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Changing Dietary Calcium-Phosphorus Level and Cereal Source Selectively Alters Abundance of Bacteria and Metabolites in the Upper Gastrointestinal Tracts of Weaned Pigs

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Several dietary ingredients may affect the bacterial community structure and metabolism in the porcine gut and may therefore influence animals' health and performance. This study investigated the effects of cereal source and calcium-phosphorus (CaP) level in the diet on bacterial microbiota and metabolites, nutrient intake, and gut environment in weaned pigs. Pigs ($n = 8$/treatment) were fed wheat-barley- or corn-based diets with an adequate or high CaP level for 14 days. Effects on microbiota in the stomach, ileum, and midcolon were assessed using quantitative PCR. Data showed that *Enterobacteriaceae*, *Campylobacter* spp., and *Helicobacter* spp., which all contain highly immune reactive lipopolysaccharide (LPS), were abundant at all gut sites. Diet effects on bacteria and metabolites were moderate and occurred mainly in the upper gut, whereas no effects on bacteria, fermentation products, and LPS could be observed in the colon. Differences in carbohydrate intake with corn versus wheat-barley diets selectively stimulated *Bifidobacterium* in the stomach and ileum. There was a growth advantage for a few bacterial groups in the stomach and ileum of pigs fed the high versus adequate CaP level (i.e., gastric *Enterobacteriaceae* and ileal *Enterococcus*, *Bacteroides-Prevotella-Porphyromonas*, and *Campylobacter*). Interestingly, gastrointestinal pH was not affected by dietary CaP level. The present findings demonstrate the stability of the bacterial community and gut environment toward dietary changes even in young pigs. The results on stimulation of gastric and ileal *Bifidobacterium* by corn diets may be employed in nutritional strategies to support gut health after weaning.

The commensal gut microbiota play an important role in the host animal and are involved in nutrient utilization, absorption, and metabolism as well as in the modulation of host immunity, thereby influencing host health and production efficiency (1). Diet is one of the major environmental factors regulating the microbial eubiosis in the porcine gut (2, 3). Therefore, to gain a better understanding on the impact of the gut microbiota upon a pig's performance and health, it is necessary to characterize the effect of dietary ingredients on the bacterial community structure and metabolites (4). Pigs reared in commercial production settings achieve only about 70% of their genetic potential for growth due to permanent immune stimulation (5). Among stressors that can attenuate a pig's growth performance are virulence factors expressed by gut bacteria (6, 7). One gut immunogenic factor of bacterial origin that is receiving increasing attention in recent years is lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria (7, 8). Depending on the lipid A fraction of the LPS molecule and thus on the bacterial origin, LPS that enters into the systemic circulation is a potent immune stimulator in pigs (7). Genera that belong to the commensal microbiota in the porcine gut with LPS of high immune reactivity are, among others, *Escherichia*, *Helicobacter*, *Campylobacter*, and *Fusobacterium* (9). Yet, comparatively little is known about the influence of dietary composition on *Helicobacter*, *Campylobacter* and *Fusobacterium* in pigs. In contrast, enterotoxigenic *Escherichia coli* abundance in the porcine gut could be associated with dietary viscous nonstarch polysaccharides (NSP) (3).

Common components of pig diets such as cereal grains largely differ in their starch, protein, and NSP fractions (10) and as a consequence may modulate the composition of the gut bacterial community and their metabolites (3, 11). Evidence is emerging that feeding minerals above an animal's nutritional requirements may also promote gut microbiota, either by meeting special microbial nutrient needs or by influencing environmental conditions in the gut lumen for microbial growth (12–14). High dietary calcium-phosphorus (CaP) levels were used to promote intestinal lactobacilli and decrease enterobacteria, including pathogens, in rats (15). However, in young pigs, high dietary Ca levels may challenge the immature gastric barrier function due to its high acid-buffering capacity (16); therefore, CaP levels above the requirement are rarely used in pig nutrition, and little is known about the ability of high CaP levels to modulate the porcine gut microbiota.

Because pigs in the postweaning period undergo substantial changes in their gastrointestinal physiology, microbiology, and immunology and are therefore more susceptible to intestinal and immunological disturbances than older animals (17), we used weaned pigs in the present research. Our objective was to assess the effects of diets differing in their cereal composition and CaP level on shifts in the gastric, ileal, and colonic bacterial communi-

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ties, including Gram-negative bacterial groups containing highly immune-reactive LPS, and their metabolic activity, nutrient intake, and gut physiological parameters in weaned pigs.

MATERIALS AND METHODS

Animals and diets. All procedures involving animal handling and treatment were approved by the institutional ethics committee of the University of Veterinary Medicine and the national authority according to §8ff of the Law for Animal Experiments, Tierversuchsgesetz (TVG) (GZ 68.205/0222-II/3b/2011). A total of 32 barrows [(Landrace × Large White] × Piétrain) from 16 litters weaned at 28 days (average body weight [BW], 8.7 ± 0.07 kg) were used in a 2-by-2 factorial arrangement of dietary treatments. The experiment was carried out in 4 replicates with 8 pigs each. Pigs were individually housed in stainless steel metabolism cages (0.85 m by 1.00 m). The cages were equipped with a heating lamp, and the room temperature was kept at 21 to 22°C. Prior to the experiment, pigs were allowed a 7-day adjustment period with *ad libitum* access to water and commercial prestarter diet. On day 7 after weaning, pigs (average BW, 9.5 ± 0.11 kg) were randomly allocated to 4 feeding groups ($n = 8$/treatment), with 2 pigs/replicate fed the same diet. Siblings did not receive the same diet. Pigs had free access to water and feed throughout the experiment.

