



Subacute ruminal acidosis phenotypes in periparturient dairy cows differ in ruminal and salivary bacteria and in the *in vitro* fermentative activity of their ruminal microbiota

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ABSTRACT

Both ruminal microbial structure and functionality might play a role in inter-individual variation in susceptibility for subacute rumen acidosis (SARA) observed in dairy cows. The aims of this study were to determine whether differences between cows with distinct SARA susceptibility were reflected in distinct (1) ruminal microbial communities, (2) salivary bacterial communities, and (3) fermentative capacity of ruminal microbiota assessed *in vitro*. To test this hypothesis, rumen samples were collected via an esophageal tube on 21 d postpartum from 38 multiparous Holstein cows, which were classified into 4 groups differing in median and mean time of reticular pH below 6 as well as area under the curve of pH below 6.0. During the 21 d postpartum, all cows within a group fulfilled following criteria: susceptible (S, $n = 10$; mean or median ≥ 180 min/d), moderately susceptible (MS, $n = 7$; $60 \text{ min/d} < \text{mean time of pH below 6} < 180 \text{ min/d}$, and median time of pH below 6 $< 180 \text{ min/d}$), moderately unsusceptible (MU, $n = 11$; $10 \text{ min/d} < \text{mean} < 60 \text{ min/d}$, and median time of pH below 6 $\leq 30 \text{ min/d}$), or unsusceptible (U, $n = 10$; median = 0 min/d, and mean $< 10 \text{ min/d}$). Groups did not differ in total daily dry matter intake nor in total, roughage, or concentrate intake during daily 6-h time intervals. Rumen bacterial α -diversity did not differ among groups, but β -diversity varied and bacterial 16S rRNA gene copy numbers were lower in S compared with U cows. The relative abundance of genera *Streptococcus*, *Sharpea*, *Prevotellaceae_YAB2003*, *Succinivibrionaceae_UCG-001*, *Ruminococcus*, and *Ruminococcaceae_UCG-001* were higher in S compared with U cows. In contrast, *Lachnospiraceae_ND3007* and *Oscillospiraceae_V9D2013* were more abundant in U

cows. Although pH-associated, inter-animal differences were also observed in the salivary bacteria, common differences in ruminal and salivary bacterial genera were limited. The functionality of the rumen microbiota was evaluated *in vitro* through exposure of the microbial inoculum of S and U cows to an anaerobic buffer at pH 5.8 and 6.8, in the presence of sterile supernatant of their own and of dry cows' rumen fluid (2×2 design). Generally, the S inoculum produced more volatile fatty acids, except at low pH with dry cows' supernatant, where volatile fatty acid production was completely impaired and lactate accumulation was highest. Compared with the microbes of U cows, microbes of S cows showed less fermentative activity in situations with 2 stress factors (low pH and an unfamiliar environment, i.e., rumen fluid supernatant of dry cows).

Key words: subacute ruminal acidosis, periparturient period, inter-animal variation, bacterial community, *in vitro* fermentative activity

INTRODUCTION

The transition around parturition in dairy cows is characterized by a decrease in DMI, which is also manifested in a decreasing share of dietary roughage (Hayirli et al., 2003), as the greatly increased energy requirements of lactation demand more energy-dense diets with a greater proportion of concentrates (Grummer, 1995). Accordingly, the higher amounts of quickly fermentable carbohydrates in combination with lower proportions of physically effective fiber in such diets put cows at higher risk of SARA. Despite several measures and guidelines to prevent SARA, incidence rates of 23.3% were reported during the first week in lactation in a recent study with 249 dairy cows (Vallejo-Timarán et al., 2020). This could be related to inter-animal variation in SARA susceptibility, resulting in part of the herd suffering from SARA when receiving the same diet and similar management (Plaizier et al.,

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2008; Mohammed et al., 2012; Jing et al., 2018). Such inter-animal variation also may reduce the effectiveness within a dairy herd of herd-based dietary interventions to prevent and control SARA (Humer et al., 2018). Thus, the early prediction and understanding of SARA susceptibility in dairy cows is paramount to our continued ability to limit its prevalence and specifically target animals at risk. In this respect, earlier work by our group indicated that the first 3 wk after calving offers a window of opportunity to identify SARA susceptibility phenotypes in dairy cows, as inter-animal differences during this period seemed to largely persist over a long period (>1 yr; Jing et al., 2018). However, as SARA prevalence is roughly 20% (Plaizier et al., 2008), experiments under practical conditions during the transition period require a much larger number of animals than traditional SARA challenge experiments. Nevertheless, such experiments are highly relevant, as the effects of most experimentally induced SARA may be more severe than those of on-farm SARA (Khafipour et al., 2009b; Plaizier et al., 2018). Accordingly, it is questionable whether inter-animal variation observed under experimentally induced SARA challenges is representative of inter-animal differences encountered during the transition period under practical management conditions (i.e., gradual build-up of increased amounts of grains accompanied with low physically effective NDF feeding).

Variation in SARA susceptibility (either inter-animal variation or dietary SARA-induction) has been related to differences in the number of rumen prokaryotes and eukaryotes (e.g., Hook et al., 2011) as well as the composition of rumen bacterial communities (e.g., Mohammed et al., 2012), as assessed by quantitative PCR (qPCR) and 16S rRNA amplicon sequencing, respectively. Moreover, these differentiating rumen microbial communities also seem to respond differently to a grain-based SARA challenge (Khafipour et al., 2009a; Chen et al., 2012; McCann et al., 2016). Accordingly, in addition to the structure of the microbiome, its functionality to cope with different disturbances during transition might also differ among animals differing in SARA susceptibility. As well as dietary shifts, other abiotic factors in the ruminal environment could also create such disturbances (Fonty et al., 1993). Obviously, low rumen pH is the most known abiotic factor influencing microbial fermentation, particularly through inhibition of pH-sensitive cellulolytic microorganisms (Chen et al., 2019). Nevertheless, other abiotic factors produced by ruminal microbiota, such as some essential growth factors (e.g., branched-chain volatile fatty acid, phenylpropanoic acid, and vitamins), can also facilitate growth and hence functionality of specific microbial communities (e.g., Fonty et al., 1993; Shaani

et al., 2018), and antimicrobial compounds excreted by microbiota can actively interfere with other species' growth (e.g., Long and Azam, 2001). Accordingly, the microbial community and its functionality are adapted to the abiotic factors of their environment, and they may be disturbed by abiotic factors to which they are not adapted.

The current study included 38 dairy cows under practical management conditions during the transition period. We hypothesized that the inter-animal variation observed in SARA susceptibility during this period is reflected in abundance of prokaryotic and eukaryotic microbiota, rumen bacterial community composition, and resilience of the rumen microbiota to withstand abiotic disturbances. As oral samples can provide accurate information on the composition of animals' rumen microbiome (Kittelmann et al., 2015; Tapio et al., 2016), inter-animal variation in SARA is assumed to be reflected in the bacterial community recovered from oral samples. Furthermore, it was hypothesized that metataxonomic analysis of the ruminal and salivary bacteria allow inference of inter-animal differences in SARA susceptibility. Finally, resilience of rumen microbial functionality against abiotic disturbances was assessed *in vitro* through exposure to various pH conditions and microbial metabolites. An *in vitro* set-up was chosen to allow assessment of microbial functionality without interaction with the host. Distinct abiotic environments were created by varying pH and through addition of sterile supernatant either of the cow's own rumen fluid or of dry cows' rumen fluid. The latter was chosen because the diet of cows in the far-off dry period differs most from that of cows in early lactation. Therefore, the aims of this study were to determine whether differences between cows with distinct SARA susceptibility were reflected in (1) distinct ruminal microbial communities, both in terms of numbers and composition (bacteria only), (2) salivary bacterial community, and (3) fermentative capacity of the ruminal microbiota as assessed by *in vitro* exposure of the microbial inoculum from cows differing in SARA susceptibility to an anaerobic buffer at pH 5.8 and 6.8, in the presence of sterile supernatant of their own and of dry cows' rumen fluid.

MATERIALS AND METHODS

Animals, Diets, and Group Assignment

All animal experimental procedures were approved by the Ethics Committee of the Flanders Research Institute for Agriculture, Fisheries and Food, Melle, Belgium (EC 2018/329 and EC 2014/241). A total of 38 multiparous Holstein-Friesian dairy cows were included

in the monitoring experiment, which took place at the research farm of the Institute for Agricultural, Fisheries and Food Research from 2 wk before the predicted calving date to 3 wk after calving in a period from March 2019 to October 2020.

From 3 wk before calving onward, cows received the same partial mixed ration as the lactating cows, supplemented with a dry cow mineral premix (Aveve) and on average 1 kg of balanced concentrate per cow per day. The partial mixed ration of the lactating cows was calculated to fulfill the needs of an average adult cow of 650 kg, producing 26 kg of fat- and protein-corrected milk and was based on maize silage, prewilted grass silage, pressed beet pulp, soybean meal, and balanced compound feed (Table 1). The latter was linearly built up from d 3 to d 20 in lactation according to the scheme shown in Table 2. Cows were given the basal diet as 2 equal offerings at roughly 0730 and 1630 h ad libitum and had free access to water.

