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Original Research Article

# Dietary citrus pectin drives more ileal microbial protein metabolism and stronger fecal carbohydrate fermentation over fructo-oligosaccharide in growing pigs

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ABSTRACT

Fructo-oligosaccharide (FOS) and pectin are known soluble dietary fibers and can influence gut microbiota and consequently modulate gut health. To understand the differential impact patterns of pectin vs. FOS in modulating gut microbiota in the small and large intestine, an ileal-cannulated pig model was adopted to compare the temporal and spatial effects of FOS and citrus pectin (CP) on the gut microbiota. Sixteen terminal ileal-cannulated pigs were randomly divided into 2 groups and fed with a standard diet supplemented with either 3% FOS or 3% CP for 28 d. The CP group and FOS group showed different microbial composition, especially in the feces, with time and location as major factors affecting microbiota in the CP group, and with only location contribution in the FOS group. In the feces, relative to the FOS group, the CP group showed higher abundance of *Christensenellaceae R-7 group* and *Ruminococcaceae UCG-010* and lower abundance of *Mitsuokella* and *Olsenella* (adjusted $P < 0.05$), a higher level of short-chain fatty acids and a lower level of lactate at both d 14 and 25 ($P < 0.05$), and more copy numbers of genes encoding key enzymes related to propionate (*mmdA*) and butyrate (*BCoAT*) production and lactate utilization (*LcdA*) ($P < 0.05$), indicating a greater degree of microbial carbohydrate fermentation. In the ileum, as compared with FOS, CP increased the bacteria with high capability of fermenting amino acids, including *Escherichia-Shigella* and *Klebsiella* (adjusted $P < 0.05$), and the expression of enzymes responsible for amino acid fermentation (i.e. lysine decarboxylase), as well as the amino acid fermentation products (cadaverine and tyramine) ($P < 0.05$), indicating a greater degree of amino acid fermentation. Overall, our results highlight a differential dynamic impact of dietary CP vs. FOS on microbial composition and metabolism in the gut. The dietary CP has a stronger ability to promote microbial amino acid fermentation in the ileum and carbohydrate fermentation in the feces than FOS. These findings provide a new insight into the role of different fibers in gut nutrition and guidelines for the choice of fibers in manipulating gut health.


## 1. Introduction

Dietary fibers are indigestible by the digestive enzymes secreted from the host, and depending on their influence on the gut microbiota, can exert physiological benefits, including intestinal development, immunity and host metabolism.

Different fibers can influence the gut microbiota differently depending on their structure and composition. Fructo-oligosaccharide (FOS) and pectin are common soluble dietary fibers used to modulate the gut microbiota in pigs. Fructo-oligosaccharide is a plant-derived oligosaccharide of fructose

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monomers linked to a terminal glucose residue via β (2-1) glycosidic bond. Pectin is a complex cell wall polysaccharide consisting of a galacturonic acid backbone with the side chain comprised of neutral sugars (arabinose, galactose, xylose and mannose) connected via an α (1-4) bond. FOS is well known as a 'bifidogenic factor' by promoting the growth of *Bifidobacterium* and *Lactobacillus* in the gut (Mao et al., 2018; Schokker et al., 2018). Pectin is also considered a prebiotic, as it stimulates the growth of beneficial bacteria, such as *Akkermansia*, *Faecalibacterium prausnitzii* and *Eubacterium eligens* in vitro (Fak et al., 2015; Min et al., 2015; Chung et al., 2017; Zhang et al., 2019). In a rat model, FOS inclusion showed greater butyrogenic capacity than pectin in the hindgut (Adam et al., 2014). Therefore, these results point to an assumption that the 2 soluble fibers exert differential patterns in modulating the microbial community and metabolism in the gut.

Fructo-oligosaccharide and pectin are known to be able to improve morphology of the small intestine in pigs (Buraczewska et al., 2007; Schokker et al., 2018). However, due to the limited accessibility of sampling under a dynamic gut environment, the impact patterns of the fibers on gut microbiota in the small intestine remain vague. The microbiota in the small intestine is markedly different from those in the large intestine (Mu et al., 2017b; Pereira and Berry, 2017) and thus, may show a different response to dietary fibers. Research showed that dietary pectin increased the relative abundances of *Prevotella* and decreased the relative abundances of *Lactobacillus* in the colon of weaning pigs (Tian et al., 2017). Some studies proposed that FOS and pectin may be partly degraded by the microbes in the foregut (Houdijk et al., 1999; Ferreira-Lazarte et al., 2019). However, it remains unclear how FOS and pectin differently influence microbial composition and function in the small intestine.

The pig has a very similar gastrointestinal tract to humans and can be easily manipulated for intestinal canulation, and therefore can be an ideal model for investigation of the compartmentalized gut microbiota (Guilloteau et al., 2010). Thus, employing a terminal ileal-cannulated pig model, this study investigated the temporal and spatial responses of gut microbial composition and metabolism to dietary citrus pectin (CP) and FOS. We hypothesized that dietary CP may affect the carbohydrate and nitrogenous substrate availability in the gut that relates to different alterations of microbial composition and function. To this end, the carbohydrate and nitrogenous substrates were measured, followed by analysis of the microbial structure and expression of functional enzymes in the ileum and feces. The results showed that the dietary fibers have different influences on microbial composition and metabolism depending on the gut location and time. Relative to FOS, dietary CP has a stronger ability to promote microbial amino acid fermentation in the foregut and carbohydrate fermentation in the hindgut, with more apparent differences occurring earlier in the feces than in the ileum. These findings provide a new insight into better modulating the microbiota through soluble dietary fibers.

## 2. Materials and methods

### 2.1. Animal ethics statement

The experiment of this study was performed with protocols approved by the Ethical Committee of Nanjing Agricultural University, Nanjing, China, in compliance with the Regulations for The Administration of Affairs Concerning Experimental Animals of China.

### 2.2. Animals and sampling

In order to dissect the temporal microbiota response to dietary fiber intervention in the small and large intestine, terminal ileal cannulated pigs were used. Sixteen growing barrows (Duroc × Landrace × Large White, aged 55 d and weighted 20 ± 1.5 kg) were selected from a commercial farm (Jiangsu Province, China) and housed individually in metabolism cages (height, 0.85 m; length, 1.70 m; and width, 0.70 m) with a feeder and an auto-drinker. The room temperature was maintained at 23 ± 2 °C. After 7 d adaptation, all pigs were fitted with a simple "T"-type fistula at the distal ileum by surgical operation according to the previous method (Li et al., 1993). Before the surgery, the pigs were fasted for 12 h. After the surgery, the pigs were injected with prophylactic antibiotics to protect against potential pathogen infections. After recuperation for 10 d, the pigs were randomly divided into 2 groups and fed with the basal diet supplemented either with 3% fructo-oligosaccharide (FOS, from Beneo-orafti, Belgium) ($n = 8$) or with 3% citrus pectin (CP, from Herbstreith & Fox, Germany) ($n = 8$). All the pigs had ad libitum access to diet and water throughout the experiment, with the diet details listed in Table S1. During the 28-d experiment, the pigs were fed twice per day at 08:00 and 17:00. At d 1, d 14 and 25, the ileal and fecal digesta samples were collected at 18:00 and immediately stored at −80 °C for further analysis of microbial composition and metabolites. The pigs' general health status and behavior were monitored and all the pigs remained healthy through the whole experimental period.

### 2.3. DNA extraction, MiSeq sequencing and data processing

Total microbial genomic DNA was extracted from 0.3 g digesta samples according to the previous method (Dai et al., 2010). Briefly, the samples were mixed with sterile cetyltrimethyl ammonium bromide (CTAB) buffer using bead-beating for splitting the cell wall of bacteria. The DNA was extracted by the phenol-chloroform method and finally suspended in TE buffer. The quantity of DNA was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and stored at −80 °C until further processing.

