Supplementation with galacto-oligosaccharides in early life persistently facilitates the microbial colonization of the rumen and promotes growth of preweaning Holstein dairy calves

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Abstract

We aimed to determine the effects of dietary supplementation with galacto-oligosaccharides (GOS) on the growth performance, serum parameters, and the rumen microbial colonization and fermentation of pre-weaning dairy calves. The study comprised 2 phases of 28 and 42 d, respectively. During phase 1, 24 newborn female Holstein dairy calves were randomly allocated to consume a diet supplemented with 10 g/d GOS (GOS, n = 12) or not (CON, n = 12). Thereafter, during phase 2, the GOS group was further divided into 2 groups: one that continued to consume GOS (GOSC, n = 6) and one that no longer consumed GOS (GOSS, n = 6), alongside the CON group. Galacto-oligosaccharides increased the average daily gain (ADG), body weight, feed efficiency, and serum high-density lipoprotein-cholesterol concentration of dairy calves during phase 1 (P < 0.05). Supplementation with GOS for the entire study reduced the incidence of diarrhea and increased the serum total protein and Ca concentrations (P < 0.05) compared with the CON group. The effect of GOS supplementation persisted after it was stopped because the ADG and final body weight of the GOSS group were higher than those of the CON group (P < 0.05). Furthermore, the GOSS group showed a persistently lower incidence of diarrhea and greater colonization of the rumen with probiotics, at the expense of less beneficial bacteria, which would promote ruminal fermentation and microbial protein synthesis. These findings provide a theoretical basis for the rational application of prebiotics and have important practical implications for the design of early life dietary interventions in dairy calf rearing.

1. Introduction

Calf health is critical to the optimal reproductive and lactation performance of adult dairy cows, which affects the sustainable development of the dairy industry. Neonatal calves are faced with a number of types of stressors between birth and weaning, and these can cause growth retardation, diarrhea, digestive disorders, and low feed efficiency (Ghoraishy and Rokouei, 2013). The most important stage of gastrointestinal development occurs in calves: the development of the rumen and the establishment of the rumen microbiota (Malmuthuge et al., 2019). It has been shown that the microbial population that first colonizes the rumen influences the later microbial ecosystem and the efficiency of rumen fermentation (Abecia et al., 2013). Therefore, early dietary interventions that aid the establishment of a healthy microbial ecosystem in the rumen promote the health and performance of adult dairy cows.

Prebiotics are functional feed additives that improve growth and health by influencing the composition of the gastrointestinal microbiota (Cangiano et al., 2020; Lopes et al., 2021). The most well-known prebiotics are indigestible oligosaccharides, including galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS), and mannan-oligosaccharides (MOS) (Uyeno et al., 2015), and these are not only added to human food, but also to animal feed (Gibson et al., 2017), especially for monogastric animals (Richards et al., 2020). However, recently, prebiotics have been shown to
improve the growth, nutrient absorption, and development of the ruminal epithelium development of calves (Ghosh and Mehla, 2012; Costa et al., 2019; Lopes et al., 2021). In addition, an in vitro study showed that oligosaccharides might represent effective means of manipulating rumen fermentation by increasing the concentrations of volatile fatty acids (VFA) and bacterial protein, and reducing that of ammonia nitrogen (NH₃—N) (Li et al., 2011, 2018). Moreover, oligosaccharides have been shown to improve rumen fermentation by specifically promoting the proliferation of particular bacterial species (Li et al., 2011).

Galacto-oligosaccharides are produced from lactose by trans-galactosylation, catalyzed by β-galactosidase, and have been included as a substitute for human milk oligosaccharides in infant formulas because of their health benefits (Botvynko et al., 2019). Robust previous findings indicate that GOS improve mineral absorption and bone health, and that a mixture of GOS and FOS increases calcium (Ca), phosphorus (P), and magnesium (Mg) absorption in growing rats recovering from protein malnutrition (Bryk et al., 2016). In addition, previous studies have shown that GOS can also promote the proliferation of probiotics, such as Lactobacillus and Bifidobacterium, which compete with pathogens for binding sites on host cell membranes, thereby preventing pathogenetic bacteria, such as strains of Escherichia coli, Salmonella, and Clostridium perfringens from forming biofilms in the intestine (Bouwhuis et al., 2017; Montozgudo-Mera et al., 2019). Furthermore, prebiotics have been shown to have long-lasting effects, even after dietary supplementation has been stopped (Gourbeyre et al., 2011). However, few studies have been performed regarding the use of GOS in dairy calves, and in particular regarding their effects on the establishment of rumen microbiota during early life. Therefore, we hypothesized that supplementation with GOS in early life may persistently affect the growth performance and serum indicators via regulating the rumen microbial colonization and fermentation of preweaning Holstein dairy calves. In the present study, we aimed to determine whether short or long-term supplementation with GOS in young dairy calves would affect their growth performance, incidence of diarrhea, serum biochemistry, mineral absorption, the establishment of the rumen microbiota, and ruminal fermentation before weaning. The findings might provide a theoretical basis for the rational application of GOS in the dairy industry.

2. Materials and methods

2.1. Animal ethic statement

This study was conducted according to the principles of the Basel Declaration and the recommendations of the Chinese Academy of Agricultural Sciences Animal Care and Use Committee (Beijing, China). All the procedures used were approved by the Ethics Committee of the Chinese Academy of Agricultural Sciences (IAS2020-102) (Beijing, China). The study was carried out at Junyuan Dairy farm (Shijiazhuang, Hebei Province, China) between October 2020 and February 2021. Animal health and welfare were monitored and recorded after birth and throughout the experimental period.