Reticular pH of all cows was monitored every 10 to 15 min using pH boluses (18 eBolut, eCow; 20 SmaXtec GmbH). The 38 animals were divided into 4 groups based on pH criteria. For this, mean and median daily

pH values were assessed against a pH threshold. A pH threshold of 6.0 was chosen in the current study, as the reticular pH is generally 0.2 units higher than the rumen pH (Beauchemin et al., 2003; Neubauer et al., 2018). Further, the mean and median duration that pH dropped below 6 was calculated for each cow on a daily basis during the 3-wk postpartum period. Then, the 38 cows were ranked from high to low based on mean and median of pH below 6 and divided into 4 approximately even-sized groups. As such, the 38 animals were classified based on the individual cow's mean or median (or both) time of pH below 6, to roughly allocate between 20 and 30% of the cows to each group:

- Susceptible group (**S**; $n = 10$): mean or median time of pH below 6 at least 180 min/d;
- Moderately susceptible group (**MS**; $n = 7$): 60 min/d < mean time of pH below 6 < 180 min/d, and median time of pH below 6 < 180 min/d;
- Moderately unsusceptible group (**MU**; $n = 11$): 10 min/d < mean time of pH below 6 < 60 min/d, and median time of pH below 6 ≤ 30 min/d;

Table 1. Ingredients and chemical composition (% of DM) of the diet offered from 3 wk before calving (Close-up) to the first 2 d of lactation as well as the diets offered on d 3 and d 20 in lactation (Lac3 and Lac20)¹

Item	Close-up	Lac3	Lac20
Maize silage	34.4	32.1	24.8
Grass silage	34.4	32.1	24.8
Beet pulp	8.42	7.84	6.07
Urea	0.140	0.130	0.101
Straw	0.957	0.892	0.690
Barley	1.10	1.02	0.789
Maize	4.16	3.88	3.00
Soybean meal	7.98	11.1	7.37
Balanced compound feed A ²	—	2.45	10.8
Covasoy ³	—	2.45	5.40
Balanced compound feed B ²	6.72	6.12	16.2
Mineral premix ⁴	1.68	—	—
Chemical composition, % of DM			
(unless noted otherwise)			
DM (% of fresh matter)	39.2	44.0	54.3
VEM ⁵ (VEM/kg of DM)	998	1,027	1,053
CP	13.8	16.0	16.8
FOM ⁶	59.2	59.1	58.7
Starch	16.8	16.4	18.0
NE _L ⁷ (MJ/kg of DM)	6.89	7.09	7.27

¹Between d 3 and d 20, a linear shift from Lac3 to Lac20 took place through build-up of balanced compound feed A and B, Covasoy (FeedValid BV) and soybean meal; see Table 2 for details.

²Balanced compound feed A and B; see Table 2 for details.

³Covasoy = formaldehyde-treated soybean meal to bypass rumen degradation.

⁴Contains 2.2 g of Ca, 39.5 g of P, 0 g of K, 81.6 g of Mg, 30.1 g of Na, 2,000 mg of choline chloride, 20 mg of calcium iodate, 15 mg of cobalt sulfate, 1,000 mg of copper sulfate, 1,250 mg of manganese oxide, 2,500 mg of zinc sulfate, 40 mg of sodium selenite, 1,000,000 IU of vitamin A, 200,000 IU of vitamin D3, 4,400 mg of vitamin E.

⁵VEM = feed unit lactation (Van Es, 1975).

⁶FOM = fermented OM (Tamminga et al., 1994).

⁷Calculated based on the Belgian-Dutch net energy evaluation system; that is, 1,000 VEM = 6.9 MJ of NE_L (Van Es, 1975).

- Unsusceptible group (U; $n = 10$): median time of pH below 6 = 0 min/d, and mean time pH below 6 < 10 min/d.

Individual daily median and mean time of pH below 6, as well as the number of days with more than 330 min with pH below 6 during the 3-wk postpartum period, are given in Table 3. Moreover, these 4 groups of cows concomitantly differed from each other in daily median and mean time of pH below 6 and mean daily area under the curve of pH below 6.0 ($P < 0.001$; Table 4). Within the first 3 wk in lactation, the U and MU groups did not show any day where time of pH < 6.0 exceeded 330 min/d, whereas a significantly larger number of such days were observed in the MS and S groups (Table 4). Three cows (S5, severe hypocalcemia; S7, severe lameness; S8, hypocalcemia) in the S group and 2 cows (MU2 and MU6, displaced abomasum) in the MU group showed some signs of clinical disease on some days during the 21-d monitoring period.

Feeding Behavior

Individual feed intake was monitored throughout the trial using roughage intake control feeding bins (Insentec, Hokofarm Group), except during the period around calving. During lactation, concentrate intake was monitored at the automatic concentrate providers (Greenfeed, C-Lock Inc.; DeLaval) and in the herringbone milking parlor (DeLaval). The raw data of roughage intake were checked for overlapping visits of the same animal as well as for outliers of visiting time or feed intake, according to the approach described by Mensching et al. (2020).

Data of 4 daily time intervals of 6 h each were compared. The time intervals were chosen in accordance with the rumen sampling time (i.e., at 0900 h). Accordingly, the following time intervals were considered: 0300 to 0900, 0900 to 1500, 1500 to 2100, and 2100 to 0300 h. For each interval the mean dry matter intake

(concentrates, roughage, and total DMI) was calculated during a 3-d period, from 2 d before sampling to the day of sampling. The number of meals was calculated based on the intake pattern at the roughage intake control bins. Visits were split into meals using a threshold of 28.7 min between visits (De Mol et al., 2016).

Collection of Rumen Samples and Saliva

On 21 d (± 1 d) postpartum, salivary samples were collected 2 h after the morning feeding using a sterile sponge (Koala Universal Sponge) according to the procedure described by Tapio et al. (2016) and immediately snap frozen in liquid nitrogen. Afterward, a stomach tube was inserted via the mouth and esophagus into the rumen, and unfractionated samples of rumen contents were collected and filtered through a sieve with a pore size of 1 mm (Geishauser et al., 2012). After homogenizing, a sterile falcon tube of 15 mL was completely filled with rumen fluid and immediately transferred to the laboratory (within 30 min) in a thermos flask filled with water at a temperature of 39°C for the *in vitro* experiment. Additionally, subsamples of rumen fluid were collected in cryovials and snap frozen in liquid nitrogen for microbial analysis. Snap-frozen saliva and rumen fluid samples were stored at -80°C until DNA extraction.

Bacterial Community Analysis Based on 16S rRNA Sequencing

DNA Extraction. A total of 1 mL of both rumen fluid and saliva samples were centrifuged at 4°C for 15 min at $16,000 \times g$ to collect the pellets before genomic DNA extraction using the repeated bead beating plus column purification method (Yu and Morrison, 2004). The concentration and quality of extracted DNA was checked in a NanoDrop spectrophotometer (VWR International BVBA).

Table 2. Linear build-up between d 3 and 20 in lactation of the supplemental part of the diet, individually supplied to the cows during milking and via the concentrate dispenser (kg/d)

Item	Amount at d 3	Build-up duration (d)	Amount at d 20
Balanced compound feed A ¹	0.2	20	2
Balanced compound feed B ²	1.5	10	4
Covasoy ³	0.2	7	1
Soybean meal	0.3	20	0.3

¹Contains (g/kg of product): dry beet pulp (100), soybean meal (270), wheat (85), maize (430), molasses (70), salt (6), feed phosphate (10), micro minerals (10), lignin-sulfonate (10), chalk (4), magnesium oxide (5).

²Contains (g/kg of product): beet pulp (370), soybean meal (210), wheat (185), maize (120), molasses (50), salt (12), soy oil (10), feed phosphate (10), micro minerals (10), lignin-sulfonate (10), chalk (8), magnesium oxide (5).

³Covasoy (FeedValid BV) = formaldehyde treated soybean meal to bypass rumen degradation.

Table 3. Median and mean diurnal time of pH below 6 (min/d) of the 3-wk postpartum period for cows grouped as susceptible, moderately susceptible, moderately unsuspensible, or unsuspensible to SARA development

Item	Cow number									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Susceptible group (S)										
Median time of pH below 6 (min/d)	660	540	480	330	315	230	195	180	150	130
Mean time of pH below 6 (min/d)	624	446	573	309	476	253	272	177	222	203
Days of time of pH below 6 > 330 min/d in 21 d	18	14	16	10	10	4	7	2	5	2
Moderately susceptible group (MS)										
Median time of pH below 6 (min/d)	MS-1	MS-2	MS-3	MS-4	MS-5	MS-6	MS-7	—	—	—
Mean time of pH below 6 (min/d)	105	80.0	60.0	60.0	10.0	10.0	0.00	—	—	—
Days of time of pH below 6 > 330 min/d in 21 d	126	147	78.6	74.3	143	72.9	83.8	—	—	—
Moderately unsusceptible group (MU)										
Median time of pH below 6 (min/d)	MU-1	MU-2	MU-3	MU-4	MU-5	MU-6	MU-7	MU-8	MU-9	MU-10
Mean time of pH below 6 (min/d)	30.0	30.0	20.0	15.0	15.0	0.00	0.00	0.00	0.00	0.00
Days of time of pH below 6 > 330 min/d in 21 d	44.8	42.9	55.2	53.6	36.4	33.6	22.9	21.4	17.1	15.2
Unsceptible group (U)										
Median time of pH below 6 (min/d)	0	0	1	0	0	0	0	0	0	0
Mean time of pH below 6 (min/d)	U1	U2	U3	U4	U5	U6	U7	U8	U9	U10
Days of time of pH below 6 > 330 min/d in 21 d	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	10.0	5.71	5.24	3.33	3.00	1.43	0.00	0.00	0.00	0.00
	0	0	0	0	0	0	0	0	0	0
Days of time of pH below 6 > 330 min/d in 21 d	—	—	—	—	—	—	—	—	—	—

Bacterial 16S rRNA Gene Amplicon Sequencing and Data Mining. Extracted gDNA was submitted to Macrogen for library preparation and bacterial 16S rRNA gene amplicon sequencing (V3–V4 region; primers: 344 forward and 806 reverse; Klindworth et al., 2013). Preparation of the amplicons barcoded library was based on the Illumina 16S metagenomic sequencing library preparation protocol (<https://support.illumina.com>), and the sequencing was performed using Illumina MiSeq V3 technology (2 × 300 bp).

