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Usability of volatile organic compounds from exhaled breath compared with those from ruminal fluid, serum, urine, and milk to identify diet-specific metabolite profiles in lactating dairy cows

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ABSTRACT

To investigate dietary influences on the volatilome, the volatile subcategory of the metabolome, we performed a comparative untargeted volatilome analysis of exhaled breath, ruminal fluid, serum, urine, and milk from lactating Holstein cows fed different diets. Thirty-two cows (83.3 ± 31.40 DIM, 30.6 ± 5.03 kg of milk/d) were assigned to 4 diets. The experiment lasted 16 wk. Throughout the experiment, half of the animals were fed a hay-based diet (HD; n = 16), and the other half were fed a silage-based diet (SIL; n = 16). In experimental wk 5 to 12, half of the animals in each group received the control concentrate (CON), and the other half was fed with the CON supplemented with a blend of essential oils (EXP). We hypothesized that the basal diet and the essential oils influence the volatile organic compound (VOC) profiles of the cows through potential changes in ruminal fermentation, digestion, and metabolism (hypothesis 1). Furthermore, we hypothesized that the potential effects of essential oils would have a delayed onset and a carryover effect (hypothesis 2). Every 4 experimental weeks (i.e., in wk 4, wk 8, wk 12, and wk 16), samples of exhaled breath, ruminal fluid, serum, urine, milk, and feed were collected for dynamic headspace extraction and gas chromatographic analysis of VOC in their gaseous phase. Milk yield, milk composition, BW, and feed intake were recorded regularly. Linear mixed models and multivariate and univariate data analyses were performed. The total DMI and basal diet intake was similar between cows fed HD and SIL diets. However, SIL cows consumed less of the concentrate, NDF, and water-soluble carbohydrates and more starch than HD cows. The SIL cows had a higher milk production than the HD cows. No effect was found regarding the concentrate type on feed intake or milk production. Irrespective of diet, 2,957 VOC were detected in the gaseous phase of serum; 2,771 in exhaled breath; 1,016 in urine; 1,001 in milk; and 921 in ruminal fluid. Across the experimental wk 4, 8, 12, and 16, the basal diet altered the VOC profiles of ruminal fluid, urine, and exhaled breath but not those of serum and milk. The concentrate type affected only the VOC profiles of the exhaled breath. Most diet-influenced VOC in the affected biological matrices were identified as dietary components. The experimental week influenced the VOC profiles of all matrices, especially those of exhaled breath. The VOC profile of exhaled breath strongly correlated with that of urine, followed by that of ruminal fluid, milk, and serum. This study provides the first description of diet- and time-specific VOC profiles from the biological matrices of dairy cows. The identified discriminatory VOC seem suitable as markers to discriminate between HD and SIL cows. Exhaled breath may be a promising, sensitive, and less invasive tool to follow diet- and time-related metabolic changes.

Key words: exhalomics, volatilome, metabolome, cattle

INTRODUCTION

The metabolome consists of the entirety of all metabolites at a certain time point (physiological state). It is captured by untargeted metabolomic approaches and provides a snapshot of an organism's metabolism (Amann et al., 2014). One subcategory of the metabolome is the volatilome. The volatilome includes the volatile fraction of the metabolites, that is, the volatile organic compounds (**VOC**), a diverse group of organic molecules with high vapor pressure, typically consisting of 5 to 20

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carbon atoms, with a molecular weight ≤500 Da and a boiling point ≤250°C, such as alcohols, amines, amides, alkaloids, and acids (Tejero Rioseras et al., 2017). Until today, the volatilome has been scarcely studied due to challenges associated with sample collection, processing, and analysis (Honeker et al., 2021). Recent analytical advances in GC-MS (Sun et al., 2015; Honeker et al., 2021) by developing the V-ITEX method (Fuchsmann et al., 2019) allow the detection of low concentrations of VOC with higher resolution and sensitivity, making them promising novel biomarkers (Honeker et al., 2021). Biologically, VOC enter the body by ingestion and inhalation (exogenous pathway) or are produced during metabolic processes (endogenous pathway). Depending on their volatility and solubility, they move through the bloodstream and body tissues, mainly by passive diffusion. Therefore, they can be found in the gaseous phase of several biological matrices, including ruminal fluid, urine, feces, saliva, blood, and others (Amann et al., 2014). Due to the passage of VOC from blood through the membrane of the pulmonary alveoli and the airway epithelium by diffusion and release from the gastrointestinal tract, VOC are also present in exhaled breath (Amann et al., 2014). The composition of an animal's exhaled breath depends on the physiological state and disease-related metabolic or inflammatory processes (Dobbelaar et al., 1996), as well as on ingested feed (Fischer et al., 2015). After ingestion, feed can be microbially degraded in the rumen into smaller molecules and VOC. Such VOC can be exhaled by the ructus (Islam et al., 2023) or absorbed through the rumen wall into the bloodstream, where they can pass the blood–lung barrier and be exhaled, thereby changing the VOC profile of exhaled breath. Therefore, in this work, we considered exhaled breath the mixture of VOC derived from the lungs and from the upper gastrointestinal tract. Hence, sampling VOC from exhaled breath as a novel and low-invasive diagnostic tool can provide information about the metabolic state of an individual and its nutrition, with the potential to detect metabolic diseases (Amann et al., 2014). Thus far, only a few earlier studies have investigated exhaled breath in ruminants. They either described the rare technical appliances and difficulties of exhaled breath sampling and analysis (Spinhirne et al., 2004; Küntzel et al., 2018), proposed exhaled breath to predict ruminal fermentation processes (Islam et al., 2024), or detected a few specific disease-related metabolites of interest in exhaled breath using targeted approaches, for example, for detecting subclinical ketosis (Dobbelaar et al., 1996). Nonpathological VOC profiles are less investigated; therefore, better knowledge of VOC profile-influencing factors is needed (Fischer et al., 2015). The aim of the present study was to evaluate the usability of VOC profiles of biological matrices (exhaled breath, ruminal fluid, serum, urine, and milk) for describing diet-specific metabolic changes in dairy cows over time in an explorative manner. For this purpose, all cows were fed over 16 wk with one of 2 basal diets (silage-based vs. hay-based), formulated to meet their nutritional requirements. A concentrate containing essential oils was added for half of the animals during wk 5 through 12. We hypothesized that the basal diet and the essential oils influence the VOC profiles of the cows through potential changes in ruminal fermentation, digestion, and metabolism (hypothesis 1). Furthermore, we hypothesized that the potential effects of essential oils would have a delayed onset and a carryover effect (hypothesis 2). Correlations of VOC among the biological matrices were also studied.