The V3–V4 regions of the bacterial 16S rRNA gene were amplified using a universal forward primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and a reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′). The amplicons were purified using a DNA Gel Extraction Kit according to the manufacturer's instructions (Axygen Biosciences; Union City, CA, USA). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform according to the standard protocols.

The raw sequence data from 16S rRNA gene MiSeq sequencing were demultiplexed and quality-filtered using QIIME. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier against the SILVA (SSU115) 16S rRNA gene database using a confidence threshold of 70%. Bacterial alpha diversity (including rarefaction curves analysis, Shannon diversity indices, Ace richness estimators) and principal coordinate analysis (PCoA) based on the unweighted distance method were assessed using MOTHUR software (Schloss et al., 2009). Significant and differential OTUs between 2 groups were determined by linear discriminant analysis (LDA) effect size (LDA score > 2 as discriminant taxa) (Segata et al., 2011). The raw reads were uploaded into the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (Accession Number: PRJNA800640).

Here the ileum and feces are defined as gut location factors, and the different sampling time points (d 1, 14 and 25) are defined as time factors. To investigate the relationship of the changes in microbial composition to gut location and time, redundancy analysis (RDA) was performed using CANOCO 5 software (Leps and

Smilauer., 2003). The gut location and time factors were regarded to significantly impact bacterial composition at $P < 0.05$.

### 2.4. Functional prediction of microbial metagenomes

The metagenomics functional predication based on 16S rRNA gene was performed with PICRUSt (Langille et al., 2013). The categories of gene were predicted at level 2 and level 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology groups (KOs) in the KEGG database (Kanehisa et al., 2012). Significant differences in key KEGG pathways and enzyme abundances were analyzed between the 2 groups in the ileum and feces.

### 2.5. Measurements of microbial metabolites

Short-chain fatty acids (SCFAs) in ileal and fecal samples were measured by gas chromatography according to our previous study (Zhang et al., 2018). Lactate was analyzed using a commercial kit according to the manufacturer's instructions (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China). The biogenic amines were measured by high-performance liquid chromatography according to our previous study (Yang et al., 2014).

### 2.6. Determination of total nitrogen and total carbohydrate contents

Dietary nitrogen and carbon are major nutrients for the microbes in the gut. To understand nitrogen and carbon availability to gut microbes, the total carbohydrate and total nitrogenous compound substrates in the ileal digesta and feces were further analyzed. The total nitrogenous compound content in the ileal digesta and feces was calculated based on the crude protein percentage (Total nitrogen $\times$ 6.25). The crude protein percentage was analyzed according to the Association of Official Analytical Chemist (AOAC) methods (International, 1995). The total carbohydrate in the ileal digesta and fecal samples was measured by a commercial kit (Cat. K645-100, Biovision, USA) according to the manufacturer's instructions.

### 2.7. Quantitative real time-PCR

To further reflect functional alterations of the ileal and fecal microbiota, the copy numbers of genes encoding key enzymes related to propionate and butyrate formation and lactate utilization were quantified by real time-PCR assay on a QuantStudio7 Flex detection system (Thermo Fisher Inc., MA, USA) according to the manufacturer's instructions using a TB Green Premix Ex Taq assay kit (Cat. RR420A; TaKaRa Biotechnology, Dalian, China). The detailed procedures, containing reaction mixtures, PCR conditions, clone library constructions and standard curve preparation, were performed according to previous methods (Mu et al., 2017c). The genes encoding methylmalonyl-CoA decarboxylase (*mmdA*) and butyryl-CoA:acetate CoA-transferase (*BCoAT*) were used for quantifying propionate- and butyrate-producing enzymes, respectively; and the gene encoding lactoyl-CoA dehydratase (*LcdA*) was used for quantifying lactate-utilizing enzyme. All the primers used were listed in Table S2.

### 2.8. Statistical analysis

All data were analyzed by using SPSS 20.0 (SPSS Inc., Chicago, USA). The differences of bacterial abundance including at the phylum and genus level were analyzed using the Mann–Whitney *U* test. To avoid type I errors during microbiota analysis, the *P*-value was adjusted with the Benjamini–Hochberg false discovery rate (FDR) multiple-testing correction, the adjusted *P*-value (*q*-value) < 0.05 was regarded as statistically significant. The data of microbial metabolite profiles, gene copy numbers, total nitrogenous compounds and carbohydrate content were analyzed with the Student's *t*-test to detect significant differences between the 2 groups. All data were visualized using Graphpad Prism version 9.0 (GRAPHPAD Software, San Diego, CA, USA).

## 3. Results

Throughout the whole experiment, none of the pigs had diarrhea or other health impairments, and no significant difference was observed between the FOS group and CP group in average feed intake, average daily gain and body weight gain.

### 3.1. The temporal and spatial effect of CP vs. FOS on the microbial composition in the gut

The dynamic effect of CP vs. FOS on the ileal and fecal microbial composition was revealed by 16S rRNA gene high-throughput sequencing. In total, 2,062,543 reads were obtained after the quality control and there were 28,646 reads per sample. The rarefaction curve of the individual sample based on the OTU numbers showed sufficient sequences for further analysis (Fig. S1). The microbiota community of the CP group and FOS group showed a significant difference in alpha-diversity (Shannon and Chao1 index) in the ileum and feces at d 25, with the value in the CP group higher than in the FOS group ($P < 0.05$; Fig. 1A). For the beta-diversity of the microbiota community, principal coordinate analysis (PCoA) showed a significant distinct between the CP group and FOS group in the ileum at d 25 and in the feces at d 14 and 25 (Fig. 1B and C), suggesting the difference in impact on microbial composition between CP and FOS occurred earlier in the large intestine than in the small intestine.

Redundancy analysis showed that in both the CP group and FOS group gut location was the major contributor shaping the gut microbiota in the growing pigs ($P < 0.05$; Fig. 2). Additionally, the time factor (d 25) had a significant impact on the microbial composition in the CP group ($P < 0.05$; Fig. 2B), but was not significant in the FOS group ($P > 0.05$; Fig. 2A). The results indicated the progressive impact of CP on the gut microbiota of the pigs.

Firmicutes and Proteobacteria were the 2 dominant phyla in both the CP group and FOS group (Fig. 3A), with lower abundance of Firmicutes and higher Proteobacteria in the ileum in the CP group than the FOS group ($q < 0.05$; Fig. 3B). At the genus level, the CP group and FOS group showed different fecal microbes at d 14 and 25 and different ileal microbes at d 25, suggesting that an early difference occurred in the feces. In the ileum, within the top 30 genera, twelve genera were different in abundance between the CP group and FOS group at d 25 (Table S3). The most abundant genus *Lactobacillus* showed markedly lower abundance in the CP group than in the FOS group at d 25 ($q < 0.05$; Fig. 3C). However, the CP group showed higher abundances in genera *Escherichia-Shigella*, *Klebsiella*, *Romboutsia*, *Enterobacter*, *Turicibacter* and *Terrisporobacter* at d 25 and lower abundance in *Sharpea* at d 14 and 25 compared with the FOS group ($q < 0.05$; Fig. 3C). In the feces, within the top 30 genera (Table S4), the CP group showed higher abundance in *Christensenellaceae R-7 group* at d 14 and 25 than the FOS group ($q < 0.05$; Fig. 3D). In addition, the CP group showed a higher abundance in *Ruminococcaceae UCG-010* and lower abundances of *Olsenella* and *Mitsuokella* at d 14 and 25 compared with the FOS group ($q < 0.05$; Fig. 3D). Further, relative to the FOS group, the CP group also showed higher abundances in *Ruminococcaceae NK4A214 group* and *Eubacterium eligens group* and lower abundances in *Megasphaera* and *Prevotella 9* at d 25 ($q < 0.05$; Fig. 3D).

