 14, 17, and 21, fecal samples were collected from each pig for determination of fecal dry matter (DM). Overall, after ETEC challenge pigs fed low CP + AA + ZnO had greater ($P < 0.05$) ADG compared with pigs fed the high CP or low CP treatments. Feed intake was greater ($P < 0.05$) for pigs fed low CP + ZnO and low CP + AA + ZnO compared with pigs fed the high or low CP diets. There was no evidence of a difference ($P = 0.640$) in the overall challenge period feed efficiency. There

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was a treatment × day interaction for fecal DM ($P < 0.0001$). On d 7 and 21, there were no differences in fecal DM between treatments. On d 10, fecal DM was greater ($P < 0.05$) for pigs fed diets containing ZnO. On d 14, pigs fed low CP + AA + ZnO had greater ($P < 0.05$) fecal DM compared with pigs fed the low CP diet. On d 17, pigs fed low CP had greater ($P < 0.05$) fecal DM compared with pigs fed high CP. In summary, inclusion of ZnO, particularly when combined with elevated AA levels in a low CP formulation strategy, enhanced growth performance in ETEC F18-challenged pigs relative to a low CP diet.

Introduction

The period immediately post-weaning is one of the most stressful times in a pig's life, where environmental and dietary changes combined with potential exposure to pathogens can lead to post-weaning diarrhea (PWD). While the cause of PWD can be multifaceted, it is commonly attributed to pathogenic bacteria like enterotoxigenic *Escherichia coli* (ETEC), which can lead to reduced growth performance, increased morbidity, and higher mortality rates. Zinc oxide (ZnO) has been relied on in the swine industry because it has been shown to mitigate the post-weaning growth lag by reducing PWD and enhancing growth performance when included at pharmacological levels (2,000 to 3,000 ppm Zn). However, scrutiny has been placed on the continued use of pharmacological Zn as a substantial proportion of consumed Zn is excreted in feces.

Newly weaned pigs often have low feed intake for 7 to 10 d after weaning; thus, a higher CP diet (20 to 23% CP) can be fed to meet protein deposition potential. However, high CP diets can result in more nitrogen and undigested protein reaching the large intestine. This substrate contributes to the proliferation of harmful bacteria that can lead to PWD. Therefore, another strategy used to mitigate PWD is to lower the dietary CP level. However, when lowering CP levels, especially when SID Lys levels are reduced, there can be negative effects on growth performance. Nutritional strategies must consider how to limit excess dietary nitrogen that contributes to PWD while still supplying adequate AAs for pigs to support growth performance. To address this, it has been proposed that increasing standardized ileal digestible (SID) Lys in low-CP diets could maintain growth while improving gut health. Additionally, it has been hypothesized that extra nitrogen entering the large intestine may modulate ZnO's efficacy by indirectly aiding in bacterial proliferation, potentially influencing its protective effects against ETEC. There is limited understanding of how dietary CP levels interact with pharmacological Zn and increased SID Lys which could offset the performance losses often associated with reduced protein. A better understanding of these relationships during an enteric challenge is critical for developing nutrition strategies that minimize PWD while maintaining performance and promoting environmental sustainability in swine production. Thus, the objective of this experiment was to evaluate the effects of low CP formulation strategies with and without pharmacological Zn from ZnO and their interactive effects on growth performance, fecal DM, and serum cytokine concentrations in enterotoxigenic *Escherichia coli* F18-challenged nursery pigs.

Procedures

The Kansas State University Institutional Animal Care and Use Committee (IACUC #5018) and Institutional Biosafety Committee (IBC-1768) approved the protocols used in these experiments. This experiment was conducted at the K-State Swine Enteric Health Research Center facility in Manhattan, KS, under biosafety level 2 (BSL-2) operating conditions. Each pen had metal tri-bar flooring, a stainless-steel self-feeder,

and nipple waterer to provide ad libitum access to feed and water. Pigs were placed in solid-sided pens to prevent cross-contamination between adjacent pens.

