Animal Nutrition 10 (2022) 223–233

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

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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ARTICLE INFO

Article history:
Received 6 October 2021
Received in revised form
18 March 2022
Accepted 10 April 2022
Available online 11 May 2022

Keywords:
Galacto-oligosaccharide
Dairy calf
Growth performance
Rumen microbiota

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 promote 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

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2022.04.009

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 (NH_3—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; Monteagudo-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 Jiuzhou 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:

$$\text{Diarrhea incidence} = \text{number of calves with diarrhea} \times \text{number of days with diarrhea}/(\text{number of calves in each group} \times \text{number of experimental days}) \times 100\%.$$

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

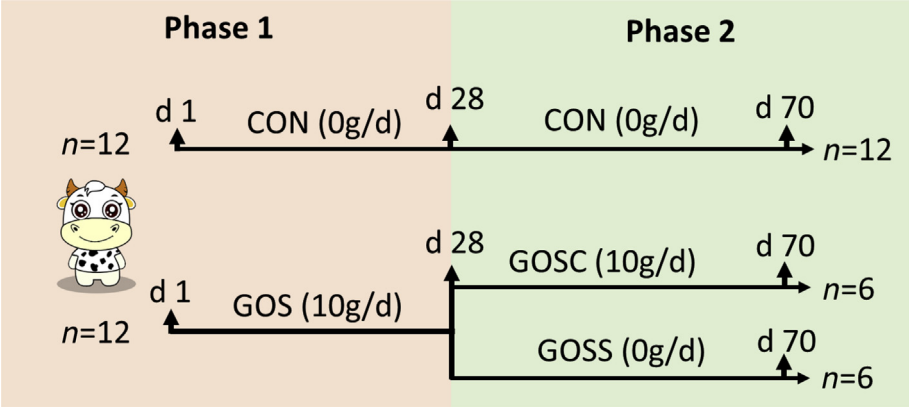

Fig. 1. Schematic diagram of experimental design. CON, the control group; GOSC, the group fed GOS for 70 d; GOSS, the group fed GOS for the first 28 d of the study.

Table 1
The nutrient compositions of the starter and milk (%).

Item	Milk, as-is basis	Item	Starter, dry matter basis
Density, kg/L	1.03	DM, %	90.1
Milk protein	3.13	CP, %DM	18.8
Milk fat	3.87	EE, %DM	5.55
Lactose	5.09	NDF, %DM	7.95
TS	12.2	ADF, %DM	3.09
SNF	9.54	Ash, %DM	8.60

TS = total solids; SNF = solids of non-fat; DM = dry matter; CP = crude protein; EE = ether extract; NDF = neutral detergent fiber; ADF = acid detergent fiber.

analysis of microbial protein (MCP), NH_3–N, and VFA content. For the NH_3–N and VFA analyses, 25% (wt/vol) metaphosphoric acid was added to the rumen filtrate, as described previously (Sun et al., 2016). The NH_3–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 derivative (PD) 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 aminotransferase (AST), and alanine transaminase (ALT) activities (Sun et al., 2017; Ma et al., 2020). 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 (QIIME, 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 (Magoč 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 Data 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 GOSS 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[1]			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^{b}	62.6^{a}		1.89	0.042
Average daily gain, g/d	704^{b}	860^{a}		37.6	0.008
Starter intake, g of DM/d	76.9	72.4		13.61	0.817
Total feed intake, g of DM/d	1,226	1,223		24.0	0.939
Feed efficiency, g of DMI/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 DMI/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^{b}	101^{ab}	111^{a}	3.52	0.044
Average daily gain, g/d	879^{b}	914^{ab}	1,018^{a}	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 DMI/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.
^{a,b}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.

Table 3
Effects of supplementation with galacto-oligosaccharides (GOS) on serum biochemical parameters in Holstein dairy calves.

