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Drinking warm water in a cold environment impairs growth performance and alters the gastrointestinal microbiota in growing calves

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Abstract: This study aimed to investigate the effects of drinking water temperature on growth performance and gastrointestinal microbiota in growing calves. Forty-five weaned male calves were randomly divided into three treatment groups (n = 15 per group). The groups were provided with drinking water at different temperatures: 5–10 °C (FC), 20–25 °C (FM), or 35–40 °C (FW). The experiment lasted for 30 days, during which all calves were housed in individual hutches. Body weight and jugular blood samples were collected on days 0, 15, and 30; body measurements were taken on days 0 and 30. On day 30, rumen fluid and faecal samples were collected for 16S rRNA gene sequencing of the V3–V4 regions. Calves in the FW group showed significantly higher water intake and diarrhoea frequency, along with significantly lower average daily gain (ADG), compared to the other groups. During the first 15 days, the ADG for the FM and FW groups was significantly lower than that of the FC group. Additionally, the FM and FW group maintained higher faecal scores throughout the experimental period. Rumen microbial analysis revealed a higher abundance of the potentially pathogenic genus *Pseudoscardovia* in the FW group compared to the FC group. In a cold winter environment, providing drinking water at 35–40 °C reduced growth performance, elevated faecal scores, and increased the abundance of harmful microorganisms in the gastrointestinal tract of growing calves.

Keywords: cold stress; diarrhoea frequency; water intake; water temperature; weaned calves

Water is an essential nutrient for organisms, playing critical roles in metabolic processes, thermoregulation, tissue lubrication, cellular structure formation, nutrient transport, and the maintenance of blood and tissue fluid circulation (Murphy 1992). Previous studies on water temperature have primarily focused on dairy cows and preweaned calves, offering insights potentially relevant to growing calves. Osborne et al. (2002) reported that lactating cows preferred drinking heated water (30–33 °C) at ambient temperatures between 7 and 15 °C, although this preference did not lead to increased milk production. Furthermore, our previous study demonstrated a consistent preference for 35 °C warm water in growing calves, independently of ambient temperature. In preweaned dairy

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calves, one study found that offering cool water (16–18 °C) resulted in higher intake than when cold water (6–8 °C) was offered, yet this increase did not improve either feed intake or growth performance (Huuskonen et al. 2011). In contrast, studies on preweaning yak calves reported that drinking warm water (20 °C) improved growth performance, likely through modulation of the rumen microbiota and enhanced nutrient digestibility (Liu et al. 2023).

Studies across species indicate that drinking water can shape the gastrointestinal microbiota by modifying key physicochemical conditions within the gut. In humans, for instance, water intake influences digestive passage rates, mixing efficiency, and water absorption, i.e. the factors that collectively reshape the microbial composition (Cremer et al. 2017). Mouse studies have shown that the pH of drinking water directly affects the structure of the intestinal microbiota (Sofi et al. 2014), and trace minerals in water can also alter the abundance of specific microbial taxa (Faulkner and Weiss 2017). Furthermore, in preweaned calves, the provision of drinking water has been found to modulate the gut microbial composition, with subsequent benefits for growth performance (Wickramasinghe et al. 2020).

However, the effect of drinking water temperature on the gastrointestinal microbiota of growing calves remains unexplored. We therefore hypothesised that providing warm water during winter would enhance the calf growth performance by beneficially remodelling the gastrointestinal microbial community. To test this hypothesis, we evaluated the impact of different drinking water temperatures on the gastrointestinal microbiota and growth performance of growing calves.

MATERIAL AND METHODS

Calves, experimental design, and sample collection. The experiment was conducted in 2022 on a farm in Helinger County, Hohhot, Inner Mongolia, P.R. China (latitude 40.17°N, longitude 111.77°E). A total of 45 Holstein growing male calves, with a mean age of 82.38 ± 2.83 days and body weight of 92.57 ± 10.55 kg, were selected and randomly assigned to one of the three treatment groups (n = 15 calves per group), differentiated by drinking water temperature: 5–10 °C (cold water, FC), 20–25 °C (moderate temperature, FM), or 35–40 °C (warm water, FW). The calves, which had entered a period of active growth by 75 days of age, were enrolled in the trial at 82 days. Fresh drinking water was provided twice daily at 08:00 and 16:00, with each access period lasting 10 minutes (Zhang et al. 2022). Water was offered in plastic buckets insulated within outer iron containers to help maintain the target temperatures.

The calves were individually housed in single pens within a designated calf-rearing area. Prior to the experiment, the pen floors were covered with clean sandy soil and a layer of straw bedding. Ambient temperature and wind speed were recorded daily at 08:00 and 14:00 using a hygrothermograph and an anemometer. These meteorological data were used to calculate the wind chill index (WCI) according to the formula established by Graunke et al. (2011):

$$WCI = 13.12 + 0.612\,5 \times T_{air} - 11.37 \times V^{0.67} + 0.396\,5 \times T_{air} \times V^{0.16} \quad (1)$$

where:

T_{air} – the air temperature (°C);

V – the wind speed (km/h).

The recorded temperature, wind speed, and calculated WCI values throughout the study period are summarised in Electronic Supplementary Material (ESM) Table S1.

Weekly feed samples were collected and stored at −20 °C for subsequent chemical analysis. The nutritional composition of the feed is presented in Table 1, analysed according to established methods (Wu et al. 2022). For water quality assessment, 100 ml samples were collected daily for the first 10 days of the trial. These daily samples were combined into a single 1 000 ml pooled composite sample, which was then analysed for key quality parameters following the procedure described by Wang et al. (2023). The water quality results are summarised in ESM Table S2.

