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Exhaled volatile fatty acids, ruminal methane emission, and their diurnal patterns in lactating dairy cows

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ABSTRACT

To date, the commonly used methods to assess rumen fermentation are invasive. Exhaled breath contains hundreds of volatile organic compounds (VOC) that can reflect animal physiological processes. In the present study, for the first time, we aimed to use a noninvasive metabolomics approach based on high-resolution mass spectrometry to identify rumen fermentation parameters in dairy cows. Enteric methane (CH_4) production from 7 lactating cows was measured 8 times over 3 consecutive days using the GreenFeed system (C-Lock Technology Inc.). Simultaneously, exhalome samples were collected in Tedlar gas sampling bags and analyzed offline using a secondary electrospray ionization high-resolution mass spectrometry system. In total, 1,298 features were detected, among them targeted exhaled volatile fatty acids (eVFA; i.e., acetate, propionate, butyrate), which were putatively annotated using their exact mass-to-charge ratio. The intensity of eVFA, in particular acetate, increased immediately after feeding and followed a similar pattern to that observed for ruminal CH_4 production. The average total eVFA concentration was 35.5 count per second (CPS), and among the individual eVFA, acetate had the greatest concentration, averaging 21.3 CPS, followed by propionate at 11.5 CPS, and butyrate at 2.67 CPS. Further, exhaled acetate was on average the most abundant of the individual eVFA at around 59.3%, followed by 32.5 and 7.9% of the total eVFA for propionate and butyrate, respectively. This corresponds well with the previously reported proportions of these VFA in the rumen. The diurnal patterns of ruminal CH_4 emission and individual eVFA were characterized using a linear mixed model with cosine function fit. The model characterized similar diurnal patterns for eVFA and ruminal CH_4 and H_2 production. Regarding the diurnal patterns of eVFA, the phase (time of peak) of butyrate occurred first, followed by that of acetate and propionate. Importantly, the phase of total eVFA occurred around 1 h before that of ruminal CH_4. This corresponds well with existing data on the relationship between rumen VFA production and CH_4 formation. Results from the present study revealed a great potential to assess the rumen fermentation of dairy cows using exhaled metabolites as a noninvasive proxy for rumen VFA. Further validation, with comparisons to rumen fluid, and establishment of the proposed method are required.

Key words: bovine exhalome, exhaled volatile fatty acids, noninvasive, secondary electrospray high-resolution mass spectrometry

INTRODUCTION

In ruminant nutrition research, the measurement of rumen fermentation parameters is crucial for characterizing and understanding nutrient digestion and utilization, the associated enteric methane (CH_4) emission, and rumen microbiome dynamics (Boadi et al., 2004; Hammond et al., 2016). The conventional approaches for assessing rumen fermentation profile are typically invasive. For example, collecting rumen contents from rumen-cannulated animals through the fistula, which requires surgical intervention, is regarded as the standard method for obtaining representative rumen digesta samples (Larsen et al., 2020). However, access to surgically modified fistulated animals is not universally adaptable and is restricted to a limited number of research facilities (Muizelaar et al., 2020). Given the increasing attention to improved animal welfare, more regulations are being implemented for use of cannulated animals worldwide. Sampling through stomach tubing (using ororuminal devices) or rumination bolus are considered less-invasive approaches but can result in saliva contamination and yield less-representative samples compared with samples obtained through a rumen cannula (Duffield et al., 2004; Geishauser et al.,

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2012). Contamination and unrepresentative samples inevitably affect the measurement of absolute concentrations of rumen fermentation metabolites and the rumen microbiome (de Assis Lage et al., 2020; Indugu et al., 2021).

The exhalome (total volatile metabolites contained in exhaled breath) of animals originates mainly from the lungs and airways, and it contains a complex mixture of inorganic gases, traces of thousands of volatile organic compounds (**VOC**), and microscopic aerosol particles (Phillips et al., 2013; Broza et al., 2014; Giannoukos et al., 2018). This breath signature reflects animal physiological changes and can serve as a tool for investigating functional biological processes. In human nutrition and clinical studies, breath analysis by secondary electrospray ionization–high resolution mass spectrometry (**SESI-HRMS**) is an emerging field of next-generation research that allows the detection of an extensive range of mass-to-charge (m/z) features in an entirely untargeted approach (Singh et al., 2019). The SESI-HRMS is a well-established and robust analytical technology specifically developed for in-depth breath metabolomics (exhalomics) characterization, and its applicability has already been demonstrated and previously published in human clinical and nutritional studies (Bregy et al., 2015; Tejero Rioseras et al., 2018; Wüthrich et al., 2022). In addition, previous clinical studies have confirmed that this method can assess biomarkers for the diagnosis of respiratory diseases, detection and monitoring of intermediate compounds, and metabolic pathways (Martinez-Lozano Sinues et al., 2014a,b; García-Gómez et al., 2016; Gaugg et al., 2019).

