Exhalomics as a non-invasive method for assessing rumen fermentation in dairy cows: Can exhaled breath metabolomics replace rumen sampling?

M. Z. Islam,† S. E. Räisänen,† A. Schudel, K. Wang, T. He, C. Kunz, Y. Li, X. Ma, A. M. Serviento, Z. Zeng, F. Wahl, R. Zenobi, S. Giannoukos,* and M. Niu*

1Department of Environmental Systems Science, Institute of Agricultural Sciences, ETH Zürich, 8092 Zürich, Switzerland
2Food Microbial Systems Research Division, Agroscope, 3003 Bern, Switzerland
3Department of Chemistry and Applied Biosciences, Analytical Chemistry, ETH Zürich, 8093, Zürich, Switzerland

ABSTRACT

Previously, we utilized secondary electrospray ionization-mass spectrometry (SESI-MS) to investigate the diurnal patterns and signal intensities of exhaled volatile fatty acids (EX-VFA) of dairy cows. The current study aimed to validate the potential of exhalomics approach for evaluating rumen fermentation. The experiment was conducted in a switchback design, with 3 periods of 9 d each, including 7 d for adaptation and 2 d for sampling. Four rumen-cannulated original Swiss Brown (Braunvieh) cows were randomly assigned to 1 of 2 diet sequences (ABA or BAB): (A) low-starch (LS; 6.31% starch of DM) and (B) high-starch (HS; 16.2% starch of DM). Feeding was 1x/d at 0800 h. Exhalome (with the GreenFeed System), and rumen samples were collected 8 times to represent every 3-h of a day, and EX-VFA and ruminal VFA (RM-VFA) were analyzed using SESI-MS and HPLC, respectively. Further, the VFA concentration in the gas phase (HR-VFA) was predicted based on RM-VFA and Henry’s Law constants. No interactions were identified between the types of diets (HS vs. LS) and the measurement methods on daily average VFA profiles [ruminal (RM) vs. exhaled (EX) or Henry’s Law (HR) vs. exhaled (EX)], suggesting a consistent performance among the methods. Additionally, when the 3-h interval VFA data from HS and LS diets were analyzed separately, no interactions were observed between methods and time-of-day, indicating that the relative daily pattern of VFA molar proportions was similar regardless of the VFA measurement method used. The results revealed that the levels of acetate sharply increased immediately after feeding, trailed by an increase in the A:P ratio and a steady increase for propionate (2 h after feeding in HS, 4 h for LS), and butyrate. This change was more pronounced for the HS than the LS diet. However, there was no overall diet effect on the VFA molar proportions, while the measurement methods affected the molar proportions. Furthermore, we observed a strong positive correlation between the levels of RM and EX acetate for both diets (HS: $r = 0.84$; LS: $r = 0.85$), RM and EX propionate ($r = 0.74$), and RM and EX A:P ratio ($r = 0.80$). Both EX-VFA and RM-VFA exhibited similar responses to feeding and dietary treatments, suggesting that EX-VFA could serve as a useful proxy for characterizing RM-VFA molar proportions to evaluate rumen fermentation. Similar relationships were observed between RM-VFA and HR-VFA. In conclusion, this study underscores the potential of exhalomics as a reliable approach for assessing rumen fermentation. Moving forward, research should further explore the depth of exhalomics in ruminant studies, to provide a comprehensive insight into rumen fermentation metabolites, especially across diverse dietary conditions. Key words: bovine exhalome, exhaled VFA, ruminal VFA, SESI-MS

INTRODUCTION

The rumen is a complex ecosystem, and its dynamic microbial community is responsible for the fermentation of feeds, producing VFA that serve as key energy sources for ruminant animals. Gaining a thorough understanding of rumen function is essential to improve nutrient utilization efficiency and promote sustainable practices in ruminant agriculture (McCann et al., 2014). All current rumen sampling techniques, such as stomach-tubing, rumen cannula, and ruminocentesis (abdominal wall puncture with a needle), have been associated with potential discomfort to animals (Cordeiro et al., 2022). In addition, other drawbacks of these methods include sample contamination by saliva in stomach tubing and its inability to capture the entire rumen ecosystem, and the restricted sample volume in...
the ruminocentesis (Shen et al., 2012). While ruminal cannulation allows for the most reliable sample acquisition (Duffield et al., 2004), it requires complex surgical procedures, making it unsuitable for commercial farm applications. Consequently, given the increasing focus on animal welfare and strict regulatory measures, developing non-invasive methods for assessing rumen fermentation and metabolic profiling is imperative.

