Effects of Diets with Different Fiber Sources and Cell Wall Degrading Enzymes on Porcine Gut Microbiota Diversity: Postprint

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Abstract

The present study aimed to investigate the effects of different fiber-source diets and cell wall-degrading enzymes on the diversity and compositional structure of porcine intestinal microbiota using terminal restriction fragment length polymorphism (T-RFLP) technique. Eight three-way crossbred growing pigs (Duroc × Landrace × Large White) with an average body weight of (35±2.5) kg were surgically fitted with terminal ileal T-cannulas and randomly assigned to 4 groups with 2 replicates per group. The experiment consisted of 4 periods; in each period, pigs in each group were fed one of four experimental diets according to a 4×4 Latin square design: wheat bran, wheat bran plus enzyme, soybean hulls, and soybean hulls plus enzyme. Each period comprised a 15-day preliminary period and a 6-day experimental period. The results demonstrated that: 1) The diversity of ileal digesta microbiota in pigs fed the soybean hulls diet was significantly higher than that in pigs fed the wheat bran diet ($P<0.05$). 2) The wheat bran diet significantly increased the abundance of Prevotella and Lactobacillus in ileal digesta ($P<0.05$), whereas the soybean hulls diet significantly increased the abundance of Ruminococcus in ileal digesta and Bacteroides and Lachnospira in feces ($P<0.05$). 3) Supplementation of cell wall-degrading enzymes significantly increased the abundance of Lachnospira and Eubacterium in ileal digesta and Lachnospira in feces across all dietary groups ($P<0.05$), but simultaneously decreased the abundance of Lactobacillus and Prevotella in ileal digesta and Lactobacillus, Bacteroides, and Klebsiella in feces ($P<0.05$). In conclusion, fiber diets can significantly increase the abundance of non-starch polysaccharide-degrading bacteria in the porcine gut, while cell wall-degrading enzymes can selectively alter the diversity and composition of the microbiota.

Full Text

Preamble

Effects of Different Dietary Fiber Sources and Cell Wall-Degrading Enzymes on Gut Microbial Diversity in Pigs

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Abstract: This study investigated the effects of different dietary fiber sources and cell wall-degrading enzymes on the diversity and composition of gut microbiota in pigs using terminal restriction fragment length polymorphism (T-RFLP) analysis. Eight growing crossbred (Duroc × Landrace × Yorkshire) pigs with an average body weight of (35±2.5) kg were fitted with a T-cannula at the terminal ileum and randomly allocated to four groups with two replicates per group. The experiment consisted of four periods, with pigs fed one of four experimental diets according to a 4×4 Latin square design in each period. The four diets included a wheat bran diet, wheat bran diet with enzyme supplementation, soybean hull diet, and soybean hull diet with enzyme supplementation. Each period comprised a 15-day adaptation phase followed by a 6-day collection phase. The results showed that: 1) The microbial diversity in ileal digesta of pigs fed the soybean hull diet was significantly higher than that of pigs fed the wheat bran diet ($P<0.05$). 2) The wheat bran diet significantly increased the abundances of *Prevotella* and *Lactobacillus* in ileal digesta ($P<0.05$), whereas the soybean hull diet significantly increased the abundances of *Ruminococcus* in ileal digesta and *Bacteroides* and *Lachnospira* in feces ($P<0.05$). 3) Enzyme supplementation significantly increased the abundances of *Lachnospira* and *Eubacterium* in ileal digesta and *Lachnospira* in feces ($P<0.05$), while decreasing the abundances of *Lactobacillus* and *Prevotella* in ileal digesta and *Lactobacillus*, *Bacteroides*, and *Klebsiella* in feces ($P<0.05$). In conclusion, dietary fiber significantly increased the abundances of non-starch polysaccharide-degrading bacteria, while cell wall-degrading enzymes selectively altered microbial diversity and composition.

Keywords: growing pigs; dietary fiber; cell wall-degrading enzymes; gut microbiota; terminal restriction fragment length polymorphism

The pig gastrointestinal tract harbors a complex and diverse microbial community [**?**]. Gut microbes ferment undigested carbohydrates and proteins from the small intestine, producing short-chain fatty acids that provide energy for the host. They also influence pig nutrition and health through vitamin and enzyme synthesis, bile acid reabsorption, and promotion of gastrointestinal development and immune system maturation [**?**, **?**]. Previous studies have shown that diet significantly affects gut microbial composition in pigs, with particular attention given to the amount and type of dietary fiber [**?**].

