J. Dairy Sci. 107:2099–2110
https://doi.org/10.3168/jds.2023-24124

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

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ABSTRACT

Previously, we used secondary electrospray ionization-mass spectrometry (SESI-MS) to investigate the diurnal patterns and signal intensities of exhaled (EX) volatile fatty acids (VFA) of dairy cows. The current study aimed to validate the potential of an 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 on a dry matter basis) and (B) high starch (HS; 16.2% starch on a dry matter basis). Feeding was once per day at 0830 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 (RM)-VFA were analyzed using SESI-MS and HPLC, respectively. Furthermore, the VFA concentration in the gas phase (HR-VFA) was predicted based on RM-VFA and Henry's Law (HR) constants. No interactions were identified between the types of diets (HS vs. LS) and the measurement methods on daily average VFA profiles (RM vs. EX or HR vs. 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 acetate:propionate ratio and a steady increase for propionate (2 h after feeding the HS diet, 4 h for LS), and butyrate. This change was more pronounced for the HS diet than the LS diet. However, there was no overall diet effect on the VFA molar proportions, although 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 acetate:propionate 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 ruminocentesis (Shen et al., 2012). Although ruminal cannulation allows for

Received September 5, 2023.
Accepted October 20, 2023.
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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 noninvasive 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 noninvasive approach to assess rumen function and diagnose diseases and metabolic health conditions in ruminant research (Reinhold et al., 2020; Islam et al., 2023a). A cutting-edge analytical platform, secondary electrospray ionization (**SESI**)-mass spectrometry (**SESI-MS**) has emerged as a robust tool for comprehensive exhalome analysis, especially for 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 nontargeted 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 (Dewhurst et al., 2001). Furthermore, a method involving proton-transfer-reaction time-of-flight MS 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 and exhaled breath (Reinhold et al., 2020). However, our recent study (Islam et al., 2023a) introduced a noncontact exhalome-sampling technique using the GreenFeed (**GF**) System (C-Lock Technology Inc., Rapid City, SD) and revealed a clear association between the rise in exhaled acetate and butyrate levels and postfeeding 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 of the cows averaged 169 ± 19.0 DIM (mean ± SD), 26.1 ± 2.30 kg/d milk yield, 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 or BAB) in a switchback design. The diets were (A) low starch (**LS**; 6.31% of DM) and (B) high starch (**HS**; 16.2% 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, including ingredients and nutrients, are listed in Table 1, with nutrient contents analyzed by standard procedures (AOAC International, 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 freestall barn with wheat straw bedding and equipped with individual feeding troughs and free access to water. Cows were milked twice daily, at 0530 and 1630 h. The cows were moved

Table 1. Ingredients and chemical composition of the experimental diets

Item	Diet	
	High starch	Low starch
Feed ingredients, % of DM		
Grass silage[1]	54.0	63.3
Maize silage[2]	15.3	17.9
Sugar-beet silage[3]	12.0	14.0
Grass hay[4]	4.20	4.90
Ground corn[5]	14.6	—
Chemical composition, % of DM		
CP	11.5	11.2
Crude fat	2.29	2.08
NDF	40.7	43.5
ADF	25.1	26.9
Starch	16.2	6.31

[1]Contained 38.9% DM and 13.2% CP, 42.7% NDF, and 1.24% starch on a DM basis.
[2]Contained 40.2% DM and 7.18% CP, 46.1% NDF, and 30.7% starch on a DM basis.
[3]Contained 39.1% DM and 9.02% CP, 40.0% NDF, and 0.12% starch on a DM basis.
[4]Contained 88.7% DM and 5.60% CP, 55.0% NDF, and 0.50% starch on a DM basis.
[5]Contained 87.6% DM and 7.56% CP, 20.8% NDF, and 73.8% starch on a DM basis.

to a tiestall barn on d 5 of each period, allowing 3 d of adaptation to the tiestall barn, which was 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 over a 3-d period, following the procedures described by Islam et al. (2023a). 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 per cow, of dehydrated alfalfa pellet (Luzatop, Désialis, Paris, France) containing 18% CP (on a DM basis) was offered. The adjacent facilitated gas sampling unit of the GF System was used to collect the exhalome samples using 1-L Tedlar gas sampling bags (Thermogreen LB-2 septa, Merck, St. Louis, MO). During each sampling event, 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), as described in Islam et al. (2023a).

