Growth, health, rumen fermentation, and bacterial community of Holstein calves fed *Lactobacillus rhamnosus* GG during the preweaning stage[1]

Liyang Zhang,[©] Xin Jiang, Xin Liu, Xuejiao Zhao, Shuai Liu, Yang Li, and Yonggen Zhang[2]

College of Animal Science and Technology, Northeast Agricultural University, Harbin, 150030, China

ABSTRACT: The aim of this study was to determine if feeding *Lactobacillus rhamnosus* GG (GG, ATCC 53013) to neonatal calves would alter their growth, health, rumen fermentation, and bacterial community composition during the preweaning stage; we hypothesized that it would. Twenty-four male Holstein calves were blocked and randomly assigned to 1 of 2 treatment groups: 1) untreated control (CON), or 2) treated with 1×10^{10} cfu/d of a GG suspension (GG). Calves received GG daily, mixed with the milk replacer in the morning feed for 6 wk. Starter and alfalfa hay intakes, as well as feces and respiratory scores, were recorded daily, while body weight and structures were measured weekly. Blood, rumen fluid, and feces samples were collected, from which relevant indicators were detected. The results showed that the administration of GG significantly increased voluntary starter intake ($P = 0.023$) and ADG ($P = 0.035$) of the calves. The fecal score ($P = 0.018$) was lower and the β-hydroxybutyric concentration in the plasma tended to increase ($P = 0.092$) in calves treated with GG. The pH of the rumen fluid in calves fed GG was lower ($P = 0.007$), which might be attributable to the tendency ($P = 0.083$) for total volatile fatty acids concentration to increase. Administration of GG significantly increased the amylase, protease activity, and the microbial protein concentrations ($P = 0.043$, $P = 0.036$, and $P = 0.044$, respectively) in the rumen fluids. Furthermore, GG treatment altered the dominant bacteria order and relative abundance of the bacteria families in the rumen fluids. The microbial diversity indices were significantly affected by GG administration. In conclusion, the neonatal calves fed GG before weaning increased their voluntary starter intake and growth performance, improved the rumen fermentation, and regulated the pattern to normally increase the propionate and butyrate concentrations. Administration of GG also diversified the bacterial community composition in the rumen, and regulated the balance of rumen and intestinal microorganisms. These results indicated that feeding calves GG were beneficial to the rumen development and early weaning.

Key words: calves, growth, *Lactobacillus rhamnosus* GG, rumen bacterial community

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INTRODUCTION

The balance of intestinal microorganisms in young animals is an important factor affecting health, especially for calves, which are prone to diarrhea and respiratory diseases that could be caused by diet changes, pathogens, viruses, or other factors before the immune system is mature (Tsuruta et al., 2010). In particular, the diarrhea,

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[2]Corresponding author: zhangyonggen@sina.com

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reduces digestibility and nutrient absorption (Lucas et al., 2007; Signorini et al., 2012), more seriously, leads to gastroenteritis or other clinical symptoms, or even death. Antibiotics are a common treatment for diarrhea, of which the commonly used include gentamicin, tetracycline, ceftiofur and amoxicillin (McConnell et al., 2008; Reiff and Kelly, 2010). Mortality rates have decreased over the past several decades, however, there is a growing concern due to residues in animal origin food products and the emergence of antibiotic-resistant pathogens, for which clinicians require alternative therapies (Ewaschuk et al., 2004).

The application of probiotics in ruminant production has been frequently reported. Despite the rumen function being powerful in ruminants, by supplementing some probiotics and optimizing the activity of dominant bacteria in the rumen, which can play a positive role in the rumen environment. Over the years, probiotics have been fed to animals as an effective antibiotic alternative, with beneficial effects for the host animals by influencing the intestinal flora (Gaggìa et al., 2010). Lactic acid bacteria, in particular, are beneficial in the improvement of intestinal health, digestive efficiency, and growth performance in calves (Frizzo et al., 2008; Frizzo et al., 2010; Morrison et al., 2010). The mechanism for lactic acid bacteria is to compete against adherent pathogenic microorganisms in the intestine by decreasing the pH, producing bacteriocins, stimulating the immune system, and improving nutrient utilization (Riddell et al., 2008; Morrison et al., 2010; Salim et al., 2013).