2.2. Experimental design, animals and diets

This study lasted 70 d and was composed of 2 phases (Fig. 1). For the 1st phase, 24 newborn female Holstein dairy calves (initial body weight, 37.8 ± 0.9 kg) were randomly allocated to 2 groups of 12 using a random number generator (Microsoft Corp., Redmond, WA, USA). The calves in the experimental group were fed a diet containing 10 g/d GOS for 28 d, whereas the diet for the control group did not contain GOS. The 2nd phase of the study lasted for 42 d, during which the calves in the experimental group were randomly allocated to 2 subgroups of 6 calves each. The calves in one of these subgroups continued to consume 10 g/d GOS, whereas the calves in the other subgroup did not consume any more GOS. Thus, the 3 groups during the 2nd phase were as follows: the control group (CON, n = 12), the group that continued to consume 10 g/d GOS (GOSC, n = 6), and the group that stopped consuming GOS on d 28 (Goss, n = 6), as illustrated in Fig. 1. Galacto-oligosaccharides (purity ≥ 90%) were provided by Quantum hi-tech Biological Co. Ltd. (Guangzhou, China).

Immediately after birth, the calves were removed from their dams and housed in individual pens (1.8 m × 1.4 m × 1.2 m, length × width × height) to avoid cross-contamination. The pens were enclosed with iron fences and bedded with straw. All the calves were provided with 4 L of colostrum within 1 h of birth. Thereafter, they were fed 2 L of colostrum from a bottle on d 2 and d 3, and 8 L of raw milk using a bucket from d 4 three times a day at 06:00, 12:00, and 18:00. All the calves received the same colostrum, raw milk, and starter concentrate.

The commercial pelleted starter concentrate was purchased from Tianjin Jiazhud Dadi Feed Co. Ltd. (Tianjin, China) and fed to the calves from d 3. The calves had free access to the starter concentrate and water. The milk and water were provided at 35 to 37 °C. The nutrient compositions of the starter and raw milk are shown in Table 1. The amount of raw milk that was provided for the calves was gradually reduced and the calves were weaned at 70 d of age.

2.3. Sample collection

The calves were weighed on d 1, 28, and 70 to calculate their average daily gain (ADG). The dry matter (DM) intake of each calf associated with the starter and milk was also recorded, and the average daily starter intake, average daily feed intake, and feed efficiency were calculated. The starter was sampled for the analysis of DM content (AOAC International, 2005; method 930.15), crude protein (CP) (AOAC International, 2000; method 976.05), and ether extract (EE) (AOAC International, 2003; method 4.5.05) using the standard procedures of the Association of Official Analytical Chemists. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were measured as reported by Van Soest et al. (1991), and expressed exclusive of residual ash. Sodium sulfite and heat-stable α-amylase (Sigma A3306,Sigma-Aldrich) were used for NDF analysis.

Fecal consistency was recorded daily on a 4-point scale, as previously reported (Teixeira et al., 2015; Chang et al., 2020). A fecal score of 3 to 4 for 2 successive days was defined as diarrhea. The incidence of diarrhea among the calves was calculated using the formula:

Diarrhea incidence = number of calves with diarrhea × number of days with diarrhea/(number of calves in each group × number of experimental days) × 100%.

Blood samples were obtained from each calf by jugular vein puncture and collected in 10 mL gel vacuum tubes (BD Biosciences, San Jose, CA, USA) before the morning feed at the end of the study. The samples were centrifuged at 3,000 × g and 4 °C for 15 min using a high-speed temperature-controlled centrifuge (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany), and the serum was collected into 2 mL centrifuge tubes (Corning, NY, US) and stored at −80 °C until assessed. Rumen fluid was collected using esophageal tubing 2 h after feeding on d 70. After discarding the initial 50 mL of each sample, which might have been contaminated with saliva, 100 mL rumen fluid samples were obtained from each calf. The rumen pH was determined using a portable pH meter (370 model pH meter, Jenway, London, UK). The samples were then filtered through 4 layers of sterile gauze and used for 16S rRNA sequencing and the
addition to the rumen analysis of microbial protein (MCP), NH₃, and VFA content. For the NH₃-N and VFA analyses, 25% (wt/vol) metaphosphoric acid was added to the rumen filtrate, as described previously (Sun et al., 2016). The NH₃-N concentration was measured using an adaptation of the phenol/hypochlorite method (Broderick and Kang, 1980) and the VFA content was assayed by gas chromatography (Stewart and Duncan, 1985). The MCP yield was determined by measuring urinary purine excretion using a previously reported method (Zinn and Owen, 1986). The MCP concentration was calculated using the ratio of purines to nitrogen in isolated bacteria, with yeast RNA as a standard (Sun et al., 2016).

### 2.4. Analysis of serum biochemical parameters and mineral elements

An automated biochemistry analyzer (Hitachi 7080, Tokyo, Japan) was used to measure serum biochemical parameters: the serum total protein (TP), albumin (ALB), triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL), low-density lipoprotein-cholesterol (LDL), urea nitrogen (UREA), and glucose (GLU) concentrations; and the serum aspartate amino transferase (AST), and alanine transaminase (ALT) activities (Sun et al., 2016). The serum concentrations of mineral elements (Ca, Cu, P, Zn, Mg, and Fe) were measured using inductively-coupled plasma optical emission spectroscopy (ICP-OES, PQ 9000, Analytik Jena, Jena, Germany), as described in the Chinese National Standards (GB 5009.268, China, 2016) and as reported previously (Wei et al., 2019).

### 2.5. DNA extraction, PCR amplification, 16S rRNA gene sequencing, and bioinformatic analysis

Microbial genomic DNA was extracted from rumen fluid samples using Mag-Bind Soil DNA Kits (Omega Bio-Tek, Norcross, GA, US), according to the manufacturer’s instructions. The quantity and quality of the extracted DNA were measured using Qubit ssDNA assay kits (Life Technologies, Carlsbad, CA, US) and agarose gel electrophoresis, respectively.