Item	Treatments[1]			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
TG, mmol/L	0.29	0.27		0.091	0.859
TC, mmol/L	2.31	3.13		0.313	0.089
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^{b}	62.8^{a}	59.8^{ab}	0.96	0.044
ALB, g/L	32.0	32.0	32.9	0.62	0.142
TG, mmol/L	0.11	0.05	0.18	0.118	0.509
TC, mmol/L	4.23	3.48	4.50	1.136	0.482
HDL, mmol/L	5.69	4.76	6.04	1.128	0.358
LDL, mmol/L	0.79	0.61	0.75	0.147	0.344
UREA, mmol/L	2.35	1.90	1.90	0.401	0.243
GLU, mmol/L	5.89	6.24	6.10	0.332	0.438
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

SEM = standard error of the mean; TP = total protein; ALB = albumin; TG = triglyceride; TC = total cholesterol; HDL = high-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol; UREA = urea nitrogen; GLU = glucose; AST = aspartate aminotransferase; ALT = alanine transaminase.
^{a,b}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.

Table 4
Effects of supplementation with galacto-oligosaccharides (GOS) on the serum mineral element concentrations of Holstein dairy calves.

Item	Treatments[1]			SEM	*P*-value
	CON	GOS			
		GOSC	GOSS		
Serum mineral element (d 28)					
Ca, mg/kg	99.0[b]	105[a]		1.90	0.032
Cu, mg/kg	0.65	0.71		0.050	0.399
P, mg/kg	140	156		6.4	0.104
Zn, mg/kg	0.80	0.98		0.069	0.101
Mg, mg/kg	18.9	17.6		0.62	0.762
Fe, mg/kg	1.44	1.23		0.359	0.682
Serum mineral element (d 70)					
Ca, mg/kg	111[b]	118[a]	112[ab]	2.0	0.030
Cu, mg/kg	0.73	0.71	0.69	0.048	0.828
P, mg/kg	162	154	166	6.0	0.402
Zn, mg/kg	1.00	0.88	0.80	0.081	0.202
Mg, mg/kg	17.2	16.5	18.6	0.944	0.366
Fe, mg/kg	2.35	1.76	2.61	0.292	0.174

SEM = standard error of the mean.
[a,b]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.

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 group 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 GOSS 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 Chao1 indices for the GOSC and GOSS groups were significantly higher than that for the CON group ($P < 0.05$). The ACE index tended to be higher for both the GOSC and GOSS groups than for the CON group ($P = 0.089$; 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.

The top ten most abundant phyla and top thirty most abundant genera in the 3 groups were compared using a taxon-dependent analysis. At the phylum level, Firmicutes was the dominant phylum, followed by Bacteroidetes, Actinobacteria, and Proteobacteria (Fig. 3A). The relative abundance of Bacteroidetes in the GOSS group was higher than in the CON group ($P < 0.05$; Fig. 3B). The relative abundance of Actinobacteria 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. 3C).

At the genus level, *Olsenella* was the dominant genus in the CON group, whereas *Prevotella* was the dominant genus in the GOSS group (Fig. 4A). The relative abundances of *Prevotella* and *Bacteroides* were higher in the GOSS group than in the CON group ($P < 0.05$; Fig. 4B and C). Longer term consumption of GOS increased the relative abundances of *Prevotella* and *Lactobacillus versus* the CON group ($P < 0.05$; Fig. 4B and D). 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).

Table 5
Effect of supplementation with galacto-oligosaccharides (GOS) on the rumen fermentation parameters of Holstein dairy calves.

Item	Treatments[1]			SEM	*P*-value
	CON	GOSC	GOSS		
pH	6.93[a]	6.78[ab]	6.42[b]	0.117	0.011
NH_3–N, mg/dL	10.4[a]	11.8[a]	6.33[b]	1.193	0.019
MCP, mg/mL	92.5[b]	171[a]	142[a]	18.33	0.013
Acetate, mmol/L	27.3[b]	36.7[a]	37.3[a]	2.70	0.031
Propionate, mmol/L	16.0[b]	21.2[a]	20.9[a]	1.41	0.033
Butyrate, mmol/L	10.4	11.3	13.4	1.36	0.353
Iso-butyrate, mmol/L	1.04	0.91	0.97	0.159	0.820
Valerate, mmol/L	2.68	2.45	3.45	0.757	0.681
Iso-valerate, mmol/L	1.63	1.37	1.62	0.313	0.810
Total VFA, mmol/L	59.9[b]	72.9[ab]	77.6[a]	5.04	0.043
Acetate/propionate ratio	1.74	1.77	1.78	0.133	0.963

SEM = standard error of the mean; NH_3–N = ammoniacal nitrogen; MCP = microbial protein; VFA = volatile fatty acid.
[a,b]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.

