Effects of Dietary Carbohydrases on Fecal Microbiome Composition of Lactating Sows and Their Piglets

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Introduction

Corn-soybean meal diets are commonly used in the pork industry as a primary source of energy and protein. However, such a diet generally contains non-starch polysaccharides (NSPs) which present a challenge in finding ways to improve their availability and digestibility. Dietary multi-carbohydrases (MCs) have been proposed as an efficient approach to utilize NSPs, and can result in improved growth performance and host intestinal fitness. In this study, we evaluated the effects of MC in lactation diets on gut microbiota composition of lactating sows and their litters. The experimental design contained two dietary treatments, a diet based on corn-soybean meal (CON), and CON supplemented with 0.01% multigrain carbohydrates (MCs). Sow and piglet fecal samples were collected on days 7 and 28 after farrowing. Based on the results from 16S rRNA gene amplicon sequencing, MC led to changes in species diversity and altered the microbial compositions in lactating sows and their piglets. Specifically, the MC treatment induced an increase in the proportions of Lactobacillus in piglets. Clostridium and Spirochaetaceae showed a significantly reduced proportion in MC-treated sows at day 28. Our results support the beneficial effects of dietary carbohydrates and their link with improved production due to better host fitness outcomes and gut microbiota composition.

Keywords: Multigrain carbohydrates, microbiome, 16S rRNA gene sequencing

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Corn-soybean meal is the primary source of energy and protein in swine diets. However, a diet generally contains non-starch polysaccharides (NSPs) which present a challenge in finding ways to improve their availability and digestibility. Dietary multi-carbohydrases (MCs) have been proposed as an efficient approach to utilize NSPs, and can result in improved growth performance and host intestinal fitness. In this study, we evaluated the effects of MC in lactation diets on gut microbiota composition of lactating sows and their litters. The experimental design contained two dietary treatments, a diet based on corn-soybean meal (CON), and CON supplemented with 0.01% multigrain carbohydrates (MCs). Sow and piglet fecal samples were collected on days 7 and 28 after farrowing. Based on the results from 16S rRNA gene amplicon sequencing, MC led to changes in species diversity and altered the microbial compositions in lactating sows and their piglets. Specifically, the MC treatment induced an increase in the proportions of Lactobacillus in piglets. Clostridium and Spirochaetaceae showed a significantly reduced proportion in MC-treated sows at day 28. Our results support the beneficial effects of dietary carbohydrates and their link with improved production due to better host fitness outcomes and gut microbiota composition.
In a previous study, we showed that addition of dietary MC in the diet of lactating sows improved the productive performance of sows and their litters and modulated their immune response [4]. Considering the importance of intestinal microbiota with respect to the availability of dietary fiber, we hypothesized that the performance improvement may be highly associated with modification of the intestinal microbiota composition. Therefore, in the current study, we evaluated the effects of exogenous carbohydases in the lactating sow diet on the changes in intestinal microbiota and individual strain composition.

Materials and Methods
Experimental Design, Animals, and Diets
A total of 12 lactating sows (Landrace x Yorkshire x Duroc; 218.37 ± 5.5 kg of average BW; 2.0 of average parity) were used in this experiment. Sows were randomly allotted to 12 pens of farrowing crates equipped with an individual feeder and drinker in an environmentally controlled farrowing room. On day 114 of gestation, sows were randomly assigned to 2 dietary treatments. Dietary treatments were a typical lactation diet based on corn and soybean meal (CON) and CON added with 0.01% of dietary multigrain carbohydase (MC). The MC (DSM Nutrition Korea Ltd., Korea) contained xylanase (2,700 units/g), glucanase (700 units/g), and cellulase (800 units/g). The dietary treatments were formulated to meet or exceed the nutrient requirement estimates of lactating sows [4]. Sows were fed 3.0 kg of the dietary treatments from farrowing until weaning. Sows were given ad libitum access to diets and water.

Sample Collection
Fecal samples of sows were collected on day 7 and 28 of lactation, and fecal samples of their litters were also collected on the same days. The samples of feces were collected from three randomly selected sows in each group and three of their randomly selected piglets by rectal palpation.

DNA Extraction
After collection, the fecal samples were stored at –20°C until analysis, at which time fecal samples (5.0 g) were bathed in Ringer's solution (Oxoid, UK) and homogenized in a stomacher for 2 min. Also, 1.0 ml subsample solutions were centrifuged at 15,000 g, at 4°C. The genomic DNA was extracted from aliquots using a Fast DNA Spin Kit (MP Bio 1052 Laboratories, USA).