Exhalomics, the entity of all exhaled volatiles (i.e., the exhalome) and their metabolomics profiles, holds immense promise as a non-invasive approach to assess rumen function and diagnose diseases and metabolic health conditions in ruminant research (Reinhold et al., 2020; Islam et al., 2023). A cutting-edge analytical platform, secondary electrospray ionization-mass spectrometry (SESI-MS) has emerged as a robust tool for comprehensive exhalone analysis, especially biomarker detection and identification. It is gaining momentum in human nutrition and clinical studies for its ability to detect a wide variety of mass-to-charge (m/z) features in a non-targeted manner (Singh et al., 2019, Wüthrich et al., 2022). In ruminant animals, microbial fermentation gives rise to thousands of volatile organic compounds (VOC) including VFA in the rumen, as well as various gases released through eructation (Kuentzel et al., 2018). Consequently, the bovine exhalome, which comprises the gaseous emissions from the lungs, airways, and rumen eructation, provides a valuable opportunity to assess rumen function and metabolic processes through exhalomics. The composition and pattern of these VOC can reflect the changes in the rumen microbiota and fermentation processes, acting as potential indicators of the underlying metabolic activities (Saleem et al., 2012).

In the past, techniques such as GC-MS have been employed for detecting ketosis in lactating dairy cows through breath analysis (Dobbelaar et al., 1996, Mottram et al., 1999) and for identifying VOC in bovine breath (Spinhirne et al., 2004). The VFA profile from rumen fluid and ruminal headspace gas was studied using selected-ion-flow-tube mass spectrometry (SIFT-MS: Dewhurst et al., 2001). Furthermore, a method involving proton-transfer-reaction time-of-flight mass spectrometry and a collection face mask was established to analyze bovine exhaled breath and effectively used to characterize breath profiles of different ruminant species (Kuentzel et al., 2018, Oertel et al., 2018). Despite these advancements, comprehensive understanding, and characterization of VFA in the exhalome, and its VOC profile, still need to be improved due to the challenges related to sample collection and separation of eructation of exhaled breath (Reinhold et al., 2020). However, our recent study (Islam et al., 2023) introduced a non-contact exhalome sampling technique using the GreenFeed (GF) System (C-Lock Technology Inc., Rapid City, SD, USA) and revealed a clear association between the rise in exhaled acetate and butyrate levels and post-feeding ruminal methane production. However, this preliminary exploration highlights the existing research gap, emphasizing the need to compare the exhaled VFA (EX-VFA) and the conventional ruminal VFA (RM-VFA) profile. This approach aims to deepen our understanding of the rumen fermentation pattern and potentially reduce the dependency on invasive sampling methods.

Consequently, to solidify the precision and applicability of exhalomics in assessing rumen fermentation parameters, it is crucial to contrast EX-VFA with RM-VFA, derived from a conventional rumen sampling method. The objective of the current study was to compare the EX-VFA profile with the RM-VFA profile and to evaluate their agreement. We hypothesized that EX-VFA exhibits similar molar proportions and diurnal patterns compared with RM-VFA, rendering EX-VFA a reliable proxy approach for evaluating rumen fermentation in dairy cows.

**MATERIALS AND METHODS**

**Animals, Experimental Design and Diet**

The experimental procedures involving animals conducted at the AgroVet-Strickhof Dairy Farm (Lindau, Switzerland) were approved by the Cantonal Veterinary Office of Zürich (ZH115/2022). Four multiparous original Swiss Brown (Braunvieh) cows were included in the study, 3 averaged 169 ± 19.0 DIM (mean ± SD), 26.1 ± 2.30 kg/d MY, and 717 ± 60.5 BW, and 1 cow (687 kg BW) was dried off at the beginning of the experiment. The study was conducted for 29 d, divided into 3 periods, each with 7 d of dietary adaptation and 2 d of sample collection. The cows were randomly assigned to 2 diet sequences (ABA/BAB) in a switch-back design. The diets were (A) low-starch (LS; 6.31% starch of DM) and (B) high-starch (HS; 16.2% starch of DM). In this context, the term ‘high-starch’ is used in relation to the lower dietary starch concentration in the LS regimen. The specific diet formulation was adopted mainly in response to the production level and stage of the experimental cows. The dietary compositions of ingredients and nutrients are outlined in Table 1, with nutrient contents analyzed by standard procedures (AOAC, 1997). The diets were offered ad libitum at 110% of the previous day’s intake; the TMR was mixed before each feeding and was delivered once per day at 0830 h. During dietary adaptation in each period, the cows were housed in a free-stall barn with wheat straw bedding equipped with individual feeding troughs and free...
access to water. Cows were milked twice daily, at 0530 and 1630 h. The cows were moved to a tie-stall barn on d 5 of each period, following 3 d of adaptation to the tie-stall barn equipped with individual feeding plates (Mettler Toledo GmbH; Greifensee, Switzerland), and 2 d for sampling.

**Exhalome and Rumen Sampling**

We collected exhalome samples using the GF System (C-Lock Technology Inc., Rapid City, SD, USA) over a 3-d period, following the procedures described by Islam et al. (2023). Samples were collected 8 times at 1000, 1600, and 2200 h (on d 1); 0400, 1300, and 1900 h (on d 2); and 0100, and 0700 h (on d 3), representing every 3-h of a 24-h period. During each sampling event (5 min/cow), a maximum of 8 feed drops, corresponding to approximately 300 g per event/cow, of dehydrated alfalfa pellet (Luzatop, Désialis, Paris, France) containing 18% CP (DM-basis) was offered. The adjacent facilitated gas sampling unit of the GF System was utilized to collect the exhalome samples using 1 L Tedlar gas sampling bags (Thermogreen LB-2 septa, Merck, St. Louis, Missouri, USA). During each sampling, the capture of an eructation event was ensured by monitoring the real-time methane emission using the mobile application ‘Control Feed’ (C-Lock Technology Inc., Rapid City, SD, USA), as described in Islam et al. (2023).

