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Response of Nursery Pigs to a Synbiotic Preparation of Starch and an Anti-*Escherichia coli* K88 Probiotic▽

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Postweaning diarrhea in pigs is frequently caused by enterotoxigenic *Escherichia coli* K88 (ETEC). The aim of this study was to test the efficacy of *E. coli* probiotics (PRO) in young pigs challenged with *E. coli* K88. We also tested the synbiotic interaction with raw potato starch (RPS), which can be used as a prebiotic. Forty 17-day-old weaned piglets were randomly assigned to four treatments: treatment 1, positive-control diet (C), no probiotics or RPS but containing in-feed antibiotics; treatment 2, probiotic (PRO), no feed antibiotics plus a 50:50 mixture of probiotic *E. coli* strains UM-2 and UM-7; treatment 3, 14% RPS, no antibiotics (RPS); treatment 4, 14% RPS plus a 50:50 mixture of probiotic *E. coli* strains UM-2 and UM-7, no antibiotics (PRO-RPS). The pigs were challenged with pathogenic *E. coli* K88 strains on day 7 of the experiment (24-day-old pigs) and euthanized on day 10 of the experiment (35-day-old pigs). Probiotic and pathogenic *E. coli* strains were enumerated by selective enrichment on antibiotics, and microbial community analysis was conducted using terminal restriction length polymorphism analysis (T-RFLP) of 16S rRNA genes. The combination of raw potato starch and the probiotic had a beneficial effect on piglet growth performance and resulted in a reduction of diarrhea and increased microbial diversity in the gut. We conclude that the use of *E. coli* probiotic strains against *E. coli* K88 in the presence of raw potato starch is effective in reducing the negative effects of ETEC in a piglet challenge model.

In weaned pigs, postweaning diarrhea (PWD) is frequently caused by enterotoxigenic *Escherichia coli* (ETEC) (19). The disease may be peracute, in which case onset is rapid and severe and is associated with edema, and mortality is typical. More commonly, animals have a diarrhea that results in morbidity but not death, and the consequences are largely economic (19). *E. coli* F4 (serotype K88) is a common ETEC serotype associated with PWD and has increased worldwide (45). *E. coli* K88 is resistant to several antimicrobials (14) that may be the result of antimicrobial use in feeds (35). Including antibiotics in feed has been the traditional way of controlling PWD but is not encouraged because of concern about antimicrobial resistance in human medicine.

Probiotics may be used to prevent PWD (18, 19) and are live microbial cells that confer a health benefit on the host (7). Probiotics potentially exclude pathogenic microorganisms from epithelial surfaces (7) by the production of natural antimicrobial compounds, like bacteriocins (15). Prebiotics are polysaccharides that are fermented by bacteria associated with gut health (44). A prebiotic gives the probiotic a competitive advantage in the gut (18).

We screened 463 strains of *E. coli* for the ability to inhibit *E. coli* K88 and utilize starch (40). In our collection, we had two strains of *E. coli* (UM-2 and UM-7) that inhibited *E. coli* K88 and grew weakly on starch, an unusual characteristic for *E. coli*. We have previously demonstrated that dietary potato starch inclusion enhanced piglet digestive health by reducing diarrhea in young pigs challenged with ETEC (4). The prebiotic effect of raw potato starch may be attributed to the fact that the starch granules are much larger than those of cereal grains (42) and consequently reach the distal small intestines and colon, where they modify fermentation. Here, we describe a feeding trial using *E. coli* UM-2 and UM-7 as probiotics in diets containing 14% RPS as the prebiotic.

MATERIALS AND METHODS

Animals, housing, and experimental design. Cotswold piglets from the University of Manitoba's Glenlea swine research farm were used. The pigs were weaned at 17 ± 1 day and had an average body weight (BW) of 4.82 ± 0.6 kg. Forty piglets were assigned to outcome groups based on body weight and sex, using a randomized complete block design, and were assigned to four treatments consisting of five replicates and two pigs per pen. Unlimited consumption of feed and water was allowed. Pig BW and feed consumption were monitored daily, and the results were used to calculate the average daily gain (ADG), the average daily feed intake (ADFI), and the gain-to-feed efficiency ratio (G:F). Room temperature was maintained at 29 ± 1°C throughout the study. The experiment was approved by the University of Manitoba Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines (8).

