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Characterizing ruminal acidosis risk: A multiherd, multicountry study

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ABSTRACT

A multicenter observational study was conducted on early lactation Holstein cows (n = 261) from 32 herds from 3 regions (Australia, AU; California, CA; and Canada, CAN) to characterize their risk of acidosis into 3 groups (high, medium, or low) using a discriminant analysis model previously developed. Diets ranged from pasture supplemented with concentrates to total mixed ration (nonfiber carbohydrates = 17 to 47 and neutral detergent fiber = 27 to 58% of dry matter). Rumen fluid samples were collected <3 h after feeding and analyzed for pH, and ammonia, D- and L-lactate, and volatile fatty acid (VFA) concentrations. Eigenvectors were produced using cluster and discriminant analysis from a combination of rumen pH, and ammonia, D-lactate, and individual VFA concentrations and were used to calculate the probability of the risk of ruminal acidosis based on proximity to the centroid of 3 clusters. Bacterial 16S ribosomal DNA sequence data were analyzed to characterize bacteria. Individual cow milk volume, fat, protein, and somatic cell count values were obtained from the closest herd test to the rumen sampling date (median = 1 d before rumen sampling). Mixed model analyses were performed on the markers of rumen fermentation, production characteristics, and the probability of acidosis. A total of 26.1% of the cows were classified as high risk for acidosis, 26.8% as medium risk, and 47.1% as low risk. Acidosis risk differed among regions with AU (37.2%) and CA (39.2%) having similar prevalence of high-risk cows and CAN only 5.2%. The high-risk group had rumen phyla, fermentation, and production characteristics consistent with a model of acidosis that reflected a rapid rate of carbohydrate fermentation. Namely, an acetate to propionate (1.98 ± 0.11), concentrations of valerate (2.93 ± 0.14 m*M*), milk fat to protein ratio (1.11 ± 0.047), and a positive association with abundance of phylum *Firmicutes*. The medium-risk group contains cows that may be inappetant or that had not eaten recently or were in recovery from acidosis. The low-risk group may represent cattle that are well fed with a stable rumen and a slower rumen fermentation of carbohydrates. The high risk for acidosis group had lower diversity of bacteria than the other groups, whereas CAN had a greater diversity than AU and CA. Rumen fermentation profile, abundance of ruminal bacterial phyla, and production characteristics of early lactation dairy cattle from 3 regions were successfully categorized in 3 different acidosis risk states, with characteristics differing between acidosis risk groups. The prevalence of acidosis risk also differed between regions.

Key words: discriminant analysis, microbiome, ruminal acidosis, valerate

INTRODUCTION

Acidosis remains a major challenge for dairy and feedlot cattle production worldwide. It is caused by the consumption of diets high in readily fermentable carbohydrates and low in effective fiber such as grains which lead to production of organic acids that exceed the buffering capacity of the rumen (Nagaraja and Titgemeyer, 2007). Current control strategies such as diet management and inclusion of feed additives to modify or buffer the rumen against a build-up of organic acids are unlikely to be effective in all cattle due to the large variation in individual cow response (Golder et al., 2014a).

Despite decades of research into acidosis and ruminant health we still lack understandings of the associations between the genetics of the host, dietary inputs, the

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rumen microbiota and their fermentation outputs, and production (Golder et al., 2018; Clemmons et al., 2019). Optimizing rumen function and controlling acidosis remain elusive challenges as a result. To achieve these goals, we need to accurately diagnose ruminal acidosis, preferably in a cost-effective and timely manner. This is critical as failure to accurately define a condition can lead to failures in its control (Lean and Golder, 2019). There is currently a lack of consistency in definition and criteria for accurate diagnosis of ruminal acidosis (Plaizier et al., 2018). Rumen pH measurements are often used as the primary diagnostic tool but may not be accurate indicators of an acidotic state (Plaizier et al., 2022), particularly when only single measurements are taken; rumen pH fluctuates throughout the day and single measures may not be representative of the entire rumen (Duffield et al., 2004; Denwood et al., 2018). Rumen pH is only one factor that may be involved in the pathology of ruminal acidosis, and individual cows on the same diet differ in pH (Denwood et al., 2018; Lean and Golder, 2019). Rumen pH also does not meet all the postulates of Evans (1976) or those modified by Lean et al. (2009) for a causal pathway with ruminal acidosis, with sensitivity, specificity, and area under the curve values of 0.74, 0.79, and 0.822 for pH measures on rumen fluid samples collected by rumenocentesis and of 0.68, 0.84, and 0.801 for those collected by stomach tube (Lean and Golder, 2019).

The focus of definitions is now shifting from rumen pH to the rumen microbiome and its metabolic activity (Bramley et al., 2008; Saleem et al., 2012; McCann et al., 2016). Compounds such as lactic acid, free AA and ammonia, bicarbonate ion, and methane could affect pH without affecting the proportion or concentration of VFA; all H^+ donors and sinks should be measured when assessing acidotic status and be supported by DMI, milk performance, and behavioral data (Nasrollahi et al., 2017). Plaizier et al. (2018) suggest an accurate diagnosis of ruminal acidosis requires a combination of clinical examinations of cows and analyses of herd management and feed quality, accompanied by rumen pH, blood, milk, urine, and fecal parameters.

To establish a more robust strategy for the diagnosis of ruminal acidosis that is not solely reliant on rumen pH, Bramley et al. (2008) collected diet, health, production, and rumen fluid data from 800 cows from 100 randomly selected commercial dairy herds across Southern Australia. The data were used to create a model by k-means cluster and discriminant analysis that classified cattle based on their rumen pH, individual VFA, ammonia, and D-lactate concentrations into one of 3 categories (1) acidotic, (2) suboptimal rumen function, or (3) normal (Bramley et al., 2008; Golder et al., 2012a). The prevalence of individual cattle in each of those 3 categories was then used to categorize the 100 herds into those 3 categories. Hence both individual and herd-level risks for acidosis were defined. Although the diet, health, and production data were not included as inputs into the model, their associations were used to support and define the characteristics of the categories (Bramley et al., 2008, 2013). The characteristics of each category for acidosis are described in Bramley et al. (2008). The model has been strongly supported by Golder et al. (2012a) using 5 independent carbohydrate challenge studies and also by the Irish data set of O'Grady et al. (2008) on a separate occasion (unpublished data). In addition, a randomized clinical study identified a profound difference in risk of acidosis, as defined by the Bramley et al. (2008) model with increased intake of concentrates (Golder et al., 2014b); therefore, meeting several of Evans' postulates as modified by Lean et al. (2009).

This is the first study in a series of 3 designed to improve our understandings of acidosis risk status and ultimately control of ruminal acidosis through evaluating associations with host genomics, diet, rumen metabolites, ruminal bacterial taxa, and production characteristics in early lactation Holstein cows from different geographical regions. The aim of this study is (1) to categorize the acidosis risk status of these cattle by using the discriminant analysis model by Bramley et al. (2008) and (2) describe the rumen metabolite, ruminal bacterial taxa, and production characteristics of cattle in each of Bramley's 3 acidosis risk categories. We hypothesize that (1) acidosis risk will differ between geographical regions and (2) rumen metabolite, ruminal bacterial taxa, and milk production values will differ between acidosis risk categories.

MATERIALS AND METHODS

This study was carried out in accordance with the recommendations of The Australian Code for Care and Use of Animals for Scientific Purposes, Scibus Animal Ethics Committee (Scibus 0618–1219), the University of California Davis Institutional Animal Care and Use Committee (protocol number #20729), the University of Wisconsin, College of Agriculture and Life Sciences Animal Care and Use Committee (approval A006225), and Animal Care Committee at the University of Guelph (Animal Utilization Protocol 4124).

Experimental Design and Study Population

This is a multicenter observational study. The target population was 320 cows with 8 cows (4 of parity 1; 4 of parities 2 to ≤4) from 40 herds [10 in California (**CA**), 10 in Canada (**CAN**), 10 in Australia (**AU**), and 10

in Wisconsin (**WI**)]. The actual population achieved was a total of 293 cows from 36 farms (10 in CA, 12 in CAN, 10 in AU, and 4 in WI). For the purposes of this paper the data from the 4 herds in WI were removed as the selection criteria for the percentage of parity 1 cows sampled was not met. Descriptive details on these herds are still included as their data were utilized in Golder et al. (2023b). The population used in this study was 261 cows from 32 farms. The herds from AU were in 4 geographical regions (Western districts of Victoria, Finley in NSW, the South Coast of NSW, and the Macarthur region of NSW).

Power Calculations

To identify a difference in risk of acidosis, a difference of 5.5 m*M* of propionate measured as a single diagnostic test with a standard deviation of 10 m*M* [the largest SD for propionate of groups in the study by Bramley et al. (2008)] would be sufficient to differentiate nonacidotic from acidotic cows. Consequently, a difference between 34.5 and 29 m*M* in propionate would require 320 cows given an intraclass correlation of 0.2.

The power calculations were performed using the *rdpower* function in Stata (version 14.2; StataCorp LLC). The study operates at 2 levels (crd2 is the code for complete randomized design at 2 levels). The effect size is 0.55, representing a 5.5 m*M* difference in propionate concentrations, as previously explained and the number of head per herd is 8 (n) and the number of herds per treatment is 20 (m). The intraclass correlation within cluster is 0.2.

There was no logical power calculation to estimate the population required to address our a priori objective to find genomic markers for ruminal acidosis. However, we found significance with 32 animals in a previous study group (Golder et al., 2018). Hence, we had a substantial probability of finding genetic markers with modest numbers of cattle.

Farm and Cow Selection

Farms were selected based on willingness to participate, had accurate details on cow identification, parentage, and calving history, had predominantly Holstein-Friesian lactating cattle, conducted herd recording, were able to provide diet details, and a sample of the diet for analysis. There were no restrictions based on herd size, production level, production system, or milking frequency per day. Details of the herds selected are summarized in Table 1.

Cows were randomly selected that met the following criteria: were Holstein-Friesian, a maximum of fourth parity (4 cows of parity 1; 4 cows of parity >1 and ≤4), were between 10 and 100 DIM, had no apparent current clinical illness, and were from different sires. This information was obtained from each herd's herd management software.

Rumen Fluid Collection

Rumen fluid collections were intended to coincide with herd testing, but this was not always practical. Rumen fluid collection was a median of 1 d after herd testing (range = 38 d before to 18 d after herd testing). Rumen fluid was collected within 3 h of feeding using a customized stomach tube and a tube approximately 3 m in length with a multiholed aluminum probe at one end into a 500-mL container. The tube was inserted to a minimum length of approximately 2 m. Rumen fluid was scored for saliva contamination as described by Bramley et al. (2008) using a 3-point scoring system (0 being the lowest and 2 being the highest level of contamination). Rumen fluid collection was repeated up to 3 times if a saliva score of >0 was observed, and the lowest scoring sample was used for pH measurement. Two of the 261 samples retained for analysis that had a saliva score of 2. Rumen fluid samples were analyzed for pH immediately after collection using a pH meter (LAQUAtwin pH-22, Horiba). Aliquots of 2 × 15 mL of raw rumen fluid were immediately stored on ice before storage at −20°C and later shipped on dry ice to Arm & Hammer Animal and Food Production (Waukesha, WI) for bacteria community analysis. A further 10-mL aliquot of raw rumen fluid was placed on ice before being centrifuged at 1,110 × *g* for 15 min at room temperature and the supernatant aliquoted into 4 × 5-mL collection tubes. Aliquots were stored −20°C and later shipped on dry ice for rumen metabolite analysis.