The amplicon sequencing data set was demultiplexed and barcodes were clipped off by the sequence provider. The amplicon sequencing data were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME2, version 2020.08; Bolyen et al., 2019). The sequences were demultiplexed, barcodes were removed, and forward and reverse reads were imported into QIIME2. The DADA2 pipeline was used to detect and correct Illumina amplicon sequences, to remove primers and chimeric reads, and for the assembly into amplicon sequence variants (ASV; Callahan et al., 2016). A further filtering step was performed to remove low-abundance sequences with frequencies below 0.01% or present in less than 2 out of the 38 samples. Finally, to normalize the number of sequences per sample, a cut-off value (30,114) was chosen based on α -rarefaction curves for all samples. Taxonomy was assigned using a naïve Bayes classifier trained on the Silva database (Bokulich et al., 2018; SILVA Release 138, <https://www.arb-silva.de/silva-license-information/>) at 99% similarity, followed by removal of the features of archaea and unassigned taxa. Sequence files associated with each sample have been submitted to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>; accession number PRJNA722858).

For saliva samples, a manual approach was applied to remove the potential oral ASV based on the description of Kittelmann et al. (2015) and published data from our group (Dewanckele et al., 2019). The removed as well as retained ASV were checked against literature to confirm that all the oral ASV were excluded, and possible ruminal ASV were retained. Two saliva samples were excluded from further analysis due to quality control failure. All potential rumen bacteria were retained. We came across many ASV of *Streptococcus* spp. Because *Streptococcus* spp. can be found in oral as well as in rumen samples, this species was initially not removed by the manual filtering approach. To determine whether the group of sequences assigned to *Streptococcus* with an unknown species affiliation could validly be eliminated from the data set, we created a phylogenetic tree of the genus *Streptococcus*, including the sequences obtained in this study and sequences downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). Based on

this phylogenetic tree, we removed the ASV clustered with known oral *Streptococcus* spp. After removal of the oral bacteria, the ASV table was normalized to 2,397 sequences per sample before further analysis in QIIME 2 (Dewanckele et al., 2019).

Microbial Population Analysis by qPCR

The abundance of 16S rRNA genes of total bacteria, mcrA genes of methanogens, 18S rRNA genes of protozoa, and 5.8S rRNA of anaerobic fungi were quantified by qPCR. The primers used for qPCR are presented in Supplemental Table S1 (<https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022). Primer sets and qPCR conditions used were the same as reported for general bacteria (Denman and McSweeney, 2006), fungi (Edwards et al., 2008), protozoa (Carberry et al., 2012), and methanogens (Denman et al., 2007). The qPCR reactions were assayed in 12.5 μ L of reaction mixture containing 6.25 μ L of Maxima SYBR Green/ROX qPCR Master Mix (2 \times) (ThermoFisher Scientific), 1 μ L of primer mixture containing 0.5 μ M concentrations of each primer, DNA (20 ng), and molecular water. Amplification of each target group was carried out in a 2-step cycling protocol (StepOne Real Time PCR System, Applied Biosystems) with the following program: initial denaturation at 95°C for 10 min, 35 cycles at 95°C for 15 s (denaturation), 60°C for 1 min for annealing and extension. The melting curve was built by measuring fluorescence emissions with increased temperature from 60 to 95°C with ramps of 0.5°C every 15 s. Duplicate qPCR quantification was performed on 20 ng of extracted DNA. A plasmid containing a single copy of the targeted gene was used as qPCR standard. The copy numbers in the standards were calculated based on the DNA concentrations determined by the NanoDrop spectrophotometer (VWR International

BVBA). External standards were prepared and used in every qPCR run to enumerate the gene copies in the samples. The absolute quantity of each group of microorganisms was calculated using the respective standards and expressed as corresponding gene copies per milliliter of sample.

In Vitro Experiment with Microbial Inoculum from S and U Cows

In vitro simulations were performed using microbes collected from SARA-susceptible ($n = 10$) and unsusceptible ($n = 10$) dairy cows to explore the fermentative capacity of these microbes when excluded from the interaction with their host. The microbes were exposed to 4 different conditions: low or normal pH (5.8 or 6.8, respectively) and sterile supernatant obtained from their own rumen fluid or rumen fluid collected from dry cows.

On the day of rumen fluid collection, an in vitro experiment was performed using inoculum collected from each cow separately. The inoculum collected from each cow was exposed to a buffer solution at pH 5.8 or pH 6.8 and the supernatant derived from either the cow's own rumen fluid or from that of a dry cow (Figure 1). At the time of inoculum collection, cows' groupings were unknown, and, hence, incubations were carried out using inoculum of each of the 38 cows. However, further analysis was performed only on the 2 extreme groups (S, $n = 10$; and U, $n = 10$).

Obtaining Sterile Supernatant from Rumen Fluid of Dry Cows. About 500 mL of rumen fluid was collected from 3 fistulated Holstein dry cows (6–7 yr old) housed in the same barn. These cows were fed 8 to 9 kg of maize silage (on DM basis) and 4.1 kg of chopped straw per day, supplemented with 1 kg of concentrate. The method to collect sterile supernatant

Table 4. Average reticular pH parameters and DMI of the 3-wk postpartum period as well as number of meals during 2 d before and the day of sampling for cows grouped as susceptible, moderately susceptible, moderately unsusceptible, or unsusceptible to SARA development¹

Item	S	MS	MU	U	SEM	P-value
Reticular pH						
Median time of pH below 6 (min/d)	321 ^a	46.4 ^b	10.0 ^c	0.0 ^d	26.75	<0.001
Mean time of pH below 6 (min/d)	356 ^a	104 ^b	32.5 ^c	2.78 ^d	26.963	<0.001
Days with time of pH below 6 > 330 min/d	8.82 ^a	1.57 ^b	0.00 ^c	0.00 ^c	0.775	<0.001
Mean daily area under curve pH below 6.0 (pH \times min)	882 ^a	293 ^b	50.1 ^c	3.85 ^d	77.548	<0.001
DMI (kg/d)	19.6	20.0	19.8	21.9	0.43	0.164
Eating behavior 2 d before and day of sampling						
Number of meals (times/d)	7.07	8.43	7.33	7.60	0.221	0.277

^{a-d}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Susceptible group (S; $n = 10$): mean or median time of below pH 6 of at least 180 min/d. Moderately susceptible group (MS; $n = 7$): 60 min/d < mean time of pH below 6 < 180 min/d, and median time of pH below 6 < 180 min/d. Moderately unsusceptible group (MU; $n = 11$): 10 min/d < mean time of pH below 6 < 60 min/d, and median time of pH below 6 \leq 30 min/d. Unsusceptible group (U; $n = 10$): median time pH below 6 = 0 min/d, and mean time pH below 6 < 10 min/d.

was based on Shaani et al. (2018). Accordingly, the collected rumen fluid was filtered through a kitchen sieve, mixed, and centrifuged at 25°C and $14,000 \times g$ for 15 min (Figure 1). The supernatant was then sterilized by filtering through a series of sterile vacuum filters (0.8-, 0.45-, and 0.2- μm filters, Sigma-Aldrich). The filtered supernatant was divided into 50 aliquots (15 mL) and stored at -20°C until use in the in vitro experiment, of which 38 aliquots were used in this study. On the incubation day, the number of aliquots required to perform the experiment were thawed and kept at 39°C under CO_2 flushing.

Obtaining Sterile Supernatant and Microbes from Rumen Fluid of Cows Sampled on 21 Days Postpartum. Rumen fluid samples collected from cows on 21 d postpartum were centrifuged at 25°C, $14,000 \times g$ for 15 min for the collection of sterile supernatant and was subjected to the same filtering process as described in the previous paragraph for dry cows. The sterile supernatant was kept in a 39°C warm water bath under continuous flushing with CO_2 until the start of the in vitro experiment.

Cleaning Microbial Pellet and Mixing with Sterile Supernatant. The pellets, containing the rumen microbes, were recovered after centrifugation (collected from 15 mL of rumen fluid) and were washed with 15 mL of autoclaved buffer, through brief vortexing and mixing the pellets with buffer through pipette trituration. Afterward, this mixture was centrifuged at 25°C, $14,000 \times g$ for 15 min, and the supernatant was removed. Oxygen exposure was minimized during all the previously mentioned steps by continuous flushing of CO_2 .

In Vitro Incubation of Mixed Microbes and Sterile Supernatant. The microbes collected from the periparturient cows were incubated with their own sterile supernatant as well as with the sterile supernatant obtained from the dry cows. Mixing the microbes with the sterile supernatant was performed under CO_2 flushing. After mixing, each of the 2 mixtures (15 mL of each microbial pellet and respective sterile supernatant) was equally divided into 2 anaerobic buffers at pH 6.8 and 5.8 [per liter: 51.59 g of 2-(N-morpholino)ethanesulfonic acid, 0.118 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 8.30 g of

