

Article

Metagenomic Insights into the Modulatory Effects of Thiamine Supplementation for Treating Subclinical Ketosis Dairy Cows

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Simple Summary

Subclinical ketosis (SCK) is commonly induced in the early lactation period due to severe negative energy balance (NEB) and the accumulation of β -hydroxybutyrate (BHBA), which disrupts the rumen microbial ecosystem and decreases milk yield. This research thoroughly investigates the alleviative effect of thiamine on SCK and its modulatory effects on rumen microbial communities. The results indicate that thiamine significantly decreased BHBA content, milk colony-forming units (CFUs), and somatic cell counts (SCCs), while it significantly increased milk yield, milk fat, acetate content, and the A/P ratio ($p < 0.05$). The microbial community results revealed that thiamine-treated cows exhibited a significantly increased relative abundance of ruminal and fecal *Proteobacteria* and significantly decreased ruminal *Firmicutes* ($p < 0.05$) and fecal *Spirochaetes* and *Cyanobacteria* ($p < 0.05$), compared with SCK cows. These findings provide a theoretical basis and a practical method for alleviating SCK in early-lactation dairy cows and increasing the milk yield of SCK cows.

Abstract

(1) Background: The objective of this study was to investigate the modulatory effects of thiamine on BHBA metabolism, milk yield, and the rumen microbial ecosystem. (2) Methods: A total of 24 SCK dairy cows with similar body conditions were selected and randomly allocated to SCK (SCK) or SCK with thiamine supplement (SCKT) treatment. Twelve healthy dairy cows served as the control (CON) treatment. Milk yield, milk quality, ruminal fermentability parameters, rumen and fecal microbial communities were further measured. (3) Results: Thiamine significantly decreased BHBA content, milk CFUs, and somatic cells, while significantly increasing milk yield, milk fat, acetate, and the A/P ratio ($p < 0.05$). Thiamine-treated cows exhibited significantly increased ruminal and fecal *Proteobacteria* but significantly decreased ruminal *Firmicutes* ($p < 0.05$) as well as fecal *Spirochaetes* and *Cyanobacteria* ($p < 0.05$), compared with SCK cows. Functional analysis showed that differential rumen bacteria exhibited high energy metabolism, nucleotide metabolism, and glycan biosynthesis and metabolism, while the metabolism of terpenoids and polyketides were the primary functional pathways of differential fecal microbiota. (4) Conclusions: Thiamine supplementation in SCK cows effectively alleviated subclinical ketosis by reducing BHBA content, enhancing ruminal fermentability, and proliferating rumen microbial communities, leading to improved milk yield in the early-lactation period.

Keywords: subclinical ketosis; dairy cows; thiamine; early lactation; beta-hydroxybutyrate



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1. Introduction

Subclinical ketosis (SCK) commonly occurs during early lactation in dairy cows due to continuous negative energy balance (NEB) and the accumulation of β -hydroxybutyrate (BHBA) catalyzed by the conversion of incompletely β -oxidized acetoacetyl-CoA [1–3]. Elevated BHBA levels disrupt the rumen microbial ecosystem, causing a significant decrease in the relative abundance of ruminal carbohydrate-utilizing bacteria, feed utilization efficiency, and production performance [4–6]. SCK has a global incidence rate of 22.7% (with a range of 15–30%) in China, which has significantly restricted dairy production and caused considerable economic loss [7]. Glucose injection is commonly used to alleviate SCK; however, this strategy fails to address the fundamental issues of BHBA breakdown and utilization, leading to recurrence [8–10]. Therefore, exploring effective methods of raising ruminal fermentability and energy provision in the early-lactation period may provide a solution to attenuate SCK.

Emerging evidence identifies succinyl-CoA as a central regulatory node in BHBA metabolism, evidenced by an inverse association with the activity of 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS)—a key enzyme in the generation of hepatic BHBA [11,12]—and a positive correlation with the peripheral enzymatic activity of succinyl-CoA:3-ketoacid CoA transferase (SCOT), the rate-limiting step for BHBA utilization. Thiamine has been found to functionally act as a coenzyme in the form of thiamine pyrophosphate (TPP) for the α -ketoglutarate dehydrogenase complex (α -KGDH), a critical rate-limiting factor in catalyzing succinyl-CoA biosynthesis [13,14]. In addition, thiamine supplementation has been reported to promote ruminal succinate-producing bacteria, including *Succinivibrionaceae*, *Succiniclaticum*, and *Succinivibrio* [15], which may further enhance succinyl-CoA generation and alleviate SCK.

Our previous studies demonstrated that supplementation with 180 mg thiamine/kg DMI effectively modulates rumen metagenomic capacity, enhances the stability of rumen microbial structure, and increases the relative abundances of fiber- and starch-degrading bacteria [16,17]. This further enhanced nutritional degradability and energy provision, thereby attenuating SCK. Therefore, in the present study, SCK dairy cows were supplemented with 180 mg thiamine/kg DMI to investigate its capability to treat SCK. We hypothesized that thiamine supplementation in SCK cows would functionally interact with succinyl-CoA biosynthesis to reduce BHBA content and help promote ruminal fermentability to enhance milk yield and milk quality.