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; Metrohm, Herisau, Switzerland). The potentiometer was fitted with suitable glass electrodes (model 6.0204.100 for pH; model 6.0506.100 for ammonia; Metrohm, Herisau, Switzerland). Subsequently, the rumen samples were centrifuged at 4,000 × *g* at 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* H_2SO_4 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 Preprocessing, 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) MS according to the method described by Islam et al. (2023a). In short, a desampling system was used to transfer exhalome molecules into the ionization chamber. After setting up the sampling bags (individually) in the desampling 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 constant temperatures 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 high-resolution (1.4×10^5) scans were performed 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 preprocessing using MATLAB (R2020b, Mathworks Inc., Natick, MA), as described in Islam et al. (2023a). Briefly, the raw MS data files were converted to mzXML format using the open-source MSConvert tool (https://proteowizard.sourceforge.io/download.html). A customized filter (height filter = 550

[threshold units not specified, typically related to the amplitude or intensity, helps to filter out noise and less significant peaks) and segmentation 0.00025 [dimensionless value used to distinguish between signal segments]) was applied to extract only the signals that increased during desampling. A peak list of 1,694 features was recorded in both positive- (847) and negative-ion (847) 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 preprocessed 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. (2023a). 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 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 note that isomers share the same m/z values with their relative compound, therefore, butyrate was considered the sum of exhaled butyrate and exhaled isobutyrate. Similarly, valerate was represented as the combined values of exhaled valerate and exhaled isovalerate, reflecting their identical m/z values. The measured m/z ratios of the targeted EX-VFA, their deviation from the theoretical masses, and the computed mass error are presented in Supplemental Table S1 (https://doi.org/10.3929/ethz-b-000638959; Islam et al., 2023b).

Henry's Law Calculations in Rumen Liquid–Gas System

We employed Henry's Law (**HR**) volatility to predict VFA concentration in the gas phase (**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, HR of solubility constant (H^{cp}) is as follows:

$$H^{cp} = C_a / P_g,$$

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 HR 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 the HR volatility constant K_H^{pc}, which is essentially the inverse of H^{cp} (Sander, 2015). The equation for this conversion is as follows:

$$K_H^{pc} = 1 / H^{cp}.$$

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

$$C_g = P_g / RT,$$

where C_g is the gas-phase concentration (mol/m^3) of VFA in the rumen environment, P_g is the partial pressure (atm) of that VFA in the gas phase under equilibrium conditions, R is the gas constant (8.312 J/mol·K), and T is the measured rumen temperature (K).

Statistical Analysis

All statistical analyses, calculations, and conversions regarding HR were performed in the R statistical programming language (version 4.3.1, 2023; R Core Team). The 3 VFA sampling and measurement methods were ruminal (**RM**), exhaled (**EX**), and HR predicted. To obtain a comprehensive numerical overview, initially we consolidated the raw data sets by transforming the 3-h intervals 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, according to the switchback experimental design description of Kuehl (2000) and deploying model 1, structured as follows:

$$Y_{ijklmn} = \mu + S_i + C_j(S_i) + P_k + D_m + M_n + D_m \times M_n + CO_l + e_{ijklmn}, \quad [1]$$

where Y_{ijklmn} was the variable of interest; μ denotes the overall mean; S_i is the random effect of treatment sequence (i = 1–2); $C_j(S_i)$ is the random effect of cow nested within the sequence (j = 1–4); P_k is the fixed effect of the period (k = 1–3); D_m is the fixed effect of treatments (m = 1–2); M_n is the fixed effect of the

VFA measurement method ($n = 1$–3); $D_m \times M_n$ is the interaction of treatment and method of measurement; CO_l is the fixed effect of the carryover effect ($l = 1$–3), considering any remaining effects from the previous period that affect the current observation (where period 1 was zero carryover effect); and e_{ijklmn} is the residual error. The preplanned contrasts were RM versus EX and HR versus EX.