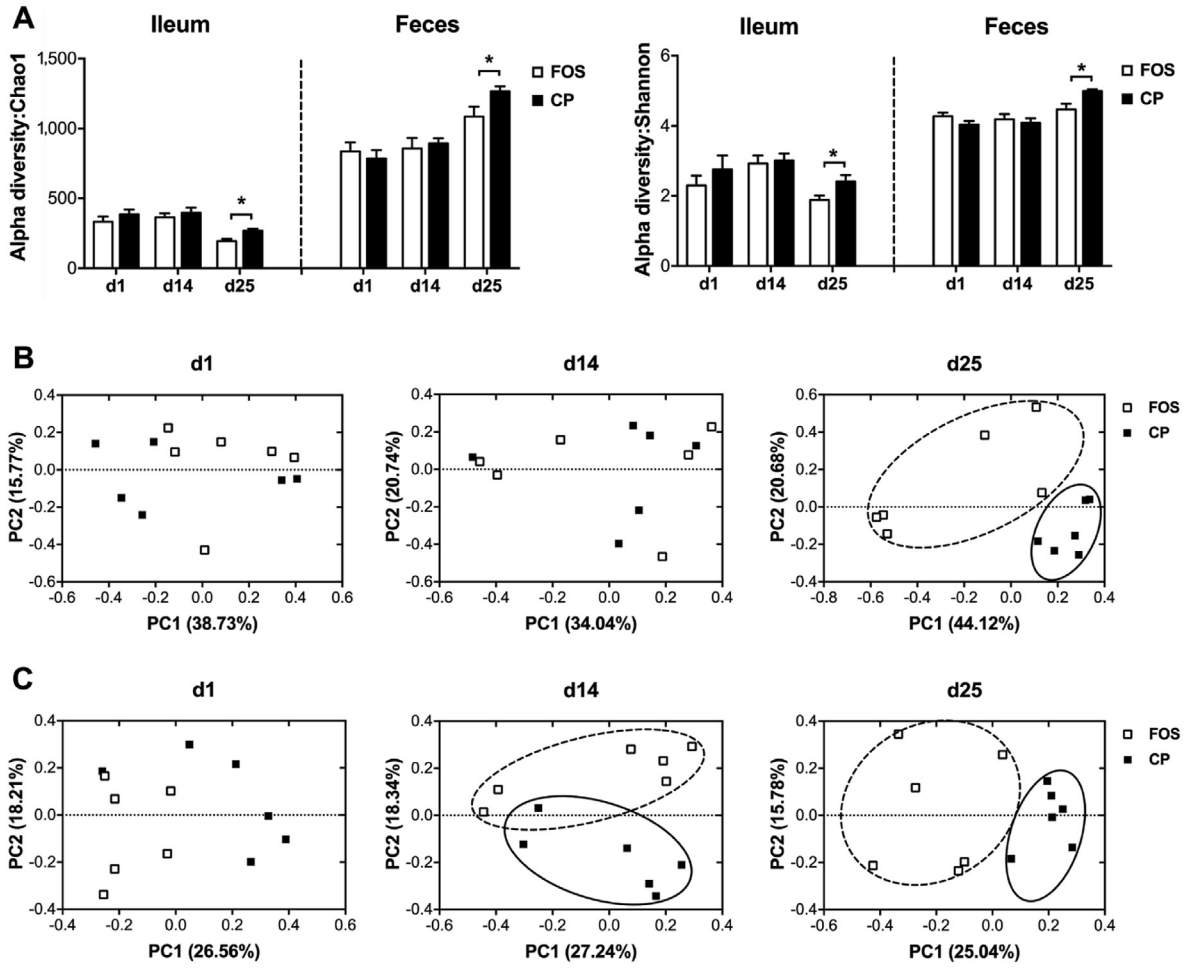


**Fig. 1.** The microbial community structure in the ileum and feces (A) The alpha diversity of the microbial community: Shannon and Chao1 index. The data are expressed as mean ± SEM ($n = 6$). Asterisks indicate a significant difference between the 2 groups ($P < 0.05$). (B and C) The principal coordinate analysis (PCoA) of ileal microbiota (B) and fecal microbiota (C). Circles with solid or dashed lines indicate that the 2 groups are significantly distinct using AMOVA analysis. CP = citrus pectin; FOS = fructo-oligosaccharide.

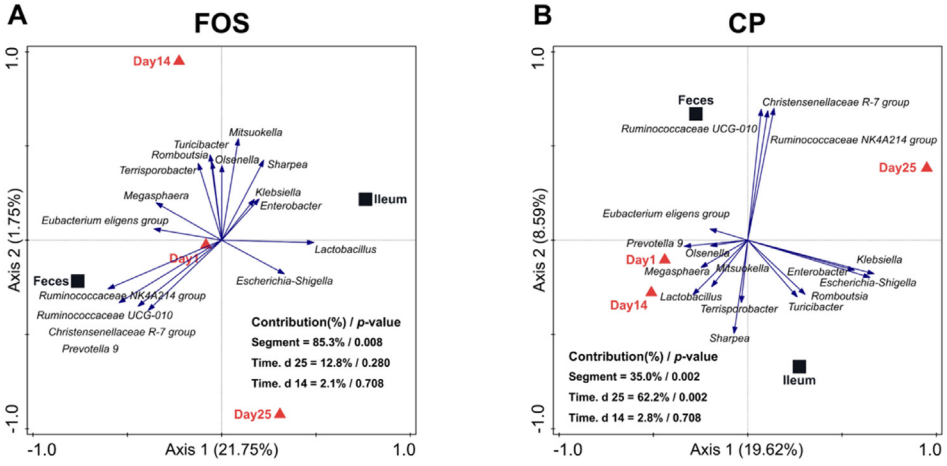


**Fig. 2.** Redundancy analysis (RDA) plots based on the relative abundance of microbiota at the genus level in the ileum and feces at different experimental time periods: RDA plots in the FOS group (A) and CP group (B). FOS = fructo-oligosaccharide; CP = citrus pectin.

At the OTU level, in the ileum, the CP group and FOS group showed only one OTU (*Sharpea azabuensis*) with a different abundance at d 14, and 9 OTUs were different at d 25, with *Lactobacillus johnsonii* higher in the FOS group and *Escherichia coli* higher in the CP group (Fig. 3E). In the feces, the CP group and FOS group showed 9 OTUs at d 14, and 11 OTUs at d 25, that differed in abundances,