Rumen Fluid Bacteria Community Analysis

Sample Processing. Rumen fluid was thawed in cold water. At least 6.0 mL of rumen fluid was poured into a filtered Whirl-Pak bag (Nasco Sampling). A one to 5 dilution was created in a sterile 1% Bacto peptone in deionized water solution (Thermo Fisher Scientific) using a DiluFlow gravimetric dilutor (Interscience) and masticated at 260 rpm for 1 min in a Stomacher 400 Circulator (Seaward Laboratory Systems Inc.) Approximately 25 mL of diluted rumen fluid was poured into a 50-mL Falcon tube (Thermo Fisher Scientific). Cells were pelleted by centrifugation at 13,880 × *g* for 10 min at 4°C, after which the supernatant was discarded. Cell pellets were resuspended in 1 mL of T_{50} E_{10} [50 m*M* Tris HCL (VWR International LLC) and 10 m*M* EDTA disodium salt dihydrate (Acros Organics)] and 1 mL of the resulting cell suspension was transferred to a

Table 1. Summary of herd test agency, number of times milked per day, herd size, farm type, feed type, minimum (Min.), median (Med.), and maximum (Max.) DIM of cows at rumen sampling and ECM (mean ± SD) of the cows at the closest herd test to the rumen sampling of herds enrolled in the study

Herd[1]	Area	Herd test agency	Times milked/d[2]	Herd size[3]	Farm type	Feed type[4]	DIM			ECM (kg/d)
							Min.	Med.	Max.	
AU1	Western Districts, VIC	National Herd Development Terang	2	372	Pasture	PC	35	65	88	23.8 ± 1.42
AU2	Macarthur region, NSW	NuGenes, Numurkah, VIC	2	200	Pasture	PC	19	55	85	27.9 ± 2.46
AU3	South Coast, NSW	Dairy Express, Armidale, NSW	2	280	Pasture	PC	52	71	90	24.4 ± 2.28
AU4	Finley, NSW	NuGenes, Numurkah, VIC	2	564	Freestall	TMR	13	57	90	42.0 ± 3.92
AU5	Western Districts, VIC	National Herd Development, Kyabram, VIC	2	604	Pasture	PC	17	35	65	33.3 ± 3.55
AU6	South Coast, NSW	Dairy Express	2	197	Pasture	PC	25	46	58	26.8 ± 2.40
AU7	Finley, NSW	NuGenes, Numurkah, VIC	2	237	Pasture	PC	32	87	97	30.6 ± 2.21
AU8	Western Districts, VIC	National Herd Development, Terang, VIC	2	188	Pasture	PC	33	53	88	29.0 ± 1.83
AU9	South Coast, NSW	Dairy Express, Armidale, NSW	3	670	Pasture	PMR	42	76	93	42.4 ± 4.48
AU10	Finley, NSW	National Herd Development, Cohuna, VIC	2	270	Pasture	PC	24	52	74	23.8 ± 1.62
CA1	Tranquillity, Fresno County	Fresno DHIA	3 F, 2 H	5,974	Freestall	TMR	37	50	72	55.9 ± 5.59
CA2	Hanford, Kings County	Kings County DHIA	3 F, 2 H	3,337	Open lot	TMR	67	83	110	41.2 ± 1.63
CA3	Tulare, Tulare County	Tulare DHIA	4 F, 2 H	1,703	Freestall	TMR	51	77	99	44.6 ± 3.68
CA4	Hanford, Kings County	Tulare DHIA	2	2,462	Freestall	TMR	63	74	83	41.3 ± 2.44
CA5	Tulare, Tulare County	Tulare DHIA	3 F, 2 H	1,698	Freestall	TMR	66	86	94	34.9 ± 1.91
CA6	Tulare, Tulare County	Tulare DHIA	3 F, 2 H	5,007	Freestall	TMR	72	82	91	41.4 ± 1.33
CA7	Hanford, Kings County	Kings County DHIA	3 F, 2 H	6,294	Freestall	TMR	49	73	83	53.0 ± 3.32
CA8	Porterville, Tulare County	Tulare DHIA	3 F, 2 H	893	Open lot	TMR	51	58	89	36.6 ± 3.43
CA9	Porterville, Tulare County	Tulare DHIA	2	1,604	Open lot	TMR	74	79	99	35.8 ± 2.15
CA10	Porterville, Tulare County	Tulare DHIA	3 F, 2 H	950	Freestall	TMR	63	91	97	42.5 ± 2.47
CAN1	Bright, ON	Lactanet, Guelph	2	255	Freestall	TMR	20	44	92	38.4 ± 3.24
CAN2	Brussels, ON	Lactanet, Guelph	Robot	450	Freestall	TMR	17	38	80	44.1 ± 2.87
CAN3	Gowanstown, ON	Lactanet, Guelph	2	57	Tiestall	TMR	26	73	102	34.1 ± 2.44
CAN4	Elora, ON	Lactanet, Guelph	2	255	Freestall	TMR	35	63	83	40.0 ± 4.19
CAN5	Fergus, ON	Lactanet, Guelph	2	115	Freestall	TMR	11	37	59	44.4 ± 3.70
CAN6	Woodstock, ON	Not tested	Robot	306	Freestall	TMR	13	44	98	
CAN7	Gowanstown, ON	Lactanet, Guelph	2	74	Tiestall	TMR	16	48	99	38.1 ± 2.68
CAN8	Listowel, ON	Lactanet, Guelph	2	126	Freestall	TMR	23	73	97	41.4 ± 2.83
CAN9	Cambridge, ON	Lactanet, Guelph	Robot	108	Freestall	TMR	13	42	84	35.5 ± 3.56
CAN10	Ingersoll, ON	Lactanet, Guelph	2	362	Freestall	TMR	12	40	98	39.5 ± 4.08
CAN11	Bright, ON	Not tested	Robot	263	Freestall	TMR	26	59	96	
CAN12	Listowel, ON	Lactanet, Guelph	2	327	Freestall	TMR	63	75	96	36.1 ± 2.58
WI1	Madison, WI	Valley Agricultural Software (VAS)	2	480	Freestall	TMR	12	40	89	36.1 ± 2.59
WI2	Madison, WI	Valley Agricultural Software (VAS)	2	84	Tiestall	TMR	12	31	95	36.1 ± 2.60
WI3	Madison, WI	Valley Agricultural Software (VAS)	3	360	Tiestall	TMR	49	84	98	36.1 ± 2.61
WI4	Madison, WI	Valley Agricultural Software (VAS)	2	132	Freestall	TMR	29	68	90	36.1 ± 2.62

[1]AU = Australia; CA = California; CAN = Canada; WI = Wisconsin.

[2]F = fresh cows; H = high cows.

[3]Herd size includes both lactating and dry cows for CA and CAN but only lactating cows for AU.

[4]PC = pasture + in parlor concentrate; PMR = partial mixed ration.

2-mL Basix Microcentrifuge Tube (Thermo Fisher Scientific). Cells were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, the supernatant was discarded, and cell pellets were stored at −20°C until DNA extraction was performed.

DNA Extraction, Library Preparation, and Sequencing. Extraction of genomic DNA was performed using the DNeasy PowerSoil HTP Kit (Qiagen) with bead beating taking place for 2 min in a Mini-Beadbeater-96 (BioSpec Products). Genomic DNA was shipped to the Functional Genomics Unit at the Roy J. Carver Biotechnology Center (The University of Illinois at Urbana-Champaign, Champaign, IL) for library preparation. The PCR was performed for 35 cycles with 16S V4 rRNA primers for total bacteria (Walters et al., 2015) using the 48 Access Array IFC (Fluidigm). The final size-selected amplicon pools were submitted to the DNA services laboratory at the Roy J. Carver Biotechnology Center. The final pools were quantitated using Qubit (Life Technologies) and then further quantitated by quantitative PCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories), then pooled evenly. The pool was loaded onto 1 lane of a 2-lane NovaSeq flowcell at a concentration of 1 n*M* for sequencing on the NovaSeq 6000 (Illumina Inc.) with version 1.5 sequencing reagents and 15% PhiX. The libraries were sequenced from both ends of the molecules to a total read length of 250 nucleotides from each end.

Sequence Analysis. Sequence analysis was performed using QIIME2 version 2021.2 (Bolyen et al., 2019). Quality filtering was performed to remove any Phred quality score less than 22 (Bokulich et al., 2013). Sequences were denoized and trimmed to 240 bp using deblur (Amir et al., 2017). Taxonomy was assigned by closed-reference clustering using VSEARCH (Rognes et al., 2016) to the EZBioCloud reference database (Kim et al., 2012) that was downloaded in 2017. All samples with fewer than 7,000 reads, and all *Archaea* and *Eukarya* were removed from the analysis.

There were 257 of the 261 cows from 32 farms that had ruminal bacterial phyla characterized as 4 had <7,000 sequences. A total of 25 phyla and one candidate phylum were identified within the ruminal bacterial population from the 16S rDNA sequences. Most of the sequences were from phylum *Firmicutes* [mean (± SD) raw relative abundance (**RA**) of 50.6% ± 15.7 per cow], followed by the phylum *Bacteroidetes* (mean raw RA of 29.1% ± 18.5 per cow). The *Actinobacteria*, *Tenericutes*, and *Proteobacteria* had mean raw RA of 8.04% ± 7.72, 4.55% ± 2.58, and 3.81% ± 5.94 per cow, respectively. The remaining bacterial phyla were present in low RA. Figure 1 shows the raw RA of the phyla per acidosis group.

Alpha and Beta Diversity. Alpha diversity was measured at the depth of 7,000 sequences. The number of observed features and acidosis risk group or region of each sample was imported into Prism (GraphPad Software). The differences in Shannon index between groups was determined by Kruskal-Wallis test. Rarefaction analysis (Hughes et al., 2001) indicated the depth of coverage of diversity of rumen bacteria within the rumen fluid samples was sufficient to evaluate bacteria community composition (**BCC**; Supplemental Figure 1; https://doi.org/10.6084/m9.figshare.c.6411203.v1; Golder et al., 2023a).

Beta diversity was examined by redundancy analysis using Canoco5 (Microcomputer Power). The amount of variation of the analysis explained by acidosis risk group or region was calculated as well as each axis. The amount of variation explained by acidosis risk group or region as well as rumen fermentation or milk measures was also calculated. The significance of each explanatory variable was determined by Bonferroni corrected *P*-value. In the biplots generated by Canoco5, categorical data are represented by a triangle or square, and numerical data are represented with an arrow indicating the direction in which the variable increases.

Rumen Fluid Metabolite Analysis

Rumen VFA concentrations were analyzed by an Agilent series gas chromatograph with HP6890 injection, 30-mm × 0.53-mm × 1.0-μm capillary column (Agilent Technologies Inc.), and ChemStation software (Agilent Technologies Inc.) based on methodology from Supelco (1975) at the Department of Primary Industries and Regional Development Diagnostic and Laboratory Services (DPIRD DDLS). The interassay coefficients of variation (**CV**) for acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, and caproate were 4.51, 2.67, 6.60, 2.09, 2.79, 3.78, and 9.87%, respectively, whereas the intraassay CV were 0.91, 0.74, 0.93, 0.67, 0.60, 1.29, and 2.46%, respectively.

