J. Dairy Sci. 108:2947–2963
https://doi.org/10.3168/jds.2024-25390


# Pathway mapping of exhaled volatile organic compounds associated with blood and ruminal fluid metabolites to describe the nutritional and metabolic status of lactating dairy cows

**J. Eichinger,[1,2] A.-M. Reiche,[1] P. Fuchsmann,[3] L. Eggerschwiler,[4] A. Münger,[1] K. Huber,[2] and F. Dohme-Meier[1]***
[1]Ruminant Nutrition and Emissions, Agroscope, 1700 Posieux, Switzerland
[2]University of Hohenheim, Institute of Animal Science, 70599 Stuttgart, Germany
[3]Human Nutrition, Sensory Analysis and Flavour, Agroscope, 3097 Bern, Switzerland
[4]Research Contracts Animals, Agroscope, 1700 Posieux, Switzerland

## ABSTRACT

Exhaled breath offers an interesting matrix for low invasive sampling of potentially relevant information about the organism's metabolism in the form of volatile organic compounds (VOC). The VOC can be exhaled by the ructus or pass the blood–lung barrier for expiration through the lungs. In this work, we consider exhaled breath as a mixture of VOC derived from the lungs and from the upper gastrointestinal tract. However, the informative value of exhaled breath in ruminants remains largely unstudied. The aim of this study was to identify exhaled VOC that could be used to assess the nutritional and metabolic status of dairy cows. To do so, we performed untargeted analysis of exhaled VOC from dairy cows, investigated their correlations with commonly analyzed blood and ruminal fluid metabolites and the calculated energy balance (EB), and explored the underlying pathways of correlated exhaled VOC. This was done as part of a feeding experiment in which 32 lactating Holstein dairy cows were assigned to 2 basal diets for 12 wk. Half of the cows were fed a hay-based diet, and the other half were fed a silage-based diet. During experimental wk 1 through 8, half of the cows in each basal diet group were supplemented with a control concentrate, and the other half received an experimental concentrate containing essential oils. During experimental wk 9 through 12, all cows received the control concentrate. Exhaled breath, blood, and ruminal fluid samples were collected every 4 experimental weeks (wk 4, 8, and 12) on 3 consecutive sampling days. Exhaled breath was analyzed for VOC, ruminal fluid for VFA and ammonia, and serum samples for albumin, total protein, urea, glucose, cholesterol, BHB, and nonesterified fatty acid (NEFA) concentrations. Pearson correlations were calculated to assess the associations between exhaled VOC and concentrations of blood and ruminal fluid metabolites and the calculated EB. Fifteen correlations were found between exhaled VOC (tetradecanal and γ-hydroxybutyrate [GHB], 3-penten-2-one, 4-hydroxy-4-methylpentan-2-one, 2-ethylhexanal, 2-ethylhexan-1-ol, p-cymene) and ruminal fluid (acetate, butyrate, valerate, and ammonia) and blood metabolite concentrations (BHB, NEFA, glucose, urea, and cholesterol) across the cow groups. The underlying pathways of 3-penten-2-one, GHB, and tetradecanal were mainly related to fat and protein catabolism and therefore to the actual animal metabolism. The correlations with the other 4 exhaled VOC, 4-hydroxy-4-methylpentan-2-one, p-cymene, 2-ethyl-hexan-1-ol, and 2-ethylhexanal, were diet- or time-related, specifically due to differences in feed ingredients. The results demonstrate the associations of single exhaled VOC with the nutritional and metabolic status of healthy dairy cows. Their potential as new biomarkers should be further investigated in cows in various nutritional and metabolic states.

**Key words:** volatilome, exhalomics, dairy cows, breath

## INTRODUCTION

Due to growing societal concerns and expectations regarding the handling of animals in livestock farming, veterinary practice and research call for noninvasive alternatives to current invasive interventions. Exhaled breath already serves as a noninvasive diagnostic tool in human medicine (Jones et al., 1992), where its analysis focuses on exhaled volatile organic compounds (**VOC**), which are small, volatile molecules (C6-C16; Schieweck et al., 2018) that are present in an organism after ingestion and inhalation or produced during metabolism (Tejero Rioseras et al., 2017). The VOC can be exhaled by the ructus (Islam et al., 2024) or passed through the blood–lung barrier for expiration through the lungs. In this work, we consider exhaled breath as a mixture of VOC derived from the lungs and from the upper gastro-

Received July 3, 2024.
Accepted November 25, 2024.
*Corresponding author: frigga.dohme-meier@agroscope.admin.ch

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

intestinal tract. The VOC are already routinely analyzed, for example, in ethanol breath tests (Jones et al., 1992) or in the diagnosis of intestinal bacterial overgrowth and lactose or fructose malabsorption in humans (Ghoshal, 2011). However, the use of exhaled VOC in ruminants and farm animals in general is still in its early stages and is mainly limited to targeted analyses. For example, exhaled very VOC (**VVOC**; <C6) may be used for ketosis detection in dairy cows by targeted analysis of exhaled acetone (Dobbelaar et al., 1996; van Erp-van der Kooij et al., 2023) and for describing ruminal fermentation through exhaled VFA during eructation from the rumen (Islam et al., 2024).

High-yielding dairy cows have high nutrient requirements, and meeting their nutritional needs is often challenging. Therefore, monitoring their nutritional and metabolic status is crucial to maintaining animal health and welfare. The nutritional and metabolic status in dairy cows is commonly evaluated by blood metabolites, which are still the most sensitive (Lee et al., 1978) compared with other matrices, such as milk or urine (Serrenho et al., 2022), but require invasive sampling. Commonly measured blood metabolites in dairy cows are blood total protein, albumin, and urea concentrations as possible indicators of protein metabolism or inflammation (Bobbo et al., 2017) and glucose, BHB, and nonesterified fatty acid (**NEFA**) concentrations for assessing energy metabolism (Peng et al., 2019). Although these blood metabolites are not purely diet-derived, they remain relevant indicators of the cows' nutritional and metabolic status. Because up to 75% of the metabolizable energy is provided by fermentation products (Bergman, 1990), these may also be used to assess a cow's nutritional and metabolic status. Fermentation end products, such as VFA and ammonia, are analyzed in ruminal fluid, which is commonly obtained through invasive sampling. Thus far, assessing the nutritional and metabolic status of dairy cows in a less-invasive manner through exhaled breath primarily targeted ketosis diagnosis by measuring the exhaled VVOC acetone. The potential informative value of exhaled VOC has been scarcely studied.

Therefore, the aim of the present study was to evaluate the informative value of exhaled VOC regarding the nutritional and metabolic status of dairy cows. We intended to achieve this by investigating correlations between exhaled VOC and metabolically relevant blood and ruminal fluid metabolites, as well as with the calculated energy balance (**EB**) and mapping their pathways. We hypothesized links between exhaled VOC and metabolite concentrations of blood and ruminal fluid and the calculated EB. Because blood and ruminal fluid metabolite concentrations are influenced by factors such as diet and physiological status such as the lactation stage (Bergman, 1990; Peng et al., 2019), we induced a certain between-animal variance by feeding the cows with 2 different basal diets. Additionally, we temporarily challenged them with a feed additive that included essential oils, which have been shown to modulate ruminal fermentation (Abdillah et al., 2024).

## MATERIALS AND METHODS

### *Animals and Experimental Design*

The experimental protocol complied with Swiss animal welfare legislation and was approved by the Animal Care Committee of the Fribourg Canton, Fribourg, Switzerland (license no. 2020–58-FR/32975). The study was part of a larger experiment conducted at Agroscope (Posieux, Switzerland) from January to April 2021 aimed at evaluating the usability of VOC of different biological matrices for describing diet-specific metabolic changes in dairy cows (Eichinger et al., 2025). The study lasted 12 wk and was a 2-factorial experiment, with the basal diet and concentrate type as the 2 factors. Thirty-two lactating primiparous (n = 16) and multiparous (n = 16, 4 ± 2 lactations) Holstein Friesian and Red Holstein dairy cows were housed in a freestall barn. At the beginning of the study, the cows were, on average, 111 ± 31.4 DIM and produced 31.3 ± 4.73 kg milk/d. Four weeks before starting the experiment, the cows were allocated to 2 basal diets, balanced for DIM, milk yield, and lactation number. Throughout the experiment, half of the cows were fed a basal diet consisting of hay (**HD**, n = 16), and the other half were fed a silage-based diet (**SIL**, n = 16) consisting of corn silage, grass silage, and hay (44%:43%:13% on a DM basis). Basal diets were offered for ad libitum intake, and fresh feed was provided 3 times a day at 0500 h, 0900 h, and 1600 h. In addition to the basal diet, the SIL cows received 1.5 kg/d of a protein concentrate (55% soybean meal, 29% corn gluten, 10% potato protein, 4% molasses, 2% animal fat). From a milk yield of 27 kg/d and 30 kg/d (for primiparous and multiparous cows, respectively), they received additionally 0.5 kg of an energy concentrate (50% corn, 30% barley, and 20% wheat) per kilogram of additional milk produced. The HD cows received 3 kg/d of the same energy concentrate, and from a milk yield of 27 kg/d and 30 kg/d (for primiparous and multiparous cows, respectively), they received 0.6 kg of the energy concentrate per kilogram of additional milk produced. The maximum amount of protein and energy concentrate mixture (**CON**) fed was set at 6 kg/cow per day. During experimental wk 1 through 8, half of the HD cows (n = 8) and half of the SIL cows (n = 8), again balanced for DIM, milk yield, and lactation number, received an experimental concentrate (**EXP**). The EXP corresponded to the CON, which was replaced at 0.34% (i.e., 0.68% of the corn) with a feed additive (Xtract

Ruminant, Pancosma, Rolle, Switzerland) containing a blend of the essential oils eugenol, cinnamaldehyde, and capsicum. Concentrates were fed via a transponder feeding station (RIC system; Insentec/Hokofarm, Marknesse, the Netherlands). During experimental wk 9 through 12, all cows received the CON concentrate. The approach of feeding the cows with the essential oils for 8 wk (wk 1–8) followed the principle of interventional studies (Aggarwal and Ranganathan, 2019), which included intervention (wk 1–8) and post-intervention (wk 9–12) study phases. The chemical compositions of the basal diets and concentrate types are shown in Table 1. All cows had free access to fresh water and received a mineral feed according to their requirements (Agroscope, 2021).