Diets. A wheat-soybean meal basal diet was formulated to meet National Research Council (NRC) (30) nutritional requirements for piglets weighing 5 to 10 kg (Table 1). Treatment 1 was a positive-control diet (C) with no probiotics or raw potato starch but containing feed antibiotics. Treatment 2 was probiotic (PRO), with no feed antibiotics, plus a 50:50 mixture of probiotic *E. coli* strains UM-2 and UM-7. Treatment 3 was 14% raw potato starch with no antibiotics (RPS). Treatment 4 was 14% raw potato starch plus a 50:50 mixture of probiotic *E. coli* strains UM-2 and UM-7 with no antibiotics (PRO-RPS). The diets were mixed 1 week before the start of the experiment using the same batch of ingredients and offered *ad libitum* in a mash form. The probiotics were not mixed into the diet but were added each morning to the mash feed. The pigs were weaned at 17 days of age (day 1 of the experiment) and placed on the four experimental diets for 7 days (until they were 24 days old; day 7 of the experiment). During this period, the prebiotic was included in the RPS and PRO-RPS

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TABLE 1. Composition and nutrient analysis of experimental diets (as-fed basis)

Parameter	Value[a]			
	C	PRO	RPS	PRO-RPS
Ingredients				
Corn	44.65	44.65	25.96	25.96
Soybean meal	32.8	32.8	39.0	39.0
Whey powder	12.0	12.0	12.0	12.0
Limestone	0.5	0.5	0.75	0.75
Dicalcium phosphate	0.75	0.76	1.0	1.0
Soybean oil	5.0	5.0	5.0	5.0
L-Lysine	0.19	0.19	0.19	0.19
Fish meal	3.0	3.0	1.0	1.0
Vitamin premix[b]	0.5	0.5	0.5	0.5
Mineral premix[c]	0.5	0.5	0.5	0.5
L-Tryptophan	0.1	0.1	0.1	0.1
ASP250[d]	0.01			
Potato starch			14.0	14.0
Calculated nutrient content				
Gross energy (kcal/kg)	3,837	3,837	3,834	3,834
Crude protein (%)	21.7	21.7	21.9	21.9
Total lysine (%)	1.5	1.5	1.5	1.5
Crude fiber (%)	2.5	2.5	2.3	2.3

[a] Diets: C, control with antibiotics; PRO, C plus probiotics without antibiotics; RPS, C plus 14% raw potato starch without antibiotics; PRO-RPS, probiotics plus 14% RPS without antibiotics.
[b] Provided per kg of diet: 9,000 IU of vitamin A, 1,500 IU of vitamin D_3, 18 mg of vitamin E, 1.5 mg of vitamin K, 250 mg of choline, 30 mg of niacin, 27.5 mg of calcium pantothenate, 9.4 mg of vitamin B_2, 2 mg of vitamin B_6, 25 μg of vitamin B_{12}, 80 μg of biotin, and 0.5 mg of folic acid.
[c] Provided per kg of diet: 18 mg Cu, 110 mg Zn, 0.2 mg I, 110 mg Fe, 50 mg Mg, and 0.3 mg Se.
[d] ASP250, Aureo SP250 (chlortetracycline, penicillin [as penicillin G Procaine], and sulfamethazine; Alpharma Inc., Fort Lee, NJ).

diets and the probiotic in the PRO and PRO-RPS diets, but the pigs were only inoculated with the pathogenic *E. coli* K88 strains on day 7 of the experiment (24-day-old pigs). The antibiotic included in the diets was Aureo SP250 (ASP250), which is a combination of chlortetracycline, penicillin (as penicillin G Procaine), and sulfamethazine.

Bacterial culture, oral challenge, and health status. The probiotic strains *E. coli* UM-2 and UM-7 were made resistant to kanamycin by repeated transfer in Luria-Bertani (LB) broth containing 1 μg kanamycin · ml^{-1}. The final MIC was 0.05 μg kanamycin · ml^{-1}. *E. coli* K88 strains (2-12, I-36, and B104) were used to infect the pigs and did not grow in the presence of 0.05 μg kanamycin · ml^{-1}. They were made resistant to 4 μg ciprofloxacin · ml^{-1} by repeated transfer in LB. By adding either kanamycin or ciprofloxacin to LB agar, it was possible to selectively enumerate *E. coli* K88 (2-12, I-36, and B104) or probiotic *E. coli* bacteria (UM-2 and UM-7) from the gut.

The probiotic strains *E. coli* UM-2 and UM-7 were grown separately overnight in 500 ml of fresh LB broth with shaking at 37°C. The final concentration of *E. coli* was approximately 3.1×10^9 CFU · ml^{-1}. Starting on day 1 of the experiment, probiotic *E. coli* was provided to the pigs in the PRO (probiotic alone) and PRO-RPS (probiotic plus raw potato starch) treatments and every day subsequently. A volume of 50 ml (25 ml of each probiotic) was mixed with 200 g of fresh feed. Once the pigs had consumed this fresh feed, dry feed was supplied *ad libitum*. To experimentally infect the pigs, three *E. coli* K88 strains (2-12, B104, and I-36) were scaled by overnight growth in 2 liters of LB broth at 37°C. On day 7 of the experiment (24-day-old pigs), each pig received 6 ml (2.3×10^9 CFU · ml^{-1}) of an *E. coli* K88 cocktail (1/3 volume of strains 2-12, B104, and I-36) using a syringe attached to a polyethylene tube held in the back of the oral cavity. A subsample was taken of the probiotic and K88 strains, serially diluted (10-fold), and plated on LB agar to obtain accurate numbers to verify inoculant concentrations. Diarrhea was scored by using the fecal consistency (FC) method (0, normal; 1, mild diarrhea; 2, diarrhea; 3, severe diarrhea) and was performed in a blinded fashion by two trained personnel with no prior knowledge of dietary treatment allocation (26). The presence of blood in feces was checked daily.

