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Predicted functional analysis of rumen microbiota suggested the underlying mechanisms of the postpartum subacute ruminal acidosis in Holstein cows

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ABSTRACT

Background: The relationships between the postpartum subacute ruminal acidosis (SARA) occurrence and predicted bacterial functions during the periparturient period are still not clear in Holstein cows.
Objectives: The present study was performed to investigate the alterations of rumen fermentation, bacterial community structure, and predicted bacterial functional pathways in Holstein cows.
Methods: Holstein cows were divided into the SARA (n = 6) or non-SARA (n = 4) groups, depending on whether they developed SARA during the first 2 weeks after parturition. Reticulo-ruminal pH was measured continuously during the study period. Reticulo-ruminal fluid samples were collected 3 weeks prepartum, and 2 and 6 weeks postpartum, and blood samples were collected 3 weeks before, 0, 2, 4 and 6 weeks postpartum.
Results: The postpartum decline in 7-day mean reticulo-ruminal pH was more severe and longer-lasting in the SARA group compared with the non-SARA group. Changes in predicted functional pathways were identified in the SARA group. A significant upregulation of pathway “PWY-6383” associated with Mycobacteriaceae species was identified at 3 weeks after parturition in the SARA group. Significantly identified pathways involved in denitrification (DENITRIFICATION-PWY and PWY-7084), detoxification of reactive oxygen and nitrogen species (PWY1G-0), and starch degradation (PWY-622) in the SARA group were downregulated.
Conclusions: The postpartum SARA occurrence is likely related to the predicted functions of rumen bacterial community rather than the alterations of rumen fermentation or fluid bacterial community structure. Therefore, our result suggests the underlying mechanisms, namely functional adaptation of bacterial community, causing postpartum SARA in Holstein cows during the periparturient period.

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Conflict of Interest
The authors declare no conflicts of interest.

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Keywords: Periparturient period; SARA; bacterial community; predicted functional pathway; dairy cows

INTRODUCTION

Periparturient feeding in dairy cows typically consists of a low-energy diet during the prepartum period, followed by a transition to a higher-energy feed postpartum [1]. A high-grain postpartum diet is accompanied by a decrease in ruminal pH caused by the increased production of volatile fatty acids (VFAs) or lactic acids, resulting in the increased occurrence and severity of ruminal acidosis and subacute ruminal acidosis (SARA), which is characterized by a ruminal pH < 5.6 for more than 3 h per day [2]. Dietary fermentation by the rumen microbial community produces various products. High-grain diets often cause dysfermentation and dysbiosis due to high acidity in the rumen [3]. However, the rumen bacterial community can mediate these changes by altering its composition, diversity, and similarity in response to dietary changes [4]. For example, rumen bacterial communities show higher total VFA and ammonia nitrogen (NH_3-N) concentrations and greater bacterial diversity during the later stages of a repeated, high-grain diet schedule [4]. These results support our hypothesis that ruminal acidosis and SARA may be unavoidable when postpartum energy demands are met.

Previously, our companion study [5] highlighted that the structural composition of the rumen bacterial community in Holstein cows was not strictly associated with transitions in parturition or diet; however, predicted functional adaption was promoted in response to an abrupt transition in the diet after parturition. A total of 53 MetaCyc pathways were upregulated at 6 weeks before compared to 3 weeks after parturition, and multiple pathways were related to intermediate metabolites of energy metabolism (pyruvate and acetyl-CoA) or vitamin synthesis (vitamin B_{12} and vitamin K_2). However, the fermentative activities of these organisms continue to provide the requisite energy for milk production after parturition to overcome these metabolic challenges, suggesting different adaptabilities of each bacterial community [5].

However, the relationships between SARA severity, rumen fluid bacterial community, and potential community functional pathways during the periparturient period are largely unclear in Holstein cows. Therefore, we investigated changes in rumen fermentation, the fluid bacterial community, and predicted bacterial functional pathways in Holstein cows based on the occurrence of SARA during the 2 weeks following parturition. We hypothesized that the occurrence of SARA postpartum may induce changes in rumen bacterial structure and its potential functions.

MATERIALS AND METHODS

Animals and group assignment

The Iwate University Laboratory Animal Care and Use Committee authorized an experimental protocol (A201452-2; Morioka, Japan). Ten multiparous Holstein cows (parity 2.6 ± 0.92; means ± SE) were housed in a commercial farm, and can freely access to water throughout the study period. Before and after parturition, the dry and lactation period diets were given *ad libitum* (**Table 1**). The requirement of the Japanese Feeding Standard for Dairy Cattle was the basis for the composition and quantity of the feed. SARA was diagnosed when

Table 1. Ingredients and nutrient composition of the dry and lactation periods on a DM basis

Items[a]	Dry period	Lactation period
Ingredients, %		
TMR[b]	90.3	
Dry period concentrate[c]	3.24	
Barley silage	4.96	
Lactation period concentrate[d]		61.3
Timothy		31.8
Corn flakes		2.95
Soybean		0.75
Feed additives[e]	1.51	3.20
Nutrient composition, %		
DM	87.0	84.8
Total digestible nutrients	59.3	74.7
Crude protein	12.7	15.0
Neutral detergent fiber	44.6	37.4
Acid detergent fiber	26.1	18.4
Starch	14.2	23.3
Calcium	1.91	0.57
Phosphate	0.34	0.41

DM, dry matter.
[a]All values are expressed on a DM basis; [b]Dry complete, Zenkoku Rakunoshiryo Co., Ltd, Tokyo, Japan; [c]Dry assist and Soychlor, Zenkoku Rakunoshiryo Co., Ltd, Tokyo, Japan; [d]Zenkoku Rakunoshiryo Co., Ltd, Tokyo, Japan; [e]Kraft pulp, calcium phosphate (dibasic), tricalcium phosphate, vitamin and mineral mix, sodium bicarbonate, and salt.

reticulo-ruminal pH was < 5.6 for more than 3 h per day [3] during the 2 weeks following parturition. Cows were divided into the SARA (n = 6) or non-SARA (n = 4) groups based on these parameters.