Furthermore, a second model was structured to ascertain the daily pattern of measured VFA using 3-h interval data. Model 2 was as follows:

$$Y_{ijklnp} = \mu + S_i + C_j(S_i) + P_k + M_n + T_p + M_n \times T_p + CO_l + e_{ijklnp}, \quad [2]$$

where $M_n \times T_p$ is the interaction of measurement methods and repeated measures of time of day ($P = 1$–8), and the remaining model components are analogous to those in model 1. In addition, we used the LSM derived from linear mixed model 2 to perform linear regressions, namely RM versus EX and HR versus 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 the *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 using model 3:

$$Y_{ijklmp} = \mu + S_i + C_j(S_i) + P_k + D_m + T_p + D_m \times T_p + CO_l + e_{ijklmp}, \quad [3]$$

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 HS levels in the current study were lower (16.2% of DM) than what usually is considered an HS diet for high-producing dairy cows in certain feeding systems. However, the difference between the 2 experimental diets was 10 percentage units, which in this context creates LS and HS 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 contents 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 in more detail in Islam et al. (2023a), 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 used 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 acetate:propionate (**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), but the levels of propionate and valerate + isovalerate were significantly lower ($P < 0.01$ and $P = 0.03$, respectively). Furthermore, 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 those on the HS diet. Additionally, the summary statistics of VFA concentrations and their molar proportions are provided in Supplemental Table S2 (https://doi.org/10.3929/ethz-b-000638959; Islam

Table 2. Interaction of the 2 dietary starch levels and 3 measurement methods employed to determine the molar proportions of VFA in cannulated dairy cows[1]

Item	Treatment LSM						*P*-value				
	HS-RM	LS-RM	HS-HR	LS-HR	HS-EX	LS-EX	SE	Diet	Method	Diet × method	RM vs. EX[2]	HR vs. EX[2]
VFA,[3] mol %												
Acetate	62.7	62.0	64.4	63.7	59.4	57.0	2.14	0.41	<0.01	0.77	<0.01	0.07
Propionate	22.2	23.8	24.4	26.1	27.1	28.0	1.44	0.19	<0.01	0.85	0.02	<0.01
Butyrate[4]	13.2	12.2	9.54	8.49	8.51	8.82	1.014	0.51	<0.01	0.30	0.69	<0.01
Valerate[5]	1.91	2.00	1.58	1.68	5.51	5.96	0.223	0.30	<0.01	0.37	<0.01	0.03
A:P[6]	3.01	2.82	2.81	2.64	2.29	2.22	0.283	0.43	<0.01	0.87	0.02	0.16
Rumen[7]												
pH	6.31	6.62	—	—	—	—	0.124	<0.01	—	—	—	—
NH_3 (m*M*)	6.07	6.93	—	—	—	—	0.306	<0.01	—	—	—	—

[1]Diets were high starch (HS, 16.2% of DM) or low starch (LS, 6.31% of DM). Measurement methods were ruminal (RM), Henry's Law predicted (HR), and exhaled (EX).

[2]Preplanned contrasts of VFA measurement methods: RM vs. EX and HR vs. EX.

[3]VFA measurement methods comparison: acetate, RM = 62.4, HR = 64.1, EX = 58.2; propionate, RM = 23.0, HR = 25.3, EX = 27.2; butyrate, RM = 12.7, HR = 9.02, EX = 8.84; valerate, RM = 1.96, HR = 1.63, EX = 5.74; and A:P, RM = 2.92, HR = 2.72, EX = 2.40. Level of significance for preplanned contrasts (RM vs. EX or HR vs. EX) was defined at $P < 0.05$, and tendency was declared at $0.05 < P \leq 0.10$.

[4]Butyrate = exhaled butyrate + exhaled isobutyrate, because isomers have m/z values that are identical to that of butyrate.

[5]Valerate = exhaled valerate + exhaled isovalerate, also considered as a sum because the isomers have the same m/z values as valerate.

[6]A:P = acetate:propionate ratio.

[7]Rumen pH and NH_3 concentrations were measured from rumen fluid.

et al., 2023b) for an overview of the VFA profiles measured using the 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 + isobutyarte, which ranged from 8.49% to 13.2%; and valerate + isovalerate, which ranged from 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% to 75%, 15% to 31%, and 10% to 24%, respectively (Dijkstra, 1994; Firkins et al., 2006; Räisänen et al., 2021), as discussed in Islam et al. (2023a). Furthermore, the EX-VFA proportions were similar to our previous study, where we characterized exhalome of dairy cows using SESI-MS (Islam et al., 2023a). 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 those of RM (62.4% and 12.7%, respectively) and HR (64.1% and 9.02%, respectively). In terms of propionate, a higher proportion was observed in EX-VFA (27.6%) 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 used 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 evidenced by the results reported here, EX-VFA molar proportions provide an accurate estimate of overall RM-VFA proportions and profiles.

