ABSTRACT

The colonization and development of the gut microbiome in dairy calves play a crucial role in their overall health and future productivity. Despite the widely proposed benefits of inulin-related products on the host, there is insufficient information about how supplementing fructo-oligosaccharides (FOS) impacts the colonization and development of the gut microbiome in calves. In a randomized intervention trial involving newborn male Holstein dairy calves, we investigated the impact of FOS on the calf hindgut microbiome, short-chain fatty acids, growth performance, and the incidence of diarrhea. The daily administration of FOS exhibited a time-dependent increase in the average daily gain and the concentration of short-chain fatty acids. Concurrently, FOS delayed the natural decline of Bifidobacterium, promoting the maturation and stabilization of the hindgut microbiome. These findings not only contribute to a theoretical understanding of the judicious application of prebiotics but also hold significant practical implications for the design of early life dietary interventions in the rearing of dairy calves. Key words: Dairy calf, fructo-oligosaccharides, hindgut microbiome, growth performance

INTRODUCTION

The colonization and development of the gut microbiome in dairy calves play a crucial role in their overall health and future productivity. Despite the widely proposed benefits of inulin-related products on the host, there is insufficient information about how supplementing fructo-oligosaccharides (FOS) impacts the colonization and development of the gut microbiome in calves. In a randomized intervention trial involving newborn male Holstein dairy calves, we investigated the impact of FOS on the calf hindgut microbiome, short-chain fatty acids, growth performance, and the incidence of diarrhea. The daily administration of FOS exhibited a time-dependent increase in the average daily gain and the concentration of short-chain fatty acids. Concurrently, FOS delayed the natural decline of Bifidobacterium, promoting the maturation and stabilization of the hindgut microbiome. These findings not only contribute to a theoretical understanding of the judicious application of prebiotics but also hold significant practical implications for the design of early life dietary interventions in the rearing of dairy calves. Key words: Dairy calf, fructo-oligosaccharides, hindgut microbiome, growth performance

Fructo-oligosaccharides supplementation enhances the growth of nursing dairy calves while stimulating the persistence of Bifidobacterium and hindgut microbiome’s maturation

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Fructo-oligosaccharides supplementation enhances the growth of nursing dairy calves while stimulating the persistence of Bifidobacterium and hindgut microbiome’s maturation
leading to a reduction in intestinal pH, and activation of the immune system (Gibson and Roberfroid, 2008). *Clostridia* and *Escherichia coli* are important pathogens causing diarrhea in calves (Muktar et al., 2015). Changes in microbial composition can influence the succession and development of the microbial community, accelerating the maturation of the microbiome. Samara and colleagues’ research revealed that the administration of probiotics increased the abundance of *Bifidobacterium*, driving the maturation of the preterm infant microbiome and enhancing intestinal immunity (Samara et al., 2022). Meanwhile, gastrointestinal bacterial composition can affect feed efficiency traits, such as average daily gain (ADG) and feed conversion ratio (FCR), and has been shown to explain 25.3% of the variation for heifer ADG (Paz et al., 2018). Despite such proposed benefits, the impact of early life FOS interventions on the assembly of the hindgut microbiome in dairy cattle remains unclear.

We hypothesized that FOS supplementation would promote the growth of *Bifidobacterium* in the feces of dairy calves, accelerate the maturation of the microbiome, thereby facilitating intestinal fermentation, promoting weight gain, and decreasing incidence of diarrhea. Thus, the specific objectives of this project were to (1) investigate the temporal response of fecal microbiota and SCFAs to FOS supplementation, (2) explore whether supplementing young dairy calves with FOS would affect their growth performance and the incidence of diarrhea.

**MATERIALS AND METHODS**

**Animals and Experimental Design**

All calves involved in our study received approval from the Nanjing Agricultural University Institutional Animal Care and Use Committee (no. SYXK-2017–0027). A cohort of 15 newborn Holstein male calves was reared in a single calf hutch at Dingzhuang Pasture, Jurong City, Jiangsu Province. Within 10 h after birth, the calves were given 2 colostrum servings (4 L for the first feeding and 2 L for the second). Subsequently, the veal calves were fed milk twice a day based on weight. The milk composition includes 3.22% protein, 3.61% fats, 5.01% lactose, 12.68% total solids, and 9.13% nonfat solids. The calves had free access to water; milk and water were provided at temperatures ranging from 35°C to 37°C.