Non-starch polysaccharides (NSP) from cereal cell walls constitute the primary fiber source in pig diets, with their content, composition, and chemical structure varying according to plant species, tissue type, and growth stage [**?**, **?**]. Most existing research has focused on mixed diets containing multiple fiber sources [**?**, **?**, **?**], while studies using single fiber sources are limited, hindering our understanding of structure-function relationships between dietary NSP chemistry and gut microbial composition.

Dietary fiber is generally resistant to degradation by endogenous pig digestive enzymes and can impair the utilization of other nutrients, thus being considered an anti-nutritional factor [**?**]. In swine production, the most common strategy to mitigate these negative effects is supplementation with exogenous enzyme preparations such as NSP-degrading enzymes [**?**, **?**]. Bedford et al. [**?**] demonstrated that exogenous NSP-degrading enzymes can promote the degradation of insoluble NSP, generating fermentable oligosaccharides that subsequently affect gut microbial composition. However, the structure of these oligosaccharides varies with dietary NSP composition and the type of exogenous enzyme applied. Moreover, different microbial species exhibit distinct capacities for utilizing these oligosaccharides [**?**]. Therefore, this study employed terminal restriction fragment length polymorphism (T-RFLP) to investigate how fiber source (wheat bran vs. soybean hull) and exogenous cell wall-degrading enzyme supplementation affect gut microbial diversity in pigs. The findings provide insights into the structure-function relationships between dietary fiber composition and gut microbiota, as well as the application potential of cell wall-degrading enzymes in feed development from cereal byproducts.

1.1 Experimental Design

Eight growing crossbred (Duroc × Landrace × Yorkshire) pigs with an average body weight of (35±2.5) kg were surgically fitted with a T-cannula at the terminal ileum (15–20 cm anterior to the ileocecal valve) and randomly divided into four groups with two replicates per group. The experiment consisted of four periods, with pigs in each group receiving one of four experimental diets according to a 4×4 Latin square design. Each experimental period lasted 21 days, including a 15-day adaptation phase during which pigs were fed a complete commercial diet (purchased from Jiangsu Haiprui Feed Co., Ltd.). On day 1 of the adaptation phase, the experimental diet began replacing the commercial diet, with complete replacement achieved within 5 days. The remaining 10 days allowed pigs to adapt to the experimental diet and stabilize gut microbiota. Fecal samples were collected via rectal palpation on days 16–18, and ileal digesta samples were collected at 11:00 and 14:00 on days 19–21.

Samples were collected in sterile bags, transferred to autoclaved 5 mL plastic centrifuge tubes, and stored at -80°C. Prior to T-RFLP analysis, samples were thawed at room temperature, and equal amounts of ileal digesta and feces collected over three days from the two pigs fed the same diet in each period were pooled as composite samples for analysis.

1.2 Diet Composition and Nutrient Levels

Experimental diets were formulated according to the Chinese Feeding Standard for Lean-Type Pigs (2004). Wheat bran and soybean hull (both purchased from Donghai Grain & Oil (Zhangjiagang) Co., Ltd.) served as single fiber sources and were combined with corn starch, fish meal, blood meal, and the indigestible marker chromium oxide (Cr O) to create two nutritionally balanced semi-purified basal diets with similar energy, protein, and fiber levels: a wheat bran diet (WB) and a soybean hull diet (SH). The composition and nutrient levels are shown in Table 1 . The same enzyme cocktail (cellulase 1,000 U/kg, xylanase 5,000 U/kg, and phytase 1,500 U/kg) was added to both basal diets, resulting in four experimental diets.

Table 1 Composition and nutrient levels of basal diets (air-dry basis) g/kg

Note: The vitamin premix provided per kg of diet: VA 10,000 IU, VD 4,000 IU, VE 32 mg, VK 4 mg, VB 3 mg, VB 8 mg, VB 5 mg, VB 0.05 mg, nicotinic acid 45 mg, D-pantothenic acid 20 mg, folic acid 2 mg, biotin 0.15 mg, choline chloride 360 mg. The mineral premix provided per kg of diet: Fe 150 mg, Cu 8 mg, Zn 100 mg, Mn 50 mg, I 0.3 mg, Se 0.3 mg. Digestible energy (DE) was a calculated value; all other values were measured.

1.3.1 DNA Extraction

Total microbial DNA from intestinal samples was extracted following the method described by Bindelle et al. [**?**].