Ammonia concentrations were measured using a direct enzymatic method utilizing the Beckman Coulter Reagent (Category no. OSR61154) provided by Beckman Coulter Australia on the AU480 clinical chemistry analyzer (NT-309) at DPIRD DDLS. The intraassay CV for drift analysis performed with an *n* of 6 at 0.57, 1.11, 2.28, 4.52, 9.00, and 16.72 m*M* were 5.58, 2.82, 2.16, 0.44, 0.28, and 0.52%, respectively. Lactate analysis was performed at Regional Laboratory Services (Benalla, VIC, Australia) after protein removal using perchloric acid precipitation. An enzymatic sample blanked end-point assay was used incorporating glutamate-pyruvate transaminase and either L-lactate dehydrogenase (Roche catalog no. 10127230001) or

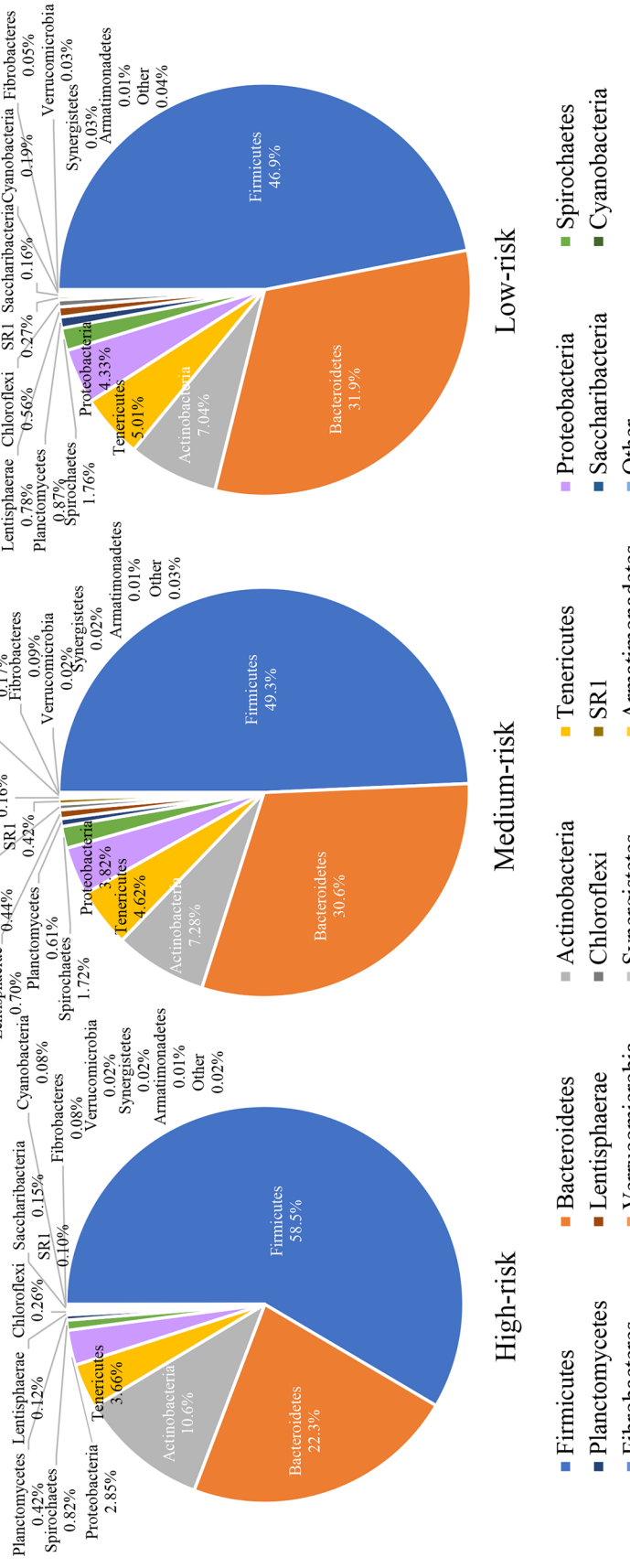

Figure 1. The raw relative abundance (%) of ruminal bacterial phyla in each acidosis group. Only phyla that had a raw relative abundance of ≥0.3% for at least one rumen sample are displayed. The raw relative abundance of all other phyla (n = 10) with minor abundances were combined and are displayed as "other," along with sequences that could not be classified, which were 0.004% for the high-risk group, 0.012% for the medium-risk group, and 0.018% for the low-risk group.

D-lactate dehydrogenase (Megazyme catalog no. E-DLDHLM), measuring change in absorbance at 340 nm as NAD is converted to NADH with quantification based on Extinction Coefficient of NADH. The interassay CV for D-lactate at 2.7 m*M* with an *n* of 18 was 6.40%. The intraassay CV for D-lactate at 2.7 m*M* with an *n* of 15 was 4.41% and L-lactate at 2.6 and 5.0 m*M* with an *n* of 17 were 2.42 and 2.28%, respectively.

Milk Collection and Analysis

Individual cow milk volume, fat, protein, and SCC values were obtained from the closest herd test to the rumen sampling date. The herd recording agencies used, the number of milkings per day, and the number of cows with a herd test result for each herd or total number of milking and dry cows at the time of rumen sampling are provided in Table 1. There were 243 out of the 261 cows that were rumen sampled that had milk measurements. Of the 18 cows without herd test results, 16 were from 2 of the initially selected and rumen sampled herds from CAN, hence an additional 2 herds had rumen collections taken to provide herd test data from 10 herds in this region. The other 2 cows without a herd test result were from 2 different herds in CA. Energy-corrected milk was calculated as ECM = [(0.3246 × milk yield) + (12.86 × fat yield) + (7.04 × protein yield)], as per NRC (2001).

Diet

The diets of the North American herds were all TMR, whereas those in AU were primarily pasture-based with supplementary concentrates or silage. A sample of TMR (~500 g) was collected from each of the herds in CA and CAN, as well as one in WI, and were analyzed at DairyOne Cooperative Inc., Forage Testing Laboratory (Ithaca, NY) according to wet chemistry AOAC International (1999) methods detailed in Golder et al. (2019). For the herds in AU ~500 g of feed components were analyzed by either wet chemistry (Golder et al., 2019) or near-infrared reflectance spectroscopy at DairyOne according to the equations based on methods detailed by Bramley et al. (2012), with the exception of NDF, which was determined as described by Van Soest et al. (1991), using heat-stable amylase without sodium sulfite and the NFC equation that was NFC = 100 − (NDF + CP + crude fat + ash). The diet chemical composition for the AU herds was then estimated using CPM Dairy Ration Analyzer (version 3.10; Cornell-Penn-Miner, Cornell University). For 3 herds from WI, their nutritionist provided details of the ration components and respective feed tests of these components for an estimation of diet chemical composition in CPM Dairy Ration Analyzer. The key nutrient composition measures for each herd are reported in Table 2.

Acidosis Risk

Acidosis risk category of each cow at the time of rumen sampling was classified by appending their individual VFA, ammonia, D-lactate, and pH values to the existing data set of Bramley et al. (2008) that was used to develop k-means cluster analysis group allocation and variables were standardized to a z-statistic. The pH values used to develop group categories in Bramley et al. (2008) were measured from samples collected by rumenocentesis. Bramley et al. (2008) tested 2, 3, and 4 clusters but 3 clusters provided the best fit for the variables. A k-nearest neighbor nonparametric discriminant analysis (*discrim knn*; Stata version 16.1; StataCorp LLC) was used to allocate cows with missing values by Bramley et al. (2008). We also used this form of discriminant analysis to classify each of the cows in this study into one of the 3 acidosis categories, category 1 "acidotic," category 2 "suboptimal rumen function," or category 3 "normal," based on the k-means clustering by Bramley et al. (2008). We have termed our categories as the following groups: "high," "medium," and "low" risk for acidosis. The probability of distance to the center for each of the 3 acidosis risk groups (high, medium, and low) was produced where values are between 0 and 1. These describe how closely the rumen metabolomic profile from each cow in the current study matches the center of the high, medium, and low group. Values approaching 1 are in the centroid of the group and represent cows that are most consistent with the characteristics defining that group. The postestimation classification table (*estat classtable;* Stata version 16.1) generated for our study indicated perfect classification and logistic regression evaluation of the categories similarly confirmed the suitability of the model.

Statistical Analysis

Cow was the experimental unit. Before any analysis all ruminal bacterial phyla that contained a raw RA of >0.3% for at least one sample in the study population were center-log-transformed in Stata version 16.1. Phyla that did not meet this threshold (n = 10) were not included in subsequent analyses.

To assess the effect of acidosis group on the probability of distance to the center of the cluster of each acidosis group (value of 0 to 1, with 1 approaching the center), rumen metabolites, and milk production a mixed model was used (Stata version 16.1) with the fixed effects of acidosis group, parity, and region

Table 2. Key diet nutrients for each of the 36 farms (% of DM)

Farm[1]	CP	ADF	NDF	NFC	Starch	Sugar	Crude fat
AU1	20.2	20.3	36.8	32.4	21.0	6.8	3.9
AU2	14.5	27.2	50.3	29.1	18.5	3.5	4.2
AU3	16.7	28.8	46.2	25.2	12.3	4.8	4.7
AU4	15.9	21.0	34.0	41.4	29.8	5.0	4.0
AU5	25.6	18.4	31.7	32.3	21.3	4.0	4.0
AU6	19.2	22.0	39.1	30.7	18.9	6.1	5.0
AU7	19.9	22.6	34.3	36.5	15.6	8.1	3.6
AU8	26.5	16.1	31.2	31.2	18.9	6.7	4.7
AU9	17.2	18.2	32.2	41.3	23.5	11.2	4.7
AU10	18.5	23.3	35.3	37.3	18.0	7.1	3.5
Mean ± SD	19.4 ± 3.95	21.8 ± 3.96	37.1 ± 6.40	33.7 ± 5.26	19.8 ± 4.70	6.32 ± 2.26	4.24 ± 0.52
CA1	17.1	21.5	30.9	41.1	20.9	4.1	5.5
CA2	16.5	23.7	32.4	37.9	18.0	8.1	5.5
CA3	17.5	20.1	30.0	41.4	23.0	6.6	4.2
CA4	15.7	21.1	27.8	43.9	22.7	8.5	4.7
CA5	15.3	22.4	27.9	46.7	23.1	9.7	3.8
CA6	16.0	19.7	27.2	44.7	26.0	5.0	5.4
CA7	15.2	23.5	31.2	40.6	21.4	6.3	5.9
CA8	16.9	21.9	29.0	42.4	21.5	8.6	4.5
CA9	16.3	20.7	30.1	42.7	23.1	6.2	5.1
CA10	15.6	22.5	28.6	44.2	23.9	8.6	4.4
Mean ± SD	16.2 ± 0.79	21.7 ± 1.35	29.5 ± 1.69	42.6 ± 2.48	22.4 ± 2.11	7.17 ± 1.80	4.90 ± 0.68
CAN1	15.6	22.6	34.1	39.7	26.4	4.1	4.7
CAN2	16.0	29.7	48.9	17.0	0.1	2.1	13.5
CAN3	16.4	26.5	39.3	33.4	23.0	2.8	3.6
CAN4	15.6	22.1	30.9	45.1	26.5	4.6	3.5
CAN5	18.6	21.8	31.6	37.3	23.6	2.4	5.1
CAN6	17.0	21.1	33.0	39.8	24.8	4.7	4.1
CAN7	16.1	22.2	32.6	41.9	28.7	4.1	3.7
CAN8	15.2	20.4	30.9	43.4	28.3	3.7	5.2
CAN9	15.2	23.7	34.1	40.2	23.1	2.8	4.0
CAN10	17.4	19.3	28.7	41.9	25.6	5.6	6.1
CAN11	15.1	24.0	34.4	38.0	22.5	2.7	5.8
CAN12	16.8	21.1	29.4	41.4	27.7	2.9	6.3
Mean ± SD	16.3 ± 1.05	22.9 ± 2.85	34.0 ± 5.44	38.3 ± 7.35	23.4 ± 7.63	3.54 ± 1.08	5.47 ± 2.71
WI1	18.4	19.8	30.3	41.5	27.0	4.3	4.8
WI2	17.6	19.5	29.7	43.0	29.5	4.2	5.4
WI3	17.3	21.7	29.5	42.0	26.4	2.7	4.6
WI4	17.2	17.4	58.0	42.9	25.0	4.8	4.0
Mean ± SD	17.6 ± 0.54	19.6 ± 1.74	36.8 ± 14.1	42.3 ± 0.72	27.0 ± 1.88	4.01 ± 0.93	4.71 ± 0.58
Total mean ± SD	17.3 ± 2.56	21.9 ± 2.87	33.9 ± 6.85	38.6 ± 6.22	22.5 ± 5.48	5.37 ± 2.27	4.88 ± 1.66
Range	14.5–26.5	16.1–29.7	27.2–58.0	17.0–46.7	0.10–29.8	2.10–11.2	3.50–13.5

