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Enumeration of Selected Anaerobic Bacterial Groups in Cecal and Colonic Contents of Growing-Finishing Pigs

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Selected anaerobic bacterial groups in cecal and colonic contents of clinically healthy pigs fed a corn-soybean meal production diet were determined at sacrifice after 4, 8, and 11 weeks on feed, corresponding to intervals within the growing-finishing growth period. By using ruminal fluid-based media, the densities of the culturable anaerobic population; the cellulolytic, pectin-fermenting, pectin-hydrolyzing, xylan-fermenting; and the xylan-hydrolyzing, sulfate-reducing, and methanogenic bacterial populations were estimated. An analysis of variance was performed on these bacterial group variables to examine the effects of phase (weeks on feed), site (cecum or colon), or the interaction of phase with site. The population of total anaerobic bacteria was twice as dense in the colon as it was in the cecum (2×10^{10} versus 1×10^{10}/g [wet weight]; $P = 0.001$). The proportion of cellulolytic bacteria was lower at 4 weeks on feed than at 8 or 11 weeks (23 versus 32%; $P = 0.026$), while the proportion of pectin-fermenting bacteria depended on the interaction of phase with site ($P = 0.021$). The numbers of sulfate-reducing bacteria were significantly higher in the colon than in the cecum (6×10^7 versus 3×10^7; $P = 0.014$), as were methanogenic bacteria (19×10^7 versus 0.6×10^7; $P = 0.0002$). The remaining bacterial groups were stable with respect to phase and site. The results suggest that except for density differences, the microbial communities of the pig cecum and colon are similar in composition throughout the growing-finishing phase.

The growing-finishing phase of hog production represents the period when roughly the last 50 to 80 kg of body weight is gained. During this time growth shifts from accretion of lean muscle mass to deposition of fatty tissue. A number of biological factors contribute to this metabolic shift, any one of which may affect the dynamics of digestion in the gastrointestinal tract, perhaps altering the composition of the intestinal microbial community. Several studies have examined the microbial population in the lower gastrointestinal tracts of normal, healthy pigs. Some have characterized isolated strains of bacteria (15–19), while others have examined the populations of microbial groups that are able to utilize selected carbohydrates (1, 21, 26–31). Although some of these were time course studies (28, 29, 31), compositional changes were monitored in the fecal flora only. Microbial populations in the cecum and colon have been enumerated far less frequently than fecal populations have, and this is usually done at only one time point in the pig growth phase (1, 29, 30). Sulfate-reducing bacteria have been found in the digestive tracts of mammals (3, 9), but in the pig they have been isolated from fecal material only (33). Additionally, although methane production has been measured (6) and methane-producing bacteria have been found in the hind gut of the pig (13, 14), this bacterial group has been enumerated only at one time point in feces (20).

We were interested in following the compositional changes of the anaerobic bacterial community of the ceca and colons of pigs fed a typical production diet over the growing-finishing phase. Because simple sugars and starch are readily digestible by the host in the stomach and small intestine, we chose to examine the bacterial groups that use the more recalcitrant, complex polysaccharides that are likely to reach the cecum and colon. We also were interested in the populations of sulfate-reducing and methanogenic bacteria. The objective of the present study was to enumerate the complex polysaccharide-degrading, the sulfate-reducing, and the methanogenic microbial populations in the cecum and colon of the growing-finishing pig to assess whether any changes occur over time, by site, or both.

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MATERIALS AND METHODS

Facilities. Pigs were raised under confinement in production facilities located at The Upjohn Co. Research Farm, Richland, Mich. Each pen had an expanded metal floor with galvanized siding and was equipped with a self-feeder and a nipple waterer. Continuous lighting was used, and adequate heating and ventilation maintained an even temperature and healthy environment for the pigs.