The V3 to V4 hypervariable regions of the 16S rRNA genes of the microbes, with lengths of approximately 464 bp, were sequenced. The primer sequences were 341F (5’-CCTACGGGNGGCWGCAG-3’) and 805R (5’-GACTACHVGGGTATCTAATCC-3’). The PCR conditions were as follows: initial denaturation at 98 °C for 2 min, followed by 25 cycles consisting of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and then a final extension of 5 min at 72 °C. The PCR reaction mixture contained 4 μL of 5 × PrimeSTAR buffer, 2 μL of 2.5 mM dNTP, 0.8 μL of each primer (5 μmol/L), 0.4 μL of PrimeSTAR heat stress DNA polymerase (TaKaRa, Dalian, China), and 20 ng of template DNA in a total volume of 20 μL. Two-percent agarose gels were used to separate the amplicons, which were purified using a DNA purification kit (Axygen, Biosciences, Union City, CA, USA). The PCR products from each sample were mixed in equal quantities and used to construct a sequencing library using an Illumina TruSeq DNA Sample Preparation Kit (San Diego, CA, USA). Finally, the V3 to V4 amplicons were sequenced on an Illumina Miseq platform. The sequences were submitted to GenBank, with the accession number SRP335459.

The 16S rRNA gene sequences were analyzed by Quantitative Insights into Microbial Ecology (QIME, v1.9.0) (Caporaso et al., 2010). Valid sequences were identified by exact matches of raw sequencing reads and barcodes were assigned to the respective samples. Low-quality sequences were filtered out if they were <150 bp long, had a quality score <Q20, or if there were >8 bp of ambiguous bases or mononucleotide repeats. Then, FLASH was utilized to merge the paired-end reads (Magoc and Salzberg, 2011). After filtering out chimeras using UCHIME (Haas et al., 2011), the valid and clean sequences were clustered into operational taxonomic units (OTU) at 97% sequence identity using UCLUST (Edgar et al., 2011). Taxonomic classification of the OTU was performed using the Ribosomal Database Project classifier, which used sets of representative sequences to search the Greengenes version 13_8 database (Wang et al., 2007). Community diversity was assessed using alpha diversity indices (Chao1, ACE, Shannon, and Simpson). Beta-diversity was estimated using the Bray–Curtis distance and visualized using principal coordinate analysis (PCoA).

### 2.6. Statistical analysis

The randomness of the initial body weight data was checked using the Durbin–Watson test, which confirmed effective
randomization. The incidence of diarrhea was analyzed using logistic regression in the GENMOD procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC, US). The growth performance data, serum mineral element and biochemical parameters, and rumen fermentation parameters were analyzed using one-way ANOVA. The data are summarized using the least square mean and standard error of the mean. Multiple comparisons were performed using Tukey’s multiple range test. Differences in alpha diversity indices and the relative abundances of ruminal bacterial phyla and genera were assessed using the non-parametric Kruskal–Wallis test. The $P$-values obtained during multiple comparisons within each analysis were adjusted to reflect the false discovery rate using the Benjamini–Hochberg (BH) algorithm. $P < 0.05$ was regarded as representing statistical significance, and $P$-values between 0.05 and 0.10 were considered to represent a statistical trend. The relationships among the various rumen microbial and phenotypic parameters were analyzed using Spearman’s rank correlation coefficients.

3. Results

3.1. Growth performance and the incidence of diarrhea

The calves showed no clinical signs indicative of ill-health during the entire study period. The ADG of the calves in the GOS group was higher than that of the CON group during phase 1 ($P = 0.008$, Table 2), resulting in a higher body weight of the former on d 28 ($P = 0.042$). However, neither the average daily starter intake nor the total feed intake was influenced by GOS supplementation, which resulted in higher feed efficiency in the GOS group than in the CON group during phase 1 ($P = 0.036$). By contrast, during phase 2, no significant differences were observed in the growth performance of the calves. However, notably, both the ADG and final body weight of dairy calves in the GOS group was higher than those of the CON group during the whole study period ($P < 0.05$), although neither the average feed intake nor the feed efficiency was affected by group.

Supplementation with GOS significantly reduced the incidence of diarrhea in the calves during phase 1 ($P = 0.003$), but no significant differences between groups were found during phase 2. The incidences of diarrhea in the GOSC and GOSS groups were lower than in the CON group during the entire study period of 70 d ($P = 0.001$).

3.2. Serum biochemical parameters and mineral elements

In comparison with the CON group, supplementation with GOS increased the serum concentration of HDL ($P = 0.027$; Table 3) and tended to increase that of TC ($P = 0.089$) in the calves during phase 1, and increased the serum TP concentration ($P = 0.044$) during the entire experimental period. However, there were no other significant differences in biochemical parameters among the 3 groups. As shown in Table 4, there were no significant differences in the serum concentrations of mineral elements between the CON and GOS groups, except with respect to Ca during phase 1, which was present at a higher concentration in the GOS group than in the CON group ($P = 0.032$). Similarly, during the entire experimental period, the serum Ca concentration of the calves in the GOSC group was higher than that of the CON group ($P = 0.030$), but there was no significant difference between the CON and GOSS groups. The serum concentrations of the other mineral elements (Cu, P, Zn, Mg, and Fe) were not significantly affected by GOS supplementation.