Four experimental diets were formulated to meet or exceed nutrient requirements (Table 1) (18). These four experimental diets differed in the cereal source (either wheat and barley or corn) and the level of CaP as follows: (i) wheat-barley diet with adequate CaP level, (ii) wheat-barley diet with high CaP level, (iii) corn diet with adequate CaP level, and (iv) corn diet with high CaP level. The CaP level of the adequate-CaP diets was formulated to contain 100% of the requirement for 10- to 20-kg pigs (18). The high-CaP diets were formulated to contain a CaP level of 190% of the Ca and P requirement for 10- to 20-kg pigs. Microbial phytase was added to the diets to compensate for different intrinsic phytase activities and thus plant P availabilities in wheat, barley, and corn (18). The experimental diets were fed *ad libitum* for 14 days. One pig fed the wheat-barley diet with a high CaP level was removed from the experiment because it developed meningitis at the commencement of the experiment. Except for the one pig with meningitis, pigs were healthy and did not develop any symptoms of enteric disease throughout the experiment.

Sample collection. From day 11 to 14 of the experiment, freshly defecated feces were collected from the cage floor and immediately frozen at −20°C. On day 15 of the experiment, 3 to 4 h after their last feeding, pigs were anesthetized and blood was collected from the heart into serum collection tubes (Primavette V Serum; KABE Labortechnik, Nürnbrecht-Elsenroth, Germany), which was followed by euthanization of pigs and exsanguination. Thereafter, the abdominal cavity was opened and the entire gastrointestinal tract was removed. To prevent mixing of digesta among gut sites, gut sites (stomach, duodenum, jejunum, ileum, cecum, and proximal, mid-, and distal colon) were immediately separated using clamps. Digesta from the stomach, ileum (20 cm before the ileo-cecal junction), and midcolon (the colon was divided into three equal parts) were aseptically collected. Digesta from each gut site were homogenized before subsamples of digesta for bacterial quantification were snap-frozen in liquid nitrogen and stored at −80°C. Other subsamples of digesta for measurements of microbial metabolites (short-chain fatty acids [SCFA], lactate, and LPS), pH, and dry matter were placed in an ice-water bath until transfer to −20°C. The ileum was empty in 4 pigs at the time of euthanization; therefore, ileal digesta could be collected from only 27 pigs ($n = 7$ for wheat-barley diet with adequate CaP level, $n = 7$ for wheat-barley diet with high CaP level, $n = 5$ for corn diet with adequate CaP level, and $n = 8$ for corn diet with high CaP level). Blood was centrifuged (10 min, 1,811 × *g*) (Eppendorf centrifuge 5810R; Eppendorf, Hamburg, Germany), and serum was stored at −20°C.

Genomic DNA extraction and quantitative PCR (qPCR). Total genomic DNA was extracted from 250-mg digesta samples from stomach, ileum, and colon using a PowerSoil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions with few modifications. To ensure proper lysis of bacteria, a heating step at 70°C for 10 min was introduced between mixing of the digesta sample with buffer C1 and bead beating. The genomic DNA concentration and purity were measured using a ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNAs from gastric, ileal, and colonic digesta samples were adjusted to concentrations of 10, 20, and 20 ng/μl, respectively.

Bacterial groups targeted in the present study were selected on the basis of recent studies, representing dominant bacterial groups of the gut microbiota in pigs (3, 13). Additionally, primers were selected to target *Helicobacter*, *Campylobacter*, and *Fusobacterium* as bacterial groups with LPS of high immune reactivity. Primers were commercially synthesized (Eurofins MWG Operon, Ebersberg, Germany), and targets of selected primers were checked and updated using Primer BLAST search of GenBank sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The quantification of DNA in digesta samples was performed using a Stratagene

TABLE 1 Ingredients and analyzed chemical compositions of the experimental diets

Parameter	Value in:			
	Wheat-barley diet		Corn diet	
	Adequate CaP	High CaP	Adequate CaP	High CaP
Ingredient (%)				
Corn			49.40	49.40
Wheat	35.20	35.20		
Soybean meal	28.00	28.00	33.50	33.50
Barley	18.20	18.20		
Dextrose	5.00	5.00	5.00	5.00
Talcum	3.32	0.27	3.43	0.40
Saccharose	3.00	3.00	3.00	3.00
Soy oil	3.00	3.00	1.50	1.50
Limestone	1.30	1.95	1.25	1.93
Salt	1.00	1.00	1.00	1.00
Monocalcium phosphate	0.75	3.15	0.85	3.20
Vitamin-mineral premix[a]	0.63	0.63	0.63	0.62
Lysine	0.35	0.35	0.24	0.25
L-Threonine	0.15	0.15	0.10	0.10
DL-Methionine	0.10	0.10	0.10	0.10
Analyzed chemical composition (dry matter basis, g/kg)				
Dry matter	929	917	921	929
Crude protein	222	223	222	218
Crude ash	94	96	95	96
Crude fiber	36	37	36	36
Xylose	16	18	11	12
β-Glucan	9	9	1	1
NDF[b]	117	122	112	104
Total starch	302	310	299	301
Ca	8.2	14.8	8.4	14.1
P	6.0	11.9	6.6	11.7
Metabolizable energy (MJ/kg)[c]	13.5	13.5	13.5	13.5

[a] Provided per kilogram of complete diet: 10,000 IU of vitamin A, 2,222 IU of vitamin D_3, 62.5 mg of vitamin E, 1.67 mg of vitamin B_1, 4.45 mg of vitamin B_2, 2.22 mg of vitamin B_6, 0.022 mg of vitamin B_{12}, 2.2 mg of vitamin K, 22.2 mg of niacin, 11.11 mg of pantothenic acid, 500 mg of choline chloride, 0.05 mg of biotin, 0.56 mg of folic acid, 25 mg of vitamin C, 44 mg of Mn (as MnO), 89 mg of Zn (as $ZnSO_4$), 153 mg of Fe (as $FeSO_4$), 13 mg of Cu (as $CuSO_4$), 0.44 mg of Se (as Na_2SeO_3), and 1.67 mg of I [as $Ca(IO_3)_2$]. Included 500 FTU phytase per kilogram of complete diet.
[b] NDF, neutral detergent fiber.
[c] Calculated values (18).

Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA). Each amplification reaction was run in duplicate in a final volume of 25 μl using Brilliant II SYBR green QPCR Low ROX master mix (Agilent Technologies) mixed with the selected primer set (see Table S1 in the supplemental material) at a concentration of 400 nmol for each primer and 1 μl of genomic DNA. All qPCR amplifications were optimized and performed in 0.2-ml 96-well plates using the following program: initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, optimal annealing temperature (see Table S1 in the supplemental material) for 30 s, and 72°C for 30 s. Fluorescence was measured at the last step of each cycle. To determine the specificity of the amplification, the dissociation characteristics of double-stranded DNA were determined by melting curve analysis. The dissociation of PCR products were monitored by slow heating with an increment of 0.1°C/s from 55 to 95°C, with fluorescence measurement at 0.1°C intervals. Correct PCR product length was checked by gel electrophoresis. Negative controls without template DNA were included in triplicate.

For quantification of total bacterial 16S rRNA gene copy numbers, standard curves were constructed by using universal primers to amplify serial dilutions of purified PCR products from genomic DNA of digesta using the universal primer set 27F-1492R as recently described by Metzler-Zebeli et al. (19). Standard curves for target bacterial groups were generated using serial dilutions (10^7 to 10^3 molecules/μl) of the purified and quantified PCR products generated by standard PCR with genomic DNA from pig gastrointestinal digesta (3, 13). Amplification efficiency was calculated according to the equation $E = 10^{-1/\text{slope}}$ (see Table S1 in the supplemental material). Gene copy numbers of total bacteria and target bacterial groups in 10, 20, and 20 ng DNA in gastric, ileal, and colonic digesta samples, respectively, were determined by relating the *Cq* values to standard curves. The final copy numbers of total bacteria and target bacterial groups per gram of digesta were calculated using the equation $(QM \times C \times DV)/(S \times V)$, where QM was the quantitative mean of the copy number, *C* was the DNA concentration of each sample, DV was the dilution volume of extracted DNA, *S* was the DNA amount (ng) subjected to analysis, and *V* was the weight of the sample (g) subjected to DNA extraction (20). Coverage of total bacteria by bacterial groups was expressed as percentage of total bacteria (20).

Chemical analyses. Diets were analyzed for dry matter, crude protein (N × 6.25), crude fiber, ether extract, crude ash, neutral detergent fiber (NDF), acid detergent fiber (ADF), xylose, β-glucan, total starch, Ca, and P. Dry matter and proximate nutrients, including Ca and P were analyzed according to VDLUFA (21). Total starch, mixed-linked β-glucan, and xylose in diets were determined using enzymatic kits (Megazyme International Ireland Ltd., Bray, Ireland). The dry matter content and pH in gastric, ileal and colonic digesta, and feces were determined. Before dry matter and pH determination, feces samples were thawed at 4°C and homogenized for each animal. The pH was measured using a Beckman Φ63 pH meter (Beckman Coulter, Fullerton, CA). Double-distilled water was added to colonic digesta and fecal samples at equal volumes to allow their pH to be read. Short-chain fatty acid concentrations in gastric, ileal, and colonic digesta were analyzed using gas chromatography as recently described (13). Concentrations of D- and L-lactate in intestinal digesta were determined using an enzymatic kit (Megazyme International Ireland Ltd., Bray, Ireland).

LAL assay. Concentrations of cell-free LPS in intestinal digesta and serum were determined using the pyrochrome *Limulus* amebocyte lysate (LAL) assay (Associates of Cape Cod Inc., East Falmouth, MA). Differences in bacterial communities in the upper and lower intestines make it necessary to investigate bacterial metabolites at different gut sites. The amount of ileal digesta collected from pigs was very small; therefore, LPS in colonic digesta and feces was determined as an example. Serum was used directly for the LAL assay, whereas colonic and fecal samples were diluted 750,000- to 1,500,000-fold using pyrogen-free LAL reagent water (Associates of Cape Cod Inc.), depending on the actual LPS concentration in the sample. Serum and diluted colonic and fecal samples were deproteinized by heating the samples for 15 min at 75°C (Thermomixer Comfort; Eppendorf, Vienna, Austria). Samples were centrifuged at 12,100 × *g* for 10 min, and 200 μl of the supernatant was used in the assay. In pyrogen-free reaction tubes (Pyrotube-K; Associates of Cape Cod Inc.), 50 μl of pyrochrome LAL reagent dissolved in Glucashield buffer (Associates of Cape Cod In.) was added to the supernatant, and the pyrochrome-sample mixture was immediately incubated at 37°C in a Pyros Kinetix Flex tube reader (Associates of Cape Cod Inc.). Using the chromogenic method, changes in the optical density of samples at 405 nm were measured against calibration curves using Pyros EQS software (Associates of Cape Cod Inc.). Reactions were run in duplicate, and the intra-assay coefficient of variation was <10%.

Statistical analysis. To compare differences among diets and gut sites, data were subjected to analysis of variance (ANOVA) using the PROC MIXED of SAS (Statistical Analysis System 9.2; SAS Institute Inc., Cary, NC, USA). For the gastrointestinal tract site effect, the model included fixed effects of intestinal site and replicate and the random effect of pig ($n = 31$) nested within sow ($n = 16$), considering the pig as the experimental unit. Data collected on the same pig and various gut sites were considered spatial repeated measures, and the covariance structure of these repeated measures was modeled separately according to the smallest values of the fit statistics based on the Bayesian information criteria (BIC). For diet effects, fixed effects in the model included the main effects of cereal and CaP level and their two-way-interactions as well as replicate and the random effect of pig ($n = 31$) nested within sow ($n = 16$), considering the pig as the experimental unit. Means were reported as least-squares mean ± standard error of the mean (SEM), with a *P* value of ≤0.05 as defined as significant and a P value of >0.05 but ≤0.10 as trends. Degrees of freedom were approximated using the Kenward-Rogers method (with ddfm = kr).

If a diet effect on bacterial groups and microbial metabolites was observed, multiple regression analysis was performed with the PROC REG of SAS using backward elimination to evaluate and quantify the relationships among independent variables (daily intakes of NDF, xylose, β-glucan, starch, Ca, or P) and the abundance of bacterial groups and concentration of microbial metabolites. To limit model overparameterization, a variance inflation factor of less than 10 (which assumes no significant multicollinearity among predictor variables tested) for every continuous independent variable tested was assumed (22).