In detail, the 15 newborn male Holstein dairy calves were randomly assigned to either consume a diet supplemented with 6 g/d FOS (n = 7) or not (CON, n = 8). The CON group served as the control, while calves in the FOS group received daily supplementation of 6 g FOS (FOS, ≥ 95% purity, degree of polymerization between 2 and 9, purchased from Shandong Bailong Chuangyuan Bio-tech Co., Ltd.) before the first feeding each day, dissolved in 20 mL of warm water, which was administered to the calf through a small feeding bottle. Antibiotic treatment for the calves was strictly prohibited throughout the experiment period. Any deviation from this protocol resulted in removing the respective calf from the experiment (Supplementary Data 1).

Power analyses based on t-test calculated a minimum sample size using pwr package in R (version 4.1.2). The parameters for power analysis were calculated using the birth weight of calves (effect size and SD). We determined that the minimum sample size per group was 6, using a power of 0.8 as the criterion.

**Sample Collection and DNA Extraction**

The calves’ weights and milk intake were measured on d 0, d 7, d 14, d 21, and d 28, and subsequently, ADG and FCR were calculated. As previously described, fecal consistency was assessed daily using a 0 to 3 scales (Renaud et al., 2018). Fecal scores of 2 and 3 were designated as indicative of diarrhea. The incidence of diarrhea among the calves was determined using the following formula: diarrhea rate (%) = [(number of cattle with diarrhea × days of diarrhea) / (number of cattle in each group × days of the experiment)] × 100.

Quadruplicate 5-mL fecal samples were collected via rectum at d 1, d 3, d 5, d 7, d 10, d 13, d 16, d 19, d 23, and d 28 before the morning feeding and promptly stored in liquid nitrogen following collection. These fecal samples were utilized for both SCFAs analyses using gas chromatography (GC-14B instrument; Shimadzu, Japan) (Lin et al., 2019) and DNA extraction. In this paper, isobutyrate, isovalerate, and valerate were collectively categorized as “other SCFAs.” Acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate were collectively categorized as “total SCFAs.”

To evaluate the significance of body weight, ADG, milk intake, FCR and concentration of SCFAs, a Linear Mixed Model (LMM) from the lmerTest package was utilized, treating group and time as fixed effects and calf as a random effect. Due to the non-normal distribution of the incidence of diarrhea (Shapiro-Wilk normality test, *P* = 0.001), we utilized Mann-Whitney U test for conducting a significance test between the 2 groups. Pearson correlation coefficients were calculated between the averaged SCFAs concentrations and ADG separately for each group. Due to the availability of data for SCFAs concentration at 10 time points and ADG represented by weekly averages over the 4 weeks, SCFAs concentration was averaged on a weekly basis (e.g., averaging data for d 1, d 3, d 5, and d 7 to rep-
resent the mean concentration of SCFAs for the first week).

**16S rRNA Gene Sequencing**

We used barcoded PCR primers F341 (5′- CCTAY-GGGRBGCASCAG-3′) and R806 (5′-GGAC- TACNNGGGTATCTAAT-3′) to amplify the V3-V4 region of the 16S rRNA gene. PCR reactions were carried out in triplicate in a 20 μL reaction mixture, comprising 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The PCR amplification conditions consisted of a preliminary denaturation step of 95°C for 5 min, followed by 27 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, concluding with an ultimate extension procedure at 72°C for 10 min. Subsequently, amplicons were purified using the AxyPrep DNA Gel Extraction Kit after being extracted from 2% agarose gels (Axygen Biosciences, Union City, CA, US) following the manufacturer’s protocols. The purified amplicons were then submitted to Shanghai Biozeron Biological Technology Co. Ltd. for sequencing on an Illumina MiSeq platform (250-bp paired-end). The raw reads have been submitted into the NCBI Sequence Read Archive under PRJNA1042798.

The raw fastq was initially demultiplexed using perl scripts based on the barcode sequences for each sample, employing Gao et al.’s criteria (Gao et al., 2021). The resulting reads were subjected to the DADA2 (QIIME2–2020.11.1). Paired reads were trimmed and filtered with up to 2 allowable errors per read. After merging paired reads and filtering chimeras, The taxonomic classification of each sequence (referred to as Amplicon Sequence Variants [ASV]) was determined by the RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU132) 16S rRNA database with a confidence threshold of 70% after merging paired reads and filtering chimeras (Amato et al., 2013).

