The Nutritional Significance of Intestinal Fungi: Alteration of Dietary Carbohydrate Composition Triggers Colonic Fungal Community Shifts in a Pig Model

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ABSTRACT Carbohydrates represent the most important energy source in the diet of humans and animals. A large number of studies have shown that dietary carbohydrates (DCHO) are related to the bacterial community in the gut, but their relationship with the composition of intestinal fungi is still unknown. Here, we report the response of the colonic fungal community to different compositions of DCHO in a pig model. Three factors, ratio (2:1, 1:1, and 1:2) of amylose to amylopectin (AM/AP), level of nonstarch polysaccharides (NSP; 1%, 2%, and 3%), and mannan-oligosaccharide (MOS; 400, 800, and 1,200 mg/kg body weight), were considered according to an L9 (3⁴) orthogonal design to form nine diets with different carbohydrate compositions. Sequencing based on an Illumina HiSeq 2500 platform targeting the internal transcribed spacer 1 region showed that the fungal community in the colon of the pigs responded to DCHO in the order of MOS, AM/AP, and NSP. A large part of some low-abundance fungal genera correlated with the composition of DCHO, represented by *Saccharomycopsis*, *Mrakia*, *Walleimia*, *Cantharellus*, *Eurotium*, *Solicoccozyma*, and *Penicillium*, were also associated with the concentration of glucose and fructose, as well as the activity of β -D-glucosidase in the colonic digesta, suggesting a role of these fungi in the degradation of DCHO in the colon of pigs. Our study provides direct evidence for the relationship between the composition of DCHO and the fungal community in the colon of pigs, which is helpful to understand the function of gut microorganisms in pigs.

IMPORTANCE Although fungi are a large group of microorganisms along with bacteria and archaea in the gut of monogastric animals, the nutritional significance of fungi has been ignored for a long time. Our previous studies revealed a distinct fungal community in the gut of grazing Tibetan pigs (J. Li, D. Chen, B. Yu, J. He, et al., *Microb Biotechnol* 13:509–521, 2020, <https://doi.org/10.1111/1751-7915.13507>) and a close correlation between fungal species and short-chain fatty acids, the main microbial metabolites of carbohydrates in the hindgut of pigs (J. Li, Y. Luo, D. Chen, B. Yu, et al., *J Anim Physiol Anim Nutr* 104:616–628, 2020, <https://doi.org/10.1111/jpn.13300>). These groundbreaking findings indicate a potential relationship between intestinal fungi and the utilization of DCHO. However, no evidence directly proves the response of intestinal fungi to changes in DCHO. Here, we show a clear alteration of the colonic fungal community in pigs triggered by different compositions of DCHO simulated by varied concentrations of starch, nonstarch polysaccharides (NSP), and oligosaccharides. Our results highlight the potential involvement of intestinal fungi in the utilization of nutrients in monogastric animals.

KEYWORDS fungi, carbohydrates, piglets, colon

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Accumulating evidence indicates that the gastrointestinal (GI) microbiota is a critical contributor to the intestinal health and homeostasis of the host (3). The composition of microbiota in the GI tract can be influenced by many factors, such as diet (4), age (5), and antibiotic administration (6), as well as the genotypes and phenotypes of the host (7). The variation of dietary components, especially carbohydrates, has been proved to directly and rapidly bring out the shifts in gut microbiota (8). The majority of existing studies focus on the relationship between bacteria and diet in the GI tract (9, 10). However, the role of fungi, or the so-called “mycobiota” that accounts for approximately 0.1% of the microorganisms in the GI tract of humans and animals (11), has been ignored for a long time. It should be noted that the real number of fungi in the GI tract may be substantially undervalued due to the underrepresentation of fungal sequences in reference databases. Although a lot of studies of human and rodent models show that fungi in the GI tract may be another class of important microorganisms associated with gut health (12, 13), the nutritional significance of gut fungi as a normal symbiont and their response to diet still remain unknown.

Carbohydrates are the main energy sources of human and most of the monogastric animals, such as pigs, accounting for 60 to 70% of total energy intake (14). The carbohydrates include polyhydroxy aldehydes, ketones, alcohols, acids, their simple derivatives, and any precursor that may be hydrolyzed to these compounds (15). According to the number of carbon atoms in the molecule, the carbohydrates may be classified into monosaccharides, oligosaccharides, and polysaccharides (16). Starch and non-starch polysaccharides (NSP) are the two members of the polysaccharides. Starch, including amylose (AM) and amylopectin (AP) (17), can be efficiently utilized by mammals. In the past a few decades, the digestion and absorption of starch has been believed to occur mainly in the small intestine of monogastric animals with the help of endogenous digestive enzymes. However, one of our studies shows that the bacterial composition in the ileum and cecum of adult pigs can be altered by diets with different ratios of AM to AP (AM/AP) (18), suggesting the participation or response of gut microorganisms to the digestion of dietary starch. NSP, a large category of dietary fiber, can be classified into soluble dietary fiber (SDF; e.g., inulin and pectin) and insoluble dietary fiber (IDF; e.g., cellulose and hemicellulose) (19). Unlike starch, NSP, especially SDF, has been widely found to shape the microbial community in the human and animal gut (20–22). Of all oligosaccharides, mannan-oligosaccharides (MOS), derived from the yeast cell wall, is the most studied functional oligosaccharide. It has been repeatedly confirmed to modify the microbial populations by stimulating the proliferation of beneficial bacteria and inhibiting the growth of pathogens in the swine gut (23). Compared with extensive research on bacteria (8, 22, 24), the influence of dietary carbohydrates (DCHO) on the community and composition of gut fungi is scarcely reported. We previously found that Tibetan pigs, a local breed adapted to grazing and abundant forage, harbors distinct fungal populations compared to Chenghua (another local breed) and Yorkshire (a popular commercial breed) pigs (1). Meanwhile, some fungal species, such as *Tomentella*, *Loreleia*, and *Nephroma*, are closely correlated with the concentration of short-chain fatty acids (SCFAs), the main metabolites of DCHO fermented by bacteria (1). Our other analysis based on the fungal sequences targeting the internal transcribed spacer 1 (ITS1) shows that Basidiomycota and Ascomycota are the two dominant fungal phyla in the hindgut of piglets (2). Compared with fungi in the GI tract of monogastric animals, fungi in rumen get more attention. One of the widespread anaerobic fungal phyla in rumen, Neocallimastigomycota, is known to play a key role in the colonization and degradation of plant cell walls (25, 26). The strong ability of such anaerobic fungi to degrade lignocellulose is attributed to a large portfolio of carbohydrate-active enzymes (CAZymes) (27, 28). Similarly, Ascomycota, a phylum containing the most species of fungi, also has strong ability to degrade lignin and cellulose. Thus, our findings indicate that the relationship between fungal community in the GI tract and DCHO deserves further study. However, most of the existing studies of human and model animals focus on the relationship between gut fungi and

diseases, ignoring the nutritional contribution of fungi to the host. Here, we hypothesize that the fungal community in the GI tract of pigs can be affected by the composition of DCHO, and the response of fungal populations to DCHO may be related to phenotypes associated with growth or intestinal health.