### Data Recording and Sampling

Silage samples were collected once a week throughout the study; concentrate and hay samples were collected every 4 wk. We reserved 200 g of all fresh feed samples at −20°C for further analysis of VOC, and the remainder was prepared for analysis of their chemical composition. Throughout the study, feed intake was continuously recorded using electronic weighing troughs with controlled-access gates (RIC system; Insentec/Hokofarm Group BV, Marknesse, the Netherlands). The cows were milked twice a day at 0500 h and 1600 h in a tandem milking parlor (Fullwood, A. Bertschy AG, Guschelmuth, Switzerland). Milk yield was recorded automatically, and twice a week, individual milk samples were collected in the evening and pooled with a milk sample from the following morning milking based on the respective milk yields. Of these pools, a 50-mL aliquot was preserved with bronopol (2-bromo-2-nitropropane-1,3-diol) for further chemical composition analysis. Every 4 experimental weeks, that is, sampling wk 4, 8, and 12 (**W4**, **W8**, and **W12**, respectively), blood, exhaled breath, and ruminal fluid samples were collected on 3 consecutive sampling days, immediately after the 0500 h milking. The same time was chosen on each sampling day to minimize the effect of feeding and circadian fluctuations on the concentration of metabolites (Couperus et al., 2021b). On the first sampling day, blood samples were collected. For this purpose, the cows were moved to a head gate in the covered outdoor area of the barn to draw blood from the jugular vein into a 9-mL serum tube containing a clot activator (Greiner Bio-One GmbH, Kremsmünster, Austria). The samples were stored for 1 h at room temperature until further preparation. The Vacuette blood collection tubes were centrifuged at 3,000 × *g* for 15 min at 5°C and afterward at 4,000 × *g* for 2 min at 5°C. The supernatant serum was transferred into 1.5-mL tubes and stored at −20°C until analysis. On the second sampling day, exhaled breath samples were collected. The breath sampling approach was based on solid-phase extraction (**SPE**) cartridges containing a highly porous polystyrene-divinylbenzene copolymer HR-P, 50 to 100 µm, 3 mL/200 mg (Macherey-Nagel, Oensingen, Switzerland). The SPE cartridges (2 per cow) were conditioned with Milli-Q ultrapure water (Millipore Sigma), methanol, acetone, and acetonitrile before use in a technical setup for sampling exhaled VOC for 3 min, following the method described by Eichinger et al. (2024). The used SPE cartridges were then stored in the refrigerator until further analysis. On the third sampling day, ruminal fluid was collected from each cow, as described in Eichinger et al. (2025). Subsequently, one 15-mL sample for further VFA analysis and a second sample for further ammonia analysis were stabilized and stored as described by Lazzari et al. (2023). A third 15-mL sample was stored on ice, and after sampling of all cows (~2 h later), the samples were frozen at −20°C until VOC analysis.

### Laboratory Analysis

***Feed Samples.*** Feed samples were ground to pass a 1.0-mm sieve (Brabender mill with titanium blades; Brabender, Duisburg, Germany) for further determination of the DM, ash, OM, NDF, ADF, water-soluble carbohydrate (**WSC**), total nitrogen, and CP contents, as described by Lazzari et al. (2023). Feed samples were analyzed in duplicate at Agroscope, Posieux, Switzerland, within its ISO 17025-accredited feed laboratory (STS077; Swiss-Accreditation-Service-SAS, 2024).

***Milk Samples.*** Bronopol-conserved milk was analyzed for fat, protein, and urea contents at SuisseLab (Zollikofen, Switzerland), using a Milkoscan FT6000 (Foss Electric, Hillerød, Denmark).

***Blood Samples.*** After thawing the serum samples at room temperature, they were analyzed using commercially available kits according to the manufacturers' instructions on a BT1500 autoanalyzer (Biotecnica Instruments Ltd., Rome, Italy) to determine albumin, total protein, urea (Greiner Diagnostic GmbH, Langenthal, Switzerland), glucose, cholesterol (Biotecnica Instruments Ltd., Rome, Italy), NEFA, and BHB (Randox Laboratories Ltd., Crumlin, UK) concentrations. The samples were analyzed in duplicate.

***Ruminal Fluid Samples.*** Ruminal VFA concentrations were analyzed by HPLC (Shodex RI, Denko KK, Minato, Japan) equipped with a refractive index detector and a polymer-based HPLC phase column (Nucleogel ION 300 OA; 300 × 7.8 mm, Macherey-Nagel, Düren, Germany). After thawing, samples were centrifuged at 1,500 × *g* for 1 min at 5°C. The supernatant was collected and 1 volume of deproteinizing solution (0.15 mol/L $NaWO_4$, $2H_2O$, and 25 mmol/L $H_2SO_4$) was added, vortexed, and centrifuged again at 10,000 × *g* for 10 min at 5°C. The

**Table 1.** Chemical composition of hay (n = 3), silage (n = 12), control concentrate (n = 3), experimental concentrate (n = 3), and protein concentrate (n = 3)[1]

| | HD | | | | SIL | | | | Energy concentrate[2] | | | | Protein concentrate | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | W4 | W8 | W12 | | W4 | W8 | W12 | | CON | | EXP | | | |
| Item (g/ kg DM) | Mean | Mean | Mean | SD[3] | Mean | Mean | Mean | SD[3] | Mean | SD[3] | Mean | SD[3] | Mean | SD[3] |
| DM (g/kg of wet weight) | 910 | 915 | 907 | 4.28 | 440 | 433 | 435 | 9.17 | 893 | 1.80 | 982 | 1.56 | 901 | 4.45 |
| OM | 887 | 904 | 900 | 8.86 | 929 | 934 | 934 | 4.29 | 980 | 2.07 | 981 | 0.52 | 955 | 1.94 |
| CP | 157 | 162 | 160 | 2.64 | 123 | 119 | 111 | 6.17 | 113 | 4.02 | 112 | 2.91 | 552 | 17.6 |
| NDF | 493 | 477 | 460 | 16.6 | 420 | 426 | 409 | 18.0 | 142 | 18.2 | 151 | 13.7 | 116 | 14.3 |
| ADF | 272 | 257 | 253 | 10.0 | 258 | 254 | 241 | 8.56 | 45.7 | 8.24 | 44.3 | 0.76 | 48.7 | 1.99 |
| Starch[4] | — | — | — | — | 178 | 177 | 194 | 16.4 | 672 | 24.1 | 663 | 10.3 | 127 | 17.2 |
| WSC[5] | 103 | 112 | 110 | 4.59 | 53 | 47 | 41 | 5.79 | 32.0 | 2.58 | 38.9 | 3.69 | 72.4 | 4.06 |
| Calculated energy and protein supply[6] (per kg of DM) | | | | | | | | | | | | | | |
| APD | 92.0 | 95.0 | 94.0 | 1.53 | 76.7 | 77.1 | 76.1 | 0.50 | 105 | 1.20 | 105 | 0.60 | 353 | 1.30 |
| NEL (MJ/kg of DM) | 5.50 | 5.70 | 5.60 | 0.10 | 6.24 | 6.31 | 6.26 | 0.04 | 8.3 | 0.10 | 8.30 | 0.10 | 8.41 | 0.10 |

[1]Given as g/ kg of DM, unless otherwise noted; means ± SD. Means for HD and SIL separately for each sampling week and for energy and protein concentrate over the 3 sampling weeks. HD = 100% hay; SIL = 44% grass silage, 43% corn silage, and 13% hay. W4, W8, and W12 = sampling wk 4, 8, and 12, respectively.

[2]CON = control concentrate; EXP = experimental concentrate containing a blend of essential oils (XtractRuminant, Pancosma, Rolle, Switzerland; 1 g/day per cow).

[3]SD was calculated from the analyzed chemical compositions of each feed sample taken.

[4]A dash (—) indicates that HD was not analyzed for starch.

[5]WSC = water-soluble carbohydrates.

[6]Absorbable protein at the duodenum (APD) and NEL was calculated according to Agroscope (2021).

concentration of $NH_3$-N in ruminal fluid was analyzed by colorimetry using a commercial test kit (Urea liquicolor 10505, Human, Wiesbaden, Germany). The samples were analyzed in duplicate.

***VOC Analysis.*** Within 2 h after exhaled breath sampling, the exhaled VOC were eluted from the SPE cartridges using 600 µL of acetonitrile. For VOC analysis of the feed samples, 200 g from each of the taken samples of each feed type (silage, hay, and concentrates) were pooled by feed type. From the pooled samples, 50 g of the respective feed samples were placed in a robust container with liquid nitrogen. After 1 min, the samples were transferred to a mixer (Robot coupe, Blixer 4 V.V, Pitec, Oberriet, Switzerland) for grinding. A total of 100 mg of the respective ground feed samples were mixed with 250 µL of water and 25 µL of an internal deuterated standard consisting of 100 µg/L dimethyl sulfide-d6 and 10 µg/L dimethyl sulfoxide-d6. Quality control (**QC**) samples of the eluted exhaled breath, ruminal fluid (after thawing at room temperature), and feed samples were prepared as described by Eichinger et al. (2024). Then, 100 µL of the samples and the QC samples were put in 20-mL headspace vials and randomized as described in Eichinger et al. (2024) for further VOC analysis. Nontargeted analyses of VOC were performed using dynamic headspace vacuum in-tube extraction (**DHS-V-ITEX**; CTC Analytics, Zwingen, Switzerland) GC/MS based on vacuum transfer in-tube extraction (**DHS-VTT**) developed by Fuchsmann et al. (2019). The DHS-V-ITEX-GC-MS consisted of an MPS Robotic autosampler (Gerstel, Sursee, Switzerland) equipped with the V-ITEX module and coupled with a vacuum pump Büchi V-300 and an interface I-300 (Büchi, Flawil, Switzerland). An Agilent 7890B GC system (Agilent Technology, Santa Clara, CA) coupled to an Agilent 5977B mass selective detector (Agilent Technology, Santa Clara, CA) completed the installation. After 10 min of incubation at 60°C, the headspace was extracted for 10 min at 60°C under vacuum (1,500 Pa) using the vacuum and ITEX tool equipped with a trap filled with a sorbent consisting of Tenax TA (2/3 bottom)/Carbosieve S III (1/3 top; ITEX2, Brechbühler, Switzerland) according to Fuchsmann et al. (2019). The trap temperature was maintained at 35°C during the whole extraction. The exhaled VOC were desorbed from the sorbent for 2 min at 300°C at a rate of 12°C/s under a nitrogen flow of 150 to 180 mL/min. The cooled injection system (CIS 4, Gerstel, Sursee, Switzerland) was equipped with a glass liner filled with Tenax TA, which was conditioned at 250°C for 60 min directly into the injector with the carrier gas helium before analysis. The injector temperature was stabilized at 10°C throughout the entire injection process using a cold air flow from compressed air at 1,500 Pa and a cryostat (D8-G, Haak, Karlsruhe, Germany) set at −20°C with propylene glycol. Next, the injector was heated to 250°C at a rate of 12°C/s to release the VOC in the column. The purge flow to the split vent was set at 100 mL/min after 2 min. The VOC were separated on an Optima-FFAP-Plus fused silica capillary column (100% polyethylene glycol with nitroterephthalic acid, bonded and cross-linked, 60 m × 0.25 mm × 0.5 µm film; Macherey-Nagel AG, Oensingen, Switzerland) with helium as the carrier gas at a flow of 0.8 mL/min (corresponding to a velocity of 23 cm/s). The oven temperature was programmed as follows: 5 min at 40°C, then heated to 230°C at a rate of 5°C/min with a final hold time of 17 min. The MS settings were as follows: transfer line at 230°C and source temperature at 230°C. The analytes were monitored in SCAN mode between 42.5 and 350 amu, with a gain at 1 and a solvent delay of 5 min. The CIS 4 injector was controlled with Maestro1 software V.1.4.8.14/3.5 (Gerstel, Sursee, Switzerland). The VOC concentrations presented in the manuscript refer to the relative concentrations determined from the VOC peak area based on the total ion count (arbitrary unit).