Beta-diversity was assessed using Principal Coordinates Analysis (PCoA) and Bray Curtis distances, was computed using normalized data in R (version 4.1.2) with the vegan package. To assess the significance of Chao 1 richness, an LMM from the lmerTest package (version 3.1.0) was used to compare the abundances of the microbiome between the CON group and FOS group, a Linear Discriminant Analysis Effect Size (LEfSe) and random forest classification model were performed.

**Correlation Between Bacteria Biomarkers and SCFAs Concentration**

The correlation between bacterial genera (with mean relative abundance >0.5%) and SCFAs concentration was determined using Pearson correlation coefficient. In the correlation matrix, only correlations that were both strong (|/rho| > 0.5) and statistically significant (P < 0.05) were retained. The result was visualized using Gephi (0.9.2).

**Identification of Microbiome Community Types**

We used the ward sum-of-squares algorithm to apply hierarchical clustering on the Bray-Curtis distance at the ASV level to evaluate microbiome maturation. The most suitable number of clusters was evaluated using Gap statistics. Community heterogeneity was assessed by calculating the inverse of the coefficient of variation (ICV) using the formula: ICV = a/b, where ‘a’ represents the mean of the relative abundance of all ASV in a particular quadrat, and ‘b’ represents the SD for the mean of the relative abundance of all ASV in that specific quadrant.

The calculation and statistical analysis of β diversity are consistent with the 16S rRNA Gene Sequencing section. A Kruskal Wallis test was used to assess bacterial richness (Chao1) and heterogeneity (ICV) in community types.

**Assessment of The Effect of FOS on Microbiome Maturation**

We estimated the state transition probabilities using the markovchain package (version 0.9.5). The Kaplan-Meier analysis with the survival package (version 3.3.5) was used to assess the time to transition to the mature community type, which was then visualized using the survminer package (version 0.4.9).

**Shotgun Metagenomics and Taxonomic Profiling**

Fecal samples from each group were randomly divided into 3 subgroups and thoroughly mixed at each designated time point, including d 1, d 7, d 16, d 23, and d 28. Consequently, a total of 30 mixed samples were obtained. Microbial DNA extraction was performed as previously described. Shanghai Biozeron Biological Technology Co. Ltd. was responsible for constructing and sequencing the metagenomic shotgun sequencing libraries. In summary, 1 μg of genomic DNA from each sample was sheared through the Covaris S220 Focused-ultrasonicator (Woburn, MA, USA), and preparing sequencing libraries, targeting a fragment length of 450
Metagenome Assembly, Taxonomic, Phylogenetic, and Functional Analyses

Clean sequence reads were assembled into contigs for each sample using MEGAHIT (version 1.2.9) with default parameters (Li et al., 2015). Subsequently, metagenome-assembled genomes (MAGs) were created by binning the assembled contigs using MetaBAT2 (version 2.12.1) (Kang et al., 2019), MaxBin (version 2.2.6) (Wu et al., 2016), and CONCOCT (version 1.0.0) (Alneberg et al., 2014). Integration of the resulting MAGs was performed using the Binning Refiner (Song and Thomas, 2017). Following filtering for completeness (>80%) and contamination (<10%), a total of 515 MAGs were taxonomically classified using the Genome Taxonomy Database Toolkit (version 1.7.0) (Chaumeil et al., 2020)

A phylogenetic tree of *Bifidobacterium* MAGs was constructed by PhyloPhAn (Asnicar et al., 2020) (version 3.0.67) and was visualized using iTOL (version 6) (Letunic and Bork, 2021). High-quality reads were mapped the dbCAN2 database (Zhang et al., 2018) (version 20230802) using diamond (Buchfink et al., 2021) (version 2.0.15.153) to annotate CAZys profile. Taking into account the interaction effect of grouping and time, differential analysis of CAZys between treatments was performed using DESeq2 (formula = ~Group + Time + Group*Time) (Love et al., 2014).

RESULTS

The Effect of FOS on Dairy Calves Growth Performance

We aimed to investigate the effects of FOS supplementation on the growth performance and incidence of diarrhea in calves. As outlined in Table 1, the ADG of calves in the FOS group exhibited a notable increase compared with CON group in wk 4 (P = 0.047), leading to a higher body weight in the former on d 28, although this difference did not reach statistical significance (P = 0.65). FOS supplementation did not significantly influence milk intake or FCR across all time points (Table 1, P > 0.05 for all). The incidence of diarrhea remained comparable between the FOS and CON groups throughout the entire study period (Table 1, P = 0.82).