Ruminants are unique among livestock animals due to the microbial ecosystem in the rumen. Volatile compounds in a ruminant exhalome, originating collectively from the lungs and airways, could contain VOC in the gaseous phase, such as ruminal CH_4 and exhaled volatile fatty acids (**eVFA**). For rumen ecosystems to function adequately, gaseous pressure needs to be maintained by releasing the gases produced during the fermentation processes via eructation. The vast majority of enteric CH_4 is emitted via the mouth and nostrils (Muñoz et al., 2012), along with other volatile compounds (Oertel et al., 2018). These VOC are produced in metabolic and biochemical processes during rumen fermentation and can be detected in different biological samples such as the exhalome (Thorn and Greenman, 2012; de Lacy Costello et al., 2014). Dobbelaar et al. (1996) investigated the detection of ketosis through the exhalome in dairy cows using gas chromatography combined with mass spectrometry. Recently, a study on ruminant animals (cattle, sheep, and goats) reported the effects of eructation events on exhaled VOC profiles through continuous real-time exhalome sampling using proton transfer reaction mass spectrometry (Oertel et al., 2018). Over the years, mass spectrometry technology has been developed to the next level (e.g., high-resolution MS, ambient ionization). For example, SESI-HRMS is able to capture a much wider range of features in exhalomics analysis, but it has not been previously used in ruminant research.

The dynamic nature of rumen fermentation and consistent eructation of fermentation gases and VOC throughout the day allows for exhalomic analysis in ruminant research as a noninvasive approach for studying the fermentation profile and daily pattern of rumen function in dairy cows. Therefore, the objectives of this study were to investigate eVFA using SESI-HRMS and their relationship with ruminal CH_4 and H_2 emissions, and to characterize their diurnal patterns. We hypothesized that the ruminal fermentation metabolites, such as VFA, can be measured through exhalome analysis using the high-resolution mass spectrometric approach, and the eVFA concentration patterns over the course of a day will be associated with the patterns of ruminal CH_4 and H_2 production.

MATERIALS AND METHODS

Animals, Experimental Design, and Diet

All experimental procedures involving animals were approved by the Cantonal Veterinary Office of Zürich (ZH207/2021). The experiment was conducted at the AgroVet-Strickhof Dairy Farm (Lindau, Switzerland). Seven multiparous lactating cows, of which 4 were Brown Swiss and 3 were Holstein, averaging (mean ± SD) 185 ± 52.0 DIM, 32.5 ± 5.21 MY, and 726 ± 48.0 BW, were enrolled in the current experiment. The experimental duration was 26 d, including 21 d of dietary adaptation and 5 d of sample collection. The current experiment was part of a larger experiment, but only ruminal gas emissions and eVFA data are presented here.

During the first 21 d of the experimental period, the cows were housed in a freestall barn with wheat straw bedding and equipped with a feeding trough (Waagen Döhrn GmbH, Germany) and Calan-gate system (American Calan Inc., Northwood, NH) for individual feeding. The diet was fed ad libitum at 110% of the previous day's intake, and the TMR was mixed before each morning feeding. Cows were milked twice daily, at 0530 and 1530 h. Cows were fed once daily at around 0830 h and received the same diet, which contained 36.1% NDF and 16.2% CP (Table 1). On d 22, the cows were moved to a tiestall barn with free access to drinking water. Two days were allowed for adaptation

Table 1. Ingredients and chemical composition of the experimental diet

Item	Value
Ingredient composition (% of DM)	
Corn silage[1]	42.8
Alfalfa hay[2]	18.8
Grass silage[3]	12.8
Energy concentrate[4]	15.0
Protein concentrate[5]	8.6
Ground corn	1.9
Salt	0.2
Chemical composition (% of DM)	
CP	16.2
Crude fat	2.79
NDF	36.1
ADF	23.4
Starch	18.6

[1]Corn silage was 39.3% DM and contained 7.06% CP, 40.8% NDF, and 35.42% starch on DM basis.

[2]Alfalfa hay (dried, 16.6% CP on DM basis), commercial product (RUMILU, Desialis, Paris, France).

[3]Grass silage was 31.3% DM and contained 13.1% CP, 51.02% NDF, and 3.3% starch on DM basis.

[4]Concentrate feed UFA 173 (UFA AG, Herzogenbuchsee, Bern, Switzerland).

[5]Concentrate feed UFA PRIMA 249 (UFA AG, Herzogenbuchsee, Bern, Switzerland).

to the tiestall barn before sampling, and samples were collected during the following 3 d.