Rumen contents were sampled manually through the cannula from the reticulum, dorsal (middle and back), and ventral sacs of the rumen immediately after exhalome sampling. The collected rumen contents were then mixed and filtered through a double layer of a 250 µm pore nylon fabric and the filtered rumen fluid was kept on ice until further processing. The pH and ammonia content of the rumen fluid were measured promptly after collection with a potentiometer (model 632 for pH; model 713 for ammonia; Metrom, Herisau, Switzerland). The potentiometer was fitted with suitable glass electrodes (model 6.0204.100 for pH; model 6.0506.100 for ammonia; Metrom, Herisau, Switzerland). Subsequently, the rumen samples were centrifuged at 4000 × g, 4°C for 5 min), and the supernatant was transferred to 2 mL Eppendorf tubes for storage at −20°C for VFA analysis. Samples were thawed (30 min, vortexed (30 s), and mixed with a quinic acid ((1S,3R,4S,5R)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid) internal standard before centrifugation. Then, VFA was analyzed using HPLC with a Bio-Rad HPX 87H ion-exchange column and a 5 m M H2SO4 eluting solvent (Ehrlich et al., 1981). Rumen temperature was obtained from rumen boluses placed in the reticulum of the cows (smaXtec bolus, Freienbach, Switzerland).

**Analysis of Exhaled VFA, Data Pre-processing, and Annotation**

To analyze exhalome samples we employed 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 according to the method described by Islam et al. (2023). In short, a de-sampling system was used to transfer exhalome molecules into the ionization chamber. After setting up the sampling bags (individually) in the de-sampling apparatus, they were pneumatically pressurized using high-purity nitrogen (99.999%, PanGas AG, Winterthur, Switzerland), and the collected sample molecules were introduced into the SESI source for ionization. The transfer line and ionization chamber were kept at a constant temperature of 130°C and 90°C respectively, to prevent water condensation and metabolite absorption. The voltage of the electrospray solution (0.1% aqueous formic acid solution) was set to ± 3.5 kV. Both positive and negative ion modes were used and performed high-resolution (1.4 × 10⁵) scans in the mass range from 50 Da to 500 Da with a maximum injection time of 500 ms.

The raw data files generated from SESI-MS were subjected to pre-processing using MATLAB (R2020b, Mathworks Inc., Natick, Massachusetts, USA), as described in Islam et al. (2023). Briefly, the raw mass
spectra data files were converted to mzXML format using the open-source MSConvert tool. A customized filter (height filter = 550, and segmentation 0.00025) was applied to extract only the signals that increased during de-sampling. A peak list of 1,694 features was recorded in both positive (847) and negative (847) ion modes. Finally, all peaks underwent baseline correction in the time dimension and were scaled to their maximum values. The obtained data set was then used for downstream statistical analysis.

The annotation of EX-VFA was conducted using the pre-processed data matrix obtained from negative ion mode, which offered enhanced sensitivity and efficient annotation of a wide range of targeted negative ions as previously described by Islam et al. (2023). Each feature of the data matrix was represented by a unique m/z and intensity, indicating the relative concentration of a specific feature as count-per-second (CPS) signal intensity. The EX-VFA, including acetate, propionate, butyrate, and valerate, were tentatively annotated using the exact m/z ratios. However, it is important to reflect the identical m/z values of exhaled valerate and exhaled isovalerate, reflecting the identical relative compound, therefore, butyrate was considered the sum of exhaled butyrate and exhaled isobutyrate. Similarly, valerate was represented as the combined m/z ratios of the targeted EX-VFA, their deviation from the theoretical masses, and the computed mass error are presented in Supplementary Table S1.

**Henry’s Law Calculations in Rumen Liquid-Gas System**

We employed Henry’s Law volatility to predict Henry’s Law VFA (HR-VFA) to understand the liquid-gas system dynamics within the rumen ecosystem. Henry’s Law is a well-established principle in chemistry that explicates the direct proportionality between the solubility of a gas in a liquid and the gas’s partial pressure present above the liquid (Sander, 2015). In the context of rumen fermentation, this law is highly pertinent. The VFA produced by the microbial communities residing in the rumen are gaseous and maintain an equilibrium between the liquid and gas phases. Consequently, the VFA concentration in the rumen fluid depends on the partial pressure of VFA in the gas phase. Therefore, Henry’s law of solubility constants, \( H^p \) is as follows:

\[
H^p = \frac{C_a}{P_g}
\]

where, \( C_a \) is the concentration (mM) of VFA in the liquid phase, and \( P_g \) is the partial pressure (atm) of that VFA in the gas phase under equilibrium conditions. The reference values of Henry’s Law of solubility constants for all VFA were obtained from von Hartungen et al. (2004). To predict the VFA concentrations in the rumen’s gas phase, we used Henry’s Law volatility constant \( K_{H}^p \), which is essentially the inverse of Henry’s law solubility constant \( \frac{C_g}{P_g} \) (Sander, 2015). The equation for this conversion is as follows:

\[
K_{H}^p = \frac{1}{H^p}.
\]