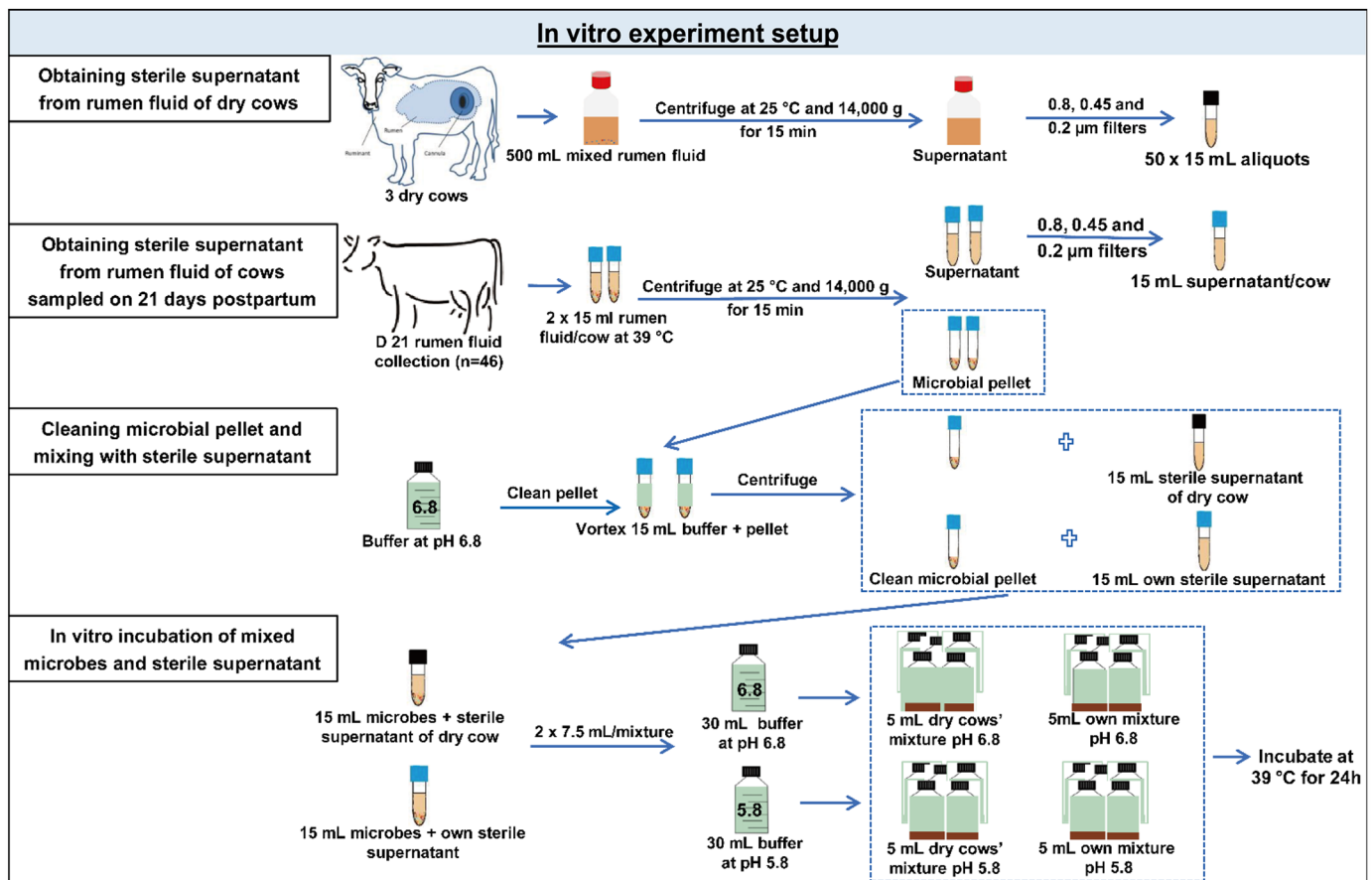


Figure 1. Scheme of the in vitro incubation set-up.

NaHCO₃, and 0.95 g of NH₄HCO₃] in a 1:4 ratio of rumen fluid to buffer. Buffer pH had been adjusted with 6 M HCl to 6.8 or 5.8. The rumen fluid-buffer mixture (5 mL) was transferred into an incubation flask under anaerobic conditions and incubated in triplicate for 24 h at 39°C in a shaking incubator (Edmund Bühler TH30; Edmund Bühler GmbH). Fifty milligrams of maize silage (proximate chemical composition, g/kg of DM: CP, 67.3; crude fat, 35.5; NDF, 451; ADF, 218; ash, 56.0), lyophilized and stored at ambient temperature, was used as substrate for the incubation. After 24-h incubation, the flasks were taken out of the incubator and kept in an ice bath to stop microbial activity. Final pH (Hanna Instruments) was measured. One milliliter of the incubation fluid was collected for analysis of VFA and acidified with 100 µL of formic acid, which contained the internal standard (10 mg of 2-ethyl butyric acid/mL of formic acid). After 15-min centrifugation at 4°C and 22,000 × g, the supernatant was filtered and an aliquot transferred into a 1.5-mL glass vial. Volatile fatty acids were analyzed through gas chromatography on an HP 7890A (Agilent Technologies) equipped with a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco) with a flame ionization detector (Dewanckele et al., 2020).

Lactate concentrations were determined according to Conway (1957). Net production of VFA and net accumulation of lactate were calculated by subtracting the amount in the incubation fluid before incubation from the amount after incubation.

Data Analysis

In Vitro Fermentation Data. Data were analyzed by R (R Core Team, 2020) using lme4 version 1.1.12 (Bates et al., 2015) according to the following model:

$$Y_{ijkl} = \mu + I_j + P_k + SUP_l + C_i(I_j) + (I_j \times P_K) \\ + (I_j \times SUP_l) + (P_K \times SUP_l) \\ + (I_j \times P_K \times SUP_l) + e_{ijkl},$$

where Y_{ijkl} = response variable (total VFA and lactate); μ = overall mean; I_j = fixed effect of inoculum group (referring to the SARA susceptibility group of the inoculum donors; j = S or U); P_k = fixed effect of pH (k = 5.8 or 6.8); SUP_l = fixed effect of supernatant (l = own or dry cow's supernatant); $C_i(I_j)$ = cow nested within inoculum group as random effect; $I_j \times P_K$ = interaction of inoculum group and pH; $I_j \times SUP_l$ = interaction of inoculum group and supernatant; $P_K \times SUP_l$ = interaction between pH and supernatant; $I_j \times P_K \times SUP_l$ = interaction of inoculum group, pH, and

supernatant; and e_{ijkl} = residual error. The fixed effects (I_j , P_k , and SUP_l) were forced into the model, and 2- and 3-way interactions were removed from the model if nonsignificant ($P > 0.05$; also based on Akaike information criterion). Normality of residuals in the model was assessed via quantile–quantile plots and Shapiro-Wilk test, and homogeneity of variances was verified based on Levene test. Variables were log-transformed to meet normality assumptions if necessary. When the lme4 test indicated a significant 2- or 3-way interaction, t -tests were performed within pH conditions for comparison between supernatants (paired) and between inoculum groups (unpaired).

Reticular pH, DMI, Feeding Behavior, and qPCR. Data of feeding behavior during 4 time intervals were analyzed by 2-way repeated-measures ANOVA of the lme4 version 1.1.12 (Bates et al., 2015) package in R (R Core Team, 2020) to examine the main effects of groups, time intervals, and their interactions, as well as random effects of cow. Data of reticular pH, DMI, and qPCR were analyzed in R (R Core Team, 2020) by one-way ANOVA of the car package (Fox and Weisberg, 2019) with groups as the main factor, after confirming normality of the data distribution assessed via quantile–quantile plots and Shapiro-Wilk test, and homogeneity of variances by the Levene test. Then, the differences between group means were assessed by Tukey's test for multiple comparisons. For data with non-normal distribution, a Kruskal-Wallis test followed by a pairwise Wilcoxon rank sum test was performed.

Bacterial Data. Bacterial sequencing profile were analyzed in QIIME2 (version 2020.08) and R (R Core Team, 2020). First, a nonparametric Kruskal-Wallis test was used to evaluate the differences of α -diversity metrics across groups (QIIME2 software; version 2020.08). Additionally, distance-based (Bray-Curtis distances) PERMANOVA was carried out to check whether bacterial composition varies between groups in QIIME2 (Kunz et al., 2019). Analysis of composition of microbiomes (Mandal et al., 2015) tests were run in R (R Core Team, 2020) at phylum, family, and genus level to determine which bacterial groups were differentially abundant between different groups of cows. The differential taxa were computed controlling for false discoveries using Benjamini-Hochberg correction at 5% level of significance. Further, the taxa that differed significantly across groups detected by the analysis of composition of microbiomes test were subject to a Wilcoxon rank sum test to assess inter-group differences. Finally, a random forest algorithm was performed for in the randomForest (Liaw and Wiener, 2002) package in R, with 10-fold cross-validation using the caret (Kuhn,

Table 5. Gene copy numbers of rumen microbes analyzed by quantitative PCR in rumen fluid sampled on d 21 postpartum in relation to variation in SARA susceptibility over the first 3 wk postpartum¹

Item (log ₁₀ /mL rumen fluid)	S	MS	MU	U	SEM	P-value
Bacteria	11.0 ^b	11.5 ^{ab}	11.2 ^{ab}	12.0 ^a	0.12	0.034
Fungi	7.48	7.31	7.33	7.47	0.102	0.917
Methanogens	10.9	10.1	10.1	10.7	0.21	0.453
Protozoa	11.6	12.7	11.2	11.0	0.30	0.319

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Susceptible group (S; n = 10): mean or median time of below pH 6 of at least 180 min/d. Moderately susceptible group (MS; n = 7): 60 min/d < mean time of pH below 6 < 180 min/d, and median time of pH below 6 < 180 min/d. Moderately unsusceptible group (MU; n = 11): 10 min/d < mean time of pH below 6 < 60 min/d, and median time of pH below 6 \leq 30 min/d. Unsusceptible group (U; n = 10): median time pH below 6 = 0 min/d, and mean time pH below 6 < 10 min/d.

2008) package in R. The number of trees in the forest was set to 500, the number of features available for splitting at each tree node (mtry) was tuned, and accuracy was used to select the optimal model using the largest value. Four saliva samples were excluded in the analysis due to the normalization of ASV table to 2,397 sequences per sample after removing potential oral bacteria, as mentioned for bacterial community analysis based on 16S rRNA sequencing. Consequently, the 4 corresponding rumen samples were also removed. In total, 16 rumen and saliva samples from the 2 extreme SARA groups (S, n = 7; and U, n = 9 cows) were used to classify the dairy cows' SARA susceptibility.

For all tests, a P -value of 0.05 was used to define significance, with trends declared at $0.05 < P$ -value < 0.10.