Gas Emission Measurements and Breath Sampling

Ruminal gaseous emissions, including CH_4 and hydrogen (H_2) concentrations, were measured using the GreenFeed (**GF**) system (C-Lock Technology Inc., Rapid City, SD). A total of 8 time points over 3 consecutive days were measured to represent every 3 h of a 24-h period at 0900, 1500, 2100 h (sampling d 1); 0300, 1200, 1800 h (sampling d 2); and 0000 and 0600 h (sampling d 3), according to the procedures described by Hristov et al. (2015). Each gas emission measurement took 5 min on average. During each sampling event, the cow received a maximum of 8 drops of bait feed (pelleted feed contained, on DM basis, 55% alfalfa hay, 25% ground corn, 10% soybean meal, and 5% molasses) in an interval of 40 s each, which resulted in a total of approximately 300 g of feed per sampling event per cow. Between each sampling event, the GF system was allowed to collect background air for 2 min and then moved in front of the cow for sampling. In parallel to the gas emission sampling, exhalome samples were collected from the adjacent facilitated gas sampling unit of the GF system (Figure 1A). Gas sampling bags (1-L Tedlar gas sampling bags with Thermogreen LB-2 septa, Merck, St. Louis, MO) were connected to the gas sampling line on the GF system (Teflon 6.35-mm polytetrafluoroethylene tubing) with a rubber grip on the opening end. A default sample flow rate of 1 L/min operated by a rotameter (10A6130 Glass Tube Purge and Low Flow Meters, Dwyer Instruments Inc.) and a 6.35-mm ball Swagelok valve (B-43F4RT; Swagelok, Solon, OH) were used to collect the exhalome samples. The sampling bag was connected to the GF for 1 min, and the cow was monitored closely to ensure that her head was inside the head chamber of the GF system for the total duration of the sampling event. If the cow removed her head during the 1-min duration, the sample was discarded, a new bag was connected, and a fresh sample was collected, to ensure that an eructation event was captured in the sampling bag. We observed the exhalome sampling event on a real-time plot of CH_4 emission using the mobile application Control Feed (C-Lock Technology Inc., Rapid City, SD), which is accessible to observe the eructation events during gaseous measurements (Figure 1B shows an exhalome collection). During exhalome sampling, the initial baseline might be noisy (no sharp peak) with y-axis values below 100 ppm attributed to background and lung emissions. The first true eructation peak was considered at >600 ppm, generated from the gas sensor voltage signal, and was used as a reference point. Thirty seconds after the first true eructation peak, the gas-releasing valve on the GF system was opened for 60 s to collect the exhalome sample.

Analysis of Exhalome Metabolites

Exhalome samples were analyzed using a commercial SESI source (Fossil Ion Tech, C. de los Cipreses, Madrid, Spain) coupled with a Q-Exactive Plus Orbitrap (Thermo Fisher Scientific, Freiburg, Germany) mass spectrometer (Figure 1C). During offline exhalomics measurements and analysis conducted in this study using SESI-MS, a custom-made de-sampling system allowed collected breath molecules in the gas sampling bags to pass through a short-length heated sample transfer line into the ionization chamber. Individual sampling bags were placed into the de-sampling box, and their inlets were connected to the outlet of that box, controlled by a 6.35-mm Swagelok perfluoroalkoxy alkanes plug valve. Upon placing the sampling bag into the de-sampling box, the box was sealed, positive pressure was applied within it, and the sampling bag was then pneumatically pressurized using compressed high-purity nitrogen, which allowed a stable de-sampling process and a constant and stable introduction of the sample into the SESI sources (Figure 1D).

The sampling line of the SESI source was constantly heated at 130°C, and the ionization chamber was kept at 90°C. The sample transfer line and the ionization