Tissue and digesta sampling. Pigs were euthanized at 34 days of age (10 days after challenge). Each pig was placed under general anesthesia with a mixture of nitric oxide and halothane and euthanized by intracardiac injection of sodium pentobarbital (50 mg · kg^{-1} BW). The proximal segments of the ileum and colon were removed, placed in sterile containers, and transported to the laboratory for measurement of mucosal attached bacteria. Digesta samples were taken from the ileum, and colon, and the pH was determined using a pH meter (AB 15; Fisher

TABLE 2. Growth performance and fecal diarrhea of weaned pigs[a]

Parameter	Value[f]				SEM[b]	*P* value
	C	PRO	RPS	PRO-RPS		
Initial BW (kg)	4.6	4.7	4.8	4.9	0.2	0.856
Final BW (kg)	5.8	5.9	5.9	6.4	0.3	0.503
ADG (g/day)						
BI[c]	109.5^{A}	118.2^{A}	54.9^{B}	122.7^{A}	15.3	0.010
AI[d]	109.1^{A}	129.2^{A}	94.4^{A}	153.3^{B}	11.3	0.014
ADFI, g/d						
BI	205.3^{A}	212.3^{A}	140.9^{B}	207^{A}	17.4	0.017
AI	225^{A}	231.4^{A}	184.7^{B}	265.1^{C}	15.1	0.083
Gain:feed						
BI	0.53^{A}	0.55^{A}	0.39^{B}	0.57^{A}	0.05	0.020
AI	0.47	0.55	0.49	0.60	0.05	0.263
Fecal score[e]						
0–48 h	0.45	0.50	0.86	0.81	0.3	0.460
48–96 h	0.99^{B}	1.23^{A}	1.39^{A}	0.99^{B}	0.1	0.084

[a] Animals were fed experimental diets from 17 to 24 days of age but were not infected with *E. coli* K88 until they were 24 days old (after 7 days on the experimental diets).
[b] Pooled standard error of the mean.
[c] BI, before inoculation, calculated from the day of weaning until inoculation (17 to 24 days of age).
[d] AI, after inoculation, calculated from the day of weaning until inoculation (24 to 34 days of age).
[e] 0, normal feces; 1, mild diarrhea; 2, diarrhea; 3, severe diarrhea.
[f] Means within rows without common letters differ ($P < 0.05$).

TABLE 3. Effects on visceral organ weights of weaned pigs fed different experimental diets

Organ	Wt (g/kg BW)[b]				SEM[a]	*P* value
	C	PRO	RPS	PRO-RPS		
Liver	28.8	25.5	28.4	28.9	1.6	0.365
Spleen	3.2	5.0	2.5	2.4	1.3	0.472
Stomach (empty)	9.3	10.5	10.3	9.2	0.6	0.381
Small intestine (full)	59.3^{A}	83.7^{B}	73.2^{B}	80.1^{B}	4.4	<0.001
Small intestine (empty)	43.8^{A}	54.6^{B}	49.8^{AB}	52.6^{B}	2.8	0.051
Colon (full)	25.8^{A}	19.4^{B}	27.4^{A}	25.3^{A}	1.9	0.022
Colon (empty)	12.2^{AB}	11.2^{B}	14.2^{A}	13.7^{A}	0.8	0.024

[a] Pooled standard error of the mean.
[b] Means within rows without common letters differ ($P < 0.05$).

Scientific, Pittsburgh, PA). Subsamples (~5 g) from each gut segment were mixed with 5 ml of 0.1 M HCl to stop microbial activity and stored at −25°C until they were analyzed for volatile fatty acids (VFA) and lactic acid using gas chromatography, as described by Erwin et al. (17). The ammonia nitrogen concentration was measured using the indole-phenol blue method (32).

Microbial population measures. Approximately 10 g of feed, feces, or ileum or colonic tissue was taken for microbial analysis. Ileum and colon tissues were washed vigorously with sterile physiological saline to remove nonattached bacteria, and a blunt knife was used to scrape off the epithelial tissue, which was weighed and homogenized with 10 volumes of anaerobic diluent and decimally diluted. In the case of feed or feces, the sample was mixed 10-fold with an anaerobic diluent, homogenized, and decimally diluted. Ten-microliter droplets of each sequential decimal dilution were pipetted onto eosin methylene blue (EMB) agar (10^{-1} to 10^{-9} dilutions) with or without 0.05 μg ciprofloxacin or kanamycin ml^{-1}, allowed to dry, and then inverted and incubated at 37 ± 1°C for 24 h. *E. coli* K88 inoculant (kanamycin resistant), probiotic *E. coli* (ciprofloxacin resistant), or generic *E. coli* (no antibiotics in EMB medium) bacteria were enumerated and expressed as CFU · g^{-1} mucosa. *E. coli* is not normally ciprofloxacin resistant, and the reason we adapted our probiotic to ciprofloxacin is that it made it easy to enumerate from the samples. A ciprofloxacin-resistant strain would not be used in a commercial setting.