Reticulo-ruminal pH, fluid, and blood analyses

Using a radio transmission device, the reticulo-ruminal pH was monitored constantly throughout the study period (YCOW-S; DKK-TOA, Japan) [6]. Oral administration of pH sensor was conducted, and calibration was done at standard pH values of 4 and 7 before and after the experiment, respectively; no change in pH was identified at calibration. Collection of reticulo-rumen fluid was performed using oral stomach tube at 3 weeks prepartum and 2 and 6 weeks postpartum. To prevent the contamination of reticulo-rumen fluid with saliva, the first fluid sample (100 to 200 mL) was discarded. Samples of reticulo-rumen fluid were utilized to examine the levels of total VFAs, specific VFAs, and NH_3-N and the bacterial community. Immediately after being collected, fluid samples were filtered through two layers of cheesecloth and kept at 80°C until use. At 3 weeks before and 0, 2, 4, and 6 weeks after parturition, blood samples were collected from the jugular vein into 10 mL evacuated serum-separating tubes and into tubes containing heparin (BD Vacutainer, USA). The serum and plasma were separated by centrifugation at 1,500 × *g*, 15 min, 4°C and then preserved at −80°C until analysis.

For VFA analyses, 5 mL of reticulo-rumen fluid was mixed with 1 mL of 25% HO_3P in 3 *N* H_2SO_4. Total VFAs and individual VFAs (i.e., acetic, propionic, and butyric acids) were separated and quantified by liquid chromatography (HPLC; Shimadzu, Japan) using a packed column (shim-pack SCR-102H, Shimadzu). Samples were examined by the steam distillation method using an NH_3-N analyzer (Kjeltec Auto Sampler System 1035 Analyzer; Tecator Inc., Sweden) to determine the content of NH_3-N in reticulo-rumen fluid. An automated biochemistry analyzer (Acecut; Toshiba Ltd., Japan) was used to measure γ-glutamyl transpeptidase (GGT), aspartate transaminase (AST), phosphate, total calcium (Ca), blood urea nitrogen, total protein (TP), albumin, cholesterol (T-CHO), total ketone body (T-KB), non-esterified fatty acid (NEFA), triglyceride (TG), and glucose (GLU) concentrations.

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DNA isolation

Reticulo-ruminal fluid samples were used to extract total bacterial DNA as described by Kim et al. [7]. Briefly, sample incubation was performed with 750 µg/mL lysozyme (Sigma-Aldrich Co.,USA) at 37°C for 90 min. The mixture was then incubated at 37°C for 30 minutes with 10 µL of pure achromopeptidase (Wako Pure Chemical Industries Ltd., Japan) at 10,000 U/mL. 1 mg/mL of proteinase K (Merck Japan Ltd., Japan) and 60 µL of 1% sodium dodecyl sulfate were added to the suspension, and it was then incubated at 55°C for 5 min. Phenol/chloroform/isoamyl alcohol (Wako Pure Chemical Industries Ltd.) and chloroform were used to rinse the lysate three times (Life Technologies Japan Ltd., Japan). The DNA was precipitated using 5M sodium chloride and 100% ethanol, followed by centrifugation at 21,900 × *g* for 15 min at 4°C. The DNA pellet was rinsed with 70% ethanol, dried, and dissolved in Tris-hydrochloride buffer. A Biospec-nano spectrophotometer (Shimadzu) was used to measure the amount of purified DNA before it was stored at 80°C for subsequent analysis.

DNA sequencing

The barcoded universal primers 515F (5′-TGYCAGCMGCCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′) were used to amplify the V4 hypervariable region of bacterial 16S rRNA gene. A 25 µL mixture containing 12.5 µL of KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA), 5 µL of each primer (1 µM), and 2.5 µL of template DNA (10 ng/µL) was used to conduct a polymerase chain reaction (PCR). 95°C for 3 min, then 25 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension of 72°C for 5 min, were the thermal cycling conditions. AMPure XP beads (Beckman Coulter, High Wycombe, United Kingdom) were used to purify amplicons in accordance with the manufacturer's recommendations. Following the manufacturer's instructions, the Nextera XT Sample Preparation Kit (Illumina, USA) was used to create libraries by ligating sequencing adapters and indices to the purified PCR products. On the Illumina MiSeq platform, paired-end sequencing (2 × 151 bp) was carried out in accordance with standard protocol. The National Center for Biotechnology Information's Sequence Read Archive has received the sequencing data, which can be accessed using the SRA BioSample accession number SAMN19291602 (https://submit.ncbi.nlm.nih.gov/subs/sra/).

Sequencing data analysis

All sequencing reads were processed using the mothur program (version 1.41.1; University of Michigan; http://www.mothur.org/wiki/; [8]), largely adhering to the MiSeq standard operating procedure (https://mothur.org/wiki/MiSeq SOP; [9]). To create a non-redundant set of sequences, the unique sequences were found, matched to the SILVA reference database (SSURef release 128; [10]), and then the sequences were compared using the Mothur Ribosomal Database Project training set (version 16).

A cutoff value of 97% similarity was used to cluster and classify the sequences into operational taxonomic units (OTUs). The 'sub.sample' command was used to standardize all samples by random subsampling (796 sequences/sample) and six samples (two samples collected at 3 weeks prepartum, one sample collected at 2 weeks, and one sample collected at 6 weeks postpartum in the SARA group, and one sample collected at 3 weeks prepartum and one sample collected at 6 weeks postpartum in the non-SARA group) were eliminated due to low sequence numbers. The 'summary.single' command calculated the OTUs, Chao1, abundance-based coverage estimator (ACE) of species richness, Shannon, and Simpson diversity indices.

Using the 'get.oturep' command, representative sequences for each OTU were identified. The sequence comparisons were carried out using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against a database of 16S ribosomal RNA sequences (Bacteria and Archaea; April 2021). Using the default pipeline of PICRUSt2 [11], predicted functional pathways were inferred based on the representative sequences and tabulated raw count data from 16S rRNA gene data. Based on the proportion of MetaCyc pathways (http://MetaCyc.org), functional differences between the groups were examined collectively.