Although not evidently reflected in the VFA proportions, a distinct diet effect on rumen pH and NH_3 occurred, highlighting dietary influence, in this case, the provision of readily fermentable carbohydrates in the form of corn, on the production of VFA and subsequently pH (Plaizier et al., 2008). However, in line with our data, Silveira et al. (2007) investigated the effects of HS and LS diets (30% vs. 23% of DM) in lactating dairy cows and reported no significant differences in total VFA concentrations between the 2 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 contents between the experimental diets. Additionally, the lack of a 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, because notably there was large variability in the lactational stage, milk yield, and DMI among the cows. As previously established, the provision of fermentable carbohydrates can decrease NH_3 production via a reduction in deamination and an increase in microbial uptake of NH_3 (Hristov et al., 2005), which was reflected in lower NH_3 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 pattern, as presented in our proof-of-concept study (Islam et al., 2023a).

Daily Pattern of VFA Profile Measured with Different Methods

Because of the lack of interaction between diet and VFA measurement method, the interaction between VFA measurement 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 Supplemental Table S3 (https://doi.org/10.3929/ethz-b-000638959; Islam et al., 2023b). 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, which was 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 2 h after feeding and continuing up to 2200 h and then decreased by midnight. Exhaled propionate discrepantly 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 for all 3 methods. Similarly for the LS diet, the VFA proportions fluctuated ($P < 0.01$) across the day. Unlike with 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, respectively (Figure 2B). The butyrate + isobutyrate showed no interaction ($P = 0.14$) with the method or 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, and 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 is presented in Supplemental Figure S1 (https://doi.org/10.3929/ethz-b-000638959; Islam et al., 2023b), and pH and rumen temperature measured in 3-h intervals are presented in Supplemental Figure S2 (https://doi.org/10.3929/ethz-b-000638959; Islam et al., 2023b).

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 the 3 methods revealed key dynamics and fluctuations influenced by the diet types, time of day, and measurement methods. There were marked differences in intraday variability of individual VFA molar proportions, regardless of method: acetate drastically increased after feeding, in accordance with the ingested feed and starch following fresh feed delivery, with a more gradual increase observed