Molecularly based analysis. DNA was extracted from the ileum and colon contents using the ZR-DNA fecal kit (Zymo Research, Orange, CA). The DNA was run on a 1% agarose gel to determine if a high-molecular-weight band indicative of intact chromosomal DNA was visible. The concentration of DNA was measured spectrophotometrically at 260 nm, and all samples were diluted to equal concentrations of 100 μg · ml^{-1} DNA. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used to assess the changes in the microbial composition in the gut (1). Primers 27f (5′-GAAG AGTTTGATCATGGCTCAG-3′) and 1100r (5′ CTGCTGCCTCCCGTAG 3′) were used in order to amplify an informative sequence of the 16S rRNA gene (22). The forward primer was fluorescently labeled (WellRED D4dye; Sigma-Proligo Co.) to allow detection of the fragments by capillary electrophoresis. The PCRs were as follows: one cycle of 94°C for 5 min and then 36 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 2 min, and a final extension

TABLE 4. VFA, pH, and ammonia N concentrations in pigs at 34 days of age

Parameter	Value[b]				SEM[a]	*P* value
	C	PRO	RPS	PRO-RPS		
Ileum VFA (Mm)						
Total	4.2	4.0	4.7	5.5	0.623	0.245
Acetate	3.9^{A}	2.9^{A}	4.1^{AB}	4.7^{B}	0.512	0.047
Propionate	0.2	1.1	0.6	0.8	0.323	0.236
Butyrate	0.00	0.00	0.01	0.01	0.003	0.658
Valeric acid	0.04	0.00	0.02	0.00	0.011	0.069
Lactic acid	13.6	18.3	12.9	20.5	3.1	0.332
Colon VFA (mM)						
Total	47.8^{A}	45.5^{A}	79.0^{B}	77.2^{B}	5.1	<0.001
Acetate	30.1^{A}	31.2^{A}	49.6^{B}	46.8^{B}	2.8	<0.001
Propionate	10.4^{A}	8.5^{A}	15.9^{B}	15.4^{B}	1.8	0.007
Isobutyrate	0.7	0.6	0.9	0.8	0.104	0.064
Butyrate	4.8^{A}	4.1^{A}	10.3^{B}	11.8^{B}	1.172	<0.001
Isovaleric acid	0.6^{A}	0.5^{A}	0.9^{B}	0.6^{A}	0.091	0.017
Valeric acid	1.1^{A}	0.9^{A}	1.3^{AB}	1.8^{B}	0.171	0.005
Lactic acid	7.6	11.3	2.5	2.0	3.012	0.106
pH						
Ileum	7.0	6.8	6.7	6.7	0.2	0.691
Colon	6.3	7.4	6.6	7.1	1.9	0.820
Ammonia N (mM)						
Ileum	28.8	28.9	24.6	30.0	2.0	0.319
Colon	34.4	33.7	52.5	41.4	7.5	0.306

[a] Pooled standard error of the mean.
[b] Means within rows without common letters differ ($P < 0.05$).

TABLE 5. *E. coli* counts in the feed, ileum, and colon[a]

Location	*E. coli* count (log CFU g^{-1})[e]				SEM[b]	*P* value
	C	PRO	RPS	PRO-RPS		
Feed (BI[c])						
Generic *E. coli*	2.62^{A}	7.95^{B}	2.58^{A}	8.11^{B}	1.56	0.021
ETEC *E. coli* K88	0.00	0.00	0.00	0.00	0.00	ND[f]
Probiotic *E. coli*	0.00^{A}	7.45^{B}	0.00^{A}	7.36^{B}	2.14	0.001
Feed (AI[d])						
Generic *E. coli*	2.48^{A}	8.00^{B}	2.60^{A}	8.00B	1.57	0.036
ETEC *E. coli* K88	0.00	0.00	0.00	0.00	0.00	ND
Probiotic *E. coli*	0.00^{A}	6.48^{B}	0.00^{A}	6.00^{B}	1.81	0.012
Ileum						
Generic *E. coli*	6.38^{AB}	4.91^{B}	6.86^{A}	5.01^{B}	0.55	0.035
ETEC *E. coli* K88	3.31^{B}	2.20^{C}	4.85^{A}	2.54^{BC}	0.23	0.001
Probiotic *E. coli*	0.00^{A}	3.70^{B}	0.00^{A}	4.03^{B}	0.16	0.001
Colon						
Generic *E. coli*	6.34	6.27	6.07	6.10	0.36	0.873
ETEC *E. coli* K88	4.99^{A}	3.17^{B}	5.17^{A}	2.45^{C}	0.19	0.001
Probiotic *E. coli*	0.00^{A}	3.43^{B}	0.00^{A}	3.94^{B}	1.06	0.043
Feces (BI)						
Generic *E. coli*	6.21	7.57	7.81	8.60	1.00	0.436
ETEC *E. coli* K88	0.00	0.00	0.00	0.00	0.00	ND
Probiotic *E. coli*	0.00^{A}	6.21^{B}	0.00^{A}	6.40^{B}	1.82	0.001
Feces (AI)						
Generic *E. coli*	7.40	6.66	6.82	6.84	0.40	0.617
ETEC *E. coli* K88	5.42^{A}	3.10^{B}	5.55^{A}	3.38^{B}	0.10	0.001
Probiotic *E. coli*	0.00^{A}	3.90^{B}	0.00^{A}	3.98^{B}	0.00	0.001