Sample collection. Throughout the trial, calves received water at their respective designated temperatures twice daily at 08:00 and 16:00. Daily water intake (DWI) was recorded for each animal. Dry matter intake (DMI) was calculated daily by weighing and subtracting the orts from the feed offered the previous day. Body weight (BW) was measured on days 0, 15, and 30 to calculate the average daily gain (ADG) and feed efficiency (gain-to-feed ratio,

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Table 1. Composition and nutrient levels of feed diets (air dry basis)

Ingredient	Percentage (%)	Nutritional level	Percentage (%)
Corn	40.00	Dry matter	88.30
Wheat bran	8.00	Metabolisable energy (Mcal/kg)	2.15
Distiller's dried grains with solubles	5.00	Crude protein	23.60
Corn skin	10.00	Ether extract	5.73
Soybean meal	16.00	Ash	8.37
Cotton meal	16.00	Neutral detergent fibre	21.70
Premix	5.00	Non-fibre carbohydrate	40.60
Total	100.00	Acid detergent fibre	12.40
		Starch	35.20

The premix provided the following per kilogram of the starter: V_A 300 000 IU, V_E 280 000 IU, V_K 80 mg, V_D 150 000 IU, V_{B1} 30 mg, V_{B2} 100 mg, V_{B6} 60 mg, V_{B12} 0.4 mg, pantothenic acid 200 mg, nicotinic acid 400 mg, folic acid 20 mg, biotin 3 mg, Mn 2 000 mg, I 10 mg, Se 10 mg, Co 10 mg

ADG/DMI). Body measurements, including height, length, and chest girth, were taken on days 0 and 30. Faecal consistency was assessed four times daily using a 4-point scale (1 = normal; 2 = soft; 3 = runny; 4 = watery) (Larson et al. 1977) and the daily faecal score and incidence of diarrhoea (score ≥3) were recorded (Wu et al. 2022).

Blood samples (10 ml each) were collected from the external jugular vein before the morning feeding on days 0 (prior to the trial), 15, and 30 using additive-free vacuum tubes. After collection, the samples were centrifuged at 1 800 × *g* for 15 min at 4 °C to obtain serum, which was aliquoted and stored at –20 °C until further analysis. A suite of serum biomarkers, including growth hormone-releasing factor (GRF), growth hormone (GH), insulin-like growth factor 1 (IGF-1), somatostatin (SS), immunoglobulins (IgA, IgM), pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), antioxidant enzymes (SOD, CAT, GSH-Px), total antioxidant capacity (T-AOC), malonaldehyde (MDA), and cortisol (COR), were quantified using specific commercial ELISA kits (Biotechnology Co., Ltd, Shanghai, P.R. China) in accordance with the manufacturer's protocols. Additionally, concentrations of glucose (GLU), triglyceride (TG), total cholesterol (TC), total protein (TP), and blood urea nitrogen (BUN) were determined automatically by spectrophotometry (Shimadzu 2100; Shimadzu Co., Kyoto, Japan) using corresponding commercial assay kits (AngleGene BioTechnology Co., Ltd., Nanjing, P.R. China).

At the conclusion of the trial (day 30), faecal samples were collected from the rectum using sterile, pyrogen-free centrifuge tubes and immediately stored at –80 °C for subsequent analysis, following the methodology of Wu et al. (2023). Rumen fluid was collected 4 h after the morning feeding via a flexible oesophageal probe (A1164K; Anscitech Co., Ltd., Wuhan, P.R. China), as described by Wu et al. (2022). To minimise salivary contamination, the initial 10 ml of fluid was discarded. Approximately 5–10 ml of subsequent rumen liquid was then collected into sterile, pyrogen-free tubes. All rumen fluid samples were rapidly frozen in liquid nitrogen and transferred to a –80 °C freezer for preservation until further processing.

DNA extraction and PCR amplification. The microbial community profiling of faecal and rumen samples was conducted through 16S rRNA gene sequencing, which involved genomic DNA extraction, PCR amplification, and sequencing. The overall methodology was adapted from a previous study (Wu et al. 2023).

Total genomic DNA was extracted from both rumen fluid and faecal samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), in accordance with the manufacturer's instructions. The quality and concentration of the extracted DNA were assessed by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, USA), respectively. DNA was stored at –80 °C until further analysis.

The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified with the primer pair 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Liu et al. 2016) using a T100 Thermal Cycler (Bio-Rad, USA). Each PCR reaction was performed in trip-

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licate with a 20 µl mixture containing 4 µl of 5 × PrimeSTAR buffer, 2 µl of dNTPs (2.5 mM), 2 µl of forward primer (5 µM), 0.8 µl of reverse primer (5 µM), 0.8 µl of PrimeSTAR hot-start DNA polymerase, and 20 ng of template DNA. The thermal cycling programme consisted of initial denaturation at 95 °C for 3 min; 29 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s; followed by a final extension at 72 °C for 10 minutes. The resulting PCR products were visualised on 2% agarose gels and purified using a commercial DNA purification kit (Axygen Biosciences, Union City, CA, USA).