Animals and diet. A total of 16 female and 16 castrated male crossbred (Yorkshire, Hampshire, and Duroc) feeder pigs (age, 8 to 9 weeks; weight, 18 to 20 kg) were used. After a 3-day period of acclimation to the study site, the pigs received a 7-day regimen of antibiotics (CSP-250, containing chlortetracycline, sulfamethazine, and penicillin at the recommended use levels of 100, 100, and 50 g/ton of feed, respectively) and dewormer (at the recommended use level of 348 g/ton of feed; dichlorvos; Atgard; Fermenta Animal Health Co.) in the feed followed by a 7-day withdrawal. The antibiotic-dewormer treatment period was used to provide uniform exposure across all pigs and to prevent avoidable diseases, especially respiratory ailments. Should a disease state have arisen in any group during the experiment, therapeutic doses of antibiotic would have had to have been administered to all pigs. This would have compromised the objective of the study. The diet during acclimation and throughout the study was a standard commercial corn-soybean ration (Table 1) fed ad libitum.

Following the 7-day withdrawal period, pigs were reweighed and randomly assigned to eight pens, with four pigs in each pen, by sex, current weight, and weight gain since the initial weighing. Two male and two female pigs were alloted to each pen. The pens were randomly assigned to one

TABLE 1. Diet composition[a]

Ingredient	Amt (%)
Corn, ground[b]	78.00
Soybean meal (48.5%)	19.00
Dicalcium phosphate	1.5
Limestone (calcium)	0.75
Salt	0.4
Dyna K[c]	0.1
Swine premix 5S[d]	0.25

[a] Crude protein, 16.1%; fat 3.1%; fiber, 2.8%; calcium, 0.68%; phosphorus, 0.62%; potassium, 0.69%; magnesium, 0.17%; lysine, 0.74%; methionine-cystine, 0.54%; energy, 3,098 cal/kg.

[b] One-third was rolled corn.

[c] Dyna K was obtained from Carl S. Akey, Inc.; it consists of 95.6% potassium chloride.

[d] Swine premix 5S was from Carl S. Akey, Inc.; and the minimum guaranteed analysis was as follows, in percent: Mg, 10.8; Zn, 3.2; Fe, 3.2; Mn, 1.6; Cu, 0.4; I, 0.04; Co, 0.016; Se, 0.012. It also contained the following, per kilogram. Vitamin A, 2,200,000 USP units; vitamin D_3, 704,000 USP units; vitamin E, 6,600 IU; vitamin K, 880 mg; riboflavin, 1,760 mg; *d*-pantothenic acid, 7,040 mg; niacin, 14,080 mg; vitamin B_{12}, 11; choline, 38,192 mg.

of three phases: three pens to phase 1, two pens to phase 2, and three pens to phase 3. Pigs in phases 1, 2, and 3 were sacrificed after 4, 8, and 11 weeks on feed, respectively. Pig weights were recorded biweekly and at the time of slaughter. Average slaughter weights at phases 1, 2, and 3 were 38, 60, and 88 kg, respectively. Slaughter weight coefficients of variation at phases 1, 2, and 3 were 3.6, 5.0, and 4.8%, respectively.

Sample collection and processing. Pigs were sacrificed individually by captive bolt. Each carcass was immediately bled out, and the intestinal tract was excised. Total cecal contents and contents from the first meter of proximal colon were collected under a flow of CO_2 gas into separate flasks, which were immediately sealed. Samples of 100 g from the cecal and colonic contents of each of the four pigs per pen were pooled into single flasks under a flow of CO_2. Since the pigs were not fed individually, cecal and colonic contents were pooled consistent with the experimental design, in which the pen was the experimental unit. Samples of 30 g from each pooled cecal or colonic sample were diluted 1:10 (wt/wt) in anaerobic dilution solution (4), blended for 1 minute under CO_2 in a Waring blender, and filtered through two layers of cheesecloth. Serial 10-fold dilutions of the filtrate were made in anaerobic dilution solution. All further manipulations of diluted cecal or colonic samples were performed in an anaerobic glove box (atmosphere of 85% N_2, 10% H_2, and 5% CO_2; ambient temperature; Coy Laboratory Products, Ann Arbor, Mich.).