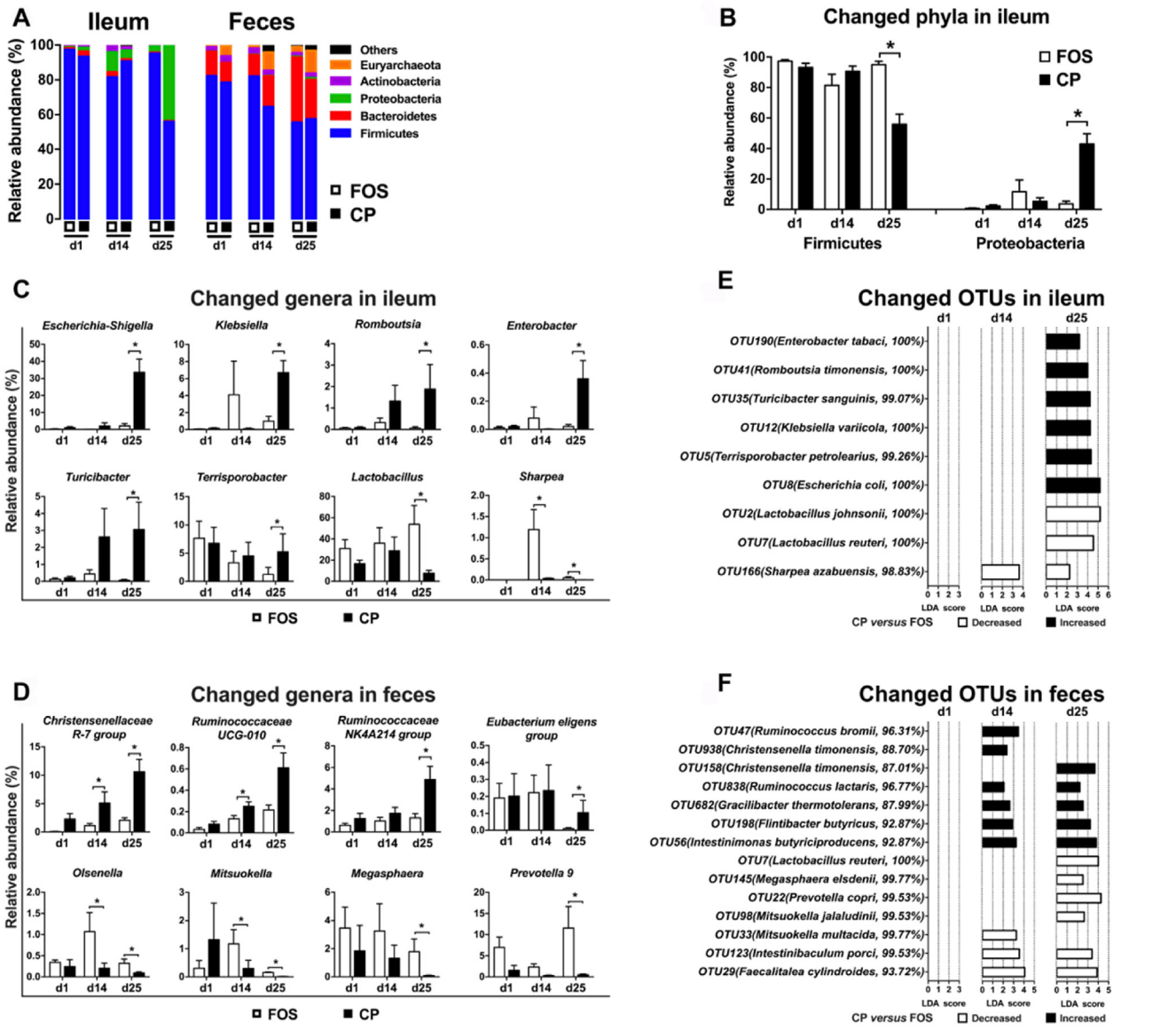


**Fig. 3.** The microbial composition in the ileum and feces. (A) The relative abundance of microbial phylum-level in the ileal and fecal digesta. (B) The significantly changed phyla in the ileal digesta. (C and D) The significantly changed genera in the ileal and fecal digesta, respectively. The data are expressed as mean ± SEM ($n = 6$). The significant difference was calculated with the Mann–Whitney *U* test. Asterisks indicated a distinct difference between FOS group and CP group ($q < 0.05$). (E and F) The significantly changed OTUs based on LDA analysis in the ileal and fecal digesta, respectively. More detailed data are shown in Table S5 and Table S6, respectively. CP = citrus pectin; FOS = fructo-oligosaccharide; LDA = linear discriminant analysis; OTU = operational taxonomic units.

with *Ruminococcus bromii*, *Christensenella timonensis*, *Ruminococcus lactaris*, *Flintibacter butyricus* and *Intestinimonas butyriciproducens* higher in the CP group and *Lactobacillus reuteri*, *Mitsuokella multacida*, *Megasphaera elsdenii*, *Prevotella copri* and *Faecalitalea cylindroides* higher in the FOS group (Fig. 3F). The results indicated that the difference in influence between dietary CP and FOS occurred earlier in the feces than in the ileum.

### 3.2. The effect of CP vs. FOS on the microbial function in the ileum and feces based on metagenomics prediction

Dietary CP and FOS showed different impacts on gut microbial function in the ileum and feces (Fig. 4). In the ileum, relative to FOS, CP mainly enriched the functional potentials related to amino acid metabolism, such as lysine, valine, leucine and isoleucine degradation ($P < 0.05$; Fig. 4A); In the feces, microbes with potential function related to carbohydrate metabolism were enriched in the CP group, like pentose and glucuronate interconversions and butanoate metabolism ($P < 0.05$; Fig. 4B). These results suggested that the 2 soluble dietary fibers exerted a compartment-specific role on microbial function in the gut. Relative to FOS, dietary CP may have a greater ability in enhancing amino acid metabolism in the ileum and promoting carbohydrate metabolism in the feces.

According to the predicted functional potentials, we further analyzed the expression abundances of the key enzymes related to amino acid metabolism and carbohydrate metabolism based on the KOs predicted. Cadaverine is a main product of microbial decarboxylation of lysine. Thus, to understand microbial lysine degradation, we focused on those enzymes predicted to participate in lysine fermentation. In the ileum, the relative expression abundance of genes encoding lysine decarboxylase was higher in

