

# Nonstarch Polysaccharides Modulate Bacterial Microbiota, Pathways for Butyrate Production, and Abundance of Pathogenic *Escherichia coli* in the Pig Gastrointestinal Tract<sup>∇†</sup>

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The impact of nonstarch polysaccharides (NSP) differing in their functional properties on intestinal bacterial community composition, prevalence of butyrate production pathway genes, and occurrence of *Escherichia coli* virulence factors was studied for eight ileum-cannulated growing pigs by use of terminal restriction fragment length polymorphism (TRFLP) and quantitative PCR. A cornstarch- and casein-based diet was supplemented with low-viscosity, low-fermentability cellulose (CEL), with high-viscosity, low-fermentability carboxymethylcellulose (CMC), with low-viscosity, high-fermentability oat  $\beta$ -glucan (LG), and with high-viscosity, high-fermentability oat  $\beta$ -glucan (HG). Only minor effects of NSP fractions on the ileal bacterial community were observed, but NSP clearly changed the digestion in the small intestine. Compared to what was observed for CMC, more fermentable substrate was transferred into the large intestine with CEL, LG, and HG, resulting in higher levels of postileal dry-matter disappearance. Linear discriminant analysis of NSP and TRFLP profiles and 16S rRNA gene copy numbers for major bacterial groups revealed that CMC resulted in a distinctive bacterial community in comparison to the other NSP, which was characterized by higher gene copy numbers for total bacteria, *Bacteroides-Prevotella-Porphoryomonas*, *Clostridium* cluster XIVa, and *Enterobacteriaceae* and increased prevalences of *E. coli* virulence factors in feces. The numbers of butyryl-coenzyme A (CoA) CoA transferase gene copies were higher than those of butyrate kinase gene copies in feces, and these quantities were affected by NSP. The present results suggest that the NSP fractions clearly and distinctly affected the taxonomic composition and metabolic features of the fecal microbiota. However, the effects were more linked to the individual NSP and to their effect on nutrient flow into the large intestine than to their shared functional properties.

The porcine intestinal microbiota change in response to dietary carbohydrate composition due to specific substrate preferences of bacteria (6). Therefore, inclusion of specific nonstarch polysaccharides (NSP) in the diet of pigs allows manipulation of the composition of the intestinal microbiota. The NSP can also reduce digestibility of nutrients in the small intestine (8). The resulting changes in nutrient flow alter the availability of fermentable substrate in the different sections of the gut and thus may modify the bacterial community structure. Differences in the fermentability levels of individual NSP may not only affect the kinetics of their degradation by intestinal bacteria but may also change the composition of the fermentation end products (49). Particularly, butyrate is an important metabolite because of its potential to affect gene

expression and to improve cellular development in enterocytes (38). The ability of gut microbiota to produce butyrate can vary considerably in response to environmental factors, such as diet composition (3). However, the number of butyrate-producing bacteria in complex fecal samples has been difficult to estimate by targeting the 16S rRNA gene, because these bacteria do not form a homogeneous phylogenetic group, and both butyrate producers and non-butyrate producers are found within the same phylogenetic clusters belonging to *Clostridium* clusters I, III, IV, XI, XIVa, XV, and XVI (27). Two alternative pathways for butyrate formation in bacteria harboring the rumen and human colon have been described (7, 26). The majority of human colonic butyrate producers use butyryl-coenzyme A (CoA) CoA transferase, whereas soil bacteria mostly utilize the butyrate kinase for the last step of butyrate formation (26, 27). However, information about the butyrate pathways used by intestinal bacteria in pigs is not available.

In addition to the effects of the functional properties of NSP on intestinal physiology and fermentation processes, selection of specific NSP fractions may also prevent or stimulate overgrowth of pathogenic bacteria. For instance, dietary inclusion of highly viscous carboxymethylcellulose (CMC) has been shown to increase fecal shedding of enterotoxigenic *Escherichia coli* in weaned pigs (15). There is a need to identify those

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dietary NSP fractions that may either increase or reduce the numbers of potential pathogenic bacteria to formulate diets exerting beneficial effects on gut health, which is particularly important in antibiotic-free feeding regimens.

Most studies pertaining on the effect of diet composition on the bacterial community in pigs have employed natural NSP sources and cereal-based diets, thereby resulting in a mixture of different soluble and insoluble NSP showing considerable interactions and modification of intestinal bacterial ecophysiology (6, 36, 37). Purified NSP fractions are increasingly available from the bioprocessing industry for use in food preparation and potentially in animal feeds, where economics and possible health benefits warrant this use. However, less is known about the fermentative properties of purified NSP fractions than about those of NSP in the grain matrix (37), which may also differ according to their origins.

The aim of the present study was to examine the effects of four purified NSP fractions differing in their functional properties, i.e., viscosity and fermentability, on the ileal and fecal bacterial community, butyrate production pathway genes, and the occurrence of virulence factor genes of swine-pathogenic *E. coli*, including enterotoxigenic and enteroaggregative *E. coli* (11, 13).