[1]AU = Australia; CA = California; CAN = Canada; WI = Wisconsin.

and their interactions with the random effects of herd nested within region in the model as follows:

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_{kl} + \omega\alpha\gamma\theta_{ijk} + f_k + r_{i:mj} + c_{l:ijkm} + \varepsilon_{ijklm},$$

where Y_{ijklm} is the observation in the mth herd on the lth cow in the kth region of the jth parity in the ith acidosis group, μ is the overall mean, α_i is the fixed effect of acidosis group (i = group 1, 2, or 3), γ_j is the fixed effect of parity (j = primiparous or multiparous), θ_{kl} is the fixed effect of region (k = AU, CA, and CAN) for cow number l (l = 1 to a max of 261), $\omega\alpha\gamma\theta_{ijk}$ are the fixed effects of interaction terms including 2- and 3-way interactions of acidosis group, parity, and region, f_k is the random effect of region, $r_{i:mj}$ is the random effect for the ith acidosis group within the mth herd and the kth region; $c_{l:ijkm}$ is the random interaction effect associated with the lth cow with the jth parity within the ith acidosis group within the mth herd and the kth region, and ε_{ijklm} is the random error term. The covariance structure was unstructured. The DIM at herd test was tested as a covariate for the milk production measures but was not significant, except for milk fat percent and did not consistently produce a significantly lower Akaike information criterion, and hence was not included in the model. Similarly, DIM at rumen fluid collection was assessed as a covariate for the distance from the centroid of each acidosis risk group and rumen metabolites but was not significant. The influence of herd and region was tested (*gllamm* Stata version

16.1) to evaluate these in allocation of cows to group categories; herd accounted for 23% and region <1% of the variance.

RESULTS AND DISCUSSION

This study, and those related, builds on the work of Golder et al. (2018) who found associations between the host genetics, acidosis risk, ruminal bacterial taxa, and rumen metabolites, albeit in a small population of nulliparous Australian cattle from a herd with closed genetics. Our findings further support the use of the model by Bramley et al. (2008) in case definition of acidosis using Evans' postulates as modified by Lean et al. (2009). The herds in this study had a range of management systems, herd sizes, production levels, and differed substantially in nutrient composition. Thus, we used an appropriate population to explore our hypotheses. A limitation is that with an observational study and rumen fluid collected at a single point in time exact interpretation of clusters is not possible and inferences are suggested only. The interval between rumen fluid collection and herd test dates was not consistent between all herds which may have influenced the milk production results; however, the median collection interval was 1 d after herd test. The study population also differed by 18 cows between the rumen fermentation and milk results.

Regional Effects

Regional differences were evident in the outcomes examined; these are, as expected, a large source of heterogeneity in this study. However, evaluation of the variance attributable to herd within region suggests that these effects are a function of herd management differences rather than region per se. The number of cows classified in each acidosis risk group by region is shown in Table 3. The interaction in mixed models between region and acidosis group allocation, rumen fermentation measures but not for production measures (Table 4) supports the need for multicenter studies to access herds with different management to address certain hypotheses.

Acidosis risk differed between region with AU (37.2%) and CA (39.2%) having similar prevalence of cows at a high risk of acidosis, whereas CAN had only 5.2% (Table 3; Figure 2A). This is also demonstrated by differences in the means of the distance from the center of the groups (Table 5).

In the correlation biplot of the redundancy analysis of bacterial phyla with respect to region, all regions were in different quadrants from each other with similar spatial separation (Figure 3C). Region accounted for 11.1% of the variation in a classified sample plot by region with CA, AU, and CAN associated with 7.8, 4.6, and 4.0% of variation, respectively, all with P-values of 0.006 (Figure 3D). This is consistent with our expectation of the variation expected for this type of study. In a biplot with respect to both region and rumen fermentation measures, the overall total variation explained approximately doubled to 21.9% with valerate (P = 0.022), butyrate (P = 0.022), propionate (P = 0.022), and ammonia (P = 0.044) associated with 5.1, 4.0, 2.9, and 1.6% of the variation, respectively (Figure 4C).

Milk yield was highest for CA (42.4 ± 1.61), followed by CAN at 38.2 ± 1.76, and AU with 30.9 ± 1.64 L/d (Table 5). The ECM, protein yield, fat yield, and TS followed a similar regional pattern to milk yield, but there was no difference between CA and CAN. Milk measures had low associations with BCC with respect to region with milk fat percentage, milk protein percentage, and fat yield explaining 1.8, 1.1, and 0.8% of the overall variation (P = 0.002, 0.016, and 0.048, respectively; Figure 4D).

The AU region was characterized by low milk yield, ECM, protein yield, fat yield, and TS (Table 5), association with decreased abundance of *Chloroflexi*, *Planctomycetes*, and *Saccharibacteria* (Figure 3B and D; Figure 4B and C), and was associated with decreased concentrations of lactate, ammonia, and acetate (Figure 4B). These lower milk measures are likely a direct response to differences in diet and management between this region and North America (Tables 1 and 2). The AU region, however, was associated with improved milk protein percentage (Figure 4D). Most of the AU herds had diets based on or containing pastures, most commonly ryegrasses, whereas the North American diets contained a larger component of maize and maize silage and were all TMR diets. The AU herds, in general, had high CP and NDF and low NFC and starch, relative to the other regions at 19.4 ± 3.95, 37.1 ± 6.40, 33.7 ± 5.26, and 19.8 ± 4.70% of DM, respectively (Table 2). This combination of diet attributes likely explains why D-lactate concentrations were 94.3% lower in the cows from AU (0.094 ± 0.436 mM) than those from CA

Table 3. The number of cows classified in each acidosis risk group by region

Acidotic risk group	Region[1]			Total
	AU	CA	CAN	
High	32	31	5	68
Medium	36	18	16	70
Low	18	30	75	123
Total cows/region	86	79	96	261

[1]AU = Australia; CA = California; CAN = Canada.

Table 4. The mean ± SE of the distance from the centroid of each acidosis group, rumen fermentation measures, and milk measures for each acidosis risk group and significances; the model includes the fixed effects of acidosis risk group, region, and parity and their interactions and the random effect of herd within region

		Acidosis risk group			*P*-value						
Variable	n	High	Medium	Low	Group (G)	Region (R)	Parity (P)	G × R	G × P	R × P	G × R × P
Distance from acidosis risk group[1]											
High	261	$0.83^{a} \pm 0.020$	$0.084^{b} \pm 0.015$	$0.051^{b} \pm 0.013$	<0.001	0.013	0.003	<0.001	0.048	0.013	<0.001
Medium	261	$0.080^{a} \pm 0.024$	$0.71^{b} \pm 0.019$	$0.10^{a} \pm 0.016$	<0.001	0.518	0.997	0.078	0.614	0.799	0.092
Low	261	$0.096^{a} \pm 0.025$	$0.20^{c} \pm 0.018$	$0.85^{b} \pm 0.015$	<0.001	0.004	0.014	0.119	0.620	0.022	0.004
Rumen fermentation measure											
pH	261	$6.51^{a} \pm 0.06$	$6.81^{b} \pm 0.05$	$6.53^{a} \pm 0.04$	<0.001	0.089	0.823	0.816	0.684	0.469	0.994
Total VFA (m*M*)	261	$109.4^{a} \pm 3.98$	$78.1^{b} \pm 2.89$	$110.1^{a} \pm 2.35$	<0.001	0.066	0.420	0.056	0.797	0.357	0.754
Acetate (m*M*)	261	$58.6^{a} \pm 2.20$	$47.8^{c} \pm 1.62$	$66.5^{b} \pm 1.33$	<0.001	0.033	0.417	0.525	0.724	0.374	0.842
Propionate (m*M*)	261	$32.8^{a} \pm 1.58$	$18.8^{c} \pm 1.19$	$24.5^{b} \pm 0.99$	<0.001	0.618	0.641	<0.001	0.970	0.739	0.877
Acetate:propionate	261	$1.98^{a} \pm 0.11$	$2.63^{c} \pm 0.091$	$2.82^{b} \pm 0.081$	<0.001	0.773	0.773	<0.001	0.130	0.891	0.804
Butyrate (m*M*)	261	$11.2^{a} \pm 0.51$	$8.82^{c} \pm 0.39$	$13.5^{b} \pm 0.32$	<0.001	<0.001	0.509	0.094	0.662	0.072	0.295
Valerate (m*M*)	261	$2.93^{a} \pm 0.14$	$1.25^{c} \pm 0.11$	$1.82^{b} \pm 0.09$	<0.001	0.063	0.012	<0.001	0.027	0.233	0.031
Iso-butyrate (m*M*)	261	$0.77^{a} \pm 0.039$	$0.63^{c} \pm 0.032$	$0.95^{b} \pm 0.028$	<0.001	<0.001	0.926	0.031	0.263	0.231	0.740
Iso-valerate (m*M*)	261	$1.43^{a} \pm 0.098$	$1.11^{b} \pm 0.076$	$1.86^{b} \pm 0.064$	<0.001	<0.001	0.132	0.011	0.101	0.013	0.028
Caproate (m*M*)	261	$0.75^{a} \pm 0.060$	$0.33^{c} \pm 0.045$	$0.50^{b} \pm 0.037$	<0.001	0.439	<0.001	<0.001	<0.001	0.238	0.003
Ammonia (m*M*)	261	$1.95^{a} \pm 0.496$	$2.41^{a} \pm 0.410$	$4.55^{b} \pm 0.365$	<0.001	0.078	0.605	0.274	0.270	0.279	0.026
D-Lactate (m*M*)	261	0.64 ± 0.476	0.64 ± 0.364	1.05 ± 0.305	0.535	0.043	0.379	0.812	0.173	0.853	0.288
L-Lactate (m*M*)	261	0.49 ± 0.349	0.53 ± 0.273	0.82 ± 0.233	0.507	0.179	0.401	0.807	0.164	0.768	0.281
Total lactate (m*M*)	261	1.14 ± 0.815	1.19 ± 0.628	1.88 ± 0.530	0.523	0.087	0.385	0.799	0.166	0.820	0.278
Milk measure											
Milk (L/d)	243	37.8 ± 1.48	37.1 ± 1.27	36.2 ± 1.13	0.571	<0.001	<0.001	0.627	0.163	0.066	0.280
ECM (L/d)	243	36.5 ± 1.54	36.9 ± 1.34	36.9 ± 1.21	0.922	<0.001	<0.001	0.169	0.370	0.088	0.298
Fat (%)	243	$3.39^{a} \pm 0.142$	$3.59^{ab} \pm 0.121$	$3.77^{b} \pm 0.107$	0.032	0.519	0.696	0.341	0.569	0.129	0.676
Protein (%)	243	3.08 ± 0.050	3.01 ± 0.044	3.07 ± 0.040	0.261	0.019	0.048	0.684	0.645	0.950	0.768
Fat (kg/d)	243	1.27 ± 0.072	1.32 ± 0.063	1.36 ± 0.056	0.458	<0.001	<0.001	0.102	0.501	0.209	0.450
Protein (kg/d)	243	1.13 ± 0.042	1.11 ± 0.036	1.10 ± 0.033	0.757	<0.001	<0.001	0.489	0.162	0.007	0.273
Fat:protein	243	$1.11^{a} \pm 0.047$	$1.20^{ab} \pm 0.040$	$1.23^{b} \pm 0.035$	0.031	0.191	0.224	0.216	0.594	0.134	0.659
TS (kg)	243	2.34 ± 0.100	2.43 ± 0.089	2.45 ± 0.079	0.829	<0.001	<0.001	0.147	0.414	0.072	0.346
ln SCC	242	3.85 ± 0.212	4.15 ± 0.160	3.92 ± 0.136	0.458	0.024	0.636	0.483	0.536	0.614	0.367

[a–c]Superscripts that differ across columns are significantly different ($P < 0.05$).