2. Materials and Methods

2.1. Animal Preparation and Experimental Design

Dairy cows were reared at the dairy farm of Modern Farming (Suqian) Co., Ltd., Suqian, China (33.82 N, 117.56 E). The animal care and experimental procedures were carried out following the Chinese Guidelines for Animal Welfare and were approved by the Animal Care and Use Committee of Jiangxi Agricultural University (approval number: JXAULL-20250425).

A total of 1126 dairy cows, all within the same lactation period and reared in the same stall, underwent blood sampling to detect subclinical ketosis (SCK), defined as a filtrate concentration of blood beta-hydroxybutyrate >1.2 mmol/L. A total of 24 SCK dairy cows with similar body weights (682 ± 37.3 kg), milk yields (45.3 ± 4.3 kg), and lactation days ($30 \text{ d} \pm 5.3 \text{ d}$) were selected to determine the alleviative effects of thiamine supplementation on SCK and were then randomly allocated to one of two groups: SCK (SCK) or SCK with thiamine supplement (SCKT) treatment. Each group contained 12 cows, and each cow was considered a replicate. Twelve healthy dairy cows with similar body weights, milk yields, and lactation days were selected as the control (CON) group. Thiamine (thiamine

hydrochloride, purity > 99%), obtained from Shandong Luyao Biotechnology Co., Ltd. (Heze, China), was provided to cows at a rate of 180 mg/kg DMI per day based on our previous study [16,17].

All selected cows were reared in an isolated barn and individually fed in separate pens, and each cow was considered a replicate. Diets were formulated according to NRC (2001) to meet the energy requirements of Holstein dairy cows yielding 40 kg of milk/day with 3.5% milk fat and 3.0% true protein. The ingredients and nutritional composition of each treatment are shown in Table 1. Thiamine was provided at a rate of 180 mg thiamine/kg DMI based on our previous study and the results of a preliminary experiment. Cows were fed two times per day at 06:00 and 18:00, and water was made available *ad libitum*. Dry matter intake and milk yield were recorded individually.

Table 1. Ingredients and chemical composition of the feedstuff.

Ingredients	Percentage (%)
Corn silage	20.5
Ground corn	19.7
Cottonseed meal	3.4
Alfalfa hay	10.5
Chinese wildrye	10.2
Distiller-Dried Grains with Solubles (DDGSs)	3.1
Steam-flaked corn	12.5
Soybean meal	12
Beet pulp	4.5
Premix ¹	3
NaCl	0.6
Total	100
Chemical composition	
DM	51.2
NE _{mf} (MJ/kg) ²	7.13
EE	4.56
CP	18.1
ADF	18.6
NDF	27.6
Starch	30.8
Ca	0.69
p	0.44

1. The components contained in the premix were as follows: Fe, 1400 mg; Cu, 1200 mg; Mn, 2400 mg; Zn, 5500 mg; Se, 40 mg; Co, 30 mg; I, 90 mg; VA, 900,000 IU; VD, 700,000 IU; VE, 9000 IU. 2. $NE_{mf} = \{322 LBW^{0.75} + [(2092 + 25.1 \times LBW) \times ADG / (1 - 0.3 \times ADG)]\} \times F$. LBW = live body weight; ADG = average daily weight gain; F = correction coefficient; DM = dry matter; EE = ether extract; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber.

2.2. Milk Yield and Milk Quality Measurement

All cows underwent milking three times per day at 07:00, 14:00, and 21:00, and milk yield was automatically recorded by the rotary milking equipment (9JRP-50P2100, Delaval, Stockholm, Sweden). Milk quality—including the milk protein, milk fat, nonfat solid content, milk density, acidity, and milk microbial colony-forming units (CFUs)—was measured during the last three consecutive days using a near-infrared analyzer (MilkoScanTM 7 RM, Foss Electric, Copenhagen, Denmark). Somatic cell count (SCC) was measured using a rapid SCC analyzer (Fossomatic 7/7 DC, FOSS).

2.3. Effects of Thiamine Supplement on Ruminal Fermentability Parameters

A rumen fluid sample was collected from each cow on the last day of the experiment through an esophageal tube and immediately divided into two portions. One was immediately used to conduct the following measurements: rumen pH was measured using a Testo 206-pH pH meter (Testo Instruments International (Shanghai) Co., Ltd., Shanghai, China); rumen volatile fatty acid (VFA) content, including acetate, propionate, and butyrate, was measured using a GC-2010 gas chromatograph (Shimadzu, Kyoto, Japan); ammonia-N (NH₃-N) content was determined via the indophenol method, with its absorbance value measured using a UV-2600 ultraviolet spectrophotometer (Tianmei Ltd., Shanghai, China) at a 700 nm wavelength; and microbial protein (MCP) was detected with a bicinchoninic acid protein quantification kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China). The other portion was frozen using liquid nitrogen and stored at −80 °C for rumen microbiota measurement.