Lactobacillus rhamnosus GG (**GG**, ATCC 53103) is a Gram-positive bacterium that was isolated from the feces of a healthy individual by Barry R. Goldin and Sherwood L. Gorbach (Silva et al., 1987). It has been used as a probiotic bacterium due to its resistance to gastric acid and bile and strong affinity for intestinal mucosal cells (Walter, 2008). *Lactobacillus rhamnosus* GG has powerful adhesive properties and can exclude or reduce pathogenic adherence as well as produce substances antagonistic to foodborne pathogens (Gorbach, 2000). Liu et al. (2010) observed a dual effect of GG, as it could increase the secretion of mucin by porcine intestinal epithelial cells and decrease rotavirus-induced mucin secretion. Nikoskelainen et al. (2001) found that the growth of pathogens was obviously inhibited by GG via nutritional competition. Further research showed that GG treatment ameliorated intestinal leakage, improved intestinal barrier function, and significantly reduced alcohol-induced oxidative stress as well as inflammation in the intestine and liver in rats (Forsyth et al., 2009). Lam et al. (2007) induced gastric kissing ulcers in rats, and GG successfully colonized in the gastric mucosa, especially at the ulcer margin. Gastric ulcer healing was enhanced via the attenuation of cell apoptosis to cell proliferation ratio and an increase in angiogenesis. Viable GG could increase the transmembrane electric resistance of intestinal epithelial cells, reduce epithelial cell damage, and maintain intestinal epithelial barrier integrity (Restalenert and Barrett, 2003; Moorthy et al., 2009; Nissen et al., 2009).

To our knowledge, Julia B. Ewaschuk and colleagues first reported that although GG did not affect D-lactic acidosis in diarrheic calves, it survived the gastrointestinal transit and could be used as a potential probiotic (Ewaschuk et al., 2004; Ewaschuk et al., 2006). However, further research is needed to study the effects of GG on calves in terms of practice. We hypothesized that neonatal calves fed GG would alter growth, health, rumen fermentation, and bacterial community composition. Therefore, a viable GG feeding trial was conducted to investigate the effects of GG on the growth, health, rumen fermentation, and bacterial community of Holstein calves during the preweaning stage.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

Lactobacillus rhamnosus GG (ATCC 53013) was purchased from the China Center of Industrial Culture Collection (Beijing, China) and originally sourced from the American Type Culture Collection. The lyophilized strain was cultured in DeMan, Rogosa, Sharpe broth (Aoboxing biotech Co., Ltd, Beijing) at 37 °C and 200 rpm for 24 h, then recultured under these conditions until a high concentration of cells (>10^{10} cells/mL) was obtained. Bacterial pellets were collected by centrifugation (3200 × *g*, 4 °C, 10 min) and washed twice with PBS (0.01 M phosphate, 0.15 M sodium chloride, pH 7.3). Bacterial cell concentration was estimated by measuring the optical density at 600 nm and comparing the results with a standard curve (obtained by combining the results from flow cytometry and spectroscopy over a range of GG concentrations; Lahtinen et al., 2004).

Animals, Experimental Design, and Diets

The experiment was conducted at the Experimental Practice and Demonstration Center of Northeast Agricultural University (Harbin,

China). The experimental protocol was conducted in accordance with the practices outlined in the *Guide for the Care and Use of Agriculture Animals in Agriculture Research and Teaching* (FASS, 2010).

Twenty-four male Holstein calves (BW = 49.91 ± 5.12 kg; age = 11.75 ± 2.71 d) were blocked according to their BW and age before being randomly assigned to 1 of 2 treatment groups (12 calves per group): 1) untreated control (**CON**), or 2) treated with 1×10^{10} cfu/d GG suspension (**GG**). Calves received GG daily, mixed with the milk replacer in the morning feed for 6 wk, until the end of the trial (before the weaning procedure).

The calves were raised in individual pens (1.5 × 2.5 m^2) on straw bedding with a front metal gate allowing access to the feed. The milk replacer (21.70% CP, 16.82% fat), mixed at a rate of 250 g to 2 L of 50 °C water, was offered at a temperature of approximately 41 °C as per the instructions from the producer (Hunneue Feed Co., Ltd, Beijing, China) and delivered 3 times daily at 0600, 1200, and 1800 h by nipple bottle. Pelleted calf starter (20.44% CP; 3.74% fat) and alfalfa hay (13.66% CP; 2.13% fat) chopped to a length of 3 cm were offered twice daily at 0600 and 1800 h. Calves were allowed ad libitum access to starter, alfalfa hay, and water throughout the feeding trial, the orts were weighed and discarded. After the trial was finished, calves were weaned gradually from 56 to 60 d of age.

Growth Measurements and Health Monitoring

Body weight (calibrated digital floor scale, P2000, Anscitech Co., Ltd, Wuhan, China), hip height, hip width, wither height (measuring stick, Anscitech) heart girth and body length (dairy calf weigh tape, Anscitech) were recorded at the beginning of the study (before GG administration) and weekly thereafter. Calculations of the ADG and stature were made from these measurements (Khan et al., 2007; Jarrah et al., 2013). Feces, nasal and ear scores were recorded daily before the morning feeding using a 0 to 3 (feces and nasal score) or 0 to 4 (ear score) scale developed by the University of Wisconsin–Madison (McGuirk, 2008). Any calf with a feces score ≥ 2 was treated with electrolytic solutions (Electrolyte Complete; DBN Technology Group Co., Ltd, Beijing, China) until the diarrhea subsided. Oral electrolyte solution was offered up to twice daily within 2 h of feeding.