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#### MATERIALS AND METHODS

**Animals and diets.** A total of 8 crossbred Duroc-Landrace pigs (average weight,  $22 \pm 1.4$  kg) from the herd of the Swine Research and Technology Centre, Edmonton, AB, Canada, were surgically fitted with a simple T-cannula at the distal ileum (23). The animal protocol was approved by the University of Alberta Animal Care and Use Committee for Livestock and followed the principles established by the Canadian Council on Animal Care (5). The pigs were assigned to one of four diets in a double 4-by-4 Latin square, resulting in 8 observations per diet. A semipurified diet based on cornstarch and casein was formulated to meet or to exceed the nutrient requirements for growing pigs (see Table S1 in the supplemental material) (34). The basal diet was supplemented with 5% active NSP ingredient of four purified NSP fractions: (i) low-fermentability, low-viscosity cellulose (CEL) (TIC pretested Ticacel MCC FG-100; TIC GUMS, White Marsh, MD), (ii) low-fermentability, high-viscosity carboxymethylcellulose (CMC) (TIC pretested Ticalose CMC 6000 F; TIC GUMS), (iii) high-fermentability, low-viscosity oat  $\beta$ -glucan (LG) (OatVantage; GTC Nutrition, Missoula, MT), and (iv) high-fermentability, high-viscosity oat  $\beta$ -glucan (HG) (Viscofiber; Cevena Bioproducts, Edmonton, AB, Canada). To reach 5% of the active NSP in the diet, the inclusion levels of the NSP fractions were 5.20, 6.25, 8.95, and 9.25% for CEL, CMC, LG, and HG, respectively. The NSP fractions were selected based on their *in vitro* viscosity levels, which were 0.3, 285, 20, and 210 mPa  $\cdot$  s for CEL, CMC, LG, and HG, respectively, as determined with 0.5% NSP solution using a rheometer (UDS 200; Paar Physica, Glenn, VA) at a shear rate of 12.9/s and 20°C. The viscosity of  $\beta$ -glucans is linked to their molecular weight and has been reported to increase about 10-fold with a doubling of molecular weight (9). Titanium dioxide was added to the diet as a digestibility marker. The pigs were allowed to consume the experimental diets at a rate of approximately 3% of their maintenance requirement for energy ( $3 \times 110$  kcal digestible energy [DE]/kg metabolic body weight [BW<sup>0.75</sup>]) (34). They were fed two equal meals in mash form twice daily (at 8 a.m. and 4 p.m.).

**Collection of intestinal samples.** Each experimental period comprised 17 days; an adaptation period of 10 days was followed by collection of feces over 3 days and collection of ileal effluent for 8 hours a day, from 8 a.m. to 4 p.m., over 4 days. Feces were collected using plastic bags attached to the skin around the anus (45). The bags were changed each time feces were voided, and after subsamples of fresh feces were taken for analysis of short-chain fatty acids (SCFA), the feces were stored at  $-20^\circ\text{C}$  until being freeze-dried. For bacterial DNA extraction, subsamples of freshly voided feces were taken and immediately stored at  $-20^\circ\text{C}$ . The ileal collection procedure was adapted from the method of Li et al. (23),

using plastic tubings attached to the barrel of the cannula by elastic bands. Twice during collection of ileal effluent (11 a.m. and 2 p.m.), subsamples of ileal effluent (approximately 50 ml) were collected for bacterial DNA analysis and immediately stored at  $-20^\circ\text{C}$ . The ileal effluent was pooled within each animal and stored at  $-20^\circ\text{C}$ . A subsample of fresh ileal effluent was stored separately for SCFA analysis. The remaining ileal and fecal samples were freeze-dried prior to analyses of dry matter and protein.

**TRFLP analysis.** Total genomic DNA was extracted from ileal effluent and feces using a FastDNA kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Partial fragments of bacterial 16S rRNA genes were amplified by PCR using universal forward primer S-D-Bact-0008-a-S-20 (AGAGTTTGATCMTGGCTCAG), labeled with 6-carboxyfluorescein (FAM), and reverse primer S-D-Bact-0926-a-S-20 (CCGTC AATTCATTGAGTTT) (37). The purified PCR product (200 ng) was digested at  $37^\circ\text{C}$  overnight using 15 U of MspI (Fermentas, Burlington, CA) in 2  $\mu\text{l}$  reaction buffer and UV-sterilized Millipore water, made up to 20  $\mu\text{l}$ . Two microliters of the digestion solution was subsequently mixed with 9  $\mu\text{l}$  of formamide and 0.5  $\mu\text{l}$  of an internal size standard (ABI GeneScan 600 LIZ size standard) and denatured at  $95^\circ\text{C}$  for 5 min, followed by cooling on ice for 2 min. Fragment sizes were analyzed using an ABI 3130xl genetic analyzer in gene scan mode and GeneMapper version 3.7 software (Applied Biosystems, Ontario, Canada). Fragments that were different at fewer than 3 bp were considered to be identical in accordance with binning criteria.

**Genomic DNA extraction for qPCR.** Nucleic acids were extracted from ileal effluents and feces of pigs by use of phenol-chloroform essentially as described by Knarreborg et al. (17). For DNA extraction, 200 mg of sample was weighed into a sterile tube containing 300 to 400 mg of sterile zirconium beads (diameter, 0.1 mm) and suspended in 1 ml of TN150 buffer (10 mM Tris-HCl, 150 mM NaCl [pH 8.0]). The suspension was vortexed and centrifuged at  $14,600 \times g$  for 5 min. The pellet was washed twice with 1 ml of TN150 buffer and was resuspended in 1 ml of TN150 buffer. The cells were lysed by physical disruption in a mini-bead beater (Biospec Products, Bartlesville, OK) at 5,000 rpm for 3 min and placed on ice to cool for 5 min. Subsequently, the sample was centrifuged at  $14,600 \times g$  for 5 min. A total of 900  $\mu\text{l}$  of the supernatant was extracted twice with 1 ml TE (10 mM Tris, 1 mM EDTA [pH 8.5])-saturated phenol, followed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acids were precipitated with 2 volumes of cold ethanol ( $-20^\circ\text{C}$ ) and 0.1 volume of 5 M potassium acetate and stored overnight at  $-20^\circ\text{C}$ . The DNA was collected by centrifugation at  $14,600 \times g$  for 20 min at  $4^\circ\text{C}$ , dried at room temperature for 1 h, and dissolved in 50  $\mu\text{l}$  TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]). Prior to quantitative PCR (qPCR), DNA was diluted 5 times with sterilized Millipore water.