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 NH_3–N concentrations ($P < 0.05$). By contrast, the relative abundances of *Prevotella* and *Bacteroides* negatively correlated with NH_3–N concentration and rumen pH, respectively ($P < 0.05$). The relative abundance of the phylum Actinobacteria negatively correlated with rumen MCP ($P < 0.05$), but positively correlated with rumen NH_3–N concentration and pH ($P < 0.05$). The relative abundance of *Olsenella* negatively correlated with serum TP concentration and the rumen concentrations of acetate and propionate ($P < 0.05$), but positively correlated with rumen NH_3–N concentration ($P < 0.05$). Finally, the relative abundance of the genus *Eubacterium* negatively correlated with the serum concentrations of Ca and TP and the rumen

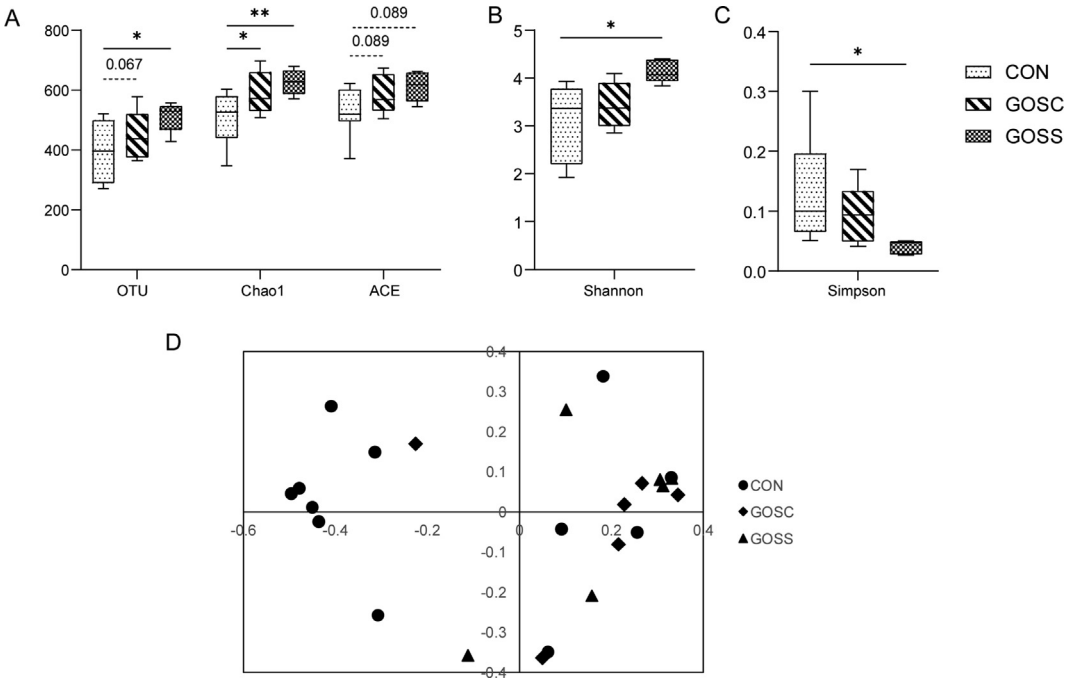

Fig. 2. Rumen microbial diversity of calves after short or long-term feeding of galacto-oligosaccharides (GOS). (A) OTU numbers and rumen bacterial richness (Chao1 and ACE indices). Rumen bacterial evenness: (B) Shannon and (C) Simpson indices. (D) Principal coordinate analysis (PCoA) profile of rumen bacterial diversity using a Bray–Curtis metric. 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, $0.05 < P < 0.1$.

