Received: 12 November 2021 | Revised: 15 March 2022 | Accepted: 1 April 2022

DOI: 10.1111/jam.15573

Journal of Applied Microbiology sfam

ORIGINAL ARTICLE

Characterization of microbial intolerances and ruminal dysbiosis towards different dietary carbohydrate sources using an in vitro model

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Funding information
This research has been supported by the Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development.

Abstract

Aim: This study aimed to characterize the critical points for determining the development of dysbiosis associated with feed intolerances and ruminal acidosis.

Methods and Results: A metabologenomics approach was used to characterize dynamic microbial and metabolomics shifts using the rumen simulation technique (RUSITEC) by feeding native cornstarch (ST), chemically modified cornstarch (CMS), or sucrose (SU). SU and CMS elicited the most drastic changes as rapidly as 4 h after feeding. This was accompanied by a swift accumulation of D-lactate, and the decline of benzoic and malonic acid. A consistent increase in *Bifidobacterium* and *Lactobacillus* as well as a decrease in fibrolytic bacteria was observed for both CMS and ST after 24 h, indicating intolerances within the fibre degrading populations. However, an increase in *Lactobacillus* was already evident in SU after 8 h. An inverse relationship between *Fibrobacter* and *Bifidobacterium* was observed in ST. In fact, *Fibrobacter* was positively correlated with several short-chain fatty acids, while *Lactobacillus* was positively correlated with lactic acid, hexoses, hexose-phosphates, pentose phosphate pathway (PENTOSE-P-PWY), and heterolactic fermentation (P122-PWY).

Conclusions: The feeding of sucrose and modified starches, followed by native cornstarch, had a strong disruptive effect in the ruminal microbial community. Feed intolerances were shown to develop at different rates based on the availability of glucose for ruminal microorganisms.

Significance and Impact of the study: These results can be used to establish patterns of early dysbiosis (biomarkers) and develop strategies for preventing undesirable shifts in the ruminal microbial ecosystem.

Parisa Kheirandish and Renee Maxine Petri share first authorship

BIOMIN GmbH, a company that produces and trades feed additives, supports financially the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock.

KEYWORDS

metabolomics, microbiota, phytogenics, RUSITEC, starch, sucrose

INTRODUCTION

Unravelling the drivers of community structure and metabolism in response to environmental changes is a central goal in microbial ecology. The rumen is a unique and complex gut ecosystem with essential roles in host nutrient provision and pathogen exclusion (Mizrahi et al., 2021; Zeineldin et al., 2018). By understanding the mechanisms controlling ecological adaptation in the rumen, it is possible to predict the responses of this ecosystem to changes in nutritional and metabolic status, as well as to predict its adaptive responses. This mechanistic knowledge provides the basis for supporting and modulating the rumen ecosystem to reach optimal nutrient utilization, and therefore, animal performance (Nagaraja, 2016).

It is well known that nutrient input is the largest contributing factor to community structure in the rumen (Henderson et al., 2015), and that the ratio of structural to nonstructural carbohydrates directly impacts ecosystem metabolism, particularly the rate of fermentation and acid production (McCann et al., 2016; Neubauer et al., 2018; Petri et al., 2018). The contribution of various non-structural carbohydrates, such as starches and sugar, to the development of dysbiosis in the rumen ecosystem has been assessed with regards to animal metabolism (Gozho & Mutsvangwa, 2008; Nozière et al., 2010), but little is understood about the progressive development of intolerances in response to increased nutrient availability. For the microbes specifically, the availability of nonstructural polysaccharides, depending on the type of linkage and enzymatic accessibility to starch, determines the rate of cleavage and the potential for environmental perturbation (Shen et al., 2020; Van Soest, 1994). However, at the host level, the rapid fermentation of glucose yields large amounts of short-chain fatty acids (SCFA) to satisfy the metabolic requirements of the host (Humer et al., 2018). Because cattle are evolutionarily adapted to the fermentation of cellulose, they have a limited ability to adapt to the rapid fermentation of starches and sugars, and prolonged exposure jeopardizes acid–base homeostasis, resulting in ruminal acidosis (Allen, 1997). The low pH resulting from increased carbohydrate input and the accumulation of acids and various deleterious microbial-derived compounds results in changes in the microbial community structure and metabolic adaptation (Dijkstra et al., 2012; Khafipour et al., 2009). However, there is a paucity of information that characterizes the progression of ruminal perturbation in response to various types of non-structural carbohydrates commonly fed to cattle. Starch and sugar, while both highly fermentable, differ in their chemical structures. Therefore, they can drive the metabolic and structural responses of the community divergently and can possess differing potentials to induce ruminal acidosis and dysbiosis (Dong et al., 2021).

While ruminal perturbations are common both due to intrinsic and extrinsic factors such as genetics and animal behaviour (Beauchemin, 2018; Stewart et al., 1997), the drivers controlling the microbial community response to nutrient and environmental perturbations are poorly understood. This is because the variation between animals has impeded the research efforts aimed to understand adaptive rumen microbial ecology in vivo (Jami & Mizrahi, 2012). As a result, research is moving towards in vitro systems to elucidate the mechanisms driving ecological responses to ruminal perturbations in order to predict responses within the in vivo system (Eger et al., 2018; Mickdam et al., 2016).

An extensive amount of research has shown the varying impacts of different phytochemicals on the rumen ecosystem and metabolic outputs (Hassan et al., 2020; Neubauer et al., 2018). However, these changes are not often seen at the taxonomic level, and information is needed on how these compounds alter the microbial metabolome. For example, previous research has shown antimicrobial effects on microbial communities, with compounds such as capsaicin (Marini et al., 2015; Patra et al., 2019) and menthol (Aperce et al., 2016), without being able to identify if these changes are due to cell apoptosis or alterations in microbial metabolism. Therefore, it is essential to assess the progressive effects of both non-structural carbohydrates and phytochemicals on the development of microbial intolerances in the rumen ecosystem in both the microbiota and the metabolome concurrently.

Thus, in this study, we aimed to develop a novel in vitro framework to conceptualize the ecological adaptation of the rumen microbiota using a liquid nutrient provision/diluted buffer model for inducing ruminal acidosis using individual carbohydrate components. Within this framework, we aimed to assess the impact of processed carbohydrate molecular structure and phytogenic substrates supplementation on the succession of acidosis through microbial community structure, predicted microbial metabolism, and in vitro rumen ecosystem metabolomic profiles. We hypothesize that carbohydrate input

perturbation, measured by community structure, microbial metabolism, and metabolite concentration, is variable based on the molecular structure, regardless of the type of non-structural carbohydrate provided (e.g., starches in the form of cornstarch [ST] or chemically modified cornstarch [CMS]). Furthermore, we hypothesize that the provision of differing antibacterial phytogenic substrates will alter microbial metabolism independent of carbohydrate type, but more predominantly in conditions of more severe environmental perturbation.

MATERIALS AND METHODS

Experimental design and treatments

The experiment was designed as an imbalanced 3 × 3 factorial design, with three carbohydrates sources and two phytogenic compounds infused in the liquid feed (Figure 1). Carbohydrate sources were chosen based on their solubility in the rumen and included sucrose (SU; AGRANA Zucker GmbH), native cornstarch (ST; MAIZENA®), and chemically modified (transglycosylated) cornstarch (CMS; AGRANA Research and Innovation Center). L-menthol (MEN; Sigma-Aldrich) and capsaicin (CAP; Sigma-Aldrich) were given as a liquid supplement. Approximately 1000 mg/L solution of each phytogenic compound was dissolved in 70% (v/v) ethanol (control, CON) at the beginning of the experiment and kept as stock solution at 4°C throughout the whole experiment. Liquid feed was prepared by mixing 25.2 g of each of carbohydrate source, 4.5 g of wheat gluten meal (AGRANA Stärke GmbH), and 0.3 g mineral premix ((RINDAVIT TMR; H. Wilhelm Schaumann GmbH & Co KG) in 50 ml ultrapure water in the evening of the day previous to the challenge and kept at 4°C. The trial was conducted in a RUSITEC system with 12 fermenters (two groups, each group contained six fermenters). A total of three experimental runs with two replicates for each treatment within each run was conducted. Each run lasted for 2 weeks and consisted of three periods: equilibration of the system (day 0–5), steady-state (day 6–9), and challenge (day 10–13).

Donor cows

Rumen fluid and solid digesta were collected on the first day of each experimental run from three rumen-cannulated non-lactating Holstein cows. The animals were fed only hay and kept at the dairy research farm (VetFarm) of Vetmeduni Vienna. Rumen fluid was obtained through the opening of the rumen cannula using a suction pump, while solid digesta was collected manually from the rumen mat. All procedures involving animal handling were performed according to Austrian guidelines for animal welfare.

Fermenter inoculation and liquid feeding

Prior to inoculation, the ruminal fluid of the donors was equally mixed and strained through four layers of medical gauze (~1-mm pore size), whereas the solid digesta was mixed and used unprocessed. Each fermenter was inoculated with 600 ml of rumen fluid and 100 ml of artificial saliva. Artificial saliva (McDougall, 1947) was continuously infused at a rate of 326 ± 19 ml/day (~2%/h) using a 12-channel peristaltic pump (model ISM932, Ismatec; Idex Health & Science GmbH). Solid digesta of all donors was pooled together, subsampled and then filled in nylon bags (160 × 70 mm, 150 µm pore size; Fa. Linker Industrie-Technik GmbH). To each fermenter, a pair of nylon bags was added, with one bag containing 12 g of dry matter (DM) of hay and the other bag containing solid ruminal digesta. After 24 h, the digesta bag was exchanged with a fresh feed bag containing the experimental hay (Table S1). Regular vertical movements of the feed containers (~7 cm and 8 cycles/min) were ensured by an electric motor and the incubation temperature was kept constant at 39.5°C. During the challenge period, acidosis was induced by infusing 50 ml of pre-warmed liquid feed three times a day (8 a.m., 12 a.m. and 4 p.m.) to each fermenter using sterile Luer Lock Solo (Omnifix BBRAUN) syringes through the tube that had been set up at the top of each fermenter. Immediately after carbohydrate infusion, 5 ml of each phytogenic solution (MEN or CAP) or CON was also infused.