### Table 2

**Effects of supplementation with galacto-oligosaccharides (GOS) on the growth performance and incidence of diarrhea in Holstein dairy calves.**

| Item | Treatments | SEM | $P$-value |
|------|------------|-----|-----------|
|      | CON | GOS | GOSC | GOSS |
| Phase 1 (d 1 to 28) | | | | |
| Initial body weight (d 1), kg | 37.1 | 38.5 | 1.23 | 0.435 |
| Body weight (d 28), kg | 56.8$^{a}$ | 62.6$^{a}$ | 1.89 | 0.042 |
| Average daily gain, g/d | 704$^{a}$ | 800$^{a}$ | 37.6 | 0.008 |
| Starter intake, g of DM/d | 76.9 | 72.4 | 13.6 | 0.817 |
| Total feed intake, g of DM/d | 1,226 | 1,233 | 24.0 | 0.939 |
| Feed efficiency, g of DM/d of gain | 1.83$^{a}$ | 1.47$^{b}$ | 0.114 | 0.036 |
| Incidence of diarrhea (d 1 to 28), % | 19.6$^{a}$ | 11.3$^{b}$ | – | 0.003 |
| Phase 2 (d 29 to 70) | | | | |
| Average daily gain, g/d | 996 | 974 | 1,099 | 60.1 | 0.394 |
| Starter intake, g of DM/d | 375 | 350 | 379 | 98.7 | 0.979 |
| Total feed intake, g of DM/d | 1,534 | 1,492 | 1,566 | 89.9 | 0.862 |
| Feed efficiency, g of DM/d of gain | 1.56 | 1.57 | 1.45 | 0.093 | 0.666 |
| Incidence of diarrhea (d 29 to 70), % | 8.93 | 7.53 | 9.13 | – | 0.520 |
| Phase 1 and 2 (d 1 to 70) | | | | |
| Final body weight (d 70), kg | 98.7$^{a}$ | 101$^{b}$ | 111$^{a}$ | 3.52 | 0.044 |
| Average daily gain, g/d | 879$^{a}$ | 914$^{a}$ | 1,018 | 40.1 | 0.046 |
| Starter intake, g of DM/d | 255 | 217 | 263 | 64.9 | 0.895 |
| Total feed intake, g of DM/d | 1,431 | 1,425 | 1,464 | 63.9 | 0.924 |
| Feed efficiency, g of DM/d of gain | 1.66 | 1.57 | 1.46 | 0.092 | 0.270 |
| Incidence of diarrhea (d 1 to 70), % | 18.6$^{a}$ | 9.52$^{b}$ | 10.2$^{b}$ | – | 0.001 |

**SEM** = standard error of the mean. $^{ab}$Values with different superscripts within a row indicate a significant difference ($P < 0.05$).

### Table 3

**Effects of supplementation with galacto-oligosaccharides (GOS) on serum biochemical parameters in Holstein dairy calves.**

| Item | Treatments | SEM | $P$-value |
|------|------------|-----|-----------|
|      | CON | GOS | GOSC | GOSS |
| Serum biochemical parameter (d 28) | | | | |
| TP, g/l | 52.2 | 51.8 | 2.69 | 0.901 |
| ALB, g/l | 25.8 | 25.2 | 1.36 | 0.763 |
| TC, mmol/L | 2.09 | 2.07 | 0.091 | 0.859 |
| HDL, mmol/L | 3.04$^{b}$ | 4.36$^{a}$ | 0.371 | 0.027 |
| LDL, mmol/L | 0.65 | 0.65 | 0.063 | 0.989 |
| UREA, mmol/L | 1.23 | 1.77 | 0.213 | 0.102 |
| GLU, mmol/L | 5.48 | 5.39 | 0.276 | 0.821 |
| AST, U/L | 26.7 | 26.0 | 1.60 | 0.781 |
| ALT, U/L | 7.34 | 10.2 | 1.75 | 0.274 |
| Serum biochemical parameter (d 70) | | | | |
| TP, g/l | 59.1$^{a}$ | 62.8$^{a}$ | 59.8$^{b}$ | 0.96 | 0.044 |
| ALB, g/l | 32.0 | 32.0 | 32.9 | 0.62 | 0.142 |
| TC, mmol/L | 11.0 | 0.05 | 0.18 | 0.118 | 0.509 |
| HDL, mmol/L | 4.23 | 3.48 | 4.50 | 1.136 | 0.482 |
| LDL, mmol/L | 5.69 | 4.76 | 6.04 | 1.128 | 0.358 |
| UREA, mmol/L | 0.79 | 0.61 | 0.75 | 0.147 | 0.344 |
| GLU, mmol/L | 2.35 | 1.50 | 1.90 | 0.401 | 0.243 |
| AST, U/L | 39.7 | 40.2 | 43.0 | 6.50 | 0.807 |
| ALT, U/L | 12.7 | 16.5 | 15.2 | 5.11 | 0.627 |

$^{ab}$Values with different superscripts within a row indicate a significant difference ($P < 0.05$).

1. CON, the control group; GOSC, the group fed GOS for 70 d; GOSS, the group fed GOS for the first 28 d of the study.
Effect of supplementation with galacto-oligosaccharides (GOS) on the serum mineral element concentrations of Holstein dairy calves.

**Table 4**

| Item                                   | Treatments | SEM | P-value |
|----------------------------------------|------------|-----|---------|
| Serum mineral element (d 28)           |            |     |         |
| Ca, mg/kg                              | CON 99.0³  | 1.90| 0.032   |
|                                        | GOS 105³   |     |         |
|                                        | GOSC 1.90  |     |         |
|                                        | GOSS 1.90  |     |         |
| Cu, mg/kg                              | CON 0.65   | 0.050| 0.399  |
|                                        | GOS 0.71   |     |         |
| P, mg/kg                               | CON 140    | 6.4 | 0.104   |
|                                        | GOS 156    |     |         |
| Zn, mg/kg                              | CON 0.80   | 0.069| 0.101  |
|                                        | GOS 0.98   |     |         |
| Mg, mg/kg                              | CON 18.9   | 0.62 | 0.762   |
|                                        | GOS 17.6   |     |         |
| Fe, mg/kg                              | CON 1.44   | 0.139| 0.682   |
|                                        | GOS 1.23   |     |         |
| Serum mineral element (d 70)           |            |     |         |
| Ca, mg/kg                              | CON 111⁴   | 2.0 | 0.030   |
|                                        | GOS 118⁴   |     |         |
|                                        | GOSC 2.0   |     |         |
|                                        | GOSS 2.0   |     |         |
| Cu, mg/kg                              | CON 0.73   | 0.048| 0.828   |
|                                        | GOS 0.71   |     |         |
| P, mg/kg                               | CON 1.00   | 0.081| 0.202   |
|                                        | GOS 0.88   |     |         |
| Zn, mg/kg                              | CON 17.2   | 0.944| 0.366   |
|                                        | GOS 16.5   |     |         |
| Mg, mg/kg                              | CON 2.35   | 0.292| 0.174   |
|                                        | GOS 1.76   |     |         |

SEM = standard error of the mean.
³Values with different superscripts within a row indicate a significant difference (P < 0.05).