### Data Processing and Statistical Analysis

Feed intake, milk yield, and BW data were averaged per week and per cow. The EB per cow averaged per week was calculated as the difference between the net energy intake from ingested feed and the energy needed for milk production (NEL) and for maintenance ($NE_M$) calculated according to Agroscope (2021). A linear mixed model (R version 4.1.3, package lme4; Bates et al., 2015) was used for the statistical analysis of EB, feed intake, milk yield, and blood and ruminal fluid metabolites. Basal diet, concentrate type, sampling week, and their interactions (basal diet × sampling week, concentrate type × sampling week, basal diet × concentrate type) were included as fixed effects and animal as a random factor. Where significant interactions were found, the Tukey test was used for comparisons between subgroups. For all analyzed parameters, nonsignificant interactions were excluded from the tables. $P$-values <0.05 were considered significant, and trends were discussed at $P < 0.10$. In the Results, the means are presented with the corresponding SEM. For VOC data, deconvolution of MS signals was performed using Masshunter Profinder software (version 10.0) in recursive mode (Agilent Technologies, Santa Clara, CA). During deconvolution, VOC with MS signals less than 3 times the median of the background peak height were eliminated. Missing values after automatic deconvolution due to signals below the detection limit were set to zero, according to Xia et al. (2009). Manual peak integration was performed using MassHunter Quantitative Analysis software (version 12.1; Agilent Technologies, Santa Clara, CA). The VOC data were normalized using probabilistic quotient normalization (Dieterle et al.,

2006). Because the variables were normally distributed, as assessed by the Shapiro-Wilk test, Pearson correlations were calculated using MetaboAnalyst (version 6.0; Xia et al., 2009) to assess the associations between each exhaled VOC and each blood (BHB, NEFA, glucose, total protein, albumin, cholesterol, and urea) and ruminal fluid (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, and ammonia) metabolite concentration and the calculated EB for each sampling week separately across the different diets.

To present only consistent correlations between exhaled VOC and ruminal fluid and blood metabolite concentrations, and the calculated EB, correlations were selected as follows. After automatic deconvolution of the MS signals, we selected correlated exhaled VOC with Pearson correlation coefficients (1) presenting the same sign (direction) in all 3 experimental weeks and (2) presenting a significant $r > |0.35|$ in at least 2 sampling weeks. This initial step revealed 124 pairs of correlations with 81 correlated exhaled VOC. To ensure correct peak identification and integration of these exhaled VOC, their GC-MS peaks were manually integrated. We redid the Pearson correlations using the integrated peaks to verify the identified correlations. Next, we selected 15 pairs of correlations with 7 correlated exhaled VOC with Pearson correlation coefficients: (3) presenting the same sign (direction) in all 3 sampling weeks and (4) presenting a significant $r > |0.35|$ in at least one sampling week. To assess the found correlations across all sampling weeks, analysis of covariance (**ANCOVA**; R version 4.3.3; package car) using the function lm() was applied using the exhaled VOC as the explained variable, the blood or ruminal fluid metabolite of interest as the explanatory variable, and basal diet, concentrate type, sampling week, and animal as covariates. Because the interaction basal diet × concentrate type was not significant ($P < 0.05$), it was excluded from the model. For all analyzed parameters, nonsignificant interactions were excluded from the tables. To differentiate between VOC derived from animal metabolism, rumen microbial metabolism, and VOC derived from feed, we investigated whether the correlated exhaled VOC were already present in feed samples. The National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17; NIST, Gaithersburg, MD) was used for peak identification of the VOC. All VOC presented in this work were identified at the identification levels 1 and 2 (Sumner et al., 2007): at level 1, VOC were identified by comparing their spectrum with the database (minimum match factor of 90%) and their calculated retention index (**RI**) with the reference RI from the literature (maximum relative difference of about ±10–15). Level 2 corresponded to spectra with a match factor >80% and a maximum relative difference in the calculated RI of ±15 of the reference RI. The RI was calculated using the temperature-programmed RI (Eichinger et al., 2024). The identified exhaled VOC were linked to their potential underlying pathways by literature research and were visualized using BioRender.com.

## RESULTS

The concentrate type had no effect on any measured trait ($P > 0.05$) and is therefore not discussed further. The data are not presented.

### Feed Intake and Milk Production

Across all experimental weeks, total DMI was 22.6 kg/d ± 0.13 and 22.9 kg/d ± 0.16, concentrate intake was 4.96 kg/d ± 0.07 and 3.92 kg/d ± 0.09, CP intake was 3.31 kg/d ± 0.02 and 3.27 kg/d ± 0.02, NDF intake was 9.34 kg/d ± 0.05 and 8.67 kg/d ± 0.07, starch intake was 2.86 kg/d ± 0.05 and 4.87 kg/d ± 0.06, and NEL intake was 149.30 ± 12.14 MJ NEL/d and 150.32 MJ NEL/d ± 14.89 for HD and SIL cows, respectively. The latter intakes were similar for the 2 concentrate types (all $P \geq 0.37$). A detailed illustration of the intake has been described previously (Eichinger et al., 2025). The daily milk yield was 30.2 kg/d ± 0.23 and was not affected by the basal diets ($P = 0.23$) or the sampling week ($P = 0.18$). Milk protein (3.56% ± 0.02) and lactose percentage (4.81% ± 0.01) were similar in the HD and SIL cows ($P = 0.59$ and $P = 0.98$, respectively). Milk protein increased over the sampling weeks ($P < 0.01$), whereas milk lactose percentage did not vary by sampling week ($P = 0.43$). The milk yield and the milk protein percentage differed between the basal diet groups and the sampling weeks, resulting in a diet × sampling week interaction ($P < 0.05$). Milk fat percentage tended ($P = 0.06$) to be lower in HD cows (4.43% ± 0.01) compared with SIL cows (4.79% ± 0.08) and was not affected by the sampling week ($P = 0.32$). The EB of the cows ranged between −30 and +34 MJ NEL and tended to be higher ($P = 0.09$) for HD cows (10.84 ± 1.30) compared with SIL cows (6.06 ± 1.50). The sampling week had no effect on EB ($P = 0.76$). In W4, W8, and W12, 14 cows (44%), 19 cows (59%), and 9 cows (28%), respectively, were in negative EB (**NEB**).

### Ruminal Fluid Metabolites

The means and ranges of ruminal fluid metabolites over the 3 sampling weeks are shown in Table 2. Total ruminal VFA concentrations were not influenced by basal diet ($P = 0.58$) and increased from W4 to W12 ($P < 0.01$). The ruminal fluid of HD cows contained higher proportions

of acetate ($P = 0.01$) and ammonia ($P < 0.01$), lower proportions of butyrate, isobutyrate, and isovalerate (all $P < 0.01$), and tended to have a lower valerate proportion ($P = 0.06$) than that of SIL cows. Proportions of isobutyrate ($P = 0.02$) and isovalerate ($P < 0.01$) varied by sampling week, whereas those of acetate, propionate, isobutyrate, valerate, and isovalerate varied by sampling week based on the basal diet without showing a distinct pattern (all interactions: $P < 0.01$).

### Blood Metabolites

All measured blood metabolite concentrations are presented in Table 3 as means and ranges. Of all the blood metabolites measured (all $P > 0.05$), only the NEFA concentration was tendentially influenced by the basal diet, tending to be lower in HD cows compared with SIL cows ($P = 0.08$). Concentrations of albumin and urea differed ($P = 0.01$) and tended to differ ($P = 0.08$), respectively, between the sampling weeks, whereas the other metabolites were not affected by them (all $P > 0.05$). No interactions between basal diet and sampling week were present (all $P > 0.05$).

### Correlations Between Exhaled VOC and Ruminal Fluid or Blood Metabolites

Of the 2,771 exhaled VOC detected, 7 exhaled VOC were correlated to ruminal fluid (acetate, butyrate, valerate, ammonia) and blood metabolite concentrations (BHB, NEFA, glucose, urea, cholesterol) in at least one sampling week. The identification characteristics of the 7 exhaled VOC are shown in Table 4. Correlations between exhaled VOC and ruminal fluid metabolites are presented in Table 5, and for one sampling week, as plots in Supplemental Figure S1 (see Notes). Negative correlations were found between exhaled 3-penten-2-one and butyrate proportions, γ-hydroxybutyrate (**GHB**) and valerate proportions, 4-hydroxy-4-methylpentan-2-one and acetate proportions, 2-ethylhexanal and butyrate proportions, 2-ethylhexan-1-ol and butyrate proportions, and between p-cymene and ammonia concentrations. Positive correlations were found between GHB and acetate proportions, and between 2-ethylhexanal and acetate proportions. When ANCOVA was applied, the associations between 3-penten-2-one and butyrate proportions, between GHB and acetate proportions, between GHB and valerate proportions, and between 2-ethylhexanal and acetate proportions (all $P \leq 0.03$) were maintained independently of the basal diet and sampling week (Table 4). Most of the correlated exhaled VOC were affected by the diet (with $P \leq 0.01$) or sampling week (with $P \leq 0.01$) or both. No correlations were found between exhaled VOC and proportions of propionate, isobutyrate, or isovalerate (data not shown).

Regarding the blood metabolites, negative correlations were found between exhaled 3-penten-2-one and NEFA concentrations, tetradecanal and BHB concentrations, GHB and glucose concentrations, and between 4-hydroxy-4-methylpentan-2-one and BHB concentrations (Table 6; Supplemental Figure S2, see Notes; for one sampling week). Positive correlations were found between tetradecanal and NEFA concentrations, GHB and urea concentrations, and between 4-hydroxy-4-methylpentan-2-one and cholesterol concentrations (Table 6, Supplemental Figure S2, for one sampling week). The results of the ANCOVA showed that the associations between 3-penten-2-one and NEFA concentrations, between tetradecanal and BHB concentrations, between tetradecanal and NEFA concentrations, between GHB and glucose concentrations, and between GHB and urea concentrations were maintained (all $P \leq 0.04$) independent of the basal diet and sampling week (Table 5). Most of the correlated exhaled VOC were affected by the diet ($P \leq 0.01$) or sampling week ($P \leq 0.04$) or both. No correlations were found between exhaled VOC and concentrations of total protein and albumin (data not shown). No correlations were found between exhaled VOC and the calculated EB (data not shown). p-Cymene and 4-hydroxy-4-methylpentan-2-one were also present in basal diet and concentrate samples, with their highest concentrations found in silage samples. 3-Penten-2-one, tetradecanal, GHB, 2-ethyl-hexan-1-ol, and 2-ethylhexanal were not detected in any of our feed samples. Furthermore, we detected 2-ethylhexanal and 2-ethylhexan-1-ol (both higher in HD cows compared with SIL cows), as well as 4-hydroxy-4-methylpentan-2-one, p-cymene, tetradecanal, and GHB (all higher in SIL cows compared with HD cows) in ruminal fluid samples of both SIL and HD cows. In contrast, 3-penten-2-one was not detected in the ruminal fluid samples.