[1]The distance from the center of the acidosis risk group as a value between 0 and 1 where 1 is the center of the group or cluster, hence higher values represent those close to the center of the group.

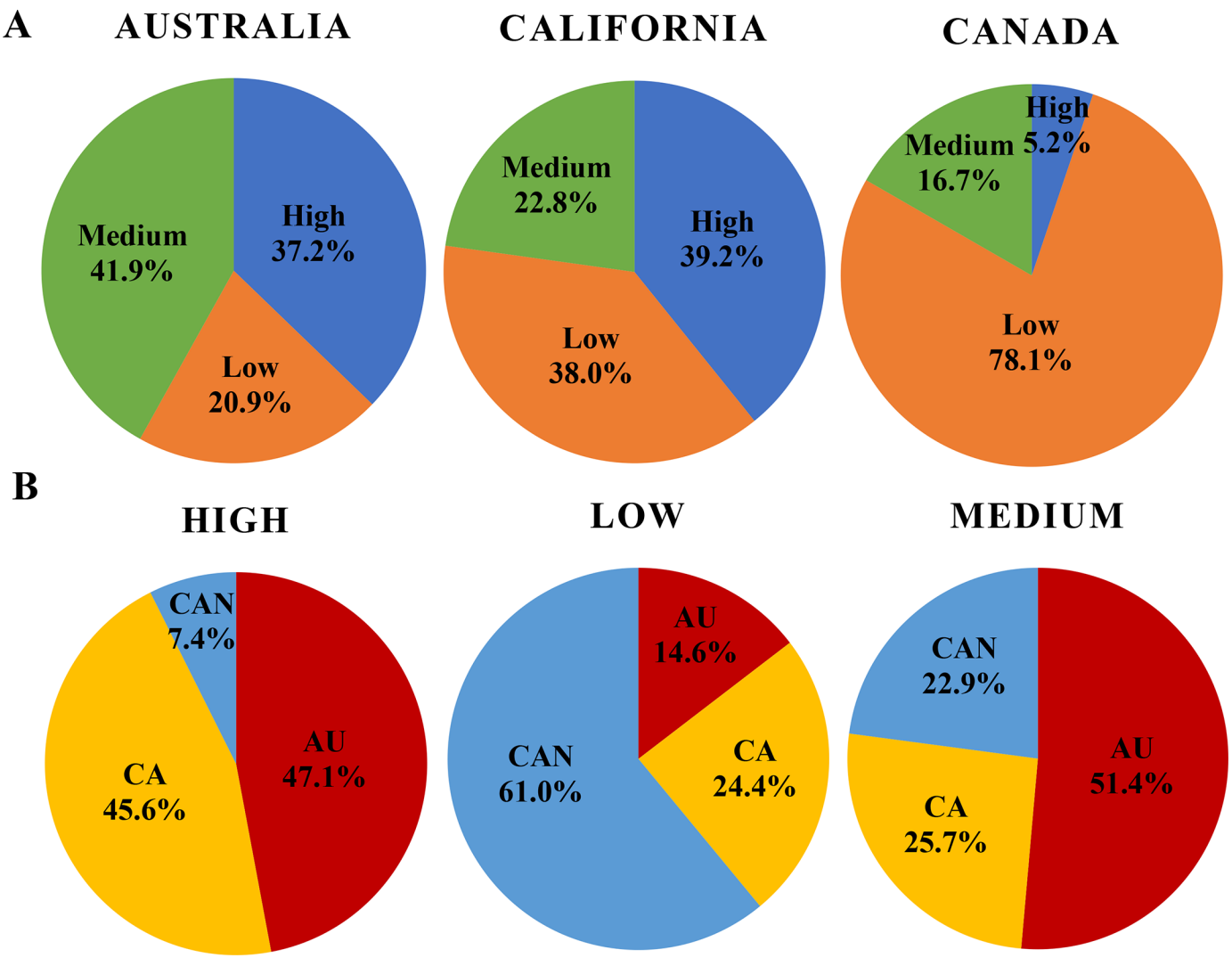

Figure 2. (A) The percentage of cows classified as high, medium, or low risk for acidosis by region, and (B) the percentage of cows from each region that contributed to each of the overall acidosis risk groups. AU = Australia; CA = California; CAN = Canada.

(1.65 ± 0.419 m*M;* Table 5) where the NFC was 42.6 ± 2.48% of DM (Table 2). D-Lactate concentrations from the cows from CAN on diets with an NFC content approximately half of the difference between that of the AU and CA diets at 38.3 ± 7.35% of DM (Table 2) were similar to those of cows from both AU and CA (Table 5).

A TMR diet facilitates simultaneous intake of forages and concentrates promoting a more consistent fermentation pattern (Humer et al., 2018); however, when early lactation cow diets exceed 26% of DM of starch, an NDF of 28 to 32% of DM, or sugars >8% of DM (Lean et al., 2014), such as for the CA herds, ruminal disturbance is more likely to occur regardless of other management. Higher frequency of feed deliveries stimulates cattle to move to the feed bunk, creates a more even distribution of feeding time across the day, promotes more stable rumen fermentation, and reduces the risk of SARA (Macmillan et al., 2017). The North American herds were also intensively managed, which allowed fresh cows to be milked up to 4 times a day.

Cows from CA were characterized by high milk yield, ln SCC, butyrate, iso-butyrate, and iso-valerate values, a low milk protein percentage (Table 5), associations with increased abundance of *Actinobacteria* and *Firmicutes*, and a decreased abundance of *Bacteroidetes*, *Cyanobacteria*, *SR1*, and *Spirochaetes* (Figure 3B). Despite statistical similarity with one other region in Table 4, high acetate and D-lactate concentrations should also be considered as characteristics for CA as supported

Table 5. The mean ± SE of the distance from the centroid of each acidosis risk group, rumen fermentation measures, and milk measures for each region and parity; the model includes the fixed effects of acidosis risk group, region, and parity and their interactions and the random effect of herd within region

		Region			Parity	
Variable	n	Australia	California	Canada	Primiparous	Multiparous
Distance from acidosis risk group[1]						
High	261	0.28[a] ± 0.017	0.29[a] ± 0.017	0.22[b] ± 0.018	0.27[a] ± 0.011	0.26[b] ± 0.011
Medium	261	0.28 ± 0.023	0.25 ± 0.022	0.24 ± 0.023	0.26 ± 0.014	0.25 ± 0.014
Low	261	0.44[a] ± 0.020	0.46[a] ± 0.019	0.54[b] ± 0.021	0.46[a] ± 0.013	0.49[b] ± 0.012
Rumen fermentation measure						
pH	261	6.60 ± 0.071	6.48 ± 0.070	6.70 ± 0.069	6.60 ± 0.042	6.61 ± 0.041
Total VFA (m*M*)	261	101.6 ± 3.07	108.4 ± 2.88	95.5 ± 3.34	101.2 ± 2.04	99.4 ± 1.93
Acetate (m*M*)	261	59.4[ab] ± 1.80	63.8[a] ± 1.71	56.1[b] ± 1.91	59.7 ± 1.17	58.3 ± 1.11
Propionate (m*M*)	261	25.6 ± 1.39	26.2 ± 1.32	23.8 ± 1.44	25.2 ± 0.87	24.9 ± 0.84
Acetate:propionate	261	1.98 ± 0.111	2.82 ± 0.081	2.63 ± 0.091	2.56 ± 0.075	2.56 ± 0.074
Butyrate (m*M*)	261	11.0[a] ± 0.46	14.0[b] ± 0.44	10.3[a] ± 0.47	11.5 ± 0.29	11.4 ± 0.27
Valerate (m*M*)	261	2.13 ± 0.116	2.02 ± 0.109	1.73 ± 0.124	2.02[a] ± 0.076	1.88[b] ± 0.072
Iso-butyrate (m*M*)	261	0.87[a] ± 0.044	0.65[b] ± 0.043	0.91[a] ± 0.043	0.79 ± 0.026	0.80 ± 0.026
Iso-valerate (m*M*)	261	1.66[a] ± 0.095	1.19[b] ± 0.092	1.73[a] ± 0.095	1.43 ± 0.058	1.52 ± 0.056
Caproate (m*M*)	261	0.44 ± 0.051	0.54 ± 0.049	0.58 ± 0.054	0.58[a] ± 0.033	0.49[b] ± 0.031
Ammonia (m*M*)	261	2.42 ± 0.579	3.24 ± 0.568	4.13 ± 0.559	3.22 ± 0.339	3.17 ± 0.334
D-Lactate (m*M*)	261	0.094[a] ± 0.436	1.65[b] ± 0.419	0.87[ab] ± 0.446	1.37 ± 0.271	0.54 ± 0.261
L-Lactate (m*M*)	261	0.13 ± 0.346	1.00 ± 0.335	0.87 ± 0.346	1.04 ± 0.210	0.45 ± 0.204
Total lactate (m*M*)	261	0.24 ± 0.771	2.65 ± 0.743	1.74 ± 0.782	2.43 ± 0.474	1.00 ± 0.459
Milk measure						
Milk (L/d)	243	30.9[a] ± 1.64	42.4[b] ± 1.61	38.2[c] ± 1.76	32.0[a] ± 1.03	42.0[b] ± 1.00
ECM (L/d)	243	30.7[a] ± 1.80	42.2[b] ± 1.78	38.3[b] ± 1.91	31.7[a] ± 1.11	42.1[b] ± 1.08
Fat (%)	243	3.51 ± 0.156	3.61 ± 0.153	3.72 ± 0.168	3.53 ± 0.098	3.66 ± 0.095
Protein (%)	243	3.10[a] ± 0.060	2.92[b] ± 0.059	3.14[a] ± 0.063	3.08[a] ± 0.037	3.02[b] ± 0.036
Fat (kg/d)	243	1.09[a] ± 0.083	1.53[b] ± 0.082	1.38[b] ± 0.088	1.13[a] ± 0.051	1.53[b] ± 0.050
Protein (kg/d)	243	0.95[a] ± 0.049	1.23[b] ± 0.048	1.18[b] ± 0.052	3.08[a] ± 0.037	3.02[b] ± 0.036
Fat:protein	243	1.14 ± 0.051	1.25 ± 0.050	1.18 ± 0.055	1.15 ± 0.032	1.22 ± 0.031
TS (kg)	243	2.04[a] ± 0.121	2.75[b] ± 0.119	2.55[b] ± 0.128	2.10[a] ± 0.075	2.78[b] ± 0.073
ln SCC	242	3.83[a] ± 0.152	4.28[b] ± 0.147	3.82[a] ± 0.192	3.95 ± 0.117	4.07 ± 0.107

[a–c]Superscripts that differ across columns are significantly different.

[1]The distance from the center of the acidosis risk group as a value between 0 and 1 where 1 is the center of the group or cluster, hence higher values represent those close to the center of the group.

by associations in Figure 4B. These concentrations are likely to reflect the low dietary NDF and high NFC of the herds in CA (Table 2).