PCR Amplification and Sequencing
Each DNA sample was adjusted to a concentration of 1 ng/μl and subjected to PCR according to the 16S Metagenomic Sequencing Library protocols (Illumina, USA). The V4 region of the 16S rRNA genes (primer set: forward, 5′-CCT ACG GGN GGC WGC AG-3′; reverse, 5′-GAC TAC HVG GGT ATC TAA TCC-3′) were analyzed using the Illumina MiSeq platform (Illumina). After measuring the concentration of the index PCR products using PicoGreen (Invitrogen, USA), equimolar PCR amplicons were pooled and sequenced using the MiSeq Reagent Kit v3 (600 cycles) for 301 paired-end bases, following the manufacturer's protocol based on the MiSeq system platform (Macrogen, Korea). The sequencing results were received in fastq file format.

Metagenomic Analysis
Fastq files obtained from MiSeq paired-end sequencing data were analyzed using the Mothur (v. 1.41) [13]. In Mothur, reads were merged using the make.contig command, and quality-filtered by the screen.seqs command. We aligned the sequences to the SILVA database v. 138, and the chimeric sequences were removed using the VSEARCH program v2.11.1 [14, 15]. Taxonomic classification was analyzed using the Greengenes-formatted database 14 released in 2013. Moreover, Chloroplast, Archaea, Mitochondria, and Eukaryota sequences were removed from the dataset. Low-abundance operational taxonomic units (OTUs) and singletons were removed using the Mothur subroutine “split.abund” 15, and the OTUs were classified using the distance 0.03 calculation (97% sequence similarity). OTUs and the taxonomy table from Mothur were further analyzed on the R platform v. 3.6.2 using the Phyloseq and Vegan packages (https://github.com/vegandevs/vegan). Analysis of similarities (ANOSIM) was conducted using the vegan package.

Statistical Analysis
Results were expressed as mean ± SD. The data were analyzed by Student's t-test using GraphPad Prism 9 (USA). Statistical significance was considered at p-value < 0.05.

Results and Discussion
Exogenous enzymes in swine diets improve the performance of growing pigs, especially in relation to factors such as NSP degradation, nutrient transit time, and subsequent changes to the gut microbial population [16]. The gastrointestinal tract of lactating piglets is particularly prone to being greatly affected by maternal nutritional status [17]. Previously, we showed that sows fed MC had less body weight loss, fewer white blood cells (WBC), and lower TGF-β1 expression during lactation than those fed CON [4]. In addition, piglets from sows fed MC had higher average weight gain, less diarrhea, fewer WBC and TGF-β1 expression, and higher IgG and IgM concentration than those from sows fed CON, indicating the beneficial effects of MCS on the productive performance and immune response of lactating sows and their piglets.
To test our hypothesis that addition of dietary MC in corn-soybean meal diets would modify the fecal bacterial communities in lactating sows and their piglets, we investigated the fecal microbiome compositions of lactating sows assigned to two dietary treatments (CON+MC) and their piglets at days 7 and 28 during lactation. The effects of MC on the fecal microbiota of sows and piglets were evaluated with 16S rRNA gene amplicon sequencing (Table 1). A total of 165,989 bacterial sequencing reads and an average of 27,665 sequencing reads per sample were obtained from all fecal samples. Fecal samples' coverage in samples was 99-100%, indicating that the majority of microbial phylotypes were detected.

|                | Sows                  | Piglets                |
|----------------|-----------------------|------------------------|
| Total no. of OTUs | Day 7                | Day 28                  |
| CON            | 242.67 ± 44.30        | 182.33 ± 109.44        |
| MC             | 260.08 ± 46.08        | 194.57 ± 109.03        |
| Chao 1         | 260.08 ± 46.08        | 194.57 ± 109.03        |
| Shannon        | 4.64 ± 0.47           | 4.32 ± 1.91            |
| Simpson        | 0.90 ± 0.04           | 0.83 ± 0.20            |
| Total no. of OTUs | Day 7                | Day 28                  |
| CON            | 93.00 ± 36.86         | 122.67 ± 35.10         |
| MC             | 98.88 ± 40.64         | 127.81 ± 34.57         |
| Chao 1         | 98.88 ± 40.64         | 127.81 ± 34.57         |
| Shannon        | 3.86 ± 1.10           | 4.68 ± 0.47            |
| Simpson        | 0.86 ± 0.11           | 0.93 ± 0.02            |