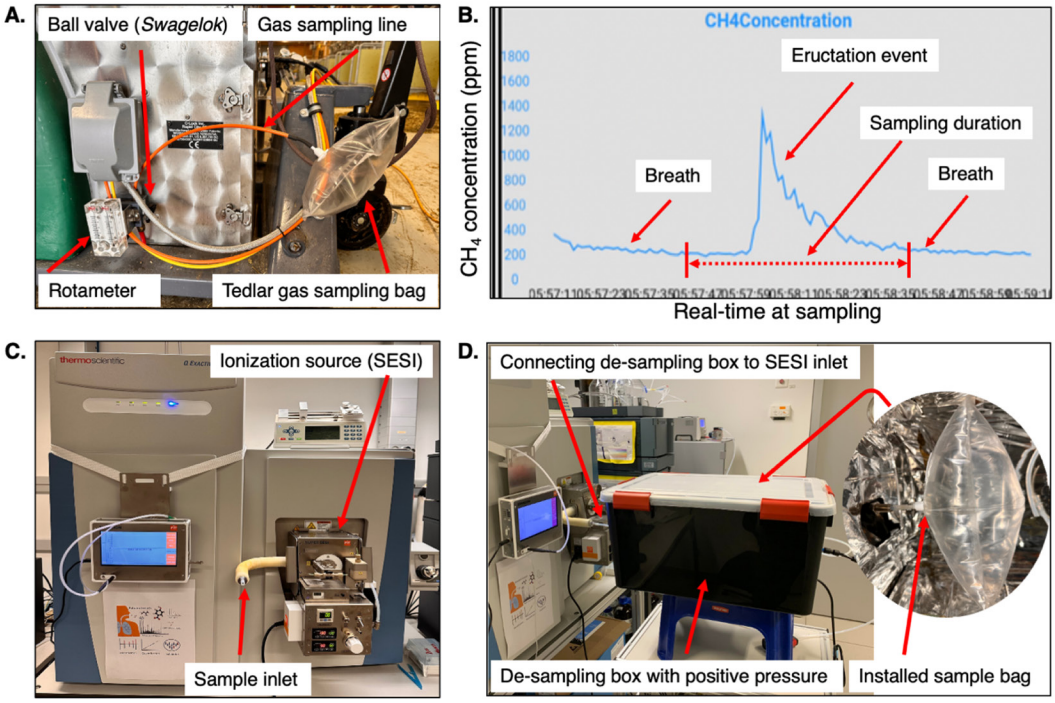

Figure 1. (A) Exhalome sampling of dairy cows using gas sampling unit of the GreenFeed system (C-Lock Technology Inc.). (B) Real-time ruminal CH_4 emission measurements (x-axis: real time of measurement; y-axis: CH_4 concentration, ppm) on the "Control Feed" mobile application (version 1.51, C-Lock Technology Inc.), showing the eructation event with an exhalome sampling duration of 1 min. (C) High-resolution mass spectrometry (HRMS) coupled with secondary electrospray ionization (SESI) source. (D) Breath sample bag installation at the de-sampling box was customized to create positive pressure.

chamber were continuously heated to prevent water condensation and absorption of low-volatility metabolites on the walls. In the SESI chamber, the electrospray solution was a 0.1% aqueous formic acid solution passing through a nanoelectrospray capillary (inner diameter of 20 μm and outer diameter of 365 μm, Fossil Ion Tech, C. de los Cipreses, Madrid, Spain) with an overpressure of 0.8 bar. In the ionization chamber, the charged electrospray droplets interact with the gaseous sample for charge transfer before introduction into the mass spectrometer for analysis. Sheath and auxiliary gas values were set to 15 psi and 2 arbitrary units, respectively. The electrospray solution voltage was ± 3.5 kV, and the MS inlet capillary was heated to 250°C. The automatic gain control target was adjusted to 3×10^6, and the maximum injection time was set to 500 ms. Scans were recorded with an m/z range of 50 to 500 and a mass resolution of 140,000. Measurements were carried out in both the positive and the negative ion modes, which allowed the determination of protonated and deprotonated molecules. During measurements, a nano-Ampere meter was used to monitor the stability of the electrospray. The mass spectrometer was calibrated and tuned on a weekly basis using commercially available standards (Pierce LTQ Velos ESI Positive Ion Calibration Solution and Pierce Negative Ion Calibration Solution, Thermo Fisher Scientific, Freiburg, Germany).

Data Pre-Processing

Data pre-processing was conducted using MATLAB (R2020b, Mathworks Inc., Natick, MA). For the mass spectral data to be readable by MATLAB, the raw files acquired by the MS were converted into the mzXML file format using the open-source tool MSConvert (ProteoWizard, version 3), according to Chambers et al. (2012). A peak list was generated by shape-preserving piecewise cubic interpolation and summation of all spectra. Afterward, the continuous mass spectra were centroided by summing the intensities around each peak within the full width at half-maximum. Spectra were then resampled to 300,000 data points by shape-preserving piecewise cubic interpolation in the peak range indices m/z 50 to 500, where only the m/z ranges corresponding to true peaks were extracted, which resulted in a reduced size of the m/z range. All mass spectra were assembled into a single 2-dimensional matrix (i.e., m/z vs. scan number) corresponding to a peak's time traces. Finally, the features above the

Table 2. Theoretical and measured m/z of the exhaled VFA detected in the negative ion mode ($[M\text{-}H]^-$) with a resolution of 140,000

Compound	Theoretical m/z $[M\text{-}H]^-$	Measured m/z $[M\text{-}H]^-$	Mass error (ppm)
Acetate	59.0139	59.0139	<0.1
Propionate	73.0295	73.0294	−1.4
Butyrate	87.0452	87.0452	<0.1

background were captured. As a result, only signals that increased during the exhalations were selected for further analysis. After a baseline correction in the time dimension for all peaks, a filter (height filter = 500, and segmentation 0.00025) was applied to extract all features that increased during the exhalations, which resulted in a peak list of 1,298 features recorded in both positive (649 features) and negative (649 features) ion mode. Finally, data obtained for each measurement were normalized to the maximum, and the obtained data set was used for downstream statistical analysis.

Annotation of Metabolites

The SESI-HRMS platform allows positive and negative electrospray ionization, providing 2 distinct runs per sample. For the present study, we collected data in both ionization modes, but for the analysis of the eVFA, we focused on the negative ion mode (Lee and Zhu, 2020). Negative-mode $[M\text{-}H]^-$ features were found to be more sensitive than positive-mode signals, as a greater number of targeted negative ions for this phase have been annotated using their measured m/z. When comparing positive and negative ionization efficiency in the electrospray ionization process, in principle, the negative ion mode provides more sensitivity (Liigand et al., 2017). The exact m/z ratios were used to putatively annotate the eVFA; that is, acetate, propionate, and butyrate. The measured m/z ratios of the targeted eVFA and their differences from the theoretical masses and the calculated mass error are presented in Table 2. Raw mass spectra from the analytical platform were processed to extract the m/z features matrix that represents the detection of the ions from the mass spectrometer. Each feature was indicated by a unique combination of its m/z and its intensity. The features' signal intensity indicates the relative concentration of a given feature as count per second (**CPS**).