[a] Animals were fed experimental diets from 17 to 24 days of age but were not infected with *E. coli* K88 until they were 24 days of age.
[b] Pooled standard error of the mean.
[c] BI, before inoculation, calculated from the day of weaning until inoculation (17 to 24 days of age).
[d] AI, after inoculation, calculated from the day of weaning until inoculation (24 to 34 days of age).
[e] Means within rows without common letters differ ($P < 0.05$).
[f] ND, not determined.

at 72°C for 5 min. To produce terminal restriction fragments (T-RFs), the base pair 27-to-1100 region of the 16S rRNA gene was digested using HhaI (10 μl of PCR product, 10 units of HhaI, 1× HhaI buffer, and 20 μg of bovine serum). In our laboratory, we have tested a range of restriction enzymes, and adding additional enzymes does not add significantly ($P < 0.05$) to the information. The mixture was adjusted to a final volume of 20 μl with MilliQ water, and the DNA was digested at 37°C for 3 h. The length of each T-RF was determined on a CEQ 8800 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA). Six microliters of fluorescently labeled fragments, 26 μl sample loading solution, and 0.5 μl of a DNA size standard (600 bp for T-RFLP) were mixed and separated.

Bioinformatics analysis of T-RFLP data. Microbial community analysis (41) Web services were used to build a putative reference database of probable T-RFs of the gut. For this purpose, we incorporated 16S rRNA gene clone libraries of nearly complete sequences of gut microorganisms found in human (16), swine (23), mouse (24), and ruminants (31, 33) into MiCA, which we called the H.Q. database. This greatly facilitated analysis by excluding the T-RFs that are unlikely to occur in the gut (16, 23, 24, 34). The fragment profiles produced by HhaI restriction of the base pair 27-to-1100 product were applied to the H.Q. database *in silico* so that a reference library for our study could be constructed and exported to the phylogenetic assignment tool (PAT) (20). Concurrently, using T-RFLP data obtained from CEQ software (fragment sizes and peak areas), various profiles of interest were developed with reference to treatment. These libraries were entered into the hierarchical browser of the ribosomal database project (RDP-II) (10) and converted to GenBank format. The resulting libraries were then assigned to the library comparison tool of RDP-II. The T-RFs of the same size were in many cases ambiguous in their assignment of taxonomic rank. To resolve this problem, the T-RFs with multiple accession numbers were assigned to a taxonomic rank according to phylum, class, order, and family, which we have previously demonstrated to be robust (39). Data were analyzed using Fisher's exact test (36).

Richness and diversity indices. A diversity index is a mathematical measure of species diversity in a community. Diversity indices provide information about community composition and take the relative abundances of different species into account. The concept of diversity has two facets: richness, or the number of taxonomic units, and evenness, or equality in the abundances of all taxonomic units. Incidence-based richness indicators—Chao2, ICE (Incidence-based Coverage Estimator), and MMMean (Michaelis-Menten Mean) function and the Shannon and Simpson diversity indices—were calculated using EstimateS 7.5 (11). Several estimators were selected, because if indices follow the same trend regardless of the calculation method, the results are likely to be robust. An upper abundance limit of 5 was used to determine rare or infrequent species. The order of the samples was randomized 500 times for each run to reduce the effect of the sample order. Tukey's multiple-comparison test (36) was applied to detect significant differences among experimental groups.

Calculations and other statistical analysis. Mucosa-attached lactic acid bacteria and *E. coli* populations were expressed as CFU per gram of intestinal tissue. The data were analyzed as a completely randomized design using the Proc Mixed procedures of SAS (36). The pen was considered the experimental unit for all response criteria measured. When a significant *F* value ($P < 0.05$) for treatment means was observed in analysis of variance, the treatments were compared using Tukey's test. A least significant difference (LSD) multiple-comparison test was used to calculate the statistical significance ($P < 0.05$) for the phylogenetic lineage in molecular analysis.