**Quantitative PCR.** Quantitative PCR was performed on a model 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) using the detection software (version 2.01; Applied Biosystems). Each reaction was run in duplicate in a volume of 25  $\mu\text{l}$  in optical reaction plates sealed with optical adhesive film (Applied Biosystems). The reaction mixture consisted of 12.5  $\mu\text{l}$  Fast SYBR green master mix (Applied Biosystems), 1  $\mu\text{l}$  (10  $\mu\text{M}$ ) of primers (Table 1), and 1  $\mu\text{l}$  of template DNA of ileal or fecal samples. To account for the degeneracy of the butyryl-CoA CoA transferase and butyrate kinase primers, higher primer concentrations were used in the reaction mixture (20  $\mu\text{M}$  each primer). For amplification of the butyrate kinase gene and virulence factor genes, 12.5  $\mu\text{l}$  QuantiFast SYBR green master mix (Qiagen, Mississauga, ON, Canada) was used. Amplification involved 1 cycle at  $95^\circ\text{C}$  for 5 min for initial denaturation, followed by 40 cycles of denaturation at  $95^\circ\text{C}$  for 15 s, primer annealing at the optimal temperatures (Table 1) for 30 s, extension at  $72^\circ\text{C}$  for 30 s, 1 cycle of  $95^\circ\text{C}$  for 1 min, 1 cycle at  $55^\circ\text{C}$  for 1 min, and a stepwise increase of the temperature from 55 to  $95^\circ\text{C}$  (at 10 s per  $0.5^\circ\text{C}$ ) to obtain melt curve data. Data were collected at the extension step. Melting curves were checked after amplification in order to ensure correct amplification results. Standard curves were generated using serial dilutions of the purified and quantified PCR products generated by standard PCR using the primers shown in Table 1 and genomic DNA from pig intestinal contents (20). The detection limits were  $10^2$ ,  $10^4$ , and  $10^3$  copies/g wet digesta for the group-specific primers, the butyrate enzyme gene primers, and the *E. coli* virulence factor primers, respectively.

**Analytical methods.** Samples of diets, freeze-dried ileal effluent, and feces were finely ground to pass through a 0.5-mm mesh screen (Lab Retsch Mill, Haan, Germany). Dry matter, crude protein, and titanium dioxide were analyzed according to the method of the AOAC (1). Feces were analyzed for SCFA by gas chromatography as described by Htoo et al. (16).

**Data presentation.** Results for terminal restriction fragment length polymorphism (TRFLP) analysis and measurement of gene copy numbers for bacterial groups, butyrate enzymes, *E. coli* virulence factors, and fecal SCFA are expressed

TABLE 1. Oligonucleotide primers used to profile intestinal samples

Targeted bacterial group (primer size [bp])	Orientation <sup>a</sup>	Primer sequence (5'-3')	Annealing temp (°C)	Reference
Domain bacteria (200)	F R	CGGYCCAGACTCCTACGGG TTACCGCGGCTGCTGGCAC	60	19
<i>Lactobacillus</i> spp. (341)	F R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	62	46 14
<i>Enterococcus</i> spp. (144)	F R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGACTTCCCATTGT	60	39
<i>Bifidobacterium</i> spp. (243)	F R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	60	39
<i>Streptococcus</i> spp. (485)	F R	AGAGTTTGATCCTGGCTCAG GTTAGCCGTCCTTTCTGG	60	33 10
<i>Clostridium</i> cluster XIVa (438-441)	F R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	60	28
<i>Clostridium</i> cluster IV (130)	F R	GCACAAGCAGTGGAGT CTTCTCCGTTTTGTCAA	60	29
<i>Clostridium</i> cluster I (120)	F R	ATGCAAGTCGAGCGAKG TATGCGGTATTAATCTYCCTTT	60	39
<i>Bacteroides-Prevotella-Porphyrionas</i> (140)	F R	GGTGTCCGGCTTAAGTGCCAT CGGAYGTAAGGGCCGTGC	60	39
<i>Enterobacteriaceae</i> family (195)	F R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	63	4
Butyryl-CoA CoA transferase (530)	F R	GCIGAICATTTACITGGAAYWSITGGCAYATG CCTGCCTTTGCAARTCIACRAANGC	53	24
Butyrate kinase (301)	F R	GTATAGACTACTIRIYIATHAAYCCNGG CAAGCTCRTCIACIACIACNGGRTCNAC	53	26
STa (193)	F R	ATGAAAAAGCTAATGTTGGC TACAACAAAGTTCACAGCAG	56	13
STb (204)	F R	AATATCGCATTTCCTTCTTGC GCATCCTTTTGCTGCAAC	56	13
LT (291)	F R	CTATTACAGAACTATGTTCCGG TACTGATTGCCGCAATTG	56	13
EAST1 (109)	F R	TGCCATCAACACAGTATATCC GCGAGTGACGGCTTTGT	56	13

<sup>a</sup> F, forward; R, reverse.

on the basis of wet weight of ileal effluent and feces rather than dry weight to illustrate the actual *in situ* situation in the gastrointestinal tract. Ileal protein flow was expressed on a dry-matter basis.

**Calculations.** Ileal flow of dry matter and crude protein represents the amounts of dry matter and protein present in ileal effluent and was calculated according to the equation  $D_O = A_I \times (I_D/I_I)$ , where  $D_O$  is the total output of a nutrient in ileal effluent (g/kg of dry-matter intake),  $A_I$  is the concentration of a nutrient in ileal effluent (g/kg of dry-matter intake),  $I_D$  is the marker concentration in the assay diet (g/kg of dry matter), and  $I_I$  is the marker concentration in ileal effluent (g/kg of dry matter). Dry-matter disappearance in the large intestine was calculated as the difference between fecal and ileal dry-matter content levels.

**Statistical analysis.** Data were analyzed according to a double 4-by-4 Latin square design using the mixed procedure (PROC MIXED) of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The fixed effects included animal and treatment effects. Instances of period and animal within a square were considered random effects, assuming a compound symmetry variance-covariance structure. To detect any influential observation on the model, a test was

performed using Cook's distance (Cook's  $D$ ) as a criterion. Any observation with a Cook  $D$  greater than 0.5 was considered influential and hence excluded from further analysis. Degrees of freedom were approximated using the Kenward-Rogers method.  $P$  values of  $\leq 0.05$  were defined as significant, whereas  $P$  values that were both  $> 0.05$  and  $\leq 0.10$  were considered representative of a trend.