CON, the control group; GOSC, the group fed GOS for 70 d; GOSS, the group fed GOS for the first 28 d of the study.

### 3.3. Rumen fermentation parameters

The rumen concentrations of acetate, propionate, and total VFA were significantly higher (P < 0.05; Table 5), but the pH and ammonia nitrogen concentration were significantly lower (P < 0.05), in the rumens of calves in the GOSS group than in those in the CON group. Similar trends were identified with respect to the GOSC group, with the rumen acetate and propionate concentrations being much higher in the GOSC than in the CON group (P < 0.05). In addition, the rumen MCP concentrations of the GOSC and GOSS groups were significantly higher than that of the CON group (P = 0.013). However, there were no significant differences in the rumen concentrations of butyrate, iso-butyrate, iso-valerate, or valerate, or in the acetate/propionate ratio, among the 3 groups.

### 3.4. Spatial differences in the diversity and composition of the rumen microbiota

After initial quality control, a total of 742,850 high-quality sequences were obtained from 24 rumen fluid samples, with a mean of 30,952 sequences per sample. On the basis of 97% species similarity, 416, 449, and 510 OTU were obtained from the CON, GOSC, and GOSS groups, respectively (Appendix Table 1). The number of OTU in the GOS group was higher than in the CON group (P < 0.05), and had a tendency to be higher in the GOSC group (P = 0.067; Fig. 2A). The Shannon index for the GOSS group was higher than that for the CON group (P < 0.05; Fig. 2B), whereas the Simpson index was lower (P < 0.05; Fig. 2C). The PCoA plot showed no significant differences among the 3 groups (Fig. 2D), and there were no significant differences in the alpha or beta diversities of the GOSC and GOSS groups.

### 3.5. Correlation analysis between the rumen microbiota and phenotypic indices

Interaction heatmaps were constructed to reflect the relationships among the phyla, genera, rumen fermentation parameters, and serum Ca and TP concentrations of each group on d 70 (Fig. 5). The relative abundance of the phylum Bacteroidetes positively correlated with the serum concentrations of Ca and TP and the rumen concentrations of MCP and acetate (P < 0.05). The relative abundance of the genus *Prevotella* positively correlated with the serum concentrations of Ca and TP and the rumen concentrations of MCP, acetate, propionate, and total VFA (P < 0.05). The relative abundance of the genus *Bacteroides* positively correlated with the rumen propionate concentration (P < 0.05). The relative abundance of *Lactobacillus* positively correlated with the serum Ca concentration and the rumen MCP, propionate, and NH₃-N concentrations (P < 0.05). By contrast, the relative abundances of *Olsenella* and *Escherichia_Shigella* were lower in both the GOSC and GOSS groups than in the CON group (P < 0.05; Fig. 4E and F). In addition, the relative abundance of *Eubacterium* was lower in the GOSS group than in the CON group (P < 0.05), and tended to be lower in the GOSC group (P = 0.083; Fig. 4G).
concentrations of MCP, acetate, propionate, and total VFA ($P < 0.05$), but positively correlated with NH$_3$-N concentration ($P < 0.05$).

4. Discussion

The health status of dairy calves is associated with their future reproductive and productive performance (Heinrichs and Heinrichs, 2011; Soberon et al., 2012; Krbalková et al., 2014). It is well known that diarrhea is one of the most important health problems in young calves, especially during the first month after birth, and it is the most frequent cause of death in calves around the world (Pempek et al., 2019). Indeed, dairy calves may show impairments in subsequent growth and adult productivity, even when they recover from diarrhea during early life (Heinrichs and Heinrichs, 2011). It has been shown that oligosaccharides reduce the incidences of diarrhea and mortality in broiler chickens and piglets (Li et al., 2008; Chang et al., 2018). In addition, Rigo-Adrover et al. (2019) demonstrated that a prebiotic mixture of short-chain GOS and long-chain FOS (scGOS/lcFOS 9:1) reduced the severity and incidence of diarrhea in rotavirus infected rats. Heinrichs et al. (2013) also reported that the addition of MOS to milk replacer improved the fecal scores of calves. Consistent with the results of these studies, in the present study, the incidence of diarrhea in dairy calves was lower in the GOSC group than in the CON group on both d 1 to 28 and d 1 to 70. Notably, even when the GOS supplementation was stopped, there was a persistent effect to reduce the incidence of diarrhea, such that there was a lower incidence of diarrhea in calves in the GOSS group than in those in the CON group, which in turn would have contributed to an improvement in growth performance.

In the present study, the body weight, ADG, and feed efficiency of the dairy calves were higher in the GOS group than in the CON group on d 28. Furthermore, there was a persistent growth-promoting effect in the GOSS group, such that the calves in this...
The group had a higher ADG and final body weight at weaning than those in the CON group. Unexpectedly, supplementation with GOS to 70 d did not further improve the growth performance of the dairy calves; there were no significant differences in the body weight, ADG, feed intake, or feed efficiency between the GOSC and CON groups at the end of the study. These results are consistent with GOS having dual effects to promote growth (Aftabgard et al., 2019; Richards et al., 2020) and prevent becoming overweight (Kavadi et al., 2017; Nie et al., 2020).