Blood, Rumen Fluid, and Feces Collection

The blood samples were collected at 0 (before GG administration), 2, 4, and 6 wk of the study from the jugular vein into heparinized tubes (Vacutainer, Huawei medical Appliances co., Ltd, Yangzhou, China) and then centrifuged (3,000 × *g*, 15 min, 4 °C). Plasma fractions were transferred into clean tubes and stored at −20 °C for later analysis. The rumen fluid samples were collected only once at 6 wk using an oral stomach tube. The first 100 mL of fluid was discarded before sample collection. The tube was cleaned with ethanol and then washed with water to prevent the contamination of rumen fluids between calves. The samples were then filtered with double-layer gauze, one tube of fluid mixed with HCl was kept at −20 °C to detect the ammonia nitrogen (**NH_3-N**); another tube was frozen immediately at −20 °C to detect the VFA and digestive enzymes activities; a third tube was instantly frozen in liquid nitrogen for further analysis of the rumen microorganisms. Feces samples were collected only once at 6 wk, the samples weighted almost 100 g were taken directly from the calves' rectum. The samples were homogenized, transferred into clean tubes, and instantly frozen in liquid nitrogen for analysis of the feces microorganisms.

Plasma Parameters Analysis

The concentrations of total protein were determined using the AU680 Beckman Coulter analyzer (Beckman Coulter Commercial Enterprise (China) Co., Ltd, Shanghai, China). The β-hydroxybutyric (**BHBA**) levels were determined using a β-Hydroxybutyric Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Commercially available RIA kits (Amresco Inc., Ohio, USA) were used to analyze the levels of hormones and immune indices as follows: triiodothyronine (HY-001), tetraiodothyronine (HY-001), growth hormone (HY-10035), insulin-like growth factor 1 (HY-10119), and immunoglobulin A/G/M (HY-755).

Rumen Fermentation and Digestive Enzymes Activity Analysis

The pH values were determined immediately after the rumen fluids were taken out using a PB-10 pH meter (Startorius AG, Göttingen, Germany). The NH_3-N concentrations were determined using the phenol hypochlorite colorimetric method developed by Broderick and Kang (1980). The concentrations of acetate, butyrate, and propionate were detected as described by Zhang et al. (2016) using a gas chromatograph (GC-2010, Shimadzu (Shanghai) Laboratory Supplies Co., Ltd, Shanghai,

China) equipped with a flame-ionization detector and capillary column (HP-Innowax, 19091N-133, Agilent Technologies, Santa Clara, CA). The rumen amylase, proteinase, carboxymethyl cellulose, and salicylase activities were determined using spectrophotometer (Martin et al., 1993, Konkit and Kim, 2016). The microbial protein (**MCP**) concentrations were determined according to Hu et al. (2005), which was estimated from the ratio of purines to N of isolated bacteria, yeast RNA was used as a standard.

Rumen and Feces Bacterial Community Composition Analysis

Rumen fluid and feces samples from 6 calves per experimental group were randomly chosen and sent to Biomarker Technologies Co., Ltd, (Beijing, China) for bacterial community composition analysis.

DNA extraction and 16S rRNA gene amplicon preparation. The total bacterial DNA was extracted using a stool DNA kit (OMEGA Bio-Tek, Norcross, GA), according to the manufacturer's instructions. The concentration of DNA was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The integrity of the extracted DNA was verified by agarose (1.5%) gel electrophoresis. Subsequently, the V3-V4 region of the 16S rRNA gene was PCR-amplified with universal primer pair(5′-ACTCCTACGGGAGGCAGCA-3′ and 5′-GGACTACHVGGGTWTCTAAT-3′) combined with adapter and barcode sequences (Jiang et al., 2017; Li et al., 2017). The PCR reactions were performed using an Applied Biosystems Veriti Thermocycler (Thermo Fisher Scientific (China) Co., Ltd, Shanghai, China) with a reaction volume of 20 µL that contained 0.5 U Taq DNA polymerase (TaKaRa Taq, Takara Biomedical Technology (Beijing) Co., Ltd, Beijing, China). The thermocycling parameters were as follows: initial denaturation at 95 °C for 2 min, 30 cycles of further denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, extension at 68 °C for 1 min, and a concluding extension at 68 °C for 7 min. All PCR reactions were performed in triplicate, from which the products were combined. The integrity of the PCR product was verified by agarose (1.5%) gel electrophoresis, then purified with the QIAquick Gel Extraction Kit (Qiagen; Venlo, Limburg, Belgium). The concentration of the PCR product was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc.), and subsequently pooled in equal proportions based on the DNA concentration.