The TRFLP results were analyzed using Statistica software (version 6.0; Statsoft, Tulsa, OK). The profiles were normalized, and only fragments with a relative peak area ratio ( $P_i$ ) of  $\geq 1\%$  were considered for further analyses. The total number of distinct fragments (richness [ $S$ ]) was counted, and Shannon's index [ $H' = -\sum_{i=1}^S (P_i)(\log P_i)$ ] and Simpson's index [ $1 - D = \sum_{i=1}^S (P_i^2)$ ] were calculated as ecological measures of the relative distribution of bacterial groups in the community. The Shannon and Simpson indices take into account the number of species and the evenness (Shannon's index) or relative distribution (Simpson's index) of the species as represented by terminal restriction fragments (TRFs). The indices are increased either by having additional unique TRFs or by having a greater evenness of TRFs. The mean values for these parameters were

TABLE 2. Characteristics of ileal effluent, feces, and fecal SCFA of pigs fed diets supplemented with viscous and fermentable nonstarch polysaccharide fractions<sup>a</sup>

Characteristic	Value for indicated supplement				Pooled (SEM)	P
	Low fermentability		High fermentability			
	Low-viscosity CEL	High-viscosity CMC	Low-viscosity LG	High-viscosity HG		
Dry-matter content (g/kg [wet wt])						
Ileal effluent	145†	55§	86‡	88‡	5.5	0.001
Feces	573†	268‡	571†	554†	30.0	<0.001
Ileal flow rate (g/kg dry-matter intake)						
Dry matter	308†	165§	242‡	277‡‡	17.4	<0.001
Crude protein	42†‡	26§	39‡	47†	2.0	<0.001
Rate of postileal dry-matter disappearance (g/kg dry matter intake)	129†	23‡	102†	126†	20.3	0.010
SCFA concn in feces (μmol/g [wet wt])						
Total	61†	28‡	69†	64†	6.9	0.005
Acetate	43†	19‡	42†	41†	4.9	0.015
Propionate	9†	5‡	10†	9†	1.2	0.038
Butyrate	5†	1‡	7†	5†	0.9	0.004
Isobutyrate	1.4‡	0.7§	2.8†	2.3†	0.22	<0.001
Valerate	1.1‡	0.5§	2.1†	1.5‡§	0.19	<0.001
Isovalerate	2.3‡	1.1‡	4.6†	3.6†	0.47	<0.001
Caproate	0.2†‡	0.2‡	0.4†	0.3†	0.06	0.159

<sup>a</sup> Data are presented as least-square means ( $n = 8$ ). Values within a row not having the same symbol are significantly different ( $P < 0.05$ ). CEL, cellulose; CMC, carboxymethylcellulose.

compared by analysis of variance (ANOVA), followed by Tukey's honestly significant difference (HSD) test. Individual TRFs were assigned to microbial species by use of the MICA II online analysis tool (<http://mica.ibest.uidaho.edu/trflp.php>), using the above-mentioned primer set and MspI for an *in silico* virtual digest against the Ribosomal Database Project (RDP) database.

The discrimination model was developed using linear discriminant analysis in the JMP software program (version 8.0.1; SAS Institute Inc., Cary, NC) to examine potential relationships between NSP fractions and TRFs and NSP fractions and gene copies for bacterial groups. Principal component analysis (PCA) was performed to examine any potential grouping of bacterial group gene copies, butyrate production pathway gene copies, *E. coli* virulence factors, ileal flow of dry matter, and postileal dry-matter disappearance according to the different diets used in this study by means of JMP software. The loading plot shows the variables responsible for the variation within the data set, and the correlations among individual variables of the first two eigenvalues (i.e., principal component 1 [PC 1] and PC 2). This gives a graphical representation of the extent to which each factor accounts for the variance in the data and shows the relationship between the different variables.

## RESULTS

**Ileal flow of dry matter and protein, dry-matter disappearances in the large intestine, and concentrations of SCFA in feces.** The dry-matter content levels of ileal effluent and feces were lower ( $P < 0.01$ ) for CMC than for LG and HG (Table 2). The levels of ileal flow of dry matter and protein were higher ( $P < 0.01$ ) for CEL, LG, and HG than for CMC. As a result, the levels of postileal dry-matter disappearance were greater ( $P = 0.01$ ) for CEL, LG, and HG than for CMC. Fecal concentrations of total SCFA, including acetate, propionate, and butyrate, were lower ( $P < 0.05$ ) in pigs fed the CMC diet than in those fed the CEL, LG, and HG diets. Inclusion of LG and HG additionally increased concentrations of isobutyrate and isovalerate in feces ( $P < 0.01$ ).

**TRFLP analysis.** The TRFLP analysis of ileal samples showed patterns dominated by relatively few major TRFs,

whereas a total of 75 different TRFs were observed in feces (see Tables S2 and S3 in the supplemental material). Measures of species richness ( $4.1 \pm 1.8$  versus  $14.5 \pm 4.3$ ), and Shannon's ( $0.34 \pm 0.21$  versus  $0.86 \pm 0.22$ ) and Simpson's ( $0.40 \pm 0.24$  versus  $0.76 \pm 0.13$ ) diversity were higher in feces than in ileum (Table 3). Among diets, diversity indices were not significantly different. However, a trend ( $P < 0.10$ ) toward higher levels of diversity for CMC than for the other treatments was observed, whereas CEL showed relatively low diversity indices (Table 3), and similar trends for ecological measures were observed in ileal effluents and fecal samples.

Terminal restriction fragments identified in ileal effluent could be assigned to *Streptococcus/Lactococcus* spp., to *Lactobacillus* spp., to *Clostridium* clusters I, XIVa, and XVIII, and to *Fibrobacter* spp. Ileal communities were dominated by TRFs representing *Streptococcus agalactiae*-like species in almost all animals. In feces, the following bacterial groups and clusters could be identified: *Streptococcus* spp., *Lactobacillus/Enterococcus/Oenococcus* spp., *Clostridium* clusters I, IV, XI, XIVa, and XVIII. Furthermore, TRFs that contributed only about 1 to 2% to all TRFs were *Corynebacterium*, *Collinsella*, *Fibrobacter*, *Selenomonas*, and *Desulfovibrio* species-like phylotypes. Two TRFs in ileal effluent and 21 TRFs in feces could not be assigned to known species.