Statistical analysis

The normality of variables was tested by Shapiro-Wilk test. To identify the significant differences between the two groups, the unpaired *t*-test was used for normal variables and the Mann-Whitney *U* test was used for non-normal variables at the same sampling period. Within-group differences were determined by a mixed-model analysis of variance (accounting for repeated measures), using time as a fixed effect, followed by Dunnett's multiple comparison method. The construction of non-metric multidimensional scaling (NMDS) plots was performed using the package 'vegan' in R software (http://www.r-project.org; R Foundation for Statistical Computing, Vienna, Austria), based on OTU abundance measures and MetaCyc pathways. Prism software (version 8.10) was used to analyze all numerical data. Significant difference was considered at $p < 0.05$. After flooring fractional counts to the nearest integer, the raw MetaCyc pathway output from PICRUSTt2 was analyzed using DESeq2 with the default parameters to determine whether the predicted functional pathway of the rumen bacterial community differed in comparison to 3 weeks prepartum within or between groups [12]. At a false discovery rate (FDR)-corrected significance level of < 0.05, the inferred probable functional pathway was determined to be differentially abundant.

RESULTS

Body weight, body condition score (BCS), dry matter intake (DMI), and milk yield and fat composition

During the periparturient period, body weight, BCS, and DMI changed significantly in the SARA and non-SARA groups ($p < 0.05$) (**Supplementary Table 1**). The body weight decreased significantly at 1–6 weeks in the SARA group and at 0–6 weeks after parturition in the non-SARA group those compared with 3 weeks before parturition ($p < 0.05$), and DMI increased significantly at 2–6 weeks after parturition in the non-SARA group ($p < 0.05$). In both groups, postpartum milk yield and milk fat changed significantly ($p < 0.05$); milk yield increased significantly at 2–6 weeks in the two groups those compared with 1 week after parturition ($p < 0.05$), and milk fat composition decreased significantly at 3–6 weeks after parturition in the SARA group and at 5–6 weeks after parturition in the non-SARA group ($p < 0.05$). However, significant difference between the two groups were not identified in these measurements ($p > 0.10$) (**Supplementary Table 1**).

Reticulo-ruminal pH, VFAs, and blood metabolites

Significant changes in the 7-day mean reticulo-ruminal pH were identified during the periparturient period in both groups ($p < 0.05$, **Table 2**). At 1 week postpartum, a significantly low 7-day mean reticulo-ruminal pH was observed in the SARA group than in the non-SARA group ($p < 0.05$). The 24-h mean reticuloruminal pH changed significantly ($p < 0.05$) during the periparturient period in the SARA and non-SARA groups, and more severe changes were observed in the SARA group (**Fig. 1**). In detail, significantly low 24-h mean reticuloruminal

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Table 2. The 7-day mean reticulo-ruminal pH and duration of time where pH was < 5.6 and < 5.8 in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

Items	Weeks after parturition									SEM	*p* value[a]
	−2	−1	0	1	2	3	4	5	6		
7-d mean ruminal pH											
SARA	6.61	6.53	6.09[b,c]	5.79[b,c]	5.98[b]	6.06[b]	6.06[b]	6.16	6.15	0.01	< 0.001
Non-SARA	6.58	6.54	6.36[b]	6.13[b]	6.22[b]	6.41[b]	6.25[b]	6.31[b]	6.41[b]	0.01	< 0.001
Duration of pH < 5.6, min/d											
SARA	0.00	0.00	158[c]	375[b,c]	270[b]	163	149	65.1	124	38.4	0.029
Non-SARA	0.00	0.00	0.95	25.2[b]	54.8	8.60	17.1	36.2	9.05	10.8	0.004
Duration of pH < 5.8, min/d											
SARA	4.90	2.04	256[c]	610[b,c]	359[b]	228	244	153	225	43.1	< 0.001
Non-SARA	3.81	0.95	47.1	229[b]	187[b]	58.6	51.4	53.8	10.5	24.6	< 0.001

SARA, subacute ruminal acidosis.
[a]Mixed effects model ANOVA, followed by Dunnet's multiple comparison method, was used to determine within-group differences; [b]Denotes significant difference (p < 0.05) compared with week −2 in each group; [c]Denotes significant difference (p < 0.05) between the non-SARA and SARA groups at the same week point.

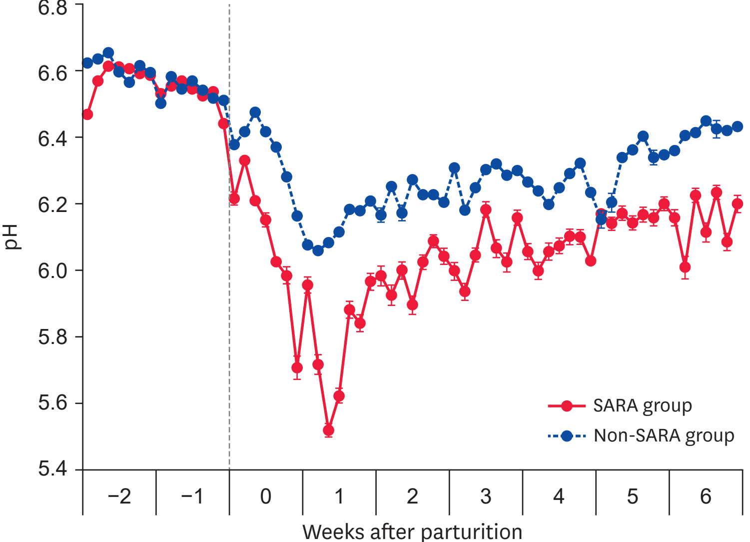

Fig. 1. The 24-h mean reticulo-ruminal pH in Holstein cows diagnosed with SARA (SARA group, n = 6) and in cows without SARA (non-SARA group, n = 4) during the 2 weeks after parturition. Negative numbers (−2 and −1) correspond to the weeks before parturition and positive numbers (0–6) to the weeks after parturition. * denotes significant difference (p < 0.05) between the SARA and non-SARA groups at each sampling time. Values represent the mean ± SE.
SARA, subacute ruminal acidosis.

pH was identified at 5, 6, 9, and 10 days after parturition in the SARA group ($p < 0.05$). The duration when reticulo-ruminal pH < 5.6 changed significantly in the SARA and non-SARA groups during the periparturient period ($p < 0.05$), and the durations at 0 and 1 week postpartum were significantly longer in the SARA group than in the non-SARA group ($p < 0.05$). In addition, the duration at which reticulo-ruminal pH was < 5.8 changed significantly in both groups during the periparturient period ($p < 0.05$), with a significantly longer duration in the SARA group than in the non-SARA group at 0 and 1 week postpartum ($p < 0.05$).