RESULTS

Daily carbohydrate intake and digesta characteristics. Daily feed intake was similar ($P > 0.1$) among dietary treatments (679, 631, 725, and 702 ± 23 g/day for wheat-barley diet with adequate CaP, wheat-barley diet with high CaP, corn diet with adequate CaP, and corn diet with high CaP, respectively). Although representing only small fractions of dietary carbohydrates, daily xylose and β-glucan intakes were higher ($P < 0.01$) for the wheat-barley diets than for the corn diets, whereas the daily starch intake tended ($P < 0.1$) to be higher for the corn diets than for the wheat-barley diets (Table 2). Accordingly, daily Ca and P intake was 4 to 5 g/day higher with the high-CaP diets than with the adequate-CaP diets. Gastric, ileal, colonic, and fecal pHs were not influenced by the cereal sources and CaP levels of the diets (Table 3); only gut site influenced gastrointestinal pH (see Table S2 in the supplemental material). Interestingly, the gastric dry matter content was lower than the dry matter content of feces, but it was higher than the dry matter content of colonic and ileal digesta ($P < 0.05$) (see Table S2 in the supplemental material). The corn diets reduced ($P < 0.05$) gastric dry matter content by 2.6% and increased colonic dry matter content by 4.3% compared to those with the wheat-barley diets (Table 3). Compared to the adequate CaP level, the high CaP level tended ($P < 0.1$) to reduce dry matter content in ileal digesta by 2.9%.

TABLE 2 Daily fiber and starch intake in weaned pigs fed cereal diets with different CaP levels[a]

Component	g/day (least-square mean) with:				Pooled SEM	*P* value		
	Wheat-barley diet		Corn diet					
	Adequate CaP	High CaP	Adequate CaP	High CaP		Cereal	CaP	Cereal × CaP
Xylose	13	13	9	10	0.5	<0.001	0.35	0.58
β-Glucan	7	7	1	1	0.3	<0.001	0.15	0.76
NDF[b]	97	92	92	88	4.1	0.30	0.31	0.97
Starch	248	225	255	255	10.7	0.09	0.29	0.29
Ca	6.8	10.9	7.0	12.0	0.4	0.14	<0.001	0.30
P	5.0	9.0	5.4	10.0	0.3	0.04	<0.001	0.46

[a] $n = 8$ pigs/treatment, except for wheat-barley diet with high CaP level ($n = 7$ pigs).
[b] NDF, neutral detergent fiber.

Gut site effect on bacterial microbiota. The bacterial microbiota in gastric and ileal digesta were dominated by *Firmicutes*, whereas *Bacteroidetes* was the main phylum in colonic digesta (Table 4). Colonic digesta contained more ($P < 0.001$) bacteria than ileal and gastric digesta. Strictly anaerobic bacterial groups, such as *Bacteroides-Prevotella-Porphyromonas* and *Clostridium* clusters IV and XIV, could be found in gastric digesta in considerable numbers and were not restricted only to the lower gut sites. Gram-negative bacterial genera that contain opportunistic pathogens and are sources of LPS with high immune reactivity, i.e., *Enterobacteriaceae*, *Helicobacter* spp., *Campylobacter* spp., could be detected at all gut sites. In contrast, *Fusobacterium* spp. could not be found in all pigs. Specifically, this group was detected in 22 out of 31 gastric digesta samples, 7 out of 27 ileal digesta samples, and 2 out of 31 colonic digesta samples. *Fusobacterium* was not detected in 9 pigs at all.

Diet effects on bacterial microbiota. Because the bacterial composition differed among gut sites and diet effects were different at the three gut sites, diet effects on the bacterial groups were separately analyzed per gut site. In gastric digesta, gene copies of *Enterobacteriaceae* increased ($P < 0.01$) in pigs fed the high versus the adequate CaP level (Table 5). As indicated by the cereal-CaP interaction ($P < 0.05$), the 0.9-log-unit increase in *Enterobacteriaceae* with a high versus adequate CaP level was observed only with the corn diet and not with the wheat-barley diet. Feeding corn versus wheat-barley diets enhanced ($P < 0.05$) gene copy numbers of *Bifidobacterium* spp. in gastric digesta by about 0.8 log unit. In ileal digesta, corn diets increased ($P < 0.01$) gene copies of total bacteria by 0.4 log unit compared to wheat-barley diets, being associated with 1.1 and 0.7 log units more gene copies of *Bifidobacterium* spp. and, as a trend ($P < 0.1$), of *Streptococcus* spp., respectively, than the wheat-barley diets. The high CaP level enhanced ($P < 0.05$) gene copies of *Enterococcus* spp., the *Bacteroides-Prevotella-Porphyromonas* group, and *Campylobacter* spp. in ileal digesta by about 0.7, 0.5, and 0.7 log units compared to those with the adequate CaP level, respectively. Interestingly, there was a trend ($P < 0.1$) for a cereal-CaP interaction for *Helicobacter* spp. in ileal digesta, indicating that the high CaP level increased *Helicobacter* sp. gene copies by 1.1 log units when combined with the wheat-barley diet but decreased *Helicobacter* sp. gene copies by 0.8 log unit when combined with the corn diet compared to the adequate CaP level. In colonic digesta, dietary cereals and CaP level interacted ($P < 0.01$) for *Clostridium* cluster I, showing that with the wheat-barley diet the high CaP level reduced their gene copies by 1.9 log units, whereas with the corn diet it increased *Clostridium* cluster I gene copies by 0.8 log unit compared to the adequate CaP level. Diets did not affect coverage of total bacteria by bacterial groups. As indicated by the SEM for the ileum (Table 5), there were some great differences in coverage among individual pigs.