**Dietary Carbohydrases Lead to Changes in Species Diversity in Lactating Sows and Piglets**

To test our hypothesis that addition of dietary MC in corn-soybean meal diets would modify the fecal bacterial communities in lactating sows and their piglets, we investigated the fecal microbiome compositions of lactating sows assigned to two dietary treatments (CON+MC) and their piglets at days 7 and 28 during lactation. The effects of MC on the fecal microbiota of sows and piglets were evaluated with 16S rRNA gene amplicon sequencing (Table 1). A total of 165,989 bacterial sequencing reads and an average of 27,665 sequencing reads per sample were obtained from all fecal samples. Fecal samples' coverage in samples was 99-100%, indicating that the majority of microbial phylotypes were detected.

**Fig. 1.** Discriminant analysis of principal components (DAPC) among fecal samples of sows (A and B) and piglets (C and D) at day 7 (A and C) and day 28 (B and D). The 6 differentially abundant bacterial genera represent the number of variables in the model. Individual pig samples for treatments are designated with the following symbols: CON (blue) and MC (red).
Overall, while the total number of OTUs was slightly lower in lactating sows fed MC compared with the CON group, the value was relatively higher in piglets with MC treatment than the control. Bacterial diversities and richness estimated by Chao1, Shannon, and Simpson diversity indices indicated a similar level of alpha diversity metrics between the MC and CON groups, but the values were lower in the sows fed MC and higher in their piglets compared with their littermates fed CON, respectively.

We further conducted the discriminant analysis of principal components (DAPC) to identify and describe clusters of microbially associated individuals (Fig. 1). Despite a widespread clustering within the samples, the fecal microbiome profiles of lactating sows and their piglets were distinct between the MC and CON groups, especially in the groups of sows at day 28 during lactation (R = 0.2889 and significance < 0.01 by ANOSIM).

The potential effects of dietary carbohydrases have been pointed out by a number of reports, in which it was indicated that feed enzymes might influence the intestinal microbiota by reducing the amount of undigested substrates or by creating short-chain oligosaccharides as prebiotics from the NSP content in the cell wall [18]. Hübener et al. reported that supplemental NSP-degrading carbohydrases such as xylanase and β-glucanase changed the composition and metabolic potential of bacterial populations in the intestine [19]. The microbial diversity in piglets was altered differently compared to the sows, which could be explained by the fact that the gastrointestinal tract of a neonatal piglet is maternally independent and affected by early-life environment factors [20]. These results suggest that MC treatment in the feed led to bacterial species diversity, while the sows and their piglets were influenced differentially during the process.

Dietary Carbohydrases Alter Microbial Compositions at the Phylum Level in Lactating Sows and Piglets

According to the OTU assignment, an average of 86.5% of the OTUs was assigned to 126 genera (Fig. S1). Thirty-eight genera including Akkermansia, Bacteroides, Bifidobacterium, Campylobacter, Clostridium, Desulfovibrio, Escherichia, Lactobacillus, Prevotella, and Ruminococcus were commonly present in sows and piglets. Several genera such as Gordonibacter and Mycoplasma were only found in sows, while some bacteria including Eggertella, Howadelia, Sutterella, Anaerovibrio, and Enterorhabdus were only detected in piglets.

To further classify the 16S rRNA gene sequencing reads into different taxonomies, relative bacterial abundance of each sample was generated at phylum and genus levels (Figs. 2 and 3). Overall, at the phylum level, Firmicutes and Bacteroidetes were dominant in fecal samples of pigs. In sows, the proportion of Bacteroidetes was higher in the MC group than in the CON group, both during lactation (37.20% ± 0.15 in MC vs. 31.30% ± 0.078 in CON) and at weaning day (33.58% ± 0.020 in MC vs. 31.49% ± 0.037 in CON, Fig. 2A). Supplement of MC in lactating diets decreased the proportion of Spirochaetes in fecal samples from both times. At the phylum levels of piglets, the litters from MC group had more Actinobacteria than those from the CON group (0.25% ± 0.00057 in MC vs. 0.63% ± 0.00057 in CON, Fig. 2B).