Utilizing this volatility constant, we were able to predict the VFA concentration in the gas phase, under equilibrium conditions using the equation:

\[
C_g = \frac{P_g}{RT},
\]

where, \( C_g \) is the gas-phase concentration (mol/m³) of VFA in the rumen environment, and \( P_g \) is the partial pressure (atm) of that VFA in the gas phase under equilibrium conditions.

**Statistical Analysis**

All statistical analyses, calculations, and conversions regarding Henry’s Law were performed in the R statistical programming language (R Core Team 2023; version 4.3.1). The 3 VFA sampling and measurement methods were ruminal (RM), exhaled (EX), and Henry’s Law predicted (HR). To obtain a comprehensive numerical overview, initially, we consolidated the raw data sets by transforming the 3-h interval into daily averages and calculated summary statistics of VFA concentrations and molar proportions for each measurement method. Subsequent statistical analyses were performed using the mixed model, utilizing the lmer procedure (Bates et al., 2014) in R. Initially, the overall effect of diets and measurement methods on the VFA profile was tested, deploying model (i) according to the switchback experimental design description of Kuehl (2000) and structured as follows:

\[
Y_{ijklmn} = \mu + S_i + C_j(S_i) + P_k + D_m + M_n + D_m \times M_n + CO_i + \epsilon_{ijklmn},
\]

where \( Y_{ijklmn} \) was the variable of interest, \( \mu \) denotes the overall mean, \( S_i \) is the random effect of treatment sequence (i = 1 to 2), \( C_j(S_i) \) is the random effect of cow nested within the sequence (j = 1 to 4), \( P_k \) is the fixed effect of the period (k = 1 to 3), \( D_m \) is the fixed effect of treatments (m = 1 to 2), \( M_n \) is the fixed effect of VFA measurement method (n = 1 to 3), \( D_m \times M_n \) is the interaction of treatment and method of measurement,
where, \( Y_{ijklmp} = \mu + S_i + C_j(S_i) + P_k + D_m + T_p + D_m \times T_p + CO_i + e_{ijklmp} \)

where, \( M_n \times T_p \) is the interaction of measurement methods and repeated measures of time of day \((P = 1\) to 8\), while the remaining model components are analogous to those in the model \((i)\). In addition, we used the LSM derived from the linear mixed model \((ii)\) to perform linear regressions, namely, RM vs. EX and HR vs. EX, respectively, to assess their agreements. In particular, interactions between EX and type of diet were included in the model and evaluated. If an interaction was identified, 95% confidence intervals of the slopes for each dietary treatment \((HS and LS)\) were calculated based on Figueiras et al. \((1998)\). If there were no interactions, the confidence intervals were calculated using \(confint\) procedure in R based on all data. This allowed us to evaluate the slope biases between different VFA measurement methods.

Finally, hourly feed intake, starch, and NDF intake data were fitted to the model \((iii)\):

\[ Y_{ijklmp} = \mu + S_i + C_j(S_i) + P_k + M_n + T_p + M_n \times T_p + CO_i + e_{ijklmp} \]

where, \( D_m \times T_p \) is the interaction of treatment and repeated measures of time of day. Statistical significance was declared at \( P < 0.05 \) for the main effect, and tendency was declared at \( 0.05 < P \leq 0.10 \). Statistical significance was declared at \( 0.05 < P \leq 0.10 \) for interactions. We implemented an iterative outlier removal approach based on the fitted mixed models. Data points with Studentized residuals outside of ± 3 were excluded from the analysis. Rarely more than 1 data point per variable was removed.

**RESULTS AND DISCUSSION**

It is important to emphasize that the high-starch levels in the current study were lower \((16.2\% of DM)\) than what usually is considered a high-starch diet for high-producing dairy cows in certain feeding systems. However, the difference between the 2 experimental diets was 10-% units, which in this context creates low and high starch diets for the purposes of this study. The primary goal of this study was to evaluate the potential of EX-VFA as a proxy for RM-VFA in the assessment of rumen fermentation with distinct VFA profiles caused by different starch content of the basal diets. Implementing an exhalomics-based approach could mitigate the need for traditional, more invasive procedures, thereby addressing ethical considerations and improving the welfare of ruminants under study. In this context, our investigation involved a comparative analysis of VFA measurement techniques across 2 dietary groups while incorporating a prediction based on Henry’s Law \((HR-VFA)\) to enhance our understanding of rumen dynamics. As described more in detail in Islam et al. \((2023)\), the unique and important benefit of our methodology includes the use of the highly sensitive SESI-MS platform, which enabled the detection of 1,694 features in the current EX samples. In addition, the EX sample collection using the head-chamber system allowed for real-time monitoring of \(CH_4\) emission, which ensures the identification of eructation events. This captures the rumen gas phase and overcomes the shortcomings \((e.g.,\ variation in sampling techniques and challenges in capturing eructation events)\) of many other exhalome sampling methods utilized previously in ruminant research \((Reinhold et al., 2020)\).