To verify our hypothesis, we employed a piglet model to examine the correlation between different carbohydrates and the composition of fungi in the colon. First, an orthogonally designed animal trial with the ratio of AM/AP and the levels of NSP and MOS as the three major factors was performed to screen the most suitable composition of DCHO meeting the optimal growth phenotypes. Then, the response of colonic fungal community to DCHO was analyzed to determine the correspondence between fungal species and certain compositions of DCHO.

RESULTS

Effect of different compositions of DCHO on the growth performance of the piglets. According to the variance analysis, although no difference was found in the average daily gain (ADG) and average daily feed intake (ADFI) of the piglets in different groups ($P > 0.05$; Table 1), diets with different DCHO compositions tended to affect the diarrhea rate of the animals ($P = 0.084$; Table 1). The range analysis (Tables 2 and 3) showed that the optimal composition of DCHO was A1-B3-C3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg body weight), A2-B3-C3 (AM/AP, 1:1; NSP, 3%; and MOS, 1,200 mg/kg), and A1-B3-C2 (AM/AP, 2:1; NSP, 3%; and MOS, 800 mg/kg), respectively, when taking ADG, ADFI, or the diarrhea rate of piglets as indicators. Further orthogonal variance analysis (Tables 4 and 5) showed that both the ratio of AM/AP and the level of dietary NSP remarkably influenced the ADG and diarrhea rate of the piglets ($P < 0.05$).

Fungal diversity in the colonic digesta of piglets fed diets with different DCHO compositions. In order to observe whether the change of DCHO composition affects the colonic fungal diversity in the piglets, high-throughput sequencing targeting ITS based on an Ion S5 XL platform was performed. After chimera checking, the total of 45 digesta samples yielded 2,116,083 sequences with an average of $47,024 \pm 3,834$ sequences per sample (see Table S1 in the supplemental material). All reads were assigned to 2,825 operational taxonomic units (OTUs), which were affiliated with 13 fungal phyla and 327 species, and 100 of these OTUs were shared by the piglets in all groups (Fig. S1). Except for observed species and Shannon index, other indices of α -diversity showed significant differences among the nine groups ($P < 0.05$; Table S2, Fig. 1), with the lowest ($P < 0.05$) α -diversity in those samples being from group 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg). The analysis of similarities (ANOSIM) based on the unweighted UniFrac distances revealed that the β -diversity of fungal community was different among the nine groups ($R = 0.203$, $P = 0.001$; Fig. 2), and the principal coordinate analysis (PCoA) analysis showed that the fungal composition in the samples from group 3 was independent of the others (Fig. S2).

Comparison of fungal species in the colon of piglets fed diets with different DCHO compositions. Based on the qualified sequences in all digesta samples, a total of 13 fungal phyla were annotated, with Ascomycota and Basidiomycota as the two predominate phyla accounting for more than 50% of the total sequences (Table 6). Although the relative abundance of these majorities in the colonic digesta showed no difference among groups ($P > 0.05$), the abundance of three minorities ($< 0.5\%$), Rozellomycota ($P = 0.037$), Aphelidiomycota ($P = 0.001$), and Blastocladiomycota ($P = 0.001$), was significantly different. Specifically, the abundance of Rozellomycota in the samples from group 1 (AM/AP, 2:1; NSP, 1%; and MOS, 400 mg/kg) was higher ($P < 0.05$) than in those from group 7 (AM/AP, 1:2; NSP, 1%; and MOS, 1,200 mg/kg), while the abundance of Aphelidiomycota and Blastocladiomycota in the samples from group 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg) was higher than in those from others ($P < 0.05$).

At a higher level of classification, all the sequences were affiliated with 318 known fungal genera, with *Kazachstania*, *Scheffersomyces*, *Bifiguratus*, *Candida*, and *Saccharomycopsis* as the five predominant genera (Table 6, Fig. 3). Of these, *Kazachstania* was the most

TABLE 1 Effect of DCHO composition on the growth performance of the pigs^a

Item ^b	Data for group:									P value
	1	2	3	4	5	6	7	8	9	
ADG (g)	343.71 ± 17.07	366.57 ± 22.41	383.62 ± 14.39	338.00 ± 16.46	365.71 ± 19.32	371.00 ± 8.62	331.00 ± 23.29	335.29 ± 14.36	325.52 ± 19.22	0.245
ADFI (g)	488.97 ± 15.14	526.70 ± 30.15	532.65 ± 23.08	497.59 ± 25.04	533.99 ± 27.07	527.23 ± 12.71	490.88 ± 30.49	474.59 ± 18.20	482.43 ± 25.85	0.460
Diarrhea rate (%)	10.64 ± 1.70	9.21 ± 1.04	7.79 ± 1.02	12.07 ± 1.57	11.43 ± 1.41	9.86 ± 1.21	13.71 ± 2.01	12.64 ± 0.49	9.76 ± 0.86	0.084

^aData are presented as means ± standard error (SEM). The carbohydrate composition in the diet of each group is shown in Table 8.^bADG, average daily gain; ADFI, average daily feed intake.

TABLE 2 Results of range analysis for the orthogonal experiment^a

Group	Carbohydrate composition			Phenotype		
	A (ratio of AM/AP)	B (level of NSP [%])	C (concn of MOS [mg/kg])	ADG (g)	ADFI (g)	Diarrhea rate (%)
1	A1 (2:1)	B1 (1)	C1 (400)	343.71	488.97	10.64
2	A1	B2 (2)	C2 (800)	366.57	526.70	9.21
3	A1	B3 (3)	C3 (1,200)	383.62	532.65	7.79
4	A2 (1:1)	B1	C2	338.00	497.59	12.07
5	A2	B2	C3	365.71	533.99	11.43
6	A2	B3	C1	371.00	527.23	9.86
7	A3 (1:2)	B1	C3	331.00	490.88	13.71
8	A3	B2	C1	335.29	474.59	12.64
9	A3	B3	C2	325.52	482.43	9.76

^a See also Table 3. ADG, average daily gain; ADFI, average daily feed intake.

abundant (61.94%) genus in samples from group 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg) but presented in extremely low ($P < 0.05$) abundance in the samples from group 1 (AM/AP, 2:1; NSP, 1%; and MOS, 400 mg/kg) and 2 (AM/AP, 2:1; NSP, 2%; and MOS, 800 mg/kg). Furthermore, the composition of DCHO also significantly ($P < 0.05$) affected the relative abundance of *Bifidobacterium*, *Saccharomycopsis*, *Wallemia*, *Dipodascus*, *Kodamaea*, *Tuber*, and *Alternaria*.