The total VFA concentration, proportions of acetic and propionic acids, ratio of acetic acid to propionic acid in both groups, and proportion of butyric acid in the non-SARA group exhibited significant changes during the periparturient period ($p < 0.05$, **Table 3**). In both groups, the total VFA concentration increased significantly at 2 weeks postpartum ($p < 0.05$), and the proportions of acetic and propionic acids increased significantly at 2 and 6 weeks

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Table 3. Total VFA, individual VFA proportions, acetic acid to propionic acid (A/P) ratio, and NH_3-N concentration in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

Items	SARA[a]					Non-SARA[a]				
	−3	2	6	SEM	*p* value[b]	−3	2	6	SEM	*p* value
Total VFA (mmol/dL)	7.04	9.04[c]	7.83	0.47	0.039	7.31	10.3[c]	9.10	0.66	0.021
Acetic acid (%)	70.6	59.6[c]	63.3[c]	0.97	< 0.001	69.7	59.2[c]	62.8[c]	1.42	0.001
Propionic acid (%)	15.7	25.4[c]	22.8[c]	1.15	0.002	16.9	27.4[c]	23.6[c]	1.81	0.003
Butyric acid (%)	12.1	12.8	11.8	1.00	0.402	11.5	11.0	10.8[c]	0.34	0.042
Others (%)	1.68	2.16	2.20	0.20	0.105	1.96	2.42	2.81	0.28	0.224
NH_3-N (mg/dL)	3.53	2.73	3.70	0.62	0.525	2.20	1.87	2.30	0.45	0.821
A/P ratio	4.56	2.45[c]	2.91[c]	0.18	< 0.001	4.14	2.17[c]	2.68[c]	0.29	< 0.001

VFA, volatile fatty acid; SARA, subacute ruminal acidosis.
[a]–3, 2, and 6 denote 3 wk before and 2 and 6 wk after parturition, respectively; [b]Mixed effects model ANOVA, followed by Dunnett's multiple comparison method, was used to determine within-group differences; [c]Denotes significant difference ($p < 0.05$) compared with wk –3 in each group.

Table 4. Biochemical analysis of peripheral blood in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

Items	SARA[a]							Non-SARA[a]						
	−3	0	2	4	6	SEM	*p* value[b]	−3	0	2	4	6	SEM	*p* value
GLU (mg/dL)	67.6	67.8	60.8	60.4	64.5	2.76	0.211	65.7	97.3	59.3	59.0	65.0	5.40	0.114
NEFA (µmol/L)	109	693[c]	234	155	163	84.0	0.003	143	600	727	207	91.3	112	0.111
T-KB (µmol/L)	595	683	473	692	513	51.9	0.038	539	531	566	547	553	73.8	0.984
T-CHO (mg/dL)	118	77.6[c]	119	169[c]	206[c]	11.5	< 0.001	116	88.0[c]	109	146[c]	190[c]	5.64	< 0.001
TG (mg/dL)	23.2	2.60[c]	6.00[c]	7.40[c]	6.00[c]	1.17	< 0.001	21.7	3.00[c]	6.67[c]	6.33[c]	6.50[c]	1.30	0.004
TP (g/dL)	7.58	7.22	7.64	7.74	7.83	0.19	0.163	7.37	7.20	7.50	7.93	7.70	0.16	0.115
ALB (g/dL)	3.86	3.86	3.78	3.84	3.90	0.07	0.903	3.80	4.00	3.90	3.97	4.05	0.09	0.347
BUN (mg/dL)	12.6	13.8	10.7	14.8	13.7	1.31	0.385	11.3	11.2	11.4	11.8	13.3	1.50	0.904
Ca (mg/dL)	10.4	9.10[c]	10.5	10.3	10.4	0.30	0.027	9.77	9.20	9.17	10.0	10.2	0.24	0.094
iP (mg/dL)	2.38	2.86	2.26	2.50	2.53	0.28	0.455	2.60	2.57	1.83	2.33	2.70	0.51	0.027
AST (IU/L)	62.0	81.2[c]	95.8[c]	85.0[c]	78.5	5.22	0.003	58.0	72.0	108[c]	93.3[c]	77.5	10.7	0.018
GGT (IU/L)	22.6	24.8[d]	24.2	25.4	26.0	1.54	0.464	17.3	18.0	18.3	27.7	20.5	2.53	0.375

SARA, subacute ruminal acidosis; GLU, glucose; NEFA, nonesterified fatty acid; T-KB, total ketone body; T-CHO, total cholesterol; TG, triglyceride; TP, total protein; ALB, albumin; BUN, blood urea nitrogen; AST, aspartate transaminase; and GGT, γ-glutamyl transpeptidase.
[a]–3, 2, and 6 denote 3 weeks before and 2 and 6 weeks after parturition, respectively; [b]Mixed effects model ANOVA, followed by Dunnett's multiple comparison method, was used to determine within-group differences; [c]Denotes significant difference ($p < 0.05$) compared with wk -3 in each group; [d]Denotes significant difference ($p < 0.05$) between the SARA and non-SARA groups at the same week point.

postpartum ($p < 0.05$) compared to those at 3 weeks postpartum. The proportion of butyric acid changed significantly in the non-SARA group during the periparturient period ($p < 0.05$), with a significantly lower proportion at 6 weeks postpartum ($p < 0.05$) than at 3 weeks postpartum. Furthermore, the proportion of butyric acid tended to be higher ($p = 0.081$) in the SARA group than that in the non-SARA group.