Sample collection

Prior to replacing the feed bag each day, the fermenter fluid was sampled through a 3-way valve using syringes for the daily measurement of pH values and redox potential (Eh) with a pH-Meter (Seven Multi TM; Mettler-Toledo GmbH) equipped with separate electrodes (InLab Expert Pro-ISM for pH and Pt4805-DPA-SC-S8/120 for redox; Mettler Toledo GmbH). Samples of the fermenter fluid were taken for pH analysis, and aliquots of samples were stored at −20°C for the analysis of fermentation parameters. For DNA extraction and sequence analysis, samples were snap-frozen in liquid nitrogen and then stored at −80°C.

Fermentation gases and effluent were collected daily by using gas-tight bags (TecoBag 8 L; Tesseraux Container GmbH) and effluent bottles kept in an ice tub. To replace

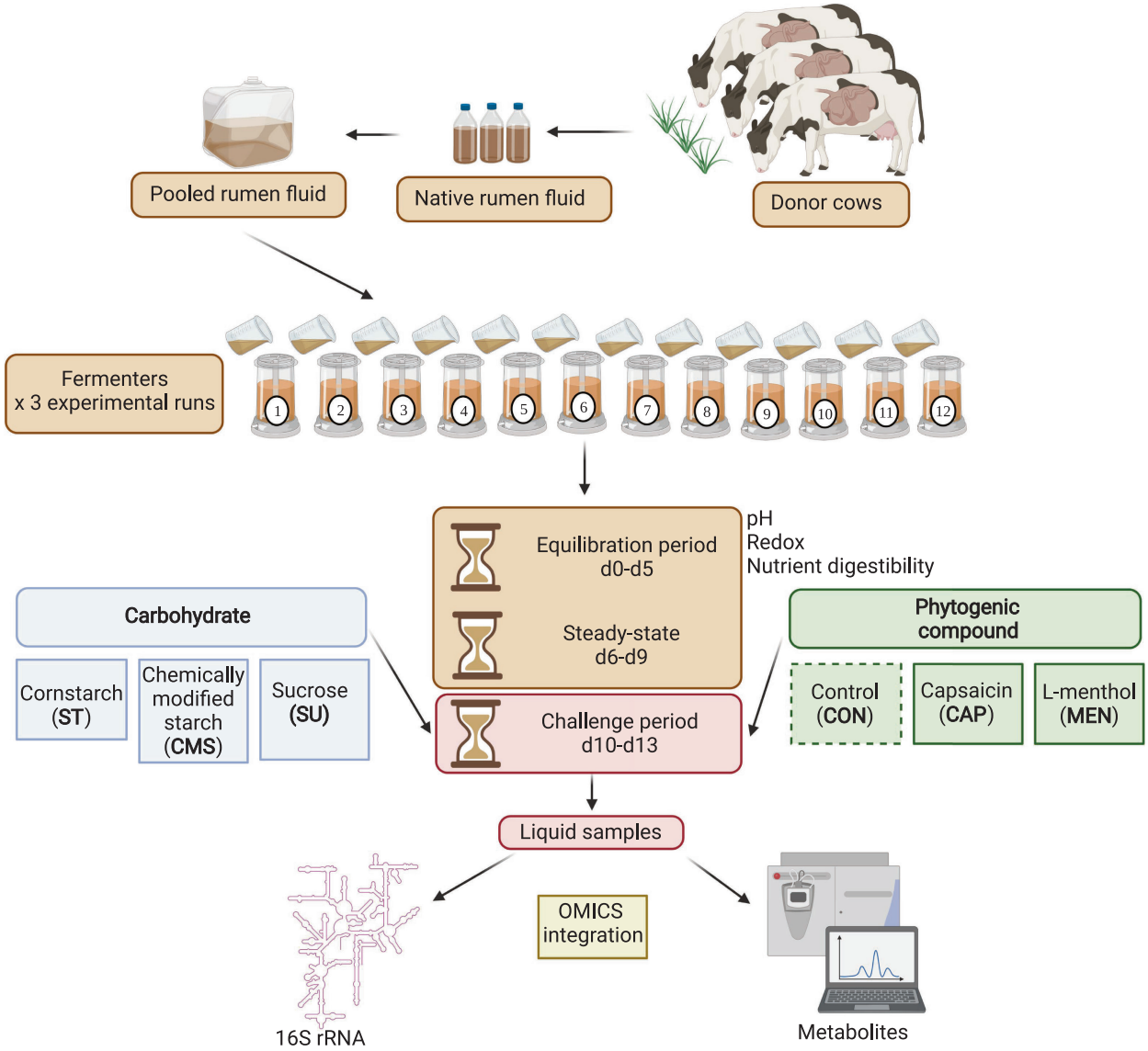

FIGURE 1 Experimental design. Three experimental runs consisted of liquid feeding with cornstarch (ST), chemically-modified starch (CMS) and sucrose (SU) and supplementation with L-menthol (MEN) and capsaicin (CAP). Created in BioRender.com

incubated feed bag after 48 h with a fresh one, nitrogen was flushed to the fermenters for 30 s. The bag was then unplugged, the fermenter was opened and the effluent was collected. The 48 h incubated feed bag was rinsed with pre-warmed buffer (artificial saliva) and squeezed to ensure microbe transfer to the fermenter and then replaced with the fresh bag. Subsequently, the fermenter was closed, nitrogen was flushed for 3 min to restore anaerobic conditions, and an empty gas bag was immediately attached. Gas composition was measured using an infrared detector (ATEX Biogas Monitor Check BM 2000; Ansyco), while gas volume measurements were performed according to the water displacement method (Soliva & Hess 2007). This procedure was done throughout the whole experiment.

During the steady-state and challenge, 48 h after incubation, feed bags were collected and washed immediately at a hand wash program (45 min, cold water with 18 g) with a washing machine and stored at −20°C. The content of the bags was freeze-dried (SANVAC CoolSafe 100-9 Pro; LaboGene) for 24 h at −40°C condenser plate temperature and a chamber pressure of less than 100 μm Hg, ground to 0.5 mm, and stored at room temperature until further chemical analysis.

Chemical composition analysis

Feed analysis was conducted on dry hay ground to 0.5 mm using a centrifugal mill (Ultra Centrifugal Mill ZM 200; Retsch). The DM of the hay was determined by oven drying (Venticell 111 eco line; BMT Medical Technology Ltd.) at 100°C for 24 h and ash content was measured by

combustion of samples at a temperature of 580°C overnight. By subtracting the value of ash, organic matter (OM), was calculated. Crude protein concentration was analysed using the Kjeldahl method (6.25×N [nitrogen content of samples], VDLUFA, 2007). The content of neutral detergent fibre (NDF) and acid detergent fibre (ADF) was quantified according to Van Soest et al. (1991) with a Fibertherm FT 12 (Gerhardt GmbH & Co. KG). The content of NDF and ADF were expressed as exclusive of residual ash. Heat-stable α-amylase and sodium sulfite were used in the NDF procedure. Ether extract was analysed using a Soxhlet extractor (Extraction System B-811; Buchi).

Fermenter fluid metabolome

Metabolome analysis was performed as described in (S. Ricci, C. Pacífico, E. Castillo-Lopez, R. Rivera-Chacon, H.E. Schwartz-Zimmermann, N. Reisinger, F. Berthiller, Q. Zebeli, R.M. Petri, unpublished). Briefly, fermenter fluid samples were collected at 0 (baseline), 4, 8, 24, and 72 h after first liquid feeding. The whole liquid samples were extracted with acetonitrile (sample to solvent ratio 1:50), centrifuged at 14,350 *g* for 10 min and measured by anion exchange chromatography on a Dionex Integrion HPIC system (Thermo Scientific) coupled to high resolution mass spectrometry. Ion chromatographic separation was performed on a Dionex IonPac AS11-HC column (250×2 mm, 4 μm) using a KOH-gradient. Analytes were ionized in negative mode and detected in full scan mode (mass range 50–750 *m/z*) on a Thermo QExactive orbitrap instrument (Thermo Scientific) at a resolving power of 70,000 full width half maximum at *m/z* 200.

Measurement of SCFA

Short-chain fatty acids including acetic acid, propionic acid, iso-butyric acid, *n*-butyric acid, iso-valeric acid and *n*-valeric acid and caproic acid were determined using gas chromatography (GC) (Klevenhusen et al., 2015). Briefly, samples were thawed and centrifuged at 20,000 *g* for 20 min at 20°C. The supernatant (0.8 ml) was diluted 1:2 with distilled water and then transferred into a fresh tube. After adding 0.2 ml of 1.2 mol hydrochloric acid and 0.2 ml of the internal standard 4-methyl valeric acid (Sigma-Aldrich), the mixture was centrifuged for several times at 20,000 *g* for 20 min at 4°C. Clear supernatant was transferred into glass vials and analysed for SCFA using a gas chromatograph equipped with a flame-ionization detector (Shimadzu GC Plus with FID detector; Shimadzu) and injector (AOC-20i Auto-Injector; Shimadzu Corp.) using a 30 m×0.53 mm capillary column (Trace TR Wax; Thermo Fisher Scientific). Helium was used as a carrier gas with a flow rate of 6 ml/min. The detector and injector were maintained at temperatures of 220°C and 170°C, respectively.