The CAN region was characterized by a predominance of cows being categorized as group 2, (78.1% of all cows sampled in this region; Figure 2A and B and Table 5). The CAN cows in the acidotic group are clustered closer to the centroid of this group than for other regions (Table 5). The cows from CAN had a greater bacterial diversity than those from either AU and CA that were similar (Figure 5B) that may have enabled the CAN cows to adapt rapidly to dietary changes. They also had an association with increased abundance of *Proteobacteria* (Figure 3B) and *Lentisphaerae* (Figures 4B and D), higher pH, and decreased concentrations of valerate and propionate (Figure 4B), and higher milk fat percentage (Figure 4D). The low dietary sugar and high fat content of the CAN herds (Table 2) may have contributed to the high proportion of cows in group 2. The lack of unique traits for this region and similarities in concentrations of acetate, butyrate, and iso-valerate and milk protein percentage, and ln SCC with AU and in ECM, fat yield, and TS with CA (Table 5) suggests that herds from this region have some diet composition characteristics in common with AU and housing and management systems in common with CA. Both diet composition and housing and management systems are more distinct between AU and CA, as supported by Tables 1 and 2.

Parity Effects

Primiparous cattle generally have a higher risk of acidosis than multiparous cattle (Krause and Oetzel, 2006; Bramley et al., 2008; Stauder et al., 2020). The opposite occurred in our study with primiparous cows predominantly classified in the low-risk group (53%), and similar percentages in the other groups (Figure 6). Risk of acidosis is often higher in primiparous because they have had less exposure to high levels of energy-dense feeds (Humer et al., 2018). Therefore, they may have fewer rumen papillae and a less-adapted rumen

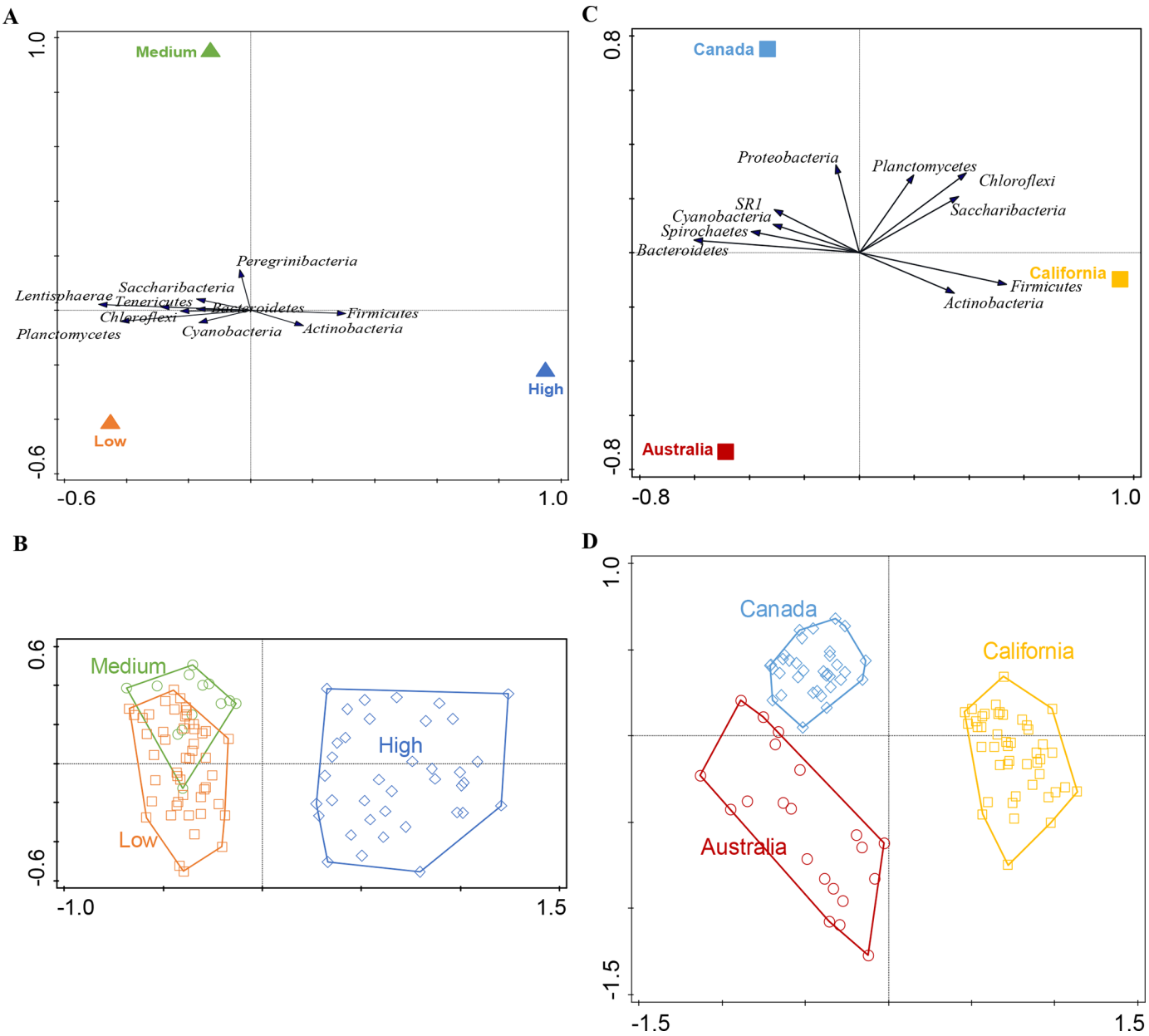

Figure 3. (A) Correlation biplot of the redundancy analysis of bacterial phyla with respect to acidosis risk. The 10 phyla best fit to acidosis risk are displayed where the size of the arrows are approximate correlation coefficients between the bacterial phyla and acidosis risk group. (B) Classified sample plot by acidosis risk where the distance between samples approximates the dissimilarity of the composition of the bacterial phyla. The total variation associated with acidosis risk is 5.3% and the eigenvalues for the first 2 axes are 0.05 and 0.003. The samples with a high risk of acidosis were different from all other samples and were associated with 4.8% of the variation ($P = 0.006$). Medium-risk acidosis samples were not different for the other groups and were associated with 0.3% of the variation ($P = 1.000$). The low risk of acidosis samples were also different than the rest of the samples ($P = 0.006$) but were associated with less of the variation (2.9%; $P = 0.006$). (C) Correlation biplot of the redundancy analysis of bacterial phyla with respect to region. The 10 phyla best fit to region are displayed where the size of the arrows are approximate correlation coefficients between the bacterial phyla and region. (D) Classified sample plot by region where the distance between samples approximates the dissimilarity of the composition of the bacterial phyla. The total variation associated with region was 11.1% and the eigenvalues for the first 2 axis are 0.079 and 0.032. Each region was different than the rest of the samples; California was associated with 7.8% of the variation ($P = 0.006$), Australia with 4.6% of the variation ($P = 0.006$), and Canada with 4.0% of the variation ($P = 0.006$).

microbiome than older cows (Penner et al., 2007; Bramley et al., 2008). Feeding behaviors and access to feed may also differ if primiparous cows are mixed with more dominant multiparous cows (Krause and Oetzel, 2006). In some herds in AU all cows regardless of parity or production may be offered the same amount of grain in the parlor, resulting in a larger percentage of grain intake on a BW basis given to primiparous cattle. This