The linear discriminant analysis effect size (LEfSe) algorithm revealed 34 significantly different (LEfSe score, >4 , $P < 0.05$; Fig. S3) fungal taxa in the digesta samples from different groups. In detail, nine taxa, including *Saccharomycopsis capsularis*, *Aspergillus flavus*, and *Wallemia canadensis*, were enriched in the samples from group 7, while seven taxa were enriched in the samples from groups 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg, i.e., *Kazachstania slooffiae*) and 5 (AM/AP, 1:1; NSP, 2%; and MOS, 1,200 mg/kg, i.e., *Kodamaea anthropila*). Four, two, and three taxa were found to be enriched in the samples from groups 1 (AM/AP, 2:1; NSP, 1%; and MOS, 400 mg/kg, i.e., *Dipodascus geotrichum*), 6 (AM/AP, 1:1; NSP, 3%; and MOS, 400 mg/kg), and 9 (AM/AP, 1:2; NSP, 3%; and MOS, 800 mg/kg, i.e., *Saccharomycopsis lassenensis*), respectively. The samples from groups 2 (AM/AP, 2:1; NSP, 2%; and MOS, 800 mg/kg) and 4 (AM/AP, 1:1; NSP, 1%; and MOS, 800 mg/kg) were enriched by only one taxon distinguished from other groups. Three taxa were enriched in the samples from groups 3

TABLE 3 Orthogonal experiment range analysis results^a

Item	Value name	Factors		
		A	B	C
ADG (g)	K1	364.63	337.57	350.00
	K2	358.24	355.86	343.36
	K3	330.60	360.05	360.11
	R	34.03	22.47	16.75
	Optimal composition	A1 (2:1)	B3 (3%)	C3 (1,200 mg/kg)
ADFI (g)	K1	516.11	492.48	496.93
	K2	519.60	511.76	502.24
	K3	482.63	514.10	519.17
	R	36.97	21.62	22.24
	Optimal composition	A2 (1:1)	B3 (3%)	C3 (1,200 mg/kg)
Diarrhea rate (%)	K1	9.21	12.14	11.05
	K2	11.12	11.09	10.35
	K3	12.04	9.14	10.98
	R	2.82	3.00	0.70
	Optimal composition	A1 (2:1)	B3 (3%)	C2 (800 mg/kg)

^a See also Table 2. ADG, average daily gain; ADFI, average daily feed intake; K, the average value of each experimental factor at the same level, used to evaluate the importance of factors; R, the range between the maximum and minimum values of K. Through the comparison of K and R, the optimal carbohydrate composition can be determined for every factor.

TABLE 4 Results of orthogonal variance analysis showing the influence of each factor (type of DCHO) on the phenotypes of the pigs^a

Group	Carbohydrate composition			Results		
	A (ratio of AM/AP)	B (levels of NSP [%])	C (the concn of MOS [mg/kg])	ADG (g)	ADFI (g)	Diarrhea rate (%)
1	A1 (2:1)	B1 (1)	C1 (400)	343.71	488.97	10.64
2	A1	B2 (2)	C2 (800)	366.57	526.70	9.21
3	A1	B3 (3)	C3 (1,200)	383.62	532.65	7.79
4	A2 (1:1)	B1	C2	338.00	497.59	12.07
5	A2	B2	C3	365.71	533.99	11.43
6	A2	B3	C1	371.00	527.23	9.86
7	A3 (1:2)	B1	C3	331.00	490.88	13.71
8	A3	B2	C1	335.29	474.59	12.64
9	A3	B3	C2	325.52	482.43	9.76

^aSee also Table 5. ADG, average daily gain; ADFI, average daily feed intake.

(*Kazachstania slooffiae*, *Pezizula*, and *Mucoraceae*), 5 (*Kodamaea anthophila*, *Bifiguratus*, and *Mucoraceae*), and 7 (AM/AP, 1:2; NSP, 1%; and MOS, 1,200 mg/kg; *Saccharomycopsis capsularis*, *Aspergillus flavus*, and *Wallemia canadensis*). Notably, no particularly enriched fungal taxon was found in the samples from group 8 (AM/AP, 1:2; NSP, 2%; and MOS, 400 mg/kg).

Comparison of fungal community derived from diet and colonic digesta samples.

Although studies of the intestinal fungi in monogastric animals are very limited, it is suspected that most of the fungal species found in the animal gut may be foodborne (29). Therefore, we used SourceTracker software to trace whether the fungal sequences obtained in the colonic digesta were diet-originated. Unlike what was found in the colonic digesta samples, the most abundant fungal genera across all diet samples were *Polythrincium* (24.45%), *Fusarium* (3.22%), *Issatchenkia* (2.97%), *Aspergillus* (2.26%), and *Diaporthe* (1.50%) (Fig. S4), and no difference was found in the abundance of fungal populations among the nine diets ($P > 0.05$; Table S3). The fungal community in the digesta samples in each group was completely different from the corresponding diet sample (Fig. 4), and most of the fungal species identified in the digesta and diet samples were nonoverlapping (shown as unknown in Fig. 4).

Interaction between the colonic fungal community and DCHO composition.

Since the SourceTracker analysis showed evidence that the fungal populations in the colon of the pigs might not foodborne, the distinct fungal community in samples from different groups may be associated with the DCHO composition. Our further canonical correspondence analysis (CCA) showed that the fungal community in the digesta samples was strongly correlated with the three DCHO compositions (the AM/AP ratio of starch, as well as the concentration of NSP and MOS). According to the CCA plot

TABLE 5 Orthogonal variance analysis results^a

Item	Source of variation	Variance analysis				
		df	SS	MS	F value	P value
ADG (g)	A	2	9,813.84	4,906.92	3.23	0.05
	B	2	4,285.53	2,142.77	1.41	0.26
	C	2	2,133.43	1,066.72	0.70	0.50
ADFI (g)	A	2	12,495.58	6,247.79	2.28	0.12
	B	2	4,224.28	2,112.14	0.77	0.47
	C	2	4,048.51	2,024.26	0.74	0.48
Diarrhea rate (%)	A	2	62.59	31.30	3.72	0.03
	B	2	69.55	34.77	4.13	0.02
	C	2	4.33	2.16	0.26	0.77

^a See also Table 4. ADG, average daily gain; ADFI, average daily feed intake; df, degrees of freedom; SS, sum of squares; MS, mean square error.

FIG 1 The α -diversity of the fungal community in the colonic digesta samples. (A) Observed species; (B) ACE; (C) Chao1; (D) Good's coverage; (E) Shannon index. *, differences are considered significant when $P < 0.05$. CD, colonic digesta.