## DISCUSSION

Our study employed a nontargeted metabolomics approach to identify correlations between exhaled VOC and commonly analyzed ruminal fluid and blood metabolite concentrations. The aim was to investigate the informative value of exhaled VOC regarding the nutritional and metabolic status of dairy cows and the underlying metabolic pathways. To achieve a certain between-animal variation in ruminal fluid and blood metabolite concentrations, the cows were fed 2 different basal diets and challenged with a concentrate containing essential oils. Interestingly, essential oil supplementation had no effect on any of the traits measured, in contrast to earlier

**Table 2.** Means, minimum, and maximum of ruminal fluid metabolites of hay (n = 16) and silage (n = 16) fed dairy cows over 3 sampling weeks[1]

| | Sampling week[2] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | W4 | | W8 | | W12 | | | *P*-value | | |
| Item | HD | SIL | HD | SIL | HD | SIL | SEM | Diet | Week | Diet × week |
| Total VFA (mmol/ L) | 74.42 [62.9–103] | 75.63 [51.9–108] | 77.05 [63.2–94.3] | 78.40 [68.3–91.1] | 87.57 [76.5–104] | 82.74 [71.5–95.1] | 1.35 | 0.58 | <0.01 | 0.56 |
| VFA (mol %) | | | | | | | | | | |
| n-Acetate | 69.34$^{bc}$ [66.9–74.7] | 67.23$^{b}$ [65.0–70.4] | 68.58$^{bc}$ [63.6–71.5] | 67.73$^{bc}$ [64.4–71.1] | 69.29$^{c}$ [66.1–72.1] | 65.35$^{a}$ [62.2–68.9] | 0.25 | 0.01 | 0.22 | <0.01 |
| n-Propionate | 16.79$^{ab}$ [14.4–18.4] | 16.49$^{a}$ [14.6–22.4] | 16.81$^{ab}$ [14.0–21.6] | 16.48$^{a}$ [14.2–20.1] | 16.69$^{ab}$ [14.9–17.9] | 18.14$^{b}$ [15.3–22.0] | 0.18 | 0.32 | 0.62 | <0.01 |
| n-Butyrate | 11.57 [8.00–13.6] | 13.24 [12.3–14.9] | 11.92 [9.51–13.6] | 12.96 [10.4–14.5] | 11.61 [9.40–13.9] | 13.11 [12.3–14.4] | 0.13 | <0.01 | 0.43 | 0.32 |
| Isobutyrate | 0.60$^{a}$ [0.51–0.88] | 0.76$^{bc}$ [0.71–0.87] | 0.71$^{abc}$ [0.52–1.02] | 0.75$^{c}$ [0.53–0.91] | 0.64$^{ab}$ [0.50–0.87] | 0.85$^{d}$ [0.76–1.09] | 0.01 | <0.01 | 0.02 | <0.01 |
| n-Valerate | 0.95$^{a}$ [0.54–1.10] | 1.07$^{a}$ [0.88–1.27] | 0.99$^{a}$ [0.83–1.17] | 1.02$^{a}$ [0.77–1.25] | 0.95$^{a}$ [0.74–1.07] | 1.26$^{b}$ [1.00–1.62] | 0.02 | 0.06 | 0.38 | <0.01 |
| Isovalerate | 0.75$^{a}$ [0.54–1.17] | 1.21$^{de}$ [0.94–1.27] | 0.99$^{bc}$ [0.64–1.70] | 1.05$^{cd}$ [0.59–1.37] | 0.82$^{ab}$ [0.60–1.12] | 1.29$^{e}$ [1.03–1.62] | 0.03 | <0.01 | <0.01 | <0.01 |
| Ammonia (mmol/L) | 2.15$^{b}$ [0.81–3.21] | 1.43$^{a}$ [1.00–2.38] | 2.39$^{b}$ [1.00–3.35] | 1.24$^{a}$ [0.46–3.6] | 2.57$^{b}$ [1.23–5.11] | 1.17$^{a}$ [0.58–2.11] | 0.09 | <0.01 | 0.03 | 0.01 |

[a–e]Means within a row with different superscripts vary due to the diet × sampling week interaction at $P < 0.05$.

[1]Arithmetic group means [minimum–maximum] of dairy cows fed a hay- (HD; n = 16) or a silage-based (SIL; n = 16) diet. HD = 100% hay-fed cows; SIL = 44% grass silage, 43% corn silage, and 13% hay-fed cows.

[2]W4, W8, and W12 = sampling wk 4, 8, and 12, respectively.

**Table 3.** Means, minimum, and maximum of blood metabolites of hay (n = 16) and silage (n = 16) fed dairy cows over three sampling weeks[1]

| Item | Sampling week[2] | | | | | | | *P*-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | W4 | | W8 | | W12 | | SEM | Diet | Week | Diet × week |
| | HD | SIL | HD | SIL | HD | SIL | | | | |
| Total protein (g/L) | 83.91 [74.9–100] | 86.16 [79.1–99.2] | 84.56 [80.1–93.6] | 87.02 [77.5–97.7] | 84.60 [69.3–97.9] | 87.59 [80.1–102] | 0.64 | 0.26 | 0.51 | 0.85 |
| Albumin (g/L) | 30.84 [25.9–34.0] | 32.07 [30.0–35.0] | 31.49 [30.0–33.5] | 32.03 [28.7–35.4] | 30.56 [26.9–35.8] | 31.14 [28.1–34.1] | 0.19 | 0.11 | 0.01 | 0.32 |
| Glucose (mmol/L) | 3.93 [3.68–4.21] | 4.08 [3.93–4.63] | 3.95 [3.73–4.29] | 4.22 [3.87–4.80] | 3.97 [3.69–4.25] | 4.13 [3.72–4.47] | 0.03 | 0.10 | 0.87 | 0.53 |
| NEFA[3] (mmol/L) | 0.09 [0.03–0.32] | 0.11 [0.03–0.36] | 0.08 [0.05–0.11] | 0.12 [0.06–0.35] | 0.08 [0.06–0.11] | 0.10 [0.06–0.16] | 0.01 | 0.08 | 0.99 | 0.91 |
| BHB (mmol/L) | 0.49 [0.35–0.63] | 0.53 [0.30–0.76] | 0.49 [0.38–0.70] | 0.50 [0.30–0.83] | 0.52 [0.40–0.65] | 0.49 [0.30–0.66] | 0.01 | 0.90 | 0.21 | 0.25 |
| Cholesterol (mmol/L) | 5.52 [4.24–7.58] | 5.39 [4.33–7.56] | 5.30 [3.83–6.76] | 5.32 [4.20–6.71] | 5.19 [3.85–7.73] | 5.09 [3.56–6.44] | 0.10 | 0.99 | 0.44 | 0.98 |
| Urea (mmol/L) | 2.72 [2.24–3.31] | 2.51 [1.98–3.21] | 2.98 [2.12–4.29] | 2.60 [2.11–4.76] | 2.88 [2.4–3.4] | 2.59 [1.81–3.26] | 0.04 | 0.10 | 0.08 | 0.71 |

[1]Arithmetic group means [minimum–maximum] of dairy cows fed a hay (HD; n = 16) or a silage (SIL; n = 16) based diet. HD = 100% hay-fed cows; SIL = 44% grass silage, 43% corn silage, and 13% hay-fed cows.
[2]W4, W8, and W12 = sampling wk 4, 8, and 12, respectively.
[3]NEFA = Nonesterified fatty acids.

reported effects on ruminal fermentation (Abdillah et al., 2024) and blood metabolites (Uyarlar et al., 2024). The varying effects could be related to differences in the composition of the essential oils used, their dosages, and the basal diets fed to the animals.

### Diet Effect on Ruminal Fluid and Blood Metabolites

As intended, feeding 2 basal diets with different forage-to-concentrate ratios caused a variation in the ruminal fluid and blood metabolite concentrations, with the influence of the basal diet being stronger on the ruminal fluid metabolites than on blood metabolites. This may be due to the direction of the nutrient flow within the organism. Because the rumen is the first organ in the nutrient flow hierarchy, feeding effects are most pronounced here. The higher NDF, ADF, and CP contents in hay compared with the silage-based diet resulted—under similar DMI intake—in a higher intake of these nutrients and consequently in a higher acetate proportion (Xie et al., 2020) and ammonia concentration (Eschenlauer et al., 2002) in the rumen fluid of HD cows compared with SIL cows. The higher starch concentration in the silage-based diet resulted in increased ruminal butyrate (Beckett et al., 2021) but not in increased propionate concentrations in SIL cows compared with HD cows. This can be explained by the counteracting effect of a higher WSC concentration in hay, resulting in higher ruminal propionate proportions in HD cows compared with SIL cows, as shown in an in vitro study by Lee et al. (2003). The variations in the ruminal VFA profile between HD and SIL cows over time could be explained by fluctuations in the chemical composition of the basal diet. Generally, the cows' blood metabolite concentrations, especially blood total protein, albumin, glucose, cholesterol, and urea, were within the reference range, irrespective of the diet, albeit varying between cows. However, at the beginning of the experiment, that is, in earlier stages of lactation (W1–8), some cows mobilized body fat, as expressed by slight elevations of BHB concentrations up to 0.83 mmol/L and NEFA concentrations up to 0.36 mmol/L, as well as by NEB. Accordingly, BHB concentrations of >0.60 mmol/L had been associated with fat catabolism (Couperus et al., 2021a).

### Exhaled VOC Related to Nutritional and Metabolic Status

We detected 2,771 exhaled VOC with our sampling technique, which allows for the detection of VOC in the range of C6 to C16. Of these, only 7 were correlated (15 correlations in total) with ruminal fluid and blood metabolites. Despite the low number of correlations, the meticulous selection of correlations allowed us to find

**Table 4.** Identification characteristics of exhaled VOC correlated with blood and ruminal fluid metabolites

| Exhaled VOC[1] | CAS-number[2] | *m*/*z*[3] | Match factor (%) | Level[4] | RI calc[5] | RI ref[6] |
|---|---|---|---|---|---|---|
| 3-Penten-2-one | 625–33–2 | 84 | 96.7 | 1 | 1,157 | 1,148 |
| Tetradecanal | 124–25–4 | 96 | 91.9 | 1 | 1,958 | 1,948 |
| γ-Hydroxybutyrate | 591–81–1 | 86 | 88.6 | 2 | 1,710 | 1,695 |
| 4-Hydroxy-4-methylpentan-2-one | 123–42–2 | 101 | 80.5 | 2 | 1,399 | 1,396 |
| 2-Ethylhexanal | 123–05–7 | 72 | 89.7 | 2 | 1,210 | 1,216 |
| 2-Ethylhexan-1-ol | 104–76–7 | 57 | 91.6 | 1 | 1,498 | 1,504 |
| p-Cymene | 99–87–6 | 119 | 93.4 | 1 | 1,301 | 1,303 |

[1]VOC = Volatile organic compounds identified using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17), match factor >80%.

[2]CAS = Chemical abstracts service registry.

[3]*m*/*z* = Mass-to-charge ratio.

[4]Level = Identification level.

[5]RI calc = Calculated retention index using the temperature-programmed Kovats index (Girard, 1996).

[6]RI ref = Reference retention index after comparison from the NIST chemistry web book (Agilent, Basel, Switzerland; polar column FFAP, ramp temperature).

correlated exhaled VOC that were consistent across the different diets. That some correlations were only present at one time point does not necessarily mean the absence of correlations at other time points (Terlouw et al., 2021) and could be related to the sampling of biological matrices on 3 consecutive days. The varying strength of the correlations over the sampling weeks presumably resulted from variations in metabolic processes during lactation (Couperus et al., 2021b). Other than being influenced by time, the detection of strong correlations might have been limited due to collecting biological matrices on consecutive, but not identical, sampling days. For future research, it might be interesting to collect all biological matrices closer in time and in relation to circadian rhythms.