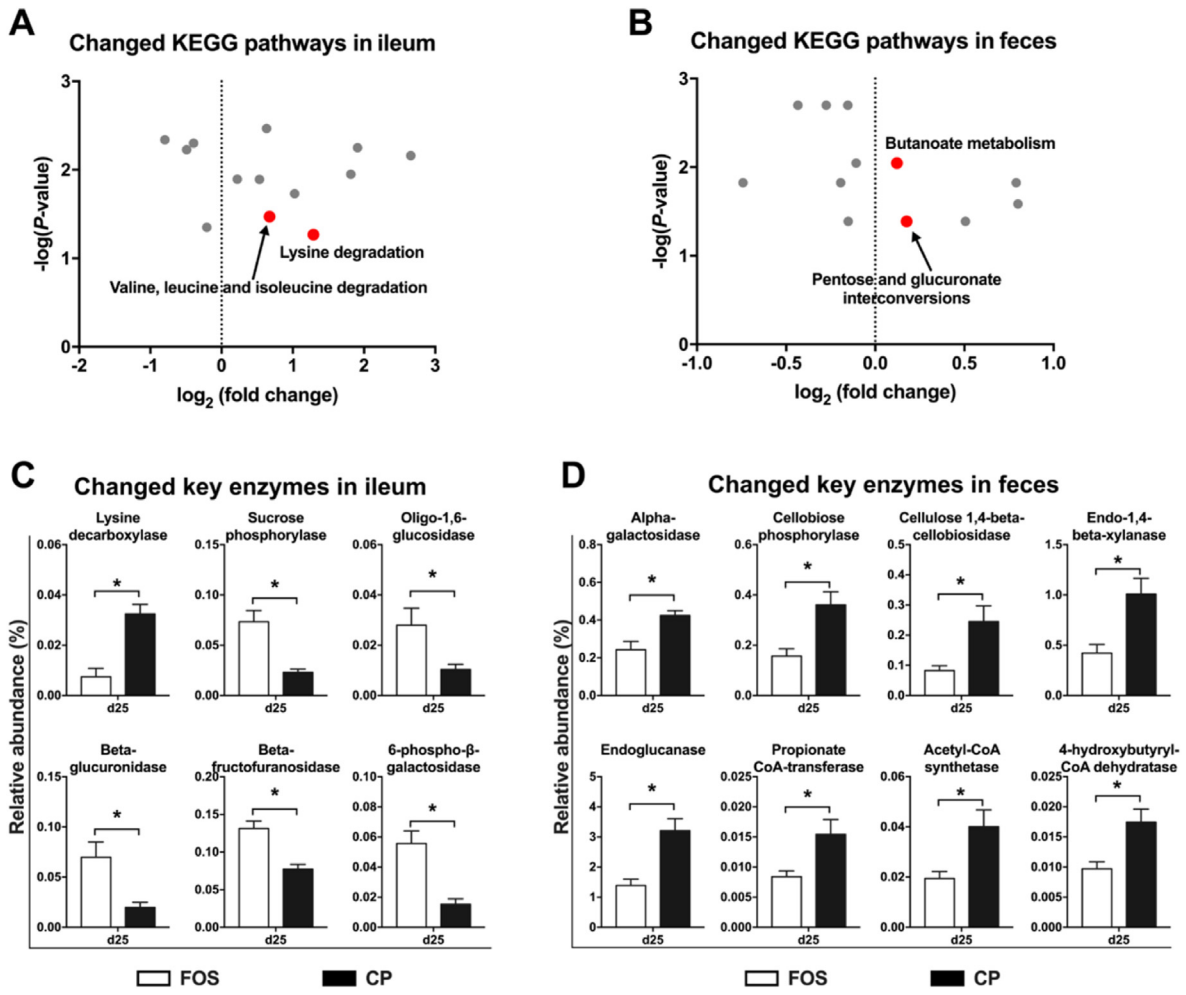


**Fig. 4.** Based on the metagenomic prediction results, the significant distinct KEGG pathways in the ileum and feces at d 25. The red solid circles showed KEGG pathways related to amino acid metabolism in the ileum (A) and related to carbohydrate metabolism in the feces (B), respectively. The changed gene expressions of key enzymes related to amino acid metabolism and carbohydrate metabolism between the 2 groups in the ileum (C) and feces (D) at d 25, respectively. The data are expressed as mean ± SEM ($n = 6$). An asterisk denotes a significant difference between the 2 groups ($P < 0.05$). CP = citrus pectin; FOS = fructo-oligosaccharide; KEGG = Kyoto Encyclopedia of Genes and Genomes.

the CP group than the FOS group at d 25 ($P < 0.05$; Fig. 4C). For the carbohydrate-active enzymes, relative to FOS, the CP group had lower relative expression abundances of genes encoding β-fructofuranosidase, β-glucuronidase, sucrose phosphorylase, oligo-1,6-glucosidase and 6-phospho-β-galactosidase ($P < 0.05$; Fig. 4C). In the feces, the CP group had higher relative expression abundances of genes encoding carbohydrate active enzymes (alpha-galactosidase, cellobiose phosphorylase, cellulose 1,4-beta-cellobiosidase, endo-1,4-beta-xylanase and endoglucanase) at d 25 than the FOS group ($P < 0.05$; Fig. 4D). Therefore, these results further supported the hypothesis that the 2 soluble dietary fibers have differing influences on microbial function in the ileum and feces.

In addition, SCFAs are the main products of carbohydrate fermentation by microbes. Thus, to understand SCFA formation, we focused on those enzymes predicted to participate in SCFA production. In the feces, the relative abundances of genes encoding enzymes associated with SCFA production (acetyl-CoA synthetase, propionate CoA transferase and 4-hydroxybutyryl-CoA dehydratase) were higher in the CP group than the FOS group ($P < 0.05$; Fig. 4D). Meanwhile, in the feces rather than the ileum, the copy numbers of functional genes encoding key enzymes involved in propionate (*mmdA*) and butyrate (*BCoAT*) production and lactate utilization (*LcdA*) were greater in the CP group at d 14 and 25 than in the FOS group ($P < 0.05$; Fig. 5). These results hinted that dietary CP may have a stronger capacity to enhance SCFA production in the hindgut than FOS.

### 3.3. The effect of CP vs. FOS on microbial metabolites in the ileal and fecal digesta

Carbohydrate substrates are fermented by the gut microbiota to produce metabolites such as SCFAs and lactate. In the ileum, the concentrations of total SCFAs and acetate were higher in the CP group than the FOS group at d 25 ($P < 0.05$; Fig. 6A). There were no significant differences in propionate, butyrate and lactate between the 2 groups ($P > 0.05$; Fig. 6A). In the fecal digesta, the CP group and FOS group showed differences at d 14 and 25, with higher concentrations of total SCFAs, acetate, propionate and butyrate and lower level of lactate in the CP group than in the FOS group ($P < 0.05$; Fig. 6A). These results indicated that relative to FOS, dietary CP showed a greater ability in promoting carbohydrate fermentation in the large intestine.

Nitrogenous compounds, especially amino acids, can be converted by microbes to biogenic amines. In the ileal digesta, the concentrations of cadaverine and tyramine were higher in the CP group at d 25 than in the FOS group ($P < 0.05$; Fig. 6B). In the feces, there was no significant difference between the 2 groups in the

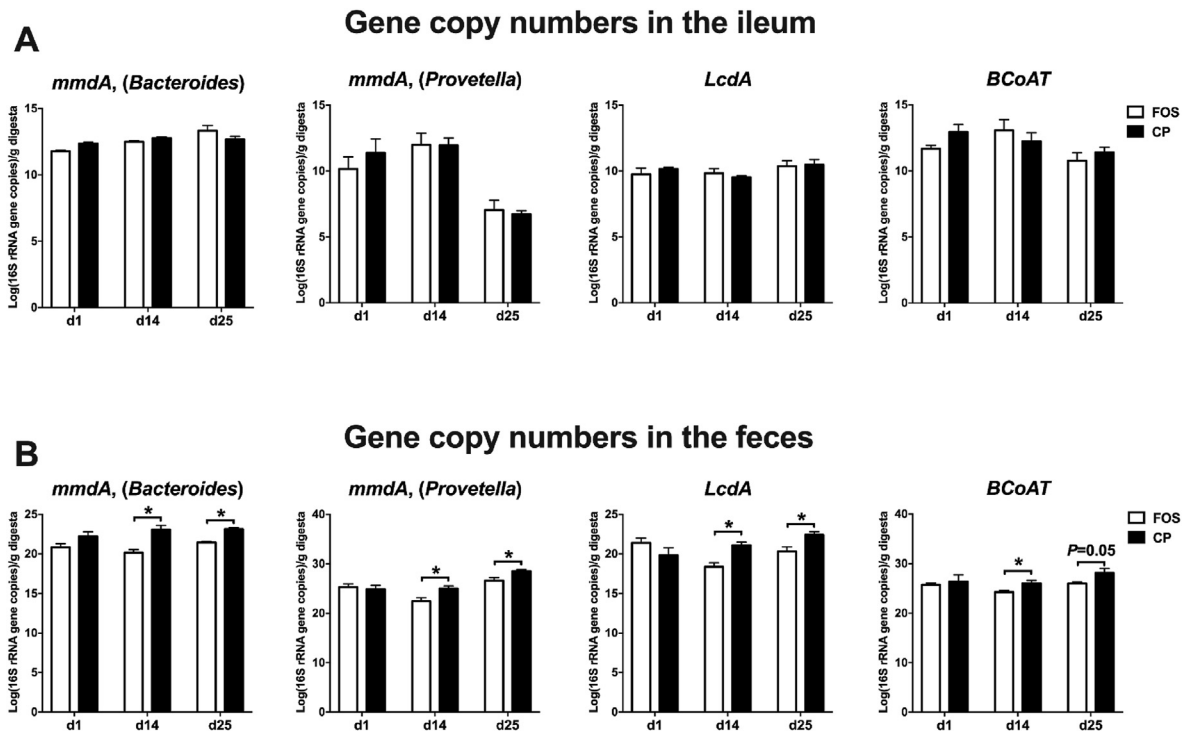


**Fig. 5.** Quantification of copy numbers of genes encoding key enzymes related to propionate- and butyrate-production and lactate utilization in the ileum (A) and feces (B). The data are expressed as mean ± SEM ($n = 8$). An asterisk denotes a significant difference ($P < 0.05$), and $0.05 < P$ value $< 0.1$ was regarded as a significant tendency between the 2 groups. *BCoAT* = butyryl-CoA:acetate CoA-transferase; CP = citrus pectin; FOS = fructo-oligosaccharide; *LcdA* = lactoyl-CoA dehydratase; *mmdA* = methylmalonyl-CoA decarboxylase.

concentration of all bioamines ($P > 0.05$; Fig. 6B). Hence, these data suggested that compared to FOS, dietary CP has a greater ability in enhancing amino acid fermentation in the ileum.