(Fig. 5), the first two discriminant functions (axes 1 and 2) contributed 85.88% (56.6% for CCA1 and 29.28% for CCA 2) of the total variation. Generally, in a CCA plot, the length of an arrow represents the relative importance of one factor (i.e., a carbohydrate's composition) in explaining the variation of microbiota. Thus, here, the correla-

FIG 2 The β -diversity of the fungal community in the digesta samples calculated using ANOSIM based on the unweighted UniFrac distances. R should be between -1 and 1 . $R > 0$ means a significant difference between groups, while $R < 0$ means that the difference within a group is greater than that between groups. When P is < 0.05 , the difference between groups was statistically significant. CD, colonic digesta.

TABLE 6 Relative abundance (%) of the main fungal phyla and genera in the colonic digesta samples^a

Fungus	Data for group: ^c									P value
	CD1	CD2	CD3	CD4	CD5	CD6	CD7	CD8	CD9	
Phyla										
Ascomycota	40.44 ± 64.08	65.37 ± 60.73	86.34 ± 20.09	43.57 ± 31.66	33.22 ± 51.77	69.79 ± 49.08	64.94 ± 26.50	72.32 ± 21.56	72.78 ± 17.39	0.166
Basidiomycota	4.81 ± 4.43	3.15 ± 12.66	0.77 ± 1.02	1.61 ± 7.47	2.02 ± 4.64	4.96 ± 10.11	4.52 ± 13.95	6.43 ± 13.60	3.12 ± 2.41	0.165
Mucoromycota	0.59 ± 17.33	0.20 ± 0.50	3.09 ± 2.66	0.74 ± 0.85	2.06 ± 50.19	0.55 ± 6.82	0.10 ± 12.53	1.08 ± 2.17	0.37 ± 2.89	0.135
Mortierellomycota	0.10 ± 1.33	0.16 ± 4.23	0.36 ± 0.52	0.48 ± 3.33	0.06 ± 3.27	0.03 ± 0.07	0.11 ± 0.78	0.12 ± 1.18	0.10 ± 0.25	0.120
Opidiomycota	0.003 ± 0.05	0.001 ± 0.06	0.39 ± 1.10	0.05 ± 0.24	0.02 ± 0.16	0.01 ± 0.02	0.00 ± 0.30	0.04 ± 0.16	0.01 ± 0.22	0.075
Glomeromycota	0.03 ± 0.53	0.04 ± 0.52	0.04 ± 0.05	0.01 ± 0.80	0.14 ± 0.53	0.01 ± 0.08	0.04 ± 0.11	0.00 ± 0.01	0.01 ± 0.02	0.115
Chytridiomycota	0.07 ± 0.63	0.01 ± 0.23	0.01 ± 0.01	0.02 ± 0.57	0.09 ± 1.25	0.03 ± 0.12	0.01 ± 0.55	0.08 ± 0.14	0.06 ± 0.15	0.664
Rozellomycota	0.002 ± 0.31 AB	0.002 ± 0.02 AB	0 ± 0.003 AB	0.09 ± 0.12 B	0.001 ± 0.04 AB	0.00 ± 0.01 AB	0 A	0.00 ± 0.12 AB	0.00 ± 0.24 AB	0.037
Neocallimastigomycota	0	0.000 ± 0.03	0	0.00 ± 0.13	0	0.00 ± 0.002	0.00 ± 0.03	0	0	0.717
Aphelidiomycota	0 A	0.000 ± 0.001 A	0.003 ± 0.01 B	0 A	0.00 ± 0.03 AB	0 A	0 A	0.00 ± 0.06 AB	0 A	0.001
Monoblepharomycota	0	0.000 ± 0.001	0	0	0.00 ± 0.05	0	0	0	0	0.128
Blastocladiomycota	0.000 ± 0.002 A	0.000 ± 0.01 AB	0.03 ± 0.02 B	0.00 ± 0.03 AB	0.00 ± 0.01 A	0 A	0 A	0 A	0 A	0.001
Kickxellomycota	0	0	0	0.00 ± 0.004	0	0	0	0	0	0.433
Others	17.68 ± 64.27	29.59 ± 51.95	8.99 ± 15.37	47.69 ± 36.49	14.07 ± 21.09	28.76 ± 54.71	12.17 ± 17.11	20.28 ± 23.00	21.93 ± 16.46	0.150
Genera										
Kazachstania ^b	0.45 ± 0.23 A	0.32 ± 0.15 A	61.94 ± 9.49 C	4.40 ± 1.82 A	1.88 ± 0.87 A	3.33 ± 1.21 A	14.35 ± 4.01 AB	25.99 ± 10.30 B	14.23 ± 4.79 AB	<0.001
Scheffersomyces ^b	22.25 ± 10.47	31.20 ± 13.21	0.02 ± 0.02	11.03 ± 4.70	14.59 ± 6.32	25.30 ± 11.84	7.10 ± 3.27	16.04 ± 6.70	28.45 ± 9.63	0.185
Bifiguratus	0.37 ± 15.69 AB	0.18 ± 0.26 AB	0 A	0.32 ± 0.32 AB	1.86 ± 50.70 B	0.26 ± 6.47 AB	0.27 ± 9.02 AB	0.16 ± 0.74 AB	0.16 ± 0.11 AB	0.003
Candida	0.19 ± 0.27	0.76 ± 0.49	0.99 ± 1.73	0.30 ± 2.61	0.75 ± 2.56	0.36 ± 19.54	0.45 ± 17.07	0.26 ± 0.74	0.16 ± 0.22	0.197
Saccharomycopsis	0.23 ± 0.23 AB	0.74 ± 7.33 AB	0.00 ± 0.01 A	0.46 ± 1.12 AB	0.61 ± 0.53 AB	1.14 ± 8.16 AB	0.28 ± 24.49 AB	2.10 ± 17.86 B	2.64 ± 4.68 B	0.002
Aspergillus	4.91 ± 6.86	0.55 ± 1.79	1.63 ± 4.07	1.68 ± 5.79	2.76 ± 4.56	1.77 ± 4.11	5.69 ± 15.18	3.01 ± 7.29	2.52 ± 13.11	0.664
Issatchenkia	1.47 ± 3.33	0.45 ± 0.89	1.88 ± 2.29	1.26 ± 7.57	5.93 ± 19.07	0.86 ± 1.13	2.78 ± 12.22	0.42 ± 5.00	1.34 ± 7.04	0.445
Wickerhamomyces	1.48 ± 3.75	0.22 ± 6.08	0.02 ± 0.30	0.03 ± 0.54	0.20 ± 4.40	0.24 ± 0.43	0.83 ± 2.62	0.05 ± 0.11	0.10 ± 10.50	0.192
Wallemia	0.05 ± 0.09 A	0.02 ± 0.91 AB	0.08 ± 0.22 AB	0.05 ± 0.61 AB	0.08 ± 0.07 AB	0.13 ± 3.67 AB	0.16 ± 11.18 AB	0.71 ± 9.09 B	0.24 ± 0.53 AB	0.009
Dipodascus	2.43 ± 14.94 AB	2.44 ± 12.89 AB	0.00 ± 0.002 A	1.46 ± 4.38 AB	2.27 ± 3.56 AB	3.76 ± 9.91 AB	0.66 ± 2.75 AB	3.08 ± 5.89 AB	3.23 ± 4.63 B	0.042
Kodamaea	0.10 ± 4.78 AB	0.71 ± 0.11 AB	0 A	0.11 ± 0.07 AB	0.81 ± 15.06 B	0.09 ± 2.07 AB	0.04 ± 2.05 AB	0.04 ± 0.25 AB	0.05 ± 0.06 AB	0.005
Sollicocozyma	0.02 ± 0.28	0.003 ± 6.58	0.002 ± 0.03	0.01 ± 0.13	0.001 ± 0.18	0.00 ± 0.11	0.004 ± 0.53	0.07 ± 0.21	0.01 ± 0.17	0.479
Penicillium	0.27 ± 0.81	0.22 ± 0.19	5.76 ± 5.59	2.18 ± 5.43	1.54 ± 3.00	0.99 ± 3.23	2.21 ± 3.60	0.72 ± 6.81	1.34 ± 1.54	0.067
Tuber	0.23 ± 0.57 AB	1.44 ± 2.85 B	0.00 ± 0.01 A	0.29 ± 0.91 AB	0.38 ± 1.49 AB	0.18 ± 6.63 AB	0.43 ± 0.77 AB	0.21 ± 0.36 AB	1.03 ± 6.10 B	0.022
Alternaria	0.10 ± 1.17	0.01 ± 0.22	0.18 ± 2.34	0.16 ± 5.43	0.13 ± 0.79	0.06 ± 0.11	0.02 ± 0.05	0.10 ± 0.16	0.07 ± 0.06	0.04
Others	36.23 ± 63.47	35.22 ± 64.74	14.15 ± 19.72	56.61 ± 29.68	20.87 ± 25.83	32.25 ± 51.59	16.41 ± 20.87	27.52 ± 28.29	27.67 ± 19.87	0.110