Statistical Analysis

Downstream statistical analyses were performed for ruminal CH_4 and H_2 emissions and eVFA data using a linear mixed model with repeated measures using the *lme4* (Bates et al., 2015) procedure of R statistical language (R Core Team, 2022, version 4.2.2). The model was as follows:

$$Y_{ij} = \mu + C_i + T_j + e_{ij},$$

where Y_{ij} is the variable of interest, μ is the overall mean, C_i is the random effect of cow ($i = 1$ to 7), T_j is the fixed effect of time ($j = 1$ to 8), and e_{ij} is the residual error. The covariance structures (unstructured [UN], autoregressive order 1 AR[1], and compound symmetry [CS]) were selected based on model fit according to Akaike's information criterion, time was the repeated variable, cow was the subject, and denominator degrees of freedom were adjusted by the Kenward-Roger method. Data are presented as least squares means. In addition, to characterize the daily patterns of gaseous emissions and eVFA, cosine function of time with a 24-h period and 12-h harmonic was fitted in a linear mixed model framework, as described by Niu et al. (2014) using the *lmer* procedure of R. The model was as follows:

$$Y_{ij} = M + C_i + A\cos\left(\frac{2\pi T_j}{24}\right) + B\sin\left(\frac{2\pi T_j}{24}\right) + C\cos\left(\frac{2\pi T_j}{12}\right) + D\sin\left(\frac{2\pi T_j}{12}\right) + e_{ij},$$

where Y_{ij} is the variable of interest, M is the midline estimating statistic of rhythm (**mesor**; indicates daily mean), C_i is the random effect of cows ($i = 1$ to 7), T_j is the time of day ($j = 1$ to 8), and e_{ij} is the residual error. Coefficients of the linear form of the cosine function within a 24-h period are indicated as A and B, and the 12-h harmonics are indicated as C and D. The cosine function model represents a daily rhythm that can differ in rhythm-adjusted daily mean (mesor), amplitude (distance of peak from the mesor), and phase (time of day at peak). Determining the model parameters describes the rhythms within a 24-h period. The amplitude, mesor, and phase were calculated from fitted values based on their definition. Amplitude was calculated as [max(fitted value) − min(fitted value)]/2, mesor (daily mean) was calculated as [max(fitted value) + min(fitted value)]/2, and the phase was determined

using *which.max* function in R and reported as phase, which is the time at which the peak of a rhythm occurs. The cosine function was fitted using ruminal CH_4 and H_2 emissions, and the eVFA profile data that was measured in 3-h intervals. A zero-amplitude *F*-test was conducted to compare the cosine fit to the linear fit, which determines the significance of the cosine fit for each variable. Statistical differences were considered significant at $P \leq 0.05$.

RESULTS AND DISCUSSION

Exhalomics (i.e., exhaled breath metabolomics) is an emerging technology in the medical research area due to its noninvasive nature and its potential capability for identifying important biomarkers. A recent survey on the human exhalome reported 1,488 trace VOC in exhaled breath, reflecting metabolic functions in the body (Drabińska et al., 2021). The application of the exhalomics approach in livestock research is scarce. To our knowledge, this study is the first attempt to use the SESI-HRMS analytical platform to characterize the VOC profile in the exhalome of dairy cows. In this work, we detected 1,298 VOC using SESI-HRMS in both negative and positive ion modes. This is a notably greater number compared with a previous study on ruminants that reported the effect of eructation events on exhaled VOC profiles using the proton transfer reaction time-of-flight MS technique (Oertel et al., 2018). They measured 36 selected VOC in exhaled breath of cattle, sheep, and goats. Of the 1,298 features detected in the current study, we focused on 3 selected features that were putatively identified based on their exact m/z values (Table 2) and annotated as acetate, propionate, and butyrate. These 3 VFA are considered the key indicators for rumen fermentation and are also linked to ruminal gas production (Moss et al., 2000). Therefore, the current experiment used ruminal CH_4 and H_2 production and their diurnal patterns to demonstrate the potential of eVFA and, further, exhalomics, as proxies to assess rumen fermentation. It should be noted that VOC, including VFA, exist in both aqueous and gaseous phases, and their concentrations are determined in part by individual Henry's law constants. The concentrations in the gaseous phase may not directly reflect the concentrations in the aqueous phase. However, based on the results from the current study described here, the molar proportions of eVFA were similar to those of ruminal VFA reported in the literature. This study provides initial data and proof-of-concept and further validation with direct comparison with ruminal VFA is needed to establish this method in ruminant nutrition research.