***Lipid and Protein Catabolism.*** Most importantly, we suspect that the revealed relationships of 3-penten-2-one, tetradecanal, and GHB are physiologically relevant, because there was no evidence suggesting that these exhaled VOC originated from feed (Supplemental Figures S1 and S2). Because 3-penten-2-one was detected only in exhaled breath and not in ruminal fluid, it is likely that it originated from the cow's endogenous metabolism (Wang et al., 2023). By contrast, the presence of tetradecanal and GHB in both exhaled breath and ruminal fluid suggests that they might be synthesized through the cow's metabolism, rumen microbial metabolism, or both. The underlying metabolic pathways of the correlated exhaled VOC are supported by the literature and are described as follows. Exhaled 3-penten-2-one, tetradecanal, and GHB were correlated with serum BHB, NEFA, glucose, and urea concentrations, which are associated with lipid and protein catabolism in dairy cows. Catabolic states occur, for example, during undernutrition or energy deficiency, which are both quite common, especially in high-yielding, early-lactating cows. Energy deficiency enhances several alternative energy-producing metabolic processes, such as glycolysis, lipolysis of triglycerides (adipose tissue), proteolysis of body proteins (muscle tissue), breakdown of amino acids and fatty acids, and, mainly linked to the latter, gluconeogenesis. Lipid catabolism in adipose tissue releases NEFA into the blood. Once taken up by the liver, NEFA are activated to fatty acyl-CoA (Rizzo, 2014) and can be further metabolized into different products, including ketones such as BHB (Marczuk et al., 2018). Other ketones can also be produced. One example is the condensation product 3-penten-2-one (Figure 1). In humans, the latter has been found in urine, feces, and exhaled breath (Raman et al., 2013). There is evidence that 3-penten-2-one is formed after sampling in biological matrices by the aldol condensation of acetaldehyde with acetone or acetoacetate. Acetaldehyde, acetone, and acetoacetate can be endogenously formed in mammals by lipid peroxidation or lipid catabolism (Jones et al., 1992; Walker et al., 2009; van Erp-van der Kooij et al., 2023), suggesting that 3-penten-2-one can serve as an indicator of metabolic processes. Therefore, it seems that 3-penten-2-one could be a marker of lipolysis. In the present study, exhaled 3-penten-2-one was negatively correlated with ruminal butyrate proportions. This can be explained by the general metabolic state during energy deficiency. There is an increased conversion of ruminal butyrate to BHB in the cells of the ruminal wall (Rico and Barrientos-Blanco, 2024), thereby lowering ruminal butyrate levels; however, NEB enhances the hepatic production of ketone bodies, including acetone and acetoacetate, which, after condensation, may be detectable in samples in the form of 3-penten-2-one (Walker et al., 2009; van Erp-van der Kooij et al., 2023).

As an alternative to the formation of ketones, fatty acyl-CoA can be reduced within the fatty alcohol cycle to fatty aldehydes, such as tetradecanal (Figure 1). The fatty alcohol cycle is recognized as a metabolic process in humans (Rizzo, 2014). The enzyme fatty acyl-CoA

**Table 5.** Correlations between exhaled VOC and ruminal fluid metabolites from dairy cows (n = 32)

| | | Pearson correlation | | | | | | ANCOVA[4] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | W4[1] | | W8[1] | | W12[1] | | | | | |
| Exhaled VOC[2] | Metabolite | r[3] | *P*-value | r[3] | *P*-value | r[3] | *P*-value | $P_{model}$ | $P_{metabolite}$ | $P_{diet}$ | $P_{week}$ |
| 3-Penten-2-one | Butyrate | −0.08 | 0.67 | −0.50 | <0.01 | −0.57 | <0.01 | <0.01 | 0.01 | 0.31 | 0.01 |
| GHB[5] | Acetate | 0.17 | 0.37 | 0.40 | 0.03 | 0.10 | 0.59 | <0.01 | 0.03 | 0.01 | 0.39 |
| | Valerate | −0.37 | 0.04 | −0.36 | 0.04 | −0.31 | 0.08 | <0.01 | 0.03 | <0.01 | 0.14 |
| 4-Hydroxy-4-methylpentan-2-one | Acetate | −0.01 | 0.98 | −0.23 | 0.22 | −0.43 | 0.01 | <0.01 | 0.49 | <0.01 | 0.33 |
| 2-Ethylhexanal | Acetate | 0.01 | 0.99 | 0.01 | 0.98 | 0.65 | <0.01 | <0.01 | <0.01 | 0.99 | 0.14 |
| | Butyrate | −0.11 | 0.57 | −0.06 | 0.74 | −0.63 | <0.01 | <0.01 | 0.62 | <0.01 | 0.74 |
| 2-Ethylhexan-1-ol | Butyrate | −0.07 | 0.73 | −0.01 | 0.97 | −0.63 | <0.01 | <0.01 | 0.68 | 0.12 | <0.01 |
| p-Cymene | Ammonia | −0.02 | 0.90 | −0.62 | <0.01 | −0.28 | 0.12 | <0.01 | 0.39 | 0.01 | <0.01 |

[1]W4, W8, and W12 = sampling wk 4, 8, and 12, respectively.

[2]VOC = volatile organic compounds identified using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17), match factor >80%.

[3]Correlation coefficient (r) = Pearson correlation coefficients after manual peak integration.

[4]$P_{model}$ = *P*-value of the overall regression model, $P_{metabolite}$ = *P*-value of the ruminal fluid metabolite, $P_{diet}$ = *P*-value of the basal diet, $P_{week}$ = *P*-value of the sampling week.

[5]GHB = γ-hydroxybutyrate.

**Table 6.** Correlations between exhaled volatile organic compounds (VOC) and blood metabolites from dairy cows (n = 32)

| | | Pearson correlation | | | | | | ANCOVA[4] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | W4[1] | | W8[1] | | W12[1] | | | | | |
| Exhaled VOC[2] | Metabolite | r[3] | *P*-value | r[3] | *P*-value | r[3] | *P*-value | $P_{model}$ | $P_{metabolite}$ | $P_{diet}$ | $P_{week}$ |
| 3-Penten-2-one | NEFA[5] | −0.46 | 0.01 | −0.23 | 0.23 | −0.19 | 0.30 | <0.01 | <0.01 | 0.81 | 0.01 |
| Tetradecanal | BHB | −0.45 | 0.01 | −0.15 | 0.44 | −0.26 | 0.14 | 0.01 | 0.03 | 0.24 | 0.03 |
| | NEFA | 0.45 | 0.01 | 0.37 | 0.04 | 0.06 | 0.75 | <0.01 | <0.01 | 0.75 | 0.26 |
| GHB[6] | Glucose | −0.08 | 0.68 | −0.18 | 0.34 | −0.38 | 0.03 | <0.01 | 0.01 | 0.47 | 0.43 |
| | Urea | 0.20 | 0.29 | 0.39 | 0.04 | 0.24 | 0.18 | <0.01 | 0.04 | 0.01 | 0.43 |
| 4-Hydroxy-4-methylpentan-2-one | BHB | −0.13 | 0.49 | −0.25 | 0.19 | −0.39 | 0.03 | <0.01 | 0.21 | 0.33 | 0.04 |
| | Cholesterol | 0.20 | 0.31 | 0.02 | 0.94 | 0.41 | 0.02 | <0.01 | 0.47 | 0.42 | 0.04 |

[1]W4, W8, and W12 = sampling wk 4, 8, and 12, respectively.

[2]VOC = volatile organic compounds identified using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17), match factor > 80%.

[3]Correlation coefficient (r) = Pearson correlation coefficients after manual peak integration.

[4]$P_{model}$ = *P*-value of the overall regression model, $P_{metabolite}$ = *P*-value of the blood metabolite, $P_{diet}$ = *P*-value of the basal diet, $P_{week}$ = *P*-value of the sampling week.

[5]NEFA = nonesterified fatty acids.

[6]GHB = γ-hydroxybutyrate.

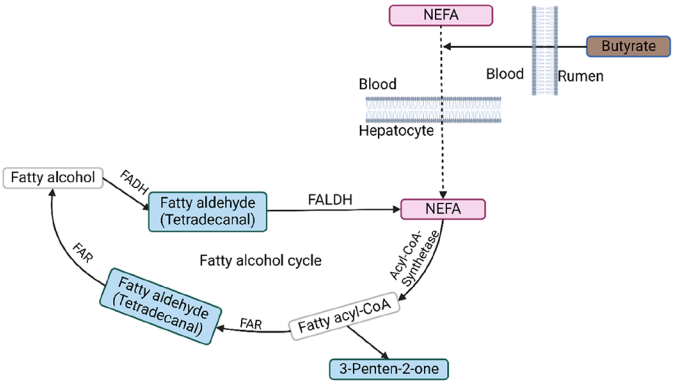


**Figure 1.** The breakdown of nonesterified fatty acids (NEFA; red boxes) and ruminal butyrate (brown box) via various intermediate products (white boxes) to volatile organic compounds (blue boxes), such as ketones (e.g., 3-penten-2-one) and fatty aldehydes (e.g., tetradecanal) in the liver. FADH = fatty alcohol dehydrogenase; FALDH = fatty aldehyde dehydrogenase; FAR = fatty acyl-CoA reductase. Figure created using BioRender.com.

reductase (**FAR**), which is relevant for this cycle, is a ubiquitously expressed membrane-bound protein found throughout mammals (Lee et al., 1980; Exner et al., 2019) and prokaryotes (Hofvander et al., 2011). For example, it has been detected in the livers of rats (Lee et al., 1980; Exner et al., 2019). Sataria et al. (2022) described elevated levels of several long-chain fatty aldehydes in exhaled breath samples of cancer patients compared with healthy individuals and hypothesized that the underlying reason was increased fatty acid breakdown and oxidative stress. These aspects are consistent with the positive correlation between serum NEFA concentrations and exhaled tetradecanal in the present study. In mice, long-chain fatty alcohols and aldehydes were excreted within minutes after dermal application of fatty alcohols, mostly via exhaled breath (>90%) over 24 h (Iwata et al., 1987). Similarly, prokaryotes of ruminal microbiota have been demonstrated to reduce fatty acids to fatty aldehydes (Hofvander et al., 2011). This may explain the presence of fatty aldehydes in ruminal fluid samples. Their ruminal formation could have contributed to the concentrations in the exhaled breath. The findings of the present study suggest that exhaled breath is also an excretion route of fatty alcohols and fatty aldehydes in dairy cows. Exhalation of fatty aldehydes is likely particularly important or intensified during energy deficiency and increased lipid catabolism. The fatty alcohol cycle is catalyzed by the enzyme FAR, which converts the fatty aldehyde to the corresponding fatty alcohol; the fatty alcohol is then converted to the fatty aldehyde by the enzyme fatty alcohol dehydrogenase (**FADH**), and then further converted to the fatty acid by the enzyme fatty aldehyde dehydrogenase (**FALDH**; Ichihara et al., 1986; Rizzo, 2014). The continuous release of fatty acyl-CoA during increased lipid catabolism inhibits fatty aldehyde-degrading enzymes (FADH and FALDH; Rizzo et al., 1987). The presence of these enzymes in the livers of mammals, including cows, has been proven earlier (Rizzo et al., 1987; Tsutsumi et al., 1988). Tetradecanal and serum BHB concentrations were probably negatively correlated because fatty acids can be—alternatively to the formation of fatty aldehydes—metabolized into ketones, such as BHB. Therefore, the more fatty acids are metabolized into BHB, the less they are converted into fatty aldehydes, and vice versa. Thus, high concentrations of exhaled tetradecanal in energy deficiency might indicate a protective alternative pathway for reducing the fatty acid and BHB load in dairy cows.