DNA extraction and 16S rRNA amplicon sequencing

Isolation and purification of microbial DNA was performed using the DNeasy PowerSoil Kit (Qiagen) with minor modifications according to Mahmood et al. (2020). Briefly, after fluid samples were thawed on ice, 800 μl of each sample was transferred to a bead beating tube provided in the kit. After adding 60 μl C1 to each sample, all samples were incubated at 95°C for 5 min. Following a centrifugation at 10,000 *g* for 2 min, supernatants were collected in fresh tubes and placed on ice for later procession. Lysozyme (100 μl of 100 mg/ml; Sigma-Aldrich) and mutanolysin (10 μl of 2.5 U/ml; Sigma-Aldrich) were added to each pellet and incubated at 37°C for 30 min. Subsequently, 21 μl of 19 mg/ml proteinase K (Sigma-Aldrich) was added to each pellet and incubated at 37°C for 1 h, followed by mechanical disruption using a homogenizer (FastPrep-24; MP Biomedical). After centrifugation, the supernatant of each sample was collected and added to the previously separated supernatant. Protein degradation, removal of PCR inhibitors and cell debris were performed by using the provided buffers C2–C5 and subsequent centrifugation steps. Finally, supernatants were loaded on silica-gel membranes and total genomic DNA was eluted in 100 μl of C6 buffer. Total DNA was measured on a Qubit Fluorometer 4.0 (Life Technologies) using the Qubit dsDNA HS Assay Kit (Life Technologies). Amplicon sequencing was performed using Illumina MiSeq paired-ends sequencing technology (Microsynth AG). Targeted amplification of the hypervariable region V4 of bacterial 16S rRNA gene (2×250 bp) was performed using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Multiplexed libraries were constructed by ligating sequencing adapters and indices onto purified PCR products using the Nextera XT Sample Preparation Kit (Illumina). Primers were trimmed and corresponding overlapping paired-end reads were stitched by Microsynth (Microsynth AG). Sequences have been submitted to the National Center for Biotechnology Information (NCBI) sequence read archive under the accession number PRJNA733994.

Bioinformatics and data analysis

A total of 6,716,224 merged reads were processed using the software package Quantitative Insights into Microbial

Ecology (QIIME2 v2020.2) (Bolyen et al., 2019). Read quality was inspected using FASTQC v. 0.11.5 (Andrews, 2010) and sequence data was quality filtered using the q-score-joined plugin with a minimum acceptable PHRED score of 20 (—-p-min-quality 20). Denoising into amplicon sequence variants (ASVs) was obtained using Deblur (Amir et al., 2017) by trimming all reads to a length of 250 nucleotides and removing low abundance ASVs (below 10). Representative sequences and ASV tables were filtered to exclude all features classified as mitochondria or chloroplast sequences, yielding a total of 3523 ASVs. All resulting filtered ASVs were aligned with mafft (Katoh & Standley, 2013) and used to construct a phylogeny with fasttree2 (Price et al., 2009). Taxonomy was assigned to ASVs using a classify-sklearn naïve Bayes taxonomy classifier trained with the 515F/806R primer set against the SILVA 132 99% operational taxonomic units reference sequences (https://www.arb-silva.de, v. 132). Rooted tree, taxonomy and filtered ASVs table were used as an input to phyloseq in R (v. 3.6.2, R Core Team, 2020). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was performed in QIIME2 in order to predict the function of microbial communities (Douglas et al., 2020).

Statistical analysis

Statistical analysis of chemical composition, SCFA, pH, redox potential, gas composition, metabolome and microbial alpha-diversity was performed using the MIXED procedure of SAS (v. 9.4; SAS Institute, Inc.). Fermenters were considered as experimental units, and measurements taken from the same fermenter throughout time were defined as repeated measures. The effects of carbohydrate, treatment, time (hours or day) and all possible interactions were considered as fixed effects, while run and fermenters were defined as random effects. Significance was declared at $p \leq 0.05$ and trends were considered at $0.05 < p \leq 0.10$. LSMEANS were compared with the PDIFF option using the Tukey post-hoc test.

To visualize the metabolome data and significant features, multivariate and univariate analysis were performed using MetaboAnalyst 4.0 software (https://www.metaboanalyst.ca; Chong et al., 2018). Data visualization was conducted in R Studio v. 1.3.1093 (R Core Team, 2020; RStudioTeam, 2020) using gplots (v. 3.1.0), dplyr (v. 1.0.2) and ggpubr (v. 0.4.0). Differential abundance of individual taxa and functional prediction was done in MaAsLin2 (Mallick et al., 2021). Data Integration Analysis for Biomarker discovery using Latent cOmponents (DIABLO) analysis was performed using mixOmics package (v. 6.14.0) (Rohart et al., 2017).

RESULTS

Ruminal pH and acidosis

Data showed that in vitro ruminal pH decreased with time after carbohydrate infusion (Figure 2a). Significant differences after 4 h for SU ($p < 0.01$) and CMS ($p < 0.01$) relative to baseline were observed in regards to pH. After 4 h, the pH in SU group decreased to 5.76 ± 0.09, indicating moderate ruminal acidosis. However, in CMS, this drop was more gradual, reaching 6.06 ± 0.09 in the 4 h post feeding. After 8 h, average pH decreased until 4.62 ± 0.09 and 4.86 ± 0.09 in the SU and CMS treatments, respectively, indicating severe acidosis. Nadir pH was reached at the end of experimental sampling with 3.95 ± 0.09 in SU and 4.27 ± 0.09 in CMS. Comparatively, a moderate pH drop was observed in ST after 8 h, with pH decreasing to 5.67 ± 0.09, and severe acidosis being reached after 24 h (4.69 ± 0.09) and maintained until 72 h (4.24 ± 0.09). No effect of the phytogenic supplementation was observed for pH ($p = 0.44$) throughout challenge.

To monitor the efficacy of the in vitro system, we monitored Eh, gas production, and nutrient degradation during the equilibration, steady-state, and feeding challenge periods. Throughout equilibration and steady-state periods, these parameters remained within the normal range for in vitro conditions, which is between 6.50–6.80 for pH and −246 to −285 mV for Eh (Mickdam et al., 2016; Orton et al., 2020; Wetzels et al., 2018), but changed after carbohydrate infusion, and often in a carbohydrate-dependent way. Nutrient degradation (DM and OM) was significantly decreased throughout the challenge ($p < 0.01$). Feeding SU and CMS led to a drop in CH_4 production throughout the challenge ($p \leq 0.05$) (Table S2).

Rumen metabolome

Several metabolites in the rumen were identified and quantified in this study using various analytical platforms. Differences at the level of the metabolites in response to carbohydrate feeding (SU, ST and CMS) during the baseline (0 h) and challenge period (4, 8, 24 and 72 h) were visualized in a clustered heatmap (Figure 3a). In response to ST (Figure 3b), CMS (Figure 3c) and SU (Figure 3d), principal component analysis clearly showed a separation according to time (0, 4, 8, 24 and 72 h).

An increase in D-lactate was visible in all three carbohydrate sources, with SU and CMS having a more pronounced effect than ST ($p \leq 0.05$). During the first hours (4 and 8 h), CMS and SU feeding increased lactate considerably, whereas lactate progression at the same time was lower for the ST group. Throughout the feeding challenge

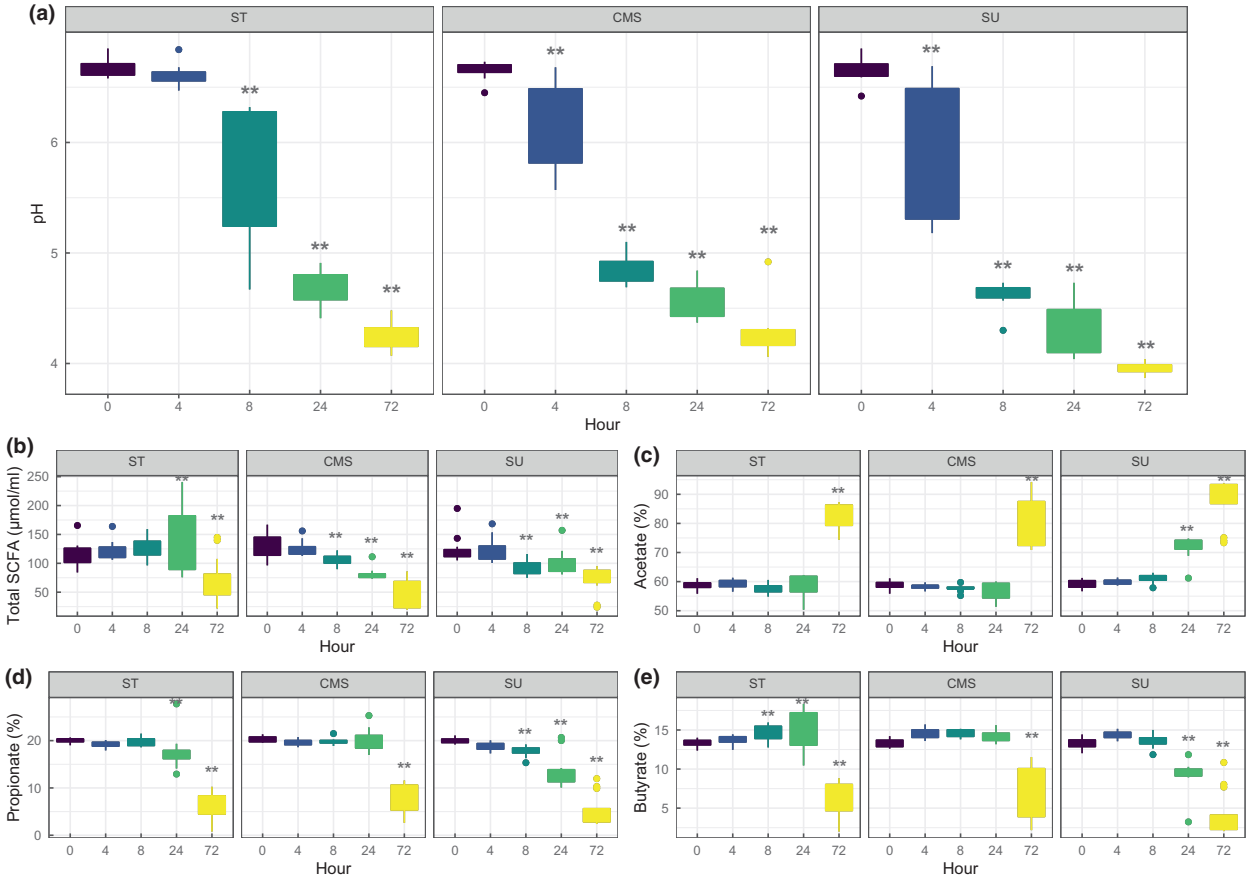

FIGURE 2 (a) pH, (b) total short chain fatty acids, (c) % of acetate, (d) % of propionate and (e) % of butyrate of fermenter fluid at 0 (baseline), 4, 8, 24 and 72 h after first liquid feeding in cornstarch (ST), chemically-modified starch (CMS) and sucrose (SU). (**) indicates significant differences between baseline and remaining time-points, within carbohydrate ($p \leq 0.05$)