2.4. Rumen and Fecal Microbial Community Measurement

A fecal sample from each cow was collected on the last day by rectal sampling and frozen at −80 °C for fecal microbial community measurement. To measure rumen and fecal microbial communities, we used the same method as in our previous study. Simply stated, DNA from each rumen fluid sample and fecal sample was extracted and purified using a Bacterial Genome DNA Extraction Kit (DP302, Tiangen, Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) and a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany), and 16s rRNA sequencing was conducted using the Illumina HiSeq 4000 platform (Illumina Incorporated Company, San Diego, CA, USA).

Quantitative Insights Into Microbial Ecology (QIIME, V2.0, San Diego, CA, USA; <https://qiime.org/>) was applied for quality-filtering raw tags according to the standard quality control process for obtaining clean, high-quality tags. The filtration method included primer/adaptor removal; accurately trimming the sequencing primer and adaptor sequences; discarding sequences that were too short or too long (typically, the length of the target variable region should be retained; in this case, it was 450 bp for the V3–V4 region); and chimera removal, which involved identifying and removing chimera sequences resulting from PCR recombination of different parent sequences. Sequences with similarity of >97% were assigned to the same operating taxonomic units (OTUs). The Green Gene Database (<http://greengenes.secondgenome.com>) (accessed on 15 September 2025), based on the SILVA classifier algorithm, was used to annotate taxonomic information. Functional predictions based on the differentially proliferated ruminal microbiota were conducted using the Tax4Fun method [18].

2.5. Statistical Analysis

The milk yield, milk quality, and rumen fermentation variables were firstly tested for distribution normality using the SAS (26.0) procedure “proc univariate data = test normal”, followed by a one-way ANOVA S-N-K test using SAS (SAS Institute, Inc., Cary, NC, USA). $p < 0.05$ indicated statistical significance; $0.05 \leq p < 0.10$ indicated a tendency. Principle coordinate analysis (PCoA) was conducted using the ggplot2 package in R software (Version 3.15.3, R Core Team, Vienna, Austria).

3. Results

3.1. Effect of Thiamine Supplementation on the Productive Performance, Milk Quality, and Fermentability Parameters of Dairy Cows with Subclinical Ketosis

The effects of thiamine supplementation on the productive performance of SCK dairy cows were investigated, and the results (shown in Table 2) demonstrate that BHBA content

was significantly higher in SCK and SCKT cows compared with CON cows ($p < 0.05$), indicating the accuracy of our sample collection. Thiamine supplementation significantly decreased the BHBA content ($p < 0.05$), confirming that it can alleviate SCK.

Table 2. Effects of thiamine supplementation on the productive performance of dairy cows with subclinical ketosis ($n = 12$).

Items	Experimental Treatments			SEM	<i>p</i> -Value
	CON	SCK	SCKT		
Milk yield (Pre)	44.75	44.26	44.13	1.36	0.674
Milk yield (Post)	44.12 ^a	40.36 ^b	43.79 ^a	1.78	0.031
DMI (Pre)	22.46	22.64	22.58	0.86	0.364
DMI (Post)	22.37	21.31	22.46	0.79	0.091
β -hydroxybutyrate (Pre) (mmol/L)	0.76 ^b	1.41 ^a	1.40 ^a	0.23	0.014
β -hydroxybutyrate (Post) (mmol/L)	0.76 ^b	1.28 ^a	0.97 ^b	0.29	0.039

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean; DMI = dry matter intake. “a” and “b” in the same row indicate a significant discrepancy between all groups.

SCK cows showed a significant decrease in milk yield ($p < 0.05$), and a trend of decreasing DMI ($0.05 < p < 0.10$) compared with CON cows, while milk yield was significantly higher in cows receiving thiamine treatment compared with SCK cows ($p < 0.05$). No other significant changes in residue parameters were observed among all treatments.

The results of the measurements of milk quality parameters, including milk fat, milk protein, nonfat solid content, CFUs, and somatic cells, are shown in Table 3. SCK cows exhibited a significant decrease in milk fat content and a significant increase in CFUs and somatic cells compared with CON cows ($p < 0.05$). Cows receiving the thiamine supplement exhibited significantly increased milk fat content and significantly decreased CFUs and somatic cells compared with SCK cows ($p < 0.05$).

Table 3. Effects of subclinical ketosis and thiamine supplementation on the milk quality of dairy cows ($n = 12$).

Items	Experimental Treatments			SEM	<i>p</i> -Value
	CON	SCK	SCKT		
Milk fat	3.86 ^a	3.62 ^b	3.81 ^a	0.16	0.016
Milk protein	3.44	3.41	3.42	0.26	0.153
Nonfat solid content	8.56	8.36	8.52	0.44	0.224
Dry matter content	12.32	11.78	12.11	0.32	0.106
Milk density	1.03	1.029	1.029	0.004	0.801
Acidity	13.2	13.1	13.1	0.055	0.534
CFUs (10^3)	6.52 ^b	12.20 ^a	7.41 ^b	1.74	0.027
Somatic cells (10^3)	5.41 ^b	7.27 ^a	5.46 ^b	0.36	0.037

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean; CFUs = colony-forming units. “a” and “b” in the same row indicate a significant discrepancy between all groups.