1.3.2 PCR Amplification

The full-length 16S rRNA gene was amplified using primers 8F (5' -AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR reactions were performed in a 25 µL volume containing 0.5 µL of each primer, 1.0 µL DNA template, 12.5 µL Taq DNA polymerase, and 10.5 µL sterile water using an ABI Veriti™ 96-well Thermal Cycler. The thermal cycling program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 30 s, and extension at 72°C for 50 s, with a final extension at 72°C for 7 min. Amplification products (5.0 µL) were verified by 1% agarose gel electrophoresis (Nanjing Keshi Instrument Research Institute EPS604), with expected amplicon size of approximately 1,500 bp.

1.3.3 Restriction Enzyme Digestion

PCR products were purified using an AxyPrep PCR purification kit (Axygen, USA). Five microliters of purified product were digested with the restriction enzyme HhaI (Takara 1056A, Japan) in a 20 µL reaction containing 5 µL PCR product, 2 µL 10× buffer, 1 µL enzyme, and 12 µL sterile water. Digestion was performed at 37°C for 2 h, followed by enzyme inactivation at 65°C for 30 min.

1.3.4 Capillary Electrophoresis of Digested Products

Purified digests were analyzed by capillary electrophoresis on an ABI 3730XL DNA Analyzer (ABI, USA) at Shanghai Sangon Biotech Co., Ltd.

1.4 Data Processing and Analysis

Electropherograms were analyzed qualitatively and quantitatively using GeneMarker V2.2.0 software to obtain terminal restriction fragment (T-RF) lengths, peak heights, and peak areas. Fragments shorter than 50 bp or longer than 600 bp were excluded. Microbial diversity indices were calculated based on peak heights. Microbial taxa corresponding to T-RFs were identified using the online MICA: T-RFLP Analysis tool [**?**]. Diversity indices and relative abundances of T-RFs were analyzed by two-way ANOVA using the GLM procedure in SPSS 18.0 to compare differences among dietary groups. Differences were considered significant at $P<0.05$ and trends at $P<0.10$.

Diversity indices were calculated as follows: - **Species richness** (number of microbial species) = number of restriction fragments - **Shannon-Weiner index** (indicates community complexity; higher values reflect greater complexity) = - P lnP - **Simpson index** (indicates dominance of major species and community evenness; higher values reflect greater dominance and lower evenness) = 1- P^2

where P represents the proportion of peak height of the i-th peak relative to total peak height.

2.1 Microbial Community Diversity

Software analysis revealed an average of 21 and 35 T-RFs (50–600 bp) per pig in ileal digesta and feces, respectively, with 13 and 17 fragments having relative abundances >1%. Microbial diversity indices in ileal digesta and feces are presented in Table 2 . Species richness in ileal digesta was significantly higher in the SH group compared to the WB group ($P<0.05$), while fiber source had no significant effect on fecal microbial richness or Shannon-Weiner index ($P>0.05$). Enzyme supplementation tended to increase the Shannon-Weiner index in ileal digesta of the SH group ($P<0.10$). The Simpson index for ileal microbial communities was affected by the interaction between fiber source and enzyme supplementation ($P<0.05$).

Table 2 Microbial diversity indices in ileal digesta and feces of pigs fed different diets

2.2 Abundance of T-RFs in Ileal Digesta

Thirteen T-RFs with relative abundance >1% were identified in ileal digesta, with corresponding taxa and relative abundances shown in Table 3 . The WB diet significantly increased the abundances of *Lactobacillus* and *Prevotella* in

ileal digesta (P<0.05), whereas the SH diet significantly increased *Ruminococcus* abundance (P<0.05). Enzyme supplementation significantly increased the abundances of undefined Proteobacteria, *Lachnospira*, and *Eubacterium* in ileal digesta (P<0.05) but decreased *Lactobacillus* and *Prevotella* abundances in the WB group (P<0.05). The abundances of *Lactobacillus* and *Prevotella* in ileal digesta were affected by the interaction between fiber source and enzyme supplementation (P<0.05).

Table 3 Bacterial abundances in ileal digesta of pigs fed different diets

T-RFs: terminal restriction fragments; ND: not detected; F: Firmicutes; P: Proteobacteria; B: Bacteroidetes. The same as below.