An alternative process for energy production is proteolysis. In the present study, exhaled GHB was negatively correlated with serum glucose concentrations and positively correlated with urea concentrations. Therefore, exhaled GHB seems to be associated with the metabolic process of protein catabolism, specifically with AA breakdown (Figure 2). During proteolysis, released AA are broken down into ammonia, which is further metabolized into urea, and the carbon skeleton of the AA, which can be used in the tricarboxylic acid cycle for energy production. For alanine, asparagine, aspartate, glutamine, arginine, histidine, and proline, this breakdown is coupled with the conversion of α-ketoglutarate to glutamate. Once released into the bloodstream, the condensation product of glutamate and ammonia—glutamine—can pass the blood–brain barrier (**BBB**). In the brain, particularly in GABA-related neurons, glutamate stimulates the formation of the neurotransmitter gamma-aminobutyric acid (**GABA**; Chapman et al., 2022), which can be further broken down into GHB, another neurotransmitter (Couper and Marinetti, 2002). Accordingly, other studies have shown increased GABA levels (Yudkoff et al., 2004; Qiao et al., 2024) and expression of glutamic acid decarboxylase, an enzyme involved in GABA synthesis (Cheng et al., 2004), as a result of a ketogenic diet in mice (Qiao et al., 2024). These processes might explain the positive correlation between GHB and urea concentrations in our study. Furthermore, the negative correlation between GHB and serum glucose concentrations could be due to lower glucose levels, which might have stimulated the described cascade of reactions, resulting in greater GHB levels. Once synthesized in the brain, GABA can cross the BBB (Shyamaladevi et al., 2002), enter the bloodstream, diffuse through the ruminal wall, and contribute to ruminal GABA concentrations. Both GHB and its precursor GABA can also be produced by ruminal microbiota during protein catabolism (Wang et al., 2023). This is in line with the detection of GHB in ruminal fluid samples, suggesting that ruminal GHB formation could have contributed to the concentrations in

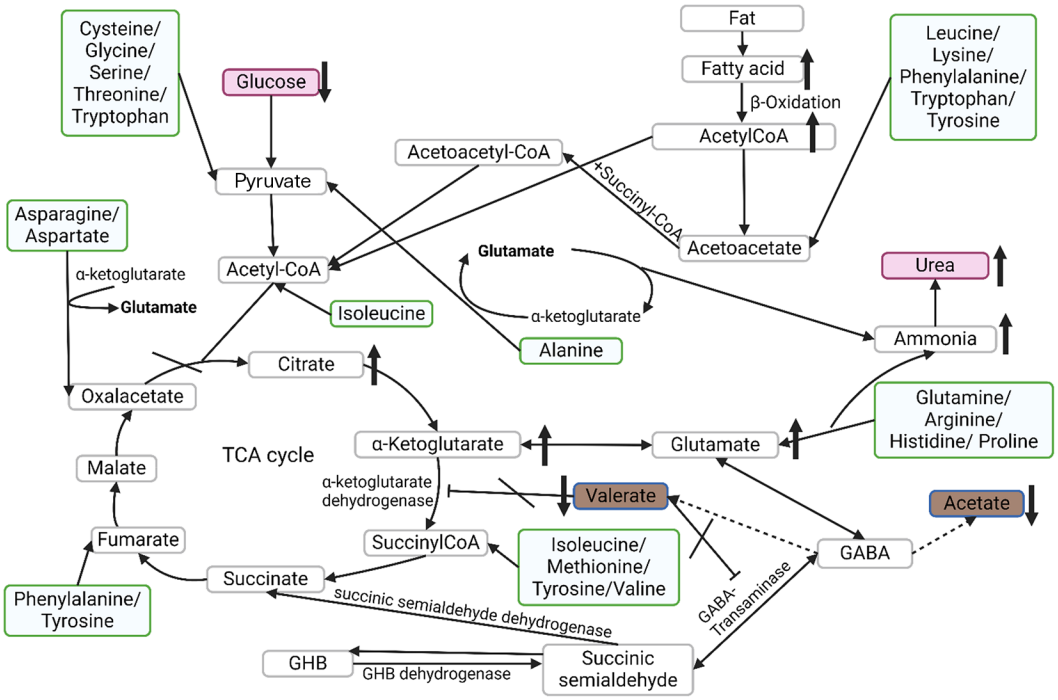


**Figure 2.** Breakdown of amino acids (green boxes) via various intermediate products (white boxes) to γ-hydroxybutyrate (GHB). Arrows pointing up and down represent the increase and decrease in metabolite levels, respectively. Dashed arrows represent the effects on ruminal acetate and valerate formation (brown boxes). Blunt arrows represent the inhibitory effects. Figure created using BioRender.com.

exhaled breath. The GABA, regardless of its origin (microbial or endogenous), can influence ruminal fermentation in an unknown mode of action (Wang et al., 2023). In vitro, adding GABA to ruminal fluid decreased the proportions of propionate and valerate and increased the proportions of acetate and butyrate in an unknown mode of action (Wang et al., 2023). These findings, although observed under in vitro conditions and supraphysiological GABA concentrations, align with the negative and positive correlations we found between GHB and valerate proportions and between GHB and acetate proportions, respectively. The process can also work the other way around. Valerate can diffuse from ruminal fluid through the ruminal wall into the bloodstream, cross the BBB, and influence GHB metabolism (Dalile et al., 2019). In rats, the intravenous injection of valerate inactivated GABA-degrading enzymes (α-ketoglutarate dehydrogenase and GABA transaminase; Monti et al., 2009), leading to increased GABA levels in neurons (Vishwakarma et al., 2016).

In the present study, we hypothesize that decreased ruminal valerate formation and, therefore, lower valerate levels in the brain might have led to increased GABA catabolism by the enzyme GABA transaminase to succinic semialdehyde, which is converted to GHB (Couper and Marinetti, 2002). The predominant way of GHB catabolism consists of 2 steps: (1) oxidation to succinic semialdehyde by the enzyme GHB dehydrogenase (Kaufman et al., 1983), and (2) oxidation of succinic semialdehyde to succinic acid by the enzyme succinic semialdehyde dehydrogenase before further breakdown in the TCA cycle (Chambliss et al., 1995). Several endogenous metabolites that are produced in excess during certain disease states have been shown to inhibit GHB dehydrogenase, the enzyme in the first step in GHB breakdown. Such metabolites include the ketone bodies BHB and acetoacetate, which are elevated during lipolysis (Kaufman et al., 1983). In the case of an impaired GHB breakdown via the TCA cycle and the impaired subsequent excretion as $CO_2$ and $H_2O$ (Maitre, 1997), GHB must instead be excreted in the urine or exhaled breath (Brenneisen et al., 2004). The absence of a correlation with EB in dairy cows may be attributed to several factors. The EB is only a predicted and calculated value, whereas blood metabolites offer more direct and reliable insights into metabolism, because they reflect the actual products and byproducts of metabolic processes. The EB and BHB levels do not contain the same information. Although EB, especially NEB, can lead to alterations in various metabolic pathways, metabolites such as BHB specifically indicate increased ketogenesis, which results from increased fat catabolism. In the present study, EB and serum BHB concentrations were not correlated (data not presented). Therefore, it is not surprising that cor-

relations between EB and exhaled VOC were absent, whereas we found correlations between serum BHB concentrations and exhaled VOC. Cows may respond differently to NEB, with some cows exhibiting changes in certain metabolic pathways, and others may shift toward alternative pathways, highlighting individual variations in metabolic responses. When cows respond differently, it becomes challenging to find correlations between EB and exhaled VOC. Finally, exploring correlations between EB and exhaled VOC might result in significant correlations in cows with a wider range of EB values, which should be addressed in future research.

***Diet and Time Aspects.*** The ANCOVA showed that some of the exhaled VOC were more related to diet-dependent variations or to nutritional or metabolic variations over time than to blood and ruminal fluid metabolite concentrations. This may explain why the correlations of these exhaled VOC were only present in one sampling week, likely due to variations in the chemical composition of the diet. 4-Hydroxy-4-methylpentan-2-one has been identified as a secondary metabolite produced by plants as a defensive secondary metabolite against bacterial infection (Tan et al., 2017). It was earlier detected in bovine urine (Nautiyal and Dubey, 2021) and in elevated concentrations in the exhaled breath samples of cattle infected with *Mycobacterium bovis* (Ellis et al., 2014). Similarly, monoterpene p-cymene is present in many plants (Marchese et al., 2017). Therefore, variations in the composition of the basal diets might be the reason for the diet-related effects. The correlation between exhaled p-cymene and ruminal ammonia concentrations in the present study is probably explained by differences in diet composition, although a potential link between exhaled p-cymene and ruminal ammonia concentrations could still exist, as other studies suggest (Chaves et al., 2008; Ribeiro et al., 2020; Abdillah et al., 2024). Abdillah et al. (2024) showed a reduction in ruminal ammonia concentration in vitro using a feed additive consisting, among others, of p-cymene. Other authors have found positive or no associations between ruminal ammonia concentrations and the use of feed additives containing p-cymene, both in vitro and in vivo (Chaves et al., 2008; Ribeiro et al., 2020). Exhaled 2-ethylhexanal and 2-ethyl-hexan-1-ol can be produced during the breakdown of CP (Sun et al., 2024). The different CP in the basal diets and its fluctuation over time may explain why these exhaled VOC are affected by diet and sampling week. However, there may be a potential link between exhaled 2-ethylhexanal and 2-ethyl-hexan-1-ol and the proportions of ruminal acetate and butyrate, because these VFA are also altered by differences in the composition of basal diets (Xie et al., 2020). Therefore, variations in the composition of the basal diets might be the reason for the correlations. This is supported by the fact that correlations between certain exhaled VOC (e.g., p-cymene, 4-hydroxy-4-methylpentan-2-one, 2-ethyl-hexan-1-ol, and 2-ethylhexanal) and metabolite concentrations of ruminal fluid and blood were observed only when data from both basal diet groups were combined, thereby capturing the broader range of metabolic responses induced by the 2 distinct diets. Furthermore, p-cymene and 4-hydroxy-4-methylpentan-2-one were already present in the basal diet and concentrate samples, with their highest concentrations in silage samples.

## CONCLUSIONS

In this study, we identified consistent physiologically relevant correlations between exhaled VOC and blood and ruminal fluid metabolite concentrations of dairy cows. The identification and mapping of the potential underlying pathways of exhaled 3-penten-2-one, tetradecanal, and GHB suggest that these exhaled VOC are associated with fat and protein catabolism of the host and rumen microbial metabolism, as supported by the existing literature. Other correlations between exhaled VOC and metabolite concentrations of blood and ruminal fluid were explained by diet-dependent (2-ethylhexanal) and time-dependent (2-ethyl-hexan-1-ol) variations or both (4-hydroxy-4-methylpentan-2-one, p-cymene). We therefore conclude that some exhaled VOC may be potential new biomarkers to describe the nutritional and metabolic status of dairy cows, identifying metabolic disorders, or conducting feed traceability. The validation of the found associations requires further investigation.

## NOTES

This study received no external funding. The authors thank Yvo Aeby and his team from Research Contracts Animals, Agroscope, Posieux, Switzerland, for their care of the cows and technical support. We also thank Paolo Silacci and his team from the Animal Biology Group, Agroscope, Posieux, Switzerland, for blood analysis, and Sébastien Dubois and his team from the Feed Chemistry, Agroscope, Posieux, Switzerland, for analysis of feed and ruminal fluid. Supplemental material for this article is available at https://doi.org/10.5281/zenodo.14000099. The experimental protocol complied with Swiss animal welfare legislation and was approved by the Animal Care Committee of the Fribourg Canton, Fribourg, Switzerland (license no. 2020–58-FR/32975). The authors have not stated any conflicts of interest.