Bacterial metabolites. Lactate was the predominant fermentation end product in gastric and ileal digesta, whereas in colonic

TABLE 3 Physicochemical characteristics of gastric, ileal, and colonic digesta and feces in weaned pigs fed cereal diets with different CaP levels[a]

Parameter	Value (least-square mean) with:				Pooled SEM	*P* value		
	Wheat-barley diet		Corn diet					
	Adequate CaP	High CaP	Adequate CaP	High CaP		Cereal	CaP	Cereal × CaP
Dry matter content (%)								
Stomach	23	21	19	19	1.2	0.04	0.42	0.51
Ileum	17	13	14	13	1.3	0.25	0.08	0.24
Colon	24	25	29	29	1.9	0.03	0.57	0.84
Feces	30	27	31	30	1.5	0.25	0.18	0.50
pH								
Stomach	3.7	3.9	3.3	3.7	0.4	0.40	0.36	0.76
Ileum	6.6	6.0	6.4	5.6	0.5	0.60	0.17	0.88
Colon	6.1	6.0	5.9	5.8	0.1	0.16	0.48	0.66
Feces	6.4	6.7	6.4	6.6	0.2	0.47	0.13	0.67

[a] For stomach, colon, and feces, $n = 8$ pigs/treatment, except for wheat-barley diet with high CaP level ($n = 7$ pigs). For ileum, $n = 7$ pigs for wheat-barley with adequate CaP level, $n = 7$ pigs for wheat-barley diet with high CaP level, $n = 5$ pigs for corn diet with adequate CaP level, and $n = 8$ pigs for corn diet with high CaP level.

TABLE 4 Comparison of 16S rRNA gene copy numbers found at different gut sites in weaned pigs[a]

Bacteria	Log_{10} gene copies/g digesta (least-square mean)[b]			Pooled SEM	*P* value
	Stomach	Ileum	Colon		
Total bacteria	8.9 b	9.1 b	10.7 a	0.08	<0.001
Firmicutes					
Lactobacillus group	8.3 b	8.1 b	9.0 a	0.11	<0.001
Streptococcus spp.	7.0	6.8	7.0	0.12	0.46
Enterococcus spp.	4.9	5.0	5.2	0.11	0.12
Clostridium cluster XIV	6.6 b	5.6 c	9.0 a	0.11	<0.001
Clostridium cluster IV	6.4 b	5.8 c	9.1 a	0.11	<0.001
Clostridium cluster I	5.3 c	6.5 b	7.2 a	0.18	<0.001
Actinobacteria					
Bifidobacterium spp.	3.6 b	6.9 a	3.7 b	0.23	<0.001
Bacteroidetes					
Bacteroides-Prevotella-Porphyromonas	7.2 b	6.3 c	10.1 a	0.12	<0.001
Proteobacteria					
Enterobacteriaceae	6.0 c	7.9 a	7.5 b	0.16	<0.001
Helicobacter spp.	4.7 c	5.6 b	6.6 a	0.18	<0.001
Campylobacter spp.	5.5 b	5.4 b	7.5 a	0.14	<0.001
Fusobacteria					
Fusobacterium spp.[c]	3.6			0.15	

[a] Mean of $n = 31$ pigs for stomach, $n = 27$ pigs for ileum, and $n = 31$ pigs for colon.
[b] Different letters within rows indicate a significant difference ($P < 0.05$).
[c] *Fusobacterium* spp. were detected in gastric digesta of 22 out of 31 pigs. Because *Fusobacterium* spp. could be found only in ileal digesta of 7 out of 27 pigs and in colonic digesta of 2 out of 31 pigs, gut site comparison was not performed for *Fusobacterium* spp.

digesta SCFA dominated (see Table S2 in the supplemental material). There were cereal-CaP interactions ($P < 0.05$) for total SCFA, acetate, and isovalerate in gastric digesta, indicating that the high CaP level increased their concentrations when combined with the wheat-barley diet but not with the corn diet (Table 6). Also, trends ($P < 0.1$) for similar cereal-CaP interactions for butyrate, isobutyrate, and valerate in gastric digesta were observed. Interestingly, gastric caproate concentrations were higher ($P < 0.05$) with the wheat-barley diets than with the corn diets. Due to a higher L-lactate concentration ($P < 0.05$), total lactate tended ($P < 0.1$) to be higher in ileal digesta of pigs fed the corn diets than in those fed the wheat-barley diets. In ileal digesta, there was a cereal-CaP interaction for caproate ($P < 0.05$) and, as a trend, for propionate ($P < 0.1$), indicating that the high CaP level decreased their concentrations in pigs fed wheat-barley diets but increased them in pigs fed corn diets. In colonic digesta, only the caproate concentration was reduced ($P < 0.05$) in pigs fed the high CaP level compared to those fed the adequate CaP level.

LPS concentrations in serum and feces were not different among the diets (Table 6). However, there was a trend for a cereal-CaP interaction ($P < 0.1$) for LPS concentrations in colonic digesta. This trend showed that with the wheat-barley diet the high CaP level seemed to reduce colonic LPS but that LPS was increased with the corn diet compared to the respective cereal diet with an adequate CaP level. When comparing colonic and fecal LPS concentrations, these were higher ($P < 0.001$) in colonic digesta than in feces.

Regression analysis. Multiple regression analysis was conducted to establish the relationships between gastric and ileal bacterial groups and metabolites (all dependent variables that were affected by diet) and the intake of dietary complex carbohydrate (NDF, xylose, β-glucan, and starch) and Ca and P. Gastric *Enterobacteriaceae* positively correlated to β-glucan and Ca intake ($y = 5.04 + 0.04 \times$ β-glucan $+ 0.09 \times$ Ca, root mean square error [RMSE] = 0.18, $r^2 = 0.88$, $P < 0.001$). Ileal abundances of *Enterococcus* ($y = 4.24 + 0.084 \times$ Ca, RMSE = 0.10, $r^2 = 0.61$, $P < 0.001$), *Bacteroides-Prevotella-Porphyromonas* ($y = 5.61 + 0.07 \times$ Ca, RMSE = 0.25, $r^2 = 0.37$, $P < 0.001$), and *Campylobacter* ($y = 4.00 + 0.15 \times$ Ca, RMSE = 0.19, $r^2 = 0.83$, $P < 0.001$) were positively linked to dietary Ca.