**Bacterial populations.** A set of 10 group-specific primers was employed to quantify bacterial populations in ileal effluent and feces. Bacterial populations in ileal effluent were only slightly affected by the NSP fractions (Table 4). The number of total bacterial gene copies in the distal ileum was lower ( $P < 0.05$ ) for LG than for CEL, CMC, and HG. *Clostridium* cluster I was detectable only in the ileal effluents of pigs fed the CEL diet. Supplementation with CMC resulted in the highest num-

TABLE 3. Species richness levels and Shannon and Simpson indices of diversity as calculated from normalized TRFLP profiles for ileal effluents and feces of pigs fed diets supplemented with viscous and fermentable nonstarch polysaccharide fractions<sup>a</sup>

Characteristic	Value for indicated supplement				Pooled (SEM)	P
	Low fermentability		High fermentability			
	Low-viscosity CEL	High-viscosity CMC	Low-viscosity LG	High-viscosity HG		
<b>Ileal effluent</b>						
Species richness	3.50	4.25	3.50	5.00	1.78	0.288
Shannon index	0.23	0.42	0.29	0.43	0.20	0.160
Simpson index	0.26	0.50	0.35	0.48	0.23	0.156
<b>Feces</b>						
Species richness	11.60	16.25	13.67	15.14	4.19	0.277
Shannon index	0.68	0.98	0.80	0.90	0.21	0.086
Simpson index	0.65	0.83	0.72	0.79	0.12	0.076

<sup>a</sup> Data are presented as least-square means ( $n = 8$ ). CEL, cellulose; CMC, carboxymethylcellulose.

ber of *Enterobacteriaceae* family gene copies in ileal effluent ( $P < 0.05$ ). In feces, the number of total bacterial gene copies was highest for CMC and lowest for LG ( $P < 0.05$ ). The number of *Clostridium* cluster XIVa gene copies in feces was increased by CMC and reduced by LG in comparison to the number obtained with CEL, whereas the numbers of *Clostridium* cluster IV gene copies in feces were lower for both CMC and LG than for CEL ( $P < 0.05$ ). Additionally, CMC and LG caused lower ( $P < 0.05$ ) numbers of *Clostridium* cluster I gene copies in feces than CEL and HG. CMC increased the number of *Bacteroides-Prevotella-Porphyrmonas* group gene copies in

feces in comparison to the other NSP fractions, whereas HG reduced the number of gene copies in feces in comparison to CEL and CMC ( $P < 0.05$ ). Finally, the number of *Enterobacteriaceae* family gene copies in feces was distinctly higher ( $P < 0.05$ ) for CMC than for LG and HG.

**Multivariate analysis.** Linear discriminant analysis of NSP fractions and TRFs (Fig. 1a) divided the effects of NSP fractions into three clusters, comprising the CEL and LG diets (overlapping in their 95% confidence intervals), the CMC diet, and the HG diet. The CEL and LG diets were more related to TRF9 (*Bacteroides* species-like phylotype), whereas the CMC

TABLE 4. Bacterial groups in ileal effluent and feces of pigs fed diets supplemented with viscous and fermentable nonstarch polysaccharide fractions<sup>a</sup>

Bacterial group	Value for indicated diet				Pooled (SEM)	P
	Low fermentability		High fermentability			
	Low-viscosity CEL	High-viscosity CMC	Low-viscosity LG	High-viscosity HG		
<b>Ileal effluent</b>						
Total bacteria	9.5†	9.8†	8.5‡	9.6†	0.18	0.001
<i>Lactobacillus</i> spp.	7.9	8.3	7.7	8.3	0.24	0.184
<i>Enterococcus</i> spp.	8.1†‡	8.0†‡	7.6‡	8.5†	0.28	0.227
<i>Streptococcus</i> spp.	8.1†	8.2†	7.8‡	8.2†	0.08	0.006
<i>Bifidobacterium</i> spp.	6.9	7.1	6.9	7.5	0.37	0.655
<i>Clostridium</i> cluster XIVa	6.8	7.3	6.4	6.9	0.36	0.387
<i>Clostridium</i> cluster IV	5.2	3.0	4.5	5.1	0.94	0.363
<i>Clostridium</i> cluster I <sup>b</sup>	6.3 ± 0.34	<2	<2	<2		
<i>Bacteroides-Prevotella-Porphyrmonas</i>	5.6	6.3	5.5	6.4	0.35	0.197
<i>Enterobacteriaceae</i> family	8.2‡	9.1†	7.6§	8.8†‡	0.27	0.006
<b>Feces</b>						
Total bacteria	10.1‡	11.3†	9.2§	9.7‡§	0.25	<0.001
<i>Lactobacillus</i> spp.	6.9†	5.6‡	6.2†‡	6.1†‡	0.41	0.202
<i>Enterococcus</i> spp.	8.1†	6.9‡§	6.6§	7.4†‡	0.29	0.010
<i>Streptococcus</i> spp.	7.8‡	8.5†	7.4‡	7.7‡	0.18	0.004
<i>Bifidobacterium</i> spp.	7.5	7.0	7.2	7.8	0.39	0.525
<i>Clostridium</i> cluster XIVa	7.7‡	8.7†	6.4§	7.1‡§	0.40	0.007
<i>Clostridium</i> cluster IV	7.8†	6.5‡	6.6‡	7.1†‡	0.33	0.059
<i>Clostridium</i> cluster I	7.2†	2.5‡	3.3‡	6.5†	0.93	0.005
<i>Bacteroides-Prevotella-Porphyrmonas</i>	8.9‡	10.5†	8.0§	8.4‡§	0.20	<0.001
<i>Enterobacteriaceae</i> family	9.0†‡	10.3†	7.0§	7.6‡§	0.58	0.004

<sup>a</sup> Data are presented as least-square means ( $n = 8$ ). Values are  $\log_{10}$  numbers of 16S rRNA gene copies/g (wet weight). Values within a row not having the same symbol are significantly different ( $P < 0.05$ ). CEL, cellulose; CMC, carboxymethylcellulose.

<sup>b</sup> The value shown for CEL is the mean ± standard error (SE). The detection limit was 2  $\log_{10}$  16S rRNA gene copies/g (wet weight).