Animals and diets

A total of 90 weanling pigs (DNA 241 × 600; initially 13.2 ± 0.80 lb BW) were used in a 21-d study using two groups (45 pigs/group). Within group, pigs were randomly allotted to one of five dietary treatments in a generalized randomized block design and grouped with four to five pigs per pen during a pre-challenge period before transfer to the BSL-2 facility for initiation of the ETEC challenge period. Diets were provided to piglets starting on d 0 and were corn-soybean meal-based with high or low CP, with or without the addition of pharmacological ZnO, synthetic amino acids, or both. Treatment diets consisted of 1) high CP diet (1.35% SID Lys, 20.7% CP), 2) low CP diet (1.17% SID Lys, 18.0% CP), 3) low CP + AA (1.35% SID Lys, 18.0% CP), 4) low CP + ZnO (1.17% SID Lys, 18.0% CP, approx. 3,000 ppm Zn from ZnO), and 5) low CP + AA + ZnO (1.35% SID Lys, 18.0% CP, approx. 3,000 ppm Zn from ZnO). All diets met or exceeded the nutrient requirement estimates of other amino acids as a ratio to Lys (NRC, 2012) for the weight ranges of the pigs (Table 1). A single 500-lb batch of feed was manufactured for each treatment and used to feed both groups. Diets were manufactured at the Kansas State University O.H. Kruse Feed Technology Innovation Center (Manhattan, KS) and fed in pelleted form. On the day of inoculation (d 7), pigs were placed in the research facility in individual pens. Pig weights were measured on d 0, 7, 14, and 21 to determine ADG, and feed intake was measured from d 7 to d 21 to determine ADFI and G:F.

Selected piglets came from sows not previously vaccinated for *E. coli* F18. Piglets were confirmed for genetic susceptibility to the F18 strain of ETEC by sequencing the (1,2) fucosyltransferase-1 (FUT1) gene via PCR at the Iowa State University Veterinary Diagnostic Laboratory (VDL). To identify susceptible piglets, an initial group of 96 piglets with similar body weights and an equal distribution of sow parities were selected within each group. A tail tissue sample was collected at litter processing from the selected piglets. Samples were frozen and shipped on dry ice. After identifying susceptible piglets (GG/AG genotype) from the initial group, 45 pigs with similar body weights, evenly distributed across litters, were randomly chosen to be used within each group of the experiment (90 pigs total with 18 replications per treatment).

The ETEC F18 strain was obtained and cultured at the Kansas State University College of Veterinary Medicine. The isolate used was an F18 fimbrial type, with genes for heat-labile enterotoxin (LT), heat-stable toxin A (STa), heat-stable toxin B (STb), and Shiga toxin type 2e (Stx2e) as determined via polymerase chain reaction (PCR) and was derived from a field clinical case submission to the Iowa State Veterinary Diagnostic Laboratory. On d 7, all pigs were inoculated with 6 mL of solution containing 10^7 CFU/mL of ETEC F18 and followed with 6 mL of phosphate-buffered saline (PBS). The inoculum was administered via orogastric gavage with a rubber catheter.

On d 7, 10, 14, 17, and 21, fecal samples were collected from each pig to determine fecal dry matter (DM). Using a cotton-tipped applicator to stimulate defecation, fecal samples were collected into plastic bags and frozen at −4°F (−20°C) until fecal DM analysis. Fecal samples were dried at 131°F (55°C) in a forced-air oven for 48 h, and the ratio of dried-to-wet fecal weight was used to determine the percentage fecal DM.

On d 7 (before challenge), 14, and 21, a blood sample was collected via jugular venipuncture into a 10-mL vacutainer tube. Samples were centrifuged (3,000 × *g* for 10 min) and serum was recovered in four separate aliquots. An aliquot of serum was analyzed using a multiplex immunoassay that quantifies 13 cytokines (Eve Technologies, Calgary, AB, Canada).