Biochemical analyses of peripheral blood revealed significant changes during the periparturient period in NEFA, T-KB, T-CHO, TG, and Ca concentrations and AST activity in the SARA group, and in T-CHO, TG, and iP concentrations and AST activity in the non-SARA group ($p < 0.05$, **Table 4**). GGT activity at 0 weeks postpartum was significantly higher ($p < 0.05$) in the SARA group than in the non-SARA group.

Bacterial diversity and abundance

In the SARA group, bacterial richness indices changed significantly (Chao1, $p < 0.05$) and showed a trend towards significant change (OTU and ACE, $p = 0.067$ and 0.065, respectively; **Table 5**). In contrast, bacterial diversity indices showed a trend towards a significant change (Shannon and Simpson, $p = 0.089$ and 0.099, respectively) in the non-SARA group. The Simpson diversity index was significantly lower at 3 weeks prepartum in the SARA group than in the non-SARA group ($p < 0.05$).

Table 5. Bacterial richness and diversity indices at the 97% similarity level in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

Items	SARA[a]					Non-SARA[a]				
	−3	2	6	SEM	*p* value[b]	−3	2	6	SEM	*p* value
OTU	182	159	142[c]	10	0.067	130	131	135	4	0.777
Chao1	299	264	229[c]	16	0.040	214	215	220	10	0.938
ACE	354	320	268[c]	23	0.065	255	249	257	18	0.959
Shannon	4.34[d]	4.11	3.97	0.14	0.277	3.62	4.05	3.82	0.12	0.089
Simpson	0.030	0.041	0.043	0.009	0.683	0.078	0.031	0.052	0.013	0.099

SARA, subacute ruminal acidosis; OTU, operational taxonomic unit; ACE, abundance-based coverage estimator.
[a]−3, 2, and 6 denote 3 weeks before and 2 and 6 weeks after parturition, respectively; [b]Mixed effects model ANOVA, followed by Dunnett's multiple comparison method, was used to determine within-group differences; [c]Denotes significant difference ($p < 0.05$) compared with wk −3 in each group; [d]Denotes significant difference ($p < 0.05$) between the SARA and non-SARA groups at the same week point.

NMDS plots of OTUs showed reasonably close similarity at 3 weeks prepartum in the non-SARA group, but not in the SARA group (**Fig. 2**). In contrast, no notable separation between the two groups was observed at 2 or 6 weeks postpartum. NMDS plots of bacterial pathways showed reasonably close similarity within and between groups at 3 weeks prepartum, but no notable separation between the two groups was identified at 2 or 6 weeks postpartum. Stress values were 0.14 and 0.10 in the OTU and MetaCyc pathway plots, respectively.

The major bacterial phyla Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria accounted for 97.1% of total rumen fluid bacteria (**Supplementary Fig. 1**), and the relative abundances of these phyla and the ratio of Bacteroidetes to Firmicutes were not affected by parturition in either group ($p > 0.10$, **Table 6**). Among the major bacterial genera (i.e., those accounting for > 1% of the total sequence; **Table 6**), the relative abundance of unclassified Porphyromonadaceae in the SARA group and *Succiniclasticum* in the non-SARA group showed significant changes during the periparturient period ($p < 0.05$), and the relative abundance of *Succiniclasticum* in the SARA group showed a trend towards a significant change ($p = 0.093$) during the periparturient period. Furthermore, the relative abundance of *Succiniclasticum* at 6 weeks postpartum tended to be higher ($p = 0.080$) in the SARA group than in the non-SARA group.

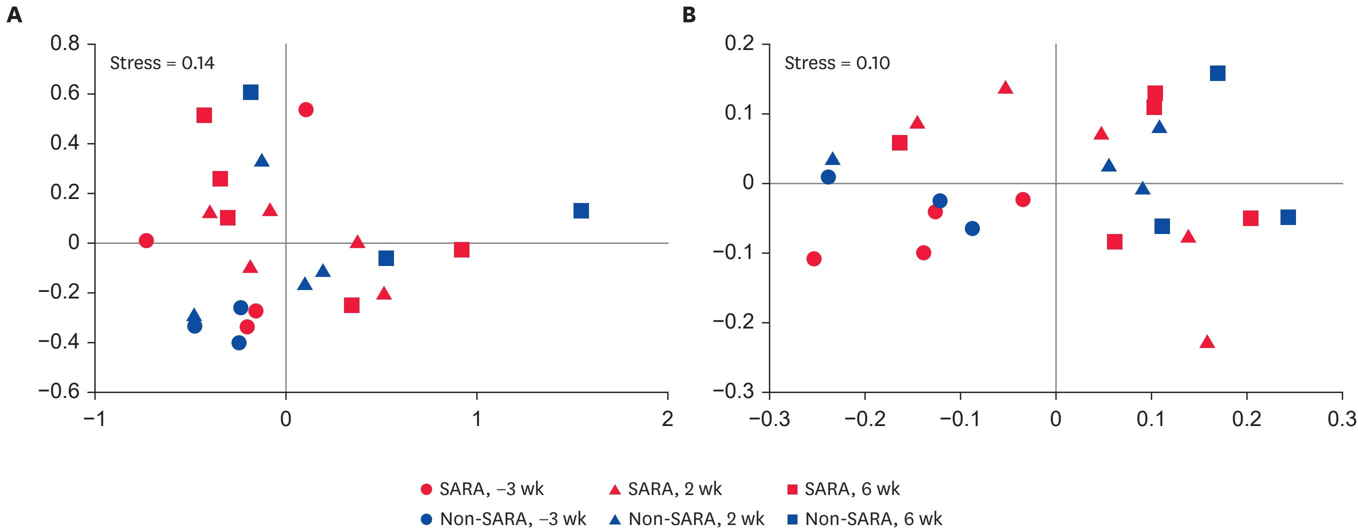

Fig. 2. NMDS plots for Holstein cows diagnosed with SARA (SARA group, n = 6) and those without SARA (non-SARA group, n = 4) during the 2 weeks after parturition. '−3 wk,' '2 wk,' and '6 wk' denote observations at 3 weeks before and 2 and 6 weeks after parturition, respectively. NMDS plots were generated for (A) bacterial OTUs by Mothur and (B) predicted functional pathways by PICRUSt2. Stress values were 0.14 and 0.10 for OUT and pathway-based ordinations, respectively.
NMDS, non-metric multidimensional scaling; SARA, subacute ruminal acidosis.