time, a significant decline in benzoic acid was also observed ($p < 0.01$), being more pronounced in SU and CMS at 4 and 8 h than in ST. Succinic and methyl malonic acid significantly increased in response to ST feeding as compared to SU and CMS ($p \leq 0.01$). Hydrocinnamic acid was lower in CMS and SU, and this decrease was smoother in ST than in the other two carbohydrate sources. Throughout the progression of time, malonic acid started to decrease until 24 h in all three carbohydrates, being more pronounced at 4 h for SU and CMS and at 8 h for ST. In response to SU, oxoglutaric acid was remarkably increased at 24 h, while for ST and CMS, no significant changes were observed throughout time.

Total SCFA (Figure 2b) decreased in SU ($p = 0.004$) and CMS ($p = 0.07$) after 8 h in comparison with ST, whereas feeding ST showed the highest values at 24 h (140 µmol/ml, $p \leq 0.05$) and lowest at 72 h (72 µmol/ml, $p \leq 0.05$). Throughout time, the relative abundance of acetate (58.85–83.36) and the acetate to propionate ratio (A:P; 2.92 to 20.62) linearly increased ($p \leq 0.05$). Furthermore, acetate (Figure 2c) was significantly affected by carbohydrate, particularly in SU versus ST ($p < 0.01$) and SU versus CMS ($p < 0.01$). Carbohydrate feeding caused a significant reduction in the percentage of propionate (Figure 2d) throughout time ($p < 0.01$). In response to SU and ST feeding, propionate decreased from 8 h ($p = 0.01$) and 24 h ($p = 0.05$), respectively. Butyrate (Figure 2e) was significantly affected by time ($p < 0.01$), carbohydrate ($p < 0.01$) and interaction between time and carbohydrate ($p < 0.01$). ST feeding caused a significant increase in the percentage of butyrate at 8 h, which then decreased at 72 h as compared to baseline ($p \leq 0.05$). In contrast, in the SU group, butyrate decreased after 24 h, while for the other two carbohydrates this was not observed before 72 h (Table S3).

In comparison to CON and CAP, MEN supplementation led to an increase in hexose phosphates (hexose-P-3*) and a decrease in uridine-5′-monophosphate ($p \leq 0.05$). MEN ($p = 0.002$) and CAP ($p = 0.06$) supplementation significantly decreased the pyruvic acid peak compared to CON. Oxoglutaric acid ($p \leq 0.05$) was decreased in MEN when compared to CON. Fumaric acid increased in response to CAP when compared with MEN ($p \leq 0.05$). Uridine 5′-monophosphate was increased in response to MEN and CAP relatively to CON ($p \leq 0.05$). Apart from caproate, total and individual (%) SCFA did not differ with phytogenic supplementation. CAP supplementation as

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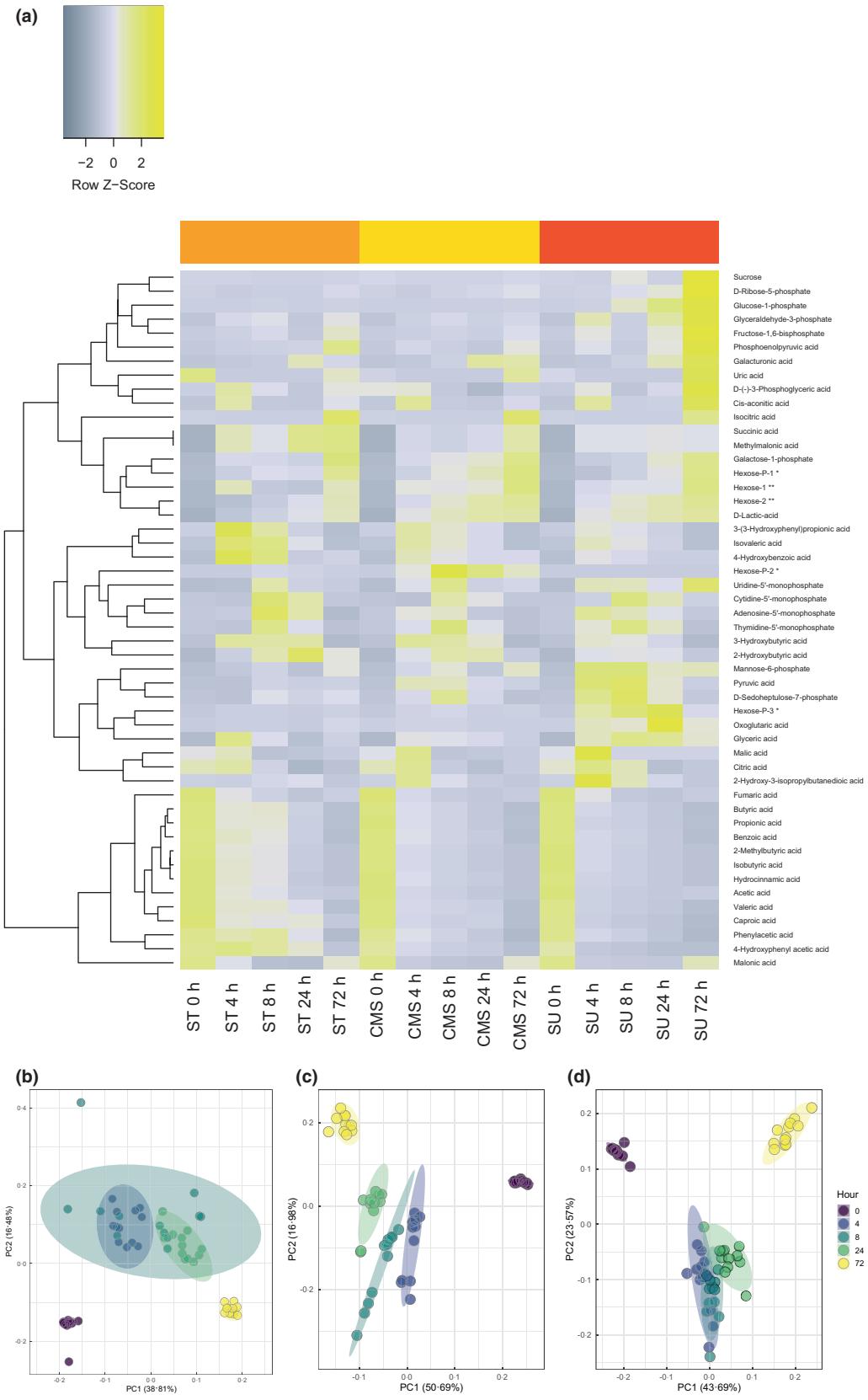

FIGURE 3 (a) Heatmap of hierarchical clustering analysis of normalized peaks of 54 metabolites detected in fermenters fluid samples of RUSITEC fermenters ($n = 12$) in response to three different substrates (cornstarch [ST], chemically-modified starch [CMS] and sucrose [SU]) feeding at 0, 4, 8, 24 and 72 h after first liquid feeding. Scores plot of principal component analysis (PCA) between principal components (PC) 1 and 2 applied to the longitudinal dataset of ferment fluid metabolome of RUSITEC fermenters according to substrates throughout time including (b) ST, (c) CMS and (d) SU. (*) exact identification (sugar moiety) was not possible due to lack of authentic reference standard. (**) the hexose was not identified

compared to MEN significantly increased the proportion of caproate ($p < 0.01$).

Microbiota composition and structure

A total of 182 samples (179 RUSITEC and three pooled donor samples) yielded 3523 different ASVs across 5,059,023 sequences (Table S4). Considering all samples, 21 phyla were identified, with Firmicutes and Bacteroidetes accounting for 78.0% and 12.1% of all reads, respectively. The bacterial composition of the pre-challenge RUSITEC samples and the donor samples based on the Aitchison distance matrix did not differ statistically (ANOSIM; $p = 0.142$).

The duration of the challenge significantly impacted bacterial diversity and richness estimates Shannon (Figure 4a, $p < 0.01$), the number of observed ASVs (Figure 4b, $p < 0.01$) and Simpson (Figure 4c, $p < 0.01$). No interaction between carbohydrate and time was found for the number of observed ASVs, Shannon and Simpson. No significant differences were found between different carbohydrates at the level of the alpha-diversity estimates Shannon, Simpson and the number of observed ASVs. A small trend was found for the Simpson index, which was slightly higher in ST when compared with CMS ($p = 0.08$). No effect of phytogenic supplementation was found at the level of the alpha-diversity estimates. However, a trend was found for the interaction between time and phytogenic supplementation for the number of observed ASVs ($p = 0.089$) and Simpson ($p = 0.075$). At the beginning of the experiment, the number of observed ASVs was approximately 963.3 ± 54.7. This parameter was significantly decreased after 24 h of incubation ($p < 0.01$), reaching its lowest value of 293.8 ± 55.4 after 72 h ($p < 0.01$). The diversity index Shannon continuously decreased from 4.37 ± 0.32 at the beginning of the experiment to 3.85 ± 0.32, 2.69 ± 0.32 and 2.35 ± 0.32 after 8 h ($p = 0.04$), 24 h ($p < 0.01$) and 72 h ($p < 0.01$), respectively. A significant decrease was found for the Simpson index already after 4 h of liquid feeding ($p = 0.005$). Overall bacterial community composition differed throughout time based on beta-diversity analysis (Figure 4d,e, ANOSIM; $p = 0.001$). Differences at the level of the bacterial community composition in regards to carbohydrate source were not detected on the Aitchison matrix (ANOSIM; $p = 0.469$).