MATERIALS AND METHODS

Animals, Experimental Design, and Diets

The experimental protocol complied with Swiss legislation for animal welfare and was approved by the Animal Care Committee of the Fribourg Canton, Fribourg, Switzerland (license no. 2020–58-FR/32975). The experiment was conducted at Agroscope (Posieux, Switzerland) from January to April 2021 and lasted 16 wk. It was based on a factorial design with 2 factors—basal diet and concentrate type (Figure 1). Thirty-two lactating primiparous (n = 16) and multiparous (n = 16, 4.00 ± 1.75 lactations) Holstein Friesian and Red Holstein dairy cows were housed in a freestall barn. At the beginning of the study, cows were, on average, 83.3 ± 31.40 DIM and produced 30.6 ± 5.03 kg of milk/d. Cows were assigned to 4 diets balanced by DIM, milk yield, and lactation number. Throughout the experiment, half of the animals were fed a basal diet comprising hay (**HD**, n = 16), and the other half were fed a silage-based diet (**SIL**, n = 16; Figure 1) consisting of corn silage, grass silage, and hay (44%:43%:13% on a DM basis). The hay was harvested from grass-rich mixed leys composed of 49.4% grasses, of which 18.5% was rye grass, 10.9% legumes, and 39.7% other plants. Basal diets were offered ad libitum, and fresh feed was provided 3 times a day at 0500 h, 0900 h, and 1600 h. Due to the assorted chemical compositions of the basal diets (Table 1), to meet the predicted nutrient requirements of the cows (Agroscope, 2021), the SIL cows received in addition to the basal diet 1.5 kg/d of a protein concentrate (55% soybean meal, 29% corn gluten, 10% potato protein, 4% molasses, 2% animal fat) besides the basal diet. In addition, from a milk yield of 27 kg/d and 30 kg/d (for primiparous and multiparous cows, respectively), they received additionally 0.5 kg/d of an energy concentrate (50% corn, 30% barley, 20% wheat) per kilogram of additional milk produced. The HD cows received 3 kg/d of the energy concentrate from a milk

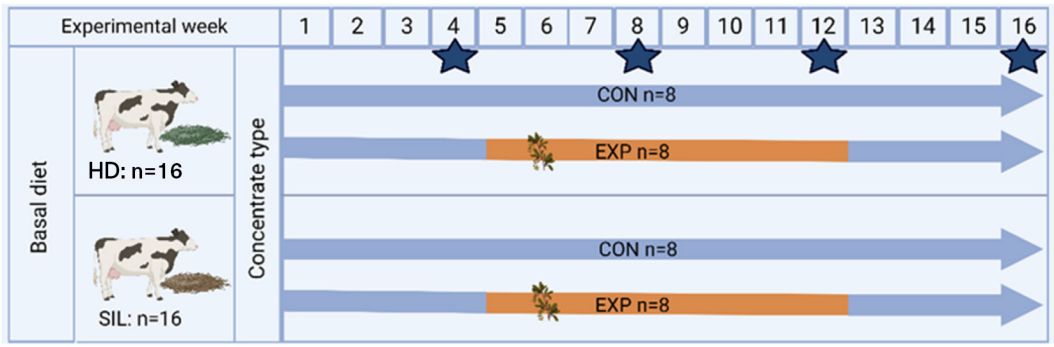

Figure 1. Schematic overview of the experimental design. Blue stars indicate the time points at which urine, blood, milk, rumen fluid, and, except for experimental wk 4, exhaled breath were sampled. HD = hay-fed cows; SIL = silage-fed cows; CON = control concentrate; EXP = experimental concentrate with an added blend of essential oils.

yield of 27 kg/d and 30 kg/d (for primiparous and multiparous cows, respectively) and 0.6 kg/d additional 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. To investigate the time effect of essential oils (see hypothesis 2), half of the HD cows (n = 8) and half of the SIL cows (n = 8) received an experimental concentrate (**EXP**) instead of CON during the experimental wk 5 through 12 (Figure 1). In EXP, 0.34% of the CON (i.e., 0.68% of the corn) was replaced by a feed additive (Xtract Ruminant, Pancosma, Rolle, Switzerland; recommended intake 1 g/d) containing a blend of the essential oils with eugenol, cinnamaldehyde, and capsicum. The 8-wk feeding duration allowed us to observe both a possible delayed onset of the essential oils' effect (several weeks), as observed in earlier studies (van Gastelen et al., 2024), and any possible carryover effect during the 4 wk following the essential oils feeding (wk 13–16). Concentrates were fed through a transponder feeding station (RIC system, Insentec/Hokofarm, Marknesse, the Netherlands). All cows had free access to fresh water and received 300 g/d of a mineral feed containing, per kilogram: $CaHPO_4$, 293.9 g; $CaCO_3$, 227.5 g; MgO, 135.5 g; NaCl, 109.8 g; oats, 80.0 g; dried apple pomace, 69.1 g; animal fat, 40 g; premix, 30 g (per kilogram: $CaCO_3$, 785.3 g; vitamin A, 17,000,000 IE; vitamin D-3, 1,350,000 IE; Zn, 100 g; vitamin E, 80 g; Mn, 20 g; Cu, 12 g; I, 1.45 g; Se, 0.75 g; and Co, 0.50 g); β-carotene, 11 g; and biotin, 3.3 g. The chemical composition of the basal diets and concentrate types are shown in Table 1.

Data Recording and Sampling

Silage samples were collected once a week throughout the experiment; concentrate and hay samples were collected every 4 wk (wk 4 [**W4**], 8 [**W8**], 12 [**W12**], and 16 [**W16**]) and stored at −20°C until analysis. Throughout the experiment, feed intake was continuously recorded using electronic weighing troughs with computer-regulated access gates (RIC system; Insentec/Hokofarm Group BV, Marknesse, Netherlands). Cows were milked twice daily at 0500 h and 1600 h in a tandem milking parlor (Fullwood, A. Bertschy AG, Guschelmuth, Switzerland). The daily milk yield was recorded automatically at each milking. Twice a week, individual milk samples were collected in the morning and pooled with a milk sample from the previous evening's milking to produce a 50-mL aliquot preserved with bronopol for further analysis of the chemical milk composition. Body weight was recorded twice daily after milking using a walkover weighing system (Insentec/Hokofarm Group BV, Marknesse, the Netherlands).