DISCUSSION

The present results showed that gastrointestinal bacterial abundances and metabolites were relatively similar among weaned pigs fed different dietary CaP levels and cereal sources. These findings demonstrate the steadiness of bacterial genera and clusters in a pig's gut in response to dietary changes, even at a young age of the pig when the bacterial community is still developing. In contrast to previous findings (23), the effect of cereal source was small in the present study, with only *Bifidobacterium* spp. being selectively promoted in the upper gut with corn versus wheat-barley diets without affecting the abundance of other bacterial groups. Accordingly, CaP increased the abundance of a few bacterial groups, but also only in the upper gut. It is surprising that bacterial numbers and fermentation end products in colonic digesta remained almost unaffected by diet.

Coverages of total bacteria by bacterial groups in gastric, ileal, and colonic digesta supported a high complexity and diversity of the gut microbiota of weaned pigs and were similar to those reported by Castillo et al. after feeding complex cereal-based diets to 14-week-old pigs (24). Recently, we showed that total bacterial gene copies in gastric, cecal, and colonic digesta of weaned pigs were covered by 80 to 100% by bacterial groups, using a similar set of primers (13). Obviously, the bacterial microbiota of the present pigs were substantially different from those of the pigs used before, which may be related to either the pigs' origin or the dietary composition. With the limitations of a targeted qPCR approach and great divergence in bacterial communities of the host species,

TABLE 5 16S rRNA gene copy numbers of bacterial groups in the stomach, ileum, and colon of weaned pigs fed cereal diets with different CaP levels[a]

Site and bacteria	Log_{10} gene copies/g digesta (least-square mean)					*P* value		
	Wheat-barley diet		Corn diet					
	Adequate CaP	High CaP	Adequate CaP	High CaP	Pooled SEM	Cereal	CaP	Cereal × CaP
Stomach								
Total bacteria	8.7	9.0	8.9	8.9	0.19	0.72	0.36	0.62
Firmicutes								
Lactobacillus group	8.2	8.5	8.2	8.4	0.27	0.95	0.45	0.86
Streptococcus spp.	6.8	7.1	6.8	7.2	0.25	0.82	0.20	0.87
Enterococcus spp.	5.1	5.7	5.6	5.6	0.31	0.59	0.36	0.34
Clostridium cluster XIV	6.3	6.7	6.5	6.9	0.29	0.43	0.21	0.92
Clostridium cluster IV	6.3	6.1	6.3	6.8	0.26	0.19	0.47	0.22
Clostridium cluster I	5.5	5.2	5.1	5.4	0.32	0.82	0.67	0.40
Actinobacteria								
Bifidobacterium spp.	5.1	5.6	6.2	6.1	0.38	0.04	0.66	0.50
Bacteroidetes								
Bacteroides-Prevotella-Porphyromonas	6.7	7.3	7.5	7.4	0.32	0.12	0.42	0.28
Proteobacteria								
Enterobacteriaceae	6.1	6.2	5.4	6.3	0.17	0.18	0.006	0.03
Helicobacter spp.	5.2	4.1	4.8	4.7	0.40	0.70	0.12	0.24
Campylobacter spp.	5.1	5.7	5.6	5.6	0.31	0.59	0.36	0.34
Fusobacteria								
Fusobacterium spp.[b]	4.7 (5)	4.9 (6)	4.8 (4)	5.1 (7)	0.21	0.55	0.22	0.95
Coverage of total bacteria (%)	57.9	54.0	42.0	49.5	8.77	0.26	0.84	0.52
Ileum								
Total bacteria	8.8	8.9	9.1	9.4	0.13	0.005	0.11	0.62
Firmicutes								
Lactobacillus group	8.0	7.9	8.4	8.2	0.22	0.12	0.40	0.84
Streptococcus spp.	6.4	6.5	6.9	7.3	0.32	0.06	0.50	0.60
Enterococcus spp.	5.2	5.8	4.8	5.8	0.29	0.51	0.02	0.50
Clostridium cluster XIV	5.2	5.7	5.7	5.6	0.18	0.29	0.23	0.09
Clostridium cluster IV	5.7	6.0	5.6	6.0	0.24	0.64	0.17	0.82
Clostridium cluster I	6.2	6.5	6.5	7.2	0.46	0.17	0.20	0.61
Actinobacteria								
Bifidobacterium spp.	4.9	5.6	6.6	6.3	0.45	0.01	0.59	0.27
Bacteroidetes								
Bacteroides-Prevotella-Porphyromonas	5.8	6.5	6.3	6.7	0.23	0.17	0.03	0.60
Proteobacteria								
Enterobacteriaceae	8.1	8.3	7.5	8.1	0.36	0.24	0.30	0.55
Helicobacter spp.	4.9	6.0	6.1	5.3	0.47	0.65	0.84	0.06
Campylobacter spp.	5.2	5.8	4.8	5.8	0.29	0.51	0.02	0.50
Coverage of total bacteria (%)	81.0	72.9	47.0	55.9	21.58	0.25	0.99	0.70
Colon								
Total bacteria	10.8	10.6	10.6	10.7	0.14	0.84	0.81	0.29
Firmicutes								
Lactobacillus group	9.2	8.9	9.0	9.1	0.18	0.98	0.71	0.27
Streptococcus spp.	6.9	7.0	6.9	7.1	0.18	0.89	0.43	0.92
Enterococcus spp.	7.7	7.1	7.5	7.6	0.23	0.41	0.26	0.16
Clostridium cluster XIV	9.0	9.0	8.8	9.1	0.16	0.73	0.49	0.33
Clostridium cluster IV	9.1	8.9	9.1	9.2	0.17	0.54	0.88	0.47
Clostridium cluster I	8.6	6.7	6.4	7.2	0.37	0.03	0.16	0.002
Actinobacteria								
Bifidobacterium spp.	6.1	6.6	6.7	6.8	0.44	0.37	0.48	0.71
Bacteroidetes								
Bacteroides-Prevotella-Porphyromonas	10.2	10.0	10.1	10.2	0.15	0.87	0.91	0.36
Proteobacteria								
Enterobacteriaceae	7.8	7.5	7.1	7.5	0.40	0.40	0.87	0.42
Helicobacter spp.	6.8	6.6	6.6	6.5	0.22	0.49	0.52	0.83
Campylobacter spp.	7.7	7.1	7.5	7.6	0.23	0.41	0.26	0.16
Coverage of total bacteria (%)	34.3	36.5	37.9	38.4	1.62	0.11	0.42	0.59