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Table 6. Relative abundances of major bacterial phyla and genera (> 1% of total sequence) in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

Items	SARA[a]					Non-SARA[a]				
	−3	2	6	SEM	*p* value[b]	−3	2	6	SEM	*p* value
Phylum										
Bacteroidetes	49.5	49.9	47.7	7.32	0.978	59.5	65.5	45.0	9.91	0.401
Firmicutes	38.5	41.8	45.7	6.46	0.796	32.2	27.8	49.6	9.74	0.336
Actinobacteria	3.13	1.72	1.75	0.78	0.410	1.12	2.35	2.71	0.68	0.120
Proteobacteria	0.44	3.07	0.65	0.89	0.412	0.68	0.73	0.21	0.18	0.253
B:F ratio[c]	1.35	1.41	1.37	0.34	0.992	2.16	2.87	1.79	0.98	0.730
Genus										
Prevotella	35.5	27.5	24.8	7.12	0.632	50.0	40.3	25.0	10.9	0.373
Unclassified *Lachnospiraceae*	8.59	14.7	11.5	4.85	0.788	5.84	8.40	20.7	4.90	0.234
Unclassified *Ruminococcaceae*	13.0	6.76	12.1	2.36	0.413	10.5	4.83	10.3	2.24	0.229
Unclassified *Bacteroidales*	2.41	8.11	7.32	1.46	0.126	1.91	5.48	7.09	1.30	0.100
Unclassified *Prevotellaceae*	2.32	4.21	6.11	1.15	0.238	1.98	13.4	4.17	3.10	0.276
Succiniclasticum	5.60	6.45	5.50	1.68	0.093	5.06	3.88	1.09[d]	0.82	0.007
Unclassified *Bacteroidetes*	6.42	4.60	5.10	1.33	0.742	4.34	3.31	3.14	1.17	0.580
Unclassified *Firmicutes*	3.17	3.05	3.02	0.90	0.994	2.16	2.00	3.55	0.85	0.349
Unclassified *Porphyromonadaceae*	1.26	2.20	2.98	0.76	0.049	0.47	1.88	4.81	1.05	0.123

SARA, subacute ruminal acidosis.
[a]-3, 2, and 6 denote 3 weeks before and 2 and 6 weeks after parturition, respectively; [b]Mixed effects model ANOVA, followed by Dunnett's multiple comparison method, was used to determine within-group differences; [c]Ratio of *Bacteroidetes* to *Firmicutes*; [d]Denotes significant difference ($p < 0.05$) compared with wk −3 in each group.

Among the 1,456 OTUs identified within the rumen bacteria, 11 were found in all samples (**Supplementary Table 2**). Among these 11 shared OTUs, the relative abundance of OTU3 (genus *Succiniclasticum*) in the non-SARA group exhibited significant changes during the periparturient period ($p < 0.05$). The relative abundance of OTU3 decreased significantly at 6 weeks postpartum in the non-SARA group ($p < 0.05$). Furthermore, the relative abundance of OTU39 (genus unclassified Firmicutes) was significantly higher in the SARA group than in the non-SARA group 2 weeks postpartum ($p < 0.05$). In addition to 11 shared OTUs (**Supplementary Table 2**), the SARA group cows had more diverse core rumen fluid bacterial OTUs, including 14 additional OTUs (**Supplementary Table 3**), than the non-SARA group cows, including 11 additional OTUs (**Supplementary Table 4**).

Predicted functional pathway analysis of OTUs

The raw MetaCyc pathways were acquired from PICRUSt2. Among the 487 KEGG pathways, 14 and 20 pathways were upregulated and downregulated at 2 weeks postpartum, respectively, and 6 pathways were significantly downregulated at 6 weeks postpartum in the SARA group compared with those at 3 weeks prepartum ($p < 0.05$, respectively; **Supplementary Table 4**). A significant upregulation of pathway "PWY-6383" [mono-*trans*, poly-*cis* decaprenyl phosphate biosynthesis; log2 fold change (FC) = 5.67] was identified ($p < 0.05$) in the SARA group compared to the non-SARA group at 3 weeks prepartum (**Table 7**). In addition, 6 significantly changed pathways in the SARA group at both 2 and 6 weeks postpartum were downregulated, including "PWY-6383" [FC = −5.68 and −5.10 at 2 and 6 weeks postpartum, respectively], "CATECHOL-ORTHO-CLEAVAGE-PWY" [catechol degradation to β-ketoadipate; FC = −3.45 and -3.00, respectively], "DENITRIFICATION-PWY" [nitrate reduction I (denitrification); FC = −1.49 and −1.84, respectively], "PWY1G-0" [mycothiol biosynthesis; FC = −4.00 and −3.49, respectively], "PWY-622" [starch biosynthesis; FC = −5.26 and −5.04, respectively], and "PWY-7084" [FC = −2.97 and −3.3, respectively; **Table 7**]. In addition to 6 pathways (**Table 7**), 453 pathways were identified using the PICRUSt2 software (**Supplementary Table 5**).

Table 7. Predicted functional pathway analysis using PICRUSt2 software in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during 2 weeks after parturition