### 3.4. The effect of CP vs. FOS on total nitrogen and total carbohydrate content in the ileum and feces

The remaining content of total nitrogenous compounds and total carbohydrates in the digesta could be used to reflect substrate utilization status in the gut. Compared to FOS, the content of total nitrogenous compounds was lower in the CP group in the ileum and feces at d 25 ($P < 0.05$; Fig. 7A). For the total carbohydrate content, there was no difference between the FOS and CP group in the ileum ($P > 0.05$; Fig. 7B). While in the feces, the total carbohydrate content was lower in the CP group than the FOS group at d 14 and 25 ($P < 0.05$; Fig. 7B). These results further demonstrated that compared to FOS, dietary CP has a stronger ability to promote ileal nitrogen fermentation and fecal carbohydrate fermentation.

## 4. Discussion

Dietary fibers have been shown to alter the microbial community and metabolism in the gut, particularly in the large intestine (Fak et al., 2015; Tian et al., 2016; Deehan et al., 2020). Citrus pectin and FOS are both soluble fibers, but differ in chemical composition. The present study aimed to explore whether CP and FOS exert differential impacts on gut microbiota by comparing their temporal and spatial influence in the small and large intestine. Results showed that these 2 soluble fibers influenced the microbial community and metabolism in different ways depending on the duration of supplementation and location in the gut, with apparent differences occurring earlier in the feces than in the ileum. Citrus pectin showed a greater ability to stimulate microbial amino acid fermentation in the ileum and microbial carbohydrate fermentation in the feces with more SCFAs in the feces than FOS.

### 4.1. Citrus pectin and FOS showed a great difference in affacting microbiota, with apparent difference occurring earlier in the feces than in the ileum

The longitudinal analysis of microbial composition and metabolism revealed the time-dependent response to fiber inclusion. In the present study, the difference in microbial composition and metabolites in the feces and ileum between the 2 groups occurred at d 14 and 25, respectively. These apparent differences occurred earlier in the feces than the ileum. These results may be attributed to the distinct species and amounts of microbiota in the small and large intestine. Our previous study reported that the intestinal microbiota consists of a relatively simple microbiota in the small intestine and a very complex microbial community in the large intestine (Mu et al., 2017b). The small intestinal microbiota consists of *Streptococcus*, *Lactobacillus* and *E. coli*, which tend to utilize simple carbohydrate, such as starch (Zoetendal et al., 2012). The large intestinal microbiota, including *Ruminococcus*, *Prevotella* and *Bacteroides*, encompasses a mass of genes encoding carbohydrate-active enzymes that enable the degradation of complex dietary glycans (El Kaoutari et al., 2013), such as pectin. Therefore, due to a greater ability to degrade dietary polysaccharides, the large intestinal microbiota can more rapidly utilize the 2 soluble fibers than the small intestinal microbiota. This may explain our finding that the difference in their impact on the microbiota between CP and FOS occurred earlier in the feces than ileum.

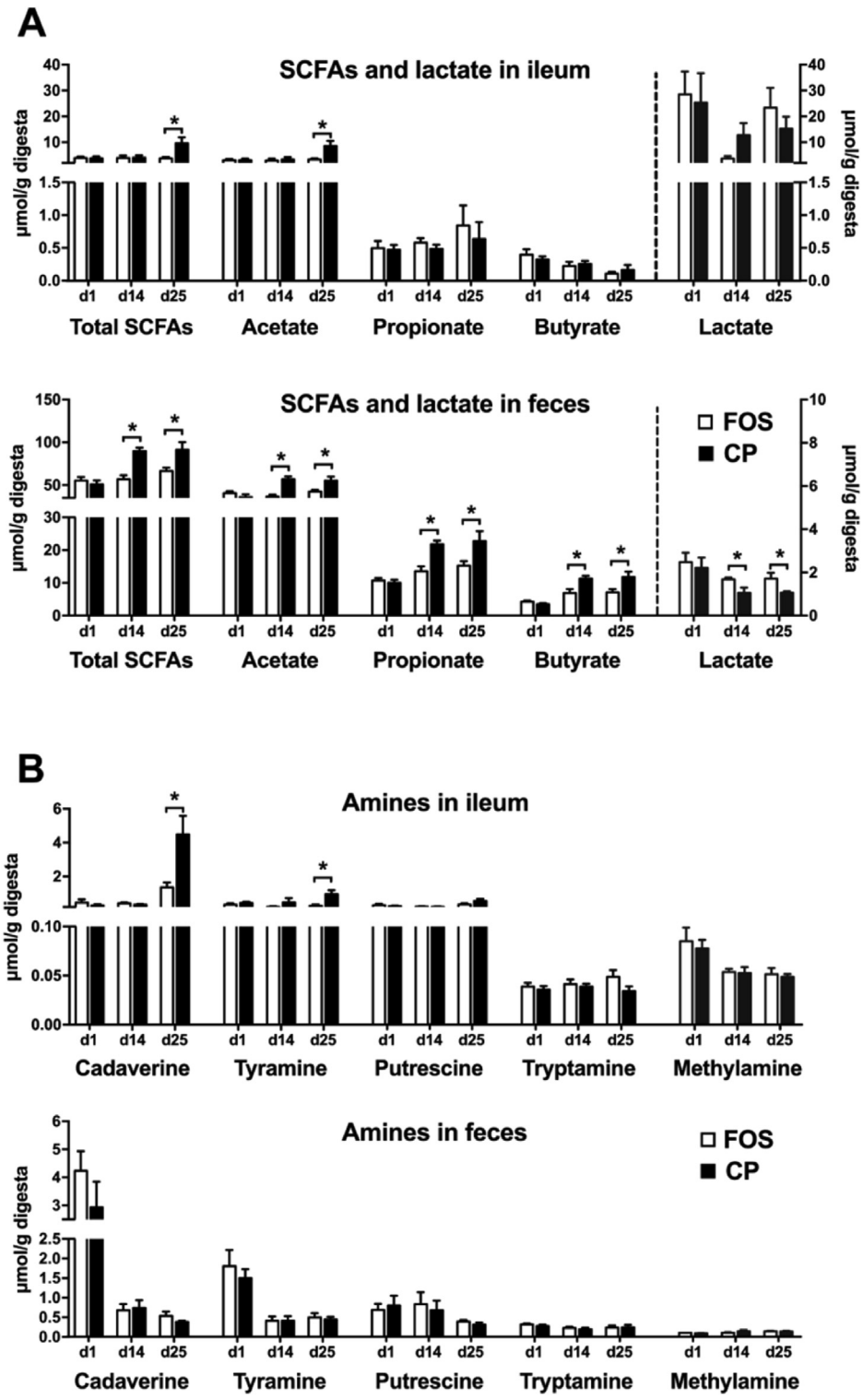


**Fig. 6.** The microbial metabolite profiles in the ileum and feces. (A) The concentration of short-chain fatty acids (SCFAs) and lactate in the ileal digesta and feces. (B) The concentration of amines in the ileal digesta and feces. The data are expressed as mean ± SEM ($n = 8$). An asterisk denotes a significant difference between the 2 groups ($P < 0.05$). CP = citrus pectin; FOS = fructo-oligosaccharide.