[a] For stomach and colon, $n = 8$ pigs/treatment, except for wheat-barley diet with high CaP level ($n = 7$ pigs). For ileum, $n = 7$ pigs for wheat-barley with adequate CaP level, $n = 7$ pigs for wheat-barley diet with high CaP level, $n = 5$ pigs for corn diet with adequate CaP level, and $n = 8$ pigs for corn diet with high CaP level.
[b] The number of positive pigs per dietary treatment in gastric digesta is given in parentheses. *Fusobacterium* spp. were detected in only 7 and 2 pigs in ileal and colonic digesta, respectively.

such as the pig, it may be advisable to include an upstream non-targeted 16S rRNA gene analysis such as terminal restriction fragment length polymorphism or high-throughput sequencing techniques in future studies.

According to the present results, bacterial groups that were previously linked to the lower gut, such as *Bacteroides-Prevotella-Porphyromonas* and *Clostridium* cluster IV and XIV, appear to tolerate diverging environmental conditions between gut seg-

TABLE 6 Concentrations of fermentation metabolites in the stomach, ileum, and colon and of LPS in sera, colonic digesta, and feces of weaned pigs fed cereal diets with different CaP levels[a]

Parameter	Value (least-square mean) with:				Pooled SEM	*P* value		
	Wheat-barley diet		Corn diet					
	Adequate CaP	High CaP	Adequate CaP	High CaP		Cereal	CaP	Cereal × CaP
Metabolite concn (μmol/g digesta)								
Stomach								
Total lactate	22.6	20.7	23.6	18.5	6.09	0.92	0.57	0.79
D-Lactate	12.9	12.8	14.6	11.5	4.19	0.97	0.71	0.73
L-Lactate	9.7	9.0	7.9	7.0	2.17	0.72	0.39	0.95
Total SCFA	4.2	16.5	14.1	8.4	4.23	0.83	0.44	0.04
Acetate	3.1	9.7	7.7	5.1	2.05	0.99	0.34	0.04
Propionate	0.4	3.9	3.2	2.4	1.40	0.66	0.33	0.13
Butyrate	0.2	1.7	2.2	0.5	0.79	0.63	0.87	0.06
Isobutyrate	0.2	0.4	0.3	0.3	0.05	0.57	0.35	0.07
Valerate	0.01	0.5	0.4	0.1	0.20	0.94	0.80	0.06
Isovalerate	<0.01	0.1	0.1	<0.01	0.02	0.58	0.58	<0.001
Caproate	0.2	0.2	0.1	0.04	0.03	0.02	0.39	0.23
Ileum								
Total lactate	19.7	22.4	46.8	39.9	12.64	0.10	0.86	0.72
D-Lactate	6.2	3.9	6.1	5.5	3.36	0.82	0.66	0.81
L-Lactate	13.5	18.5	40.7	34.4	11.78	0.09	0.96	0.65
Total SCFA	13.5	14.0	7.2	16.6	3.09	0.56	0.13	0.18
Acetate	10.1	11.8	5.3	12.9	2.93	0.54	0.1	0.33
Propionate	1.6	0.9	0.9	1.7	0.38	0.82	0.87	0.07
Butyrate	0.8	0.8	0.5	1.1	0.19	0.78	0.14	0.15
Isobutyrate	0.4	0.3	0.2	0.3	0.06	0.23	0.83	0.16
Valerate	0.1	0.03	0.1	0.2	0.05	0.48	0.84	0.19
Isovalerate	0.2	0.1	0.1	0.2	0.04	0.69	0.58	0.11
Caproate	0.3	0.1	0.1	0.2	0.06	0.96	0.69	0.04
Colon								
Total lactate	0.4	1.9	0.6	0.3	0.69	0.35	0.39	0.21
D-Lactate	0.2	1.0	0.3	0.2	0.34	0.31	0.32	0.16
L-Lactate	0.2	0.9	0.3	0.2	0.35	0.39	0.47	0.28
Total SCFA	130.6	140.5	139.1	130.5	8.89	0.93	0.94	0.31
Acetate	65.6	72.5	73.2	71.6	4.62	0.48	0.57	0.37
Propionate	38.1	37.9	40.1	36.2	3.00	0.96	0.50	0.54
Butyrate	19.5	18.6	19.6	17.0	1.52	0.62	0.26	0.60
Isobutyrate	1.3	1.1	0.9	1.0	0.14	0.15	0.88	0.31
Valerate	4.3	3.5	4.0	3.5	0.61	0.80	0.26	0.83
Isovalerate	1.2	1.0	0.8	0.9	0.16	0.11	0.85	0.25
Caproate	0.6	0.2	0.6	0.3	0.11	0.93	0.003	0.45
Serum LPS (EU/ml)	0.08	0.31	0.21	0.23	0.31	0.63	0.32	0.40
LPS in colonic digesta (EU/g)[b]	3.72E+06	2.45E+06	1.74E+06	3.31E+06	0.12	0.42	0.71	0.07
LPS in feces (EU/g)[b]	1.05E+06	7.94E+05	1.02E+06	1.07E+06	0.10	0.55	0.61	0.46

[a] For stomach, colon, and feces, $n = 8$ pigs/treatment, except for wheat-barley diet with high CaP level ($n = 7$ pigs). For ileum, $n = 7$ pigs for wheat-barley with adequate CaP level, $n = 7$ pigs for wheat-barley diet with high CaP level, $n = 5$ pigs for corn diet with adequate CaP level, and $n = 8$ pigs for corn diet with high CaP level. EU, endotoxin units.
[b] The lipopolysaccharide concentration in feces was lower than that in colonic digesta (colon, 2.69E+06 EU/ml; feces, 9.77E+05; SEM = 0.06; $P < 0.001$).