Progression of dysbiosis and role of phytogenic supplementation

Both the progression of ruminal acidosis and dysbiosis was different depending on the carbohydrate source. All changes at the phylum, family and genus levels are given in detail in Table S5. Accordingly, the dysbiotic changes in SU were already visible after 4 h of feeding, with a marked decrease in Bacteroidetes ($Q = 0.02$). In ST, after 8 h, an increase in Proteobacteria and a decrease in Euryarchaeota were observed ($Q < 0.1$). In CMS, the first phyla to be affected was Fibrobacteres after 8 h ($Q < 0.01$). In fact, the rate by which Fibrobacteres started to decline differed among the three carbohydrates tested. Fibrobacteres population started declining in SU after 8 h and in ST only after 24 h ($Q < 0.01$). In SU, nine other phyla changed after 8 h. Interestingly, Elusimicrobia, Planctomycetes and Synergistetes increased ($Q < 0.07$).

In response to ST feeding, Dysgonomonadaceae decreased after 4 h ($Q = 0.04$), and methanogenic families such as Methanomethylophilaceae and Methanomicrobiaceae decreased after 8 h ($Q < 0.05$). ST feeding led to increases in Bifidobacteriaceae, Lactobacillaceae, Prevotellaceae and Succinivibrionaceae ($Q < 0.01$) after 24 and 72 h. The families that mostly declined after 24 h were Fibrobacteraceae, Anaerolineaceae, Marinilabiliaceae, Peptococcaceae and Pirellulaceae ($Q < 0.05$). Rhodospirillaceae, Bacteroidaceae, AKAU3644 and Vibrionaceae increased after 72 h ($Q < 0.08$). After this time point, the families that decreased the most in ST were Fibrobacteriaceae, Erysipelotrichaceae, vadinBE97 and Clostridiales vadinBB60 group ($Q < 0.05$).

In CMS, Peptococcaceae increased immediately after 4 h ($Q < 0.05$) and kept increasing until 8 h, while Fibrobacteraceae and Puniceicoccaceae decreased at this time point ($Q < 0.05$). Similarly, to ST, Lactobacillaceae and Bifidobacteriaceae are among the families that most increased after 24 h. However, in CMS, a high increase in Bacteroidaceae and Dysgonomonadaceae was also detected ($Q < 0.05$) after 24 h, while Fibrobacteraceae, Erysipelotrichaceae, Bacteroidales RF16 group and Elusimicrobiaceae were the families that mostly decreased ($Q < 0.05$). After 72 h, Lactobacillaceae, Bacteroidaceae, Dysgonomonadaceae, Bifidobacteriaceae and Leuconostocaceae are among the families that mostly increased in CMS ($Q < 0.05$), while Fibrobacteraceae, Clostridiales vadinBB60 group, Erysipelotrichaceae, Bacteroidales RF16 group and vadinBE97 are among those that mostly declined ($Q < 0.05$).

In SU, several bacterial families were affected immediately after 8 h. Bacteroidaceae, Lactobacillaceae, Peptococcaceae, PeH15, Endomicrobiaceae, Desulfuromonadaceae and Marinilabiliaceae were found to increase ($Q < 0.08$), while Fibrobacteraceae, Prevotellaceae and Spirochaetaceae decreased ($Q < 0.08$). Bifidobacteriaceae is only found to increase after 72 h ($Q < 0.05$). A significant increase in Enterobacteriaceae was only found for SU after 72 h ($Q < 0.05$).

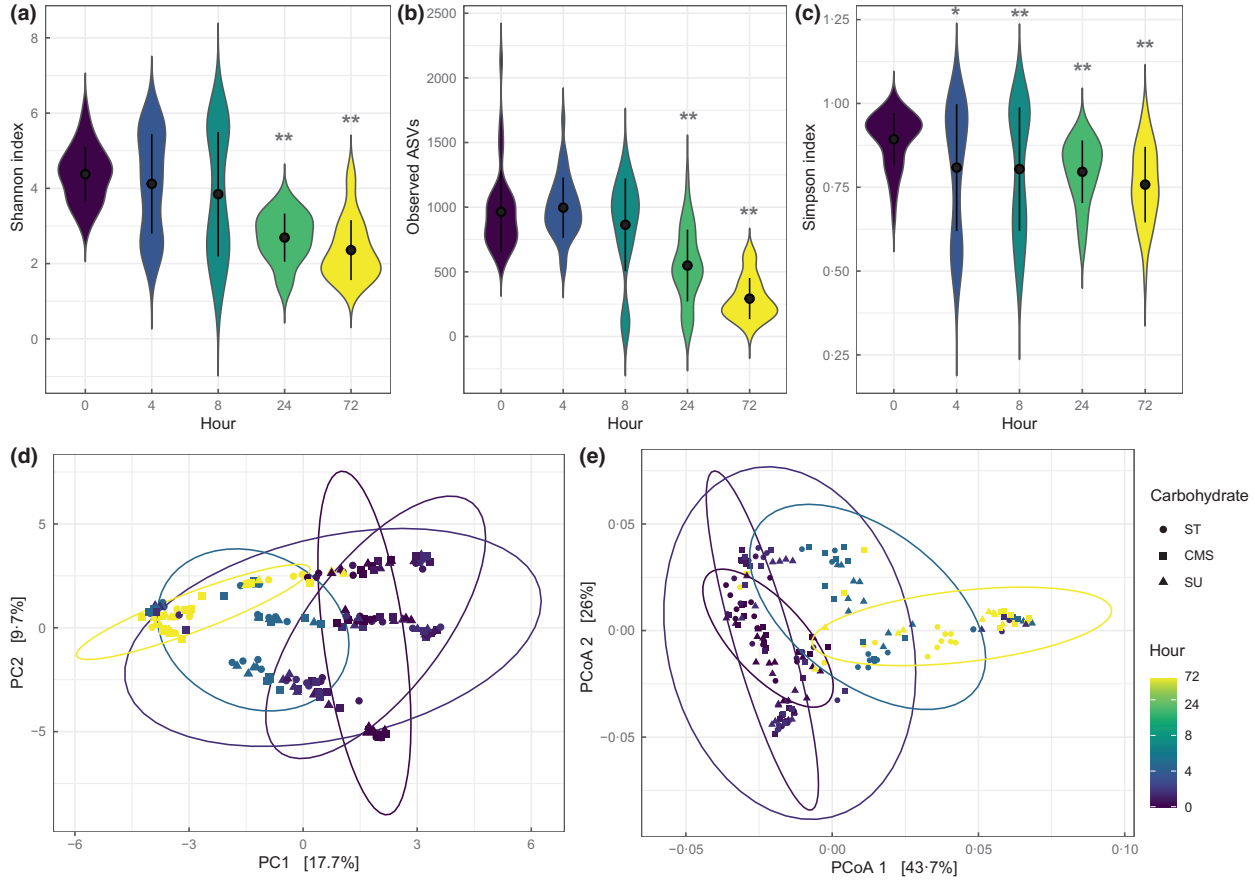

FIGURE 4 Alpha-diversity indexes (a) Shannon, (b) observed ASVs and (c) Simpson of the of fermenter fluid microbiota at 0 (baseline), 4, 8, 24 and 72 h after first liquid feeding. Beta-diversity matrices according to (d) Aitchison and (e) weighted UniFrac. Time is given in the colour gradient, while carbohydrates are specified by the different shapes (cornstarch [ST]—circles, chemically-modified starch [CMS]—squares and sucrose [SU]—triangles). (*) indicates a difference by trend ($0.05 < p \leq 0.10$) and (**) indicates significant differences between baseline and remaining time-points ($p \leq 0.05$). ASV, amplicon sequence variant

The most pronounced changes at the genus level for ST, CMS and SU are given in Figure 5a–c. *Fibrobacter* was shown to decrease in ST after 24 h ($Q < 0.05$), while decreasing in CMS and SU already after 8 h ($Q < 0.05$). In ST, *Bifidobacterium* was the bacterial genus that most increased after 24 and 72 h ($Q < 0.05$). It was also shown to increase in CMS and SU after 24 and 72 h, but behind *Lactobacillus* and *Bacteroides* ($Q < 0.05$). Both *Lactobacillus* and *Bacteroides* were shown to increase in SU immediately after 8 h and in CMS after 24 h ($Q < 0.05$). *Bacteroides* only increased in ST after 72 h ($Q < 0.06$). *Ruminococcus* 1, *Fibrobacter* and *Kandleria* were the bacterial genera that mostly decreased with ST infusion after 72 h ($Q < 0.05$). These bacteria also decreased in CMS and SU ($Q < 0.05$).

CAP and MEN supplementation led to a decrease in Proteobacteria in the ST group ($Q < 0.05$). In SU, CAP and MEN had a higher abundance of Veillonellaceae than CON ($Q < 0.05$). An additional decrease in X0319-6G20 was also observed in the MEN group ($Q < 0.03$). At the genus level, *Anaerovibrio* increased in MEN while Ruminococcaceae UCG-012, *Selenomonas* and *Selenomonas* 1 increased in CAP in the SU group ($Q < 0.1$). In the CMS group, MEN promoted the growth of *Methanosphaera* and *Selenomonas* ($Q < 0.03$), while CAP led to an increase in *Synergistes* ($Q < 0.03$). CAP decreased *Ruminobacter*, *Selenomonas* and *Eubacterium saphenum* group in ST ($Q < 0.09$), while MEN increased DNF00809 ($Q = 0.07$).