Illumina MiSeq sequencing and sequence analysis

The purified 16S rRNA gene amplicons were sequenced using a paired-end method with the Illumina Hiseq 2500 system (Illumina Inc., San Diego, CA). The resulting sequences were then screened and filtered for quality and length. Overlapping paired-end reads of the DNA fragments (minimum overlap of 10 bp, maximum mismatch rate set 0.2) were merged using FLASH v1.2.7 (Magoč and Salzberg, 2011). Low-quality sequences were trimmed using a minimum average quality score of 20, with a window size of 50 bp. Chimeric reads were identified and removed using UCHIME v4.2 to obtain effective tags (Edgar, 2010). Subsequently, the sequences were processed and analyzed using QIIME, v1.8.0 according to Caporaso et al., (2010). Singletons were removed from further analysis (Bokulich et al., 2013). High-quality sequences were clustered into operational taxonomic units (OTUs), defined by a 97% similarity. Representative OTU were assigned a taxonomy by the RDP Classifier and then aligned to the Greengenes reference database. For community analysis, the number of sequences in each sample was normalized to standardize sequencing effort across samples and minimize any bias due to different number of total sequences. MOTHUR v1.3.0 was used to calculate α-diversity indicators of diversity (Shannon and Simpson), richness (Ace and Chao1), and rarefaction curves. It should be noted that the Simpson's results calculated by MOTHUR mean the probability that 2 sequences are randomly selected from the sequencing data of sample, which belong to the same OTU (Desantis et al., 2006; Edgar, 2010).

Statistical Analysis

The data were analyzed using SAS/STAT software (version 9.2, SAS Institute Inc., Cary, NC). Before analysis, daily measurements of intake and health scores were adjusted to weekly means. All data were tested for normality using the UNIVARIATE procedure. The data of intake, growth, health scores and plasma biochemical parameters were analyzed as a randomized complete block design with repeated measures when applicable by a MIXED model that included the fixed effects of treatment, week, and treatment × week

interaction, the random effects of block, and calf within block. The data for body structure measurements and plasma biochemical parameters obtained before GG administration were added to the model as a covariate in the statistical analysis concerned. Data collected on the fermentation parameters, digestive enzyme activities and the MCP concentrations of rumen were analyzed using Student's *t*-test. The relative abundance (%) of the dominant bacteria families (accounting for > 0.5% of the total) and *Lactobacillaceae* in the rumen fluids and feces of calves were reported. The bacterial diversity indices (Shannon and Simpson) and richness (Chao1 and ACE) were also analyzed using the Student's *t*-test procedure. Significance was declared when $P \leq 0.05$, with trends toward significance declared when $0.05 < P \leq 0.10$. Least squares means with the standard error of the means are reported.

RESULTS AND DISCUSSION

Feed Intake, Growth Performance, and Health Status

All calves received an equal amount of milk replacer throughout the trial, but the starter and alfalfa hay were offered ad libitum. Table 1 shows the results of feeds and nutrients intake. Administration of GG significantly increased the starter intake of calves ($P = 0.023$), but no difference was observed in alfalfa hay intake ($P = 0.294$). As a result, the CP intake tended to be higher for calves fed GG ($P = 0.062$), whereas the fat, NDF, and ADF intake were not different ($P = 0.361$, $P = 0.846$, and $P = 0.766$, respectively). In addition, a difference was noted for week for all feeds and nutrients intake ($P < 0.001$), and the mean daily feed intake of the calves in both groups increased with age. Increasing the starter intake was conducive to minimize the calves' weight loss and distress during the transition from liquid milk to solid feed. Henderson et al. (2016) demonstrated that the best studied and most important factor affecting rumen fermentation and bacterial community development is the diet, as it determines the substrates available for rumen fermentation.

The BW of calves at the beginning of our study was similar between the 2 groups. We hypothesized that BW, ADG, and body structure measurements would be affected by the treatment, calf age, or the interaction of treatment and age. Table 2 shows the results of ADG and body structure measurements. Administration of GG significantly increased the ADG of calves ($P = 0.035$). No significant differences were observed for body structures ($P > 0.05$). Similar to the results of intake, a difference was noted for week for the body structure measurements ($P < 0.001$). Intensive researches have revealed that feeding calves probiotics could improve their growth performance. Zhang et al. (2016) found that oral *Lactobacillus plantarum* (1.7×10^{10} cfu/d) could improve the growth performance and nutrient digestibility of calves. The results from Frizzo et al. (2010) showed that dairy calves supplemented with bovine origin composed of *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T, and *Pediococcus acidilactici* DSPV 006T increased starter intake and growth performance of dairy calves exposed to nutritional stress.