FOS Stimulated Short-chain Fatty Acid Production

Subsequently, we investigated the impact of FOS on metabolism by assessing SCFAs production. Fecal concentrations of acetate, propionate, and total SCFAs were significantly higher in the FOS group compared with the CON group, particularly from d 16 onward (Figure 1, P < 0.05). However, the 2 groups had no significant differences in the fecal concentrations of butyrate and other SCFAs (Figure 1, P > 0.05). We observed a higher correlation between the ADG and fecal concentrations of acetate and propionate, and total SCFAs in the FOS group compared with the CON group (Table 2). These results suggested that a diet supplemented with FOS may enhance calf growth by increasing acetate and propionate concentration time-dependently.

FOS Supplementation Delayed the Extinction of *Bifidobacterium* in Nursing Calves

Next, we sought to elucidate how increased FOS altered the global structure of the hindgut microbiota. We collected 140 fecal samples covering 10 time points from 15 newborn Holstein male calves (Supplementary Data1). After initial quality control, a total of 8.77 million high-quality 16S rRNA sequences with an average length of 417 bp were obtained (Supplementary Data 2).

The FOS group exhibited a notable reduction in α diversity, as measured by Chao 1 richness at the end of the intervention (d 28), compared with the CON group (Figure 2A, P = 0.027). This indicated that FOS supplementation had a mild, rather than a dramatic, effect on the α diversity of the microbiome. Subse-
quently, we analyzed the microbiome structure using PCoA and PERMANOVA test based on Bray-Curtis distance. The overall structure of the fecal microbiota displayed significant alterations in both groups, with FOS supplementation emerging as a significant grouping factor in discriminating metagenomes (Figure 2B, \( P = 0.018 \)).

A total of 36 phyla, including Firmicutes, Bacteroidota, Actinobacteriota, and Proteobacteria, were detected across both groups. Firmicutes (55.29%) and Bacteroidota (15.70%) constituted the 2 most abundant phyla, accounting for over 70% of the total sequences (Supplementary Figure 1A). Notably, the abundance of Proteobacteria gradually decreased over time (Supplementary Figure 1A). At the genus level, Lactobacillus (15.40%) was the dominant genus of the microbiota, followed by Bacteroides (12.77%), Escherichia Shigella (7.51%), Fusobacterium (6.38%), and Bifidobacterium (5.83%) in both the CON and FOS groups during early life (Supplementary Figure 1B). Consistent with the overall dynamics of Proteobacteria, the relative abundance of Escherichia Shigella showed a gradual reduction (Supplementary Figure 1B).

We further performed LefSe analysis to identify microbiotas at the genus level closely associated with FOS supplementation (Supplementary Figure 1C). A random forest classification model was also applied to find bacterial biomarkers discriminating FOS supplementation using 10-fold cross-validation for 10 replicates (Supplementary Figure 1D). Through these 2 approaches, we identified 5 bacterial biomarkers between the FOS group and the CON group. Among them, Bifidobacterium, Dorea, and Alloprevotella were significantly enriched with the FOS diet, whereas Clostridium sensu stricto 1 and Peptostreptococcus were more abundant in the CON group (Figure 2C).

To further explore key microbes at the species level, we employed shotgun metagenomic sequencing on feces, including 30 samples from 5 time points: d 1, d 7, d 16, d 23, and d 28. During the initial data pre-processing, a direct comparison of sequences indicated that the sequencing depth of raw sequences per sample was 43.84 million (median; interquartile range [IQR] = 11.58) reads. Following quality control, this was reduced to 29.41 million (median; IQR = 11.92), a significant difference as determined by the Mann-Whitney U test \( (P < 0.001) \). Our data pre-processing procedure notably enhanced the quality of metagenomic sequences (Supplementary Figure 1E).

By applying differential abundance analysis with LEfSe, we identified Bifidobacterium pseudocatenulatum as the dominant species promoted by FOS (Figure 2D, \( P = 0.002 \)). Consistent with the metagenomic data sets, our genome-centric analysis of Bifidobacterium revealed that all MAGs were classified into Bifidobacterium longum (10 MAGs, 60% of them from the CON group, 40% from the FOS group) and Bifidobacterium.