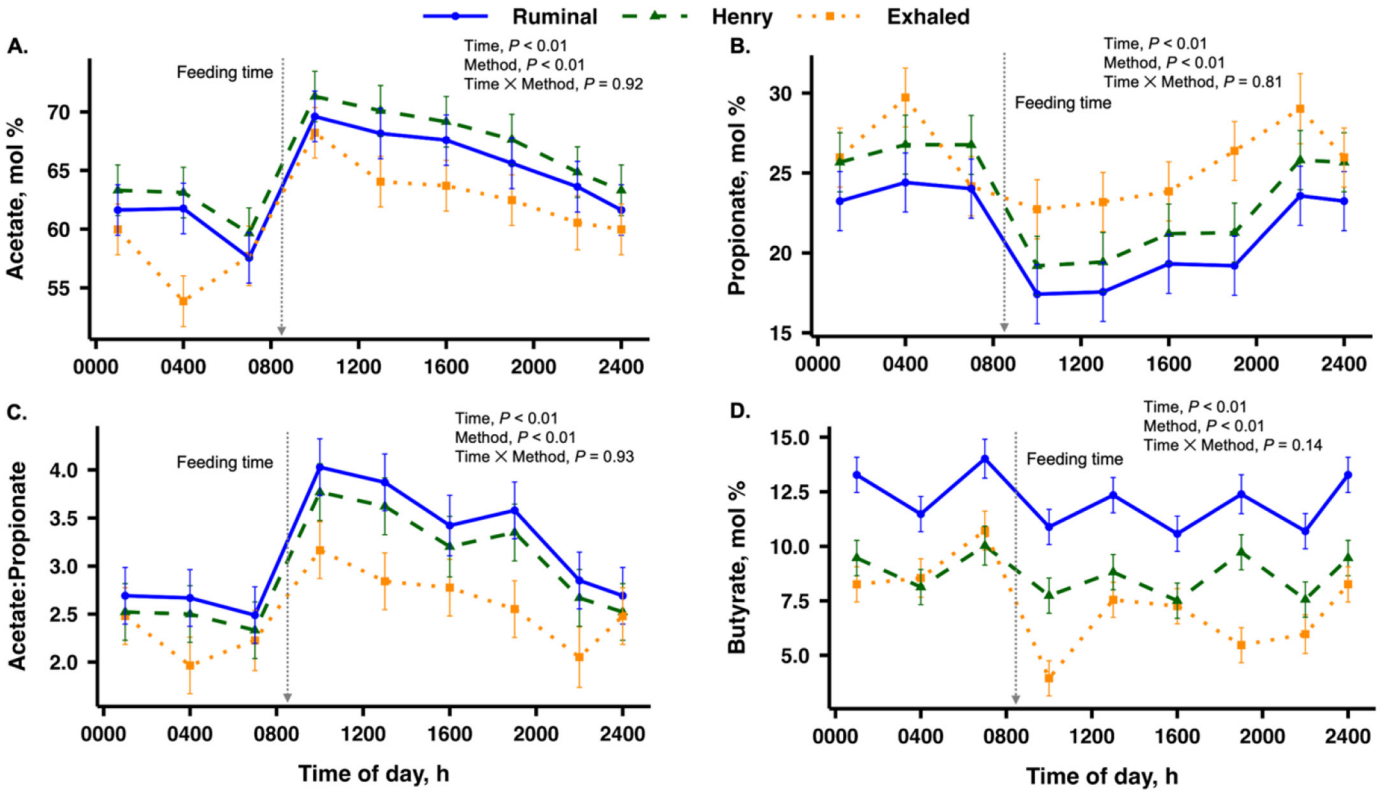

Figure 1. Molar proportions of (A) acetate, (B) propionate, (C) acetate:propionate ratio, and (D) butyrate measured in dairy cows fed a high-starch diet. These measurements were recorded in 3-h intervals from different methods and sources: ruminal (rumen fluid), Henry (predictions based on Henry's Law), and exhaled (exhalome). Cows were fed at 0830 h.

for propionate, A:P ratio, and butyrate + isobutyrate for the 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., 2023a). Furthermore, the ruminal pH data (Supplemental Figure S2A) supports the intraday fluctuations in the VFA proportions, especially acetate, regardless of method; the lowest pH coincided with the greatest acetate concentration and was greatest before feed delivery 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), which was also observed in our study (Supplemental Figure S1A). As follows, in the current study, the conditioned starch intake after feeding (Supplemental 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 intraday pattern, reflecting the rumen fermentation dynamics (Mao et al., 2008), as discussed in detail by Islam et al. (2023a). 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 diets, and the fluctuations were less pronounced with the LS diet given its extremely low starch content (6.31%). This can be expected because the rate of starch intake was notably lower postfeeding and throughout the day, which eventually affects the rate of fermentation and production of VFA (Hatew et al., 2015).

Furthermore, 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 varies 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 thus their concentrations in the gas phase of the rumen (Oertel et al., 2018). However, the relative abundance