^aThe nonnormally distributed data are presented as median ± interquartile range (IQR).^bThe normally distributed data which are presented as means ± standard error (SEM). Different uppercase letters (A, B, C, AB) represent a significant difference ($P < 0.05$). "Others" means those phyla/genera could not be identified as any known phyla/genera. Only the main genera with an average abundance of more than 1.00% are listed. The carbohydrate composition in the diet of each group is shown in Table 8.^cCD, colonic digesta.

FIG 3 The heatmap of the fungal composition in the colonic digesta samples from the piglets (genus level). Only the top 30 genera are shown. The highest and lowest abundances are shown in red and blue, respectively. CD, colonic digesta.

tion between the three DCHO and the fungal community in the digesta samples can be arranged in the following order: MOS > AM/AP > NSP.

Spearman correlation analysis (Fig. 6) showed that the relative abundances of genera *Saccharomycopsis*, *Mrakia*, *Wallemia*, and *Plectosphaerella* were negatively correlated with the AM/AP ratio of dietary starch ($P < 0.05$ or $P < 0.01$), while the abundances of the genera *Chaetomium* and *Cantharellus* were negatively related to the concentration of dietary NSP ($P < 0.05$), and the abundance of the genus *Dipodascus* was negatively related to the concentration of dietary MOS ($P < 0.01$). In contrast, the

FIG 4 The corresponding comparison of the fungal community in diets and colonic digesta of the piglets in different groups analyzed by SourceTracker. Genera classifications of OTUs were assigned to sinks from the respective source using SourceTracker. The dietary samples in each category were assigned as a source and the digesta samples used as a sink.

abundance of the genus *Eurotium* was positively correlated with the concentration of NSP ($P < 0.05$), while the abundances of the genera *Cornuversica*, *Meyerozyma*, *Penicillium*, and *Issatchenkia* were positively correlated with the concentration of MOS ($P < 0.05$).

Concentration of saccharides and the activity of CAZymes in the colonic digesta of piglets fed diets with different DCHO composition. Complex carbohydrates that cannot be digested in the foregut can be used as growth substrates by microorganisms secreting corresponding CAZymes in the hindgut of monogastric animals (30, 31). Many fungi found in rumen and the environment have a strong ability to decompose lignocellulose (32, 33). Thus, we further investigated the relationship between the concentration of the main saccharides as well as CAZymes and the abundance of fungal species in the digesta samples. We found (Table 7) that the concentration of fructose was higher in the samples from group 7 (AM/AP, 1:2; NSP, 1%; and MOS, 1,200 mg/kg) than in those from groups 1 (AM/AP, 2:1; NSP, 1%; and MOS, 400 mg/kg), 8 (AM/AP, 1:2; NSP, 2%; and MOS, 400 mg/kg), and 9 (AM/AP, 1:2; NSP, 3%; and MOS, 800 mg/kg) ($P < 0.01$). Meanwhile, the activity of amylase in the digesta samples from group 7 also was increased compared to those from groups 1 and 9 ($P < 0.01$). The activity of β -D-glucosidase in the samples from groups 3, 5 (AM/AP, 1:1; NSP, 2%; and MOS, 1,200 mg/kg), and 7 was remarkably higher than that of others ($P < 0.05$). The correlation analysis (Fig. 7) showed that the abundance of five fungal genera, *Eurotium*, *Microascus*, *Penicillium*, *Xerochrysium*, and *Cantharellus*, was positively ($P < 0.01$ or 0.05) correlated with the concentration of glucose in the digesta. Additionally, the abundance of *Saccharomycopsis* and *Wallemia* was negatively ($P < 0.05$) correlated with the activity of β -D-glucosidase, while the abundance of *Solicoccozyma* was negatively correlated with both the concentration of fructose ($P < 0.01$) and the activity of amylase ($P < 0.05$).

FIG 5 The relationship between the DCHO (starch, NSP, and MOS) and the fungal community in the colonic digesta samples from the piglets calculated with the canonical correspondence analysis (CCA). Each symbol represents one group, each arrow line in the CCA chart represents the corresponding factor (starch, NSP, or MOS), and the length of each arrow line represents the degree of correlation between each factor and the fungal community. An acute angle between the two arrow lines indicates a positive correlation between the two corresponding environmental factors. Conversely, an obtuse angle indicates a negative correlation. Axes 1 and 2 contributed 85.88% of the total variation, with 56.6% for CCA1 and 29.28% for CCA2. CD, colonic digesta.