**Effect of VFA Measurement Method and Dietary Starch Content**

No interactions were observed between diet types \((HS and LS)\) and VFA measurement methods \((RM, HR, and EX; Table 2)\). Although no diet effect was found for any VFA variables, the measurement method had an effect \((P < 0.01)\) on VFA molar proportions. Based on the preplanned contrasts, acetate and the A:P ratio were greater \((P < 0.01, and P = 0.02, respectively)\), and conversely, propionate was lower \((P = 0.02)\), when measured with RM compared with EX. In HR compared with EX, the acetate levels and the A:P ratio closely resembled each other \((P = 0.07 and P = 0.16, respectively)\), while the levels of propionate and valerate+isovalerate were significantly lower \((P < 0.01 and P = 0.03, respectively)\). Further, we observed a diet effect on rumen pH \((P < 0.01)\) and \(NH_3\) \((P < 0.01)\); the cows receiving the LS diet had a higher rumen pH and greater \(NH_3\) concentration compared with the HS diet. Additionally, the summary statistics of VFA concentrations and their molar proportions are provided in Supplemental Table S2 for an overview of the VFA profiles measured using 3 methods.

Acetate, one of the key VFA, exhibited the greatest molar proportion averaging 61.6%, with consistent
deviations between different diets across different measurement methods. It was followed by propionate, which ranged from 22.2 to 28.0%, butyrate+isobutyrate 8.49 to 13.2%, and valerate+isovalerate 1.58 to 5.96%. The lack of interaction and measured molar proportions reflect the robustness of the measurement methods. Henry’s Law constants and measured RM-VFA concentrations were used to estimate VFA concentrations and their molar proportions in the gas phase of the rumen. The HR-VFA showed a greater acetate proportion than EX-VFA, and values between RM-VFA and EX-VFA for the other VFA proportions and the A:P ratio. Factors affecting the profile of VFA in the gas phase include feed intake rate, pH, and rumen temperature (Russell, 2002), which are further discussed below in the context of daily patterns of VFA.

Overall, these molar proportions of the main rumen VFA, acetate, propionate, and butyrate+isobutyrate, align well with the previously reported range of 51–75%, 15–31%, and 10–24% respectively (Dijkstra, 1994; Firkins et al., 2006; Räisänen et al., 2022), as discussed in Islam et al. (2023). Further, the EX-VFA proportions are similar to our previous study, where we characterized exhalome of dairy cows using SESI-MS (Islam et al., 2023). In general, VFA proportions depend on the animal’s basal diet, metabolic status, and the timing and frequency of the rumen sampling (Dijkstra, 1994, Firkins et al., 2006). In addition, the molar proportions of acetate and butyrate were shown to be slightly lower in rumen headspace gas than in rumen fluid samples, changing with increasing chain length, but overall VFA molar proportions were similar between the 2 sample types (Dewhurst et al., 2001). This aligns well with our current findings where EX acetate (58.2%) and butyrate+isobutyrate (8.84%) were slightly lower than that of RM (62.4% and 12.7%) and HR (64.1% and 9.02%), respectively. In terms of propionate, a higher proportion was observed in EX-VFA (27.2%) compared with RM-VFA (23.0%), which is slightly different from the results reported by Dewhurst et al. (2001); they observed a similar propionate proportion in rumen headspace gas and rumen liquid samples in contrast to our finding. This discrepancy may be due to the different sampling methods and analytical platforms used in the experiments. Overall, the molar proportions of VFA were strikingly similar among the methods in the current study. Therefore, given the high variability in the amount of liquid in rumen digesta and ruminal pool sizes, VFA molar percentages, rather than their concentrations, are considered a more reliable assessment of ruminal fermentation and its dynamics caused by dietary treatments (Hall et al., 2015). As evident by the results reported here, EX-VFA molar propor-

<table>
<thead>
<tr>
<th>Items</th>
<th>VFA, mol %</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Butyrate+isobutyrate</th>
<th>Valerate+isovalerate</th>
<th>A:P</th>
<th>Rumen pH</th>
<th>NH3 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-RM</td>
<td>62.7</td>
<td>63.7</td>
<td>13.2</td>
<td>1.91</td>
<td>3.01</td>
<td>6.31</td>
<td>6.62</td>
<td>0.69</td>
</tr>
<tr>
<td>HS-HR</td>
<td>62.2</td>
<td>63.6</td>
<td>13.3</td>
<td>1.91</td>
<td>3.00</td>
<td>6.31</td>
<td>6.62</td>
<td>0.69</td>
</tr>
<tr>
<td>HS-EX</td>
<td>61.7</td>
<td>63.0</td>
<td>12.9</td>
<td>1.94</td>
<td>2.99</td>
<td>6.31</td>
<td>6.62</td>
<td>0.69</td>
</tr>
</tbody>
</table>