ments, such as gastrointestinal pH (25). Gastric and colonic dry matter contents were oppositely affected by dietary cereal composition, suggesting differences in the availability of water-binding substrates in digesta at various gut sites and thus progression of digestion and fermentation (25, 26). The most important difference between the wheat-barley and corn diets was the complex carbohydrate fraction being reflected by the higher intake of arabinoxylan and β-glucan by pigs fed wheat-barley diets. Also, pigs fed corn diets tended to consume more starch and, as corn possesses a higher amylose/amylopection ratio than wheat and barley, possibly more resistant starch (10, 27). Certain *Bifidobacterium* species are actively amylolytic (28) and thus might have been promoted in gastric and ileal digesta by the higher starch intake of pigs fed the corn diets. Particularly, starch escaping digestion by host enzymes may promote *Bifidobacterium* in the distal small intestine, as previously shown for high-amylose starch diets in pigs (29). Despite representing only a small bacterial proportion in gastric and ileal digesta, selective promotion of *Bifidobacterium* may contribute to gut health after weaning. Beneficial bacteria such as lactobacilli and bifidobacteria can establish an efficient barrier to the invasion and colonization of the gut by pathogenic bacteria (30). Nutritional concepts after weaning especially target these bacterial groups (17), and they can be complemented by the present findings.

In contrast to previous assumptions (16), gastric pH was not buffered by high dietary Ca, thereby bringing into question a pH-related promotion of *Enterobacteriaceae* in the present study. Also, the cereal-CaP interaction and regression analysis may indicate that the growth advantage was not solely related to Ca^{2+} or phosphate ions but that carbohydrate composition, specifically, a greater presence of β-glucan in the diet, may have supported enterobacterial abundance. In line with our present findings, dietary supplementation with oat β-glucan recently was found to promote bacterial growth, including that of enterobacteria, in gastric digesta of weaned pigs (13). Because enterobacteria do not express β-glucanase, an overall β-glucan-related improved bacterial acid tolerance might constitute a possible mode of action (31). In contrast, Ca and P are needed for a variety of metabolic processes in the bacterial cell (32, 33).

Increased CaP intake promoted ileal abundances of *Enterococcus* spp., *Bacteroides-Prevotella-Porphyromonas*, and *Campylobacter* spp. These data are in contrast to recent findings in growing pigs fed approximately similar amounts of dietary CaP (34), demonstrating that the gut microbiota of weaned pigs may respond differently to dietary interventions than those of growing pigs, likely due to intestinal maturity (35, 36). Whereas CaP exists mostly in its dissociated form at acidic gastric pH values, CaP in ileal and colonic digesta can be found in a dynamic equilibrium between dissociated and undissociated forms, with the latter being an amorphous Ca-phosphate complex that forms at pH values above 6 (37). This complex has buffering abilities and thus may have protected acid-sensitive bacteria against organic and bile acids in ileal digesta (15). Because *Campylobacter* species, e.g., *Campylobacter coli*, may cause watery and bloody diarrhea in pigs (38), and as a source of highly immune potent LPS, dietary promotion of *Campylobacter* spp. should be avoided. Promotion of ileal *Enterococcus* spp. by high CaP may be ambiguous regarding intestinal eubiosis without further information on the species or strains present. Some *Enterococcus* species produce bacteriocins and are used as probiotics in pig nutrition (17), whereas others may cause disease in pigs (39). Promotion of intestinal lactobacilli and reduction in enterobacteria, as observed in rats (15), could not be found in the present study, underlining that observations made in one monogastric species cannot be unconditionally extrapolated to another species due to species-specific physiological and microbial differences (40).

Changes in dietary cereal source and CaP level mainly affected gastric fermentation. Apparently, bacterial communities of pigs fed wheat-barley diets were stimulated in their metabolic activity by more CaP in gastric digesta, whereas communities in pigs fed corn diets were inhibited in their activity by a higher gastric CaP availability. Another interesting finding was the diet effect on gastric, ileal, and colonic caproate, which differed among gut sites, indicating that different caproate-producing bacteria dominated at the three gut sites. Caproate-producing bacteria can be found within different *Clostridium* clusters, such as clusters I, IV, XI, and XIV (41–45).

Lipopolysaccharide is released during bacterial growth, division, and death, making it a ubiquitous contaminant (7). Therefore, Gram-negative bacterial genera with LPS of high immune reactivity may impair host health and performance without expression of other bacterial virulence factors such as enterotoxins. The current abundances of *Enterobacteriaceae*, *Helicobacter* spp., *Campylobacter* spp., and *Fusobacterium* spp. indicated that sources of highly immune-reactive LPS are present throughout the gut, even in the stomach. As an example, we measured cell-free LPS in colonic digesta and feces. Because feces can be collected noninvasively, data on fecal LPS might be used as predictor of LPS accumulation in the lower guts of weaned pigs. A lower LPS concentration in feces than in colonic digesta was not expected, but it indicates that a certain degradation or disappearance of LPS occurs during the passage through the distal large intestine. The present serum LPS concentrations support that a very small translocation of LPS from the intestinal lumen to the systemic circulation occurs in young healthy pigs (46). Nevertheless, given that the LAL test does not differentiate between LPS of different immune reactivities, intestinal LPS values should be presented together with dominant Gram-negative bacterial groups to estimate the origin of LPS.

In conclusion, the present results demonstrated that the investigated bacterial community in gastrointestinal digesta of weaned pigs was mostly stable against changes in dietary cereal source and CaP level. Yet, certain bacterial groups were selectively promoted in the upper gut, such as *Bifidobacterium* spp. in gastric and ileal digesta by corn diets and ileal *Bacteroides-Prevotella-Porphyromonas*, *Campylobacter* spp., and *Enterococcus* spp. by a high CaP level. Information on selective stimulation of certain beneficial bacterial groups may be useful for diet formulation for weaned pigs. The presence of Gram-negative bacterial groups belonging to *Proteobacteria* and containing LPS of high immune reactivity at all gut sites indicated that more attention should be paid to these bacteria regarding gut health and performance.

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