Predicted metagenome profiles

PICRUSt2 allowed us to infer the functional potential of the microbiota based on the 16S rRNA composition. Most pronounced changes are given in Figure 5a–c. After 4 h, visible changes were found in SU and CMS. Pathways involved in tricarboxylic acid (TCA) cycle (partial TCA cycle, PWY-5913) and benzoate degradation (meta-cleavage pathway of aromatic compounds, PWY-5430) were enriched in SU when compared with CON, while polysaccharide degradation (β-[1,4]-mannan degradation, PWY-7456), reductive TCA cycles (incomplete reductive TCA cycle, P42-PWY), sugar acid degradation

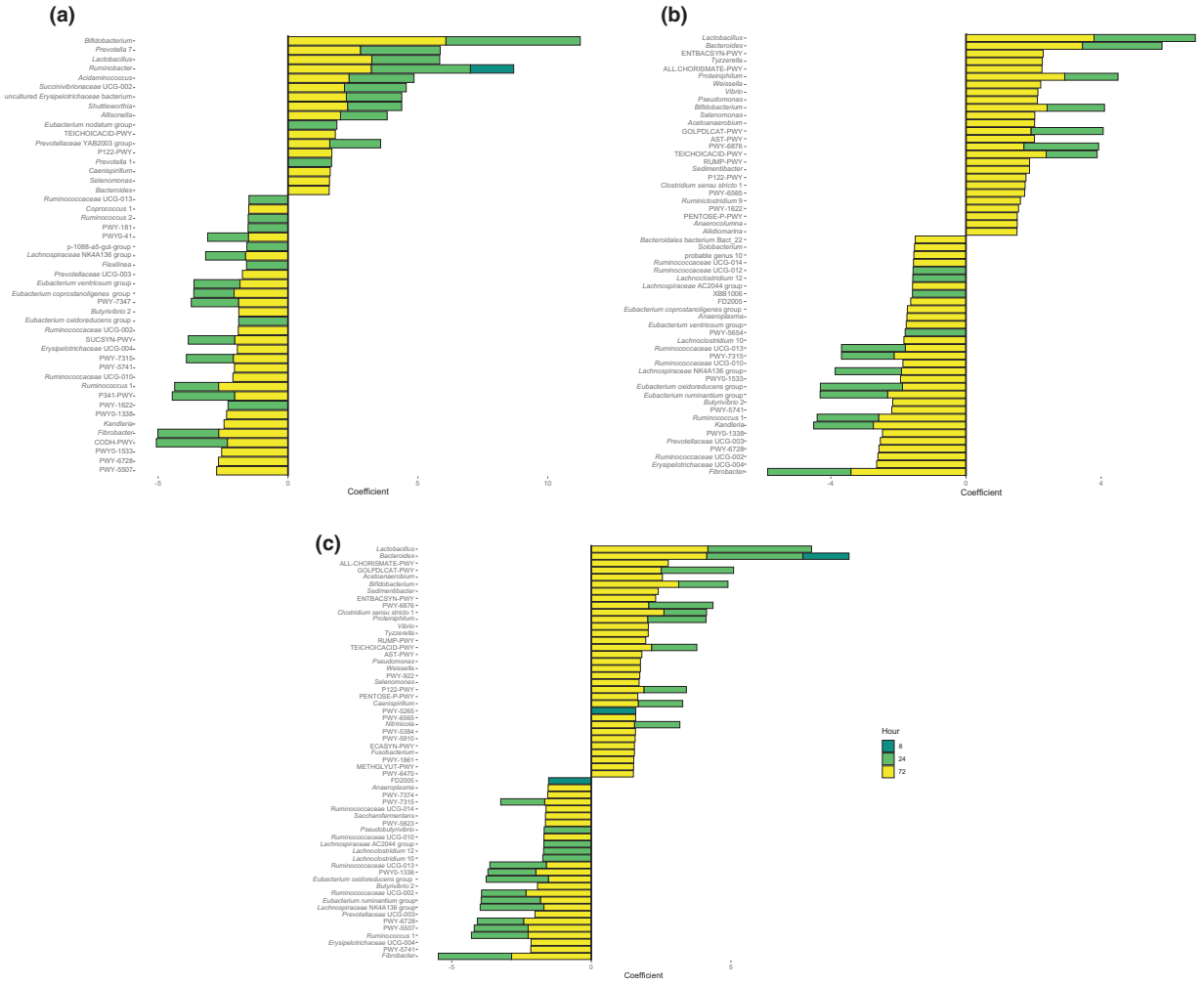

FIGURE 5 Most pronounced modifications in bacteria at the genus level and metabolic pathways (coefficient >1.5) in cornstarch (ST) (a), chemically-modified starch (CMS) (b) and sucrose (SU) (c). Reference is 0 h (baseline)

(D-fructuronate degradation, PWY-7242) and L-rhamnose degradation (L-rhamnose degradation I, RHAMCAT-PWY) were decreased ($Q < 0.1$). In the CMS group, after 4 h, glycolysis (glycolysis V, P341-PWY) and alcohol degradation (superpathway of glycol metabolism and degradation, GLYCOL-GLYOXDEG-PWY) increased. On the contrary, pathways associated with the generation of precursor metabolites and energy (superpathway of glycolysis and the Entner-Doudoroff pathway, GLYCOLYSIS-E-D) decreased ($Q < 0.09$). No changes in pathways were observed in ST after 4 h.

After 8 h, 20, 3 and 85 pathways underwent alterations in response to ST, CMS and SU feeding, respectively. Partial TCA cycle (PWY-5913) increased in CMS, while nitrate reduction VI (PWY490-3) and superpathway of sulfur oxidation (PWY-5304) decreased ($Q < 0.1$). In ST, L-arginine degradation (L-arginine degradation II, AST-PWY), aldehyde degradation (superpathway of methylglyoxal degradation, METHGLYUT-PWY), D-galactarate degradation (D-galactarate degradation I, GALACTARDEG-PWY), superpathway of D-glucarate and D-galactarate degradation (GLUCARGALACTSUPER-PWY) and purine nucleotide degradation (inosine 5′-phosphate degradation, PWY-5695) are among the pathways that mostly increased ($Q < 0.08$). Peptidoglycan biosynthesis (peptidoglycan biosynthesis II, PWY-5265), glycolysis (glycolysis V, P341-PWY), allantoin degradation (allantoin degradation IV, PWY0-41), glycerol degradation (superpathway of glycerol degradation to 1,3-propanediol, GOLPDLCAT-PWY) and glycosaminoglycan degradation (chondroitin sulfate degradation I, PWY-6572) were the pathways that mostly increased in SU after 8 h ($Q < 0.09$). Overall, the most pronounced changes occurred after 24 and 72 h in all three carbohydrate feed groups (Table S5).

Regarding phytogenic supplementation, in ST, MEN led to an increase in L-arginine degradation (ARGORNPROST-PWY), 2-methylcitrate cycle (2-methylcitrate cycle II, PWY-5747), fermentation to alcohols (glycerol degradation to butanol, PWY-7003) and a decrease in pyrimidine deoxyribonucleotide de

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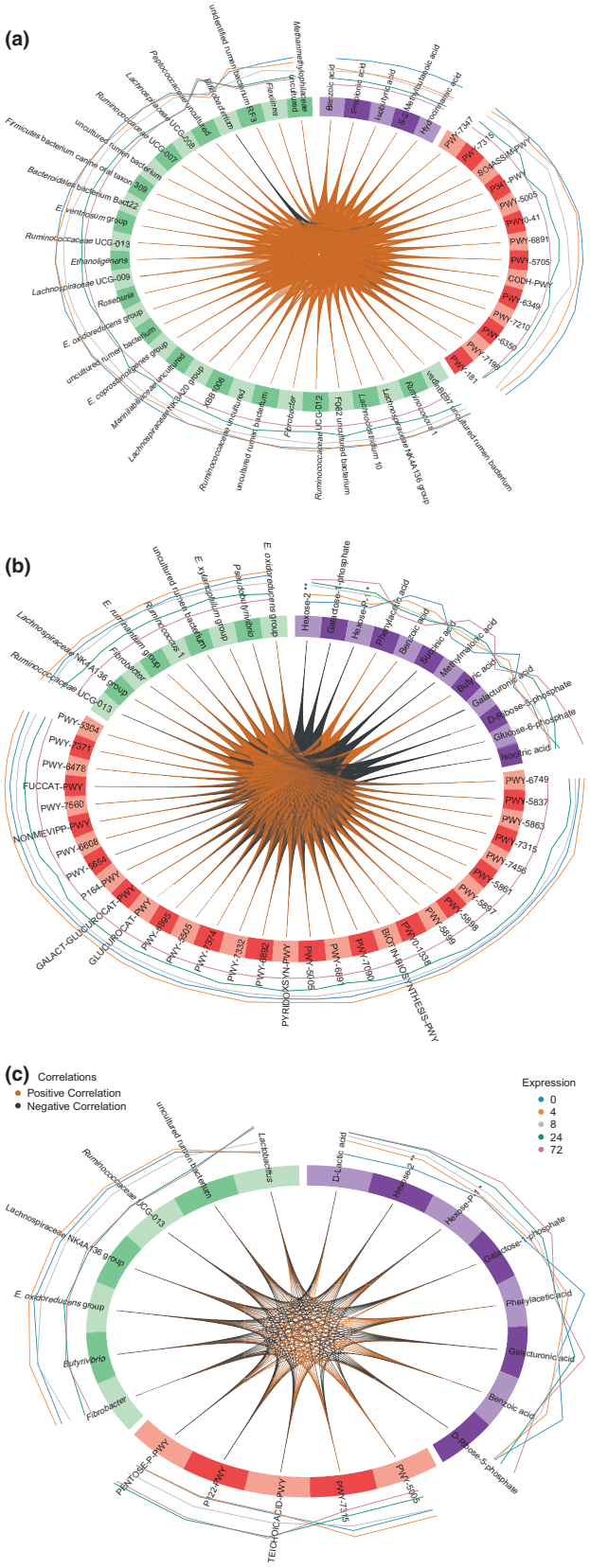