We observed a significant decrease ($P < 0.001$) in the fecal scores with age for week. Administration of GG significantly ($P = 0.018$) decreased the feces scores (Table 1). Additionally, there was no difference ($P > 0.05$) in the respiratory scores between the 2 groups for the duration of the study (data not shown). Kim et al. (2011) showed that direct-fed of

Table 1. Least squares means of feed intake, nutrients intake and feces scores for calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			Week							*P*-value		
Item	CON	GG	SEM	1	2	3	4	5	6	SEM	Treatment	Week	Treatment × Week
Milk replacer intake, DM, kg/d	0.75	0.75	-	0.75	0.75	0.75	0.75	0.75	0.75	-	-	-	-
Starter intake, DM, kg/d	0.57	0.68	0.03	0.11^{f}	0.21^{e}	0.40^{d}	0.64^{c}	1.03^{b}	1.34^{a}	0.03	0.023	<0.001	0.052
Alfalfa hay intake, DM, kg/d	0.26	0.28	0.03	0.02^{d}	0.03^{d}	0.07^{d}	0.20^{c}	0.45^{b}	0.85^{a}	0.01	0.294	<0.001	0.357
CP intake, kg/d	0.31	0.34	0.01	0.19^{e}	0.21^{e}	0.25^{d}	0.32^{c}	0.44^{b}	0.55^{a}	0.01	0.062	<0.001	0.382
Fat intake, kg/d	0.15	0.16	0.001	0.13^{e}	0.13^{e}	0.14^{d}	0.15^{c}	0.17^{b}	0.19^{a}	0.001	0.361	<0.001	0.145
NDF intake, kg/d	0.29	0.32	0.01	0.04^{e}	0.07^{e}	0.15^{d}	0.28^{c}	0.51^{b}	0.80^{a}	0.01	0.846	<0.001	0.931
ADF intake, kg/d	0.16	0.17	0.005	0.02^{d}	0.03^{d}	0.07^{d}	0.14^{c}	0.28^{b}	0.47^{a}	0.004	0.766	<0.001	0.904
Feces score, 0–3	1.16	1.04	0.03	1.50^{a}	1.60^{a}	1.28^{b}	0.93^{c}	0.67^{d}	0.62^{d}	0.06	0.018	<0.001	0.383

[a–f]Weekly means with different superscript letters are significantly different ($P \leq 0.05$).

[1]CON = untreated control; GG = treated with 1×10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

probiotic mixed products as an antibiotic alternative could reduce the incidence of diarrhea in calves with no adverse effect on the growth performance. A variety of microbial species including *Bacillus spp.*, *Enterococcus spp.*, and *Saccharomyces spp.* have been used in animals as probiotic products. Ewaschuk et al. (2004) demonstrated that GG survived the gastrointestinal transit in young calves and could be administered in an oral rehydration solution. *Lactobacillus rhamnosus* GG showed powerful adhesive properties and could exclude or reduce pathogenic adherence, as well as produce substances antagonistic to foodborne pathogens (Gorbach, 2000). The results of the fecal scores further confirmed the potential of GG in preventing diarrhea in calves during the preweaning stage.

Plasma Parameters

The results of the plasma parameters are reported in Table 3. The concentration of BHBA tended to be higher in the GG treated calves ($P = 0.092$). Bayatkouhsar et al. (2013) stated that the rumen in neonatal calves is metabolically non-functional, thus the concentration of BHBA is low until rumen function improves. The BHBA is formed mainly by the conversion of butyrate in the rumen wall before release into the portal circulation. After the starter intake initiation and subsequent establishment of rumen fermentation and microbial population, the development of physically and metabolically rumen occurs and the rumen epithelium becomes the primary location of BHBA production (Bayatkouhsar et al., 2013). Quigley et al. (1991) suggested that the BHBA concentration in plasma could be considered as an indicator of rumen development and reflected concentrate intake. Our results indicated that the calves fed GG promote the early development of rumen. This result was in agreement with the report published by Bayatkouhsar et al. (2013), who found that rumen development was promoted by feeding calves with lactic acid bacteria. No significant difference was observed for the total protein, triiodothyronine, tetraiodothyronine, growth hormone, insulin-like growth factor 1, and immunoglobulin A/M/G levels ($P > 0.05$) (Table 3), which suggested that the GG treatment had no effect on immune function or secretion of growth related hormones.

Table 2. Least squares means of ADG (kg/d) and body structure measurements (cm) for calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			Week							*P*-value		
Item	CON	GG	SEM	1	2	3	4	5	6	SEM	Treatment	Week	Treatment × Week
ADG	0.62	0.70	0.03	0.47^{d}	0.50^{d}	0.57^{d}	0.66^{c}	0.80^{b}	0.94^{a}	0.02	0.035	<0.001	0.112
Hip height	83.9	85.72	6.75	70.96^{e}	77.51^{d}	80.67^{d}	86.94^{c}	93.46^{b}	99.34^{a}	4.05	0.102	<0.001	0.864
Hip width	18.76	19.4	1.27	15.70^{f}	17.13^{e}	18.26^{d}	19.28^{c}	21.10^{b}	23.01^{a}	0.93	0.108	<0.001	0.424
Wither height	80.89	82.61	4.75	70.85^{f}	74.34^{e}	79.01^{d}	83.05^{c}	85.96^{b}	97.30^{a}	5.76	0.119	<0.001	0.191
Heart girth	91.95	93.54	7.05	79.58^{f}	85.31^{e}	88.51^{d}	95.30^{c}	101.72^{b}	106.04^{a}	8.25	0.295	<0.001	0.799
Body length	87.48	90.52	6.54	71.81^{f}	78.36^{e}	86.38^{d}	92.9^{c}	98.51^{b}	106.64^{a}	8.54	0.175	<0.001	0.081

$^{a-f}$Weekly means with different superscript letters are significantly different ($P \leq 0.05$).