Every 4 wk, that is, the sampling weeks W4, W8, W12 and W16, milk, urine, blood, exhaled breath, and ruminal fluid samples were collected on 4 consecutive sampling days. On the first sampling day, individual milk samples were collected in the morning and pooled with a milk sample from the previous evening's milking to make an aliquot of 100 mL. Fifteen milliliters of this aliquot were stored at −20°C until VOC analysis. On the second sampling day, urine and blood samples were collected. Urine samples were collected immediately after the milking at 0530 h by either spontaneous or stimulated micturition. For this purpose, the cows were attached to the cubicles of the freestall barn. Immediately after sampling, 30 mL of urine per cow was stored on ice and then at −20°C until analysis. Subsequently, cows were moved to the head gate in the covered outdoor area of the barn to take blood from the jugular vein into a 9-mL serum tube containing clot activator (Greiner Bio-One GmbH, Kremsmünster, Austria). After centrifugation at 2,000 × *g* for 15 min at 4°C, the serum samples were stored in a 2-mL tube at −80°C until VOC analysis. On the third sampling day in W8, W12, and W16, but not W4 due to the unavailability of the sampling device in W4,

Table 1. Chemical composition of the basal diets HD (n = 5) and SIL (n = 16) and control concentrate (n = 4) and experimental concentrate (n = 4)[1]

Item	Diet[2]		Energy concentrate[3]		Protein concentrate[4]
	HD	SIL	CON	EXP	
DM (g/kg of wet weight)	911 ± 5.8	439 ± 9.9	891 ± 3.5	890 ± 2.8	899 ± 4.3
OM	902 ± 9.3	931 ± 4.2	980 ± 1.7	980 ± 1.2	954 ± 2.0
CP	153 ± 11	118 ± 6.2	114 ± 3.4	112 ± 2.4	559 ± 20
NDF	488 ± 20	415 ± 13.0	148 ± 20	161 ± 22	113 ± 13
ADF	261 ± 7.3	247 ± 7.7	45.6 ± 6.7	44.0 ± 0.7	48.0 ± 2.1
Starch	—	180 ± 15	674 ± 20	664 ± 8.7	120 ± 19
WSC[5]	117 ± 18	52.6 ± 4.9	31.7 ± 2.2	40.0 ± 3.8	72.7 ± 3.4
Ca	5.85 ± 1.1	4.64 ± 0.3	1.00 ± 0.4	1.00 ± 0.1	2.00 ± 0.2
P	3.65 ± 0.4	2.85 ± 0.5	3.57 ± 0.2	4.00 ± 0.1	6.00 ± 0.3
Mg	1.65 ± 0.1	1.68 ± 0.1	1.00 ± 0.1	1.00 ± 0.3	2.00 ± 0.1
K	27.1 ± 1.4	19.9 ± 0.9	5.00 ± 0.4	4.00 ± 0.1	15.0 ± 0.1
Na	0.26 ± 0.2	0.29 ± 0.2	0.15 ± 0.0	0.63 ± 0.0	0.39 ± 0.1
Calculated energy and protein supply[6] (per kg of DM)					
APD	98.0 ± 6.5	70.1 ± 3.2	105 ± 1.2	105 ± 0.6	353 ± 1.3
N_{EL} (MJ)	6.10 ± 0.1	6.28 ± 0.1	8.30 ± 0.1	8.30 ± 0.1	8.41 ± 0.1

[1]Values are given as grams per kilogram of DM, unless otherwise noted, and means (over all experimental weeks) ± SD.

[2]HD = 100% hay; SIL = 44% grass silage, 43% corn silage, and 13% hay.

[3]CON = control concentrate (50% corn, 30% barley, and 20% wheat); EXP = experimental concentrate (49.32% corn, 30% barley, 20% wheat, and 0.34% essential oils [XtractRuminant, Pancosma, Rolle, Switzerland; 1 g/d per cow]).

[4]Protein concentrate (55% soybean meal, 29% corn gluten, 10% potato protein, 4% molasses, and 2% animal fat).

[5]WSC = water-soluble carbohydrates.

[6]Absorbable protein at the duodenum (APD) and N_{EL} were calculated according to Agroscope (2021).

exhaled breath was collected directly after the 0530 h milking. Before the exhaled breath sampling, solid-phase extraction (**SPE**) cartridges (2 per cow to have a technical replica) were conditioned with a rinse consisting of nanopure water, methanol, acetone, and acetonitrile (3 × 3 mL each). All required chemicals were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) with the following solvent purities: acetonitrile (HPLC grade 99.9%), acetone (HPLC grade 99.8%), methanol (HPLC grade 99.9%), and water (mili-Q ultrapure grade). The cartridges were then dried under nitrogen (purity level 5: 99.999%) for 20 min. For exhaled breath sampling, each cow was moved to the head gate. A tight-fitting face mask (Air One, Hippomed/Neu-Tec GmbH, Steinhagen, Germany) was manually held over the nostrils and mouths of the cows for 3 min by experienced personnel, as described by Küntzel et al. (2018). The face mask was connected to a silicone tube with a vacuum pump (Type HN 726.3FT.18, Neuberger, Balterswil, Switzerland) at its end to transport the exhaled breath through a bottle containing 1 mL of an internal deuterated standard solution (100 µg/L dimethylsulfide-d6, 10 µg/L dimethylsulfoxide-d6 in acetonitrile; the presence of the latter 2 was ensured by DHS-V-ITEX-GC-MS analysis) and afterward over the 2 SPE cartridges simultaneously, each containing a highly porous polystyrene–divinylbenzene copolymer Chromabond HR-P (Macherey–Nagel, Oensingen, Switzerland; Eichinger et al., 2024). For sampling the next cow, the used cartridges were replaced by 2 new cartridges. Within 2 h after sampling the exhaled breath from all cows, the SPE cartridges were dried under a flow of nitrogen for 3 min, and the captured VOC were eluted with 600 µL of acetonitrile. After 5 min, the cartridges were flushed with air using a 20-mL syringe to recover the concentrated VOC and stored in 1.5-mL glass vials at −40°C until VOC analyses. On the fourth sampling day, 15 mL of ruminal fluid was collected from each cow immediately after the 0530 h milking via an oral stomach tube (SELEKT, Qidee GmbH, Homberg, Germany) with an inner tube connected over a liquid trap to a vacuum pump. Samples were taken from the trap after discarding the first 0.5 to 1 L of pumped liquid. After collection, samples were stored on ice and then at −20°C until VOC analysis. Samples of urine, blood, exhaled breath, and ruminal fluid were each taken directly after the 0530 h milking to disregard the influence of diurnal variability.