FIGURE 6 Integration of multiple OMICS datasets using DIABLO. Data are visualized according to the cornstarch (ST) (a), chemically-modified starch (CMS) (b) and (c) sucrose (SU) group. Circos plots display the strongest correlations between microbiota, metabolome and PICRUSt predictions generated from the first component with links between features indicating positive (brown) or negative (black) correlations. The blue, orange, grey, green and pink lines represent 0 (baseline), 4, 8, 24 and 72 h after first carbohydrate feeding. (*) exact identification (sugar moiety) was not possible due to lack of authentic reference standard. (**) the hexose was not identified

novo biosynthesis (superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis, PWY-7211) when compared with CON ($Q < 0.1$). CAP led to an increase in nitrate reduction (nitrate reduction VI, PWY490-3, $Q = 0.08$). In the CMS group, CAP led to a decrease in catechol degradation (catechol degradation II, PWY-5420; catechol degradation to 2-hydroxypentadienoate II, PWY-5419, $Q < 0.06$). CAP led to a decrease in peptidoglycan biosynthesis (peptidoglycan biosynthesis II, PWY-5265) and an increase in 1,4-dihydroxy-6-naphthoate biosynthesis (1,4-dihydroxy-6-naphthoate biosynthesis II, PWY-7371), UDP-sugar biosynthesis (superpathway of UDP-glucose-derived O-antigen building blocks biosynthesis, PWY-7328) and sugar degradation (glucose and glucose-1-phosphate degradation, GLUCOSE1PMETAB-PWY) in the SU group ($Q < 0.1$). MEN increased amine and polyamine biosynthesis (superpathway of polyamine biosynthesis II, POLYAMINSYN3-PWY) in SU ($Q < 0.1$).

Relationship between metabolome and microbiota

Integrative analysis was done to determine comparisons between multiple OMICS datasets for ruminal fluid metabolites, rumen microbes and their predicted pathways. Strong interactions ($r > 0.7$) between genera, metabolites and pathways within each carbohydrate are given in Figure 6a–c. In ST, *Flexilinea*, *Fibrobacter*, *Eubacterium oxidoreducens* group, *Eubacterium ventriosum* group, *Lachnoclostridium* 10, *Lachnospiraceae* NK3A20 group, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-008, *Lachnospiraceae* UCG-009, *Roseburia*, *Eubacterium coprostanoligenes* group, *Ethanoligenens*, *Ruminococcaceae* UCG-007, *Ruminococcaceae* UCG-012, *Ruminococcaceae* UCG-013 and *Ruminococcus* 1 were all found to be strongly positively correlated with the metabolites propionate, isobutyrate, S-2-methylbutanoic acid, benzoic acid and hydrocinnamic acid, which were all positively correlated with 14 metabolic pathways associated with, for example, production of acetyl-CoA, glycolysis and sulfate reduction. Interestingly, an inverse result was obtained for *Bifidobacterium* and all above-mentioned metabolites and pathways (Figure 6a). In the CMS group, several positive correlations were observed between microbiota, pathways and metabolites. Butyric acid,

phenylacetic acid and benzoic acid were positively correlated to *Fibrobacter*, *Eubacterium oxidoreducens* group, *Lachnospiracea* NK4A136 group and *Ruminococcaceae* UCG-013, showing some similarity between ST and CMS, regarding SCFA-associated bacteria (Figure 6b). In regards to the SU group, *Fibrobacter*, *Butyrivibrio*, *Eubacterium oxidoreducens* group, *Lachnospiraceae* NK4A136 group and *Ruminococcaceae* UCG-013 were positively correlated with propionic acid, butyric acid, valeric acid and phenylacetic acid and negatively correlated with lactic acid and two hexoses, while *Lactobacillus* had the reverse correlation profile. *Lactobacillus* was positively associated with P122-PWY, TEICHOICACID-PWY and lactate (heterolactic fermentation) and PENTOSE-P-PWY (pentose phosphate pathway) and had a negative correlation with several pathways, such as FUCCAT-PWY (fucose degradation), PWY-5005 (biotin biosynthesis II), PWY-5654 (2-amino-3-carboxymuconate semialdehyde degradation to 2-hydroxypentadienoate) (Figure 6c).

DISCUSSION

Effect of carbohydrate source on severity of ruminal acidosis

By feeding different carbohydrate sources in the same amount, the initial aim of the study was to simulate progressive ruminal acidosis models of various severities and durations that allow deeper characterization of the related dysbiosis. Indeed, we found that SU feeding caused a drop of ruminal pH to 5.76 ± 0.09 after 4 h, a pH level considered as moderate acidosis (Plaizier et al., 2008; Mickdam et al., 2017; Zebeli et al., 2008), and that D-lactate accumulation was also more evident in SU and CMS than ST. Lactate is less protonated than SCFA and thereby accumulates in the rumen, contributing actively to the acidification of the rumen environment, which partially explains the severity of acidosis observed in CMS and SU. Previous research has shown that the feeding of CMS increased lactate levels and decreased pH compared to purified cornstarch (Newman et al., 2018), which is in agreement with the current findings. With the progression of acidosis, SCFA concentrations decline dramatically because of the destruction of the normal bacterial flora (Huber et al., 1976). After 8 h, total SCFA decreased in SU and CMS, indicating an arrest in fermentation and a disruption of the state of homeostasis in the rumen. After 8 h, severe acidosis was already observed in SU and CMS, with pH reaching 4.62 ± 0.09 and 4.86 ± 0.09, respectively. A decrease in pH was also recently reported in vitro after an increase in the levels of sugar, as a rapid pH decline associated with the lack of epithelial absorption of SCFA due to the in vitro conditions of the study might explain the severity of dysbiosis observed (Dong et al., 2021).

SU and CMS, the groups with stronger effects on pH, were also those that elicited a greater increase in Eh. This result agrees with previous in vitro studies, which have also reported increased redox potentials with the decrease of pH (Orton et al., 2020). Our results showed that, while each carbohydrate tested resulted in an acute acidosis after 72 h, the rate at which this was achieved varied among substrates. SU, followed by CMS, were the substrates that most rapidly induced perturbations in the in vitro system.

Loss of bacterial diversity and richness and progression of dysbiosis

The main aim of the study was to characterize the progression of dysbiosis and changes in bacterial diversity and richness in response to individual carbohydrate components. Dysbiosis is typically described as an unbalanced microbial community that undergoes metabolic perturbations generally after dietary changes. Ruminal SCFA alternation, low pH and loss of diversity (Brown et al., 2012; Hawrelak & Myers, 2004) have been shown to negatively affect animal health by promoting the proliferation of opportunistic microbes and their products (Plaizier et al., 2008; Tao et al., 2017). A deep characterization of dysbiosis is thus important for its early diagnosis. In this context, an important finding of our study was that dysbiosis is expressed differently based on the substrate availability. Accordingly, feeding SU, which has the highest solubility, caused a microbial shift immediately after 4 h by decreasing the *Bacteroidetes* population. Increased availability of fermentable carbohydrates, such as starch and sugars, leads to rapid availability of glucose, which stimulates growth rates of all microbes resulting in an overall increase in SCFA production as seen, shortly after carbohydrate feeding. However, this is only maintained as long as there is no accumulation of inhibitory metabolites. In our study, rapid changes in the ecosystem were mirrored in microbial diversity and richness estimates, which declined independently of the type of carbohydrate. Several studies have previously reported that acidosis induction lowered the bacterial diversity and richness (Khafipour et al., 2009; Nagata et al., 2018; Petri et al., 2013). This is likely because diversity can be affected by the ruminal pH (Nagata et al., 2018), and the difference of pH between carbohydrates tested in this experiment was not strong enough to make a significant alteration in bacterial community diversity and richness. This is in agreement with previous observations, as feeding starch and sugar did not significantly affect Chao1, Simpson, and Shannon indices (Dong et al., 2021).

Bacterial changes associated with ruminal acidosis primarily include shifts in the populations of amylolytic, maltose-, glucose- and lactic acid-fermenting bacteria (Nagaraja & Titgemeyer, 2007). In our study, these shifts in environmental conditions and in nutrient availability preceded changes in differential abundance of 44 bacterial genera 8 h after feeding in the SU group. At 8 h post challenge, only eight bacterial genera were affected in ST and CMS, evidencing the higher degree of perturbation caused by sucrose when compared with starches. A severe acidosis after 8 h in the CMS group was not reflected in such an abrupt shift in microbial composition. This might be due to the fact that CMS is a transglycosylated cornstarch, which is often used in monogastric diets to reduce the rate of enzymatic digestion (Newman et al., 2018). Therefore, the current findings may be associated with a delay in microbial activity due to the crosslinking bonds within CMS, rendering the substrate unavailable for microbial degradation. In contrast to the alpha-diversity data, weighted UniFrac distances were significantly impacted by the carbohydrate type, and a difference between carbohydrates can be observed in the clustering of the microbial data after 72 h. Interestingly, in the metabolome data, this clustering in the metabolites occurs in a different manner, with SU clustering separately from the starches. This might be associated with robust survival mechanisms (Liu et al., 2015) of the acid-tolerant microbes that thrive in these conditions.