Statistical analysis

Data were analyzed using the GLIMMIX procedure of SAS OnDemand for Academics (SAS Institute Inc., Cary, NC). Individual pig served as the experimental unit, with dietary treatment as a fixed effect and BW block and group included as random intercepts. Serum cytokine data were log-transformed for analysis and back-transformed for presentation of least squares means. Following implementation of the base statistical model for each response, data points with absolute studentized residuals greater than 4 were removed from data analysis. When treatment was a significant source of variation, differences were determined by pairwise comparison using the Tukey-Kramer multiplicity adjustment to control for type I error. Results were considered significant at $P \leq 0.05$ and were considered marginally significant at $P \leq 0.10$.

Results and Discussion

Growth performance

On d 7, following consumption of the treatment diets for 7 d, there were no observed differences in BW (Table 2). Pigs were challenged with ETEC on d 7, and from d 7 to 14, pigs fed either diet with pharmacological Zn from ZnO had greater ($P < 0.05$) ADG than pigs fed each treatment diet without ZnO, regardless of the CP formulation strategy. Pigs fed diets with pharmacological Zn, regardless of the CP formulation strategy, had greater ($P < 0.05$) ADFI compared with pigs fed a high CP diet, with other treatments being intermediate. Pigs fed the low CP + ZnO diet had improved ($P < 0.05$) compared with those fed either a high CP diet or low CP diet without pharmacological Zn. Also, pigs fed pharmacological Zn in the low CP diet with elevated synthetic amino acids had improved ($P < 0.05$) G:F compared with pigs fed low CP diet without pharmacological ZnO.

From d 14 to 21, there was a tendency for a treatment effect ($P = 0.060$) for ADG, but no mean separation between treatments was detected. Pigs fed the low CP diet formulated with additional amino acids and pharmacological Zn had increased ($P < 0.05$) ADFI compared with those fed a high CP or low CP diets without pharmacological ZnO. Pigs fed a low CP diet with pharmacological ZnO had reduced ($P < 0.05$) G:F compared with those fed a high CP, low CP, or low CP + AA diet.

During the challenge period (d 7 to 21), pigs fed the low CP + AA + ZnO diet had greater ($P < 0.05$) ADG compared with pigs fed the high CP or low CP treatments. Feed intake was greater ($P < 0.05$) for pigs fed low CP + ZnO and low CP + AA + ZnO compared with pigs fed the high or low CP diets. There was no evidence of a difference for feed efficiency from d 7 to d 21.

Fecal dry matter

There was a significant treatment × day interaction for fecal DM ($P < 0.0001$). On d 7, there was no evidence of a difference in fecal DM between treatments. By d 10, fecal DM was greater ($P < 0.05$) for pigs fed diets containing ZnO compared with pigs fed the high or low CP treatments. On d 14, pigs fed low CP + AA + ZnO had greater

($P < 0.05$) fecal DM compared with pigs fed the low CP diet, with the other treatments being intermediate. On d 17, pigs fed low CP had greater ($P < 0.05$) fecal DM compared with pigs fed high CP, with the other treatments being intermediate. By d 21, there was no evidence of a difference in fecal DM between dietary treatments.

Serum cytokines

Serum cytokine concentrations are presented in Table 3. There was a significant difference ($P = 0.001$) in IL-8, where pigs fed the high CP diet had increased IL-8 compared with those fed a low CP diet with ZnO, regardless of amino acid inclusion. Pigs fed the low CP diet had significantly increased ($P < 0.05$) levels of IL-1RA compared with pigs fed a low CP + ZnO diet, with the other treatments being intermediate. There was no evidence of differences between treatments for other measured serum cytokines.

In conclusion, lowering CP resulted in modest improvements in fecal dry matter 10 d post-ETEC challenge compared with pigs fed diets with greater SID Lys and CP. Inclusion of ZnO, particularly when combined with elevated AA levels in a low CP formulation, enhanced growth performance in ETEC F18-challenged pigs relative to a low CP diet.