Ruminal Gaseous Emissions and Exhaled VFA

The 3-h interval data on ruminal CH_4 and H_2 emissions and the eVFA are presented in Figure 2. (The mean values of the 3-h interval data are presented in Supplemental Table S1; https://doi.org/10.3929/ethz-b-000621565). Figure 2A shows that the ruminal CH_4 emission increased immediately after feeding (at around 0830 h). The emission of CH_4 remained elevated until 1800 h, and an overall lower emission was observed during the overnight period (between 2100 h and 0800 h). For H_2 emission we observed a slight increase between 0600 and 1800 h, where the greatest emission was observed at 1800 h. The lowest H_2 emission was observed at around 2100 h, and the overall lowest emission overnight at 2100 and 0300 h. The eVFA concentrations (signal intensity as CPS) also changed over the course of the day in relation to feeding time; they started to increase and reached the highest concentration after fresh feed delivery (Figure 2B). The daily average (mean ± SD) concentration of total eVFA was 35.5 ± 18.32 CPS, and the greatest mean concentration of the individual eVFA was for acetate (21.3 ± 13.94 CPS), followed by exhaled propionate (11.5 ± 3.10 CPS), and lowest for exhaled butyrate (2.67 ± 2.459 CPS).

Ruminal CH_4 production from ruminant animals is intermittent (Hegarty, 2013) and fluctuates according to feeding time and frequency, which establishes a strong positive correlation between CH_4 emission and DMI (Crompton et al., 2011; Brask et al., 2015). Indeed, recent meta-analyses have established DMI as the main driver of ruminal CH_4 production (Hristov et al., 2018; Niu and Harvatine, 2018a,b). In addition, it has been shown that a large proportion of daily feed consumption occurs within 2 h after feeding (Niu et al., 2014, 2017, 2018) with subsequent production of rumen fermentation metabolites, which can be captured in the exhalome as VOC together with CH_4 and H_2. Marked differences in individual intra-day variability of gaseous emissions and eVFA concentrations occurred in the current experiment, all following a similar pattern, with a sharp increase directly after feeding. We observed the greatest eVFA concentrations and ruminal CH_4 production during the day (i.e., greater fermentation rate) and the lowest eVFA concentrations and CH_4 emissions overnight. Similarly, Hristov and Melgar (2020) reported a greater CH_4 emission from 2 h after feeding and lower emissions during the overnight period.

Concurrent with the decrease in ruminal CH_4 production and eVFA concentrations, H_2 concentration increased slightly between 6 and 9 h after feeding, with a subsequent sharp reduction in H_2 emissions after 1800 h, whereas CH_4 and eVFA remained stable. The fluc-

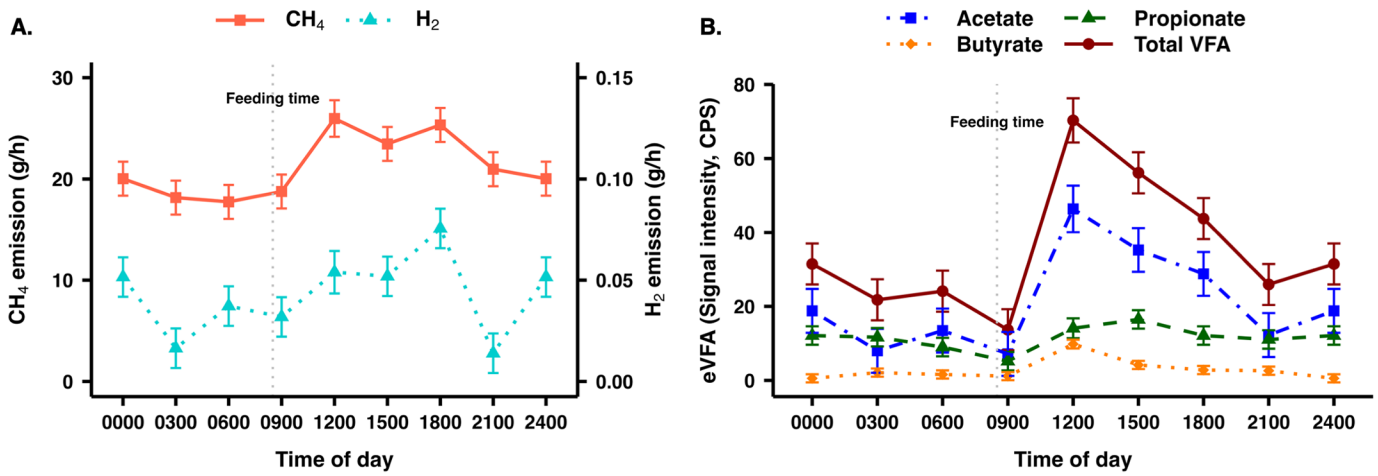

Figure 2. (A) Ruminal CH_4 and H_2 emissions (g/h) measured every 3 h with the GreenFeed system (C-Lock Technology Inc.). (B) Exhaled volatile fatty acids (eVFA) in dairy cows measured every 3 h using secondary electrospray ionization high-resolution mass spectrometry. Signal intensity (CPS = count per second) indicates the relative concentrations of eVFA in the exhalome samples. Data are presented as LSM ± SE (n = 7). Feeding time was at 0830 h.

tuations in H_2 emission are reflective of its production in several pathways of glucose fermentation (Pereira et al., 2022) as well as the role of H_2 as the main determinant of the rate of CH_4 production and VFA formation (Janssen, 2010), being the primary energy substrate, together with CO_2, for ruminal CH_4 formation (Wolin, 1981). The sharp reduction in H_2 emission observed in the current study indicates a redirection of H_2 from methanogenesis to alternative H_2 sinks (Janssen, 2010; Pereira et al., 2022). Overall, H_2 emission fluctuations over the course of the day correspond well with the fluctuations in eVFA concentrations and follow the well-established dynamics of H_2 in relation to rumen fermentation and CH_4 production (Janssen, 2010).