TABLE 6. T-RFLP analysis-based hierarchical microbial composition of ileum digesta in early-weaned pigs fed different experimental diets

Taxon	Microbial level (%)[b]				SEM[a]
	C	PRO	RPS	PRO-RPS	
Phylum *Bacteroidetes*	30.4	30.2	30.9	29.0	0.40
Class unclassified *Bacteroidetes*	1.4	1.2	1.9	0.0	0.40
Phylum *Firmicutes*	63.1	62.3	62.1	64.6	0.57
Class *Bacilli*	9.7	13.6	9.7	12.1	0.96
Order *Lactobacillales*	9.7	13.6	9.7	12.1	0.96
Class *Clostridia*	80.3	75.7	80.6	80.5	1.19
Order *Clostridiales*	61.0^{A}	61.5^{A}	56.5^{AB}	47.7^{B}	3.20
Order unclassified *Clostridia*	19.3^{AB}	14.2^{A}	24.1^{BC}	32.9^{C}	3.98
Class unclassified *Firmicutes*	3.1	3.0	1.9	2.0	0.32
Phylum *Actinobacteria*	0.3	0.0	0.0	0.7	0.17
Class *Actinobateria*	0.3	0.0	0.0	0.7	0.17
Subclass *Coriobacteridae*	0.3	0.0	0.0	0.7	0.17
Order *Coriobacteriales*	0.3	0.0	0.0	0.7	0.17
Phylum *Proteobacteria*	4.8	5.9	5.6	4.0	0.43
Class *Epsilonproteobacteria*	0.7	1.2	0.9	0.0	0.25
Order *Campylobacterales*	0.7	1.2	0.9	0.0	0.25
Class *Deltaproteobacteria*	0.7	0.0	0.9	0.0	0.23
Order *Desulfovibrionales*	0.7	0.0	0.9	0.0	0.23
Class *Gammaproteobacteria*	1.7	2.4	1.4	1.3	0.25
Order *Pasteurellales*	0.3	0.0	0.5	0.0	0.12
Order *Enterobacteriales*	1.4	2.4	0.9	1.3	0.32
Class *Betaproteobacteria*	1.4	1.8	1.9	2.0	0.13
Order *Burkholderiales*	1.0	1.2	1.4	1.3	0.09
Order unclassified *Betaproteobacteria*	0.3	0.6	0.5	0.7	0.09
Class *Alphaproteobacteria*	0.0	0.0	0.0	0.0	0.00
Order unclassified *Alphaproteobacteria*	0.0	0.0	0.0	0.0	0.00
Class unclassified *Proteobacteria*	0.3	0.6	0.5	0.7	0.09
Phylum *Lentisphaerae*	0.3	0.6	0.5	0.7	0.09
Class *Lentisphaerae*	0.3	0.6	0.5	0.7	0.09
Order *Victivallales*	0.3	0.6	0.5	0.7	0.09
Phylum unclassified *Bacteria*	0.3	0.0	0.0	0.0	0.08

[a] Pooled standard error of the mean.
[b] Means within rows without a common letters differ ($P < 0.05$).

RESULTS

Piglet performance, fecal score, and organ weights. The initial BWs of the piglets did not differ ($P > 0.05$) among dietary treatments (Table 2). The ADG was lowest ($P < 0.05$) for the RPS treatment before and after inoculation and highest for the PRO-RPS treatment. The ADFI was highest ($P < 0.08$) for the RPS-PRO diet. The feed intake of the RPS treatment was lowest before *E. coli* K88 inoculation ($P < 0.05$). The G:F ratio ($P < 0.05$) was lowest for RPS before inoculation, but there were no other treatment differences. Fecal scores were highest ($P < 0.05$) for the PRO and RPS diets at 48 to 96 h (Table 2). Dietary treatments had no affect on the weights of the liver, spleen, or empty stomach but did affect the weights of the small intestine and colon ($P < 0.05$) (Table 3).

Digesta pH, ammonia N, volatile fatty acids, and lactic acid concentrations. The digesta pH, ammonia N, and lactic acid concentrations of the ileum and colon contents were not affected by dietary treatments ($P > 0.05$) (Table 4). The dietary treatments had no effect on ileal VFA concentrations, except for acetic acid ($P < 0.05$). In the colonic digesta, total VFA, acetate, propionate, and butyrate concentrations were higher ($P < 0.05$) in the raw potato starch-containing diets (Table 4).

Microbial measurements. LB agar containing ciprofloxacin was used to selectively enumerate the probiotic bacteria, and LB containing kanamycin was used to selectively enumerate the *E. coli* K88 inoculant. Before inoculation with *E. coli* K88, no kanamycin-resistant *E. coli* bacteria were found in the feces or feed (Table 5). No ciprofloxacin-resistant *E. coli* bacteria were found in nonprobiotic treatments at any time during the experiment, indicating that no cross-contamination with the probiotic had occurred. The *E. coli* K88 strain persisted in the digestive tract, and counts were lowest in dietary treatments that contained RPS or RPS plus the *E. coli* probiotic (Table 5).