16S rRNA gene sequencing. The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered using fastp (v0.20.0), and merged with FLASH (v1.2.7) following previously described criteria. Operational taxonomic units (OTUs) were clustered at a 97% similarity threshold using UPARSE (v7.0), and chimeric sequences were identified and removed during this process (Chen et al. 2018). Taxonomy was assigned to each OTU by classifying its representative sequence against the SILVA 16S rRNA database (SSU123) using the RDP classifier algorithm with a confidence threshold of 70% (Wang et al. 2007).

Beta-diversity across samples was assessed using the principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity with the Vegan package (v2.5-3). Permutational multivariate analysis of variance (PERMANOVA), implemented in the same package, was used to evaluate the effect of treatment on the microbial community composition. To identify the bacterial taxa (from phylum to genus) that were significantly enriched in different groups, we performed the linear discriminant analysis effect size (LEfSe) (Segata et al. 2011), with a linear discriminant analysis (LDA) score threshold of >2 and statistical significance of $P < 0.05$.

Statistical analysis. The calf was considered the experimental unit for all analyses, which were performed using SAS v9.4 (SAS Institute Inc., Cary, NC). Data on serum parameters, body weight (initial and final), DWI, ADG, feed efficiency, and diarrhoea frequency were tested for normality of residuals and homoscedasticity using the Shapiro–Wilk and Levene's tests, respectively. A mixed-effects model was applied that included the fixed effects of treatment and day, the random effect of calf, and the residual error. Results are presented as least squares means with the standard error of the mean (SEM), and effects were considered significant at $P \leq 0.05$. Differences in α-diversity indices were evaluated using the Kruskal–Wallis test, while β-diversity differences were assessed by the permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. A two-matrix correlation heatmap (Pearson) was generated on the Majorbio Cloud Platform to examine the relationship between ruminal and intestinal microbiota. In addition, Pearson's correlation coefficients were calculated to assess associations between ruminal and intestinal microbiota (genus level) and growth-related indices in calves.

RESULTS

The environmental conditions recorded at 08:00 and 14:00 are summarised in ESM Table S1. At 08:00, air temperature ranged from –28 °C to –5 °C, wind speed from 0.37 km/h to 4.13 km/h, and the WCI from –32.45 to –3.08. The respective values at 14:00 were –20 °C to 4 °C for temperature, 2.77 km/h to 10.11 km/h for wind speed, and –28.51 to 1.12 for WCI.

Water intake. Water intake increased with drinking water temperature throughout the experimental period. Calves in the FW and FM groups consumed significantly more water than those in the FC group ($P < 0.01$; Table 2). Over the entire trial, the average daily water intake was 10.22 l for the FC group, 11.17 l for the FM group, and 15.02 l for the FW group.

Growth performance and health. Over the entire trial period, DMI was significantly lower in the FW group than in the FC and FM groups ($P < 0.01$). In the first 15 days, the ADG of calves in the FM and FW groups was significantly lower than that in the FC group ($P < 0.01$). From day 16 to 30, however, ADG was not significantly affected by the water temperature ($P > 0.05$). Across the entire experimental period, the FW group exhibited a significantly lower overall ADG than the FC group ($P < 0.01$). Feed efficiency remained comparable among all treatment groups ($P > 0.05$; Table 2).

Drinking water temperature had no significant effect on chest girth or body length ($P > 0.05$). However, the final body height and the average daily increase in body height were significantly lower in the FM and FW groups than in the FC group ($P < 0.05$ and $P < 0.01$, respectively; Table 2).

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Table 2. Effect of drinking water temperature on the growth performance of growing calves

Item	Treatment			SEM	*P*-value
	FC	FM	FW		
DWI (l/d)					
d 0–15	8.33[c]	9.42[b]	13.59[a]	0.290	<0.001
d 16–30	12.2[c]	13.1[b]	16.6[a]	0.271	<0.001
Whole trial	10.2[c]	11.2[b]	15.0[a]	0.223	<0.001
BW (kg)					
d 0	93.8	91.7	92.20	2.78	0.856
d 15	117	113	111.43	2.96	0.393
d 30	137	133	129.03	3.59	0.314
ADG (kg/d)					
d 0–15	1.64[a]	1.38[b]	1.30[b]	0.070	0.008
d 16–30	1.31	1.44	1.23	0.080	0.256
Whole trial	1.49[a]	1.42[a]	1.26[b]	0.040	0.001
DMI (kg/d)					
d 0–15	3.21[a]	2.97[b]	2.79[c]	0.120	<0.001
d 16–30	4.29[a]	3.88[b]	3.56[c]	0.130	<0.001
Whole trial	3.77[a]	3.43[b]	3.18[c]	0.110	<0.001
Feed efficiency					
d 0–15	0.511	0.481	0.472	0.030	0.441
d 16–30	0.300	0.350	0.330	0.020	0.301
Whole trial	0.400	0.410	0.400	0.010	0.406
Chest circumference (cm)					
d 0	109	108	109	0.960	0.958
d 30	120	120	119	0.960	0.790
Average daily growth	0.37	0.38	0.34	0.022	0.204
Body height (cm)					
d 0	87.2	87.5	87.4	0.66	0.964
d 30	97.9[a]	95.4[b]	95.7[b]	0.745	0.044
Average daily growth	0.361[a]	0.260[b]	0.282[b]	0.023	0.001
Body length (cm)					
d 0	87.8	88.7	88.7	0.752	0.631
d 30	101	101	100	0.790	0.846
Average daily growth	0.442	0.391	0.400	0.021	0.183
Faecal score					
d 0–15	1.46[b]	1.46[b]	1.61[a]	0.042	0.005
d 16–30	1.41[c]	1.59[b]	2.05[a]	0.040	<0.001
Whole trial	1.44[c]	1.53[b]	1.84[a]	0.030	<0.001
Diarrhoea frequency (%)	17.3[b]	19.1[b]	43.2[a]	6.53	0.033