HR-VFA showed a greater acetate proportion than EX-VFA, and values between RM-VFA and EX-VFA for the other VFA proportions and the A:P ratio. Factors affecting the profile of VFA in the gas phase include feed intake rate, pH, and rumen temperature (Russell, 2002), which are further discussed below in the context of daily patterns of VFA. Overall, these molar proportions of the main rumen VFA, acetate, propionate, and butyrate+isobutyrate, align well with the previously reported range of 51–75%, 15–31%, and 10–24% respectively (Dijkstra, 1994; Firkins et al., 2006; Räisänen et al., 2022), as discussed in Islam et al. (2023). Further, the EX-VFA proportions are similar to our previous study, where we characterized exhalome of dairy cows using SESI-MS (Islam et al., 2023). In general, VFA proportions depend on the animal’s basal diet, metabolic status, and the timing and frequency of the rumen sampling (Dijkstra, 1994, Firkins et al., 2006). In addition, the molar proportions of acetate and butyrate were shown to be slightly lower in rumen headspace gas than in rumen fluid samples, changing with increasing chain length, but overall VFA molar proportions were similar between the 2 sample types (Dewhurst et al., 2001). This aligns well with our current findings where EX acetate (58.2%) and butyrate+isobutyrate (8.84%) were slightly lower than that of RM (62.4% and 12.7%) and HR (64.1% and 9.02%), respectively. In terms of propionate, a higher proportion was observed in EX-VFA (27.2%) compared with RM-VFA (23.0%), which is slightly different from the results reported by Dewhurst et al. (2001); they observed a similar propionate proportion in rumen headspace gas and rumen liquid samples in contrast to our finding. This discrepancy may be due to the different sampling methods and analytical platforms used in the experiments. Overall, the molar proportions of VFA were strikingly similar among the methods in the current study. Therefore, given the high variability in the amount of liquid in rumen digesta and ruminal pool sizes, VFA molar percentages, rather than their concentrations, are considered a more reliable assessment of ruminal fermentation and its dynamics caused by dietary treatments (Hall et al., 2015). As evident by the results reported here, EX-VFA molar propor-
Methods

Daily Pattern of VFA Profile Measured with Different Carbohydrates

As previously established, the provision of fermentable carbohydrates in the form of corn, on the production of VFA and as follows pH (Plaizier et al., 2008). However, in line with our data, Silveira et al. (2007) investigated the effects of high and low-starch diets (30% vs. 23% of DM) in lactating dairy cows and reported no significant differences in total VFA concentrations between the two groups, which had similar DMI. However, they observed differences in the molar proportions of acetate and propionate, in contrast to our data, while the proportions for butyrate+isobutyrate and valerate+isovalerate remained unchanged. A larger sample size and extended study period in the current experiment would have been needed to observe a response in the VFA profile induced by the different starch content between the experimental diets. Additionally, the lack of diet effect on VFA molar proportions can be partly attributed to the individual variability of cows (Thomas and Martin, 1988), especially in regard to DMI and subsequent VFA profile, as notably there was large variability in the lactational stage, MY, and DMI between the cows. As previously established, the provision of fermentable carbohydrates can decrease NH₃ production via a reduction in deamination and an increase in microbial uptake of NH₃ (Hristov et al., 2005), which was reflected in lower NH₃ concentration in the rumen fluid of cows fed the HS diet.

Overall, the findings in the current experiment align well with prior studies that have reported a range of ratios between the 3 main VFA (e.g., acetate, propionate, and butyrate+isobutyrate), as noted above. These observations underscore the potential of the proposed exhalomics approach to assess rumen fermentation patterns, as presented in our proof-of-concept study (Islam et al., 2023).

Daily Pattern of VFA Profile Measured with Different Methods

Due to the lack of interaction between diet and VFA measurement method, the interaction between VFA method and time of day was analyzed independently for HS and LS. Consequently, the daily pattern of VFA molar proportions measured in 3-h intervals are presented in Figures 1 and 2 for HS and LS diets, respectively, in addition, the numerical values are presented in Supplementary Table S3. No interaction was observed between the measurement methods and time of day for either diet. However, all VFA molar proportions differed (P < 0.01) across the day. For the HS diet, the acetate molar proportions drastically increased after feeding and peaked at 1000 h as observed by all 3 methods (Figure 1A). Afterward, a steady decline was observed until 0400 h, marked by a sudden drop in EX acetate. Following an interval of 2 h, a sharp decrease was observed around 0700 h for both HR and RM acetate, right before morning feeding. For propionate, a gradual slow increase was observed starting 2h after feeding and continued up to 2200 h, then decreased by midnight. Exhaled propionate discrepancy peaked at 0400 h (Figure 1B). For butyrate+isobutyrate, the highest molar proportion was observed at 0700 h, and was 14.0% for RM, 10.7% for EX, and 10.0% for HR. The butyrate+isobutyrate proportions started increasing around 2 h after feeding and fluctuated across the day with a higher proportion observed for RM butyrate+isobutyrate. The A:P ratio was highest at 1000 h in all 3 methods. Similarly for the LS, the VFA proportions fluctuated (P < 0.01) across the day. Unlike, the HS diet, acetate reached its peak at 1300 h for all 3 methods (Figure 2A) and EX propionate showed the highest proportion at 1600 h and 1900 h for the RM and HR methods (Figure 2B). The butyrate+isobutyrate showed no interaction (P = 0.14) with the method and by time-of-day, and peaked at 0700 h for all measurement methods. Similar to acetate, the A:P ratio peaked at 1300 h in all 3 methods, where the proportion of EX A:P ratio remained lower across the day (P < 0.01). The daily pattern of feed, starch, and NDF intake rates are presented in Supplementary Figure S1, and pH and rumen temperature measured in 3-h intervals are presented in Supplementary Figure S2.