### *4.2. Dietary CP upregulated foregut amino acid fermentation and hindgut carbohydrate fermentation relative to FOS*

Microbiota in the foregut and hindgut are well known to have a different composition and function. Herein, we further demonstrated that the effect of dietary CP and FOS differed depending on location in the gut, characterized by greater amino acid fermentation in the foregut and stronger carbohydrate fermentation in the hindgut with CP than with FOS.

Microbial utilization of amino acids is of great physiological relevance in terms of amino acid metabolism. In the ileum, dietary CP showed a greater abundance of the bacteria within

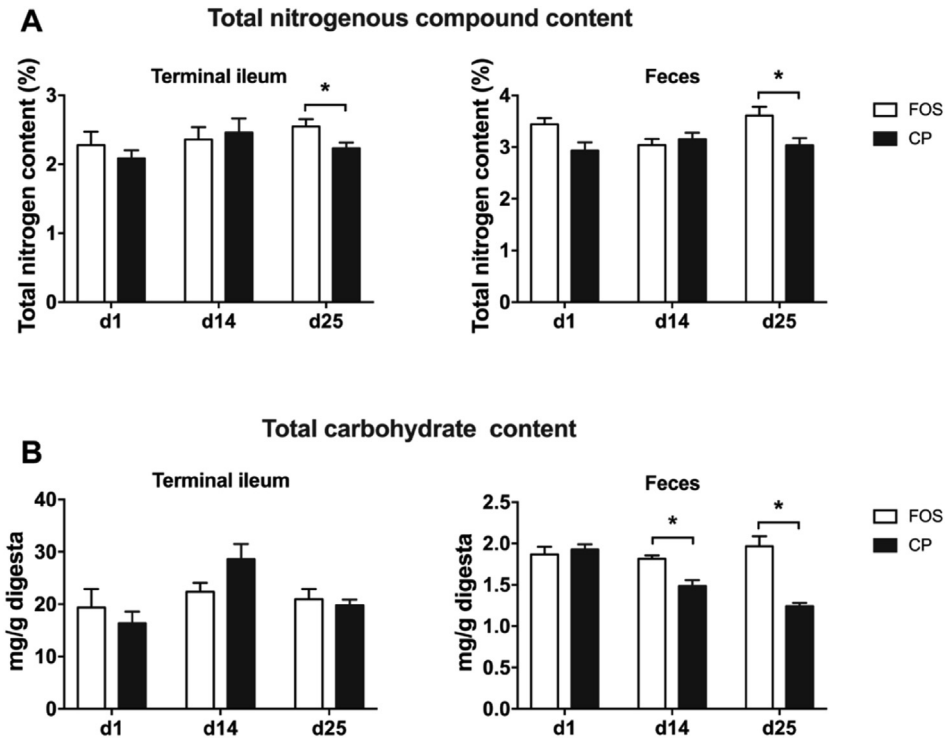


**Fig. 7.** The total nitrogenous compound content (A) and total carbohydrate concentration (B) in the terminal ileum and feces. The data are expressed as mean ± SEM ($n = 8$). An asterisk denotes a significant difference between the 2 groups ($P < 0.05$). CP = citrus pectin; FOS = fructo-oligosaccharide.

Proteobacteria (e.g. *Escherichia-Shigella* and *Klebsiella*) and a larger amount of metabolites (e.g. cadaverine and tyramine) involved in amino acid decarboxylation. The mechanism behind is complex. Pectin could increase the viscosity of digesta in the small intestine (Owusu-Asiedu et al., 2006; Hooda et al., 2011), which could subsequently increase retention time and accessibility of gut microbes to nutritional substrates, such as protein and amino acids. The genera *Escherichia-Shigella* and *Klebsiella* are commonly recognized as amino acid-fermenting bacteria in the small intestine (Dai et al., 2010), which are capable of metabolizing lysine and tyrosine to cadaverine and tyramine, respectively, via decarboxylation (Louis et al., 2007; Pieper et al., 2016; Xu et al., 2020). Recently, it has been reported that *Romboutsia*, *Enterobacter*, *Turicibacter* and *Terrisporobacter* are commensal bacteria known to be associated with amino acid fermentation in the foregut (Luna et al., 2017; Gao et al., 2019; Hu et al., 2019). The present study found an increase in the relative abundances of these taxa in the CP group relative to FOS group. Ultimately, an increase in fermentation of amino acids may lead to the decrease of total nitrogenous substrates in the ileum. Cadaverine and tyramine have been reported to promote gut development and exert an anti-inflammatory effect (Patel et al., 2019; Bekebrede et al., 2020). It is of great interest for future study to investigate how trace amines are involved in gut health regulated by pectin.

Differing from the impact in the ileum, the dietary pectin stimulated carbohydrate fermentation in the hindgut, as revealed by increased pectinolytic microbes and upregulated key enzymes. In the present study, relative to FOS, the supplementation of CP increased *Ruminococcaceae UCG-010, Christensenella R-7 group* and *Eubacterium eligens group* in the feces. Bacteria in *Ruminococcus* and *Eubacterium* were mainly responsible for the degradation of complex polysaccharides in the large intestine (Chung et al., 2016; Koh et al., 2016). A metagenomics analysis of the fecal microbiota in goats showed that *Ruminococcus* contained genes encoding partly pectinase/esterase to catabolize pectin (Peng et al., 2021). *Eubacterium eligens group*, contains pectinolytic bacteria which could utilize pectin by encoding a broad repertoire of pectinolytic enzymes, including a highly abundant pectate lyase (Chung et al., 2017). Consistently, our results based on metagenomic functional prediction also demonstrated that the relative expression abundances of genes encoding carbohydrate-active enzymes were higher with dietary CP supplementation than with FOS, such as endoglucanase and endo-1,4-xylanase, which have an important role in catabolizing complex polysaccharides (Cantarel et al., 2012). Therefore, the increase in bacteria by CP supplementation may accelerate the degradation of complex polysaccharides and lead to the promotion of carbohydrate fermentation and the decrease of total carbohydrate substrates in the feces.

Additionally, we observed an increase in the abundance of Euryarchaeota and *Methanobrevibacter* in the feces. *Methanobrevibacter* is well known to utilize hydrogen and produce methane, which reduces intestinal hydrogen pressure and ultimately accelerates carbohydrate fermentation (Ungerfeld, 2015; Ruaud et al., 2020). Given this functional potential, the increase of *Methanobrevibacter* further provides evidence of increased carbohydrate fermentation in the feces.

### 4.3. Fiber type-oriented gut microbiota: CP as a stronger trigger of SCFA-producing capability than FOS

Dietary fibers are important carbon sources for microbial fermentation to produce SCFAs, especially in the hindgut. In the present study, compared with FOS, dietary CP increased the production of SCFAs in the feces, including acetate, propionate and butyrate. These results are mainly attributed to the increase in SCFA-producing bacteria, such as *Ruminococcus* and *Eubacterium* (Louis et al., 2007; Xu et al., 2020). *F. butyricus* and

*I. butyriciproducens*, shown with greater abundance in the CP group relative to the FOS group, were recognized as the butyrate-producing bacteria in gut (Lagkouvardos et al., 2016), which may participate in butyrate formation. Furthermore, the chemical composition of fibers has been reported to be associated with SCFA production (Wang et al., 2019). Generally, the aldehydes, such as glucose, galactose, mannose and xylose, are mainly fermented to produce acetate and butyrate; whereas propionate is produced by ketone fermentation, such as fructose, arabinose and tagatose (Hu et al., 2013). Therefore, the chemical composition of fibers could partly explain our finding that the supplementation of CP rather than FOS promoted higher SCFA production in the feces. Both *mmdA* and *BCoAT* are functional genes involved in propionate- and butyrate-producing pathways in bacteria (Pryde et al., 2002; Mu et al., 2017a), respectively. The increase of copy numbers of *mmdA* and *BCoAT* in the CP group also supported the increased production of SCFAs in the large intestine.