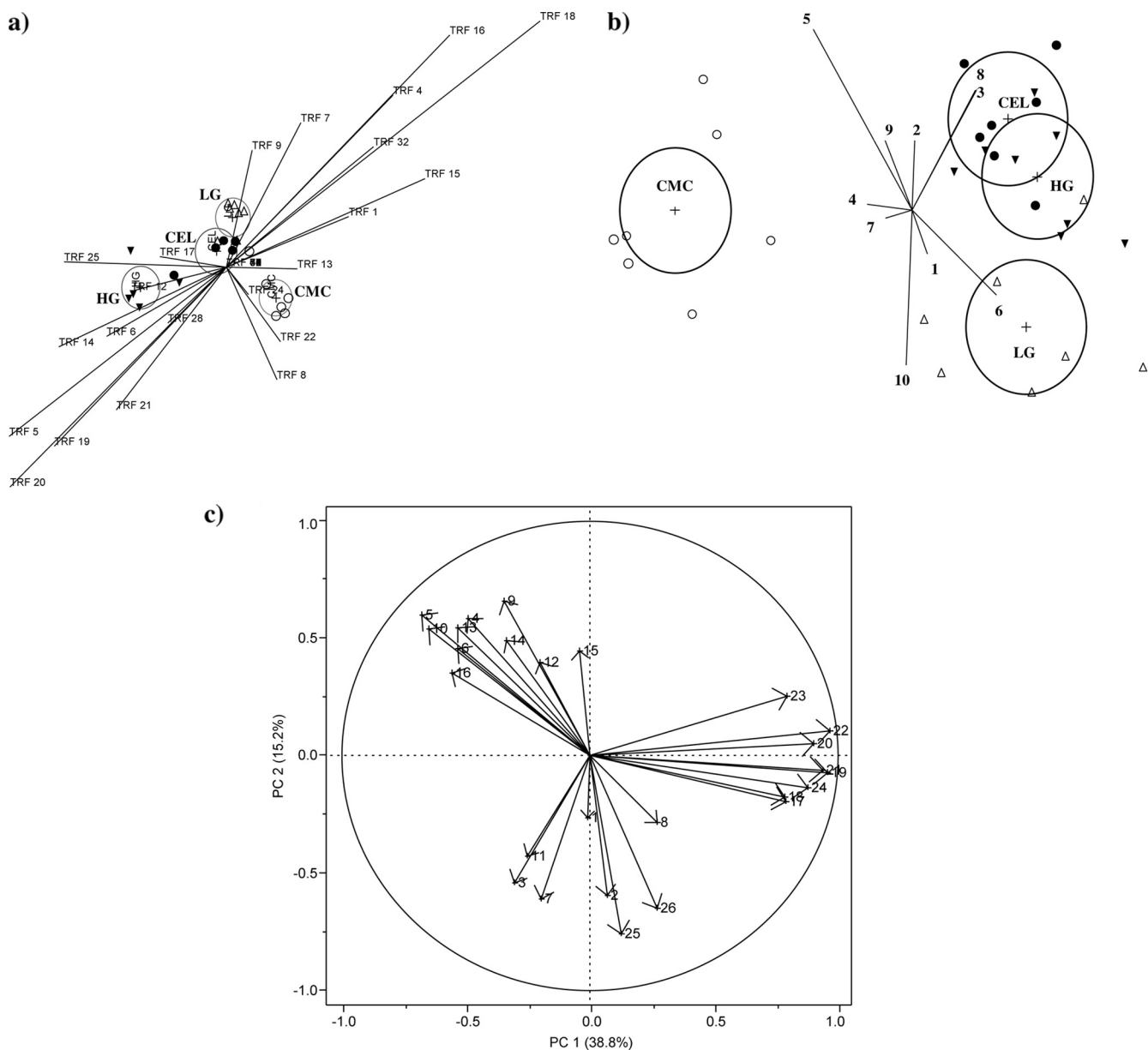


FIG. 1. (a, b) Linear discriminant analysis of the NSP fractions and TRFs (a) and gene copies for bacterial groups (b): cellulose (CEL; ●), carboxymethylcellulose (CMC; ○), low-viscosity oat β-glucan (LG; △), and high-viscosity oat β-glucan (HG; ▼). (c) Loading plot showing the correlations among gene copies for bacterial groups in feces, *E. coli* virulence factors in feces, butyryl-coenzyme A (CoA) CoA transferase and butyrate kinase in feces, fecal SCFA and ileal flow, and disappearance of fermentable substrate in the large intestines of the first two eigenvalues (PC 1 and PC 2). 1, *Lactobacillus* spp.; 2, *Enterococcus* spp.; 3, *Bifidobacterium* spp.; 4, *Streptococcus* spp.; 5, *Bacteroides-Prevotella-Porphyromonas*; 6, *Clostridium* cluster XIVa; 7, *Clostridium* cluster IV; 8, *Clostridium* cluster I; 9, *Enterobacteriaceae*; 10, total bacteria; 11, butyryl-CoA CoA transferase; 12, butyrate kinase; 13, EAST1; 14, STa; 15, STb; 16, LT; 17, acetate; 18, propionate; 19, iso-butyrate; 20, butyrate; 21, isovalerate; 22, valerate; 23, caproate; 24, total SCFA; 25, ileal flow of dry matter; and 26, postileal dry-matter disappearance.

diet was linked to TRF22 (*Clostridium polysaccharolyticum*-like phylotype). The HG diet was more related to TRF14 (*Clostridium innocuum*-like phylotypes). In accordance with the linear discriminant analysis of NSP fractions and gene copy numbers for bacterial groups (Fig. 1b), samples were also divided into three clusters; however, CEL and HG formed a single cluster, as indicated by the intersecting 95% confidence intervals. The results for the LG diet were different from those for the CEL, HG, and CMC diets, whereas the results for CMC

were drastically different from those for the other NSP fractions. Here, the gene copy numbers for *Bifidobacterium* spp. and *Clostridium* cluster I discriminated best for CEL and HG, whereas those for *Clostridium* cluster IV and *Streptococcus* spp. discriminated best for CMC and those for *Clostridium* cluster XIVa for LG.