[a–c]Treatment effects with different superscripts within a row are significantly different ($P < 0.05$)

ADG = average daily gain; BW = body weight; d = experimental day; DMI =dry matter intake; DWI = daily water intake; FC = 5 °C to 10 °C; feed efficiency = ADG/DMI; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C; SEM = standard error of the mean

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Regarding the gastrointestinal health, calves in the FW group demonstrated significantly higher faecal scores ($P < 0.01$) and a greater incidence of diarrhoea ($P < 0.05$) throughout the trial compared to the FM and FC groups (Table 2).

Blood parameters. Serum BUN levels were significantly lower ($P < 0.05$) in the FW group compared to the FC and FM groups. No significant differences were observed between groups for the other serum nutritional metabolites measured ($P > 0.05$; Table 3).

On day 30, serum CAT concentrations were significantly lower in the FW and FM groups than in the FC group ($P < 0.05$). On day 15, MDA levels were significantly elevated in the FM and FW groups compared to the FC group ($P < 0.05$). Furthermore, GSH-Px activity on day 15 was significantly lower in the FM group than in both the FC and FW groups ($P < 0.05$; Table 4).

No effect of drinking water temperature on serum immune, inflammatory factors, and growth-related hormones was found ($P > 0.05$; ESM Table S3 and S4).

Sequencing information and diversity of the ruminal microbiota. A total of 670 265 high-quality sequences were obtained from rumen fluid samples after quality filtering (ESM Table S5). The bacterial communities in all groups were sufficiently captured, as indicated by Good's coverage values exceeding 99.7%. The adequacy of the sequencing depth was further supported by rarefaction curves that approached a saturation plateau (ESM Figure S1A), demonstrating that the majority of the microbial diversity was represented.

As shown in Table 5, the drinking water temperature did not significantly affect the α-diversity or community composition (β-diversity) of the ruminal microbiota ($P > 0.05$; ESM Figure S2A).

Table 3. Effect of drinking water temperature on serum nutritional metabolism indexes of growing calves

Item	Treatment			SEM	*P*-value
	FC	FM	FW		
GLU (mmol/l)					
d 0	9.75	8.70	8.47	0.711	0.604
d 15	11.8	10.0	10.1	0.792	0.241
d 30	11.7	11.6	11.4	0.833	0.967
TP (g/l)					
d 0	63.2	63.3	58.6	2.79	0.469
d 15	75.8	73.2	66.6	4.32	0.327
d 30	80.0	70.5	78.8	4.14	0.240
BUN (mmol/l)					
d 0	4.23	5.32	4.12	0.580	0.355
d 15	4.78[a]	5.27[a]	3.63[b]	0.360	0.024
d 30	3.52	3.96	3.75	0.490	0.821
TG (mmol/l)					
d 0	0.30	0.38	0.38	0.041	0.235
d 15	0.25	0.32	0.30	0.054	0.598
d 30	0.25	0.25	0.30	0.051	0.745
TC (mmol/l)					
d 0	1.51	2.35	1.95	0.280	0.166
d 15	1.48	1.98	1.89	0.182	0.138
d 30	1.60	1.80	1.45	0.251	0.629

[a,b]Treatment effects with different superscripts within a row are significantly different ($P < 0.05$)

BUN = blood urea nitrogen; d = experimental day; FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C; GLU = glucose; SEM = standard error of the mean; TC = total cholesterol; TG = triglyceride; TP = total protein

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Table 4. Effects of drinking water temperature on serum oxidative stress indexes of growing calves

Item	Treatment			SEM	*P*-value
	FC	FM	FW		
SOD (U/ml)					
d 0	17.4	15.9	16.0	1.34	0.711
d 15	19.0	16.5	15.8	1.62	0.369
d 30	22.4	18.5	18.9	1.84	0.290
CAT (U/ml)					
d 0	26.6	23.6	24.2	1.83	0.522
d 15	31.0	24.7	27.7	2.09	0.133
d 30	35.5[a]	28.6[b]	30.2[b]	1.59	0.019
MDA (nmol/ml)					
d 0	1.85	2.05	2.05	0.08	0.163
d 15	1.36[b]	1.86[a]	1.71[a]	0.09	0.003
d 30	1.19	1.42	1.45	0.11	0.183
GSH-Px (U/ml)					
d 0	357	352	372	13.5	0.601
d 15	402[a]	368[b]	400[a]	9.52	0.044
d 30	422	376	448	37.5	0.412
T-AOC (U/ml)					
d 0	22.0	17.4	18.8	1.69	0.199
d 15	24.4	20.9	20.8	1.67	0.245
d 30	25.1	22.8	24.5	1.89	0.691
COR (ng/ml)					
d 0	77.1	71.3	76.0	4.86	0.709
d 15	86.7	83.1	86.1	4.74	0.849
d 30	102	91.5	92.7	4.08	0.165

[a,b]Treatment effects with different superscripts within a row are significantly different ($P < 0.05$)

CAT = catalase; COR = cortisol; d = experimental day; FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C; GSH-Px = glutathione peroxidase; MDA = malondialdehyde; SEM = standard error of the mean; SOD = superoxide dismutase; T-AOC = total antioxidant capacity

The microbial composition at the phylum and genus levels is depicted in Figure 1. Bacillota and Bacteroidota collectively constituted over 85% of the total sequences at the phylum level (Figure 1A). At the genus level, the dominant taxa were Prevotella_7, Erysipelotrichaceae_UCG-002, and Eubacterium_ruminantium_group (Figure 1B).