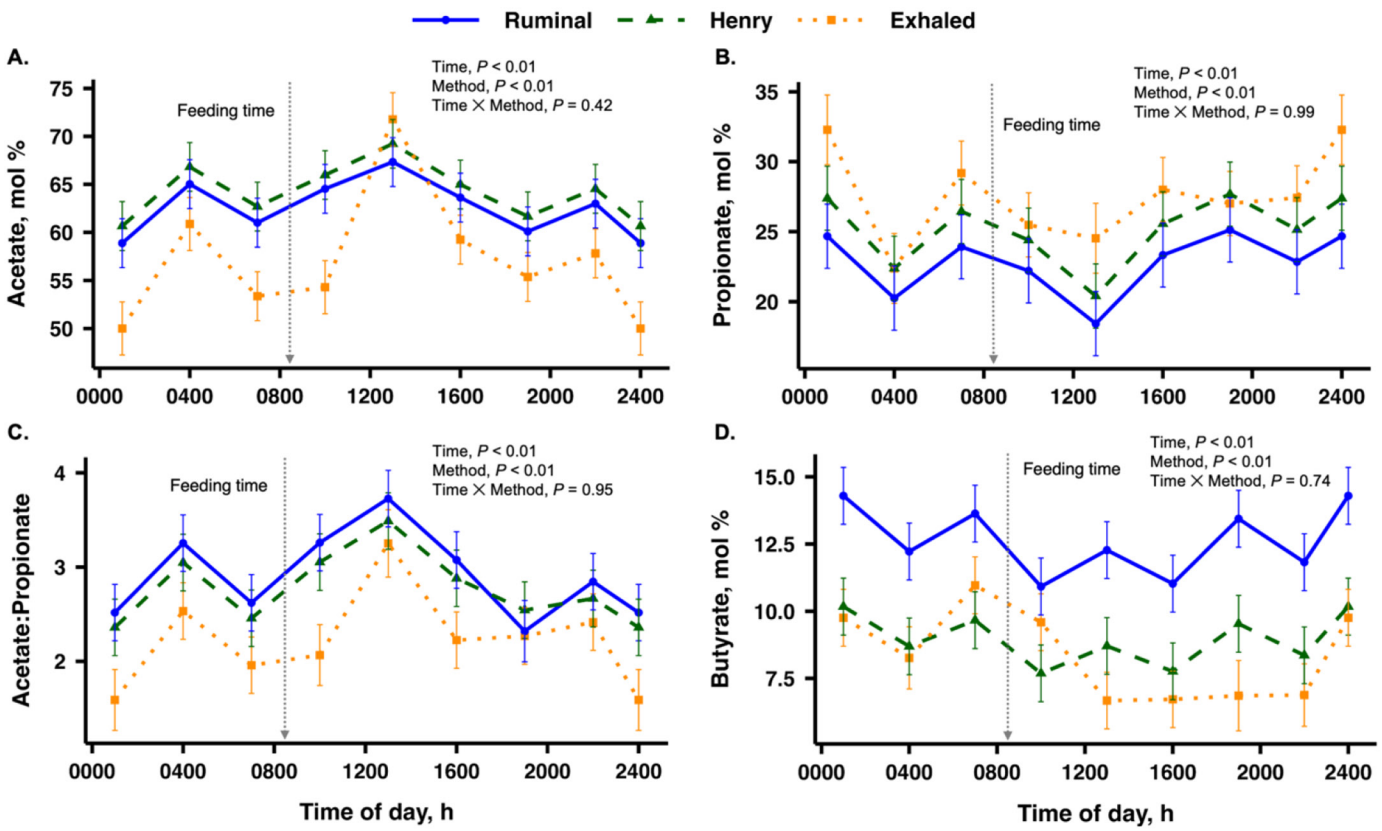

Figure 2. Molar proportions of (A) acetate, (B) propionate, (C) acetate:propionate ratio, and (D) butyrate measured in dairy cows fed a low-starch diet. These measurements were recorded in 3-h intervals from different methods and sources: ruminal (rumen fluid), Henry (predictions based on Henry's Law), and exhaled (exhalome). Cows were fed at 0830 h.

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 for acetate and A:P ratio in the current study, as well as in our previous study, especially with the HS diet (Islam et al., 2023a).

Additionally, 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 affecting 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 VFA. This highlights the multifaceted relationship between VFA concentration in the rumen and actual production rates (Dijkstra et al., 1993; Dijkstra,1994). According to Henry's Law, the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas above the liquid (Sander, 2015). Therefore, 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). Furthermore, 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 (Supplemental Figure S2). Thus the presence of each VFA in the rumen headspace, and subsequently in the EX samples, can differ in accordance with the factors mentioned above, thereby 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 the 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 and reveal 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 posi-