2.3 Abundance of T-RFs in Feces

Seventeen T-RFs with relative abundance >1% were identified in feces, with corresponding taxa and relative abundances shown in Table 4 . The WB diet significantly increased the abundances of *Megamonas* and *Mitsuokella* in feces (P<0.05), while *Lactobacillus* and *Klebsiella* tended to be higher compared to the SH group (P<0.10). The SH diet significantly increased the abundances of *Bacteroides* and *Lachnospira* in feces (P<0.05). Enzyme supplementation significantly increased the abundances of undefined Proteobacteria and *Lachnospira* in feces (P<0.05) while decreasing *Lactobacillus* and *Klebsiella* abundances (P<0.05) and *Bacteroides* abundance in the SH group (P<0.05). The abundance of *Eubacterium* in feces was affected by the interaction between fiber source and enzyme supplementation (P<0.05).

Table 4 Bacterial abundances in feces of pigs fed different diets

3.1 Effects of Dietary Fiber Source and Cell Wall-Degrading Enzymes on Gut Microbial Diversity

Overall, fecal samples exhibited higher species richness than ileal digesta samples, indicating greater microbial diversity and more complex community structure in the large intestine, with a lower proportion of dominant taxa. Dietary fiber source and enzyme supplementation differentially affected ileal microbial diversity but had no significant effects on fecal diversity indices. These results suggest that the small intestinal microbiota is more susceptible to dietary factors, whereas the large intestinal microbiota, with its more complex and balanced composition, remains relatively stable. Generally, microbial growth and development in the gastrointestinal tract are related to the types and amounts of available substrates in the diet [**?**]. Although the neutral detergent fiber content was similar between the two fiber diets, the NSP composition differed markedly. Wheat bran NSP consisted primarily of arabinose, xylose, and glucose (arabinoxylans and cellulose), whereas soybean hull contained higher levels of uronic acids (pectic substances) and greater total NSP content. Additionally, soluble NSP content is significantly higher in soybean hull than in wheat bran

[?, ?]. While Liu et al. [?] reported that high-pectin and high-arabinoxylan diets did not significantly affect gut microbial diversity, Ivarsson [?] found that wheat bran stimulated *Lactobacillus* growth in the porcine ileum, hypothesizing that this genus produces antimicrobial compounds that inhibit other bacteria, thereby reducing microbial diversity. The lower species richness observed in the WB group in our study may be related to the limited substrate diversity in wheat bran and the selective stimulation of *Lactobacillus* by arabinoxylans.

3.2 Effects of Dietary Fiber Source and Cell Wall-Degrading Enzymes on Gut Microbial Composition

Lamendella et al. [?] noted that most gut microbes in pigs are associated with carbohydrate metabolism, primarily Gram-positive Firmicutes and Gram-negative Bacteroidetes. In our study, T-RFs identified as *Clostridium*, *Lactobacillus*, *Enterococcus*, *Eubacterium*, *Megamonas*, *Mitsuokella*, *Lachnospira*, and *Ruminococcus* belonged to Firmicutes, accounting for 72% and 65% of total microbial abundance in ileal digesta and feces, respectively. These results align with findings from Lamendella et al. [?] and Niu et al. [?] in pigs. Most Firmicutes secrete extracellular polysaccharide-degrading enzymes, and some genera (e.g., *Ruminococcus* and *Clostridium*) possess complex multifunctional cellulosomes capable of degrading insoluble xyloglucans, xylans, and cellulose to release soluble oligosaccharides, though some species (e.g., *R. thermocellum*) cannot utilize pentose degradation products [?, ?]. Bacteroidetes bacteria (e.g., *Prevotella*) have sophisticated and comprehensive carbohydrate-degrading systems that are highly adaptable and substrate-regulated, primarily degrading oligosaccharides, starch, pectin, xyloglucans, and xylans, but show poor cellulose-degrading capacity [?].

The predominance of Firmicutes (1.53%–19.28% Bacteroidetes) in our study may be attributed to the high proportion of insoluble polysaccharides in both wheat bran (84% of total NSP) and soybean hull (77% of total NSP). However, Bacteroidetes abundance increased markedly from small to large intestine, likely due to increased solubilization of dietary NSP or generation of soluble oligosaccharides through bacterial and exogenous enzymatic action [?], providing suitable substrates for Bacteroidetes. Proteobacteria abundance in ileal digesta was notably higher than reported in previous studies. For example, *Klebsiella*, associated with xylan degradation, typically shows low relative abundance [?]. Most Proteobacteria are involved in protein fermentation [?], and their elevated abundance may be attributed to the high inclusion of animal protein sources (fish meal and blood meal) used to balance protein-to-energy ratios among fiber diets. As most dietary protein is digested in the small intestine [?], Proteobacteria abundance decreased significantly from ileum to feces, supporting this hypothesis. Similarly, as dietary starch is almost completely digested before the ileum [?], the abundance of *Lactobacillus* and *Enterococcus* (major starch-degrading bacteria [?, ?]) decreased markedly in feces, with *Enterococcus* being undetectable. Thus, available nutrients appear to play a decisive role in

shaping gut microbial composition.