In the current study, the lack of interaction between methods and time of day implies that VFA measurements from these 3 methods were changing in the same direction across the day. The daily pattern of VFA molar proportion measured with 3 methods revealed key dynamics and fluctuations influenced by the diet types, time of day, and measurement methods. There were marked differences in intra-day variability of individual VFA molar proportions, regardless of method, where acetate drastically increased after feeding, in accordance with the ingested feed and starch following fresh feed delivery, with a more gradual increase observed for propionate, A:P ratio, and butyrate+isobutyrate for HS diet. A corresponding pattern of EX acetate and butyrate+isobutyrate immediately after delivery of fresh feed was reported in our previous proof-of-concept study (Islam et al., 2023). Further, the ruminal pH data (Supplementary Figure S2A) supports the intra-day fluctuations in the VFA proportions, especially acetate, regardless of method; the lowest pH coincided with the greatest acetate concentration, and being greatest be-
fore feeding when acetate concentration was at its lowest. A significant portion of daily feed intake occurs within 2 h after fresh feed delivery (Niu et al., 2014, 2017), also observed in our study (Supplementary Figure S1A). As follows, in the current study, the conditioned starch intake after feeding (Supplementary Figure S1B) resulted in higher production of primary ruminal fermentation end products, particularly acetate, propionate, and butyrate (Dijkstra et al., 2012; Hatew et al., 2015). These end products have a variable intra-day pattern, reflecting the rumen fermentation dynamics (Mao et al., 2008), as discussed in detail by Islam et al. (2023). Interestingly, the overall pattern of VFA was observed as diet-specific, showing how the peak times for acetate, propionate, and butyrate+isobutyrate varied across the day between HS and LS, and the fluctuations were less pronounced with the LS diet given its extremely low starch content (6.31%). This can be expected as the rate of starch intake was notably lower post-feeding, and throughout the day, which eventually impacts the rate of fermentation and production of VFA (Hatew et al., 2015).

Further, our findings revealed minor discrepancies regarding the EX acetate and propionate for the HS diet right before feeding time, the former being at its lowest, and the latter at its highest 2 h before the lowest and highest points for RM, respectively. This discrepancy can be attributed to the 1 to 2 min travel time of the eructation gas (from the rumen headspace through the esophagus), whereby the volume of each eructation vary between each event (Reinhold et al., 2020). This in turn could lead to slight variations in the daily fluctuations in VFA proportions between measurements methods. In addition, the water solubility of VFA is very high and differs between the individual VFA which could influence their volatility and as follows their concentrations in the gas phase of the rumen (Oertel et al., 2018). However, the relative abundance of VOC measured in GC-MS, particularly the VFA has been compared in the rumen gas of steers before and after feeding, and a sharp increase in after-feeding samples was reported (Cai et al., 2006), which is also in line with the observed response in acetate and A:P ratio in the current study, especially with the HS diet, as well as our previous study (Islam et al., 2023).

Further, the variations in individual VFA proportions regarding the time of day, diet, and measurement method may have been affected by factors such as rumen temperature and pH. Indeed, pH can influence the production and metabolism pathways of VFA, thereby impacting the relationship between VFA in the rumen and the eructated gases (Russell, 1998, Dijkstra et al., 2012). Additionally, the pH of the rumen fluid plays a pivotal role in VFA absorption rates, with increasing pH leading to reduced fractional absorption of specific VFAs. This highlights the multi-faceted relationship between VFA concentration in the rumen and actual production rates (Dijkstra et al., 1993,1994). In addition, according to Henry's Law principle, the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas above the liquid (Sander, 2015). Accordingly, the proportion of VFA in the rumen and in eructated gases is directly related, although both temperature and pH can affect the solubility of a gas (Sander, 2015). Further, as demonstrated by ALZahal et al. (2008) rumen temperature and pH are inversely related, which aligns with the pH and rumen temperature data in the current study (Supplementary Figure S2). Thereby the presence of each VFA in the rumen headspace, and subsequently in the EX samples, can differ in accordance with the factors mentioned above affecting the rumen fermentation profile and volatilization of VOC. However, the overall proportions of VFA closely reflect the corresponding proportions in the rumen liquid, as was demonstrated by Dewhurst et al. (2001), and EX vs. RM data in the current study.