The effects of thiamine supplementation on the ruminal fermentability parameters of SCK dairy cows, including the VFAs, rumen MCP, and rumen $\text{NH}_3\text{-N}$, are shown in Table 4. Acetate, propionate, and butyrate contents significantly decreased in SCK dairy cows compared with CON cows ($p < 0.05$). Cows supplemented with thiamine exhibited significantly increased acetate content compared with SCK cows ($p < 0.05$); however, no significant discrepancies were observed in propionate and butyrate contents. A significant increase in the A/P ratio was detected in SCKT treatment dairy cows compared with the

CON and SCK groups ($p < 0.05$). MCP content, ammonia content, and ruminal pH showed no significant differences among all dairy cows groups.

Table 4. Effects of subclinical ketosis and thiamine supplementation on ruminal fermentability of dairy cows ($n = 12$).

Items	Experimental Treatments			SEM	<i>p</i> -Value
	CON	SCK	SCKT		
Ruminal pH	5.97	6.01	6.02	0.094	0.216
MCP (mg dL ⁻¹)	24.16	23.23	23.34	0.741	0.143
Acetate (mmol/L)	47.24 ^a	44.26 ^b	46.27 ^a	1.273	0.028
Propionate (mmol/L)	17.49 ^a	16.11 ^b	16.13 ^b	0.632	0.027
Acetate/propionate	2.70 ^b	2.74 ^b	2.87 ^a	0.113	0.043
Butyrate (mmol/L)	12.77 ^a	11.28 ^b	11.75 ^{ab}	0.579	0.036
Ammonia-N (mg/100 mL)	12.49	13.54	12.77	0.981	0.106

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean; MCP = microbial protein. “a” and “b” in the same row indicate a significant discrepancy between all groups.

3.2. Effect of Thiamine Supplementation on Ruminal and Fecal Microbial Diversity of Dairy Cows with Subclinical Ketosis

A total of 16 phyla and 1674 genera of rumen microbiota, as well as 12 phyla and 1076 genera of fecal microbiota, were identified after quality control. Table S1 presents the identified microbial communities, all of which were first subjected to α -diversity analysis, and the results are shown in Table 5.

Table 5. Effects of thiamine supplementation on the microbial α -diversity of dairy cows with subclinical ketosis ($n = 12$).

Items		Experimental Treatments			SEM	<i>p</i> -Value
		CON	SCK	SCKT		
Rumen	Shannon	7.85	7.46	7.66	0.11	0.182
	Simpson	0.98	0.98	0.98	0.001	0.342
	Ace	2356.5 ^a	2171.4 ^b	2214.4 ^b	52.34	0.018
	Goods_coverage	0.99	0.99	0.99	0.001	0.101
	Chao1	2256.3 ^a	2116.6 ^b	2187.4 ^{ab}	31.24	0.032
	Observed_species	1934.9 ^a	1772.5 ^b	1822.4 ^b	44.84	0.017
	PD_whole_tree	182.36 ^a	171.98 ^b	171.98 ^b	2.94	0.021
Fecal	Shannon	6.922	6.914	7.053	0.131	0.512
	Simpson	0.944	0.93	0.937	0.006	0.386
	Ace	2232.7 ^a	2034.2 ^b	2123.4 ^{ab}	62.7	0.025
	Goods_coverage	0.991	0.991	0.992	0.001	0.339
	Chao1	2133.5 ^a	1919.5 ^b	2009.5 ^b	57.6	0.019
	Observed_species	1807.7 ^a	1664.6 ^b	1744.6 ^{ab}	58.6	0.011
	PD_whole_tree	145.2 ^a	134.4 ^b	141.4 ^a	4.61	0.009

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean. “a” and “b” in the same row indicate a significant discrepancy between all groups.

3.2.1. α -Diversity

Table 4 presents the results of the α -diversity analysis, including the Chao1, Shannon, Simpson, Goods_coverage, Observed_species, Ace, and PD_whole_tree indexes. SCK cows showed a significant decline in ruminal α -diversity, evidenced by the significant decrease in the Ace, Chao1, Observed_species, and PD_whole_tree indexes, compared with CON cows

($p < 0.05$). None of the α -diversity indexes showed a significant increase after thiamine supplementation ($p > 0.05$).

In addition, SCK cows showed significant decreases in fecal microbial α -diversity, including the Ace, Chao1, Observed_species, and PD_whole_tree indexes, compared with CON cows ($p < 0.05$). The PD_whole_tree index significantly increased after thiamine supplementation ($p < 0.05$); however, no significant differences were observed in the Chao1, Shannon, and Simpson indexes among the CON, SCK, and SCKT treatment groups.

3.2.2. β -Diversity

The beta-diversity of rumen and fecal microbial communities among the CON, SCK, and SCKT groups was assessed through principal coordinate analysis (PCoA). Figure 1A shows the discrepancies in ruminal microbiota, where it can be seen that PCoA axes 1 and 2 accounted for 20.9% and 9.83% of the total discrepancies, respectively. The microbial communities of SCK cows are clearly separated from those of CON cows on PCoA axes 1 and 2, while the microbial communities of thiamine-supplemented cows show strong cohesiveness compared with those of the other two groups, and a significant discrepancy from those of SCK cows, on PCoA axes 1 and 2. Figure 1B displays the results of the differential analysis of fecal microbiota, where it can be seen that PCoA axes 1 and 2 accounted for 21.38% and 10.92% of the total discrepancies, respectively. The microbial communities of SCK cows are clearly separated from those of CON cows on PCoA axes 1 and 2, while the microbial communities of thiamine-supplemented cows are distributed between the CON and SCK groups.