Figure 1c depicts the loading plot showing the individual qPCR data, fecal SCFA, ileal flow, and disappearance of dry matter in the large intestine responsible for the variation of the

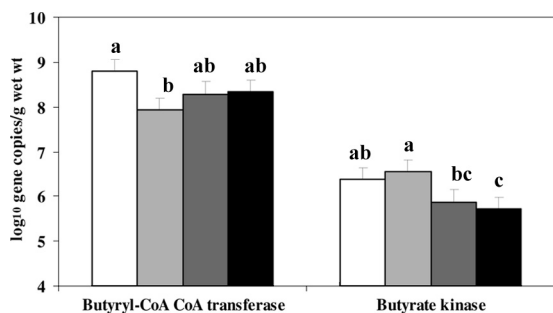


FIG. 2. Gene copy numbers for butyryl-coenzyme A (CoA) CoA-transferase and butyrate kinase in feces of pigs fed diets supplemented with cellulose (white bars), carboxymethylcellulose (light gray bars), low-viscosity oat  $\beta$ -glucan (dark gray bars), or high-viscosity oat  $\beta$ -glucan (black bars). The detection limit was 4  $\log_{10}$  gene copies/g (wet weight).

first two eigenvalues (PC 1 and PC 2) and the correlations among individual variables. The first PC component accounted for 38.8% of the variation, and PC 2 explained 15.2%. The SCFA formed a cluster located on the right part of the graph and were highly influenced by PC 1. *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Clostridium* clusters I and IV, and butyryl-CoA CoA transferase formed a second cluster at the bottom of the loading plot and were negatively correlated with PC 2. A third cluster was formed by total bacteria, *Enterobacteriaceae*, the *Bacteroides-Prevotella-Porphyrromonas* group, *Streptococcus* spp., *Clostridium* cluster XIVa, *E. coli* virulence factors, and butyrate kinase, which were influenced by both PC 1 and PC 2 and were negatively correlated with the other two clusters (with angles of  $>90^\circ$  between the arrows for these clusters). Variables within these three clusters were positively related among each other, as indicated by the small angles between the arrows for these variables ( $<90^\circ$ ).

**Butyrate production pathway genes.** Butyrate-producing bacteria in feces were determined by targeting genes for the enzymes butyryl-CoA CoA transferase and butyrate kinase (Fig. 2). The gene copy numbers for butyryl-CoA CoA transferase were higher (7.9 to 8.8  $\log_{10}$  DNA gene copies/g [wet weight]) than those for the butyrate kinase (5.7 to 6.6  $\log_{10}$  DNA gene copies/g [wet weight]). Dietary supplementation with CMC resulted in a lower ( $P < 0.05$ ) gene copy number for the butyryl-CoA CoA transferase than supplementation with the CEL diet. In contrast, CMC increased ( $P < 0.05$ ) the gene copy number for the butyrate kinase in comparison to LG and HG.

***Escherichia coli* virulence factors.** The heat-stable enterotoxin of enteroaggregative *E. coli* was the dominating virulence factor detected in both ileal and fecal samples, ranging from 6.0 to 8.9  $\log_{10}$  DNA gene copies/g (wet weight). In ileal effluent, EAST1 levels were affected by NSP ( $P = 0.026$ ) and were significantly higher for CMC and HG than for LG (6.8, 7.5, 6.0, and  $7.3 \pm 0.35$   $\log_{10}$  DNA gene copies/g [wet weight] for CEL, CMC, LG, and HG, respectively). The levels of virulence factors of enterotoxigenic *E. coli* heat-stable toxin A (STa), STb, and heat-labile toxin (LT) were below the detection limits in the ileal effluent. In feces, the EAST1 levels were higher by 1.4 to 2.5 log units for CMC than for the other NSP fractions ( $P < 0.05$ ) (Fig. 3). The gene copy numbers for STa

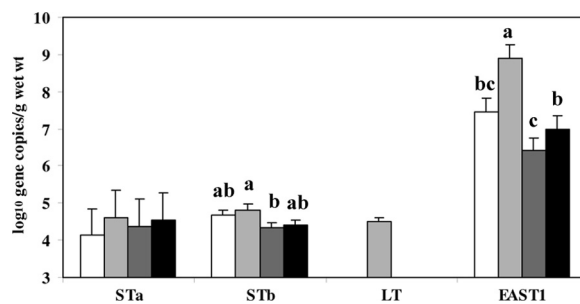


FIG. 3. Gene copy numbers for virulence factors (heat-stable enterotoxins [STa and STb], heat-labile enterotoxin [LT] of enterotoxigenic *Escherichia coli*, and heat-stable enterotoxin [EAST1] of enteroaggregative *E. coli*) in feces of pigs fed diets supplemented with cellulose (white bars), carboxymethylcellulose (light gray bars), low-viscosity oat  $\beta$ -glucan (dark gray bars), or high-viscosity oat  $\beta$ -glucan (black bars). The detection limit was 3  $\log_{10}$  gene copies/g (wet weight).

did not differ in feces, whereas the numbers of STb copies were higher ( $P < 0.05$ ) for CMC than for LG. The LT was detectable only in the feces of pigs fed the CMC diet.

## DISCUSSION

In the present study, we used a polyphasic approach to study the effects of four purified NSP fractions of low and high viscosity and fermentability on the taxonomic composition of the ileal and fecal microbiota and, at a metabolic level, on butyrate-producing bacteria and *E. coli* virulence factors, using TRFLP and qPCR. Because purified NSP fractions may affect the bacterial community structure in a different way when added to a cereal-based diet due to the NSP in the grain matrix (36), a semipurified diet was employed in the present experiment.

The NSP fractions differently affected the small intestinal digestion and markedly changed the availability of fermentable substrate in the large intestine. However, there was no evidence that the shared functional properties affected digestive processes and endogenous nitrogen losses (44) consistently among the NSP fractions, suggesting that the specific chemical structures of the NSP are as relevant as shared rheological properties (8, 48). Correspondingly, consistent effects of viscosity and fermentability were not observed for gene copies for bacterial groups in ileal effluent. Cellulose resulted in faster transit than CMC and HG (data not shown); thus, besides the retention time in the small intestine, the accessibility of dietary nutrients appeared to be a critical factor for bacterial growth. Increased digesta viscosity impairs intestinal contractions (21), thereby preventing mixing of digesta and bacteria and thus access of bacteria to new substrate. Low-viscosity CEL, in turn, likely did not impair intestinal contractions and hence digesta mixing. According to the TRFLP profiles, *Streptococcus agalactiae*-like phylotypes dominated the ileal microbiota, followed by phylotypes belonging to *Clostridium* cluster XIVa. Surprisingly, TRFs representing *Enterobacteriaceae* species were not detected in ileal effluent or feces, which can be likely associated with the utilization of only one restriction enzyme (30), whereas the high rRNA gene copy numbers for *Entero-*

*bacteriaceae* produced with qPCR confirmed the prevalence of this bacterial group in the guts of pigs (22, 41).