### Table 1. Effects of supplementation with fructo-oligosaccharides (FOS) on the growth performance and incidence of diarrhea

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatments</th>
<th>SEM</th>
<th>( P )-value</th>
<th>( P )-value</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>FOS</td>
<td></td>
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<td></td>
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<tr>
<td>Body weight, kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 0</td>
<td>42.9</td>
<td>1.21</td>
<td>0.31</td>
<td>&lt;0.001</td>
<td>0.55</td>
</tr>
<tr>
<td>d 7</td>
<td>47.2</td>
<td>1.16</td>
<td>0.31</td>
<td></td>
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<tr>
<td>d 14</td>
<td>51.4</td>
<td>1.20</td>
<td>0.41</td>
<td></td>
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<tr>
<td>d 21</td>
<td>57.4</td>
<td>1.16</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d 28</td>
<td>64.2</td>
<td>1.51</td>
<td>0.65</td>
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<tr>
<td>Average daily gain, kg/d</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>wk 1</td>
<td>0.607</td>
<td>0.058</td>
<td>0.98</td>
<td>&lt;0.001</td>
<td>0.18</td>
</tr>
<tr>
<td>wk 2</td>
<td>0.720</td>
<td>0.062</td>
<td>0.77</td>
<td></td>
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</tr>
<tr>
<td>wk 3</td>
<td>0.847</td>
<td>0.083</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 4</td>
<td>0.980</td>
<td>0.082</td>
<td>0.047</td>
<td></td>
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<tr>
<td>Milk intake, kg/d</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>wk 1</td>
<td>10.0</td>
<td>0.381</td>
<td>0.80</td>
<td>&lt;0.001</td>
<td>0.92</td>
</tr>
<tr>
<td>wk 2</td>
<td>10.0</td>
<td>0.021</td>
<td>0.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>wk 3</td>
<td>12.0</td>
<td>0</td>
<td>0.99</td>
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<tr>
<td>wk 4</td>
<td>12.1</td>
<td>0.042</td>
<td>0.89</td>
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<td>Feed conversion ratio</td>
<td></td>
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<td></td>
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<tr>
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<td>15.0</td>
<td>1.38</td>
<td>0.43</td>
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<tr>
<td>wk 2</td>
<td>18.3</td>
<td>2.52</td>
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<tr>
<td>wk 3</td>
<td>17.4</td>
<td>2.66</td>
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<tr>
<td>wk 4</td>
<td>13.5</td>
<td>1.28</td>
<td>0.37</td>
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<tr>
<td>Incidence of diarrhea day1 to day28, %</td>
<td>0.164</td>
<td>0.034</td>
<td>0.82</td>
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</table>

Values are expressed as mean values; SEM = standard error of the mean.
CON, the control group; FOS, the group supplementation with FOS.
**Correlation between Bacteria Biomarkers and SCFAs Concentration**

To investigate whether FOS-induced shifts in SCFAs output were associated with bacteria biomarkers, a co-occurrence network was constructed to illustrate the relationships between the relative abundance of differential microbes and SCFAs concentrations (Figure 3). Notably, the relative abundance of *Bifidobacterium* showed a significant positive correlation with the concentrations of acetate and propionate (Figure 3, $P < 0.05$). Similar positive correlations were observed between *Dorea* and propionate concentrations (Figure 3, $P < 0.05$). In contrast, the relative abundance of *Clostridium sensu stricto 1* negatively correlated with the concentration of butyrate (Figure 3, $P < 0.05$). Additionally, a significant positive correlation was detected between *Peptostreptococcus* and the concentrations of propionate (Figure 3, $P < 0.05$).

**FOS Accelerates Microbiome Maturation and Stabilization**

As the age of calves increases, the diversity and stability of the gut microbiota gradually increase (Ma et al., 2020). However, the ecological impacts on the assembly and successional trajectory of prebiotics on the gut microbiota of calves have not been systematically determined. To address this, we employed a hierarchical clustering method on the microbiome data gathered over time during the study. According to a PERMANOVA test, the bacterial communities among the 7 clusters exhibited significant dissimilarities at the ASV level (Figure 4A and Supplementary Figure 2A, $P = 0.001$). The dominant community type transitioned from C1 at birth to C5, and finally shifted to C7 by the end of the d 28 experimental period (Figure 4 B). A stepwise increase in $\alpha$ diversity (Chao1) occurred as
the microbiota matured from C1 to C7 (Figure 4C, \( P < 0.001 \)) and minimal heterogeneity in C7 (Supplementary Figure 2B, \( P < 0.001 \)). Furthermore, the C7 community type was distinguished by high abundance of \textit{Faecalibacterium} while the less mature community types were predominantly by \textit{Peptostreptococcus} and \textit{Clostridium sensu stricto 1}.