Depending on the basal diet, metabolic status of animals, as well as timing and frequency of rumen sampling, the molar proportions of rumen VFA are variable and can range from 51 to 75%, 15 to 31%, and 10 to 24% for acetate, propionate, and butyrate, respectively (Dijkstra, 1994; Firkins et al., 2006; Räisänen et al., 2021). The proportions of eVFA in the current experiment were within the above-mentioned ranges, which indicates that the VFA are present in the exhalome in a similar proportion as in the rumen fluid. The changes in eVFA concentrations aligned well with the emission of CH_4 from the rumen, further indicating the potential of exhaled breath measurements to be used for the assessment of rumen function. These findings are further supported by Børsting et al. (2020) and Zhang et al. (2022), who reported that rumen acetate, propionate, total VFA, and the ratio of acetate to propionate follow a consistent daily pattern, in which greater concentrations occur during the day than at night. Theoretically, VFA in the ruminal fluid have been related to ruminal CH_4 production using stoichiometric equations in which the conversion of pyruvate to acetate in the rumen results in the formation of H_2, whereas the conversion of pyruvate to propionate involves the utilization of H_2 (Moss et al., 2000; Holtshausen et al., 2009; Cabezas-Garcia et al., 2017). Indeed, recently Williams et al. (2019) reported with data from 215 cows across 24 diets that CH_4 yield was positively correlated with ruminal acetate and butyrate concentrations but had a negative correlation with propionate concentration. Overall, the characterization of eVFA concentrations, and their relationships with ruminal CH_4 and H_2 emissions reported in our study were in accordance with previous studies.

Diurnal Pattern of CH_4 and eVFA

To characterize the daily patterns of ruminal CH_4 and H_2 emissions, and eVFA, we fitted the cosine function in a linear mixed model. The 24-h diurnal patterns of the key variables for rumen fermentation are presented in Table 3 and Figure 3. The fitted cosine function model demonstrated a daily pattern ($P < 0.05$) for ruminal CH_4 and H_2 emissions and eVFA concentrations over a 24-h period. Comparisons of the diurnal parameters between different variables are on a numerical basis. The mesors for the eVFA were similar to the daily averages computed from 8 measurements throughout the day, as reported above. Based on the cosine function model, the phases of total eVFA and exhaled acetate, propionate, and butyrate were at 1354 h, 1348 h, 1448 h, and 1312 h, respectively, whereas the peak of CH_4 occurred at 1436 h and of H_2 at 1600 h (Table 3).

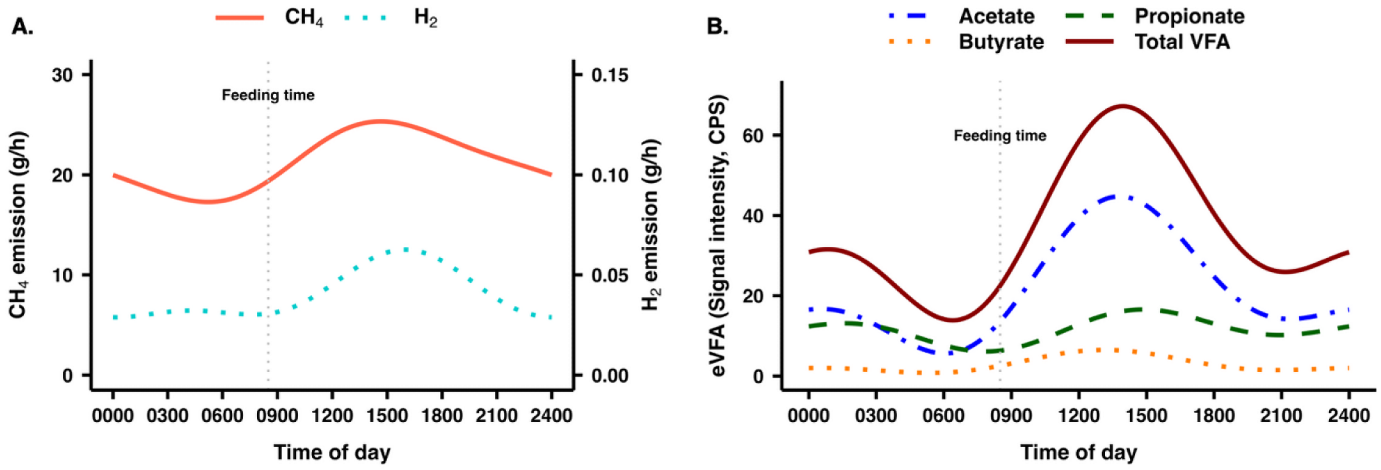

Figure 3. (A) Fitted 24-h diurnal patterns of ruminal CH_4 and H_2 emissions. (B) Exhaled volatile fatty acid (eVFA) concentrations based on the cosine function model (n = 7; CPS = count per second). Feeding time was at 0830 h.