DISCUSSION

DCHO not only provide animals with calories but also affect the microbial community and metabolism in the GI tract of mammals (34, 35). The influence of carbohydrates, such as dietary fiber, oligosaccharide, and starch, on the bacteria in the gut of monogastric animals has been extensively studied in the past decade (18, 22, 24). For example, we reported that compared with growing pigs fed a diet with high AM/AP, pigs fed a diet with low AM/AP harbor a higher abundance of *Bacteroidetes* and *Prevotella* in the ileum (18). NSP can also modulate the intestinal microbiota in pigs. SDF, such as inulin, is proved to improve the number of bifidobacteria and lactobacilli (36–38), while IDF may promote the proliferation of cellulose-degrading bacteria, such as *Clostridium* sp. and *Eubacterium* sp. (39). Some functional oligosaccharides such as MOS also interact with gut microbiota, especially in increasing the concentration of butyrate in the cecum and the abundance of *Bacteroidetes* (40), yet the interaction between DCHO and intestinal fungi still remains unknown. Many studies of ruminants show that anaerobic fungi in the GI tract have a strong ability to utilize complex carbohydrates (41, 42). We also report diverse fungal communities in the hindgut of piglets

FIG 6 The heatmap of the correlation between the main fungal genera and DCHO (starch, NSP, and MOS). The heatmap was created based on the result of Spearman correlation analysis. Positive correlations are represented in red, while negative correlations are represented in blue. The depth of the color represents the degree of correlation. The significant correlations are presented as asterisks (*, $P < 0.05$; **, $P < 0.01$).

(2) and adult pigs (1), showing a potential close relationship between some fungal genera and bacterial metabolites, SCFAs, which indicates a possible interaction between fungi and the fermentation of dietary carbohydrates in the hindgut of pigs. It is important to note that carbohydrates in animal feed are multisourced rather than single-sourced. Therefore, mixed DCHO were considered in the current study to simulate the natural composition of DCHO as much as possible. To the best of our knowledge, this is the first report on the relationship between DCHO composition and the fungal community in the hindgut of pigs.

Generally, orthogonal design is suitable to screen the optimal combination meeting certain objective(s). Here, the best composition of DCHO for ADG was determined as group 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg), and the diarrhea rate of the pigs in this group was the lowest among the nine groups. Coincidentally, the α -diversity of the fungal community in the colon of these pigs was remarkably lower than that of other pigs, with a completely distinct fungal composition which was contributed by some dominant genera (i.e., *Scheffersomyces* and *Kazachstania*) and multiple scarce genera (i.e., *Alternaria*, *Tuber*, and *Mrakia*). Because the Q20 values (a measure of read quality, indicating the probability of having no more than one error in 100 bases) fell in a reasonable range (Table S1), these analyses and conjectures are credible. Thus, these findings can be considered evidence that the composition of DCHO affects the fungal community in the colon of piglets. If we subdivide such effects into each DCHO, the effect of MOS on the colonic fungal community was greater than that of AM/AP, followed by NSP. In line with this, the abundance of the fungal genera associated with MOS and AM/AP was higher than those correlated with NSP. Interestingly, none of these genera were dominant, suggesting that these less-abundant fungi, but not the dominants, in the colon of the piglets may have a greater response to the change of DCHO composition. It is worth mentioning that the fungal species found in the colon of these piglets were not foodborne according to our SourceTracker analysis, which further confirmed that the identified fungal species in the current study were autochthonous in the GI tract. This provides more powerful evidence for the influence of DCHO composition on the colonic fungal community. Although dietary AM/AP and/or NSP level was found to markedly influence the ADG and diarrhea rate of piglets, no direct correlation was found between host phenotypes and fungal diversity or species (data not shown), suggesting that alteration in the DCHO composition may directly lead to the change of phenotypes and colonic fungal community, but the interaction of fungal population and the phenotypes of the host require a lot of discussion.

Because of the large number of possible molecular configurations and structures, carbohydrates are the most heterogeneous and diverse group of associated molecules found in nature (8), which is closely related to their utilization efficiency in the animal gut. As a well-known example, starch with high amylopectin, but not amylose, can be easily digested in the foregut (43). Excess dietary amylose enters the hindgut and becomes the substrate of microorganisms. The utilization of dietary fibers and

TABLE 7 Effect of different DCHO compositions on the concentration of saccharides and the activity of digestive enzyme in colonic digesta of the piglets^{a,b}

Item ^e	Data for group: ^d									P value
	CD1	CD2	CD3	CD4	CD5	CD6	CD7	CD8	CD9	
Total protein ^c (gprot/ liter)	1.057 ± 0.181 A	0.801 ± 0.102 BCD	0.690 ± 0.045 ABC	0.667 ± 0.042 AB	0.720 ± 0.049 BC	0.624 ± 0.083 AB	0.391 ± 0.060 A	0.998 ± 0.164 CD	0.917 ± 0.036 BCD	0.001
Glucose ^e (mg/dl)	28.760 ± 6.205	10.968 ± 1.994	23.512 ± 5.430	29.094 ± 6.669	28.968 ± 4.184	25.555 ± 5.502	23.762 ± 3.969	17.431 ± 2.118	15.256 ± 2.941	0.071
Total polysaccharide ^c (mg/g)	0.419 ± 0.030	0.621 ± 0.081	0.375 ± 0.094	0.476 ± 0.057	0.511 ± 0.062	0.530 ± 0.048	0.459 ± 0.026	0.475 ± 0.041	0.440 ± 0.018	0.161
Fructose ^c (mg/gprot)	0.839 ± 0.502 A	1.038 ± 0.601 AB	1.172 ± 0.320 AB	1.150 ± 0.250 AB	1.139 ± 0.276 AB	1.114 ± 0.832 AB	1.871 ± 1.681 B	0.753 ± 0.695 A	0.825 ± 0.167 A	0.005
Amylase (U/mgprot)	1.114 ± 0.854 A	1.081 ± 0.820 AB	1.464 ± 0.582 AB	1.820 ± 0.238 AB	1.579 ± 0.424 AB	1.898 ± 1.308 AB	2.650 ± 2.585 B	1.038 ± 1.365 AB	1.281 ± 0.127 A	0.003
cellulose (U/mgprot)	0.079 ± 0.137	0.156 ± 0.057	0.079 ± 0.131	0.048 ± 0.226	0.068 ± 0.079	0.108 ± 0.301	0.083 ± 0.082	0.013 ± 0.071	0.103 ± 0.069	0.150
β-o-Glucosidase (U/mgprot)	1919.780 ± 623.35	1708.85 ± 1759.43 B	3430.64 ± 4391.15 C	1499.14 ± 1736.08 AB	3356.72 ± 2484.15 C	1247.60 ± 1576.04 AB	2536.94 ± 1527.98 C	848.37 ± 553.91 A	813.13 ± 764.11 A	0.027

^aThe nonnormally distributed data are presented as median ± interquartile range (IQR).
^bDifferent uppercase letters (A, B, C, AB) represent a significant difference ($P < 0.05$).
^cThe normally distributed data which are presented as means ± standard error (SEM).
^dCD: colonic digesta. The carbohydrate composition in the diet of each group is shown in Table 8.
^egprot, gram protein concentration; mgprot, milligram protein concentration.