**Nonstandard abbreviations used:** ANCOVA = analysis of covariance; APD = absorbable protein at the duodenum; BBB = blood–brain barrier; CON = control protein and energy concentrate mixture; DHS-V-ITEX =

dynamic headspace vacuum in-tube; DHS-VTT = vacuum transfer in-tube extraction; EB = energy balance; EXP = experimental concentrate mixture in which 0.68% of the corn was replaced with an essential oil blend; FADH = fatty alcohol dehydrogenase; FALDH = fatty aldehyde dehydrogenase; FAR = fatty acyl-CoA reductase; GABA = gamma-aminobutyric acid; GHB = γ-hydroxybutyrate; HD = Hay-fed cows; NEB = negative energy balance; NEFA = nonesterified fatty acids; QC = quality control. RI = retention index; SIL = silage-fed cows; SPE = solid-phase extraction; VOC = volatile organic compounds; VVOC = very volatile organic compounds; W4 = sampling wk 4; W8 = sampling wk 8; W12 = sampling wk 12; WSC = water-soluble carbohydrates.

## REFERENCES


Abdillah, A. E., D. Sarah, A. Ardian, M. Anas, M. Aprianto, C. Hanim, A. Kurniawati, Muhlisin, and L. M. Yusiati. 2024. Effect of nutmeg essential oil (*Myristica fragrans* Houtt.) on methane production, rumen fermentation, and nutrient digestibility in vitro. Sci. Rep. 14:3554. https://doi.org/10.1038/s41598-024-52532-3.

Aggarwal, R., and P. Ranganathan. 2019. Study designs: Part 4—Interventional studies. Perspect. Clin. Res. 10:137–139. https://doi.org/10.4103/picr.PICR_91_19.

Agroscope. 2021. Feeding recommendations and nutrient tables for ruminants (in German). Accessed Feb. 7, 2024. https://www.agroscope.admin.ch/agroscope/fr/home/services/ soutien/aliments-pour-animaux/rapports-alimentaires-recommandes-ruminants.html.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48. https://doi.org/10.18637/jss.v067.i01.

Beckett, L., C. B. Gleason, A. Bedford, D. Liebe, T. T. Yohe, M. B. Hall, K. M. Daniels, and R. R. White. 2021. Rumen volatile fatty acid molar proportions, rumen epithelial gene expression, and blood metabolite concentration responses to ruminally degradable starch and fiber supplies. J. Dairy Sci. 104:8857–8869. https://doi.org/10.3168/jds.2020-19622.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70:567–590. https://doi.org/10.1152/physrev.1990.70.2.567.

Bobbo, T., E. Fiore, M. Gianesella, M. Morgante, L. Gallo, P. L. Ruegg, G. Bittante, and A. Cecchinato. 2017. Variation in blood serum proteins and association with somatic cell count in dairy cattle from multi-breed herds. Animal 11:2309–2319. https://doi.org/10.1017/S1751731117001227.

Brenneisen, R., M. A. Elsohly, T. P. Murphy, J. Passarelli, S. Russmann, S. J. Salamone, and D. E. Watson. 2004. Pharmacokinetics and excretion of gamma-hydroxybutyrate (GHB) in healthy subjects. J. Anal. Toxicol. 28:625–630. https://doi.org/10.1093/jat/28.8.625.

Chambliss, K. L., D. L. Caudle, D. D. Hinson, C. R. Moomaw, C. A. Slaughter, C. Jakobs, and K. M. Gibson. 1995. Molecular cloning of the mature NAD(+)-dependent succinic semialdehyde dehydrogenase from rat and human. cDNA isolation, evolutionary homology, and tissue expression. J. Biol. Chem. 270:461–467. https://doi.org/10.1074/jbc.270.1.461.

Chapman, C. A., J. L. Nuwer, and T. C. Jacob. 2022. The yin and yang of GABAergic and glutamatergic synaptic plasticity: Opposites in balance by crosstalking mechanisms. Front. Synaptic Neurosci. 14:911020. https://doi.org/10.3389/fnsyn.2022.911020.

Chaves, A., M. He, W. Yang, A. Hristov, T. McAllister, and C. Benchaar. 2008. Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. Can. J. Anim. Sci. 88:117–122. https://doi.org/10.4141/CJAS07061.

Cheng, C. M., K. Hicks, J. Wang, D. A. Eagles, and C. A. Bondy. 2004. Caloric restriction augments brain glutamic acid decarboxylase-65 and -67 expression. J. Neurosci. Res. 77:270–276. https://doi.org/10.1002/jnr.20144.

Couper, F. J., and L. J. Marinetti. 2002. γ-Hydroxybutyrate (GHB) - Effects on human performance and behavior. Forensic Sci. Rev. 14:101–121.

Couperus, A. M., F. Schroeder, P. Hettegger, J. Huber, T. Wittek, and J. R. Peham. 2021a. Longitudinal metabolic biomarker profile of hyperketonemic cows from dry-off to peak lactation and identification of prognostic classifiers. Animals (Basel) 11:1353. https://doi.org/10.3390/ani11051353.

Couperus, A. M., F. Schroeder, R. Klukas, J. Huber, T. Wittek, and J. R. Peham. 2021b. Influence of different lactation stages on circadian rhythmicity of metabolic biomarkers in dairy cows: A pilot study. Animals (Basel) 11:1043. https://doi.org/10.3390/ani11041043.

Dalile, B., L. Van Oudenhove, B. Vervliet, and K. Verbeke. 2019. The role of short-chain fatty acids in microbiota–gut–brain communication. Nat. Rev. Gastroenterol. Hepatol. 16:461–478. https://doi.org/10.1038/s41575-019-0157-3.

Dieterle, F., A. Ross, G. Schlotterbeck, and H. Senn. 2006. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1H NMR metabonomics. Anal. Chem. 78:4281–4290. https://doi.org/10.1021/ac051632c.

Dobbelaar, P., T. Mottram, C. Nyabadza, P. Hobbs, R. J. Elliott-Martin, and Y. H. Schukken. 1996. Detection of ketosis in dairy cows by analysis of exhaled breath. Vet. Q. 18:151–152. https://doi.org/10.1080/01652176.1996.9694638.

Eichinger, J., A.-M. Reiche, F. Dohme-Meier, and P. Fuchsmann. 2024. Optimization of volatile organic compounds sampling from dairy cow exhaled breath using polymer-based solid-phase extraction cartridges for gas chromatographic analysis. J. Breath Res. 18:036001. https://doi.org/10.1088/1752-7163/ad38d5.

Eichinger, J., A.-M. Reiche, A. Münger, L. Eggerschwiler, G. Pimentel, P. Fuchsmann, K. Huber, and F. Dohme-Meier. 2025. Usability of volatile organic compounds from exhaled breath compared to those from ruminal fluid, serum, urine, and milk to identify diet-specific metabolite profiles in lactating dairy cows. J. Dairy Sci. 108:1242–1260. https://doi.org/10.3168/jds.2023-24579.

Ellis, C. K., R. S. Stahl, P. Nol, W. R. Waters, M. V. Palmer, J. C. Rhyan, K. C. VerCauteren, M. McCollum, and M. D. Salman. 2014. A pilot study exploring the use of breath analysis to differentiate healthy cattle from cattle experimentally infected with *Mycobacterium bovis*. PLoS One 9:e89280. https://doi.org/10.1371/journal.pone.0089280.

Eschenlauer, S. C., N. McKain, N. D. Walker, N. R. McEwan, C. J. Newbold, and R. J. Wallace. 2002. Ammonia production by ruminal microorganisms and enumeration, isolation, and characterization of bacteria capable of growth on peptides and amino acids from the sheep rumen. Appl. Environ. Microbiol. 68:4925–4931. https://doi.org/10.1128/AEM.68.10.4925-4931.2002.

Exner, T., I. Romero-Brey, E. Yifrach, J. Rivera-Monroy, B. Schrul, C. C. Zouboulis, W. Stremmel, M. Honsho, R. Bartenschlager, E. Zalckvar, M. Poppelreuther, and J. Füllekrug. 2019. An alternative membrane topology permits lipid droplet localization of peroxisomal fatty acyl-CoA reductase 1. J. Cell Sci. 132:jcs223016. https://doi.org/10.1242/jcs.223016.

Fuchsmann, P., M. Tena Stern, P. Bischoff, R. Badertscher, K. Breme, and B. Walther. 2019. Development and performance evaluation of a novel dynamic headspace vacuum transfer "In Trap" extraction method for volatile compounds and comparison with headspace solid-phase microextraction and headspace in-tube extraction. J. Chromatogr. A 1601:60–70. https://doi.org/10.1016/j.chroma.2019.05.016.

Ghoshal, U. C. 2011. How to interpret hydrogen breath tests. J. Neurogastroenterol. Motil. 17:312–317. https://doi.org/10.5056/jnm.2011.17.3.312.

Girard, B. 1996. Retention index calculation using Kováts constant model for linear temperature-programmed gas chromatography. J. Chromatogr. A 721:279–288. https://doi.org/10.1016/0021-9673(95)00790-3.

Hofvander, P., T. T. P. Doan, and M. Hamberg. 2011. A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. FEBS Lett. 585:3538–3543. https://doi.org/10.1016/j.febslet.2011.10.016.

Ichihara, K., E. Kusunose, Y. Noda, and M. Kusunose. 1986. Some properties of the fatty alcohol oxidation system and reconstitution of microsomal oxidation activity in intestinal mucosa. Biochim. Biophys. Acta Lipids Lipid Metab. 878:412–418. https://doi.org/10.1016/0005-2760(86)90250-X.

Islam, M. Z., 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. 2024. Exhalomics as a non-invasive method for assessing rumen fermentation in dairy cows: Can exhaled breath metabolomics replace rumen sampling? J. Dairy Sci. 107:2099–2110. https://doi.org/10.3168/jds.2023-24124.

Iwata, Y., Y. Moriya, and T. Kobayashi. 1987. Percutaneous absorption of aliphatic compounds. Cosmetics and Toiletries 102:53–68.

Jones, A. W., K. M. Beylich, A. Bjørneboe, J. Ingum, and J. Mørland. 1992. Measuring ethanol in blood and breath for legal purposes: Variability between laboratories and between breath-test instruments. Clin. Chem. 38:743–747. https://doi.org/10.1093/clinchem/38.5.743.

Kaufman, E. E., N. Relkin, and T. Nelson. 1983. Regulation and properties of an NADP+ oxidoreductase which functions as a gamma-hydroxybutyrate dehydrogenase. J. Neurochem. 40:1639–1646. https://doi.org/10.1111/j.1471-4159.1983.tb08137.x.

Lazzari, G., A. Münger, L. Eggerschwiler, D. Borda-Molina, J. Seifert, A. Camarinha-Silva, S. Schrade, M. Zähner, K. Zeyer, M. Kreuzer, and F. Dohme-Meier. 2023. Effects of *Acacia mearnsii* added to silages differing in nutrient composition and condensed tannins on ruminal and manure-derived methane emissions of dairy cows. J. Dairy Sci. 106:6816–6833. https://doi.org/10.3168/jds.2022-22901.

Lee, T.-C., V. F. N. Stephens, and F. Snyder. 1980. Activities of enzymes involved in the metabolism of ether-linked lipids in normal and neoplastic tissues of rat. Biochim. Biophys. Acta Lipids Lipid Metab. 619:420–423. https://doi.org/10.1016/0005-2760(80)90091-0.