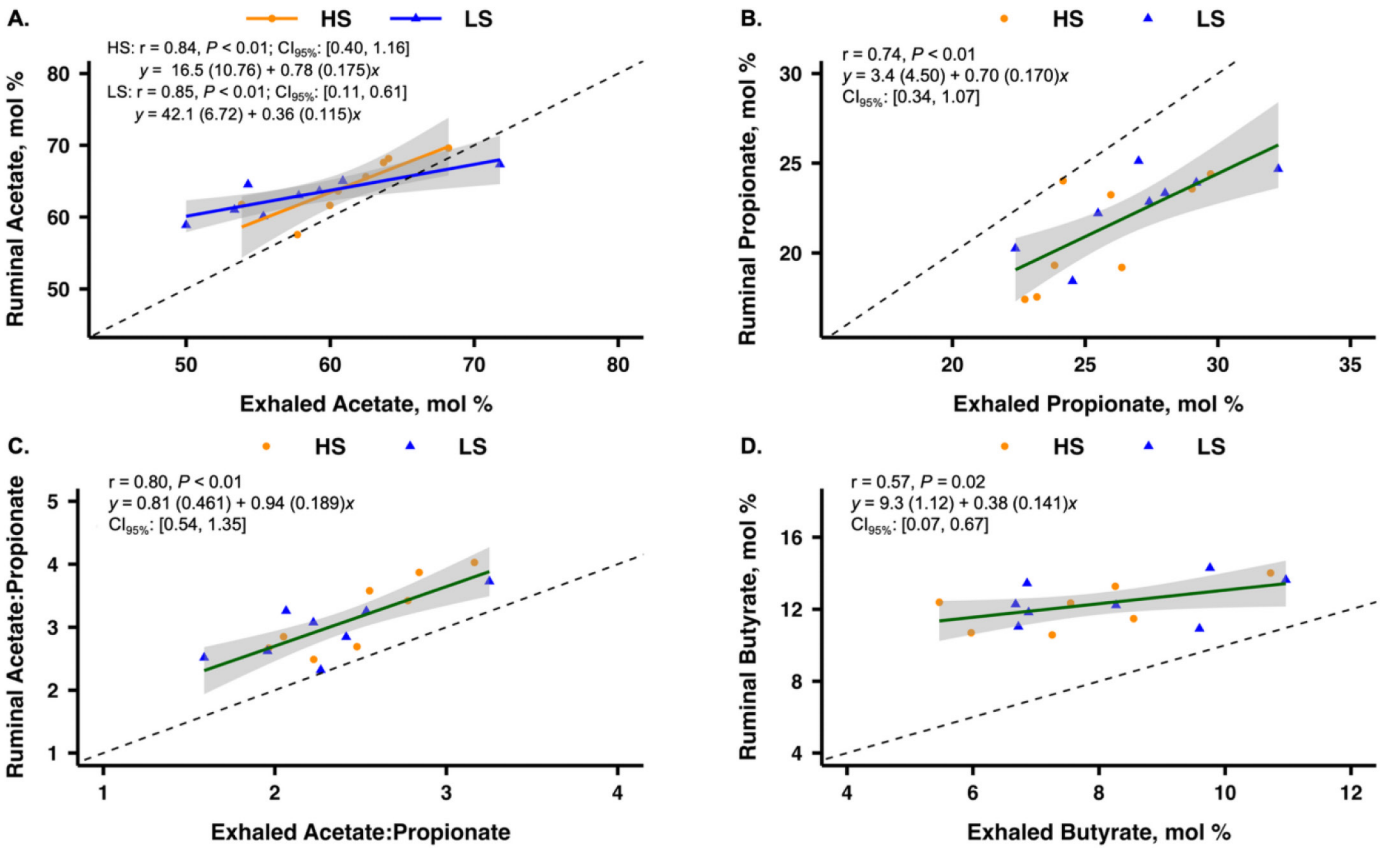

Figure 3. Correlation between the molar proportions of ruminal VFA and exhaled (EX) VFA, (A) acetate, (B) propionate, (C) acetate:propionate ratio, and (D) butyrate, using LSM from mixed model 2 outputs. The significant interaction term between diet (HS = high starch and LS = low starch) and EX-VFA is 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:propionate ratio ($P = 0.18$), and butyrate ($P = 0.74$). The black dashed line represents the identity relationship ($y = x$). The shaded area indicated 95% CI.

tive 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 ranged from 0.40 to 1.16 and from 0.11 to 0.61 for HS and LS diets, 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 existed between RM and EX (r = 0.57; $P = 0.02$). We extended a similar analysis by examining the relationship between HR-VFA and EX-VFA profiles (Supplemental Figure S3; https://doi.org/10.3929/ethz-b-000638959; Islam et al., 2023b) and found similar and stronger associations for acetate, propionate, and their ratios.

Further supporting the data discussed above, 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., 2023a). In particular, *1* (the 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 Supplemental Figure S3), further demonstrating the applicability of the exhalomics-based approach as a noninvasive 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 postfeeding, 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 noninvasively. To further establish this method, future research should focus on exploring diverse dietary treatments to characterize a broader range of rumen fermentation profiles.

ACKNOWLEDGMENTS

The research leading to these results has received funding from the NutriExhalomics project (grant agreement no. 15668, Agroscope, Bern, Switzerland). We extend our heartfelt appreciation to the dedicated barn staff led by Mirjam Klöppel (Strickhof, Lindau, Switzerland) and the research team led by Melissa Terranova (AgroVet-Strickhof, Eschikon, Lindau, Switzerland). Additional thanks go to Sirui Yang, Peng Rong, Meiqing Wang, and Xiaoge Sun (Animal Nutrition Group, ETH Zürich, Switzerland) for their assistance during the sampling process. The authors have not stated any conflicts of interest.

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