[1]CON = untreated control; GG = treated with 1×10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

Table 3. Least squares means of the plasma parameters for calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			*P*-value		
Item[2]	CON	GG	SEM	Treatment	Week	Treatment × Week
TP, g/L	61.67	65.24	4.84	0.107	<0.001	0.900
BHBA, mM	0.31	0.42	0.03	0.092	<0.001	0.784
T3, ng/mL	0.83	0.80	0.09	0.564	<0.001	0.918
T4, ng/mL	61.43	60.68	2.52	0.836	<0.001	0.308
GH, ng/mL	4.06	4.32	0.22	0.418	<0.001	0.499
IGF-1, ng/mL	162.30	167.70	9.73	0.428	<0.001	0.635
IgA, g/L	0.72	0.73	0.12	0.694	<0.001	0.301
IgG, g/L	10.13	10.47	1.19	0.214	0.015	0.989
IgM, g/L	2.36	2.49	0.09	0.121	0.007	0.107

[1]CON = untreated control; GG = treated with 1×10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

[2]TP = total protein; BHBA =β-hydroxybutyric; T3 = triiodothyronine; T4 = tetraiodothyronine; GH = growth hormone; IGF-1 = insulin-like growth factor 1; IgA/M/G = immunoglobulin A/M/G.

Rumen Fermentation Parameters

The least squares means of the treatment effects on rumen fermentation parameters, enzymes activity, and MCP concentrations are presented in Table 4. The pH of rumen fluids in the calves fed GG was lower ($P = 0.007$), which might be attributable to the tendency ($P = 0.083$) for the total VFA concentration in rumen fluids to increase. In addition, a significant increase ($P = 0.023$) in the propionate concentration, as well as the tendency ($P = 0.077$) for an increased butyrate concentration were observed in rumen fluids of calves with GG treatment. The results of rumen fermentation were related to the difference in starter intake between treatments. Growing calves will gradually increase their voluntary starter intake over time (Yohe et al., 2015). Feeding more starter will regulate the rumen fermentation pattern to normally increase the propionate and butyrate concentration, and change the proportion of VFA composition, which is more conducive to the absorption of lactate and propionate by the rumen epithelial cells. Butyrate, produced from the fermentation of concentrate, plays an important role in stimulating the development of rumen mucosa, which is more effective than roughage (Mentschel et al., 2001, Moolchand et al., 2013).

Administration of GG significantly increased the amylase activity ($P = 0.043$), protease activity ($P = 0.036$) and MCP concentration ($P = 0.044$) in the rumen fluids. However, the carboxymethyl cellulase and salicylase activity were not different between the 2 groups ($P = 0.258$ and $P = 0.210$, respectively). Our results were consistent with Agarwal et al. (2010), who found that the amylase and protease activities were increased in the rumen fluid of neonatal crossbred calves fed *Lactobacillus acidophilus*. This may be due to a more stable rumen internal environment from the increased production of VFA, which decreased the rumen pH in calves after GG treatment (Abd El-Tawab et al., 2016). Most of the digestive enzymes are secreted by various bacteria in the rumen and convert ingested rations to VFA and MCP, which provide approximately 70–85% of the nutrients absorbed by the ruminant (Bergman, 1990). Our results further confirmed the modulation effects of GG on rumen microbial activity, especially for bacteria producing amylase and protease. The increase of the MCP concentration was related to the higher levels of hydrolyzed proteins and energy in the rumen fluid. In current studies, administration of GG significantly increased starter intake, enzymatic activity and provided more substrates and energy, which contributed to MCP synthesis.

Table 4. Least squares means of rumen pH, ammonia nitrogen, volatile fatty acids, and enzyme activity for calves fed *Lactobacillus rhamnosus* GG

	Treatment[2]			
Item[1]	CON	GG	SEM	*P*-value
pH	6.31	5.83	0.14	0.007
Ammonia nitrogen, mg/dL	13.97	13.07	1.62	0.697
Acetate, mM	36.20	34.11	2.14	0.269
Propionate, mM	13.36	16.14	1.13	0.023
Butyrate, mM	4.23	5.11	0.34	0.077
Total VFA, mM	56.19	59.82	3.48	0.083
Amylase	1.27	1.66	0.09	0.043
Proteinase	3.03	4.25	0.55	0.036
Carboxymethyl cellulose	1.24	1.39	0.09	0.258
Salicylase	2.14	2.74	0.33	0.210
Microbial protein, mg/dL	142.04	168.47	12.37	0.044

[1]Units of enzyme activity are: amylase (μmol glucose/min/mL); proteinase (μg hydrolyzed protein/min/mL); carboxymethyl cellulose (μmol glucose/min/mL) and salicylase (μmol glucose/min/mL).