The extent of NSP degradation by gut microbes depends on both physicochemical properties (monosaccharide composition, degree of polymerization, solubility) [?] and interactions among microbial genera [?]. *Clostridium* is a major fiber-degrading genus in the human gut that cooperates with *Eubacterium* and *Prevotella* to effectively degrade cellulose and hemicellulose [?]. In our study, *Clostridium* showed the highest relative abundance across all dietary groups and was unaffected by fiber source or enzyme supplementation. *Lactobacillus* and *Bacteroides* were the next most abundant, but *Lactobacillus* was significantly more abundant in ileal digesta and feces of WB-fed pigs, whereas *Bacteroides* was only detected in feces and was more abundant in SH-fed pigs. Sanchez et al. [?] reported that certain *Lactobacillus* species secrete arabinoxylan hydrolases and grow on xylo- or arabinoxylan-based media. Ivarsson [?] also reported that wheat bran promoted *Lactobacillus* growth in the pig gut, consistent with our findings. *Prevotella* and *Bacteroides*, both Bacteroidetes, were detected exclusively in ileal digesta and feces, respectively, possibly because *Bacteroides* primarily utilizes soluble polysaccharides and oligosaccharides [?]. Similarly, *Megamonas* and *Mitsuokella*, both lactate-utilizing bacteria, showed contrasting distribution patterns: *Megamonas* was abundant in ileal digesta but decreased significantly in feces, whereas *Mitsuokella* was detected only in feces, coinciding with the appearance of an unknown butyrate-producing bacterium, suggesting microbial interactions in substrate degradation and metabolite utilization. Weber et al. [?] reported that *Lachnospira* is a major pectin-degrading genus in the human gut. In our study, *Lachnospira* was significantly more abundant in feces of SH-fed pigs, consistent with higher pectin content in soybean hull. *Lachnospira* was undetectable in ileal digesta without enzyme supplementation but increased significantly with enzyme addition, indicating that cell wall-degrading enzymes promoted pectin solubilization or degradation. However, since pectin components in both wheat bran and soybean hull are primarily fermented in the hindgut [?, ?], fecal abundance of this genus was significantly higher than in ileal digesta. Similarly, because exogenous cell wall-degrading enzymes accelerated starch digestion in the small intestine [?], reducing available substrates for the starch-degrading genus *Lactobacillus*, its abundance decreased, demonstrating the inhibitory effect of exogenous enzymes on starch-degrading bacteria in the small intestine as described by Bedford et al. [?].

Although T-RFLP offers advantages in speed, cost-effectiveness, and high throughput, classification based solely on restriction fragment length rather than sequence information may not accurately distinguish microbial species. Results can be influenced by enzyme choice, digestion time, electropherogram quality, and database coverage, potentially leading to misclassification. For example, T-RF 370 was assigned to *Enterobacter* in Jensen et al. [?], whereas the one-base-pair difference T-RF 371 was assigned to *Klebsiella* in our study. To validate our T-RFLP results, we performed additional Illumina MiSeq high-throughput sequencing. Preliminary results indicated that while high-throughput sequencing identified more microbial taxa and enabled genus-level

resolution, both methods yielded consistent results regarding microbial diversity and fiber-associated dominant genera. Thus, our T-RFLP-based results provide valuable reference information on gut microbial diversity.

In summary: 1) T-RFLP analysis revealed that fiber source significantly affected microbial diversity in ileal digesta but not in feces. 2) The wheat bran diet increased abundances of hemicellulose-degrading bacteria (*Lactobacillus*, *Prevotella*, *Megamonas*, and *Mitsuokella*), whereas the soybean hull diet increased abundances of pectin-degrading (*Lachnospira*), cellulose-degrading (*Ruminococcus*), and *Bacteroides* populations. 3) Enzyme supplementation selectively altered the abundances of *Lachnospira*, *Eubacterium*, *Lactobacillus*, *Prevotella*, and *Bacteroides* in the pig gut.

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Note: Figure translations are in progress. See original paper for figures.