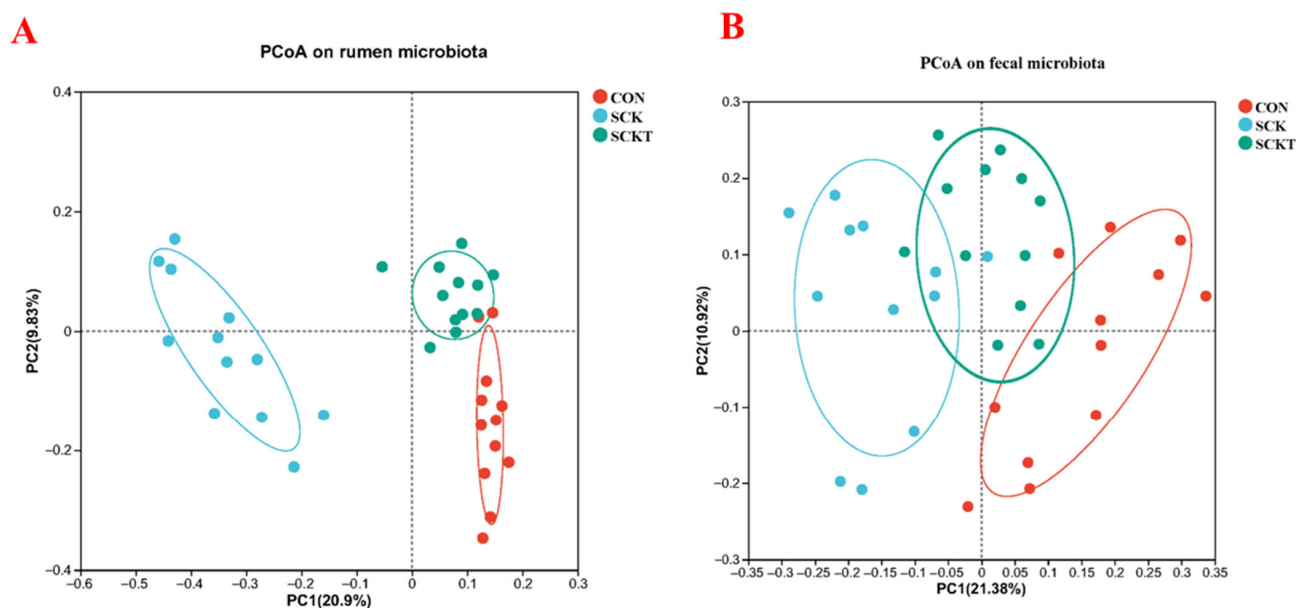


Figure 1. Principal coordinate analysis (PCoA) of the community structures of the (A) ruminal and (B) fecal microbiota among the CON, SCK, and SCKT groups. CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement.

The results of the differential analysis for the relative abundance of rumen and fecal microbial communities among the CON, SCK, and SCKT groups are shown in Tables 6 and 7 and Figure 2.

Table 6 displays the relative abundances of rumen microbial communities at the phylum level in the SCK and SCKT treatment groups. *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* represented the three most abundant microbial communities and accounted for 95% of the total rumen microbial biomass. SCK cows exhibited significantly increased relative abun-

dances of Firmicutes and significantly decreased *Fibrobacteres* and *Actinobacteria* ($p < 0.05$) compared with CON cows. Thiamine-supplemented cows exhibited significantly increased *Proteobacteria* and significantly decreased *Firmicutes* ($p < 0.05$) compared with SCK cows. However, no significant differences were observed in *Fibrobacteres* and *Actinobacteria* after thiamine treatment, and no other significant changes were observed among all treatments ($p > 0.05$).

Table 7 displays the relative abundances of fecal microbial communities at the phylum level in the SCK and SCKT treatment groups. Unlike the rumen microbiota, *Firmicutes* contributed most of the total biomass, and *Firmicutes*, *Bacteroidetes*, and *Spirochaetes* represented the three most abundant microbial communities. SCK cows exhibited significantly increased relative abundances of *Firmicutes* and significantly decreased *Bacteroidetes* ($p < 0.05$) compared with CON cows. Thiamine-supplemented cows exhibited significantly increased *Proteobacteria* and significantly decreased *Spirochaetes* and *Cyanobacteria* ($p < 0.05$) compared with SCK cows. No other significant changes were observed among all treatments ($p > 0.05$).