While the drinking water temperature did not induce any significant shifts at the phylum level, several differences were observed at the genus level. The abundances of the *Pseudoscardovia* and Lachnospiraceae_FCS020_group were significantly altered in the FM and FW groups compared to the FC group ($P < 0.05$). Specifically, *Pseudoscardovia* was enriched, whereas the Lachnospiraceae_FCS020_group was reduced. Additionally, the abundance of Family_XIII_UCG-001 was significantly higher in the FW group than in the FM and FC groups ($P < 0.05$; Table 5). LEfSe revealed one order, one family, and four genera as discriminative taxa (Figure 2A). Among these, the genus *Pseudoscardovia* was enriched in the FM group, while norank_f_Eubacterium_coprostanoligenes_group and Family_XIII_UCG-001 were enriched in the FW group. In contrast, an uncultured genus of the family Eggerthellaceae was significantly enriched in the FC group.

Metagenomic analysis of faecal samples yielded 784 024 high-quality reads (ESM Table S6). The sequencing depth was deemed sufficient, as re-

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Table 5. Effect of drinking water temperature on the α-diversity and taxonomic analysis (relative abundance, %) at the genus level of ruminal microbiota in growing calves

Item	Treatment			SEM	*P*-value
	FC	FM	FW		
α-diversity					
Community diversity					
Shannon	3.36	2.90	3.53	0.22	0.153
Simpson	0.091	0.140	0.101	0.030	0.446
Community richness					
Sobs	235	230	273	28.1	0.522
Ace	277	280	337	31.5	0.348
Chao1	285	284	337	32.3	0.439
Genus level					
Pseudoscardovia	0.011^{b}	5.36^{a}	1.34^{a}	3.11	0.011
Norank_f__Eubacterium_coprostanoligenes_group	1.440^{a}	0.670^{b}	1.530^{a}	0.280	0.042
Family_XIII_UCG-001	0.090^{b}	0.080^{b}	0.160^{a}	0.020	0.028
Unclassified_o__Coriobacteriales	0.04^{a}	0.01^{b}	0.02^{b}	0.010	0.029
Unclassified_k__norank_d__Bacteria	0.021^{a}	0.002^{b}	0.012^{ab}	0.003	0.031
Uncultured_f__Eggerthellaceae	0.007^{a}	0.002^{ab}	0.000^{b}	0.002	0.040
Lachnospiraceae_FCS020_group	0.003^{a}	0.000^{b}	0.000^{b}	0.001	0.030

^{a,b}Treatment effects with different superscripts within a row are significantly different ($P < 0.05$)
FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C; SEM = standard error of the mean

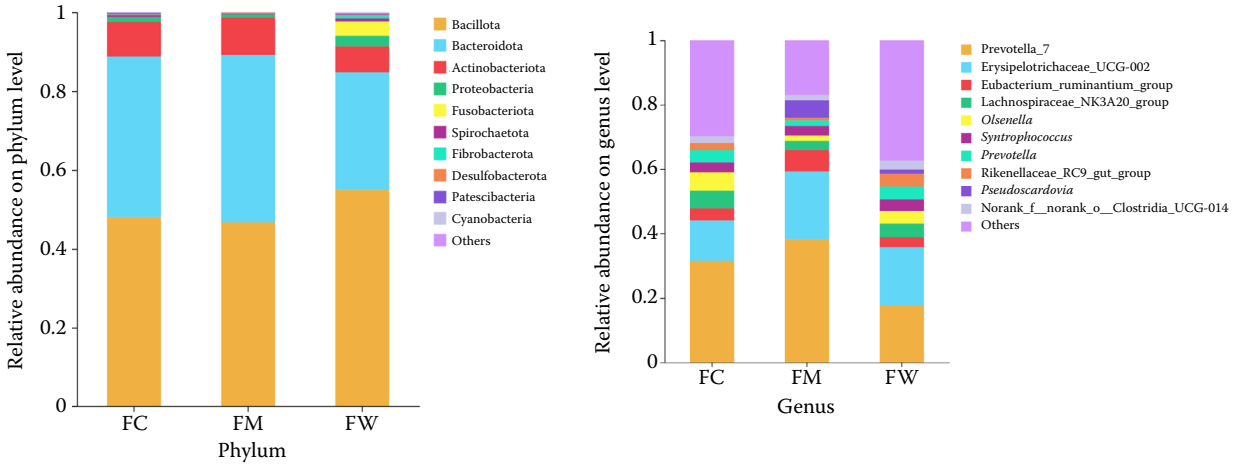

Figure 1. Composition of the ruminal microbiota at phylum and genus levels at different drinking water temperatures
FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C

flected by Good's coverage values exceeding 99.4% for all groups and rarefaction curves approaching a plateau (ESM Figure S1B). As summarised in Table 6, the drinking water temperature did not significantly influence the α-diversity or community composition (β-diversity) of the intestinal microbiota ($P > 0.05$; ESM Figure S2B). The overall microbial structure was dominated at the phylum level by Bacillota and Bacteroidota, which together accounted for over 90% of the community (Figure 3A). At the genus level, the most abundant taxa were UCG-005, Rikenellaceae_RC9_gut_group, norank_f_Muribaculaceae, and unclassified_f_Lachnospiraceae (Figure 3B).