Laboratory Analysis

Feed Samples. Feed samples were ground to pass a 1.0-mm sieve (Brabender mill with titanium blades; Brabender, Duisburg, Germany). Dry matter and ash contents were determined by drying at 105°C for 3 h (prepASH 340, Precisa, Dietikon, Switzerland), followed by incineration at 550°C until a constant weight was reached. The distinction between DM and ash content was defined as OM. The NDF (method 16472:2006; ISO, 2006) and ADF (method 13906:2008; ISO, 2008)

contents were determined with a Fibertherm (Gerhardt, Konigswinter, Germany) and corrected for ash content. The NDF content was analyzed with the addition of heat-stable amylase and sodium sulfite. Water-soluble carbohydrates (**WSC**) were determined according to Hall et al. (1999). The total nitrogen content was determined using the Kjeldahl method (AOAC, 1990; method 988.05). The CP content of the feed items was calculated by multiplying the N content by 6.25.

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

Before VOC analyses of exhaled breath, ruminal fluid, serum, urine, milk, and feed, the respective samples were thawed on ice. The refractive index of the urine samples was determined (refractometer RE40, Mettler Toledo, Switzerland) according to Weeth et al. (1969). To standardize the urine samples, they were diluted with ultrapure water to a common refractive index of 1.3341 at 20°C, corresponding to a specific gravity of 1.00083 according to Pimentel et al. (2020). For feed sample preprocessing, 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 were prepared for each biological matrix and for the supernatant of the feed samples by pooling all samples of one matrix at equivalent volumes (Dunn et al., 2011). Afterward, 100 µL of each sample (including the QC samples) were put in a 20-mL headspace vial, hermetically sealed with a silicone and Teflon septum (Macherey–Nagel, Oensingen, Switzerland), and stored at 4°C for a few minutes until analysis.

The prepared samples of each matrix were randomized using the Excel function RAND to avoid systematic bias and were analyzed in the following order: exhaled breath, feed, serum, milk, urine, and ruminal fluid. One analytical batch contained 5 QC samples (first and every tenth sample) and 32 samples of the respective matrix. Untargeted analyses of VOC were performed using dynamic headspace vacuum in-tube extraction (**DHS-V-ITEX**, CTC Analytics, Zwingen, Switzerland) gas chromatography–mass spectrometry based on the vacuum transfer in-tube extraction (**DHS-VTT**) developed by Fuchsmann et al. (2019). The DHS-V-ITEX-GC–MS consisted of an MPS2 autosampler (Gerstel, Sursee, Switzerland) equipped with a V-ITEX module and an Agilent 7890B GC system (Agilent Technology, Santa Clara, CA) coupled to an Agilent 5977B mass selective detector (Agilent Technology, Santa Clara, CA). After 10 min of incubation at 60°C, the headspace was extracted for 10 min at 60°C under a vacuum (1,500 Pa) using a vacuum pump (Büchi V-300 and Interface I-300, Büchi, Flawil, Switzerland) and in-tube extraction materials equipped with a trap filled with ITEX2 Carbosieve S III/Tenax TA (Tenax TA 2/3 bottom)/Carbosieve S III (1/3 top; BGB Analytics, Boeckten, Switzerland) according to Fuchsmann et al. (2019). The VOC were desorbed from the sorbent for 2 min at 300°C under a nitrogen flow of 220 to 250 mL/min at 35°C. The programmable temperature vaporizing injector was equipped with a glass liner filled with Tenax TA and conditioned for 60 min at 320°C. The injector was heated to 250°C at a rate of 12°C/s. 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, Oensingen, Switzerland) with helium as the carrier gas at a flow of 2.1 mL/min (37 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 29 and 250 atomic mass units with a gain of 15 without solvent delay. The autosampler was controlled by Cycle Composer V. 1.5.4 (CTC Analytics, Zwingen, Switzerland), and the CIS 4 injector was controlled by Maestro1 software V.1.4.8.14/3.5 (Gerstel). Because the analysis was semiquantitative, the reported VOC concentrations are relative, determined from the total ion count (**TIC**) for the VOC peak area (arbitrary unit).

Data Processing and Statistical Analysis

Feed intake, milk yield, and BW data were averaged per week and cow. For VOC data, the automatic deconvolution of MS signals was performed using Masshunter Profinder software (Version 10.0) in recursive mode (Agilent Technologies, Santa Clara, CA). During deconvolution, VOC were eliminated if their MS signals were less than 3 times the median of the background peak height. Missing values after automatic deconvolution due to signals below the detection limit were replaced by zero, according to Xia et al. (2009). The data were normalized using probabilistic quotient normalization (Dieterle et al., 2006).

As mentioned previously, the experiment was a 2-factor factorial experiment with the 2 factors basal diet and concentrate type (Figure 1). To investigate both the potential effects of the basal diet and concentrate type on animal production variables to provide a basis for interpreting diet-related changes in VOC profiles (hypothesis 1) and the time effect of the essential oils (hypothesis 2), a linear mixed model (R Version 4.1.3, package nlme; Pinheiro and Bates, 2000) was used for

the statistical analysis of BW, feed intake, and milk production variables. The complete data set was analyzed together, and the animal served as experimental unit. Basal diet, concentrate type, experimental week, and their interactions (basal diet × experimental week, concentrate type × experimental week, basal diet × concentrate type × experimental week) were included as fixed effects. Because the interaction basal diet × concentrate type was not significant, the interaction was removed from the statistical model. The animal was used as a random factor. An autoregressive structure (AR1) was included to consider that data points closer in time are typically more correlated than those further apart. Where significant interactions were found the Tukey test was used for comparisons between subgroups. *P*-values <0.05 were considered statistically significant. For VOC data analyses, multivariate and univariate analyses were performed. To discriminate between the 2 different basal diets and between the 2 different concentrate types, partial least squares discriminant analysis (**PLS-DA**), and sparse partial least squares discriminant analysis (**sPLS-DA**) were performed per biological matrix and sampling week separately. To discriminate between the 4 sampling weeks, PLS-DA and sPLS-DA were performed for each biological matrix. The R packages used were mixOmics (Kim-Anh Le Cao, 2016), ropls (Thévenot et al., 2015), and MetaboAnalyst (Xia et al., 2009). To evaluate the quality of the PLS-DA models, the goodness-of-fit (**R2**) and predictive ability parameter (**Q2**) were calculated using the ropls package (using Q2 > 0.5, R2 > 0.8 as validity thresholds; Wold et al., 2001). Additionally, the classification error rates (**ER**) of sPLS-DA models, that is, the percentage of misclassified samples across 999 cross-validated bootstrapping runs, were calculated using mixOmics. An ER <0.5 indicates that the model performs better than a random model (Triba et al., 2015; Singh et al., 2019; Pimentel et al., 2020). For each PLS-DA model, the variable importance in projection (**VIP**) scores of VOC were calculated using MetaboAnalyst (https://www.metaboanalyst.ca/) to select VOC that contribute the most to samples discrimination. The univariate analyses included Wilcoxon's signed-rank tests and nonparametric longitudinal data analyses. Wilcoxon's tests were conducted across all sampling weeks for each biological matrix, adjusted for multiple testing by Benjamini Hochberg's correction. Nonparametric longitudinal data analysis was performed across all sampling weeks for each biological matrix using the nparLD R package (Noguchi et al., 2012). Finally, to investigate the magnitude of diet-related differences, fold changes of VOC's TIC area were calculated between means of HD and SIL and means of CON and EXP cows across sampling weeks per biological matrix. Correlations (correlation cutoff r = 0.7) between the first axes of PLS-DA models from each of the biological matrices were presented using data integration analysis for biomarker discovery (DIABLO; Singh et al., 2019) from the R package mixOmics.