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

Ingredient, %	High CP	Low CP	Low CP + AA	Low CP + ZnO	Low CP + AA + ZnO
Corn	54.92	61.79	62.65	61.36	62.22
Soybean meal, 47.7% CP	20.18	13.32	11.41	13.35	11.44
Whey powder	14.00	14.00	14.00	14.00	14.00
Soy protein concentrate[2]	6.25	6.25	6.25	6.25	6.25
Soybean oil	1.00	1.00	1.00	1.00	1.00
Calcium carbonate	0.50	0.48	0.47	0.48	0.47
Monocalcium phosphate, 21.5% P	0.98	1.08	1.11	1.08	1.11
Salt	0.73	0.73	0.73	0.73	0.73
L-Lys-HCl	0.41	0.40	0.69	0.40	0.69
DL-Met	0.18	0.14	0.26	0.14	0.26
L-Thr	0.19	0.17	0.32	0.17	0.32
L-Trp	0.06	0.06	0.11	0.06	0.11
L-Val	0.10	0.09	0.26	0.09	0.26
L-Ile	---	0.01	0.15	0.01	0.15
L-His	---	---	0.11	---	0.11
Trace mineral premix	0.15	0.15	0.15	0.15	0.15
Vitamin premix	0.25	0.25	0.25	0.25	0.25
Choline chloride, 60%	0.05	0.05	0.05	0.05	0.05
Phytase[3]	0.06	0.06	0.06	0.06	0.06
ZnO	---	---	---	0.40	0.40
Total	100	100	100	100	100
Calculated analysis					
SID amino acids, %					
Lys	1.35	1.17	1.35	1.17	1.35
Ile:Lys	58	58	58	58	58
Leu:Lys	117	121	101	121	101
Met:Lys	35	34	38	34	37
Met and Cys:Lys	56	56	56	56	56
Thr:Lys	64	64	64	64	64
Trp:Lys	21	21	21	21	21
Val:Lys	70	70	70	70	70
His:Lys	35	35	35	35	35
SID Lys:CP	6.51	6.51	7.51	6.51	7.51
NE, kcal/lb	1,143	1,160	1,169	1,155	1,164
SID Lys:NE, g/Mcal	5.36	4.57	5.24	4.59	5.26
CP, %	20.7	18.0	18.0	18.0	18.0
STTD P, %	0.54	0.54	0.54	0.54	0.54
Ca:P	1.00	1.00	1.00	1.00	1.00

[1] Treatment diets were fed in a pellet form from d 0 to 21. All diets were manufactured at the O.H. Kruse Feed Technology Center in Manhattan, KS.

[2] AX3 Digest; Protekta, Newport Beach, CA.

[3] Ronozyme HiPhos 2700 (DSM-Firmenich; Parsippany, NJ) provided an estimated release of 0.12% STTD P with 2,027 FYT/kg.

Table 2. Effects of low crude protein formulation strategies with and without pharmacological ZnO on nursery pig growth performance following enterotoxigenic *Escherichia coli* F18 challenge