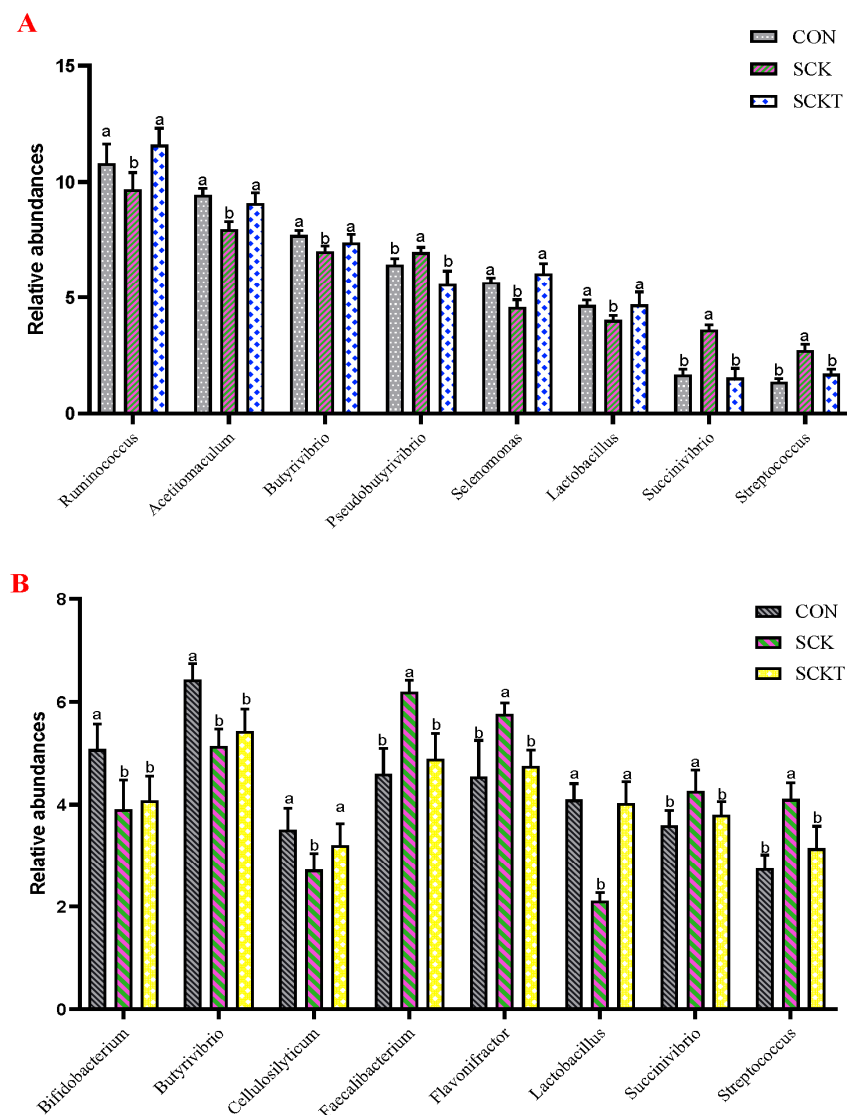


Figure 2. Differential analysis of the relative abundances of (A) ruminal and (B) fecal microbiota among the CON, SCK, and SCKT groups ($n = 12$). CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement. “a” and “b” indicate a significant discrepancy between CON, SCK and SCKT treatments.

Table 6. Effects of thiamine supplementation on rumen bacterial composition of dairy cows with subclinical ketosis (at the phylum level, $n = 12$).

Items	Experimental Treatments			SEM	<i>p</i> -Value
	CON	SCK	SCKT		
<i>Bacteroidetes</i>	41.78	40.21	40.51	1.274	0.441
<i>Firmicutes</i>	38.26 ^b	42.12 ^a	39.57 ^b	0.935	0.031
<i>Proteobacteria</i>	12.06 ^{ab}	11.03 ^b	12.83 ^a	0.769	0.016
<i>Fibrobacteres</i>	1.99 ^a	1.13 ^b	1.64 ^{ab}	0.138	0.028
<i>Spirochaetes</i>	0.95	0.91	1.04	0.166	0.187
<i>Actinobacteria</i>	2.42 ^a	1.12 ^b	1.71 ^b	0.244	0.008
<i>Verrucomicrobia</i>	0.32	0.32	0.34	0.031	0.407
<i>Chlamydiae</i>	0.44	0.51	0.59	0.059	0.101
<i>Cyanobacteria</i>	0.19	0.14	0.14	0.056	0.081
<i>Tenericutes</i>	0.14	0.18	0.16	0.017	0.301
<i>Fusobacteria</i>	0.07	0.08	0.12	0.011	0.158
Others	1.88	2.25	1.95	0.316	0.312

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean. "a" and "b" in the same row indicate a significant discrepancy between all groups.

Table 7. Effects of thiamine supplementation on fecal bacterial composition of dairy cows with subclinical ketosis (at the phylum level, $n = 12$).

Items	Experimental Treatments			SEM	<i>p</i> -Value
	CON	SCK	SCKT		
<i>p__Firmicutes</i>	29.34 ^b	32.24 ^a	30.35 ^b	1.31	0.024
<i>p__Bacteroidetes</i>	26.57 ^a	23.24 ^b	26.17 ^a	1.12	0.038
<i>p__Spirochaetes</i>	8.02 ^{ab}	8.68 ^a	7.36 ^b	0.41	0.033
<i>p__Tenericutes</i>	7.96	8.03	7.55	0.43	0.201
<i>p__Actinobacteria</i>	6.47	5.46	6.51	0.76	0.129
<i>p__Proteobacteria</i>	5.94 ^{ab}	5.12 ^b	6.67 ^a	0.45	0.034
<i>p__Fibrobacteres</i>	1.48	1.33	1.42	0.42	0.486
<i>p__Cyanobacteria</i>	2.88 ^a	3.24 ^a	2.15 ^b	0.34	0.035
<i>p__Patescibacteria</i>	5.52	6.01	5.88	0.53	0.266
Others	5.86	6.65	5.94	0.62	0.322

CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement; SEM = standard error of the mean. "a" and "b" in the same row indicate a significant discrepancy between all groups.