T-RFLP analysis. After correction for background values and base pair variation, five phyla were observed in the digestive tract (Tables 6 and 7). The dominant phylum was *Firmi-*

TABLE 7. T-RFLP analysis-based hierarchical microbial composition of colon digesta in early-weaned pigs fed different experimental diets

Taxon	Microbial level (%)[b]				SEM[a]
	C	PRO	RPS	PRO-RPS	
Phylum *Bacteroidetes*	29.4	30.5	30.3	30.4	0.25
Class unclassified *Bacteroidetes*	0.4	1.5	1.3	1.4	0.25
Phylum *Firmicutes*	65.6	62.0	63.9	63.8	0.74
Class *Bacilli*	9.9	9.5	8.7	10.0	0.30
Order *Lactobacillales*	9.9	9.5	8.7	10.0	0.30
Class *Clostridia*	82.1	79.4	81.2	79.6	0.65
Order *Clostridiales*	58.2	59.9	68.1	57.4	2.46
Order unclassified *Clostridia*	23.8[A]	19.5[A]	13.1[B]	22.1[A]	2.35
Class *Mollicutes*	0.0	0.0	0.4	0.0	0.10
Order *incertae sedis* 8	0.0	0.0	0.4	0.0	0.10
Class unclassified *Firmicutes*	3.7	3.1	3.5	4.2	0.23
Phylum *Actinobacteria*	0.0	0.4	0.0	0.0	0.10
Class *Actinobateria*	0.0	0.4	0.0	0.0	0.10
Subclass *Coriobacteridae*	0.0	0.4	0.0	0.0	0.10
Order *Coriobacteriales*	0.0	0.4	0.0	0.0	0.10
Phylum *Proteobacteria*	4.0	5.0	4.4	4.8	0.22
Class *Epsilonproteobacteria*	0.0	0.8	0.9	0.7	0.20
Order *Campylobacterales*	0.0	0.8	0.9	0.7	0.20
Class *Deltaproteobacteria*	0.4	0.8	0.4	0.7	0.10
Order *Desulfovibrionales*	0.4	0.8	0.4	0.7	0.10
Class *Gammaproteobacteria*	1.8	1.9	2.2	1.7	0.10
Order *Pasteurellales*	0.4	0.4	0.4	0.3	0.03
Order *Enterobacteriales*	1.5	1.5	1.8	1.4	0.09
Class *Betaproteobacteria*	1.5	1.5	0.9	1.3	0.14
Order *Burkholderiales*	1.1	1.1	0.4	1.0	0.17
Order unclassified *Betaproteobacteria*	0.4	0.4	0.5	0.3	0.04
Class *Alphaproteobacteria*	0.4	0.0	0.0	0.0	0.10
Order unclassified *Alphaproteobacteria*	0.4	0.0	0.0	0.0	0.10
Class unclassified *Proteobacteria*	0.0	0.4	0.0	0.3	0.10
Phylum *Lentisphaerae*	0.0	0.4	0.4	0.0	0.12
Class *Lentisphaerae*	0.0	0.4	0.4	0.0	0.12
Order *Victivallales*	0.0	0.4	0.4	0.0	0.12
Phylum unclassified *Bacteria*	0.0	0.8	0.0	0.0	0.20

[a] Pooled standard error of the mean.
[b] Means within rows without common letters differ ($P < 0.05$).

cutes, followed by *Bacteroidetes*. Comparing taxa across dietary treatments indicated that only the orders *Clostridiales* and unclassified *Clostridia* (based on RDP classification) showed significant ($P < 0.05$) differences in the ileum (Table 6). Only the unclassified *Clostridia* were significantly ($P < 0.05$) different in the colon (Table 7).

Diversity indices. In the ileum, the Chao2 and MMMean indices were highest for PRO and PRO-RPS. In the colon, all three richness indices were highest for the probiotic-containing treatments (Table 8). There were no differences in the Shannon and Simpson diversity indices for the ileum, but the Simpson index was highest for the PRO-RPS treatment, and both the probiotic treatments were significantly different ($P < 0.05$) from the control.

DISCUSSION

In the ileum, colon, and feces, there was an inverse relationship between the presence of probiotic *E. coli* and pathogenic *E. coli* K88 (Table 5). This suggests that the inhibitory effect of the *E. coli* probiotics UM-2 and UM-7 against *E. coli* K88 observed previously *in vitro* (40) are also exhibited *in vivo*. The greatest ADG was observed when the probiotics and potato starch (PRO-RPS) were both present in the diet (Table 2), and the lowest ADG was seen when the probiotics were absent and only potato starch was included (RPS). The second-highest ADG was seen with the probiotic alone, and there appeared to be a biological interaction between the potato starch and the probiotics.

The site of digestion of the RPS is the colon (5), and our results suggest that this is because of the increased colonic contents and tissue weights for the RPS and RPS-PRO treatments, which indicate that there was more starch to ferment (Table 3). The VFA concentration was also higher in the colon than in the ileum for the RPS treatments (Table 4). In particular, the concentration of butyric acid increased significantly in the RPS-containing diets (Table 4). Microorganisms are often

TABLE 8. Effect on richness and diversity indices of ileum and colon digesta in early weaned pigs fed different experimental diets[1]