RESULTS

Reticular-Ruminal pH Parameters, DMI, and Eating Behavior

Grouping of the animals was based on the time below pH 6 during the first 21 d postpartum, with groups concomitantly differing in daily median and mean time of pH below 6, number of days with time of pH below 6 exceeding 330 min/d, and mean daily area under the curve of pH below 6.0. However, average daily DMI over this period did not differ. Additionally, DMI data of the days around sampling were assessed in some more depth: number of meals during the 2 d before sampling and the day of sampling did not differ between groups ($P > 0.05$), whereas total DMI tended to decrease in the S group during the time intervals of 0900 to 1500 h ($P = 0.084$; Supplemental Table S2; <https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022) and 2100 to 0300 h ($P = 0.051$; Supplemental Table S2). In contrast, no differences were found in roughage DMI and concentrate DMI throughout the day nor in total

DMI during the time blocks when the basal diet was distributed (i.e., 1500–2100 and 0300–0900 h).

Rumen Bacteria, Fungi, Methanogens, and Protozoa Quantified by qPCR

Gene copy numbers of rumen bacteria, fungi, methanogens, and protozoa assessed by qPCR are shown in Table 5. The 16S rRNA gene copy numbers of bacteria were significantly greater in the U cows than in the S cows ($P = 0.034$), whereas the copy numbers of fungi, methanogens, and protozoa did not differ between groups ($P > 0.05$).

Rumen Bacterial Community Composition

α -Diversity indices of rumen bacterial community (observed ASV, Faith_{pd}, evenness, and Shannon index) did not differ ($P > 0.05$) among groups (Supplemental Table S3; <https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022). With regard to β -diversity compared by PERMANOVA, the Bray-Curtis distances of the U cows differed from the S cows ($P = 0.031$; Figure 2a and 2c), whereas the U and MU cows tended to differ ($P = 0.062$; Figure 2c).

Relative abundances of bacterial families and genera, differing between cows varying in SARA susceptibility during the 3-wk postpartum period, are shown in Table 6. Relative abundances of phyla to which these bacterial families or genera belong are also reported in Table 6. Overall, a total of 18 phyla were identified in 38 animals (Supplemental Table S4; <https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022); only 4 phyla exceeded a relative abundance of 1%, with *Firmicutes* ($48.2 \pm 6.08\%$) and *Bacteroidota* ($42.4 \pm 6.23\%$; previously known as *Bacteroidetes*) representing the 2 dominant phyla. At genus level, *Prevotella* ($28.7 \pm 0.66\%$; Supplemental Figure S1a; <https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022) is the

dominant genus, followed by *Succinivibrionaceae* ($7.36 \pm 0.493\%$; Supplemental Figure S1a). The relative abundances of the 2 predominant phyla and genera did not depend on the SARA grouping (P -value adjusted for false discovery rate, $P_{\text{adj}} > 0.05$).

However, at the family and genus levels, 3 families and 8 genera differed between SARA groups (Table 6; $P_{\text{adj}} < 0.05$). Briefly, at family level, the relative abundances of *Ruminococcaceae*, *Streptococcaceae*, and *vadinBE97* were higher in S and MS cows compared with MU and U cows ($P_{\text{adj}} < 0.05$). At genus level, *Ruminococcus*, *Sharpea*, *Coprococcus*, *Prevotellaceae_YAB2003*, and *Succinivibrionaceae_UCG-001* were more abundant in S cows and, in some cases in the MS cows, compared with U cows ($P_{\text{adj}} < 0.05$). Besides these differences, which were in accordance with the SARA classes, the genera *Ruminococcaceae_UCG-001* and *Streptococcus*, within the phylum *Firmicutes*, were below the detection limit in the MS and MU cows, respectively, but the SARA

extremes (S vs. U cows) did not differ. Additionally, differences in relative abundance of the *Lachnospiraceae_ND3007* group and *Oscillospiraceae_V9D2013* within the phylum *Firmicutes* did not follow the gradual SARA class differences.

Classification of SARA Susceptibility in Dairy Cows by Bacterial Community Using Random Forest Model. After manually removing potentially oral bacteria, a principal coordinate analysis was used to visualize the similarity of the saliva bacterial community and the rumen bacterial community (Figure 2). Despite the separation between the samples from saliva and rumen, some similarities were observed between the bacterial communities of these 2 sample types. Similar to the rumen bacterial community, *Firmicutes* ($37.9 \pm 5.41\%$) and *Bacteroidota* ($55.2 \pm 4.08\%$) represented the 2 dominant phyla in samples from saliva (Supplemental Table S5; <https://doi.org/10.6084/m9.figshare.19158098>; Yang et al., 2022). At genus level, 8 of the

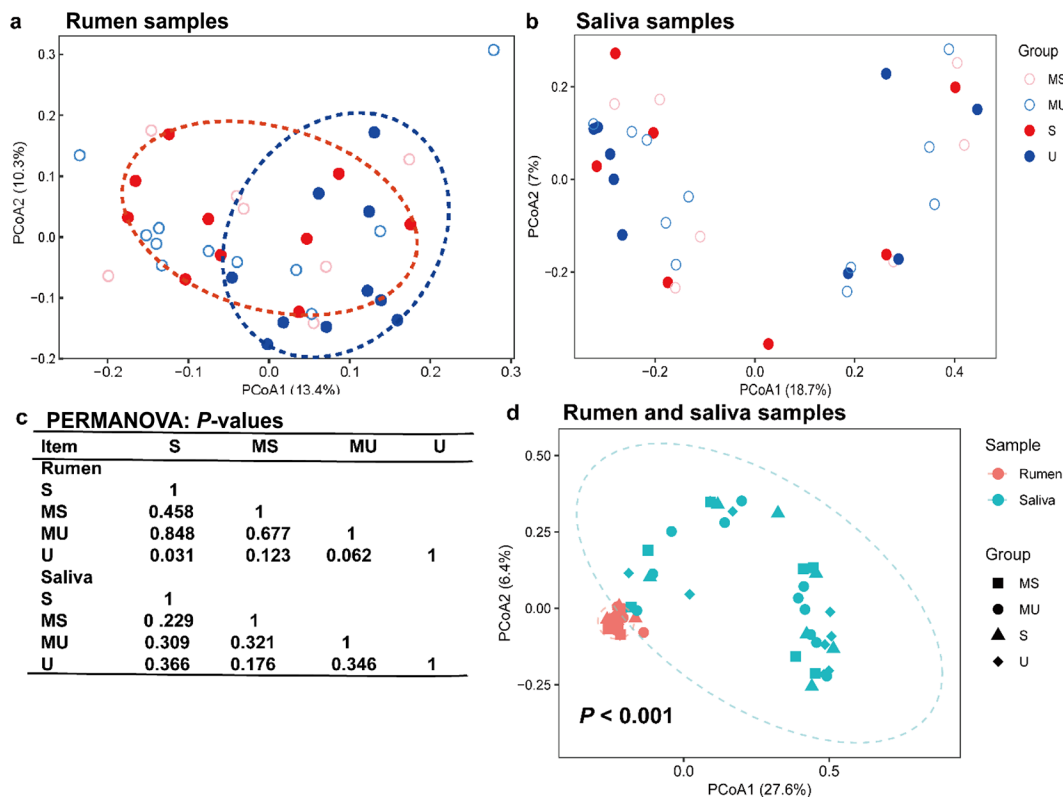


Figure 2. Principal coordinate analysis (PCoA) of the effect of sample type (a, rumen; b, saliva; d, rumen and saliva) on Bray-Curtis dissimilarities and P -values of the PERMANOVA model assessing differences in β -diversity (c) of the bacterial communities. Individual points in each plot represent the microbiome of an individual dairy cow; different colors represent samples from saliva or the rumen. Percentages shown along the axes represent the proportion of dissimilarities captured by PCoA in the 2-dimensional coordinate space. Susceptible group (S; $n = 10$): mean or median time of below pH 6 of at least 180 min/d. Moderately susceptible group (MS; $n = 7$): 60 min/d < mean time of pH below 6 < 180 min/d, and median time of pH below 6 < 180 min/d. Moderately unsuitable group (MU; $n = 11$): 10 min/d < mean time of pH below 6 < 60 min/d, and median time of pH below 6 \leq 30 min/d. Unsuitable group (U; $n = 10$): median time pH below 6 = 0 min/d, and mean time pH below 6 < 10 min/d.

Table 6. Average relative abundance (%) of rumen bacterial groups that differ among groups of dairy cows varying in SARA susceptibility over a 3-wk postpartum period¹

Phylum	Family	Genus	S	MS	MU	U	SEM	P_{adj}^2
Firmicutes	Ruminococcaceae	<i>Ruminococcus</i> <i>UCG-001</i>	42.1	44.2	41.8	41.6	1.11	0.340
			4.19 ^a	4.24 ^a	4.16 ^a	3.22 ^b	0.122	0.035
			3.18 ^a	3.24 ^a	2.96 ^b	2.45 ^b	0.117	0.023
	Streptococcaceae	<i>Streptococcus</i> <i>Coproccoccus</i>	0.018 ^a	0.000 ^b	0.029 ^a	0.012 ^{ab}	0.0056	<0.001
			0.010 ^a	0.006 ^{ab}	0.000 ^b	0.004 ^b	0.0020	<0.001
	Lachnospiraceae	<i>Streptococcus</i> <i>ND3007_group</i>	0.002 ^a	0.001 ^{ab}	0.000 ^b	0.001 ^{ab}	0.0005	<0.001
			0.120 ^a	0.129 ^a	0.122 ^{ab}	0.071 ^b	0.0227	0.014
	Erysipelatoclostridiaceae	<i>Sharpea</i> <i>V9D2013_group</i>	0.102 ^b	0.141 ^a	0.097 ^b	0.208 ^a	0.0132	0.002
			0.132 ^a	0.043 ^a	0.002 ^{ab}	0.000 ^b	0.0345	<0.001
	Oscillospiraceae		0.002 ^b	0.013 ^a	0.028 ^a	0.017 ^a	0.0056	0.014
Bacteroidota			48.8	45.5	47.5	50.4	1.08	0.537
Proteobacteria	Prevotellaceae		0.201 ^a	0.117 ^{ab}	0.186 ^{ab}	0.084 ^b	0.0217	0.028
			3.04	3.92	4.57	2.01	0.522	0.730
Verrucomicrobiota	Succinivibrionaceae		1.32 ^a	2.63 ^a	2.64 ^a	0.281 ^b	0.402	0.001
	<i>vadinBE97</i>		0.127	0.078	0.151	0.188	0.0144	0.343
			0.010 ^a	0.006 ^{ab}	0.000 ^b	0.000 ^b	0.0026	<0.001

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).¹Only differentially abundant family and genera are included, and numbers are presented as relative abundance. Susceptible group (S; $n = 10$); mean or median time of below pH 6 of at least 180 min/d. Moderately susceptible group (MS; $n = 7$); 60 min/d < mean time of pH below 6 < 180 min/d, and median time of pH below 6 < 180 min/d. Moderately unsuceptible group (MU; $n = 11$); 10 min/d < mean time of pH below 6 < 60 min/d, and median time of pH below 6 \leq 30 min/d. Unsuceptible group (U; $n = 10$); median time pH below 6 = 0 min/d, and mean time pH below 6 < 10 min/d.² P_{adj} = P -value adjusted for false discovery rate.

top 10 genera from saliva as also belonged to the top 10 genera in rumen samples (Supplemental Figure S1) with *Prevotella* ($28.7 \pm 6.33\%$; Supplemental Figure S1b) being the dominant genus irrespective of SARA grouping, followed by *Streptococcus* ($15.7 \pm 3.59\%$; Supplemental Figure S1b).