	Dietary treatment[1]						
Item	High CP	Low CP	Low CP + AA	Low CP + ZnO	Low CP + AA + ZnO	SEM	*P* =
BW, lb							
d 0	13.1	13.2	13.2	13.2	13.2	0.80	1.000
d 7	14.3	14.5	14.8	14.4	14.6	0.90	0.874
d 14	15.9^{c}	15.4^{c}	16.3^{bc}	18.4^{ab}	18.7^{a}	1.31	< 0.0001
d 21	20.7^{b}	21.7^{ab}	22.4^{ab}	22.3^{ab}	24.2^{a}	1.38	0.018
d 0 to 7							
ADG, lb	0.17	0.19	0.23	0.18	0.21	0.030	0.584
d 7 to 14							
ADG, lb	0.22^{b}	0.10^{b}	0.20^{b}	0.56^{a}	0.58^{a}	0.216	< 0.0001
ADFI, lb	0.46^{b}	0.47^{ab}	0.56^{ab}	0.67^{a}	0.66^{a}	0.130	0.006
G:F	0.177^{bc}	-0.122^{c}	-0.152^{c}	0.976^{a}	0.819^{ab}	0.4047	< 0.0001
d 14 to 21							
ADG, lb	0.70	0.60	0.87	0.59	0.78	0.132	0.060
ADFI, lb	0.75^{b}	0.72^{b}	0.99^{ab}	0.99^{ab}	1.15^{a}	0.158	0.001
G:F	0.973^{a}	0.860^{ab}	0.977^{a}	0.588^{c}	0.688^{bc}	0.0688	< 0.0001
Challenge period (d 7 to 21)							
ADG, lb	0.44^{b}	0.39^{b}	0.56^{ab}	0.58^{ab}	0.68^{a}	0.071	0.001
ADFI, lb	0.59^{b}	0.58^{b}	0.76^{ab}	0.83^{a}	0.91^{a}	0.070	< 0.0001
G:F	0.776	0.728	0.719	0.715	0.756	0.0505	0.640
Fecal dry matter, %[2]							
d 7	14.7	16.9	17.0	14.0	18.2	1.62	0.317
d 10	10.7^{c}	10.0^{c}	13.0^{bc}	20.5^{a}	17.1^{ab}	1.65	< 0.0001
d 14	16.7^{ab}	14.5^{b}	18.8^{ab}	20.8^{ab}	22.1^{a}	1.95	0.024
d 17	15.5^{b}	25.5^{a}	18.7^{ab}	20.1^{ab}	21.2^{ab}	2.36	0.027
d 21	18.0	22.1	22.9	18.7	19.5	1.71	0.115

[1A] total of 90 pigs were used with 1 pig per pen and 18 replicates per treatment. Dietary treatments consisted of: high CP (1.35% SID Lys, 20.7% CP), low CP (1.17% SID Lys, 17.9% CP), low CP + AA (1.35% SID Lys, 17.9% CP), low CP + ZnO (1.17% SID Lys, 17.9% CP, approx. 3,000 ppm Zn from ZnO), and low CP + AA + ZnO (1.35% SID Lys, 17.9% CP, approx. 3,000 ppm Zn from ZnO). Treatment diets were fed in a pellet form from d 0 to 21. On d 7, pigs were challenged with 6 mL of solution containing 10^{7} CFU/mL of ETEC F18 and followed with 6 mL of phosphate buffered saline. On d 21, all pigs were euthanized.

[2] Treatment × day interaction, $P < 0.0001$.

Table 3. Effect of low crude protein formulation strategies with and without pharmacological ZnO on serum cytokine concentrations (fluorescence intensity values) of F18 *E. coli* challenged nursery pigs[1]

	Dietary treatment						
Item	High CP	Low CP	Low CP + AA	Low CP + ZnO	Low CP + AA + ZnO	SEM	*P* =
GM-CSF	15.8	19.7	16.7	17.9	21.0	3.48	0.186
IFNγ	38.6	47.0	33.9	40.4	42.2	17.08	0.736
IL-1α	44.9	65.0	49.7	41.4	63.4	63.42	0.677
IL-1β	69.0	108.1	72.5	71.3	104.4	53.48	0.319
IL-2	35.8	50.4	39.9	38.3	53.4	35.54	0.772
IL-4	47.0	66.9	51.7	45.2	65.3	39.77	0.693
IL-6	48.3	75.3	52.7	52.1	83.8	44.85	0.293
IL-8	374.3[a]	340.6[ab]	269.6[abc]	95.7[c]	120.6[bc]	148.70	0.001
IL-10	54.2	70.6	59.5	48.8	76.0	43.51	0.693
IL-12	971.7	948.6	896.0	1097.9	986.9	94.65	0.327
IL-18	66.4	84.8	61.7	70.7	92.3	41.01	0.562
TNFα	26.7	40.6	32.2	26.4	38.1	13.56	0.328
IL-1RA	227.0[ab]	537.2[a]	306.6[ab]	161.6[b]	223.9[ab]	145.97	0.014

[1] A total of 90 pigs were used with 1 pig per pen and 18 replicates per treatment. Blood samples were collected on d 14 and serum cytokines were analyzed at Eve Technologies, Calgary, AB, Canada using a multiplex immunoassay that quantifies 13 cytokines.