FIG 7 The correlation between the main fungal genera in the colon of the piglets, as well as the concentration of saccharides and carbohydrate active enzymes. Positive correlations are represented in red, while negative correlations are represented in blue. The depth of the color represents the degree of correlation. The significant correlations are presented as asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

oligosaccharides by monogastric mammals largely depends on their resident intestinal microorganisms (44) producing CAZymes. Multiple fungal species in rumen and the environment can secrete a variety of highly active CAZymes (45, 46). For instance, glycoside hydrolases, polysaccharide lyases, carbohydrate esterases, glycosyltransferases, and carbohydrate-binding modules have been systemically identified from the predicted proteomes of 103 representative fungi belonging to the Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (47). We therefore detected the activity of enzymes involving the degradation of starch and polysaccharides, as well as the concentration of saccharides, the degradation products of carbohydrates, in the colonic digesta of the piglets. We found that the concentration of fructose and the activity of amylase and β -D-glucosidase in the colon of piglets showed clear variability. Some low-abundance genera, such as *Eurotium*, *Microascus*, *Penicillium*, *Xerochrysium*, and *Cantharellus*, were associated with the concentration of glucose in the colonic digesta of piglets, while other genera, such as *Saccharomycopsis*, *Wallemia*, and *Sollicoccozyma*, showed a correlation with the activity of β -D-glucosidase. Interestingly, some of these fungi overlapped with those related to carbohydrate composition, including *Saccharomycopsis*, *Wallemia*, *Cantharellus*, *Eurotium*, and *Penicillium*. Such results suggest that these low-abundance fungi rather than those that are dominant, may be important for the utilization of carbohydrates in the hindgut of pigs. In the current study, Ascomycota and Basidiomycota were identified as the two predominant phyla and accounted for more than 40% of all obtained sequences, which was consistent with previous studies (2, 48). Ascomycota is known as the largest phylum in the fungal kingdom and plays an important role in the cycling of nutrients due to a variety of high-activity CAZymes from different isolates (49). Fungi belonging to the Basidiomycota not only produce unique small- and medium-molecule metabolites, such as uncommon phenylhydrazines (50), volatile flavors (51), azo pigments (50), antibiotics (52), and glucans (53), but also secrete a specific set of lignin-modifying enzymes, enabling them to thrive on lignocellulose (54). The preponderance of these two fungal phyla in the colon of piglets suggests their potential in the fermentation and utilization of polysaccharides in the swine hindgut. It is noteworthy that the relative abundance of *Kazachstania*, characterized by the species *Kazachstania slooffiae*, was found to be remarkably higher in the colon of piglets in group 3 (AM/AP, 2:1; NSP, 3%; and MOS, 1,200 mg/kg) than that of the pigs in other groups. *K. slooffiae* is considered a natural inhabitant in the GI tract of pigs (48, 55), and the supplementation of active *K. slooffiae* cells (oral, once a day for 3 days) to weaning piglets has been

FIG 8 Diagrammatic sketch showing the response of the colonic fungal community to different DCHO composition in piglets. A solid arrow indicates that there is a correlation between the two terminals. The fungal genera associated with DCHO, monosaccharides, or glycoside hydrolases are highlighted in yellow.

reported to significantly affect the composition of intestinal microbiota to influence the metabolism of carbohydrates in the host (56). Considering these characteristics, the large amount of amylose, NSP, and MOS in the diet may contribute to the increased *K. slooffiae* in the colon of these pigs.

In summary (Fig. 8), our study shows that alteration of DCHO composition may trigger the response of colonic fungi in pigs. Taking ADG as the reference, AM/AP of 2:1, NSP of 3%, and MOS of 1,200 mg/kg may be the optimum carbohydrate composition for the piglets harboring the lowest diversity and unique composition of fungi in the colon, with *K. slooffiae* as the representative species. Among the three carbohydrate factors, the level of dietary MOS had the greatest effect on the colonic fungal community, followed by AM/AP and NSP. In accordance with this, the fungal genera have the greatest correlation with the level of dietary MOS. A large part of low-abundance fungi associated with carbohydrate composition overlapped those related to the concentration of monosaccharide and β -D-glucosidase in the colonic digesta, suggesting a possible important role of these fungal genera for the degradation of complex carbohydrates in the hindgut. These findings suggest that fungi in the colon of pigs should have irreplaceable significance for the digestion and utilization of DCHO. The shifts in the fungal community due to the alteration of DCHO composition may be (indirectly) associated with the phenotypes of the host, which provides a new idea to modify the growth phenotype by shaping the gut microbiota.

MATERIALS AND METHODS

All experimental procedures and animal care were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Sichuan Agricultural University, and all animal protocols were approved by the Animal Care and Use Committee of Sichuan Agricultural University under permit number DKY-B20172008.

Animal trial and sample collection. A total of 135 cross-bred (Duroc $\times$ Landrace $\times$ Yorkshire) pigs (weaned at 21 days) with an initial body weight of 6.71 ± 0.23 kg were selected as experimental animals and randomly assigned to 9 groups with 5 pens per group and 3 pigs per pen for an orthogonal array design (OAD) using an OA_9 (3^4) matrix with three factors (Table 8), the ratio of AM/AP, the level of NSP (mixture of inulin with microcrystalline cellulose by 1:1), and the concentration of MOS (57). All pigs were housed in a temperature- and light-controlled nursery house with completely slotted floors (opened pigsty). Experimental diets (Table S4) were formulated to meet the nutrient recommendations

TABLE 8 The matrix of orthogonal array design^a

Group	Factor		
	A (the ratio of amylose/amylopectin)	B (%) (level of NSP)	C (mg/kg) (level of MOS)
1	2:1 (A1)	1 (B1)	400 (C1)
2	2:1	2 (B2)	800 (C2)
3	2:1	3 (B3)	1,200 (C3)
4	1:1 (A2)	1	800
5	1:1	2	1,200
6	1:1	3	400
7	1:2 (A3)	1	1,200
8	1:2	2	400
9	1:2	3	800

^aNSP, nonstarch polysaccharides; MOS, mannan-oligosaccharides. The NSP used in the current study was mixed with inulin and microcrystalline cellulose in a ratio of 1:1.

of the National Research Council (NRC) 2012 guidelines for 10- to 20-kg pigs (58), and the levels of main nutrients in the diet were analyzed using the standard method recommended by the Association of Official Analytical Chemists (AOAC) (59). The diets for all piglets were antibiotics and growth promoter free, and no fungal growth promoters or additives were supplemented. The amylose, amylopectin, cellulose, inulin, and MOS utilized in the current study were purchased from Kang Biological Products Co., Ltd. (Chicago, USA), Fuyang Biotechnology Co., Ltd. (Dezhou, China), Shandong Tianli Pharmaceutical Co., Ltd. (Qufu, China), VILOF Group Co., Ltd. (Beijing, China), and Alltech Biological Products Co., Ltd. (Beijing, China), respectively, and their purity was 68.5%, 95%, 99%, 85%, and 95%, respectively. The experimental diets and water were available *ad libitum* during the entire experimental period, and the trial lasted 28 days. All pigs were weighed at the beginning and end of the trial to calculate the ADG. Feed intake of each individual was recorded every day to calculate the ADFI. At the end of the experiment, one pig from each pen was sacrificed with an intravenous injection of pentobarbital sodium (59 mg/kg body weight). Approximately 4 g digesta from middle colon of each pig was collected and immediately transferred into sterile 2-ml tubes and frozen at -80°C for further analysis.