**Correlation Between Ruminal and Exhaled VFA**

The Pearson correlations between RM-VFA and EX-VFA are depicted in Figure 3, revealing strong positive relationships between RM-VFA and EX-VFA across dietary categories. There was an interaction ($P = 0.07$) between EX-VFA and diet for acetate, but strong positive correlations were observed for acetate in both HS and LS diet groups ($r = 0.84$ and 0.85, respectively). The slopes were 0.78 and 0.36, respectively ($P < 0.01$), and 95% confidence intervals ranging from 0.40 to 1.16, and from 0.11 to 0.61 for HS and LS, respectively (Figure 3A). There were no interactions for propionate, A:P ratio, or butyrate+isobutyrate. For both propionate and the A:P ratio, strong positive correlations were observed ($r = 0.74$, and 0.80, respectively), with slopes of 0.70 and 0.94, respectively ($P < 0.01$). The 95% confidence intervals for these relationships were 0.34 to 1.07 for propionate and 0.54 to 1.35 for the A:P ratio (Figure 3B and 3C). For butyrate+isobutyrate, depicted in Figure 3D, a moderate level of positive association between RM and EX butyrate+isobutyrate ($r = 0.57$; $P = 0.02$). We extended a similar analysis by examining the relationship between HR-VFA and EX-VFA profiles (Supplementary Figure S3) and found similar and stronger associations for acetate, propionate, and their ratios.

Further supporting the above-discussed data, there were strong correlations between RM-VFA and EX-VFA, especially in acetate, propionate, and A:P ratio, demonstrating that EX-VFA could serve as a
noninvasive proxy for RM-VFA. The data from the current study supports the proposed hypothesis that EX-VFA mirror the proportions of RM-VFA (Islam et al., 2023). In particular, 1 (slope of the identity line, y = x) fell right within the 95% confidence intervals of propionate, the A:P ratio, and acetate (HS only) for both preplanned contrasts (Figure 3 and Figure S3), further demonstrating the applicability of exhalomics-based approach as a non-invasive indicator of rumen fermentation profile. The observed slope biases and relatively wide confidence intervals for some VFA parameters imply some degree of variability in the VFA profiles between the measurement techniques and call for further investigations with a larger sample size and a wider range of VFA profiles induced by different feeding regimens with higher starch content. This would strengthen the current findings and further application of the proposed measurement technique. Collectively, the results from the current experiment substantiate a significant association between RM-VFA and EX-VFA, suggesting the potential utility of exhalomics in assessing the rumen fermentation profile.

CONCLUSIONS

The present study confirms the potential of a noninvasive exhalomics approach using SESI-MS to monitor and assess ruminal fermentation. Our findings confirm no interaction between the VFA measurement methods and the diets or time of day. This indicates that the analysis of exhalome can serve as a reliable method to characterize the ruminal VFA profile and its diurnal pattern with relative fluctuations post-feeding, making it a promising tool for dietary management and rumen health assessment in ruminants. The significance of these findings lies in the ability to evaluate diet-induced changes in ruminal fermentation non-invasively. To further establish this method, future research should focus on exploring diverse dietary treatments to characterize a broader range of rumen fermentation profiles.

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Figure 1. Molar proportions of (A) acetate, (B) propionate, (C) the ratio of acetate-to-propionate, and (D) butyrate measured in dairy cows fed a high-starch diet. These measurements were recorded in 3-h intervals from different methods and sources: ruminal (rumen fluid), Henry (predictions based on Henry’s Law), and exhaled (exhalome). Cows were fed at 0830 h.
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Figure 3. Correlation between the molar proportions of ruminal VFA and exhaled VFA, (A) acetate, (B) propionate, (C) acetate-to-propionate ratio, and (D) butyrate using LSM from mixed model (ii) outputs. The significant interaction term between diet (HS = High-starch, and LS = Low-starch) and exhaled VFA is depicted by 2 fitted lines for acetate (Diet × EX-VFA, P = 0.07) with no interaction confirmed by a common slope across both diets (green fitted line) for propionate (P = 0.54), acetate-to-propionate ratio (P = 0.18), and butyrate (P = 0.74).

The black dashed line represents the identity relationship (y = x).
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ORCIDS

M. Z. Islam https://orcid.org/0000-0002-6672-1121
T. He https://orcid.org/0000-0001-5643-2062
C. Kunz https://orcid.org/0000-0002-1907-9527
Y. Li https://orcid.org/0000-0001-9371-9978
X. Ma https://orcid.org/0000-0002-1652-9035
A. M. Serviento https://orcid.org/0000-0002-2166-0791
R. Zenobi https://orcid.org/0000-0001-5211-3558
S. Giannoukos https://orcid.org/0000-0002-5066-533X
M. Niu https://orcid.org/0000-0003-4484-4611

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