Significantly differentially proliferated microbial communities at the genus level are listed in Figure 2. Figure 2A shows that the relative abundances of ruminal *Ruminococcus*, *Acetitomaculum*, *Butyrivibrio*, *Selenomonas*, and *Lactobacillus* significantly decreased in SCK cows compared with CON cows, while they significantly increased after thiamine supplementation ($p < 0.05$). Relative abundances of ruminal *Pseudobutyrvibrio*, *Succinivibrio*, and *Streptococcus* significantly increased in SCK cows compared with CON cows, while they significantly decreased after thiamine supplementation ($p < 0.05$). Figure 2B shows the differentially proliferated communities of fecal microbiota. Relative abundances of *Bifidobacterium*, *Butyrivibrio*, *Cellulosilyticum*, and *Lactobacillus* significantly decreased in SCK cows compared with CON cows, while those of *Faecalibacterium*, *Flavonifractor*, *Succinivibrio*, and *Streptococcus* significantly increased ($p < 0.05$). Thiamine supplementation significantly proliferated the relative abundances of *Cellulosilyticum* and *Lactobacillus*, while it significantly decreased those of *Faecalibacterium*, *Flavonifractor*, *Succinivibrio*, and *Streptococcus* ($p < 0.05$). No other significant changes were observed among all treatments.

3.3. Functional Prediction of Significantly Differentially Proliferated Microbiota in Response to Thiamine Supplementation

The functional prediction results are shown in Figure 3. Metabolism, genetic information processing, environmental information processing, and cellular process are the primary functions predicted to be enriched in the differential proliferated microbial communities. The metabolic processes of carbohydrate metabolism, amino acid metabolism, and metabolism of cofactors and vitamins were found to be the predominant functional pathways in ruminal and fecal microbial communities. Specifically, the functions of differentially proliferated rumen bacteria were primarily enriched in energy metabolism, nucleotide metabolism, and glycan biosynthesis and metabolism, while the metabolism of terpenoids and polyketides, as well as other amino acids and lipids, were the primary functional pathways enriched in differential fecal microbiota.

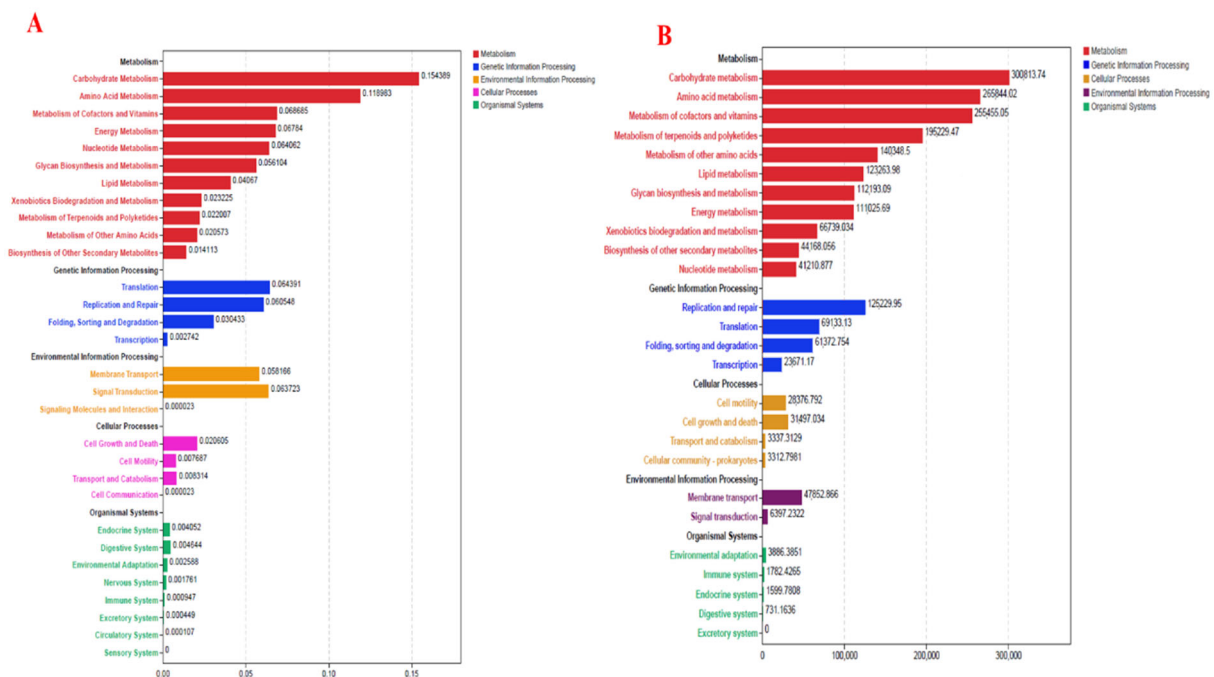


Figure 3. Tax4Fun functional prediction of the differential (A) ruminal and (B) fecal microbial communities among the CON, SCK, and SCKT groups ($n = 12$). CON = control treatment; SCK = subclinical ketosis; SCKT = subclinical ketosis with thiamine supplement.