It has been well documented that oligosaccharides not only promote animal growth (Abecia et al., 2013; Richards et al., 2020), but regulate body weight by directly or indirectly affecting lipid metabolism (Williams and Jackson, 2002; Overduin et al., 2013). For instance, Overduin et al. (2013) found that GOS had a direct effect on fat metabolism, including lipid oxidation, as illustrated by a significant reduction in epididymal fat-pad mass in rats that consumed GOS. In the present study, the serum HDL concentration of the GOS-fed dairy calves was higher during the first phase of the study. HDL is a vascular scavenger that limits cholesterol accumulation in arterial walls and transports cholesterol from the peripheral tissues to the liver for utilization and excretion in the bile (Kudinov et al., 2020), which helps prevent obesity. The increased

Fig. 4. Rumen microbial profiles of the groups at the genus level, according to taxon-based analysis. (A) Bacterial composition of the groups at the genus level. The abundances of specific genera were compared among the groups: (B) Prevotella, (C) Bacteroides, (D) Lactobacillus, (E) Olsenella, (F) Escherichia_Shigella, and (G) Eubacterium. CON, the control group; GOSC, the group fed GOS for 70 d; GOSS, the group fed GOS for the first 28 d of the study. Asterisk (*) and solid line, \( P < 0.05 \); double asterisks (**) and solid line, \( P < 0.01 \); numbers and dotted lines, \( 0.05 < P < 0.1 \).
concentration of serum HDL promoted the metabolism of TC (tended to be higher in the serum of GOS-fed calves), which helped promote the growth of the calves. Similar results were also observed by Bandyopadhyay et al. (2021), who demonstrated that supplementation with FOS for 30 d increases serum HDL concentration in Swiss albino mice. However, the underlying mechanism of GOS on serum HDL still needs further study.

Recent studies have shown the effects of prebiotics on mineral metabolism and skeletal health, especially on Ca metabolism, which manifests as high bone mineral density (Rastall, 2010; Whisner and Castillo, 2017), Santos et al. (2011) showed that GOS metabolism and skeletal health, especially on Ca metabolism, of GOS on serum HDL still needs further study.

Interaction heatmaps reveal associations of the relative abundances of rumen bacterial phyla and genera with fermentation parameters and the serum Ca and TP concentrations on 70 d. Spearman’s correlation analysis was used. * P > 0.20, P < 0.05. TP – total protein; MCP – microbial protein; VFA – volatile fatty acid; NH₃–N – ammoniacal nitrogen.

Fig. 5. Interaction heatmaps reveal associations of the relative abundances of rumen bacterial phyla and genera with fermentation parameters and the serum Ca and TP concentrations on 70 d. Spearman’s correlation analysis was used. * P > 0.20, P < 0.05. TP – total protein; MCP – microbial protein; VFA – volatile fatty acid; NH₃–N – ammoniacal nitrogen.

Weaver et al. (2011) also found that GOS improved mineral absorption and bone properties in growing rats. Although the mechanism for the effect of prebiotics on Ca absorption is not clear, it has been hypothesized that the fermentation of prebiotics might increase the absorption of essential minerals (Rastall, 2010; Weaver et al., 2011). Consistent with the results of these previous studies, in the present study, the consumption of GOS resulted in a significant increase in serum Ca concentration in dairy calves on 28 and 70 d, which suggests that supplementation with GOS promotes Ca absorption in dairy calves. Different from monogastric animals, the rumens of the young calves develop progressively, and GOS may either undergo rumen fermentation or be absorbed in the intestine, which is a complicated process. Further studies are required to determine the mechanism whereby GOS promotes Ca absorption in ruminants.

Early rumen development is critical for efficient fermentation, and supplementation with GOS promoted rumen fermentation in the dairy calves in the present study, even when GOS supplementation was stopped at a relatively young age. The rumen acetate and propionate concentrations were higher in calves in the GOSC and GOSS groups than in the CON group. The Shannon index is increased and the Simpson index is decreased by GOS (Wang et al., 2019; Tran et al., 2020). In the rumen, the MCP concentration may have been lower in the GOSS group than in the GOSC group because the rumen NH₃ concentration was lower in the former group.

The manipulation of the rumen microbiota is another effective approach to the stimulation of rumen fermentation (Eisler et al., 2014). Yáñez-Ruiz et al. (2008) demonstrated that the rumen microbial population can be modified by nutritional interventions during early life. Another previous study showed that supplementation with GOS can increase the alpha diversity of the colonic microbiota of suckling piglets, indicated by a high Chao1 index (Wang et al., 2019), which is consistent with the present finding of higher Chao1 index in the GOSC and GOSS groups than in the CON group. The Shannon index is increased and the Simpson index is decreased by GOS (Wang et al., 2019; Tran et al., 2020). In the present study, GOS supplementation had persistent effects that increased the Shannon index and reduced the Simpson index, which implies that feeding GOS until weaning increases the number of rumen bacterial species, and that temporary supplementation with GOS has a persistent effect to increase the diversity of the rumen microbiota.
The strictly anaerobic Bacteroidetes is one of the predominant bacterial phyla in the gastrointestinal tract of dairy calves (Kim et al., 2021). They metabolize a variety of complex carbohydrates through glycolysis, including cellulose, hemicelluloses, and starch, producing acetate and propionate (Martens et al., 2009; Wu et al., 2021), and GOS have been reported to facilitate the growth of Bacteroidetes in the gastrointestinal tract (Tran et al., 2020). In the present study, the phylum Bacteroidetes was enriched in the GOS group. The genus Prevotella belongs to the Bacteroidetes and was also enriched in the GOSS and GOSC groups. Prevotella is a key rumen microbial genus and its relative abundance can be 48.9% at the age of 2 months (Jami et al., 2013). This genus ferments carbohydrates to produce succinate and acetate (Ramakrishna, 2013; Nográsek et al., 2015). Consistent with the present findings, Zhai et al. (2019) reported that GOS consumption increases the relative abundance of Prevotella in the fecal microbiota of mice. These findings imply that supplementation with GOS for 28 d has a persistent effect to promote rumen fermentation by increasing the relative abundances of Bacteroidetes and Prevotella, which positively correlated with serum Ca concentration in the present study. The reason that GOS increased Ca absorption might be that there was greater fermentation of the prebiotics by saccharolytic microbes (Whisner and Castillo, 2017).