Media and enumeration methods. Total culturable bacterial populations were determined on agar plates with an RGCA medium modified from the media of Bryant and Burkey (4) and Bryant and Robinson (5). The medium that was used contained the following, in percent: mineral 1 and 2, 3.75 each; clarified ruminal fluid, 30; resazurin (0.1% [wt/vol]), 0.1; hemin (0.01% [wt/vol]), 1; glucose, 0.05; cellobiose, 0.05; soluble starch, 0.05; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.5; agar, 2; and distilled water, 57. After the medium was autoclaved and cooled, sterile solutions of Na_2CO_3 (8% [wt/vol]) and L-cysteine hydrochloride (2.5% [wt/vol]) were added at 5 and 2%, respectively. Pectin-fermenting, pectin-hydrolyzing, xylan-fermenting, and xylan-hydrolyzing bacterial populations were estimated on agar plates containing differential carbohydrate media as described by Leedle and Hespell (12) and Leedle et al. (10). Fiber-hydrolyzing bacterial populations were determined by using plates of the carboxymethyl cellulose (CM-cellulose) medium described by Teather and Wood (23), as modified by Leedle and Butine (11). Plates were incubated for 2 days at 39°C under CO_2 at 5 lb/in^2 (135 kPa) in stainless steel vessels described previously (11). Hydrolysis zones overlapped if plates were allowed to incubate for longer than 2 days.

Methanogenic bacteria were enumerated in a ruminal fluid-based broth medium adapted from Balch et al. (2) containing the following, per liter: minerals 1 and 2, 50 ml each; resazurin (0.1%), 1 ml; trace minerals (modified from Balch et al. [2] by adding 0.1 g of $NiCl_2 \cdot 6H_2O$ and 0.01 g of sodium selenite per liter), 10 ml; trace vitamins (modified from Balch et al. [2] by adding 5 mg each of sodium ascorbate, choline chloride, myoinositol, niacinamide, and pyridoxal per liter), 10 ml; $NaHCO_3$, 5 g; NH_4Cl, 0.5 g; sodium acetate, 2.5 g; sodium formate, 2.5 g; yeast extract, 2 g; Trypticase (BBL Microbiology Systems), 2 g; clarified ruminal fluid (autoclaved at 121°C for 15 min; centrifuged at 16,300 × *g* for 20 min), 200 ml; and $Na_2S \cdot 9H_2O$–L-cysteine hydrochloride solution (2.5%/2.5%, sterile and anaerobic), 10 ml. The medium was prepared in crimp-sealed serum tubes. Methanogenic populations were estimated by using a most probable number (MPN) determination in the medium described above. A 1:5 dilution series containing 10 levels with five replicates each was initiated at a dilution of 10^{-5}. All tubes were pressurized to 30 lb/in^2 (207 kPa) with H_2-CO_2 (80:20) and incubated at 37°C for 10 days. The headspace gas was analyzed for the presence of methane by using a gas chromatograph (model 311; Carle). Tubes with methane concentrations greater than 100 ppm (μg/ml) were considered positive for methanogenic bacteria. Positive responses at each dilution level were counted, and the MPNs were calculated (7).

Sulfate-reducing bacteria were enumerated in a broth medium adapted from the medium of Widdel and Pfennig (34) containing the following, per liter: KH_2PO_4, 0.2 g; NaCl, 7 g; $MgCl_2$, 0.5 g; NH_4Cl, 0.3 g; KCl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; $FeSO_4 \cdot 7H_2O$, 2.78 g; yeast extract, 0.1 g; sodium acetate, 2.5 g; trace element solution (modified from the medium of Widdel and Pfennig [34] by omitting $FeCl_2 \cdot 2H_2O$ and adding 0.01 g of Na_2SeO_3 per liter), 1 ml; vitamin solution (2), 2 ml; resazurin (0.1%), 1.0 ml; $NaHCO_3$ (added after the medium was boiled and cooled), 5 g; and L-cysteine hydrochloride (2.5% sterile, anaerobic solution; added to sterile medium), 20 ml. The numbers of sulfate-reducing bacteria were estimated by MPN determination in multiwell microtiter plates. The last medium described above was dispensed into the microtiter plate wells, and dilution series were initiated by using the 10^{-5} cecal or colonic sample dilutions. A total of 8 dilution levels (1:5) with 12 replicates each were used for samples from the first two pens of pigs, and 12 dilution levels (1:3) with 8 replicates each were used for samples from subsequent pens. After inoculation, the microtiter plates were placed in stainless steel vessels and incubated at 35°C under CO_2 at 5 lb/in^2 (135 kPa) for 7 days. Wells containing a black FeS precipitate were considered positive for sulfate-reducing bacteria and were counted, and the MPNs were calculated (7).