MetaCyc pathways[a]	FC[b]			FC[b]		FC[b]	
	SARA vs. Non-SARA			SARA		Non-SARA	
	−3[c]	2	6	2	6	2	6
PWY-6383	5.67[d]	0.19	−0.27	−5.68[e]	−5.10[e]	−0.19	0.84
CATECHOL-ORTHO-CLEAVAGE-PWY	1.81	0.57	−0.70	−3.45[e]	−3.00[e]	−2.21	−0.50
DENITRIFICATION-PWY	0.46	−0.07	0.15	−1.49[e]	−1.84[e]	−0.96	−1.53
PWY1G-0	3.06	−0.58	0.32	−4.00[e]	−3.49[e]	−0.36	−0.76
PWY-622	0.96	−1.26	−0.45	−5.26[e]	−5.04[e]	−3.04	−3.63
PWY-7084	0.41	−0.24	−0.47	−2.97[e]	−3.30[e]	−2.32	−2.42
1CMET2-PWY	−0.21	0.02	−0.03	0.37[e]	0.23	0.14	0.06
CENTBENZCOA-PWY	0.87	0.05	0.68	−1.82[e]	−1.07	−1.00	−0.88
FASYN-INITIAL-PWY	0.07	0.18	0.28	0.81[e]	0.45	0.70	0.24
FOLSYN-PWY	−0.16	0	0.06	0.58[e]	0.35	0.43	0.12
LIPASYN-PWY	0.07	−1.60	1.08	−3.40[e]	−2.48	−1.73	−3.49
METHYLGALLATE-DEGRADATION-PWY	1.23	2.01	0.03	3.79[e]	1.22	3.02	2.42
P184-PWY	0.91	1.95	−0.09	3.85[e]	1.10	2.81	2.10
P241-PWY	0.58	−0.09	0.39	−2.90[e]	−1.79	−2.23	−1.60
P281-PWY	−0.17	0.89	0.33	2.68[e]	0.92	1.62	0.42
PROTOCATECHUATE-ORTHO-CLEAVAGE-PWY	0.26	−0.46	0.79	−1.66[e]	−1.28	−0.94	−1.81
PWY0-862	0.12	0.21	0.34	0.90[e]	0.55	0.81	0.32
PWY-3661	0.38	1.92	0.75	5.66[e]	2.20	4.12	1.84
PWY-5941	−0.26	−0.90	0.36	−2.08[e]	−1.41	−1.44	−2.02
PWY-5971	−0.08	0.33	0.33	0.77[e]	0.41	0.37	0
PWY-5989	0.12	0.20	0.34	0.92[e]	0.55	0.83	0.33
PWY-6185	1.29	−0.24	−0.36	−2.10[e]	−1.69	−0.57	−0.04
PWY-6282	0.13	0.23	0.34	0.91[e]	0.54	0.80	0.33
PWY-6338	0.84	2.02	−0.22	4.17[e]	1.23	2.98	2.29
PWY-6612	−0.14	0	0.08	0.64[e]	0.38	0.51	0.16
PWY-6876	1.61	2.86	0.86	5.02[e]	1.33	3.77	2.08
PWY-6944	1.39	2.83	0.88	5.28[e]	1.54	3.85	2.06
PWY-7090	0.31	−0.35	0.05	−0.89[e]	−0.45	−0.23	−0.18
PWY-7097	0.84	2.02	−0.22	4.17[e]	1.23	2.98	2.29
PWY-7098	0.83	2.10	−0.30	4.31[e]	1.30	3.05	2.43
PWY-7347	0.11	−0.40	−0.17	−1.50[e]	−0.95	−0.99	−0.67
PWY-7664	0.12	0.23	0.33	0.87[e]	0.53	0.76	0.31
PWYG-321	0.13	0.20	0.34	0.77[e]	0.44	0.70	0.24
SUCSYN-PWY	−0.03	−0.41	−0.17	−1.26[e]	−0.83	−0.87	−0.70

SARA, subacute ruminal acidosis.
[a]Representative sequences and tabulated raw count data were analyzed using PICRUSt2 algorithm to assign metagenomic functions; [b]log2 fold change was calculated by comparisons of −3 weeks and 2 or 6 weeks after parturition in each group and comparison of the SARA and Non-SARA groups at the same week point; [c]−3, 2, and 6 denote 3 weeks before and 2 and 6 weeks after parturition, respectively; [d]Denotes significant difference ($p < 0.05$) between the SARA and non-SARA groups at the same week point; [e]Denotes significant difference ($p < 0.05$) compared with wk −3 in each group.

DISCUSSION

We evaluated SARA-affected Holstein cows for 2 and 6 weeks after parturition to classify changes in rumen fermentation, fluid bacteria, and predicted bacterial functional pathways relative to the control. During the postpartum period, we observed a decrease in reticuloruminal pH in both groups, but the changes in the SARA group were more severe. In the SARA group, the mean reticulo-ruminal pH exceeded the criteria for SARA at 1 and 2 weeks postpartum (reticulo-ruminal pH < 5.6 lasting 384.9 and 269.6 min/day, respectively), and the duration of SARA was significantly longer in the SARA group than the non-SARA group. In contrast to the significant differences in reticuloruminal pH between the groups at 5, 6, 9, and 10 days after parturition, no differences were observed in rumen fermentation parameters at 2 weeks postpartum, and we observed a slightly higher total VFA concentration in the non-SARA group. Although a decrease in the acetate-to-propionate ratio suggested

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concentrate diet-induced propionate production in both groups, the postpartum findings in the SARA group were not consistent with previous research on changes in ruminal pH [4,13,14], in which the total VFA concentration was determined to be a major determinant of ruminal pH [15]. Our findings suggest that SARA-induced ruminal dysfermentation and dysbiosis [3] may promote irregular diurnal variation in total VFA production or physiological responses, such as increased passage rate, absorption, and acid buffering, which are associated with severe decreases in reticulo-ruminal pH [16,17]. Furthermore, the lactic acid concentration, which is 10 times less protonated than VFA in the rumen (pKa 4.9 vs. 3.9), and the major determinants of ruminal pH simultaneously with VFA [18] were not measured in the present study. Although our findings provide evidence that SARA affects ruminal fermentation, namely dysfermentation and dysbiosis, rumen fluid collection and pH measurements were performed at different time points in our study. Therefore, future studies should address the timing discrepancy between organic acids (e.g., VFA and lactic acid) and pH measurements to validate the causal relationships among reticulo-ruminal pH, fermentation, and parturition.