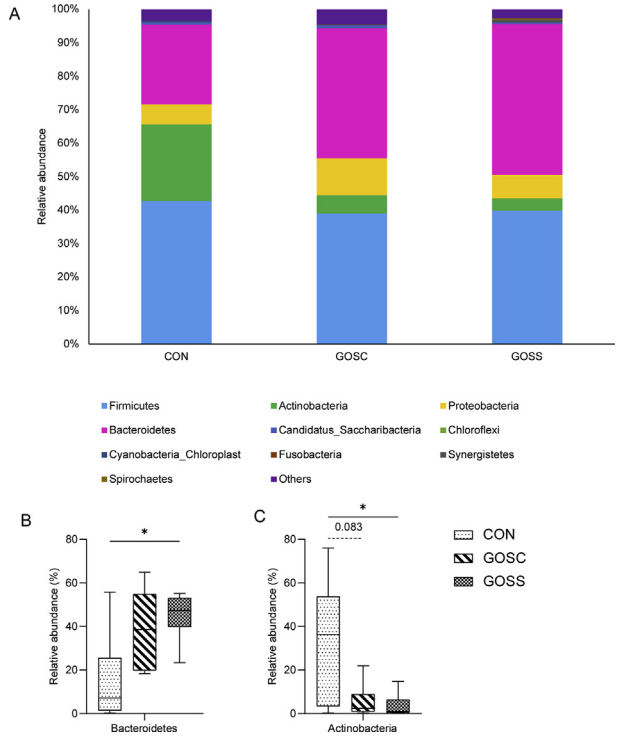

Fig. 3. Rumen microbial profiles of the groups at the phylum level, according to taxon-based analysis. (A) Bacterial composition of the groups at the phylum level. The abundances of specific phyla were compared among the 3 groups: (B) Bacteroidetes and (C) Actinobacteria. CON, the control group; GOSC, 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, $0.05 < P < 0.1$.

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; Krpálková 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 over 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