Sequencing information and diversity of the intestinal microbiota. Significant differences

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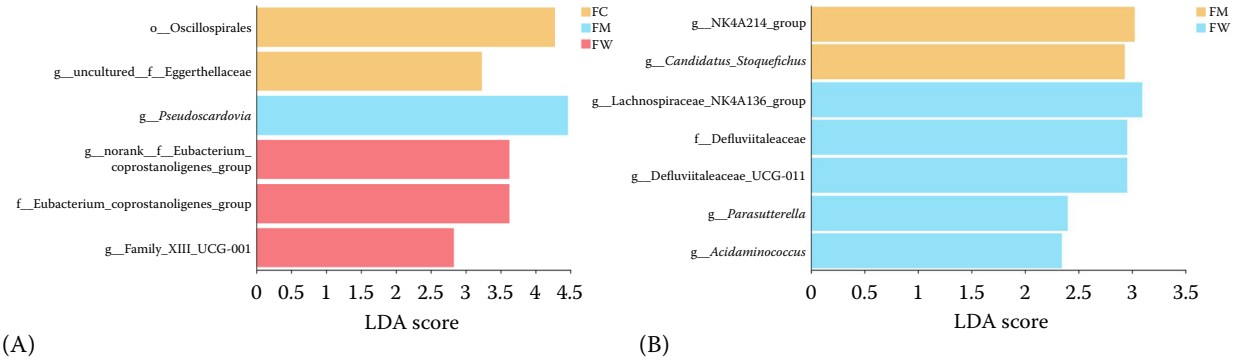

Figure 2. Linear discriminant analysis effect size (LEfSe) analysis of gut microbiota between three groups
Only taxa with LDA scores >2.0 are shown; The length of the bar represents the effect size (LDA score)
(A) Rumen; (B) Faeces
FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C

Table 6. Effect of drinking water temperature on the α-diversity and taxonomic analysis (relative abundance, %) at the genus level of faecal microbiota in growing calves

Item	Treatment			SEM	*P*-value
	FC	FM	FW		
α-diversity					
Community diversity					
Shannon	4.38	4.49	4.59	0.091	0.343
Simpson	0.041	0.042	0.030	0.012	0.797
Community richness					
Sobs	522	552	559	22.3	0.491
Ace	625	650	630	24.9	0.755
Chao1	662	658	639	26.2	0.803
Genus level					
NK4A214_group	0.180^{b}	0.370^{a}	0.370^{ab}	0.052	0.048
Lachnospiraceae_NK4A136_group	0.110^{b}	0.140^{b}	0.320^{a}	0.051	0.023
Candidatus_Stoquefichus	0.036^{b}	0.234^{a}	0.160^{ab}	0.060	0.048
Defluviitaleaceae_UCG-011	0.059^{b}	0.101^{b}	0.220^{a}	0.030	0.019
Unclassified_f__Butyricicoccaceae	0.021^{a}	0.010^{b}	0.021^{a}	0.004	0.024
Acidaminococcus	0.003^{b}	0.002^{b}	0.02^{a}	0.007	0.020

^{a,b}Treatment effects with different superscripts within a row are significantly different ($P < 0.05$)
FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C; SEM = standard error of the mean

in specific bacterial genera were observed in the faecal microbiota. The abundances of NK4A214_group and *Candidatus_Stoquefichus* were significantly higher in the FM group than in the FC group ($P < 0.05$). In the FW group, the abundances of Lachnospiraceae_NK4A136_group and Defluviitaleaceae_UCG-011 were significantly elevated compared to the FM and FC groups ($P < 0.05$; Table 6). LDA identified one bacterial family and six bacterial genera as discriminative features (Figure 2B). Specifically, the genera NK4A214_group and *Candidatus_Stoquefichus* were significantly enriched in the FM group, while Lachnospiraceae_NK4A136_group, Defluviitaleaceae_UCG-011, *Parasutterella*, and *Acidaminococcus* were enriched in the FW group.

Correlation analysis between the microbiota and serum parameters further revealed several significant associations (Figure 4). In the rumen, the genus Lachnospiraceae_NK3A20_group

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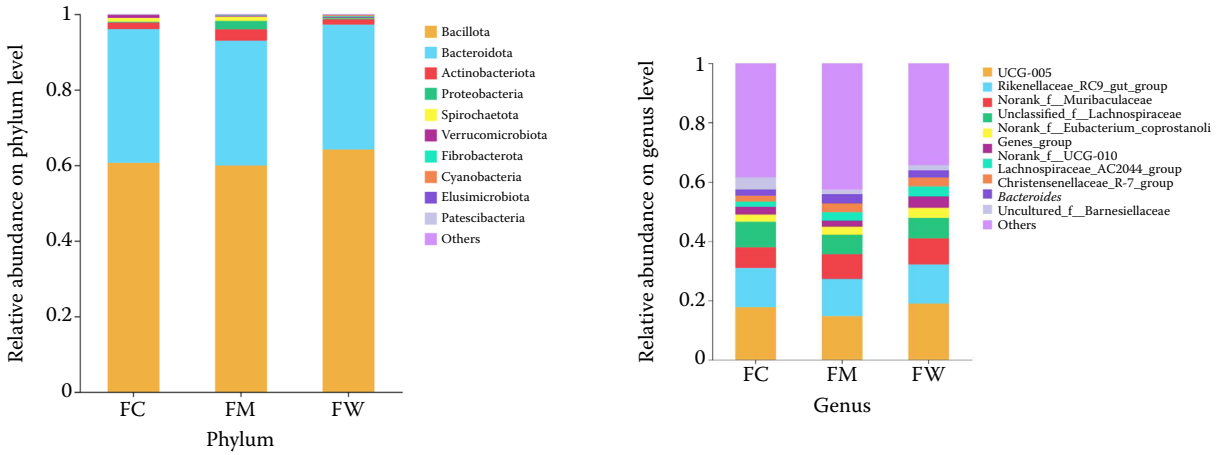