[2]CON = untreated control; GG = treated with 1×10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

Bacterial Community Composition in Rumen Fluids and Feces

In rumen fluid. The relative abundance (%) of the dominant bacteria families (accounting for > 0.5% of the total) in the rumen fluids were identified (Table 5). The most abundant bacteria family for the CON calves was *Succinivibrionaceae*, which accounted for approximately 38.35% of all sequences, followed by *Prevotellaceae* (31.60%), *Lachnospiraceae* (5.75%), *Veillonellaceae* (4.88%), and the *Bacteroidales* family S24-7 (2.98%), etc. However, the order and relative abundance of the dominant bacteria families were inconsistent between the CON and GG calves. The bacteria family with the highest relative abundance in GG calves was *Prevotellaceae* (55.67%), instead of *Succinivibrionaceae*, and the relative abundance increased by 76.18% compared with that of the CON calves. It was followed by the *Bacteroidales* family S24-7 (9.23%), *Succinivibrionaceae* (6.35%), *Rikenellaceae* (4.30%), and *Lachnospiraceae* (3.66%), etc. Among these bacteria, the relative abundance of *Prevotellaceae* ($P < 0.001$) and *Bacteroidaceae* ($P = 0.011$) increased significantly while *Succinivibrionaceae* decreased significantly ($P < 0.001$) in the rumen fluids of calves treated with GG. In addition, although the relative abundance of *Lactobacillaceae* was relatively low in the rumen fluid, it was significantly increased after GG

Table 5. Relative abundance (%) of the dominant bacteria families (accounting for >0.5% of the total) and *Lactobacillaceae* in rumen fluids of calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			
Item	CON	GG	SEM	*P*-value
Prevotellaceae	31.60	55.67	3.91	<0.001
Succinivibrionaceae	38.35	6.35	5.58	<0.001
Bacteroidales family S24-7	2.98	9.23	1.04	0.011
Lachnospiraceae	5.75	3.66	1.04	0.340
Veillonellaceae	4.88	2.63	1.02	0.486
Rikenellaceae	1.53	4.30	0.77	0.067
*Bacteroidales*_BS11_gut_group	2.04	3.17	0.98	0.587
Erysipelotrichaceae	0.56	3.78	0.80	0.037
Acidaminococcaceae	2.90	0.45	0.55	0.017
Ruminococcaceae	1.63	1.60	0.27	0.956
*Bacteroidales*_RF16_group	1.51	0.99	0.27	0.374
Spirochaetaceae	0.73	1.66	0.44	0.321
*Bacteroidales*_Incertae_Sedis	0.91	1.42	0.30	0.418
uncultured_rumen_bacterium	1.75	0.41	0.60	0.289
Others	2.59	2.81	0.80	0.278
Unclassified	0.29	1.13	0.37	0.275
Lactobacillaceae	0.008	0.067	0.014	0.030

[1]CON = untreated control; GG = treated with 1 × 10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

Table 6. Relative abundance (%) of the dominant bacteria families (accounting for >0.5% of the total) and *Lactobacillaceae* in feces of calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			
Item	CON	GG	SEM	*P*-value
Ruminococcaceae	37.42	28.76	1.73	0.004
Rikenellaceae	6.80	25.80	3.35	<0.001
Bacteroidaceae	13.50	9.81	0.83	0.017
Lachnospiraceae	10.90	9.11	0.59	0.137
Bacteroidales family S24-7	4.87	3.55	0.72	0.385
*Bacteroidales*_RF16_group	3.97	1.90	1.02	0.332
Christensenellaceae	3.23	2.25	0.34	0.153
Porphyromonadaceae	2.34	1.93	0.24	0.412
*Clostridiales*_vadinBB60_group	1.30	2.38	0.25	0.023
Prevotellaceae	2.24	1.49	0.26	0.152
Succinivibrionaceae	1.79	1.84	0.33	0.944
*Bacteroidales*_Incertae_Sedis	0.90	2.20	0.41	0.123
Verrucomicrobiaceae	1.83	1.09	0.32	0.270
Family_XIII	1.48	0.92	0.10	0.001
Acidaminococcaceae	0.75	1.41	0.19	0.078
uncultured_bacterium_o_Mollicutes_RF9	1.65	0.45	0.36	0.092
Alcaligenaceae	0.35	0.82	0.19	0.246
Elusimicrobiaceae	0.72	0.34	0.27	0.504
Others	3.34	3.33	0.78	0.192
Unclassified	0.60	0.64	0.07	0.797
Lactobacillaceae	0.006	0.013	0.005	0.002