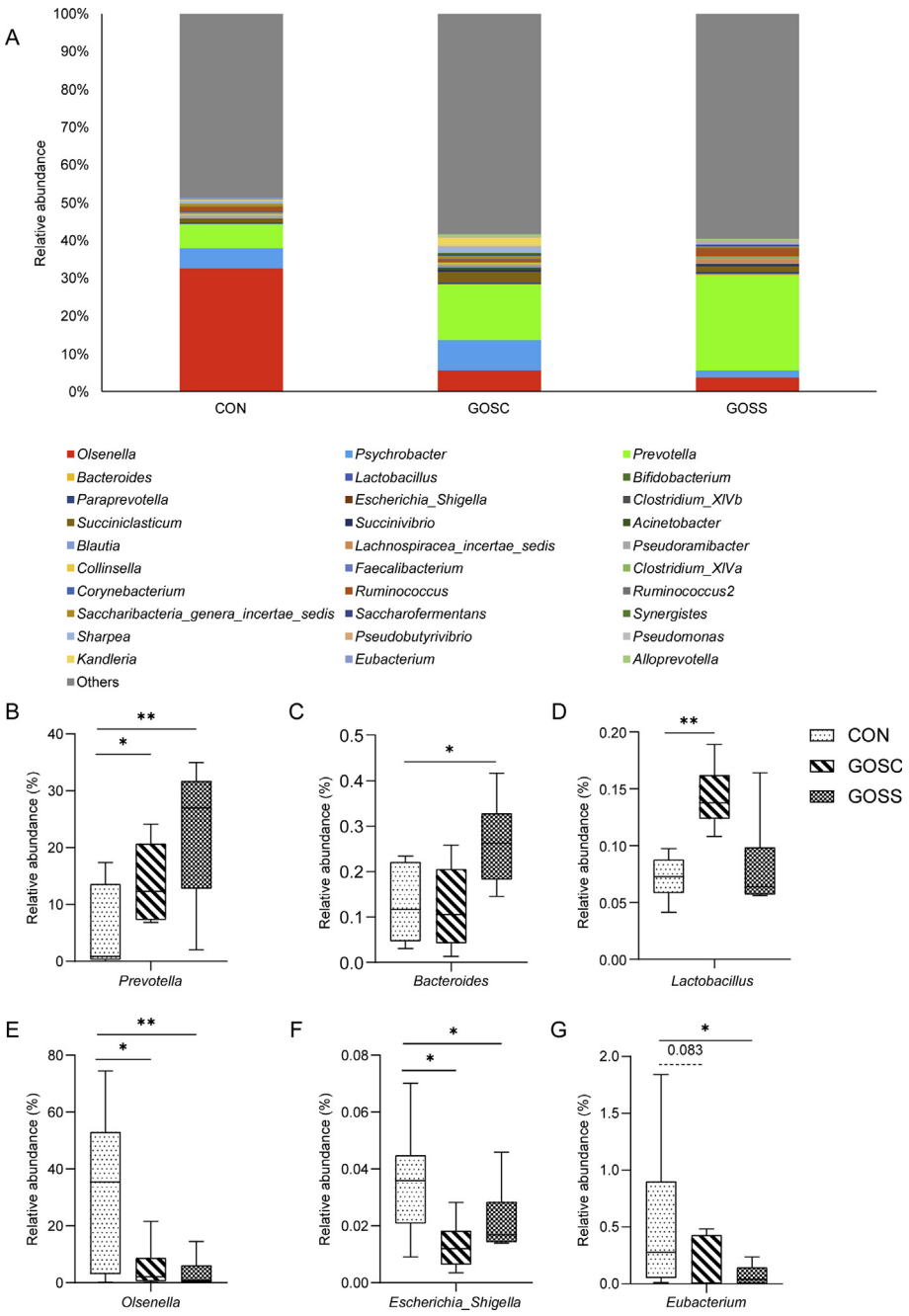

Fig. 4. Rumen microbial profiles of the groups at the genus level, according to taxon-based analysis. (A) Bacterial composition of the groups at the genus level. The abundances of specific genera were compared among the groups: (B) *Prevotella*, (C) *Bacteroides*, (D) *Lactobacillus*, (E) *Olsenella*, (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, $0.05 < P < 0.1$.

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

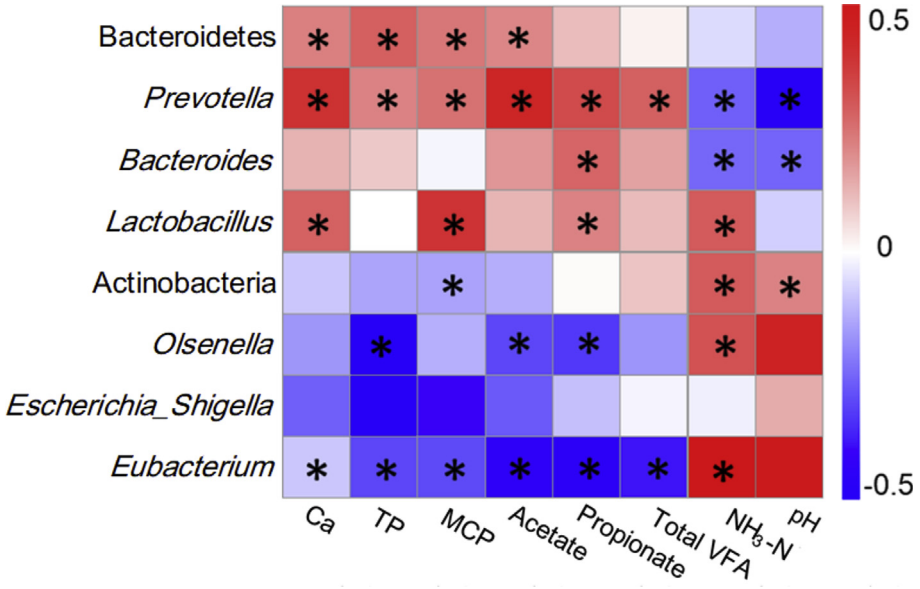

Fig. 5. Interaction heatmaps reveal associations of the relative abundances of rumen bacterial phyla and genera with fermentation parameters and the serum Ca and TP concentrations on 70 d. Spearman's correlation analysis was used. *, $R > 0.20$, $P < 0.05$. TP = total protein; MCP = microbial protein; VFA = volatile fatty acid; NH_3–N = ammoniacal nitrogen.

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 consumption increases Ca absorption in both normal and gastrectomized rats. 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. Previous studies have also shown that prebiotics, such as oligosaccharides, are capable of increasing rumen short chain fatty acid (SCFA) concentrations by promoting microbial fermentation in the gastrointestinal tract (Yu et al., 2019; Tran et al., 2020). Specifically, oligosaccharides may be utilized by rumen bacteria to produce SCFA (Cotta, 1993). Consistent with the present findings, in vitro fermentation studies have shown that GOS stimulate SCFA production, including that of acetic acid and propionic acid (Tran et al., 2020).