Similar to the limited changes observed in rumen fermentation between the groups, few changes were observed in the rumen fluid bacterial community diversity, similarity, or composition. Although our subsampling level was slightly lower than those in previous reports [19,20], rumen bacterial richness (SARA group) and diversity (non-SARA group) tended to be affected by parturition in the present study. Previouly, Ogata et al. [21] reported that approximately 20 months of long-term concentrate diet feeding from 10 to 30 months of age induced low ruminal pH (SARA challenge level) in Japanese Black beef cattle. In their study, OTU, Chao1, and ACE indices (bacterial richness indices) decreased gradually in the later fattening stages, but Shannon, Simpson, and Heip indices (bacterial diversity indices) did not differ among the fattening stages. Moreover, higher bacterial diversity and richness indices before parturition in the SARA group might be consistent with the significantly increased bacterial diversity and richness during the later period of the four consecutive SARA challenges [4]. Therefore, we assumed that the SARA group cows in this study might have experienced SARA or rumen acidosis before pH measurement [4], and the preserved bacterial diversity is an adaptation to maintain their fermentation ability against the rumen bacterial dysbiosis [21]. Moreover, the NDMS plots for both groups indicated a highly dispersed distribution during the periparturient period. In contrast, SARA and non-SARA cows showed different profiles in additional shared OTUs (14 and 11, respectively) during the periparturient period, suggesting distinctive core bacterial communities.

At the genus level, the relative abundance of *Succiniclasticum* decreased significantly in the non-SARA group after parturition. Moreover, OTU3 (a genus of *Succiniclasticum*) showed similar changes in the non-SARA group and was identified as a *Succiniclasticum ruminis* strain at a 95.8% identification level using BLASTn software. *S. ruminis* is a gram-negative, non-motile, non-spore-forming, anaerobic, rod-shaped bacterium [22]. *Succiniclasticum* spp. are prevalent in dairy cattle, along with *Prevotella* spp. [23], and commonly detected in the rumens of pasture-fed yellow cattle [22]. Furthermore, Xin et al. [23] suggested that the decreased distribution of *Prevotella* spp. and *Succiniclasticum* spp. in the Shenza yak and Zhongba yak was likely due to forage quality. Therefore, the decreased relative abundances of *Succiniclasticum* and OTU3 in the non-SARA group cows might have been affected by dietary transition in the present study.

Prevotella bacteria are the most dominant bacterial species in the rumen [14,24,25]. All shared OTUs (OTU1, OTU2, and OTU10) classified as *Prevotella* were assigned to a *Prevotella ruminicola* strain that comprised 0.63–42.3% of rumen bacteria during the periparturient period, in contrast to a previous finding that *P. ruminicola* accounted for only 1.7% of the rumen bacterial rRNA gene copies in Holstein cows [25]. *Prevotella* (a genus of ruminal species) can utilize starches, non-cellulosic polysaccharides, and simple sugars [26] and was the most abundant in our study (34.6% of the total sequence; data not shown). Our results suggest that *Prevotella* (likely *P. ruminicola*) may play an important role in rumen fermentation of fermentable materials such as starch, sugar, and crude protein [26,27] during the periparturient period.

Although the PICRUSt2 pipeline has not been specifically developed for animals, recent studies have applied it to ruminants [28,29]. A total of 34 (20 upregulated) and 6 (all downregulated) pathways at 2 and 6 weeks postpartum were up- or downregulated in the SARA group, respectively, and significant differences between the two groups were identified at 3 weeks prepartum. Specifically, mono-*trans*, poly-*cis* decaprenyl phosphate biosynthesis in the SARA group (log2 FC = 5.67, FDR-adjusted $p = 0.014$) is associated with Mycobacteriaceae species and plays a central role in the biosynthesis of essential mycobacterial cell wall components, such as the mycolyl-arabinogalactan-peptidoglycan complex and lipoarabinomannan [30]. Among the significant pathways in the SARA group at both 2 and 6 weeks postpartum, "DENITRIFICATION-PWY" and "PWY-7084" were involved in the denitrification of nitrite and ammonia, respectively, to nitrogen molecules. The "PWY-622" pathway produces glucose-based polysaccharide (starch), and then biosynthesized starch source goes into the starch degradation II (PWY-6724) pathway. Furthermore, mycothiol produced by the "PWY1G-0" pathway is used as a protectant against electrophilic compounds as well as for detoxification of reactive oxygen and nitrogen species [31], and catechol acts as an antioxidant and is metabolized by the denitrifying bacterium in the rumen [32]. Therefore, downregulation of the functional pathways that play an important role in the rumen might have a significant influence on the regulation of rumen-produced organic acids and SARA-induced ruminal dysfermentation and dysbiosis identified in the SARA group.

Although it is difficult to directly apply the PICRUSt2 analysis results to rumen bacterial function, our results suggest underlying differences in fermentation or functional adaptation between the two groups of Holstein cows during the periparturient period. Combined with periparturient period dietary transition and changes in reticulo-ruminal pH, the result of predicted functional pathway analysis may indicate the underlying mechanisms of postpartum SARA occurrence in Holstein cows.

SUPPLEMENTARY MATERIALS

Supplementary Table 1

Changes in body weight, body condition score, dry matter intake, milk yield, and milk fat composition in Holstein cows diagnosed with SARA (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the periparturient period

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Supplementary Table 2

Relative abundances and taxonomic classification of operational taxonomic units (OTU; shared by all samples) in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

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Supplementary Table 3

Additional profile of relative abundances and taxonomic classification of taxonomic units (OTU; shared by all samples in the SARA group) in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

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Supplementary Table 4

Additional profile of relative abundances and taxonomic classification of taxonomic units (OTU; shared by all samples in the non-SARA group) in Holstein cows diagnosed with (SARA group, n = 6) or without SARA (non-SARA group, n = 4) during the 2 weeks after parturition

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Supplementary Table 5

Additional profiles of predicted functional pathways analyzed by PICRUSt2 software in Holstein cows diagnosed with SARA (SARA group, n = 6) or without SARA (non-SARA group, n = 4) for 2 weeks after parturition

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Supplementary Fig. 1

Relative abundances of the four major bacterial phyla and genera in Holstein cows diagnosed with SARA (SARA group, n = 6) and those without SARA (non-SARA group, n = 4) at 2 weeks after parturition. “−3,” “2,” and “6” denote observations at 3 weeks before and 2 and 6 weeks after parturition, respectively. Data are shown as a percentage of the total identified sequences per sampling period in each group (SARA and non-SARA groups).

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