[1]CON = untreated control; GG = treated with 1 × 10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

Table 7. Alpha diversity indices for bacterial communities in the rumen fluids and feces of calves fed *Lactobacillus rhamnosus* GG

	Treatment[1]			
Item	CON	GG	SEM	*P*-value
Rumen fluid				
Reads	73,164.17	66,240.33	3,219.39	0.304
Total species, OTU	460.67	593.5	21.99	0.049
Shannon	3.32	4.14	0.22	0.027
Simpson	0.14	0.04	0.01	0.040
Chao1	566.38	721.28	29.47	0.051
Ace	557.12	703.23	36.53	0.052
Feces				
Reads	69,423.50	7,6347.50	2,636.49	0.203
Total species, OTU	672.00	753.33	30.51	0.089
Shannon	4.70	4.85	0.12	0.398
Simpson	0.02	0.03	0.01	0.744
Chao1	759.24	860.72	38.98	0.095
Ace	750.17	830.02	33.85	0.126

[1]CON = untreated control; GG = treated with 1 × 10^{10} cfu/d *Lactobacillus rhamnosus* GG suspension.

OTU = operational taxonomic unit.

administration (P = 0.030). In addition, there were 75 families collectively detected in both groups, 11 of these had a relative abundance greater than 1.0%, which accounted for approximately 86.29% and 86.65% of the sequences for the CON and GG calves, respectively.

The establishment of rumen function is associated with internal microorganisms, as their fermentation products play an important role in stimulating the growth of the rumen epithelium (Beharka et al., 1998). The relative abundance of *Prevotellaceae* and *Bacteroidaceae* in the rumen of neonatal calves increased with age (Jami et al., 2013; Rey et al., 2014). Our results showed that the relative abundance of these 2 bacteria families were higher in calves treated with GG, especially for *Prevotellaceae*, which is the most abundant family in all bacteria and widely distributed in the rumen (Rey et al., 2014). Starch and plant cell wall polysaccharides such as xylan, pectin, and hemicellulose can be degraded and utilized as substrates by the *Prevotellaceae* (Castillo-Lopez et al., 2018). *Prevotella ruminicola* and *Prevotella bryantii* can produce xylanase and carboxymethyl cellulose. In addition, *Prevotellaceae* plays an important role in the degradation of diets protein, MCP synthesis and the absorption and fermentation of peptides in rumen. The increase in the relative abundance of *Prevotellaceae* in the rumen fluid would be conducive to increase the degradation rate of diets, especially for the concentrate portion.

The results of the microbial diversity indices for the bacterial communities in rumen fluids of calves are shown in Table 7. The rarefaction curve analysis suggested that the sequencing depth in this study was adequate (data not shown) and the common microbial diversity indices including Shannon, Simpson, Ace, and Chao1 were evaluated. Administration of GG significantly affected the OTU-level microbial diversity in the rumen microbiome ($P = 0.049$). The Shannon and Simpson indices were significantly increased ($P = 0.027$) and decreased ($P = 0.040$), respectively, for calves fed GG. In addition, both the Chao1 and Ace indices tended to increase ($P = 0.051$ and $P = 0.052$, respectively). The Shannon and Simpson indices are the most widely accepted measures of ecological diversity. A higher Shannon index and lower Simpson index indicate higher bacterial community diversity. Therefore, the results obtained in our study indicated that the administration of GG increased microorganism diversity in the rumen.

In feces. The relative abundance (%) of the dominant bacteria families (accounting for > 0.5% of the total) in the feces of calves were reported (Table 6). The most abundant bacteria family in the feces of the CON calves were *Ruminococcaceae* (37.42%), *Bacteroidaceae* (13.50%), *Lachnospiraceae* (10.90%), *Rikenellaceae* (6.80%), and the *Bacteroidales* family S24-7 (4.87%). Administration of GG significantly increased the relative abundance of *Rikenellaceae* from 6.80 to 25.81% ($P < 0.001$). The relative abundance of *Ruminococcacea* ($P = 0.004$) and *Bacteroidaceae* ($P = 0.017$) decreased significantly. The results of the microbial diversity indices (Table 7) for bacterial communities in feces showed that the total species (OTU) and Chao1 index tended to increase with GG supplementation ($P = 0.089$ and $P = 0.095$, respectively). There were no differences observed in the Shannon, Simpson and Ace indices between the 2 groups ($P > 0.05$).

CONCLUSIONS

The results of this study indicated that neonatal calves fed GG before weaning increased their voluntary starter intake and growth performance, improved the amylase and protease activities, increased MCP concentration in the rumen fluid, and regulated rumen fermentation pattern to normally increase the concentration of propionate and butyrate. Administration of GG also diversified the bacterial community composition in the rumen, and regulated the balance of rumen and intestinal microorganisms. These results were beneficial to the rumen development and early weaning of calves.

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