The increased production of organic acids is essentially because of the establishment of an acid-tolerant bacterial population and inactivation of lactate-fermenting bacteria due to disruptive environmental conditions (Therion et al., 1982). Under normal conditions (pH >6.5), most rumen microbes grow well and lactate is rapidly metabolized by lactate utilizers, such as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Nocek, 1997). In our study, *Megasphaera* was proliferating in the ST group after 24 and 72 h and in SU after 24 h. *Selenomonas* was also increased after 72 h in all three carbohydrates, highlighting that despite acidosis, the growth of these microorganisms was still sustained and they are probably more resilient than previously thought. The low pH in our experiment (<5.5) could have reduced the growth/ increased apoptosis in Gram-negative bacteria, including lactate-utilizing bacteria such as *M. elsdenii* and *S. ruminantium* (Chen et al., 2016; Hernández et al., 2014). Gram-positive bacteria such as *Streptococcus bovis*, a prominent lactate-producing bacteria, drives lactate accumulation in the rumen which consequently results in metabolic acidosis (Nocek, 1997; Russell & Hino, 1985). *Lactobacillus*, *Bacteroides* and *Bifidobacterium* are among the bacteria that mostly increased in our study. However, the rate at which each bacterium increased in time is highly variable and seems to reflect substrate preference and metabolic capacity to degrade starches or use up sugars. The predominant amylolytic, amylodextrin-, and maltose-utilizing bacteria in the ruminal ecosystem include *Bifidobacterium*, *Butyrivibrio*, *Eubacterium*, *Lactobacillus*, *Mitsuokella*, *Prevotella*, *Ruminobacter*, *Selenomonas*, *Streptococcus*, *Succinimonas* and *Succinivibrio* (Chesson & Forsberg, 1997; Kotarski et al., 1992; Stewart et al., 1997). In the ST group, *Ruminobacter* increased immediately after 8 h; 16 h later, *Bifidobacterium*, *Ruminobacter*, *Prevotella* 7 and *Lactobacillus* were the most differentially abundant microbes at the expense of *Fibrobacter*, *Eubacterium* and *Ruminococcus*. In our study, *Bacteroides* increased at 8 h in SU and 24 h in CMS, while only at 72 h in ST. *Bacteroides* is normally seen as a commensal microorganism in the gut and possesses a wide array of sugar utilization enzymes. It has been found to be associated with high caloric diets of animal protein, sugar, starch and fat in European children (De Filippo et al., 2010). This genus was predominant through the adaptation period in sheep feeding an energy dense diet, fermented glucose and produced lactate in a small amount and acetate as a main product (Mackie & Gilchrist, 1979). *Fibrobacter*, a well-known fibrolytic bacterium, was shown to decrease in ST only after 24 h, while decreasing in CMS and SU already after 8 h.

Correlations between datasets and functional profiles

Grain-rich diets and resulting acidotic conditions have been shown to increase the abundance of Bifidobacteria (Trovatelli & Matteuzzi, 1976), whose main pathway for carbohydrate fermentation is phosphoketolase, yielding acetate and potentially lactate instead of propionate and butyrate. In ST, *Bifidobacterium* was found to be negatively correlated with propionate and isobutyrate, which were found to decrease throughout time in all carbohydrate groups. This known amylolytic and maltose utilizing bacteria (Nagaraja & Titgemeyer, 2007) was also found to be negatively associated with several fibrolytic genera. This microorganism cannot use starch directly but is able to metabolize maltose and glucose which are outputs of starch degradation (Biavati & Mattarelli, 1991; Glor et al., 1988). Despite increasing in the three carbohydrate groups after 24 and 72 h, the highest coefficient was found in ST. This suggests that the chemical modification of CMS has much likely rendered the substrate inaccessible for degradation by *Bifidobacterium*, which were able to effectively proliferate when native cornstarch was available.

In both ST and SU, propionate was found to be highly correlated with biotin biosynthesis II (PWY-5005), which decreased after 24 and 72 h in all three carbohydrates.

The end product of this pathway is biotin, which is a crucial co-enzyme for acetyl-CoA carboxylase complex, propionyl-CoA carboxylase, and pyruvate carboxylase activities (Weiss, 2001). Propionyl-CoA carboxylase is responsible for converting (S)-methylmalonyl-CoA to propanoyl-CoA and afterwards propanoyl-CoA by succinate utilization can produce propionate (Streit & Entcheva, 2003). Bacteria also require biotin to produce propionate in the rumen (Weiss, 2001). A decrease in this pathway is consistent with an overall decrease in propionate in our study, which was particularly evident in ST and SU. Furthermore, this pathway is overall positively correlated with several SCFA, *Fibrobacter*, *Butyrivibrio*, *Lachnospiraceae* NK4A136 group, *Ruminococcaceae* UCG-013 and *Eubacterium oxidoreducens*, but negatively associated with *Lactobacillus*, *Bifidobacterium*, lactic acid and hexoses. *Bifidobacterium* and *Lactobacillus* lack a pathway of biotin production, but express a free biotin transporter, which suggests that these bacteria compete with the host by utilizing bacterial-derived biotin. As biotin is necessary for survival and growth of microbes, a drop in biotin production may lead to gut dysbiosis and has been shown to be associated with overgrowth of *Lactobacillus murinus* (Hayashi et al., 2017). A positive correlation between *Lactobacillus*, poly (glycerol phosphate) wall teichoic acid biosynthesis (TEICHOICACID-PWY) and lactate was discovered with DIABLO analysis in the SU group. Teichoic acids, which are covalently connected to the peptidoglycan of cytoplasmic membrane of Gram-positive bacteria, comprise a huge percentage of the Gram-positive cell wall and are highly important for cell wall integrity (Colagiorgi et al., 2015; Pooley & Karamata, 1994). An increase in the concentrations of potentially inflammatory substances, such as lipopolysaccharides (LPS) and lipoteichoic acids (LTA) is normally associated with microbial dysbiosis (Garcia et al., 2017). The pathway of LTA biosynthesis was shown to increase after 24 h in CMS and SU and after 72 h in ST, indicating again a higher potential of CMS and SU in inducing dysbiosis when compared with ST. Additionally, LTA is associated with various inflammatory pathologies and is known to trigger innate immune responses and develop adaptive immunity (Ginsburg, 2002). Despite not being as recognized as LPS when it comes to its probable immunogenic function, the role of LTA in inflammation in cattle connected to diet needs to be further elucidated. Pathways associated with benzoic acid degradation and TCA cycle were upregulated after 4 h in the SU group. An accentuated decline in benzoic acid was observed in SU and CMS at 4 and 8 h. Anaerobic microorganisms typically metabolize benzoate into TCA cycle intermediates through the anaerobic benzoyl-CoA degradation pathway (Valderrama et al., 2012). Benzoic acid was further found to be positively associated with several fibrolytic bacteria in our study, including *Fibrobacter*, *Lachnospiraceae* NK4A136 group, *Eubacterium oxidoreducens* group and *Ruminococcaceae* UCG-013, all found to decrease at some point during SU feeding.

Phytogenic supplementation with CAP or MEN

Phytogenic supplementation did not elicit major changes in the microenvironment, contrarily to our expectations. However, ANOSIM highlighted an effect of phytogenic in all distance matrices calculated, suggesting nevertheless an impact in the microbial community structure. Microbial and pathway abundances were also affected by phytogenic supplementation, but these changes did not result in significant modifications in the microenvironment. No effects on fermentation parameters or pH were found, which was unexpected. A positive influence of capsaicin on chewing activity during feeding of high-concentrate diets has been previously reported by our team (Ricci et al., 2021). As artificial saliva was kept at a constant flow rate in our study, it is very likely that the effect of capsaicin in salivary dynamics was neglected. Previous studies in beef cattle have shown a successful modulation of rumen fermentation by capsaicin, by decreasing acetate and increasing ammonia production (Fandiño et al., 2008; Rodríguez-Prado et al., 2012). In our study, however, only caproate was modified by phytogenic supplementation. In dairy cows, previous studies have found no modulatory effects of oleoresin supplementation, whose active compound is capsaicin (Giallongo et al., 2015; Tager & Krause, 2011). It was expected that the phenolic group of capsaicin may act by suppressing bacterial growth and decreasing membrane stability because of its hydrophobicity (Burt, 2004). In fact, *Ruminobacter* and *Selenomonas* (ST group) were significantly reduced by CAP. Further studies are necessary to understand the molecular mechanisms of phytogenic supplementation in specific rumen microbes.

Our study has shown that in order to improve animal production and health, by reducing dysbiosis and production related diseases, it is important to determine the impact of nutrients at the molecular level. Altering the chemical bonds in a single substrate within the diet can alter the microbial metabolism and the resulting development of dysbiosis due to system intolerances to SCFA and microbial toxin accumulation. Our study also revealed that different carbohydrate sources with different availability caused dysbiotic modifications at the metabolome and microbiome levels, which seems to be highly substrate solubility-dependent. Further research is needed to address the opportunities and challenges of using modified energy sources on the microbial metabolism and

metabolome. The advancements of technology require that nutritional research focus on the microbes (at deeper taxonomic levels) as the primary target for improved nutritional strategies.

ACKNOWLEDGEMENTS

We thank the staff of the dairy farm of Vetmeduni Vienna for their help. The assistance of Sabine Leiner, Suchitra Sharma, Anita Dockner, Marco Reiter, Markus Aigensberger and Patrick Rennhofer in sample analysis and support in the laboratory is very much appreciated. We are also thankful to Claudia Lang for her help in sampling. We acknowledge the supply of the substrates from AGRANA Research and Innovation Center (Tulln, Austria).

CONFLICT OF INTEREST

The authors have not stated any conflict of interest.

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How to cite this article: Kheirandish, P., Petri, R.M., Sener-Aydemir, A., Schwartz-Zimmermann, H.E., Berthiller, F. & Zebeli, Q. et al. (2022) Characterization of microbial intolerances and ruminal dysbiosis towards different dietary carbohydrate sources using an in vitro model. *Journal of Applied Microbiology*, 133, 458–476. Available from: https://doi.org/10.1111/jam.15573