Furthermore, we have shown a persistent effect of GOS supplementation after it was stopped because the total rumen concentration of VFA was higher in the GOSS group than in the CON group, which resulted in a lower pH in the rumen. The increase in VFA concentration would have contributed to the synthesis of MCP, which was also greater in the GOSC and GOSS groups than in the CON group. It is known that the VFA produced by rumen fermentation can be used as carbon skeletons, along with ammonia nitrogen, to synthesize MCP (Welch and Hooper 1993), which provides 50-90% of the rumen bypass protein and optimizes protein availability in dairy cows (Xie et al., 2019). Rumen MCP is digested in the more distal parts of the gastrointestinal tract and the products are transported in the circulation to tissues and organs to provide quality protein, which might explain the higher serum TP concentration in the GOSC group. In the rumen, the MCP concentration is in equilibrium with the NH_3–N concentration, meaning that a lower NH_3–N concentration affects the production of MCP. Thus, the MCP concentration may have been lower in the GOSS group than in the GOSC group because the rumen NH_3–N 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.

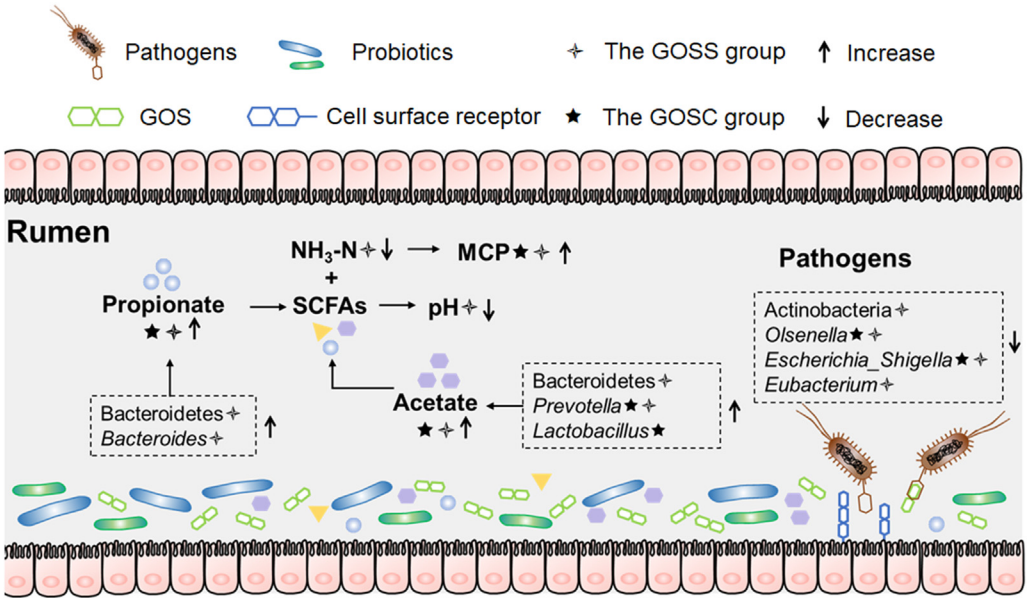

Fig. 6. An overview of the means whereby galacto-oligosaccharides (GOS) supplementation might facilitate optimal rumen microbial composition and fermentation. Probiotic bacteria ferment GOS to produce SCFA, which reduces the pH of the rumen. SCFA and NH_3–N are utilized to synthesize MCP. In addition, GOS have some structural similarity to cell-surface receptors and inhibit the adhesion of pathogens to cells. Simultaneously, the adhesion of probiotic bacteria to cell-surface receptors inhibits colonization by pathogens. GOSC, the group fed GOS for 70 d; GOSS, the group fed GOS for the first 28 d of the study. SCFA = short chain fatty acids; NH_3–N = ammoniacal nitrogen; MCP = microbial protein.

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 GOSS 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; Nograšek 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 GOSS 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 efficiency 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

The16S rRNA sequencing data for all samples have been deposited into the NCBI Sequence Read Archive database (project number, PRJNA760240 and accession number, SRP335459).

Acknowledgments

This study was supported by the Agricultural Science and Technology Innovation Program, Beijing, China (cxgc-ias-07), and the National Program for Support of Top-notch Young Professionals, Beijing, China.

Appendix. Supplementary data

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

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