Lee, M. R. F., R. J. Merry, D. R. Davies, J. M. Moorby, M. O. Humphreys, M. K. Theodorou, J. C. MacRae, and N. D. Scollan. 2003. Effect of increasing availability of water-soluble carbohydrates on in vitro rumen fermentation. Anim. Feed Sci. Technol. 104:59–70. https://doi.org/10.1016/S0377-8401(02)00319-X.

Lee, A. J., A. R. Twardock, R. H. Bubar, J. E. Hall, and C. L. Davis. 1978. Blood metabolic profiles: Their use and relation to nutritional status of dairy cows. J. Dairy Sci. 61:1652–1670. https://doi.org/10.3168/jds.S0022-0302(78)83780-1.

Maitre, M. 1997. The γ-hydroxybutyrate signalling system in brain: Organization and functional implications. Prog. Neurobiol. 51:337–361. https://doi.org/10.1016/S0301-0082(96)00064-0.

Marchese, A., C. Arciola, R. Barbieri, A. S. Silva, S. Nabavi, A. Tsetegho Sokeng, M. Izadi, N. J. Jafari, I. Süntar, and M. Daglia. 2017. Update on monoterpenes as antimicrobial agents: A particular focus on p-cymene. Materials (Basel) 10:947. https://doi.org/10.3390/ma10080947.

Marczuk, J., P. Brodzki, A. Brodzki, and Ł. Kurek. 2018. The concentration of free amino acids in blood serum of dairy cows with primary ketosis. Pol. J. Vet. Sci. 21:149–156. https://doi.org/10.24425/119033.

Monti, B., E. Polazzi, and A. Contestabile. 2009. Biochemical, molecular and epigenetic mechanisms of valproic acid neuroprotection. Curr. Mol. Pharmacol. 2:95–109. https://doi.org/10.2174/1874467210902010095.

Nautiyal, V., and R. C. Dubey. 2021. FT-IR and GC-MS analyses of potential bioactive compounds of cow urine and its antibacterial activity. Saudi J. Biol. Sci. 28:2432–2437. https://doi.org/10.1016/j.sjbs.2021.01.041.

Peng, Z., X. Li, Z. Wang, G. Liu, and X. Li. 2019. The effects of non-esterified fatty acids and β-hydroxybutyrate on the hepatic CYP2E1 in cows with clinical ketosis. J. Dairy Res. 86:68–72. https://doi.org/10.1017/S0022029919000025.

Qiao, Y.-N., L. Li, S.-H. Hu, Y.-X. Yang, Z.-Z. Ma, L. Huang, Y.-P. An, Y.-Y. Yuan, Y. Lin, W. Xu, Y. Li, P.-C. Lin, J. Cao, J.-Y. Zhao, and S.-M. Zhao. 2024. Ketogenic diet-produced β-hydroxybutyric acid accumulates brain GABA and increases GABA/glutamate ratio to inhibit epilepsy. Cell Discov. 10:17. https://doi.org/10.1038/s41421-023-00636-x.

Raman, M., I. Ahmed, P. M. Gillevet, C. S. Probert, N. M. Ratcliffe, S. Smith, R. Greenwood, M. Sikaroodi, V. Lam, P. Crotty, J. Bailey, R. P. Myers, and K. P. Rioux. 2013. Fecal microbiome and volatile organic compound metabolome in obese humans with nonalcoholic fatty liver disease. Clin. Gastroenterol. Hepatol. 11:868–875.e3. https://doi.org/10.1016/j.cgh.2013.02.015.

Ribeiro, A. D. B., M. Ferraz Junior, D. Polizel, A. Miszura, J. P. Barroso, A. R. Cunha, T. T. Souza, E. Ferreira, I. Susin, and A. Pires. 2020. Effect of thyme essential oil on rumen parameters, nutrient digestibility, and nitrogen balance in wethers fed high concentrate diets. Arq. Bras. Med. Vet. Zootec. 72:573–580. https://doi.org/10.1590/1678-4162-11322.

Rico, J.-E., and M.-A. Barrientos-Blanco. 2024. *Invited review:* Ketone biology—The shifting paradigm of ketones and ketosis in the dairy cow. J. Dairy Sci. 107:3367–3388. https://doi.org/10.3168/jds.2023-23904.

Rizzo, W. B. 2014. Fatty aldehyde and fatty alcohol metabolism: Review and importance for epidermal structure and function. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1841:377–389. https://doi.org/10.1016/j.bbalip.2013.09.001.

Rizzo, W. B., D. A. Craft, A. L. Dammann, and M. W. Phillips. 1987. Fatty alcohol metabolism in cultured human fibroblasts. Evidence for a fatty alcohol cycle. J. Biol. Chem. 262:17412–17419. https://doi.org/10.1016/S0021-9258(18)45394-X.

Sataria, S. R., S. S. Gori, J. D. Morris, Z. Xie, X.-A. Fu, and M. H. Nantz. 2022. Lipid peroxidation produces a diverse mixture of saturated and unsaturated aldehydes in exhaled breath that can serve as biomarkers of lung cancer—A review. Metabolites 12:561. https://doi.org/10.3390/metabo12060561.

Schieweck, A., J. Gunschera, D. Varol, and T. Salthammer. 2018. Analytical procedure for the determination of very volatile organic compounds (C3–C6) in indoor air. Anal. Bioanal. Chem. 410:3171–3183. https://doi.org/10.1007/s00216-018-1004-z.

Serrenho, R. C., M. Williamson, O. Berke, S. J. LeBlanc, T. J. DeVries, B. W. McBride, and T. F. Duffield. 2022. An investigation of blood, milk, and urine test patterns for the diagnosis of ketosis in dairy cows in early lactation. J. Dairy Sci. 105:7719–7727. https://doi.org/10.3168/jds.2021-21590.

Shyamaladevi, N., A. R. Jayakumar, R. Sujatha, V. Paul, and E. H. Subramanian. 2002. Evidence that nitric oxide production increases γ-amino butyric acid permeability of blood-brain barrier. Brain Res. Bull. 57:231–236. https://doi.org/10.1016/S0361-9230(01)00755-9.

Sumner, L. W., A. Amberg, D. Barrett, M. H. Beale, R. Beger, C. A. Daykin, T. W. Fan, O. Fiehn, R. Goodacre, J. L. Griffin, T. Hankemeier, N. Hardy, J. Harnly, R. Higashi, J. Kopka, A. N. Lane, J. C. Lindon, P. Marriott, A. W. Nicholls, M. D. Reily, J. J. Thaden, and M. R. Viant. 2007. Proposed minimum reporting standards for chemical analysis. Metabolomics 3:211–221. https://doi.org/10.1007/s11306-007-0082-2.

Sun, C., S. Zhang, B. Gao, T. Yang, Y. Wang, H. Cheng, H. Zhou, and Z. Chen. 2024. Conversion of lignocellulosic biomass-derived compounds to 2-ethylhexanol: A review. Environ. Chem. Lett. 22:1093–1113. https://doi.org/10.1007/s10311-024-01710-w.

Swiss-Accreditation-Service-SAS. 2024. Search accredited bodies SAS. Accessed May 5, 2024. https://www.sas.admin.ch/sas/en/home/akkreditiertestellen/akkrstellensuchesas.exturl.html/aHR0cHM6Ly9zYXNkYi5jbGllbnRzLmxpaXAuY2gvc2VhcmNoLm/h0bWw=.html?csrfmiddlewaretoken=UVLK0g4093lLLwak9J7QAMt7wUc89GTNM4CrrJ7dGE7Xz9AzSojeuiJnOQL6jH9Q&lang=en&search_term=0077&accreditation_type=&submit=Start+search.

Tan, Y., D. Li, J. Hua, S. Luo, Y. Liu, and S. Li. 2017. Localization of a defensive volatile 4-hydroxy-4-methylpentan-2-one in the capitate glandular trichomes of *Oenothera glazioviana*. Plant Divers. 39:154–159. https://doi.org/10.1016/j.pld.2017.05.004.

Tejero Rioseras, A., D. Garcia Gomez, B. E. Ebert, L. M. Blank, A. J. Ibáñez, and P. M. Sinues. 2017. Comprehensive real-time analysis of the yeast volatilome. Sci. Rep. 7:14236. https://doi.org/10.1038/s41598-017-14554-y.

Terlouw, E. M. C., B. Picard, V. Deiss, C. Berri, J. F. Hocquette, B. Lebret, F. Lefèvre, R. Hamill, and M. Gagaoua. 2021. Understanding the determination of meat quality using biochemical characteristics of

the muscle: Stress at slaughter and other missing keys. Foods 10:84. https://doi.org/10.3390/foods10010084.

Tsutsumi, M., A. Takada, S. Takase, and K. Sugata. 1988. Hepatic aldehyde dehydrogenase isoenzymes: Differences with respect to species. Alcohol 5:33–38. https://doi.org/10.1016/0741-8329(88)90040-7.

Uyarlar, C., A. Rahman, E. E. Gultepe, I. S. Cetingul, and I. Bayram. 2024. Effect of a dietary essential oil blend in dairy cows during the dry and transition period on blood and metabolic parameters of dams and their calves. Animals (Basel) 14:150. https://doi.org/10.3390/ani14010150.

van Erp-van der Kooij, E., J. Derix, S. van Gorp, A. Timmermans, C. Krijnen, I. Fodor, and L. Dingboom. 2023. Breath analysis for early detection of rising ketone bodies in postpartum dairy cows classified as at risk of ketosis. Ruminants (Basel). 3:39–54. https://doi.org/10.3390/ruminants3010005.

Vishwakarma, S., R. Goyal, V. Gupta, and K. L. Dhar. 2016. GABAergic effect of valeric acid from *Valeriana wallichii* in amelioration of ICV STZ induced dementia in rats. Rev. Bras. Farmacogn. 26:484–489. https://doi.org/10.1016/j.bjp.2016.02.008.

Walker, V., G.-A. Mills, and E.-M. Stansbridge. 2009. 3-Penten-2-one, a novel aldehyde adduct, is a biomarker for increased acetaldehyde in urine. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 877:784–790. https://doi.org/10.1016/j.jchromb.2009.02.017.

Wang, Y.-L., Z.-H. Zhang, W.-K. Wang, Q.-C. Wu, F. Zhang, W.-J. Li, S.-L. Li, W. Wang, Z.-J. Cao, and H.-J. Yang. 2023. The effect of γ-aminobutyric acid addition on in vitro ruminal fermentation characteristics and methane production of diets differing in forage-to-concentrate ratio. Fermentation (Basel) 9:105. https://doi.org/10.3390/fermentation9020105.

Xia, J., N. Psychogios, N. Young, and D. S. Wishart. 2009. MetaboAnalyst: A web server for metabolomic data analysis and interpretation. Nucleic Acids Res. 37(Suppl. 2):W652–W660. https://doi.org/10.1093/nar/gkp356.

Xie, B., W. Huang, C. Zhang, Q. Diao, K. Cui, J. Chai, S. Wang, X. Lv, and N. Zhang. 2020. Influences of starter NDF level on growth performance and rumen development in lambs fed isocaloric and isonitrogenous diets. J. Anim. Sci. 98:skaa093. https://doi.org/10.1093/jas/skaa093.

Yudkoff, M., Y. Daikhin, I. Nissim, A. Lazarow, and I. Nissim. 2004. Ketogenic diet, brain glutamate metabolism and seizure control. Prostaglandins Leukot. Essent. Fatty Acids 70:277–285. https://doi.org/10.1016/j.plefa.2003.07.005.