**Figure 2.** Temporal response of fecal microbiome to FOS supplementation. (A) Boxplots represent the \( \alpha \) diversity measured by microbiome Chao 1 richness. (B) PCoA based on Bray–Curtis distance, illustrating the variances in microbial structure. (C) Differential abundance of genera over time. (D) Relative abundance dynamics of \textit{Bifidobacterium pseudocatenulatum} in the 2 groups over time. (E) A phylogenetic tree of \textit{Bifidobacterium} MAGs.
(Supplementary Figure 2C, LefSe, \( P < 0.05 \)), indicating earlier phases in the progression of microbiome development (Brown et al., 2018; Song et al., 2018; Husso et al., 2021; Du et al., 2023). These results affirmed that the identified community types in calves represent progressive phases of hindgut microbiota maturation.

An examination of the influence of FOS on community maturation indicated no disparity in the distribution of community types between the FOS and CON groups at the initiation of the experiment on d 1, as both groups comprised C1 (Figure 4B). As the experiment progressed, the more mature C7 type dominated in the FOS group from d 16 onward, while in the control group, it dominated after d 23 (Figure 4B). The hastening of microbiome maturation with FOS supplementation was also evident in the Bray-Curtis analysis, as the distance to d 28 microbiomes was reduced on d 13 \( (P = 0.027) \), d 16 \( (P < 0.001) \), and d 19 \( (P < 0.001) \) (Figures 4D, LMM).

Using Markov chain analysis to determine the probability of transitions between community types, the results showed that the FOS group had a higher probability of transitioning to C7 (1.63 vs. 0.71) and maintaining C7 status (0.58 vs. 0.44), indicating higher community stability (Figures 4E). A time-to-occurrence investigation validated that FOS-supplemented calves were more likely to mature to C7 earlier than the CON group, although no statistically significant differences were observed (Supplementary Figure 2D, log-rank, \( P = 0.2 \)). This analysis demonstrated that FOS supplementation for calves accelerates the microbiota toward a more mature and temporally stable development of early life hindgut microbiota.

**Shift in CAZys Profile of Hindgut Microbiome in Response to FOS**

As the relative abundance of bacteria enriched in the FOS group was significantly correlated with SCFAs concentration, a shift in CAZys profile was characterized. Although we did not observe differences in CAZys, FOS-degrading enzyme (GH68) was more prominent in the FOS group (supplementary Figure 3).
Figure 4. FOS accelerates microbiome maturation and stabilization. (A) Hierarchical clustering based on Bray-Curtis dissimilarity matrix revealed 7 distinct hindgut microbiome community types. (B) Distribution of microbiome community types across different time points in the CON and FOS groups. (C) Comparison of bacterial richness (Chao1) in different community types. (D) The Bray-Curtis distance of microbiome to the d 28 microbiome at each time point. (E) Probability of transition between community types assessed by Markov chain modeling in the CON and FOS groups.
DISCUSSION

The establishment and maturation of the gut microbiome play a pivotal role in the growth and health of calves. Therefore, understanding and modulating the establishment and maturation of the gastrointestinal microbiota in calves is crucial. The current research examined the impacts of supplementing FOS on dairy calf growth performance, the rate of diarrhea, SCFAs production, and the early assembly of the hindgut microbiota. Although no significant difference in ADG was observed between the 2 groups during the first 3 wk, we found that the ADG of the calves in the FOS group was higher than that of the CON group in wk 4, suggesting that supplementation with FOS during the entire rearing period of calves led to an increase in ADG with a time-effect relationship. Although a trend of promoting calf growth was observed in our experiment with FOS, research needs to further determine the optimal dosage and duration of supplementation.