The demonstrated daily patterns of ruminal CH_4 and H_2 emissions and eVFA in our study agree with previous experiments. Brask et al. (2015) measured rumen fermentation parameters every 2 h over a 24-h period. They reported a diurnal pattern of CH_4 and total VFA, which was closely related to feeding time (feeding at 0500 and 1700 h), both peaking around 2 h after feeding. In addition, Söllinger et al. (2018) and Shaani et al. (2018) reported the greatest total VFA concentrations at 3 and 4 h after feeding (feeding time at 0700 and 1000 h), when rumen samples were collected 1, 3, and 5 or 1, 4, and 7 h after feeding, respectively. In the current experiment, the greatest ruminal CH_4 emission and eVFA peaked around 3.5 h after feeding, based on the measured concentrations with 3-h sampling intervals (discussed above). Overall, the discrepancies in the timing of the most abundant rumen fermentation parameters between experiments stem from the variable feeding time, feeding frequency, and sampling intervals. This highlights the usefulness of parameterizing the daily patterns of rumen fermentation to better describe their fluctuations relative to feeding and dietary factors. Indeed, based on the fitted cosine function model, the phase of ruminal CH_4 and H_2 emissions and eVFA occurred around 4 to 6 h after feeding, which lagged approximately 2 h behind the reported peak time based on our sampling schedule. Such a modeling approach allows for a more robust comparison of rumen function and related daily patterns within and across experiments regardless of timing and frequency of sample collection. The sharp increase in fermentation products directly after feeding will shift the apparent peak of the fermentation pattern earlier than a fitted curve, which in turn, smoothens the extremes over the 24-h period, and thereby shifts the peak to a later time point.

In general, the increased concentration of fermentation products (i.e., VFA and CH_4) results from the rapid increase in carbohydrate fermentation and is related to

Table 3. The fit of a cosine function with a 24-h period on key variables of rumen fermentation in lactating dairy cows (n = 7)

Item	Mesor,[1] CPS[2]	Amplitude,[3] CPS	Phase[4] (time of day, h)	*P*-value[5]
Exhaled VFA				
Acetate	25.2	19.6	1348	<0.01
Propionate	11.3	5.22	1448	0.02
Butyrate	3.65	2.85	1312	<0.01
Total	40.5	26.7	1354	<0.01
Methane production, g/h	21.3	4.02	1436	<0.01
Hydrogen production, g/h	0.046	0.017	1600	<0.05

[1]Mesor (midline estimating statistic of rhythm) is the daily mean of the fitted curve.

[2]CPS = count per second.

[3]Amplitude is distance from mesor to peak or trough of cosine function.

[4]Phase is the time of the peak of cosine function.

[5]Indicates the significance ($P < 0.05$) of the cosine function model for diurnal pattern.

the conditioned feed intake directly after feeding (Brask et al., 2015; Söllinger et al., 2018). This in turn has been linked to the abundance of rumen microbes, which increases relative to feeding time (Shaani et al., 2018). Further, Söllinger et al. (2018) reported a close link between the transcript abundances of VFA production pathway enzymes and VFA concentration patterns, each being reflected in the increased activity of methanogens and subsequent ruminal CH_4 production. Both the activity of methanogens and related rumen VFA and CH_4 production expressed a daily pattern relative to feeding. In the current experiment, eVFA concentrations reached their lowest points in the early morning hours before feed delivery. Previous experiments have also reported a high concentration of plasma nonesterified fatty acid (an indicator of energy balance), suggesting increased lipid mobilization to meet energy demands during the overnight low-intake period (Niu et al., 2014, 2017). Characterizing and understanding the daily patterns of feed intake and rumen fermentation will allow for dietary and management interventions to optimize the rumen function to provide adequate energy and nutrient supply to animals. This will have potentially positive effects on nutrient utilization, feed efficiency, and metabolic health of lactating dairy cows.

CONCLUSIONS

In the present study, we identified and estimated the concentrations of exhaled VFA eructed through the breath of dairy cows. The daily patterns of eVFA matched well with ruminal CH_4 production, indicating that eVFA could be used as a proxy for rumen VFA. Further, the relative profiles of eVFA were reflective of their expected concentrations, being the greatest for acetate, followed by propionate, and lowest for butyrate. Overall, the data from the current study revealed a great potential of exhalomics using high-resolution mass spectrometry to assess rumen function in dairy cows. Further research is needed to validate and establish the method as a noninvasive tool for evaluating rumen fermentation parameters.

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