Statistical analysis. All bacterial populations estimates were calculated and reported on a per-gram (wet weight) basis of cecal or colonic contents. The experimental design was a repeated measures design in which two sites (cecum and colon) were sampled from experimental units (pens) that were randomly assigned to the whole plot treatments (phases = weeks on feed). By using a significance level of $P \leq 0.05$,

TABLE 2. Analysis of variance *P* values associated with measurement of selected bacterial groups in cecal or colonic samples from growing-finishing pigs

Bacterial group	*P* values for:		
	Phase	Site	Phase by site
Total culturable anaerobic population	0.105	0.001	0.303
CM-cellulose hydrolyzing	0.026	0.135	0.593
Pectin fermenting	0.410	0.818	0.021
Pectin hydrolyzing	0.111	0.820	0.659
Xylan fermenting	0.502	0.794	0.682
Xylan hydrolyzing	0.172	0.093	0.720
Sulfate reducing	0.838	0.014	0.434
Methane producing	0.085	0.0002	0.359

an analysis of variance (22) was performed on the bacterial group variables to determine the effect of phase (weeks on feed), site (cecum or colon), or their interaction (phase by site). For comparison, these data also were analyzed on a per-gram (dry weight) basis. The patterns of significant differences between sites and phases were found to be the same. The results are presented on a wet weight basis.

RESULTS

Results from the analysis of variance (*P* values) are presented in Table 2 for all bacterial group variables with respect to phase (4, 8, or 11 weeks on feed), site (cecum or colon), or their interaction (phase by site). Table 3 shows the population size estimates and the estimate of error associated with measuring each of the bacterial groups examined. Within each group, like values for phases and (or) sites are reported as their overall means. Note that the proportions of the carbohydrate-degrading bacterial groups are based on the total culturable population represented by their appropriate phase(s) and (or) site(s) (Table 3).

TABLE 3. Population size estimates and relative proportions of selected bacterial groups in cecal or colonic samples from feeder pigs over three growth phases

Bacterial group	Phase[a]	Population density in[b]:		MSE[c]
		Cecum	Colon	
Total culturable	1, 2, 3	10.3	20.1	0.0043
CM-cellulose hydrolyzing	1	2.4	5.2 (23)[d]	0.0078
	2, 3	3.3	6.2 (32)	
Pectin fermenting	1	8.9 (86)	11.3 (50)	0.0043
	2, 3	9.3	17.4 (90)	
Pectin hydrolyzing	1, 2, 3	2.4	4.7 (23)	0.0237
Xylan fermenting	1, 2, 3	8.6	17.0 (83)	0.0031
Xylan hydrolyzing	1, 2, 3	1.0	2.0 (10)	0.0136
Sulfate reducing	1, 2, 3	2.9[e]	5.7[e]	0.0237
Methane producing	1, 2, 3	0.6[e]	19.0[e]	0.0891

[a] Phases 1, 2, and 3 represent 4, 8 and 11 weeks on feed, respectively.
[b] Values are least-square mean numbers (10^{-9} per g [wet weight]), except as noted.
[c] MSE, Log_{10} mean square errors.
[d] Values in parentheses are the percentage of the respective total culturable population [across indicated phase(s) and (or) by site(s)] represented by the bacterial group.
[e] Values are least-square mean numbers (10^{-7} per g [wet weight]).

The population of culturable anaerobic bacteria was significantly different by site ($P = 0.001$; Table 2), with the population being twice as dense in the colon as in the cecum (20.1×10^9 versus 10.3×10^9 bacteria per g [wet weight]; Table 3). The proportion of CM-cellulose-hydrolyzing bacteria was significantly lower in phase 1 pigs ($P = 0.026$; Table 2) than in phase 2 and 3 pigs (23 versus 32%, respectively; Table 3). In the pectin-fermenting group, there was a phase by site interaction ($P = 0.021$; Table 2), in which the cecal and colonic populations could be differentiated in phase 1 pigs (86 versus 50%; Table 3) but not in phase 2 or 3 pigs (90% in both sites; Table 3). No significant differences were observed for the proportions of pectin-hydrolyzing, xylan-fermenting, or xylan-hydrolyzing bacterial groups which represented 23, 83, and 10% of their total culturable populations, respectively (Table 3).