4. Discussion

Restoring positive energy balance in SCK cows, especially for energy generated through carbohydrate utilization, is the primary factor in alleviating SCK [19]. In the present study, carbohydrate metabolism was enriched due to a significant increase in carbohydrate-degrading bacteria, including *Ruminococcus*, *Acetivomaculum*, *Butyrivibrio*, *Selenomonas*, and *Lactobacillus*, after thiamine supplementation; therefore, thiamine supplementation may represent a key factor in improving energy supply, reversing NEB status, and alleviating SCK [20–22]. The modulatory pathways may attributed to the following.

4.1. The Potentially Modulatory Effects on BHBA Metabolism

The results of the present study indicated an effective reduction in BHBA content and significantly alleviated SCK symptoms after thiamine supplementation. The reductive effects of thiamine supplementation on BHBA (as the key initiator of SCK in dairy cows) may be the primary reason for its alleviative effect. The potential pathways that modulate BHBA metabolism are as follows.

Thiamine functionally triggered the utilization of acetyl-CoA, a cofactor of TPP in the hepatic ecosystem; promoted the conversion of acetoacetyl-CoA to acetyl-CoA; and consequently reduced BHBA generation [23,24]. Elevated hepatic thiamine levels stimulate the conversion of alpha-ketoglutarate to succinyl-CoA, which was found to be negatively correlated with BHBA synthesis, potentially leading to decreased hepatic BHBA production [25,26]. Additionally, in peripheral tissues, thiamine may enhance BHBA utilization by increasing succinyl-CoA [13], which catalyzes the conversion of BHBA to acetoacetyl-CoA, improves BHBA utilization, and further alleviates SCK.

4.2. Modulatory Effects of Thiamine on Ruminal Fermentability and Milk Quality of SCK Cows

Thiamine supplementation effectively enhanced the content of acetate and butyrate, which served as the primary energy-supplying substrates for biological processes in dairy cows, which aligns with the results of Jiang (2022) [27]. The increases in acetate and butyrate were primarily due to the activation of the carbohydrate-catalytic enzyme pyruvate formate-lyase (PFL), a central enzyme in the degradation of pyruvate to acetyl-CoA, assisted by the cofactors of thiamine diphosphate (TPP) [28–30]. Acetyl-CoA in the rumen was further converted into acetate and underwent transmembrane transport into tissues to maintain fundamental bioprocess. Moreover, the increased content of acetate also made it the key substrate in milk fat synthesis, which may account for the increase in milk fat content after thiamine supplementation in SCK dairy cows.

Additionally, thiamine is indispensable for the growth and proliferation of key acetate-producing rumen bacteria, such as *Ruminococcus albus*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens*, which were significantly proliferated after thiamine supplementation and further promoted ruminal fermentation activity, leading to increased rumen VFA content [31,32]. Mammary de novo synthesis required acetate, which is primarily generated in rumen fermentation and transported through blood circulation, and represents a primary component of milk fat. Therefore, the milk fat content was significantly increased in the thiamine-supplemented group compared with the SCK group.

Thiamine supplementation was also found to significantly increase *Megasphaera elsdenii* (lactate utilizers that convert ruminal lactate into propionate) and inhibit *Streptococcus bovis* (lactate producing bacteria), which aligns with the results of the present study [33]. These alterations in the microbiota may help to reduce ruminal lactate content and inflammatory responses and to proliferate the diversity of the rumen microbiome. A higher ruminal thiamine concentration significantly proliferated rumen α -diversity and consolidated the ruminal microbial ecosystem [34], which improved the degradation of fermentable carbohydrates, consequently stabilizing ruminal pH and promoting ruminal fermentability.

5. Conclusions

In summary, thiamine supplementation in SCK cows effectively alleviated subclinical ketosis by reducing BHBA content and enhanced ruminal fermentability by increasing the relative abundances of rumen microbial communities, which consequentially enhanced milk yield in the early-lactation period.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ani16040680/s1>, Table S1: Microbial communities of each rumen and fecal sample.

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F.Z.; resources, B.X.; data curation, F.X.; writing—original draft preparation, F.X.; writing—review and editing, B.X.; visualization, F.Z.; supervision, B.X.; project administration, B.X.; funding acquisition, B.X. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in the study are deposited in the Supplementary Materials and will be further deposited into NCBI.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

SCK	subclinical ketosis
NEB	negative energy balance
BHBA	β -hydroxybutyrate
TPP	thiamine pyrophosphate
α -KGDH	α -ketoglutarate dehydrogenase complex
SCOT	succinyl-CoA:3-ketoacid CoA transferase
HMGCS	3-hydroxy-3-methylglutaryl-CoA synthase
SCC	somatic cell count

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