DNA extraction and sequencing. Total genomic DNA of each sample was extracted using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany), and the concentration and purity of DNA were detected using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). Each DNA sample was diluted to a final concentration at 1 ng/ μl using sterile double-distilled water. Specific primers, ITS5-1737F/ITS2-2043R (ITS5-1737F: GGAAGTAAAGTCGTAACAAGG; ITS2-2043R: GCTGCGTCTTCATCGATGC), were used to amplify the ITS1 region of fungi. The 30- μl PCR included 15 μl of Phusion high-fidelity PCR master mix (New England Biolabs), 0.2 μM each forward and reverse primer, and 10 ng of template DNA. Thermal cycling consisted of an initial denaturation at 98°C for 1 min and 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s, as well as a final extension at 72°C for 5 min. Three parallel PCRs were conducted for each DNA sample, and the PCR products were detected with electrophoresis using 2% agarose gel. PCR products for each sample were mixed in equidensity ratios, and the mixture was then purified with a GeneJET gel extraction kit (Thermo Scientific, United States). Sequencing libraries were generated using an Ion Plus fragment library kit 48 rxns (Thermo Scientific) following the manufacturer's recommendations. The library quality was assessed on the Qubit 2.0 fluorometer (Thermo Scientific). Finally, the library was sequenced on an Ion S5 XL platform, and 400- to 600-bp single-end reads were generated.

Bioinformatics analysis. Single-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequences. Quality filtering of raw reads was performed according to the Cutadapt v1.9.1 quality control process (60) to obtain the high-quality reads. The chimera sequences in the qualified reads were then removed using the UCHIME algorithm to obtain clean reads (61). Sequence analysis was performed using Uparse software v7.0.1001 (62). Generally, sequences with $\geq 97\%$ similarity were assigned to the same OTU. For the representative sequence of each OTU, the Unite Database was used as a reference database, and the BLAST algorithm calculated with QIIME software v 1.9.1 was performed to annotate the taxonomic information. Finally, the 45 collected digesta samples yielded a total of 2,149,187 sequence reads, and 2,116,083 high-quality reads (98.46%) were used for the analysis of OTUs. The average length of sequences for each sample was 168 to 291 bp. The Q20 and the GC content for each clean read were also calculated. All downstream analyses were carried out based on the obtained high-quality clean data (Table S1).

The α -diversity of each sample was reflected by 5 indices, observed species (observed OTUs), Chao1, Shannon, ACE, and Good's coverage, which were calculated with QIIME v 1.7.0 and visualized with R software v 2.15.3. The β -diversity of each sample was calculated based on the Unifrac (both unweighted and weighted) distances. The PCoA was performed to obtain and visualize principal coordinates from complex and multidimensional data, and the plot was constructed based on the unweighted Unifrac distance between samples. The differences between intergroup samples and within intragroup samples were compared using a multiresponse permutation procedure (MRPP) with 1,000 permutations. The ANOSIM was used to evaluate group differences using unweighted UniFrac distance metrics. The

ANOSIM *R* value indicated the extent to which the groups differed (*R* value > 0, indicating significant differences between groups). ANOSIM analyses were performed with the R package in vegan.

Source tracking analysis. At present, it is still controversial whether the fungal populations in the GI tract of humans and animals are residents or passers-by (29); SourceTracker v 1.0.0 software (63) was utilized to estimate the proportion of fungal OTUs sourced from diets in the current study. Briefly, the relative abundance of each OTU within both the colonic digesta and diet samples of each group were compared to calculate the probability that each OTU found in the colon of the piglets was sourced from diet.

Concentration of saccharides and the activity of digestive enzymes in the colonic digesta samples. The concentration of saccharides and the activity of digestive enzymes in frozen colonic digesta were determined using commercial kits. In detail, the colonic digesta sample for each piglet was thawed and homogenized after dilution with saline (1:9), followed by a centrifugation at 2,000 rpm at 4°C for 10 min, and the supernatant was collected for subsequent analysis. The concentration of total polysaccharides (Shanghai mlbio Biotechnology Co., Ltd., Shanghai, China), glucose (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and fructose (Nanjing Jiancheng Bioengineering Institute), as well as the activity of enzymes, including amylase (Nanjing Jiancheng Bioengineering Institute), cellulase (Nanjing Jiancheng Bioengineering Institute) and β -D-glucosidase (Shanghai mlbio Biotechnology) in the supernatant was detected using commercial kits with an ultraviolet-visible (UV-VIS) spectrophotometer (UV1100; Mapada, Shanghai, China) according to the manufacturer's protocols.

Statistical analysis. All data were first evaluated for normal distribution using the statistical software SPSS 20.0 (SPSS, Inc., Chicago, IL, USA). For the nonnormally distributed data, a nonparametric Kruskal-Wallis (K-W) test was used to assess the differences in the data among groups, and then a pairwise Mann-Whitney U test was used to evaluate the differences within the groups. Both one-way analysis of variance (ANOVA) and Duncan's multiple-range test were used to compare the differences of the normally distributed data among groups. The phenotypic data (ADG, ADFI, and diarrhea rate) were analyzed with the general linear model (GLM) procedures of SAS 9.2 (SAS Institute, Inc., Cary, NC, USA), and the range and orthogonal variance analyses were also performed. Differences were considered significant when $P < 0.05$, while the differences were defined as not significant when $P \geq 0.05$. A significant trend was defined when $0.05 < P < 0.1$.

Data availability. All sequences obtained in the current study were deposited at the National Center for Biotechnology Information (NCBI) and can be accessed in the Sequence Read Archive (SRA) under the BioProject numbers [PRJNA689380](#) (colonic digesta samples) and [PRJNA689672](#) (diet samples).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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We declare that we have no competing interests.

We hereby certify that the current study shares an animal trial with our published study (57), but the number of animals, as well as the data regarding growth performance and diarrhea rate included in the final statistics in these two studies, is different.

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