Figure 3. Composition of faecal microbiota at the phylum and genus levels at different drinking water temperatures
FC = 5 °C to 10 °C; FM = 20 °C to 25 °C; FW = 35 °C to 40 °C

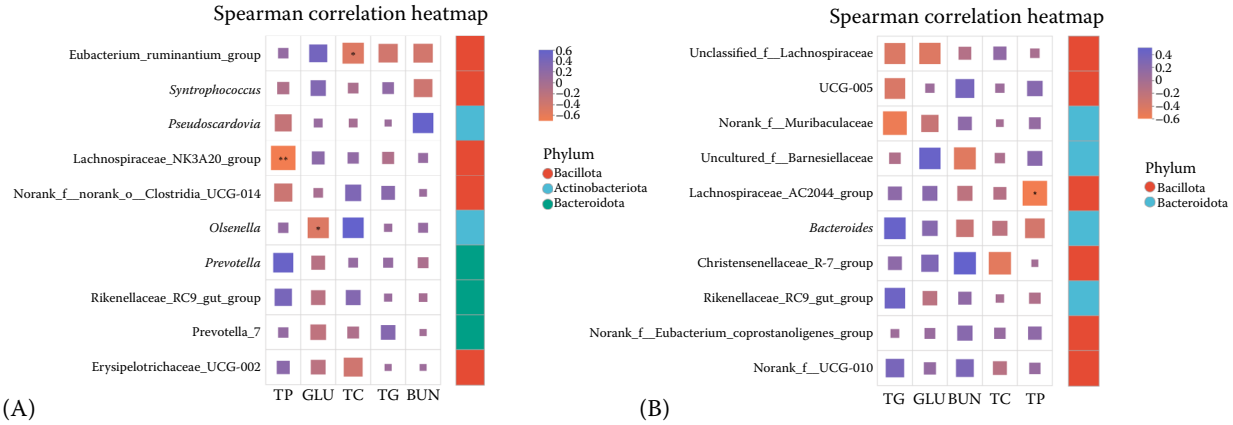

Figure 4. Generation of heatmaps to describe the relationship between blood parameters and microbiota genera in the rumen (A) and faeces (B)
Differences were defined as significant with $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 15$

showed a significant negative correlation with TP ($r = -0.7$, $P = 0.004$). In faecal samples, the genus Lachnospiraceae_AC2044_group was negatively correlated with blood TP ($r = -0.525$, $P = 0.044$).

A two-matrix correlation heatmap (Pearson) was employed to evaluate the relationship between ruminal and intestinal microbiota within each treatment group, based on α-diversity indices (Figure 5).

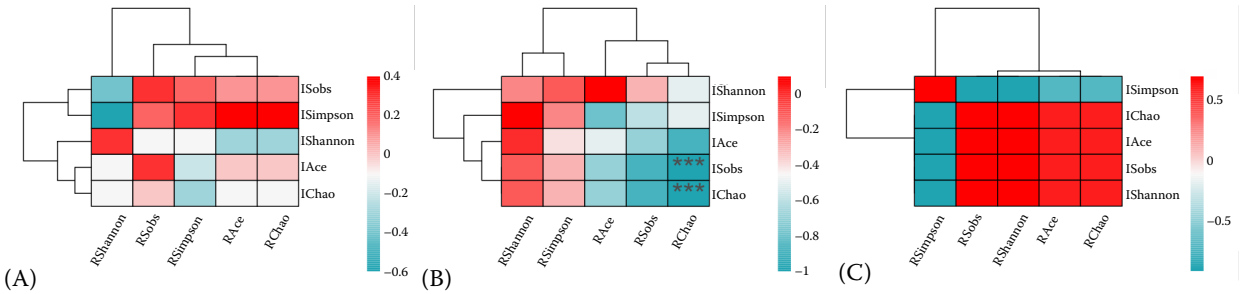

Figure 5. The correlation analysis of the correlation between ruminal and faecal microbiota in each group using Two-Matrix Correlation Heatmap (Pearson) to analyse the α-diversity index of microbiota
The correlation heatmaps for each group show the 5 °C to 10 °C group (A), 20 °C to 25 °C group (B), and 35 °C to 40 °C group (C). R represents the ruminal outcome, while I represents the faecal outcome. Significance was established at $^{*}P < 0.05$; $^{***}P < 0.001$; $n = 5$

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The corresponding correlation coefficients (r) and P-values are detailed in ESM Table S7. Notably, a significant negative correlation was observed between the ruminal and intestinal microbial communities in the FM group (Figure 5B). Specifically, the ruminal Chao1 index was strongly negatively correlated with the intestinal Chao1 index ($r = -0.921$, $P < 0.001$). In contrast, no significant correlations were detected between the ruminal and intestinal microbiota in the FC and FW groups (Figure 5A and 5C).