The possibility of correctly classifying cows into the S or U categories based on the bacterial community of saliva or rumen sampled 3-wk postpartum was tested through random forest classification (Figure 3 and 4). The 20 most predictive genera for classification of S versus U cows, as assessed by the decrease in mean classification accuracy, showed some similarities between saliva and rumen samples (Figure 3). Among these 20 most predictive genera, saliva and rumen samples showed 3 common genera, such as uncultured *Erysipelotrichaceae*, *Prevotella*, and *Anaerovibrio* (Figure 3a and 3c). These genera predominated in saliva and rumen samples of S cows (Figure 3b and 3d), although differences between S and U cows in *Prevotella* abundance of rumen samples were not clearly distinguished by the color code of the heatmap. The most predictive genus in rumen samples was *Succinivibrionaceae_UCG-001*, whereas *Bacteroides* was the most predictive genus in saliva samples. The accuracy of the classification was 75% when relying on rumen bacterial genera (Figure 4a) and 62.5% when relying on the bacterial genera of saliva samples (Figure 4b).

In Vitro Experiment with Microbial Inoculum from S and U Cows. Generally, microbes collected from SARA-susceptible cows produced more total VFA (TVFA) than microbes originating from SARA-unsusceptible animals (Figure 5; $P_{\text{Group}} = 0.049$). Also, TVFA was higher at pH 6.8 than in incubations with a buffer solution at pH 5.8 (Figure 5; $P_{\text{pH}} < 0.001$). Exposure to dry cows' sterile supernatant rather than their own sterile supernatant reduced the fermentative capacity of the microbes, except when microbes from the U group were incubated at pH 5.8, where a limited TVFA production was maintained, as observed in the pH 5.8 incubations with cows' own sterile supernatant (Figure 5; $P_{\text{Supernatant} \times \text{Group} \times \text{pH}} = 0.054$). On the contrary, introduction of dry cows' sterile supernatant instead of cows' own in incubations at pH 5.8 with microbes from the S group completely stopped TVFA production (Figure 5; $P_{\text{paired t-test}} < 0.001$; 29.9 ± 9.58 $\mu\text{mol/flask}$).

Hardly any net lactate accumulation was observed in incubations with microbes from unsuceptible cows incubated with their own supernatant at pH 6.8 (Figure 6). Irrespective of the microbial origin (U or S group), introduction of dry cows' sterile supernatant (Figure 6; $P_{\text{Supernatant}} = 0.028$) as well as reduction of the buffer pH (Figure 6; $P_{\text{pH}} = 0.003$) enhanced net lactate accu-

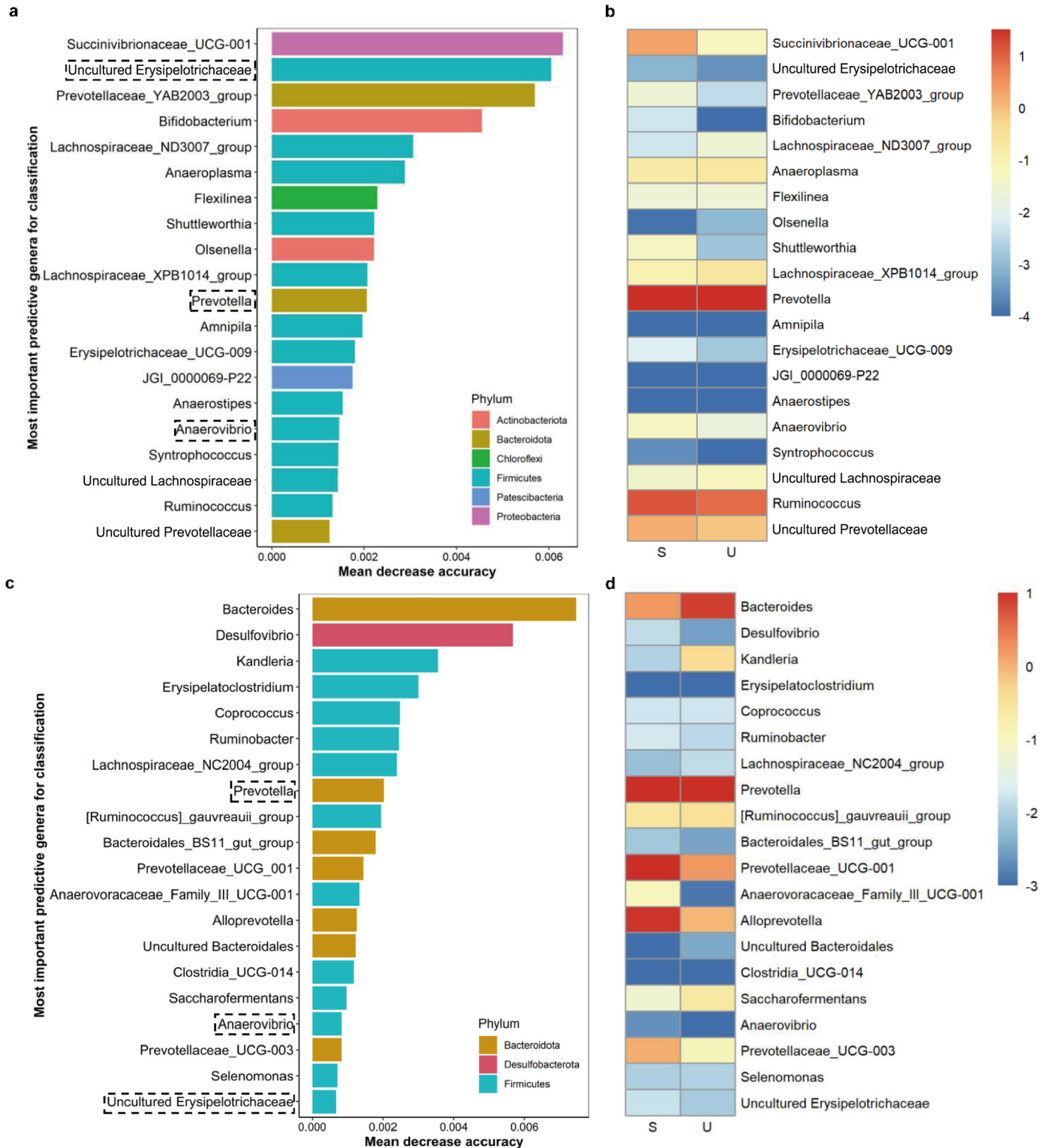


Figure 3. The 20 most predictive genera to classify samples of SARA-susceptible (S) versus unsusceptible (U) cows based on bacterial analysis of rumen (a and b) and saliva samples (c and d). SARA susceptibility classification was based on time of pH below 6 during the first 3 wk postpartum (Table 3). Predictive importance of the bacterial genera was based on the mean decrease in accuracy of the random forest classification (a and c) of cows that were susceptible (S, n = 7) or unsusceptible (U, n = 9) to SARA. Heat maps (b and d) indicate the log relative abundances of these bacterial genera. S cows: mean or median of time of pH below 6 > 180 min/d; U cows: median and mean of time of pH below 6 equals 0 and does not exceed 10 min/d, respectively. The common genera between rumen and saliva samples are indicated by dotted squares.

mulation, with the highest accumulation in incubations where both were combined (Figure 6; $P_{\text{Supernatant} \times \text{pH}} = 0.045$).

DISCUSSION

Inter-individual variation in SARA susceptibility has been shown among dairy cows (e.g., Mohammed et al., 2012; Khiaosa-ard et al., 2018). The origin of the inter-individual variation could be multifactorial, as previous studies have related inter-animal variation in SARA susceptibility to feeding behavior (Khiaosa-ard et al., 2018; Plaizier et al., 2018; Stauder et al., 2020), parity (Stauder et al., 2020; Khorrami et al., 2021), dietary factors (Khorrami et al., 2021), rumen microbial composition (Khafipour et al., 2009a; Chen et al., 2012; McCann et al., 2016), and genetics (Plaizier et al., 2018). Although variation in SARA susceptibility has been studied in relation to shifts in the rumen microbial community, these studies particularly followed a SARA induction protocol (e.g., Khafipour et al., 2009a; Chen et al., 2012; McCann et al., 2016). On the contrary, knowledge on inter-animal differences in the rumen microbial population under practical management conditions, during the SARA-risky transition period, is still lacking.