Identification of Discriminatory Volatile Organic Compounds

To determine discriminatory VOC of valid PLS-DA models discriminating between diets within each of the 4 sampling weeks, we followed a selection procedure that was based on 4 conditions (Supplemental Figure S1A; see Notes): First, we selected VOC that had (1) a VIP score >2 on at least one of the first 2 axes of the PLS-DA model in at least one sampling week. Second, VOC had to be significantly ($P < 0.05$) different between diets in (2) a Wilcoxon's signed-rank test adjusted by Benjamini Hochberg's correction in at least one sampling week and (3) a nonparametric longitudinal analysis across all sampling weeks. Finally, the VOC's TIC area had to (4) demonstrate a fold change greater than 1.4 between diets across sampling weeks. Because the sampling week consist of 4 levels, slightly different statistical tests were used. The determination of discriminatory VOC of valid PLS-DA models discriminating between sampling weeks involved a slightly different selection procedure (Supplemental Figure S1B). The adjustment was necessary because the factor "sampling week" had 4 levels, unlike the factor "diet" having only 2 levels. We selected VOC that had (1) loading weights < −0.2 or >0.2 on at least one of the first 2 axes of the sPLS-DA model. Additionally, these VOC had to be significantly ($P < 0.05$) different between sampling weeks in (2) a Kruskal−Wallis test corrected for false discovery rate and (3) a nonparametric longitudinal analysis. For the peak identification of the selected VOC, the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (**NIST17**; NIST, Gaithersburg, MD) was used. The identification of VOC was performed considering the identification levels defined by the metabolomics standards initiative (Sumner et al., 2007). Both level 2–identified VOC and tentatively identified VOC were considered. Level 2 corresponds to spectra with a match factor greater than 80% and a maximum relative difference in the calculated retention index (**RI**) of ± 15 of the reference RI. Tentatively identified VOC corresponds to spectra with a match factor greater than 80% and a reference RI not defined in reference databases. The RI was calculated using the temperature-programmed Kovats index (Girard, 1996). The identified VOC were classified into their chemical compound groups: aldehydes, alcohols, alkanes, alkenes, amides, anhydrides, azines, azoles, carboxylic acids, esters, ethers, pyridines, ketones, nitriles, terpenes, and sulfur compounds.

RESULTS

Body Weight, Milk Production, and Feed Intake

Body weight was not affected by the basal diets (P = 0.99), but increased continuously from one experimental week to another (P < 0.01; Table 2). The SIL cows produced more ECM (P = 0.03) than HD cows. No interaction between basal diet and experimental week was found for BW and ECM (P > 0.05). Compared with HD cows, the SIL cows ingested similar amounts of the respective basal diet (P = 0.14) but, due to the concentrate allocation scheme, less (P < 0.01) concentrate, which did not affect total DMI (P = 0.56). The SIL cows had a greater CP intake in W4 and W8 and a lower CP intake in W12 and W16 than HD cows (interaction P = 0.02). The ADF intake was similar for HD and SIL cows (P = 0.47). The intakes of basal diet, concentrate, total DMI, CP, and ADF varied by basal diet depending on the experimental week (all interactions P < 0.01), without showing distinct patterns. Compared with HD cows, SIL cows had a lower NDF intake (P < 0.01), which was more pronounced in W4 and W8 (interaction effect P < 0.01). The SIL cows had further a greater starch intake and lower WSC intake, which fluctuated by experimental week with no clear pattern observed (both interactions P < 0.01). Feed and nutrient intake varied among experimental weeks (all P < 0.01) without a distinct pattern. No differences in terms of BW, ECM, and feed and nutrient intake (all P > 0.05) were observed between CON and EXP cows (Table 3; Supplemental Table S1; see Notes). Finally, no interactions between concentrate type and experimental week and between basal diet, concentrate type, and experimental week were found (Table 3).

Dietary Influences on Volatile Organic Compounds

Across all sampling weeks and diets, 2,771 VOC were detected in exhaled breath, 921 in ruminal fluid, 2,975 VOC in serum, 1,016 in urine, and 1,001 in milk. The HD and SIL cows differed in their VOC profiles of exhaled breath in W12 and W16 (Figure 2A) and across all sampling weeks in those of ruminal fluid (Figure 2B) and urine (Figure 2C). No differences were found in the VOC profiles of serum and milk according to a basal diet (Supplemental Figures S2A and S2B; see Notes). Within exhaled breath, ruminal fluid, and urine, 21 VOC were selected as discriminatory based on our 4 conditions and, therefore, occurred in diverse concentrations or were present—or not—according to fed basal diets (Table 4). These VOC were identified, and all were present in the corresponding feed samples. Fifteen discriminatory VOC were more abundant (all P < 0.01) in SIL cows than in HD cows (5 in ruminal fluid, 6 in exhaled breath, and 4

Table 2. Effects of basal diet HD (n = 16) and SIL (n = 16) on feed intake and milk production