Lactate can be converted to propionate by lactate-utilizing bacteria via the acrylate pathway, which is mediated by the key enzyme LcdA (Koh et al., 2016). The gene *LcdA* encodes a key enzyme which catalyzes lactate to form propionate (Reichardt et al., 2014). In the present study, dietary CP showed a lower concentration of lactate and greater copy numbers of the *LcdA* gene in the feces than FOS supplementation. These results suggested that the supplementation of CP in diet promoted lactate utilization towards propionate production. Moreover, compared with FOS supplementation, the supplementation of CP showed a lower abundance of *Lactobacillus* and *Olsenella*, both of which are lactate-producing bacteria (Mao et al., 2015). Thus, the reduction in lactate-producing bacteria may also explain the decrease in lactate.

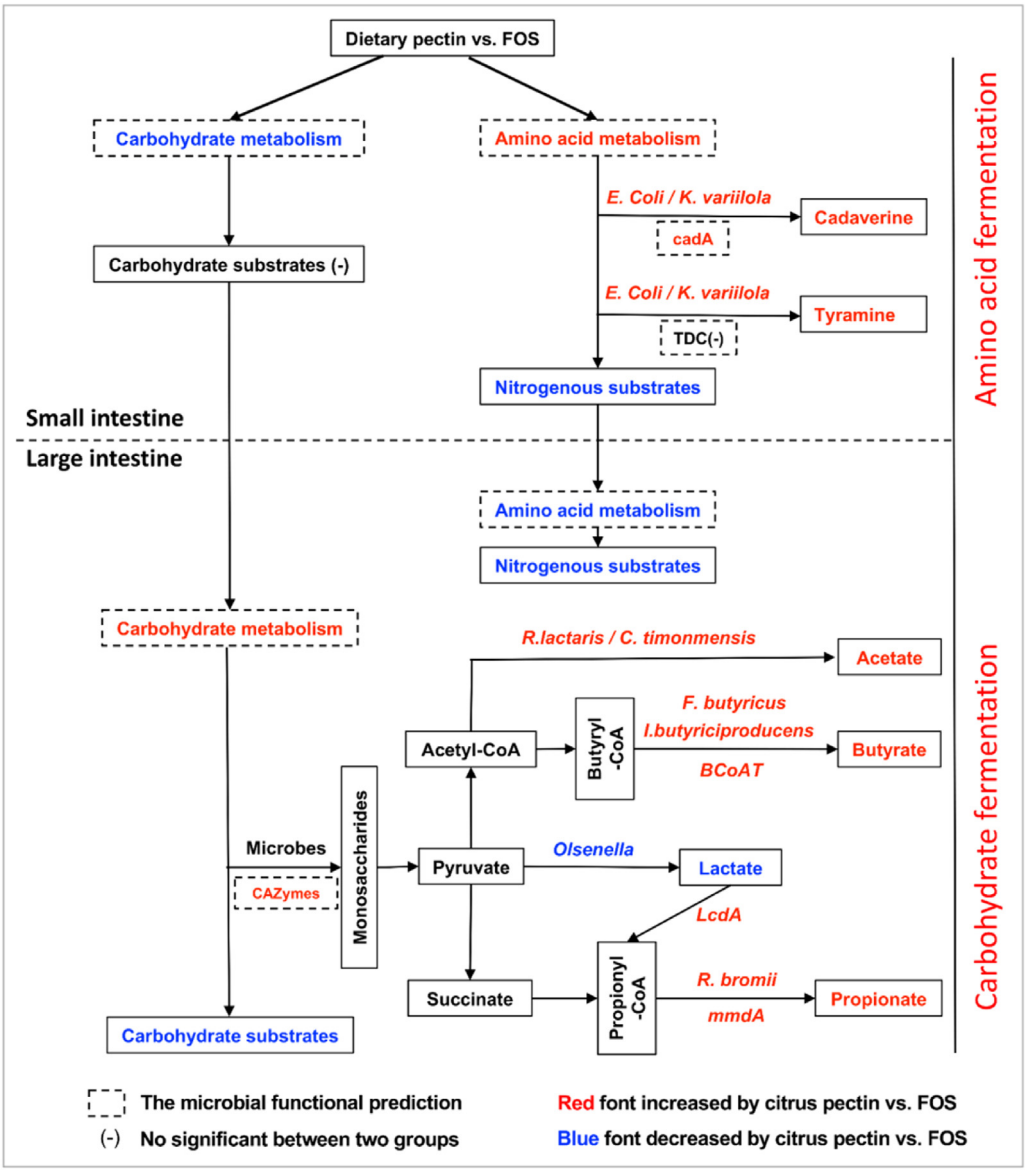


**Fig. 8.** Proposed model of the impact of dietary citrus pectin (CP) vs. fructo-oligosaccharide (FOS) on modulating the microbial composition and metabolism in the foregut and hindgut. Compared with FOS, dietary CP enhanced amino acid metabolism with a higher amine concentration in the small intestine, allowing a smaller amount of nitrogenous compounds to enter the large intestine, leading to a lesser degree of nitrogenous metabolism in the large intestine; while in the large intestine, dietary CP promoted carbohydrate metabolism and fermentation, with a higher expression abundance of predicted carbohydrate-active enzymes and a higher concentration of short-chain fatty acids, leading to lower excretion of total carbohydrate content. *BCoAT* = butyryl-CoA: acetate CoA-transferase; *cadA* = lysine decarboxylase; *LcdA* = lactoyl-CoA dehydratase; *mmdA* = methylmalonyl-CoA decarboxylase; *TDC* = tyrosine decarboxylase.

Collectively, compared with FOS, the gut microbiota with dietary CP supplementation displayed a stronger ability to produce SCFAs.

To summarize the responses of the ileal and fecal microbes and microbial metabolism to the soluble dietary fibers in growing pigs, we proposed a functional model as shown in Fig. 8. Compared with FOS, dietary CP promoted amino acid fermentation and increased the relative abundances of *E. coli* and *K. variilola, cadA* (lysine decarboxylase) and cadaverine and tyramine concentration, and decreased the total nitrogenous substrates in the ileum. In the feces, dietary CP enhanced carbohydrate fermentation and increased the relative abundances of *R. bromii*, *F. butyricus* and *C. timonmensis*, and the copy number of functional genes involved in propionate (*mmdA*) and butyrate (*BCoAT*) production and lactate utilization (*LcdA*) and SCFAs, and decreased the total carbohydrate substrates. Taken together, dietary CP and FOS elicited a different effect on the ileal and fecal microbial community and metabolism, with CP enhancing ileal amino acid fermentation and promoting fecal carbohydrate fermentation.

## 5. Conclusions

The current study revealed temporal and spatial impacts of soluble dietary fibers on the microbial community and metabolism in the small and large intestine. Dietary CP and FOS showed progressive and different influences on microbial composition in the ileum and feces, with apparent differences occurring earlier in fecal microbiota than in ileal microbiota. Meanwhile, relative to FOS, dietary CP displayed a stronger ability to enhance amino acid fermentation in the foregut and promote carbohydrate fermentation in the hindgut. Collectively, the present study highlights the temporal and spatial responses of the microbiome in the small and large intestine to different types of soluble dietary fibers with CP as a stronger trigger SCFAs than FOS, which provides a new insight for modulating the microbiota community and metabolism through dietary fibers.

## Author contributions

Yanan Zhang conducted the animal experiment, analyzed the data and wrote the manuscript. Chunlong Mu provided advice on the methodology and conceptualization and reviewed the manuscript. Shuai Liu conducted the animal experiment. Weiyun Zhu supervised the study, reviewed and edited the manuscript, and secured funding for the study. All authors read and approved the final manuscript.

## Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.08.005.

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