The sulfate-reducing and methanogenic bacterial populations were significantly different between sites (Table 2). Sulfate-reducing bacteria were approximately twice as dense in the colon as they were in the cecum (5.7×10^7 versus 2.9×10^7 bacteria per g [wet weight]; Table 3), and methane-producing bacteria were over 30 times as dense in the colon as they were in the cecum (19.0×10^7 versus 0.6×10^7 per g [wet weight]; Table 3).

DISCUSSION

The total culturable anaerobic bacterial populations in the pig cecum and colon observed in this study were within an order of magnitude of those reported by other investigators (1). However, we found significantly higher populations in the colon than in the cecum. We also found that the number of total culturable anaerobic bacteria was stable over the 11 weeks, consistent with the data of studies that followed the size of the fecal population from pigs fed a conventional diet over a 3- to 4-month period (29, 30).

In contrast to other investigations (1, 26–30), in which the cellulolytic bacteria in the cecum, colon, and rectum of pigs fed a standard high-concentrate ration were never higher than 5% of the total population, we found the CM-cellulose-hydrolyzing populations from these sites ranged from 23 to 32% of the total culturable population. This difference may simply reflect the nature of the substrates used to enumerate the fiber-hydrolyzing group. We used CM-cellulose, which has been shown to detect other enzyme activities in addition to cellulase (24). This may inflate our estimates of fiber-hydrolyzing populations relative to those estimated with filter paper. However, previous work in our laboratory showed that ball-milled cellulose or CM-cellulose medium yields comparable estimates of ruminal cellulolytic populations (11). Given that the nature and amount of fiber reaching the lower tract of the pig may differ from cellulosic fiber in the rumen, however, the CM-cellulose method may be more appropriate for estimating fiber-degrading bacteria in the pig lower intestinal tract. This speculation is supported by recent work suggesting that pebble-milled filter paper fails to detect a portion of the cellulolytic bacteria in human feces (32).

An increase in the proportion of cellulolytic bacteria has been well documented in pigs that were switched from a standard commercial (low-fiber) diet to a high-fiber diet (27–30), suggesting that the gut microflora adapts to the change in feed composition. In the present study we found an increase in the proportion of CM-cellulose-hydrolyzing

bacteria in pigs fed a standard diet throughout the growing-finishing period. An explanation is the enlargement of the digestive tract as the pig matures. This results in slower rates of digesta passage or longer residence times, consistent with the inverse correlation between the rate of cellulose fermentation and residence time reported by Van Soest (25). Thus, a larger fiber-hydrolyzing population can be supported.

The pectin-fermenting bacterial profile was complicated by the significant phase by site interaction in phase 1, although the proportion of bacteria that was able to hydrolyze pectin remained a constant 23% at each site and phase (Table 3). A total of 82% fermented xylan, and 10% hydrolyzed xylan, a pattern similar to that reported by Henning (8) for ruminal fluid samples. Henning (8) suggested that the difference reflects the proportions of bacteria with bound xylanases and those with freely diffusible xylanases; both groups are able to degrade xylan, but only those with diffusible xylanases are able to produce zones of hydrolysis. Thus, the xylan-utilizing bacterial profiles of the porcine lower intestinal tract appear to be similar to those of the bovine rumen.

The cecal and colonic populations of sulfate-reducing bacteria were within the range reported for human feces by Beerens and Romond (3) and, as with the population of methanogenic bacteria, were stable over the growing-finishing period. The methanogenic population, however, was more than 30 times as dense in the colon as in the cecum, a finding that was corroborated by estimates of in vitro rates of methanogenesis in pig gut samples. Rates of methane production of colonic samples have been found to be ninefold higher than those of cecal samples (J. A. Robinson and W. J. Smolenski, personal communication).

In summary, this study documents the complex polysaccharide-degrading, sulfate-reducing, and methanogenic anaerobic bacterial groups present in the microbial communities of the porcine cecum and colon during the growing-finishing period. Few differences were observed over the length of time that the pigs were fed the production-type corn-soybean diet, suggesting that host metabolic changes during growth do not greatly affect the indigenous gut microbial composition. The similar proportions of bacteria between sites suggests that the cecum and colon share common characteristics for supporting similar microbial communities. The much denser colonic than cecal community probably results from host-dependent chemical or physical factors such as substrate availability or longer residence times.

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