Inter-animal variation in reticular pH was observed among cows in the 3-wk postpartum period in the current study, which allowed us to classify cows into 4 groups based on reticular pH: S, MS, MU, and U cows

(Table 3). Nevertheless, it should be noted that not all cows classified in the susceptible group experienced SARA, according to the definition proposed by Zebeli et al. (2008)—that is, rumen pH below 5.8 (equivalent to reticular pH below 6.0) for more than 330 min/d. Over the entire 3-wk postpartum period, only 4 out of the 10 susceptible cows showed a median or mean time of reticular pH below 6 (equivalent to rumen pH 5.8) exceeding 330 min/d, whereas all susceptible cows suffered from SARA (defined as reticular pH below 6.0 for more than 330 min/d) at least 2 d of the 21-d measuring period.

Although only a limited number of animals met SARA conditions, *sensu stricto*, over the whole 3-wk postpartum period, great inter-animal variation in reticular pH was observed, and the 4 groups differed in pH characteristics. Accordingly, we aimed to investigate the rumen bacterial composition in these 4 groups of cows. A higher bacterial copy number was observed in rumen fluid sampled 21 d postpartum from U cows. In contrast, none of the groups differed in α -diversity indices, which was similar to the results found in cows differing in risk of SARA development undergoing a high-grain challenge (Mohammed et al., 2012; Wetzel et al., 2017; Dewanckele et al., 2019). However, β -diversity analysis revealed significant differences in the rumen bacterial community between S and U cows. Higher relative abundances of the genera *Prevotellaceae_YAB2003*, *Ruminococcus*, and *Ruminococcaceae_UCG-001*, *Prevotella*, and the family *Ruminococ-*

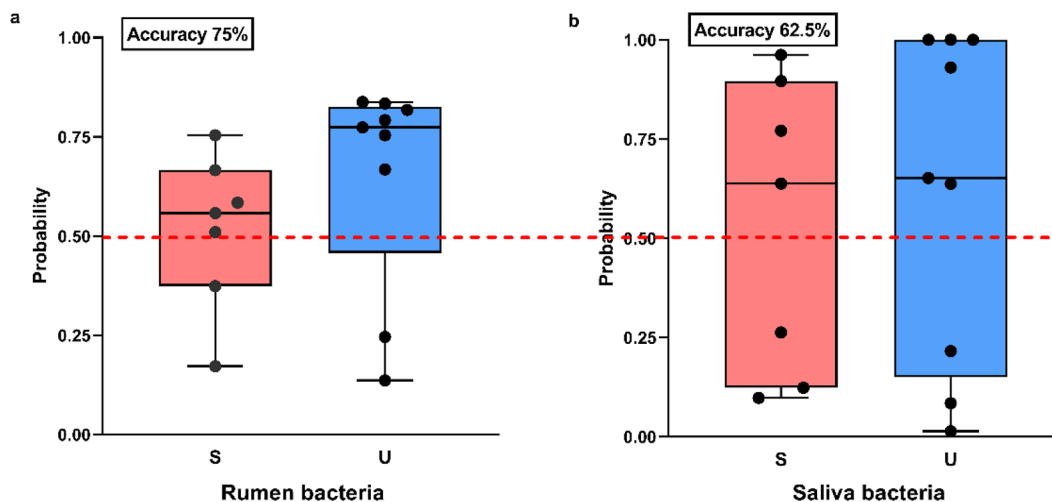


Figure 4. Predicted probability of the random forest model based on the bacterial community of ruminal fluid (a) and saliva (b) to discriminate cows that were susceptible (S, $n = 7$) or unsusceptible (U, $n = 9$) to SARA. Dots represent the probability of correct classification through the random forest model for each individual cow (10-fold cross-validation). SARA susceptibility classification was based on time of pH below 6 during the 3-wk postpartum period. S cows: mean or median of time of pH below 6 > 180 min/d; U cows: median and mean of time of pH below 6 equals 0 and does not exceed 10 min/d, respectively. The upper and lower edges of boxes represent the upper and lower quartiles of the interquartile range, and the midline is the median. The whiskers show the upper and lower values within 1.5 times the interquartile range from the upper and lower quartiles.

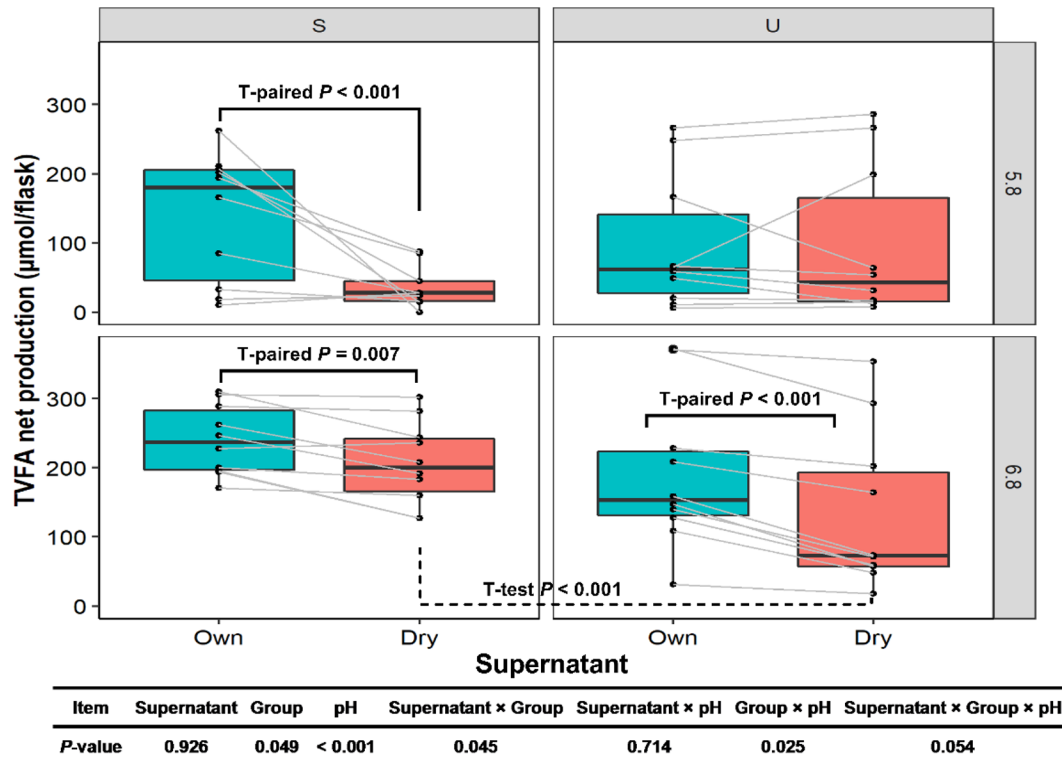


Figure 5. Net in vitro total volatile fatty acid (TVFA) production by microbes from cows classified as susceptible (S, n = 10) or unsusceptible (U, n = 10) to SARA during the first 3 wk postpartum. In vitro incubation was performed either with dry cows' or with lactating cows' own sterile supernatant at pH 5.8 or 6.8. As significant 2- and 3-way interactions occurred, treatments were compared within pH conditions. When significant, differences between supernatants (Own vs. Dry, paired *t*-test within inoculum) and inoculum (S vs. U, unpaired *t*-test within supernatant) are indicated with solid and dotted lines, respectively. The upper and lower edges of boxes represent the upper and lower quartiles of the interquartile range, and the midline is the median. The whiskers show the upper and lower values within 1.5 times the interquartile range from the upper and lower quartiles. The dots represent individual values.

caceae were observed in the rumen bacterial community of S cows. Starch-degrading bacteria have often been reported to be included in these genera and families (Strobel, 1992; Klieve et al., 2007; Mu et al., 2021). In line with this, McCann et al. (2016) also found an increase in the abundance of the family *Prevotellaceae* and the genus *Prevotella* in cows with rumen pH below 5.6 for more than 3 h under a SARA challenge including 1-d feed restriction followed by increased concentrate intake. Nevertheless, in our case, differences between groups were not provoked by changes in total DMI or intake of roughage or concentrate, nor by differences in diurnal intake pattern. In addition to starch degraders, known lactate producers, such as *Streptococcus*, *Sharpea*, and *Succinivibrionaceae_UCG-001* were higher in S cows compared with U cows, which could have been expected given their tolerance to low pH, probably linked to the enhanced activity of lactate dehydrogenase in bacterial cells of these genera under low-pH circumstances (Asanuma and Hino, 1997; Kamke et al., 2016; Dai et al., 2020). Increased abundance of lactate-producing bacteria in SARA-susceptible cows also has

been reported in previous studies in which increased abundance of uncultured *Streptococcaceae* and *Streptococcus bovis* were observed in cows experiencing a reticular pH below 6.0 (equivalent to rumen pH below 5.8) of at least 90 min/d, with a group mean of 7.54 h/d (Dewanckele et al., 2019), as well as in cows with mean time of rumen pH below 5.6 of at least 337 min/d (Khafipour et al., 2009a). However, higher abundance of the lactate utilizers (i.e., *Megasphaera*) in S cows was not observed in our study, whereas such difference was reported by Dewanckele et al. (2019). As the higher relative abundance of *Megasphaera* in SARA-susceptible cows was observed during both the low- and the high-starch periods in the study by Dewanckele et al. (2019), this seemed to be an animal characteristic rather than directly related to the rumen pH conditions. However, in the study by Khafipour et al. (2009a), *Escherichia coli*, associated with endotoxin release, also increased in the severe-SARA cows (rumen pH below 5.6 for at least 337 min/d) under a 60% grain-induced SARA challenge. In our study U and S cows did not differ in abundance of the genus *Escherichia*, to which this

species belongs. However, only 4 out of the 10 S cows suffered from SARA over the entire 3-wk postpartum period in the current study. Within these 4 cows, only in 1 was *Escherichia-Shigella* detected, with a relative abundance of 0.073%. Similarly, in the mild-SARA cows (rumen pH below 5.6 for at least 217 min/d) reported by Khafipour et al. (2009a), no increase in *Escherichia coli* has been observed. Accordingly, *Escherichia coli* proliferation seems an indication of harsh rumen pH conditions under a SARA challenge. Hence, in terms of changes in bacterial families and genera with starch degraders and lactate producers, inter-animal variation of periparturient cows under practical management seems comparable to changes observed in cows under SARA challenges. However, *Escherichia coli* seems an indicator for harsher pH conditions.