Bacteroides is another genus of the phylum Bacteroidetes that also makes a substantial contribution to the production of acetate, succinate, and propionate (Krieg et al., 2010). Recent scientific literature show that Bacteroides metabolizes oligosaccharides and polysaccharides to provide nutrients, including vitamins, to the host and other intestinal microbial residents. A deficiency in Bacteroides spp. is often associated with diseases such as inflammatory bowel disease and tumors (Zafar and Saier, 2021). In the present study, the relative abundance of Bacteroides was increased in the GOSC group and positively correlated with the rumen propionate concentration. In addition, it is known that GOS can be selectively utilized by probiotics, such as Lactobacillus, to benefit the host (Marquez, 2014; Bouwhuis et al., 2017; Gibson et al., 2017; Zhai et al., 2019). In the present study, Lactobacillus showed high relative abundance in the GOSC group. Lactobacillus produce essential SCFA, including lactate and acetate (Hati et al., 2019), which facilitate MCP production in the rumen. An in vitro study reported that Lactobacillus strains improve Ca transport and uptake in intestinal cells (Raveschot et al., 2020). In the present study, the relative abundance of Lactobacillus positively correlated with the serum concentration of Ca and rumen MCP concentration.

Prebiotic oligosaccharides, especially GOS, have some structural similarities to cell surface glycoproteins and are postulated to inhibit the adhesion of pathogens to cells (Monteagudo-Mera et al., 2019). Previous studies have shown that GOS reduces the adherence of enteropathogenic E. coli microcolonies and has superior effects to other oligosaccharides, such as FOS, inulin, lactulose, and raffinose (Tzortzis et al., 2005; Shoaf et al., 2006). Consistent with previous studies, in the present study, the relative abundance of Escherichia_Shigella was decreased in the GOSC and GOSS groups. Similar differences in the relative abundances of the phylum Actinobacteria and of the genus Olsenella have also been shown previously (Long et al., 2019). Olsenella is one of the most abundant genera in the phylum Actinobacteria and is closely associated with rumen acidosis (Petri et al., 2013), which suggests that the consumption of GOS might prevent rumen acidosis by reducing the relative abundances of the Actinobacteria and Olsenella. In addition, in the present study, the relative abundance of Eubacterium in the GOS groups was low and tended to be lower in the GOSC group than in the CON group. A recent study showed that Eubacterium rectale contributes to the etiology of colorectal cancer by promoting colitis (Wang et al., 2021), and a positive correlation of the abundance of Eubacterium spp. has been reported with obesity and being overweight (Jones et al., 2019). These results suggest that supplementation with GOS inhibits the colonization of deleterious bacteria in the rumen, and that this effect persisted after the end of GOS supplementation.

5. Conclusion

In summary, the present study showed that GOS supplementation had persistent effects that increased ADG and body weight at
weaning, reduced the incidence of diarrhea, and improved the rumen microbial structure and promoted rumen fermentation by increasing the relative abundances of Bacteroidetes and Prevotella and reducing the abundance of deleterious bacteria. An overview of the means whereby GOS supplementation facilitated optimal rumen microbial composition and fermentation is shown in Fig. 6. The present findings suggest that early dietary interventions by GOS help the establishment of a healthy microbial ecosystem in the rumen, which potentially benefits the efficacy of rumen fermentation and promotes the growth performance of preweaning dairy calves. Further study is needed to investigate the exact mechanisms by which GOS can increase the relative abundance of Prevotella and promote rumen development in preweaning dairy calves.

**Author contributions**

**Meinan Chang:** investigation, visualization, writing – original draft, conceptualization, methodology. **Peng Sun:** supervision, writing – review & editing, funding acquisition, project administration, conceptualization. **Feifei Wang, Fengtao Ma and Yuhang Jin:** investigation, methodology.

**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.

**Availability of data and materials**

The 16S rRNA sequencing data for all samples have been deposited into the NCBI Sequence Read Archive database (project number, PRJNA760240 and accession number, SRP335459).

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**Appendix. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.04.009.

**References**

Abeica L, Martin-Garcia A L, Martinez-Fernandez G, Newbold C J, Yanez-Ruiz D R. Nutritional intervention in early life to manipulate rumen microbial colonisa- tion and methane output by kid goats postweaning. J Anim Sci 2011:91: 4832–40.

Aftabguard M, Salarzadeh A, Mohseni M. The effects of a symbiotic mixture of galacto-oligosaccharides and bacillus strains in caspian salmon, salmon trutta caspius fingerlings. Probiotics Antimicrob 2019:11:1300–8.

AOAC International. Official Methods of Analysis. 18th ed. Washington, DC: AOAC International, 2005.

AOAC International. Official Methods of Analysis. 17th ed. Gaithersburg, MD: AOAC International, 2003.

AOAC International. Official Methods of Analysis. 16th ed. Arlington, VA: AOAC International, 2000.

Bandopadhyay B, Mandal V, Mandal NC. Partial characterization of novel inulin-like prebiotic fructooligosaccharides of Sechium edule (Jacq.) Sw. (Cucurbita-ceae) tuberous roots. J Food Biochem 2021:45:e13764.