Considering the link between feeding calves large amounts of milk and causing diarrhea (Fischer et al., 2019), we controlled calves’ milk intake, allowing us to observe relatively consistent feed intake in both groups. The relatively short trial period may be a possible reason for the absence of significant differences in body weight and FCR (Table 1). As previous research has shown, the duration of prebiotic supplementation is an important factor in the efficacy of prebiotics (Liu et al., 2020; Perini et al., 2020). Although FOS has been shown to reduce the incidence of diarrhea in piglets (Xu et al., 2005; Zhang et al., 2022), we did not find a significant improvement in diarrhea incidence in calves.

The more direct effect of FOS is that microorganisms utilize them to produce SCFAs (Yao et al., 2022). In our study, supplementation with FOS promoted hindgut fermentation in dairy calves, especially increasing acetate and propionate production. SCFAs are quantitatively important substrates in ruminant energy metabolism (Kristensen et al., 1998). The substantial quantities of acetate produced through microbial fermentation are employed for energy production and fat synthesis (Kaneko, 2008), supporting animal growth and weight gain. Meanwhile, propionate, captured by the portal circulation, plays an active part in gluconeogenesis (Pascale et al., 2018).

We observed a positive association between the ADG and fecal concentrations of acetate, propionate, and total SCFAs, especially in FOS group. Based on these results, we concluded that FOS supplementation could promote the growth of calves by regulating the production of acetate and propionate. The changes in SCFAs levels were attributed to variations in the microbial community (Martin-Gallausiaux et al., 2021). Therefore, next investigated the effects of FOS on the hindgut microbiome. Our 16S rRNA analyses showed that FOS decreased the feces microbiome’s α diversity at the intervention’s end. This decrease may be due to the stimulation of particular bacterial species (e.g., Bifidobacterium) by FOS and the subsequent increase in metabolite production, leading to a decrease in pH inhibiting some species (Han et al., 2021; Mahalak et al., 2023).

Bifidobacterium gradually increased in the first week and began to decrease gradually from d 7 to d 21 (Rada et al., 2006). Notably, FOS supplementation delayed the natural decline of Bifidobacterium, particularly Bifidobacterium pseudocatenulatum. Bifidobacterium is closely linked to gut health and is commonly used as a probiotic to promote health (Yakoob and Pradeep, 2019). FOS is considered to promote the proliferation of Bifidobacterium and produce acetate (Rossi et al., 2005). On the other hand, some microorganisms specialize in the degradation of high molecular weight polysaccharides, and the pathway leading from polysaccharides to SCFAs forms an intricate and indirect metabolic network (Sanders et al., 2019). In this respect, lactate and acetate produced by Bifidobacterium could serve as substrates for bacteria that produce propionate, such as Bacteroides. In the present study, we also found a significant positive correlation between the relative abundance of Bifidobacterium and the concentration of acetate and propionate, indicating that FOS may contribute to the increase in calf weight gain by selectively promoting the propagation of Bifidobacterium that favors acetate and propionate production.

Furthermore, we observed that FOS accelerated the maturation and stabilization of the calf hindgut microbiome, which is beneficial for the production efficiency, nutritional, and immune system development of calves (Du et al., 2023). Investigations into preterm infants indicated that those receiving probiotic supplementation showed increased probabilities of transitioning to the most mature community type and greater stability, in contrast to the placebo group (Samara et al., 2022; Bargheer et al., 2023). Our study observed that the microbial structure of the FOS group was closer to the full-term community structure earlier. Meanwhile, supplementation with FOS decreased the abundance of Peptostreptococcus and Clostridium sensu stricto 1, which commonly dominate in the less mature community types (Brown et al., 2018; Song et al., 2018; Husso et al., 2021; Du et al., 2023). The possible reason is the resistance of Bifidobacterium to colonization by pathogens. Above all, our study provides new insight into the link between prebiotic administration and calves’ accelerated microbiome maturation, which is closely associated with the calf’s ability to utilize fibrous sub-
stances. This may serve as a reference for the early weaning of dairy calves.

CONCLUSIONS

The present study demonstrated that FOS supplementation had time-dependent effects, increasing the ADG and promoting hindgut fermentation in calves. Meanwhile, the findings suggest that early dietary interventions with FOS help delay the natural decline of Bifidobacterium and accelerate maturation and stabilization, potentially benefiting the efficiency of hindgut fermentation and promoting growth performance. Overall, our study provides new insights into the impact of early prebiotic interventions on the colonization and development of the microbiome in calves, contributing to establishing a healthy microbial ecosystem to improve calf health and growth.

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