Item	Experimental week										*P*-value[1]		
	W4		W8		W12		W16						
	HD[2]	SIL[2]	HD	SIL	HD	SIL	HD	SIL	SEM	Basal diet	Experimental week	Basal diet × exp. week
BW (kg)	669	666	681	676	697	695	704	709	5.87	0.99	<0.01	0.09
ECM[3] (kg/d)	29.9	34.5	31.6	34.3	32.1	34.0	30.8	33.4	0.40	0.03	0.18	0.24
Feed intake (kg DM/d)												
Basal diet	17.1	18.8	17.0	17.7	18.2	19.3	17.9	19.3	0.20	0.14	<0.01	<0.01
Concentrates	4.40	4.26	5.06	4.13	5.00	3.89	4.77	3.67	0.11	<0.01	<0.01	<0.01
Total DMI	21.5	23.0	22.1	21.8	23.2	23.2	22.7	23.0	0.19	0.56	<0.01	<0.01
CP	3.06	3.32	3.18	3.22	3.45	3.28	3.35	3.08	0.03	0.02	<0.01	<0.01
ADF	4.85	4.71	5.01	4.78	5.05	5.20	4.90	5.04	0.05	0.47	<0.01	<0.01
NDF	9.54[a]	8.32[b]	9.36[a]	7.99[b]	9.63	9.16	9.17	8.75	0.09	<0.01	<0.01	<0.01
Starch	2.46[a]	4.80[b]	2.91[a]	4.76[b]	2.87[a]	4.76[b]	2.72[a]	4.87[b]	0.11	0.42	<0.01	<0.01
WSC[4]	2.12[a]	1.32[b]	1.98[a]	1.18[b]	2.25[a]	1.11[b]	2.18[a]	0.94[b]	0.05	<0.01	<0.01	<0.01

[a,b]Within rows, means with different superscripts within one experimental week are statistically different in the post hoc test (Tukey's HSD).
[1]*P*-values for the effects of the basal diet, experimental week, and the basal diet × experimental week interaction.
[2]HD = cows fed 100% hay as a basal diet; SIL = cows fed 44% grass silage, 43% corn silage, and 13% hay as a basal diet.
[3]Here defined as milk yield (kg) × [0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%)]/3.14.
[4]WSC = water-soluble carbohydrates.

Table 3. Effects of the concentrate type, CON (n = 16) and EXP (n = 16), on BW, feed intake, and milk production and their means during experimental wk 5–12

	Weeks 5–12[1]					*P*-value[2]		
	HD[3]		SIL[3]					
Item	CON	EXP	CON	EXP	SEM	Concentrate type	Concentrate type × exp. week	Basal diet × concentrate type × exp. week
BW (kg)	678	691	670	691	2.97	0.41	0.55	0.79
ECM[4] (kg/d)	31.5	32	35.3	33.3	0.20	0.06	0.06	0.06
Feed intake (kg DM/d)								
Basal diet	17.3	17.9	17.7	19.4	0.10	0.81	0.60	0.67
Concentrate	5.16	4.81	4.31	3.74	0.05	0.37	0.51	0.36
Total DMI	22.5	22.7	22.0	23.1	0.10	0.90	0.80	0.68
CP	3.28	3.33	3.24	3.35	0.05	0.86	0.66	0.63
ADF	4.97	5.09	4.84	5.26	0.02	0.90	0.67	0.74
NDF	9.38	9.58	8.26	8.9	0.04	0.98	0.72	0.73
Starch	2.97	2.79	4.77	4.66	0.05	0.74	0.88	0.30
WSC[5]	2.08	2.12	1.14	1.21	0.02	0.78	0.63	0.96

[1]Experimental wk 5–12, CON cow fed the control concentrate and EXP cows fed the experimental concentrate containing a blend of essential oils (XtractRuminant, Pancosma, Rolle, Switzerland; 1 g/day per cow).

[2]*P*-values for the effects of the concentrate type, the concentrate type × experimental (exp.) week, and the basal diet × concentrate type × experimental week interaction calculated for data from the experimental weeks (wk 5–12).

[3]HD = cows fed 100% hay as a basal diet; SIL = cows fed 44% grass silage, 43% corn silage, and 13% hay as a basal diet.

[4]Here defined as milk yield (kg) × [0.38 × fat (%) + 0.24 × protein (%) + 0.17 × lactose (%)]/3.14.

[5]WSC = water-soluble carbohydrates.

in urine). Six VOC were more abundant (all $P < 0.03$) in HD cows compared with SIL cows (4 in ruminal fluid and 2 in urine). The abundance of VOC in serum and milk did not differ ($P > 0.05$) between HD and SIL cows (Supplemental Figures S2A and S2B). The VOC profiles of the exhaled breath of cows fed the differing EXP and CON concentrates in W8 are shown in Figure 3; the VOC profiles of exhaled breath in W12 and of ruminal fluid, serum, urine, or milk did not differ in any of the sampling weeks (Supplemental Table S2; see Notes). In W8, 2 discriminatory VOC, namely, one aldehyde and one alcohol, were found. Both were more abundant in the EXP than in the CON cows and, except for the aldehyde, in the EXP feed sample (Table 5).

Influence of the Sampling Week on Volatile Organic Compounds

The sampling week had the greatest effect on the VOC profile of exhaled breath, followed by that of urine, ruminal fluid, milk, and serum (Figure 4). A total of 52 VOC were selected as discriminatory based on our 4 conditions and, thus, occurred in disparate concentrations between sampling weeks. These VOC were identified and are displayed in Table 6.

Relationships of Volatile Organic Compounds of Ruminal Fluid, Urine, Exhaled Breath, Serum, and Milk

Figure 5 shows the correlations among the VOC of the biological matrices for each sampling week. The correlated VOC remained unidentified. Most were more abundant in SIL cows compared with HD cows, and most correlations were positive. The Pearson correlations between the PLS axes ranged from 0.60 (between VOC from exhaled breath and milk) to 0.98 (between VOC from ruminal fluid and urine; Figure 6). The strongest correlations for exhaled breath were found with urine, with correlation coefficients ranging from 0.70 to 0.86, followed by ruminal fluid, with correlation coefficients ranging from 0.69 to 0.84, milk with correlation coefficients ranging from 0.60 to 0.76, and serum with correlation coefficients ranging from 0.69 to 0.78.

DISCUSSION

To the best of our knowledge, our study is the first to evaluate the suitability of VOC profiles from exhaled breath for describing dietary effects over time. Comparing VOC profiles to profiles obtained from ruminal fluid, serum, urine, and milk enabled correlations of VOC between varying biological matrices of dairy cows to hypothesize about interconnecting pathways.