DISCUSSION

Our previous study demonstrated that growing calves exhibit a preference for warm water (approximately 35 °C) in cold environments (Zhang et al. 2022). The present findings are consistent with this fact, showing that under free-drinking conditions, calves offered water at 35–40 °C (FW group) consumed significantly more water (15.02 l/d) than those receiving water at 5–10 °C (10.22 l/d, FC group) or 20–25 °C (11.17 l/d, FM group).

The increased water intake in the FW group was, however, accompanied by a significant reduction in DMI. We speculate that the large volume of consumed warm water may have occupied the rumen space, enhanced satiety, and potentially relayed inhibitory signals to feeding centres in the hypothalamus, thereby suppressing appetite (Yousefvand and Hamidi 2021). Consequently, ADG was significantly lower in the FW group. This contrasts with a study by Liu et al. (2023), who reported improved ADG in winter-housed calves drinking 20 °C water, which was attributed to increased DMI. The discrepancy highlights that the benefits of warm water may exist within an optimal temperature range, and the 35–40 °C range used in our study might be excessive, leading to suppressed feed intake and growth.

Furthermore, the high water intake in the FW group was associated with elevated faecal scores and diarrhoea frequency. Excessive water consumption can dilute intestinal contents and increase water excretion via urine, respiration, and faeces (Gurung and Acharya 2018), placing additional stress on the urinary system and potentially leading to digestive disturbances.

Regarding the metabolic and oxidative status, the FW group exhibited significantly lower serum BUN on day 15, suggesting impaired protein utilisation (Gurung and Acharya 2018). While we found no effects on serum GLU or TC, the FW and FM groups showed altered oxidative stress markers, including reduced CAT and GSH-Px activities and elevated MDA concentrations, indicating a compromised antioxidant capacity.

This finding contrasts with a report on beef cattle where warmer water reduced oxidative stress (He et al. 2023), suggesting species- or condition-specific responses. Notably, the drinking water temperature did not affect the serum COR levels, implying that the post-weaning transition to warm water did not induce a measurable stress response in these calves.

Collectively, these results indicate that while calves prefer warm water, excessive consumption at 35–40 °C in winter suppresses feed intake, retards growth, disrupts digestive health, and induces oxidative stress. Furthermore, the warm water group (35–40 °C) exhibited an increased abundance of potential gastrointestinal pathogens and a reduction in beneficial bacteria, a pattern not observed in the cold water group (5–10 °C). In the present study, Bacillota and Bacteroidota were identified as the dominant phyla in the rumen, which aligns with our previous findings in post-weaned calves (Wu et al. 2023). At the genus level, Prevotella_7 and Erysipelotrichaceae_UCG-002 were the predominant ruminal taxa.

Notably, the abundance of *Pseudoscardovia* in the rumen was significantly higher in the 35–40 °C and 20–25 °C groups than in the 5–10 °C group. *Pseudoscardovia*, a genus within the Bifidobacteriaceae family originally isolated from the wild boar intestine (Killer et al. 2014), has been positively associated with several disease states, including abnormal liver metabolism (Henning et al. 2018) and tuberculosis (Maji et al. 2018).

In addition, the abundance of Family_XIII_UCG-001 was significantly increased in the rumen of calves drinking water at 35–40 °C. Interestingly, this genus has been correlated with higher rumen keratinisation scores (Maji et al. 2018), yet other studies suggest it may help alleviate lipid metabolic disorders (Zhong et al. 2025). The reason for this apparent functional discrepancy remains unclear and warrants further investigation.

No significant effect of drinking water temperature was observed on the α-diversity or β-diversity of the intestinal microbiota in calves. At the ge-

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nus level, the dominant taxa were UCG-005 and Rikenellaceae_RC9_gut_group.

Notably, the abundance of NK4A214_group – a rumen bacterium involved in cellulose degradation (Guo et al. 2022), was significantly higher in the intestine of the 20–25 °C group than in the 5–10 °C group. In poultry, this genus has also been recognised for its role in maintaining the microbial balance (Zhang et al. 2023). Similarly, *Candidatus_Stoquefichus*, a member of the Erysipelotrichaceae family, was most abundant in the 20–25 °C group. This genus has been reported to negatively correlate with serum inflammatory cytokines in mice (Yang et al. 2021) and is implicated in host lipid metabolism and inflammatory regulation (Kaakoush 2015). Furthermore, *Candidatus_Stoquefichus* may help suppress inflammatory cytokine production and support the intestinal barrier function, thereby facilitating the host energy absorption (Zhou et al. 2023).

An interesting finding concerns the relationship between ruminal and intestinal microbial communities. While one study reported a significant negative correlation between these communities in untreated control calves (Wu et al. 2022), we observed a similar negative correlation specifically in the 20–25 °C group. By contrast, no such rumen-gut correlation was detected in calves drinking water at 5–10 °C or 35–40 °C. This suggests that drinking water temperatures at either extreme may disrupt the functional synergy between the ruminal and intestinal microbiota.

CONCLUSION

In conclusion, providing warm drinking water (35–40 °C) in a cold winter environment adversely affects the growth performance and enriches the gastrointestinal tract with potentially pathogenic microorganisms of growing calves. These findings provide a theoretical basis for optimising water management strategies for calves.

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Conflict of interest

The authors declare no conflict of interest.

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