Noninvasive oral samples have been reported as an attractive alternative to sampling of the ruminal bacterial community at a large scale (Kittelman et al., 2015; Tapio et al., 2016). Accordingly, we hypothesized that the bacterial genera, discriminating between S and U

cows, would be the same in rumen and saliva samples. However, the top 20 most predictive genera in the random forest classification of S and U cows showed only 3 common genera in both sample types: uncultured *Erysipelotrichaceae*, *Prevotella*, and *Anaerovibrio*. Strikingly, the genus *Streptococcus* was one of the dominant genera in the saliva samples, although not within the top 10 of most abundant rumen genera. This may be related to the difficulty of manually removing some of the oral ASV from this genus. As such, this also could have affected the classification accuracy. As a result, in the current study, the bacterial community in saliva seemed less accurate than the rumen bacteria in classifying S and U cows (62.5% vs. 75% accuracy, respectively). Obviously, these results should be interpreted with caution, as the number of observations included in the current study is relatively limited, particularly for the random forest classification methodology. Nevertheless, in former studies applying this technique to classify animals according to their health status or diets based on their gastrointestinal microbiota, the number

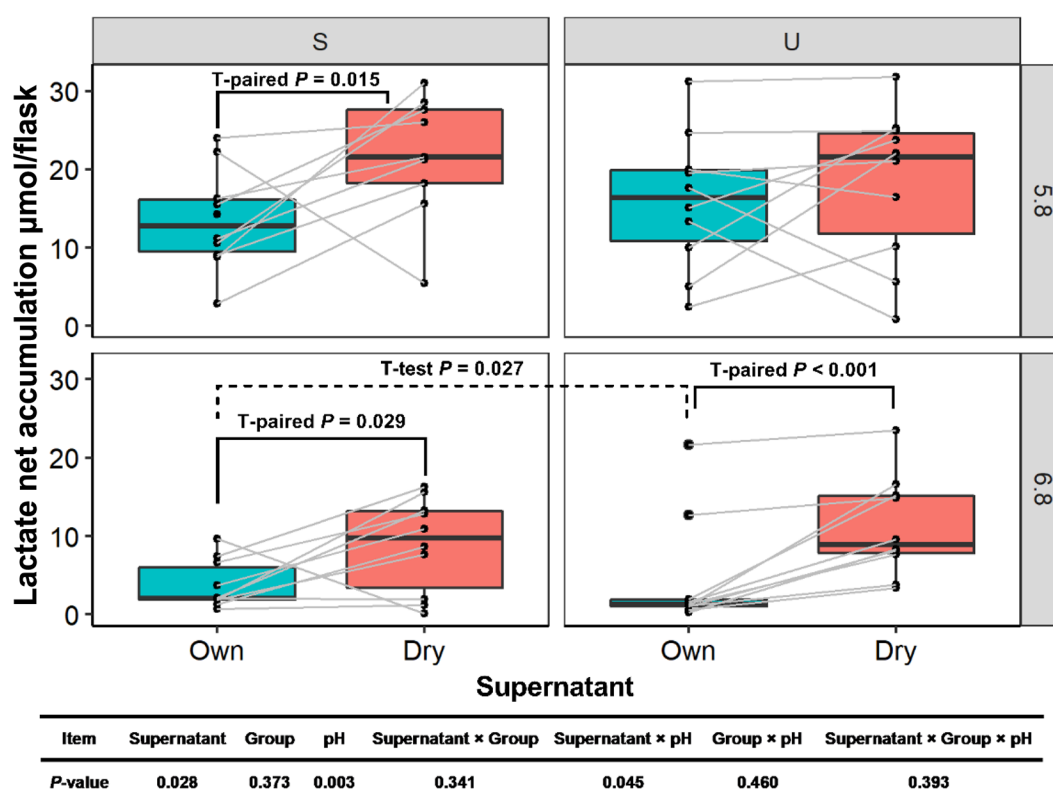


Figure 6. Net in vitro lactate accumulation by microbes from cows classified as susceptible (S, n = 10) or unsusceptible (U, n = 10) to SARA during the first 3 wk postpartum. In vitro incubation was performed either with dry cows' or with lactating cows' own sterile supernatant at pH 5.8 or 6.8. As significant 2-way interaction occurred, treatments were compared within pH conditions. As significant 2- and 3-way interactions occurred, treatments were compared within pH conditions. When significant, differences between supernatants (Own vs. Dry, paired *t*-test within inoculum) and inoculum (S vs. U, unpaired *t*-test within supernatant) are indicated with solid and dotted lines, respectively. The upper and lower edges of boxes represent the upper and lower quartiles of the interquartile range, and the midline is the median. The whiskers show the upper and lower values within 1.5 times the interquartile range from the upper and lower quartiles. The dots represent individual values.

of animals included was not always higher (16, 12, and 39 in the studies by Xin et al., 2021; Liu et al., 2020; and Ma et al., 2020, respectively).

As microbiota carry out various metabolic functions, variations in the bacterial community structure in cows differing in reticular pH profile might affect the functionality of microorganisms to cope with different disturbances during transition. In the current study, the functionality of the rumen microbiota was evaluated in vitro through exposure of the microbial inoculum of S and U cows to an anaerobic buffer at different pH (5.8 and 6.8) and in the presence of sterile supernatant of their own and of dry cows' rumen fluid. Ruminant pH is a critical factor affecting and potentially disturbing metabolic activity of rumen microbiota (Chen et al., 2019; Khiaosa-ard et al., 2020). At low pH, in situ fiber degradation and overall rumen fermentation were reduced by alteration of metabolic pathways associated with glycolysis and pyruvate fermentation (Pourazad et al., 2017; Li et al., 2021). In the current study as well as in the study by Khiaosa-ard et al. (2020), this reduced fermentation was also observed when exposing rumen inoculum to lower pH conditions in vitro. Nevertheless, the reduction in TVFA concentration (i.e., 7.8 mmol/L, from 92.5 to 84.7 mmol/L) from high-pH to low-pH conditions in the study of Khiaosa-ard et al. (2020) was less severe than the reduction observed in the current study. This may be related to the milder acidotic conditions (pH 6.0 vs. a standard pH of 6.6) in Khiaosa-ard et al. (2020) compared with the stronger pH reduction (pH 6.8 to pH 5.8) induced in the current study. Further, exposure to dry cows' rumen fluid was chosen because the diet of cows in the far-off period differentiates most from that of cows in early lactation. As such, it could be hypothesized that exposure to this type of supernatant requires more adaptive abilities of the ruminal microbes, as compared with exposure to their own supernatant. When exposed to no stressor (pH of 6.8 and own supernatant) or one stressor (i.e., either pH of 5.8 with own supernatant or dry cows' supernatant at pH 6.8), microbial inoculum collected from S cows produced more TVFA than microbes originating from U cows. Previous in vivo studies have also reported an increase of the TVFA concentration in the rumen of SARA-susceptible steers compared with unsusceptible steers in an 85%-grain SARA challenge (Chen et al., 2012; Schlau et al., 2012). Obviously, in vivo, final TVFA concentrations are determined by the balance between the amount of TVFA produced during rumen fermentation and their removal and neutralization. Nevertheless, Gao and Oba (2016) suggested no difference in TVFA absorption rate between susceptible and unsusceptible cows, which would imply that higher

ruminal TVFA concentrations could have been particularly provoked by a higher TVFA production.

Interestingly, when exposed to a combination of stressors, such as low pH (5.8) and external supernatant (dry cows' sterile supernatant), microbes obtained from U cows were capable of maintaining TVFA production at the level of incubations with their own sterile supernatant at pH 5.8 without increase in lactate accumulation. By contrast, TVFA production was completely stopped and lactate accumulation was highest in pH 5.8 incubations with external supernatant and microbes collected from S cows. The reduced resilience of SARA microbiota to a combination of disturbances was also observed by Rabaza et al. (2020): in their study, 20 animals suffering from SARA before the experimental period were exposed to a variable length of feed withdrawal (0, 12, 24, or 36 h) followed by a resumption of the feed supply. Total rumen VFA concentrations decreased, and lactate accumulation increased with increasing length of feed withdrawal. Although, in that study, no comparison was made with animals that did not suffer from SARA before feed withdrawal, nevertheless, both their study and ours could suggest an association between the inter-animal variation in SARA susceptibility and the capacity of their ruminal microorganisms to adapt to external stressors.

CONCLUSIONS

Variation in reticular pH was shown among cows housed under the same conditions during a 3-wk postpartum period. Cows highly susceptible to SARA (S group), experiencing low pH for a longer period, differed in ruminal bacterial copy numbers and composition from those that hardly experienced any pH drop below 6.0 during this postpartum period (U cows). More specifically, the relative abundance of lactate producers, such as *Streptococcus*, *Sharpea*, and *Succinivibrionaceae_UCG-001*, as well as starch degraders of the genera *Prevotellaceae_YAB2003*, *Ruminococcus*, and *Ruminococcaceae_UCG-001*, were increased in the rumen bacteria of S cows. In contrast, the genera *Lachnospiraceae_ND3007* and *Oscillospiraceae_V9D2013* were more abundant in rumen samples from U cows. Additionally, pH-associated inter-animal differences were observed in the salivary bacteria, but common differences in ruminal and salivary bacterial genera were limited. Furthermore, inter-animal reticular pH differences were reflected in changes in the fermentative activity of microorganisms upon in vitro exposure to pH of 6.8 or 5.8 and sterile supernatant of their own or dry cows' rumen fluid. The S inoculum produced more TVFA, except at low pH with the dry cows'

supernatant, where VFA production was completely impaired and lactate accumulation was highest. Taken together, SARA-susceptible cows showed differences in the rumen microbial community, salivary bacterial community, and ruminal microbial fermentative capacity in vitro.

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


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