The effects of the different purified NSP fractions on the formation of fermentation end products and bacterial numbers in feces mostly depended on changes in the ileal flow of dry matter (i.e., NSP fractions and other nondigested dietary ingredients) into the large intestine. In this context, low-fermentability CEL resulted in levels of postileal dry-matter disappearance and SCFA concentrations in feces similar to those obtained with high-fermentability LG and HG and significantly higher than those obtained with low-fermentability CMC.

Diversity indexes indicated that CMC supported a higher level of fecal bacterial diversity than the other NSP. Linear discriminant analysis of qPCR and TRFLP data confirmed that the fecal bacterial community structure in pigs fed the CMC diet differed from that observed in pigs fed the other NSP fractions. For instance, cellulolytic *C. polysaccharolyticum*-like phylotypes (TRF22) (47) discriminated best for the CMC diet, whereas fibrolytic and amylolytic *Bacteroides* species-like phylotypes (TRF9) (42) represented the best discrimination variable for CEL and LG. This may indicate that not only the NSP fractions but also the starch content in digesta may have modulated the bacterial community in pigs fed these diets.

Among the NSP fractions, low-fermentability CMC resulted in the highest numbers of total bacterial gene copies in feces. Generally, *Clostridium* clusters IV and XIVa and the *Bacteroides-Prevotella-Porphyromonas* group are the dominating strictly anaerobic bacterial groups in the large intestines of pigs (22). Carboxymethylcellulose clearly promoted the growth of *Clostridium* cluster XIVa and particularly of the *Bacteroides-Prevotella-Porphyromonas* group and *Enterobacteriaceae* in comparison to the other NSP. In contrast, the CEL diet favored the growth of *Clostridium* cluster IV. In addition to cellulose, the availability of other easily fermentable substrates in the ileal effluent, such as starch and protein, may have supported the higher numbers for *Clostridium* cluster IV, as this cluster contains both fibrolytic and nonfibrolytic species (25, 27), including some butyrate-producing bacterial species, such as *Butyrivibrio fibrisolvens* (2).

Cellulose, LG, and HG caused higher butyrate concentrations in feces than CMC. However, the measurement of butyrate in colonic digesta and portal blood is insufficient, as butyrate is mainly catabolized by colonocytes (3) and the various *Clostridium* clusters contain both butyrate producers and non-butyrate producers (27). In humans, butyryl-CoA CoA transferase and butyrate kinase genes are used as marker genes to detect butyrate-producing bacteria in the colon (24, 26). Similar to what was found for human butyrate producers (26), the main route of butyrate formation in the hindguts of pigs is the butyryl-CoA CoA transferase pathway. Moreover, the loading plot of PCA indicated that the ileal flow of dry matter into the large intestine was positively correlated with the number of butyryl-CoA CoA transferase gene copies, suggesting that the availability of not only the NSP fractions but also fermentable substrate was important for butyrate producers using the butyryl-CoA CoA transferase pathway. Similarly, butyryl-CoA CoA transferase correlated with lactic acid-producing groups, such as lactobacilli, bifidobacteria, and enterococci. This may be related to cross-feeding of butyrate-producing bacteria with lactate (27). The butyrate kinase was negatively

correlated with the ileal flow of fermentable substrate, and its gene copy numbers were increased by CMC.

The CEL and HG diets markedly raised the gene copy numbers for *Clostridium* cluster I. Although this cluster contains fibrolytic and butyrate-producing bacteria (e.g., *Clostridium cellulovorans*), other members, such as *Clostridium perfringens*, may be harmful for the host (32). A TRF representing a *C. perfringens*-like phylotype was identified in feces, and a TRF was recognized as a *Clostridium bifermentans*-like phylotype that represents a potential pathogenic bacterium belonging to *Clostridium* cluster XI (40). However, adverse effects of high-viscosity NSP on gut health are mainly attributed to pathogenic *E. coli* (18, 43). High-viscosity CMC favored growth of pathogenic *E. coli* in weaning pigs (15, 31); however, these effects were generally confined to the immediate period after weaning (12, 15). The loose feces in combination with the high numbers of *Enterobacteriaceae* rRNA gene copies in the feces and ileal effluents of growing pigs used in the present study indicate that older pigs are also susceptible to overgrowth of enteropathogenic bacteria when the diet contains CMC. Quantitative PCR of virulence factors revealed that gene copies for particularly enteroaggregative *E. coli* bacteria were present in higher numbers in the distal ileum and in feces. Moreover, the heat-labile enterotoxin LT was exclusively detectable in feces of pigs fed the CMC diet and not in those of pigs fed diets supplemented with the other NSP. The similar gene copy numbers for EAST1, STa, and STb in feces of pigs fed CEL, LG, and HG did not cause any signs of diarrhea. The lower numbers observed for *Enterobacteriaceae* and *E. coli* virulence factors with the use of high-viscosity HG suggest that factors other than viscosity are involved in the stimulation of pathogenic *E. coli*. The CMC diet may have influenced the proliferation of pathogenic *E. coli* through changes in the mucus composition and the amount of mucus produced (35).

In conclusion, this study disclosed that the intestinal bacterial community, genes of alternative pathways of butyrate production, and *E. coli* virulence factors are specifically modulated by supplementing a semipurified diet with CEL, CMC, LG, or HG. Changes may be attributable to bacterial fermentation of NSP; additionally, NSP altered the ileal flow of nutrients into the large intestine. Effects of the NSP fractions were linked to the individual NSP fractions rather than to their shared functional properties, i.e., viscosity and fermentability. Comparable to what was observed for human colonic microbiota, the gene copy numbers for butyryl-CoA CoA transferase were higher than those for butyrate kinase, indicating that this pathway is the dominant butyrate production pathway in the large intestines of pigs. Although increasing intestinal viscosity was generally associated with impaired gut health (18, 43), only CMC increased the susceptibility of pigs to overgrowth of pathogenic *E. coli*, suggesting that the use of CMC in diets for growing pigs is detrimental compared to the use of the other NSP fractions investigated.

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