Diet-Specific Metabolic Effects

Hay- Versus Silage-Fed Cows. In the present study, the basal diet influenced both performance traits and VOC profiles. Across all experimental weeks, SIL cows consumed similar amounts of the respective basal diet but less concentrate compared with HD cows, which did not affect total DMI but resulted in a slight difference in

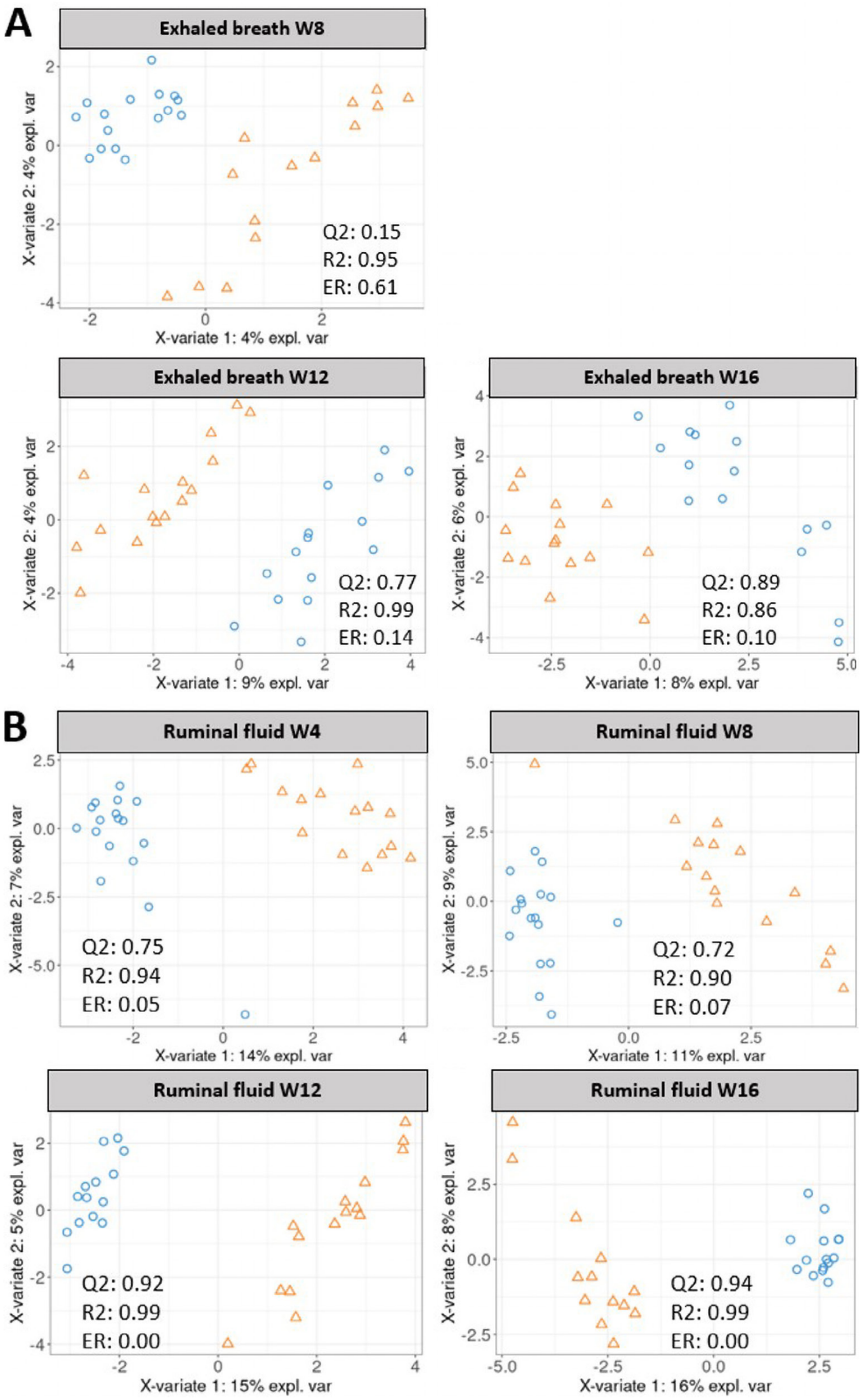

Figure 2. Partial least squares discriminant analysis (PLS-DA) individual plots of axes one and 2, presenting samples of (A) exhaled breath, (B) ruminal fluid, and (C) urine from hay-fed (HD; n = 16; blue circles) and silage-fed (SIL; n = 16; orange triangles) cows in the sampling weeks (W4, W8, W12, and W16; for exhaled breath from W8 onward). Q2 = predictive ability parameter of the PLS-DA models, R2 = the goodness-of-fit value of the PLS-DA models, ER = error rate of the sparse PLS-DA (sPLS-DA) models, and expl. var = explained variance.