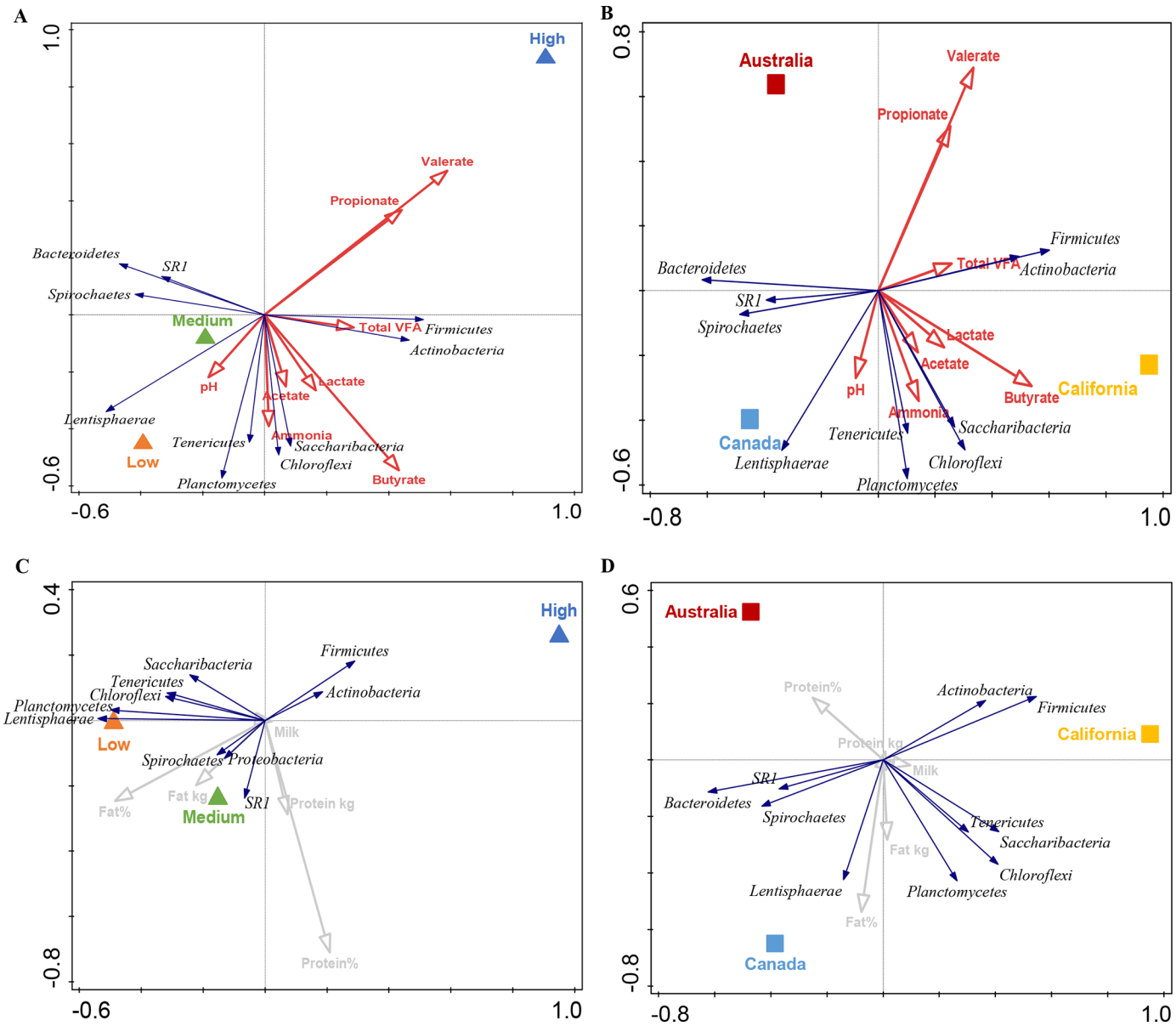

Figure 4. Correlation biplot of the redundancy analysis of bacterial phyla with respect to (A) acidosis risk group and rumen fluid-related explanatory variables. The 10 phyla best fit to the model are displayed where the size of the arrows are approximate correlation coefficients between the bacterial phyla and the explanatory variables. The total variation explained is 18.7%, and the eigenvalues for the first 2 axes are 0.09 and 0.07. The high- and low-risk acidosis groups were significantly different ($P = 0.022$) and associated with 4.8 and 2.9% of variation, respectively. The medium-risk acidosis group was not significantly different ($P = 1.000$) and was only associated with 0.3% of the variation. Valerate, butyrate, propionate, and ammonia were significantly different ($P = 0.022$), associated with 5.1, 4.0, 2.9, and 1.6% of the variation, respectively. Lactate tended to be different ($P = 0.088$) and was associated with 1.2% of the variation. Total VFA ($P = 0.220$), pH ($P = 0.418$), and acetate ($P = 1.000$) were not different and were associated with 1.0, 0.9, and 0.7%, respectively. (B) Region and rumen fluid-related explanatory variables. The total variation explained is 21.9% and the eigenvalues for the first 2 axes are 0.11 and 0.08. California, Australia, and Canada were significantly different ($P = 0.022$) and associated with 7.8, 4.6, and 4.0% of variation, respectively. Valerate ($P = 0.022$), butyrate ($P = 0.022$), propionate ($P = 0.022$), and ammonia ($P = 0.044$) were significantly different, associated with 5.1, 4.0, 2.9, and 1.6% of the variation, respectively. Lactate ($P = 0.110$), total VFA ($P = 0.308$), pH ($P = 0.418$), and acetate ($P = 0.924$) were not different and were associated with 1.2, 1.0, 0.9, and 0.7% of variation, respectively. (C) Acidosis risk and milk-related explanatory variables. The total variation explained is 9.5% and the eigenvalues for the first 2 axes are 0.07 and 0.01. The high- and low-risk acidosis groups were significantly different ($P = 0.028$) and were associated with 4.8 and 2.9% of variation, respectively. The medium-risk acidosis group was not significantly different ($P = 0.482$) and only associated with 0.3% of the variation. Fat percentage (Fat%) was associated with 1.8% of the variation ($P = 0.002$), protein percentage (Protein%) with 1.1% ($P = 0.016$), and fat yield (Fat kg) with 0.8% of the variation ($P = 0.048$). (D) Region and milk-related explanatory variables. The total variation explained is 17.1% and the eigenvalues for the first 2 axes are 0.10 and 0.06. California, Australia, and Canada were significantly different ($P = 0.016$) and associated with 8.8, 4.7, and 4.3% of variation, respectively. Fat percentage (Fat%) was associated with 1.8% of the variation ($P = 0.002$), protein percentage (Protein%) with 1.1% ($P = 0.008$), and fat yield (Fat kg) with 0.8% of the variation ($P = 0.058$).

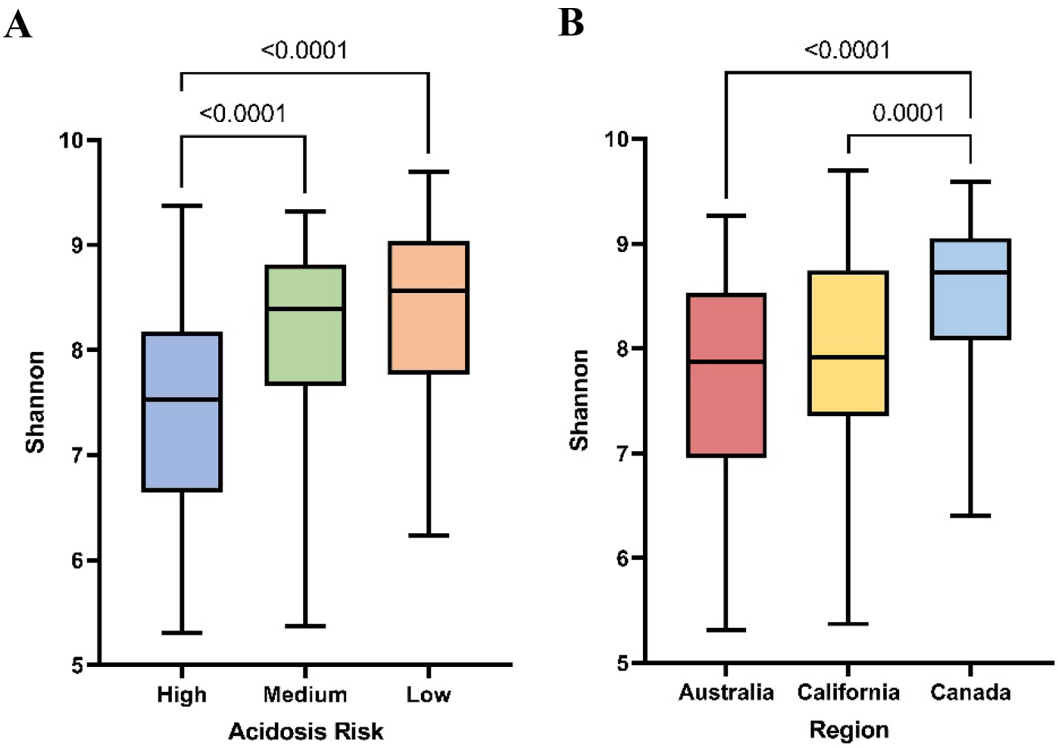

Figure 5. Boxplots of Shannon diversity index by (A) acidosis risk group and (B) region with significance levels indicated. High, n = 67; medium, n = 68; low, n = 117; Australia, n = 85; California, n = 77; Canada, n = 90. The lower whisker is the minimum Shannon index, and the upper whisker is the maximum. The lower end of the box represents the first quartile, the line in the box is the median, and the upper end of the box represents the third quartile.

likely explains why primiparous cows from AU were closer to the center of the high-risk acidosis group than primiparous cows from CA; however, the distance to the centroid was similar between AU and CAN (Table 4 and Supplemental Figure S2; https://doi.org/10.6084/m9.figshare.c.6411203.v1; Golder et al., 2023a).

We hypothesized that parity differences would occur for rumen bacteria and fermentation profiles based on diet preferences, and differences in feeding behaviors, feed intakes, and rumen microbiome adaptation. This was not the case, with limited differences occurring. Parity and G × P only influenced valerate and caproate concentrations with primiparous cows having 0.14 m*M* (7%) more valerate and 0.09 m*M* (15.5%) more caproate than multiparous cows. These very few interactions with parity, suggest that confounding was not an issue. The higher amount and percentage of valerate measured from the primiparous cattle compared with the multiparous is likely responsible for their closer distance to the centroid of the high-risk group than the multiparous cows, but the difference in distance was minor (Table 5). Bramley et al. (2008) showed that valerate was the most important factor in determining classification. Golder et al. (2012a) also demonstrated using the model by Bramley et al. (2008) that valerate concentration is both a sensitive (0.90) and specific (0.90) indicator of ruminal acidosis and has a high area under the curve (0.954). Nasrollahi et al. (2017) showed valerate was positively associated with SARA susceptibility in high producing mid-lactation Holsteins fed a high concentrate diet. Further, substantial variation in absorption of rumen valerate among primiparous cattle during the first 5 wk postpartum was reported by Stone et al. (2003), relating to health disorders in the transition period and may indicate susceptibility to acidosis during high concentrate feeding (Stone, 2004).

As expected, multiparous cows had higher milk yield, ECM, fat yield, and TS and lower protein percent and yield than primiparous cows (Tables 4 and 5). There was a significant region by parity interaction for protein yield where multiparous cows from CA had lower protein yield than all other comparisons except for the primiparous cows from that region (Supplemental Figure S3; https://doi.org/10.6084/m9.figshare.c.6411203.v1; Golder et al., 2023a).

Acidosis Risk Groups

There were 261 cows from 32 farms classified into 1 of the 3 acidosis risk groups, 26.1% of the cows classi-

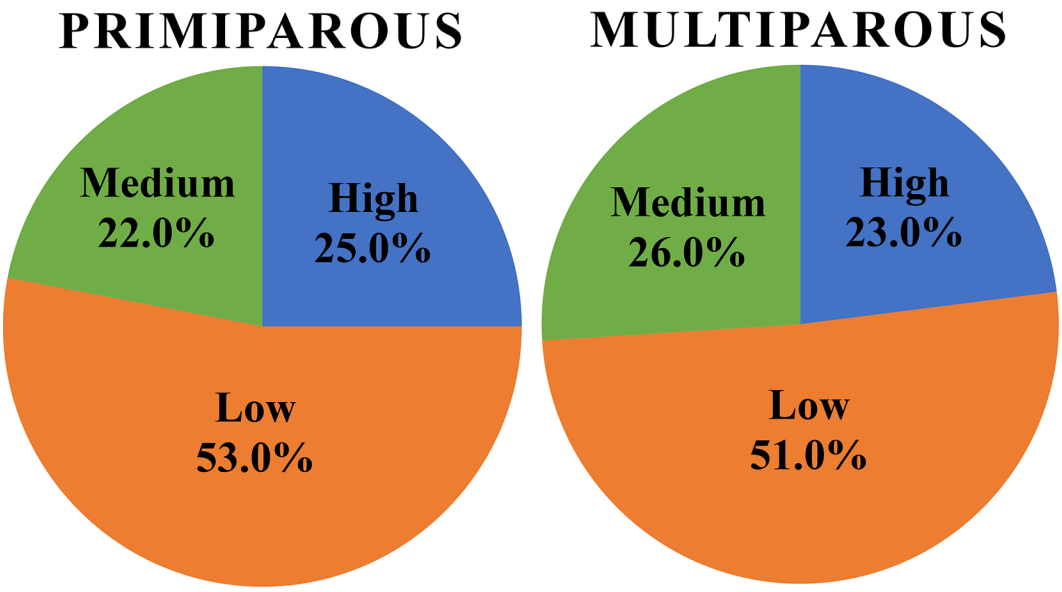

Figure 6. The percentage of primiparous and multiparous cows classified as high, medium, or low risk for acidosis.

fied as high risk, 47.1% as medium risk, and 26.8% as low risk for acidosis (Table 3). Bramley et al. (2008) had 10.2% of cows categorized as category 1 "acidotic," 29.9% as category 2 "suboptimal rumen function," and 60% as category 3 "normal." The difference is proportions between the studies likely reflects the different study populations, housing and management systems, and diets among the herds within regions.

The probabilities or distance from the center of the cluster for each of the 3 respective acidosis groups were influenced by the 3-way interaction between group (G), region (R), and parity (P) for the high- and low-risk groups, and a tendency for the medium-risk group (Supplemental Figure S2); several 2-way interactions and main effects of group, region, and parity were also significant for these groups (Table 4). The distances to the center of the cluster of the high-risk group differed for the high-risk group compared with the medium- and low-risk groups, and the medium- and low-risk groups were similar. Distances to the medium-risk group were similar for the high-risk group and low-risk group but both differed to the medium group distances to the low-risk group differed for each group.

We expect the categorization of cattle to be relatively dynamic throughout early and into mid-lactation with cattle moving in and out of acidotic states. Bramley et al. (2008) found that 10% of cows had acidosis within the first 100 DIM. Golder et al. (2014a) evaluated change in DMI following acidosis challenge and found that a case of acidosis lasted approximately 2 d. Based on these figures, Lean and Golder (2019) estimated an incidence of ~1,500 cases over a 300-d lactation per 100 cows. Therefore, it is reasonable to assume that some phenotypes in our data may have rumen fermentation states that are in a phase of transitioning into one of the other groups. Notwithstanding this, we hypothesize that cattle may be genetically predisposed to a particular acidosis risk group, which is the focus of our unpublished data.

Group was significantly different for all rumen measures except lactates (Table 4). Differences occurred between each of the 3 groups for acetate, propionate, their ratio, butyrate, valerate, iso-butyrate, iso-valerate, and caproate concentrations. There was a 3-way interaction (G × R × P) for valerate, iso-valerate, caproate, and ammonia concentration (Supplemental Figures S4 and S5; https://doi.org/10.6084/m9.figshare.c.6411203.v1; Golder et al., 2023a). The G × R was significant for propionate (Supplemental Figure S5E), acetate: propionate (Supplemental Figure S5F), iso-butyrate, valerate, iso-valerate, and caproate and R × P for iso-valerate concentration. The ruminal total lactate concentrations in this study are low (Table 4), as values of <5 m*M* are generally considered normal (Nagaraja and Titgemeyer, 2007), thus the risk of ruminal acidosis is our study is predominantly being driven by the VFA.