Broderick GA, Kang JH. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J Dairy Sci 1980:63:64–75.

Botvynko A, Bednarová A, Henke S, Shahknh N, Curda L. Production of galactooligosaccharides using various combinations of the commercial β-galacto-sidase from Aspergillus niger. Biochem Biophys Commun 2019:519:167–72.

Bouwhuys MA, Middel Ch, Sweeten T, Mukhopadhyaya A, O’Shea CJ, O’Doherty JV. Seaweed extracts and galacto-oligosaccharides improve intestinal health in pigs following Salmonella Typhimurium challenge. Animal 2017:11:1488–96.

Bryk G, Neeckel MZ, Lugo M, C. Mamede R. A mixture of galacto-oligosaccharides/fructo-oligosaccharides (GOS/FOS) in calcium absorption and retention during recovery from protein malnutrition: experimental model in growing rats. Eur J Nutr 2016:55:2445–58.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010:7:135–6.

Cangiano LR, Vohe TT, Steele MA, Renaud DL. Invited review: use of microbial-based probiotics and prebiotics in dairy calf rearing. Anim Appl Sci 2020:36:1–21.

Chang MN, Wei YJ, Hao LY, Ma FT, Li HY, Zhao SC, et al. Effects of different types of zinc supplement on the growth, incidence of diarrhea, immune function, and rectal microflora of newborn dairy calves. J Dairy Sci 2020:103:6100–13.

Chang MN, Zhao Y, Qin GX, Zhang XD. Fructo-oligosaccharide alleviates soybean-induced anaphylaxis in piglets by modulating gut microbes. Front Microbiol 2018:9:2769.

Costa NA, Pansani AP, de Castro CH, Colugnati BD, Xaxier CH, Guimaraes KC, et al. Milk restriction or oligosaccharide supplementation in calves improves compensatory gain and digestive tract development without changing hor- mone levels. PLoS One 2019:14:e0214626.

CotTA MA. Utilization of xylooligosaccharides by selected ruminal bacteria. Appl Environ Microbiol 1993:59:3557–63.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 2011:27:2194–200.

Eisler MC, Lee MR, Tarlton JF, Martin GB, Beddington J, Dungait JA, et al. Agriculture: steps to sustainable livestock. Nature 2014:507:32–4.

Ghosh S, Mehla RK. In: Gibson G, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Sanders SJ. Expert consensus document: the international scienti- fic standards for probiotics. Nat Rev Gastroenterol Hepatol 2017:14:491–507.

Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011:89: 41–6.

Ghosh S, Melha RK. Influence of dietary supplementation of prebiotics (man- nanooligosaccharide) on the performance of crossbred calves. Jop Anim Health Prod 2012:44:017–22.

Gibson G, Rutkis R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ. Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol 2017:14:491–507.

Goutrey P, Denery S, Bodinier M. Probiotics, prebiotics, and symbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011:89: 41–6.

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, et al. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 2011:21:494–504.

Hati S, Patel M, Mishra BK, Das S. Short-chain fatty acid and vitamin production potentials of Lactobacillus isolated from fermented foods of Khalsa Tribes, Meghalaya, India. Ann Microbiol 2019:69:1191–9.

Heinrichs AJ, Heinrichs BS. A prospective study of calf factors affecting first- lactation and lifetime milk production and age of cows when removed from the herd. J Dairy Sci 2011:94:336–41.

Heinrichs AJ, Heinrichs BS, Jones CM. Fecal and saliva IgA secretion when feeding a diet containing 5% fructose and 5% inulin: effects on growth, performance, and speed of chimera detection. Bioinformatics 2011:27:2194–200.

Heinrichs BS. A prospective study of calf factors affecting first-lactation and lifetime milk production and age of cows when removed from the herd. J Dairy Sci 2011:94:336–41.

Jones RB, Alderete TL, Kim JS, Millstein J, Gilliland FD, Goran MI. High intake of dietary fructose in overweight/obese teenagers associated with depletion of Eubacterium and Streptococcus in gut microbiome. Gut Microbes 2019:10: 1–8.

Kavadi PK, Pothuraju R, Chagalamari J, Bhakri G, Mallepogu A, Sharma RK. Dietary incorporation of whey protein isolate and galactooligosaccharides exhibits improvement in glucose homeostasis and insulin resistance in high fat diet fed mice. J Intercol Ethnopharmacol 2017:6:326–32.

Kim HS, Whon TW, Sung J, Jeong YS, Jung ES, Shin NR, et al. Longitudinal evalu- ation of fecal microbial transplantation for ameliorating calf diarrhea and improving growth performance. Nat Commun 2021:12:161.

Krieg NR, Ludwig W, Euzéby J, Whitman BW. Phylum XIV. Bacteroidetes phyl. nov. In: Bergey’s manual of systematic bacteriology. New York: Springer; 2000.

Krpálekova L, Cabrera VE, Kwapil J, Burdych J, Crump P. Associations between age at first calving, rearing average daily weight gain, herd milk yield and dairy herd performance, reproduction, and profitability. J Dairy Sci 2014:97:6573–82.

Kudrović IJ, Aleksicev OV, Tadić-Radončević TL, Bostenaric R, Radojevic RS, et al. High-density lipoproteins as homeostatic nanoparticles of blood plasma. Int J Mol Sci 2020:21:8737.

Li GH, Ling BM, Qu MR, You JM, Song XZ. Effects of several oligosaccharides on ruminal fermentation in sheep: an in vitro experiment. Rev Med Vet 2011:162: 192–7.

Li X, Liu LQ, Xu CL. Effects of supplementation of fructooligosaccharide and/or Ba- culius subtilis to diets on performance and intestinal microflora in broilers. Arch Tierz 2008:51:64–70.