Bacteroidetes produce short-chain fatty acids and create an acidic environment, which could inhibit the growth of intestinal pathogens such as pathogenic Escherichia coli, Salmonella spp., and Clostridium spp. [21]. Bacteroidetes are also able to digest indigestible nutrients, which in turn provides extra energy to their host [22]. With respect to the decreased abundance of Spirochaetes in the MC-treated sows, Spirochaetes has the ability to degrade fibers such as hemicellulose [23]. From these findings, it is clear that exogenous carbohydrases in the lactating sow diet induce shape changes of the intestinal microbiota.

Fig. 2. Taxonomic classification of total bacteria at phylum level retrieved from pooled DNA amplicons from feces of CON (n = 3) and MC (n = 3) groups of sows (A) and piglets (B) at day 7 and 28.
When looking at the differences in relative abundances of bacterial genera, *Bacteroides* and *Lactobacillus* were dominant in fecal samples of sows on days 7 and 28, respectively (Fig. 3A). With respect to the MC supplement, the proportions of *Barnesiella* (2.76% in MC vs 7.36% in CON), *Lactobacillus* (9.79% in MC vs 12.97% in CON), and *Treponema* (9.87% in MC vs 14.23% in CON) decreased with the MC treatment compared with the control at day 7 in sows, while *Christensenella* (2.94% in MC vs 1.47% in CON), *Lactobacillus* (18.34% in MC vs 11.45% in CON), and *Treponema* (15.57% in MC vs. 8.59% in CON) increased at day 28. The treatment of MC decreased the relative abundances of *Turicibacter* (4.18% in MC vs. 11.07% in CON) and *Clostridium* (4.10% in MC vs. 10.31% in CON) in sows at day 28. Similar to sows, piglets also possessed *Bacteroides* and *Lactobacillus* as dominant fecal bacteria (Fig. 3B). The proportions of *Prevotella*, *Lactobacillus*, and *Treponema* were higher in the piglets from the MC-treated sows at both days than those from the control diet-fed sows.

Among the various bacterial taxa present in swine fecal samples, specifically *Clostridiales* (OTUs 29), *Clostridium* (OTUs 35) and *Sporochaeaceae* (OTUs 77) showed significantly different relative abundances when...
treated with MC than the control sows at day 28 (Fig. 4). Especially, the MC treatment reduced the abundances of *Clostridium* and *Spirochaetaceae* at the level of 2.5-fold and 14.1-fold compared with the values of control, respectively.

*Lactobacillus* spp. or lactic acid bacterium create an acidic environment in the intestine and inhibit the growth of intestinal pathogens, conferring gut health benefits on the host [24]. Supplementation of MC is predicted to change the profile of available nutrients, mainly carbohydrate-asagenetides and oligosaccharides, in the gut [25]. It is also well known that maternal diets affect maternal milk composition and gut microbiota in offspring [26]. From the crosstalk between the gut nutrients and the milk composition, it can be speculated that MC may increase availability of milk oligosaccharides, and the increase would promote the growth of health-promoting lactic acid-producing bacteria, further resulting in better growth performance. In association with the current findings, the proportions of bacterial strains such as *Treponema* and *Prevotella* were higher with the MC treatment in the lactating sows or their piglets. *Treponema* is a genus of spiral-shaped bacteria called spirochetes and has been found in the gut microbiome of traditional rural human populations [27]. This microbe is mainly involved in the digestion of dietary polysaccharides, which are fiber, including cellulose and lignin, and the production of significant amounts of short-chain fatty acids. *Prevotella* is a gram-negative bacterium which also can use soluble fiber and is considered an important organism for biodegrading complex sugars in non-ruminant animals [28]. Taken together, the increase in available nutrient sources produced by MC would afford the ability to use the diet sources more easily while also enriching bacterial diversity. Considering the fact that they are largely involved in degrading fiber, their increase would possibly result in improvement of growth performance and immune modulation activities.

To our knowledge, the present study is the first to show the effects of dietary multigrain carbohydrides in sow diets during lactation. Our results clearly demonstrated that dietary enzymes lead to changes in species diversity and alter microbial compositions at the phylum and genus levels in lactating sows and their piglets. In order to fully understand the mechanisms of action of MC on sows and their litters, it will be necessary to further elucidate the detailed biochemical and microbial mechanisms from a productive performance and health standpoint.

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**Conflict of Interest**

The authors have no financial conflicts of interest to declare.

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