The mean rumen pH and total VFA for each of the groups were higher than those of Bramley et al. (2008), which were 5.74 ± 0.47, 6.18 ± 0.44, and 6.33 ± 0.43, and 100.74 ± 23.22, 94.79 ± 18.13, and 62.81 ± 15.65 m*M*, for categories 1 to 3, respectively. Perhaps this reflects the more stable rumen fermentation of the TMR-fed herds which dominated our data set and that were likely to have a higher DMI. As the rumen pH values

reported by Bramley et al. (2008) were from samples collected by rumenocentesis and the R^2 value between these values and those by stomach tube was only 0.2, we compared our mean values to the mean stomach tube pH values for Bramley's groups; they were similar with only CA differing from the Bramley et al., (2008) group. In contrast to our study, Bramley et al. (2008) had higher ammonia concentrations at 2.46 ± 2.02, 7.79 ± 3.75, and 3.64 ± 2.03, for categories 1 to 3, respectively and D-lactate concentrations that were lower; 0.34 ± 0.86, 0.28 ± 0.97, and 0.12 ± 0.51, respectively. It is apparent that our study population differed in some aspects to that of Bramley et al. (2008).

The 3 acidosis risk groups were in different quadrants of the correlation biplot of the redundancy analysis of bacterial phyla with respect to acidosis risk groups (Figure 3A) indicating differences in BCC. The high-risk acidosis group was associated with 5.3% of the variation in a classified sample plot by acidosis group (Figure 3B), which is approximately half of that explained by region (Figure 3D). This result is to be expected as the rumen is dynamic and well evolved to accommodate change in diet and substrate. There was considerable overlap in the 10 most influential phyla in correlation biplots of BCC constrained by acidosis group or region (Figure 3A and C).

Samples from the high- and low-risk groups were each different from all other samples in terms of BCC, despite overlap observed for the medium- and low-risk groups (Figure 3B). The high-risk group was associated with 4.8% of variation ($P = 0.006$), the low-risk group with 2.9% ($P = 0.006$), and medium-risk group with 0.3% ($P = 1.00$; Figure 3B).

Rumen fermentation metabolites and milk measures had differing influences on BCC associations in both acidosis risk group and region plots as observed by changes in the identity of the top 10 most influential phyla, their magnitude, and direction. The rumen fermentation metabolites were more associated with the BCC than the milk measures for both acidosis risk group and region plots, as observed by the greater magnitude of the rumen measure arrows, larger axes, and total variation associated (Figure 4).

Concentrations of valerate and butyrate had the largest magnitude of association ($P = 0.022$) in redundancy correlation biplots of bacteria with respect to acidosis group, associating with 5.1 and 4.0% of the variation, respectively (Figure 4A). Propionate and ammonia concentrations were also significant ($P = 0.022$), lactate had a tendency ($P = 0.088$), whereas total VFA, pH, and acetate were not significant ($P > 0.05$; Figure 4A).

High-Risk Group. The rumen metabolism and milk production measures that characterized the high-risk group were highest propionate, valerate, and caproate concentrations and lowest milk fat percentage (Table 4), compared with other groups, consistent with Bramley et al. (2008). Our low ammonia and ratio of milk fat to protein for the high-risk group demonstrate further consistency in trends with Bramley et al. (2008). In contrast, Bramley et al. (2008) showed high iso-valerate and D-lactate concentrations in the equivalent group, category 1, and rumen pH, ammonia concentration, and ratio of milk fat to protein were low. The combination of rumen and milk characteristics for this group are consistent with high rates of carbohydrate fermentation as suggested by Bramley et al. (2008). In terms of BCC the high-risk group was different from the low-risk group ($P = 0.022$) and was associated with increased concentrations of rumen valerate and propionate, decreased pH, and reduced abundance of *Lentisphaerae* (Figure 4A).

The Bramley et al. (2008) herds that had a high proportion of cows that were acidotic were fed more highly readily fermentable carbohydrates than the other herds and had the lowest NDF, potentially favoring bacteria that ferment starch and sugars. The magnitude and direction of the arrows for the associated abundance of *Firmicutes* and *Bacteroidetes* in the biplots (Figures 3A and 4A, C) support this hypothesis. The gram-positive *Firmicutes* may displace the gram-negative *Bacteroidetes* during ruminal acidosis (Nagaraja and Titgemeyer, 2007; Plaizier et al., 2022). Other less abundant bacterial phyla the *Chloroflexi*, *Lentisphaerae*, *Planctomycetes*, *Saccharibacteria*, and *Tenericutes* had decreased associated abundance with the high-risk group than the other groups, whereas the associated abundance of the *Actinobacteria* was increased (Figures 3A and 4A, C). The lower diversity of bacteria in this group may be unfavorable.

The higher propionate and valerate concentrations in the high-risk acidosis group are likely to be the main factors that influenced classification to this group as they are strong diagnostic measures for acidosis risk with sensitivity, specificity, and area under the curve values of 0.93, 0.90, and 0.954, respectively, for propionate, and 0.90, 0.90, and 0.955 for valerate, respectively (Lean and Golder, 2019). Both VFA can be produced from lactate and are safer hydrogen sinks for the ruminant than lactate (Lean and Golder, 2019). Propionate, one of the end products of both the succinate and the acrylate pathways, is a key source of energy production for ruminants and its increase and shifts in the acetate to propionate ratio are associated with increased risk of ruminal acidosis (Ørskov, 1986; France and Dijkstra, 2005). The acrylate pathway converts lactic acid, an unsafe sink electron sink, into propionate, which is a safe electron sink, thereby, lowering the risk of ruminal damage. Valerate is usually present in relatively low

rumen concentrations but when lactate is available valerate can be one of the products formed from this substrate (Stewart et al., 1997).

Valerate and lactate concentrations are expected to correlate; however, in our study, this is not the case. Golder et al. (2012b) found that lactate concentrations peaked within 20 min of feeding sugar but declined rapidly. Our rumen samples were not collected immediately after initial feeding, and it is possible that if lactate was generated, it had already been metabolized with portions converted into propionate, acetate, butyrate, iso-butyrate, valerate, and caproate that is synthesized from 3 lactate molecules (Annison and Lewis, 1959; Huntington, 1988; Marounek et al., 1989; Stewart et al., 1997). Our lactate concentrations were low as expected when not evaluating clinical acidosis or after feeding challenge diets containing sugars or starch.

The low ammonia concentrations are consistent with our findings in other studies (Golder et al., 2012b; 2014a,b). In some cases, protein-sparing effects can be beneficial to the host but are likely to be detrimental if microbial protein synthesis is impaired (Golder et al., 2014a). Monensin was present in many of the diets and can reduce ammonia concentrations (Plaizier et al., 2000; Ghorbani et al., 2011). Bramley et al. (2008) proposed that the relatively low ammonia concentrations in their acidotic cattle may have reflected either increased microbial protein synthesis and ammonia assimilation, more rapid clearance from the rumen, or failure of proteolytic bacteria to deaminate available protein.

The lower milk fat percentage for acidotic cattle was associated with higher concentrate feeding and lower NDF, peNDF, and rumen pH in Bramley et al. (2008) and is likely to be consistent with our study. Dietary peNDF has been linked to milk fat percentage and rumen pH and fermentation (Allen, 1997; Mertens, 1997; Zebeli et al., 2012; Nasrollahi et al., 2017).

Medium-Risk Group. The medium-risk group was characterized by the highest rumen pH and lowest total VFA, acetate, propionate, butyrate, valerate, iso-butyrate, iso-valerate, and caproate concentrations (Table 4). The mean total VFA of 78.1 ± 0.05 is considerably lower than the recommended optimum of >95 m*M* (Leng and Brett, 1966). The medium group had similar low ammonia concentrations to the high-risk group (Table 4). We propose that the equivalent group in Bramley et al. (2008) that had low total VFA, D-lactate, adequate ammonia, and high rumen pH is category 2, which were suggested to have suboptimum rumen function. In contrast to our study, where the medium group had similar milk measures to both other groups, cattle in the suboptimum rumen function group had lower milk production than category 3 "normal" cows (Bramley et al., 2008).

Bramley et al. (2008) proposed that the herds that had the most cows in this suboptimum rumen function group may have had different feeding management to those categorized as acidotic as their diets were lower in ME, NFC, and higher in NDF content that possibly allowed a slower rumen fermentation, or a rapid clearance from the rumen, resulting in lower total VFA concentrations and higher pH, which could also be the case in our study. Therefore, a reduced risk of acidosis compared with the high group. In addition, a mismatch in energy and protein inputs could also have occurred resulting in less ammonia being used in microbial protein. The reduced fermentation from the proposed lower amount of readily fermentable carbohydrates and potentially a mismatch in substrates most likely resulted in the associated changes to the BCC that differed between the medium- and high-risk groups (Figures 3A, B and 4A, C). We propose that the medium-risk group may contain underfed cows, cows that are inappetant or had not eaten recently or were in recovery from acidosis or an illness.

Low-Risk Group. The low-risk group were characterized by the highest acetate, acetate: propionate, butyrate, iso-butyrate, iso-valerate, and ammonia concentrations (Table 4) and the greatest association with abundance of *Lentisphaerae*, compared with both other groups. The high- and low-risk groups had similar total VFA concentrations, as well as similar rumen pH indicating that they likely had similar hydrogen and electron generation or accumulation, but their source and sinks differed, with safe sinks likely to be predominant in the low-risk group. The higher ammonia concentration in the low group compared with the high group was likely to be protective and suggested that there was a greater synergy of energy and protein in the low group, so they were able to slow the overall rate of fermentation of organic acids. The rumen was more stable and acetate and butyrate fermentation was favored, providing "safer" electron sinks as opposed to fermentation to propionate and valerate, which are "safer" electron sinks than lactate but less so than acetate and butyrate. This hypothesis is supported by Table 4.

The low-risk group produced 0.38 percentage points higher milk fat than the high-risk group, which represents an increase of 10.1% for milk fat percent and contributes to the 9.8% higher fat to protein ratio for the low-risk group than the high-risk group ($P = 0.032$ and 0.031, respectively; Table 4). The high acetate and butyrate concentrations in the low-risk group most likely explain their higher milk fat percentage than for the high group, as the mammary gland uses these for energy and the production of milk fat (Rook, 1976).

The numerically highest lactate concentrations in the low group, compared with both other groups, coupled with the mid-range propionate and valerate concentrations may suggest that as the rumen is relatively stable the amount of accumulation of lactate in the rumen requires minimal use of the acrylate pathway to convert lactic acid, which can lower ruminal pH to a greater extent than VFA, as it has a pKa of 3.0, compared with a pKa of 4.8 for ruminal VFA (Oetzel, 2003). However, ruminal pH is not always correlated with ruminal lactate concentrations ($r = -0.14$; Britton et al., 1989).

The low-risk group possibly represents cattle that are well fed with a stable rumen and a slower rumen fermentation of carbohydrates.

CONCLUSIONS

Acidosis risk differed between region with AU and CA having similar prevalence of high-risk cows for acidosis and CAN having a lower prevalence, supporting our hypothesis of regional differences in acidosis risk. Our second hypothesis that rumen metabolite, ruminal bacterial taxa, and milk production characteristics would differ between acidosis risk categories was also supported. The high-risk group for acidosis had rumen phyla, fermentation, and production characteristics consistent with a model of acidosis that reflected a rapid rate of carbohydrate fermentation. The medium-risk group may contain cows that are inappetant or that had not fed recently or were in recovery from acidosis. The low-risk group possibly represents cattle that are well fed with a stable rumen and a slower rumen fermentation of carbohydrates. There is a need to characterize the lower taxonomic levels of bacteria of each of the acidosis groups, identify bacteria and feed nutrients that may predict ruminal acidosis, and determine if there is a heritable component of acidosis susceptibility.

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