Parameter	Value[b]				SEM[a]	*P* value
	C	PRO	RPS	PRO-RPS		
Ileum						
Richness						
ICE	223.3	307.9	266.4	241.0	30.2	0.157
Chao 2	154.8^{A}	259.3^{BC}	214.9^{B}	277.5^{C}	17.6	0.002
MMMean	178.2^{A}	375.2^{B}	281.1^{C}	297.8^{BC}	25.4	0.006
Diversity						
Shannon	4.1	3.8	4.1	4.0	0.18	0.593
Simpson	154.7	148.3	89.5	109.6	19.5	0.201
Colon						
Richness						
ICE	169.4^{A}	295.7^{B}	256.3^{C}	514.4^{D}	8.5	<0.001
Chao 2	146.9^{A}	253.7^{B}	217.2^{B}	436.5^{C}	13.1	<0.001
MMMean	171.9^{A}	285.8^{B}	245.1^{C}	414.0^{D}	7.4	<0.001
Diversity						
Shannon	4.4	4.6	4.4	4.7	0.091	0.100
Simpson	113.3^{A}	177.5^{B}	155.5^{AB}	250.7^{C}	15.2	<0.001

[a] Pooled standard error of the mean.
[b] Means within rows without common letters differ ($P < 0.05$).

inhibited by high concentrations of VFA, but in the RPS diet, the total *E. coli* count was not affected (Table 5); *E. coli* K88 proliferated (Table 5) and contributed to an increase in diarrhea in these pigs (Table 3).

E. coli Nissel 1917 has been licensed in Europe for use in cattle, mice, and humans for a number of years (21). *E. coli* probiotics have also been shown to prevent acute secretary diarrhea in pigs (38), reduce shedding of *E. coli* O157:H7 in cattle (37), and retard ETEC excretion in calves with experimentally induced colibacillary diarrhea (43). The specific modes of action of *E. coli* probiotics are not known, but studies have demonstrated that *E. coli* Nissel 1917 modifies intestinal motility (3), modulates Th2 responses of the immune system (6), and mediates antimicrobial human beta-defensin synthesis and secretion in the feces (29).

Our results demonstrate the efficacy of an *E. coli* probiotic that was selected based on its ability to produce colicins active against pathogenic *E. coli* K88 and tested in young pigs experimentally infected with *E. coli* K88, even though we are not certain that this was the mode of action *in vivo*. In addition, we selected probiotics that could ferment starch, or at least the by-products of starch. Colicin-like products from *E. coli* H22 were shown to inhibit the growth of pathogenic *E. coli in vitro* and *in vivo* in mice (12). Schamberger et al. (37) demonstrated a reduction in *E. coli* O157:H7 in cattle when colicin E7-producing *E. coli* strains were used as a probiotic. Cutler et al. (13) observed a decline in piglet diarrhea caused by the pathogenic F18-producing *E. coli* bacteria when isolated colicin E1 was included in the feed. The selection of a combination of probiotics that can produce colicins and ferment starch by-products was effective. The results for growth performance and diarrhea (Table 3) were superior when RPS and PRO were fed together.

In general, there was an increase in richness and diversity in the colon when RPS and PRO were present (Table 8). These diversity effects are not easily seen in the T-RFLP data (Tables 6 and 7) because the values are raw T-RF richness data while the diversity indices take into account the structure of the microbial community as a whole. In particular, the richness and diversity of the PRO-RPS treatment for the colon were higher than for other treatments (Table 8). This observation is consistent with the idea that when RPS and PRO are both present, the pathogenic role of *E. coli* K88 declines. We have demonstrated (39) that a reduction in microbial richness and diversity is related to poor gut health. A reduction in ecosystem diversity is associated with the concepts of ecosystem instability and reduced resilience (27). These concepts have been well developed in macroecosystems (25) but may have utility in microbial ecosystems of the gut and potentially provide a means of assessing the gut health benefits of dietary ingredients.

Recently, Bailey et al. (2) demonstrated that mice that were stressed, had lower gut microbiome diversity, and this was positively correlated with increased susceptibility to infection with *Citrobacter rodentium*. Metzler-Zebeli et al. (28) also demonstrated that microbial diversity in pigs can be modified by changing the fermentability and viscosity of carbohydrates fed to the pigs. Low fermentability with high viscosity resulted in increased diversity. Although these authors did not experimentally infect animals, they did demonstrate that there was an increase in the abundance of *E. coli* virulence factors in pigs fed higher levels of carboxymethyl cellulose, a diet that had low fermentability and high viscosity. Raw potato starch has much higher viscosity than most other starches and is relatively low in fermentability (International Starch Institute [http://www.starch.dk/isi/profile/home.asp]). Thus, based on the work of Metzler-Zebeli, it is not implausible to expect an increase in microbial diversity in the colon. An important property of our probiotics was that they could ferment starch or at least the

by-products of starch and thus had the added benefit of being able to multiply in the gut, while *E. coli* is not usually able to grow on starch.

In conclusion, we have demonstrated *in vivo* that the selection of probiotic *E. coli* strains against *E. coli* K88 is effective in preventing diarrhea in piglets when fed in conjunction with raw potato starch. A benefit of selecting probiotic strains of the same genus and species as the pathogen one is trying to exclude is that the organisms typically occupy the same niche in the gut (19). The disadvantage of using *E. coli* as a probiotic is that it is not generally considered safe and regulatory hurdles are higher when trying to gain approval for its inclusion in animal feeds (9).

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