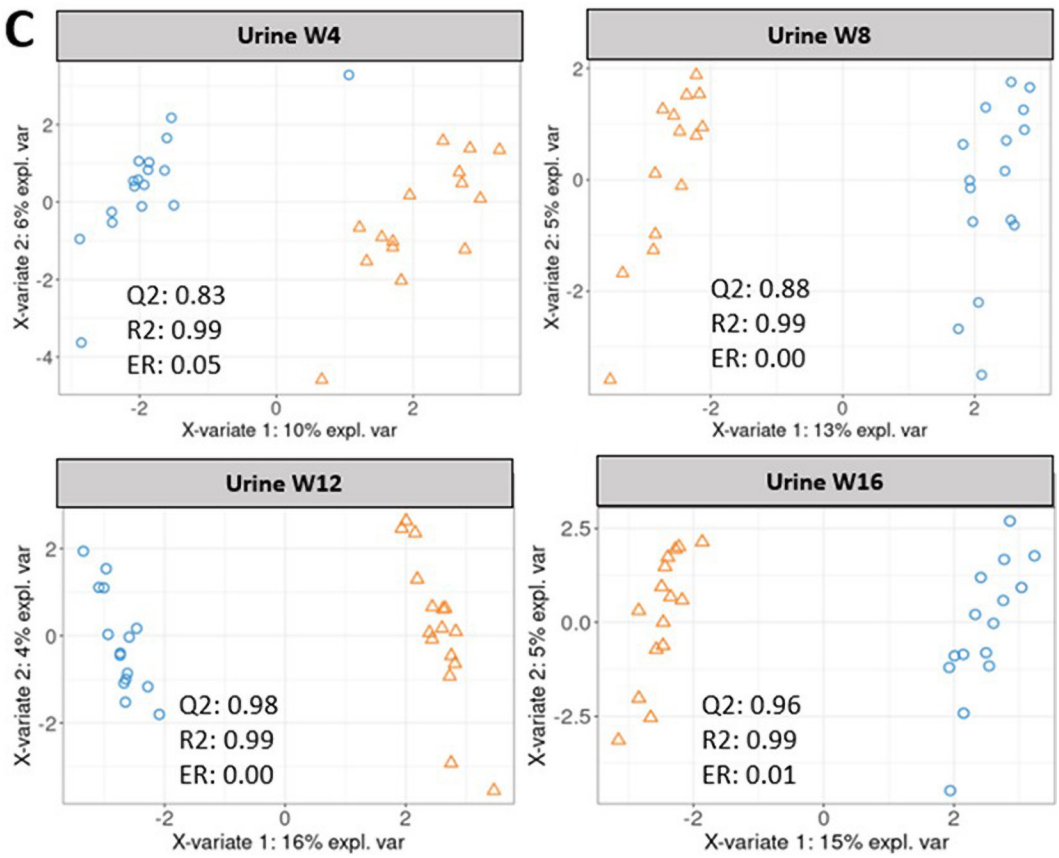

Figure 2 (Continued). Partial least squares discriminant analysis (PLS-DA) individual plots of axes one and 2, presenting samples of (A) exhaled breath, (B) ruminal fluid, and (C) urine from hay-fed (HD; n = 16; blue circles) and silage-fed (SIL; n = 16; orange triangles) cows in the sampling weeks (W4, W8, W12, and W16; for exhaled breath from W8 onward). Q2 = predictive ability parameter of the PLS-DA models, R2 = the goodness-of-fit value of the PLS-DA models, ER = error rate of the sparse PLS-DA (sPLS-DA) models, and expl. var = explained variance.

forage-to-concentrate ratio (72:28 and 80:20 for HD and SIL cows, respectively). This is explained by the lower energy content of hay compared with silage and, consequently, the higher amounts of concentrate fed to HD cows to provide similar amounts of energy. The lower intakes of NDF, WSC, and CP in W12 and W16, as well as the greater ingested amounts of CP (W4 and W8) and starch (all experimental weeks) of SIL cows compared with HD cows related to fluctuations in the nutritional values of hay and silage. The VOC profiles, including many individual VOC of exhaled breath in W12 and W16 and of ruminal fluid and urine across all sampling weeks were influenced by the basal diet. This supports the hypothesis that the basal diet influences VOC profiles (hypothesis 1), at least those of exhaled breath, ruminal fluid, and urine. Milk and serum VOC were not influenced, indicating the poor suitability of milk and serum VOC to discriminate between cows fed diverse basal diets. This contrasts with milk and serum being very suitable for detecting metabolic disorders or nutritional imbalances, for example, by non-VOC such as milk fat, milk protein and MUN, and serum glucose, nonesterified fatty acids, BHB, and urea (Andjelić et al., 2022).

In the present study, most identified discriminatory VOC in exhaled breath (6), ruminal fluid (9), and urine (6) related to distinctions in feed ingredients, composition, and processing. The higher levels of p-cresol in urine and ruminal fluid and indole in the ruminal fluid of HD cows (W12, W16) may partly be explained by the higher CP intake of these cows compared with SIL cows. Higher CP intake can increase p-cresol and indole concentrations in ruminal fluid in vitro (Schreurs et al., 2003) due to tryptophan and tyrosine degradation by *Lactobacillus* strains (Schreurs et al., 2003; Rivaroli et al., 2019). This relationship has not yet been studied in cows but has been demonstrated in sheep (Schreurs et al., 2003). Other than CP intake, the increased p-cresol and indole levels in HD cows may be related to disparities in protein solubility and degradability in the rumen after hay ingestion. Tavendale et al. (2006) demonstrated higher p-cresol and indole concentrations in the rumen of dairy cows, presumably due to AA degradation after

Table 4. Discriminatory VOC of exhaled breath, ruminal fluid and urine of hay- (n = 16) compared with silage-fed (n = 16) cows across sampling weeks and of hay (n = 1) and silage (n = 1) feed samples

					Cows[1]				Feed[2]	
Biological matrix	Chemical group	Volatile organic compound[3]	RI calc[4]	RI ref[5]	TIC[6]	TIC[6]	SEM	*P*-value[7]	TIC[6]	TIC[6]
Exhaled breath	Alkene	**p-(1-Propenyl)-toluene**	1,478	ND	653,299	765,576	35,062	<0.01	—	15,356,818
	Ester	Diethyl butandioate	1,703	1,694	323,415	492,419	59,202	<0.01	—	26,383,194
		Ethyl acetate	887	890	1,349,343	2,204,743	260,873	<0.01	2,101,432	79,422,854
	Sulfone	Dimethyl sulfone[8]	1,882	1,890	9,788,452	15,140,850	942,415	<0.01	386,179	841,070
	Sulfoxide	Dimethyl sulfoxide	1,552	1,549	20,303,889	29,436,758	2,035,027	<0.01	—	915,276
	Terpene	p-Cymene	1,300	1,293	865,878	1,017,309	47,079	<0.01	525,610	43,272,319
Ruminal fluid	Aldehyde	Hexanal	1,099	1,088	306,277	570,818	22,818	<0.01	5,565,441	24,122,430
	Alkane	**Propyl-cyclopropane**	1,363	ND	796,910	1,554,291	92,039	<0.01	—	18,990,664
	Alcohol	p-Cresol	2,123	2,126	1,406,312	708,279	85,837	<0.01	1,940,018	1,732,972
		2-Undecanone	1,736	1,722	113,994	73,878	6,100	<0.01	1,545,701	—
	Ketone	2-Butanone	912	900	1,047,500	613,710	31,171	<0.01	1,075,778	—
		6,10,14-Trimethyl-pentadecan-2-one	2,145	2,131	906,403	472,182	32,194	0.03	16,233,283	10,385,794
		2-Tridecanone	1,831	1,828	396,918	920,137	20,085	<0.01	17,041	418,273
	Heterocyclic aromatic amine	Indole	2,435	2,446	2,358,349	529,040	63,204	<0.01	433,433	127,741
		Dimethyl sulfone[8]	1,882	1,890	5,918,701	12,936,397	813,734	<0.01	386,179	841,070
Urine	Alcohol	α, 4-Dimethyl-benzenmethanol	1,689	ND	126,663	877,688	10,003	<0.01	99,502	987,999
		p-Cresol	2,123	2,126	310,336	90,780	16,756	<0.01	1,940,018	1,732,972
	Ketone	1-(2,6,6-Trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one	1,835	1,822	384,858	3,946,986	105,702	<0.01	37,375,668	—
		2-Acetylfuran	1,521	1,512	134,634	605,439	22,855	<0.01	18,090,434	225,129
		4-Hexen-2-one	1,229	ND	58,427	505,086	25,231	<0.01	24,209,020	—
		Isophorone	1,591	1,605	1,760,252	778,930	94,968	<0.01	11,786,192	720,685

[1]HD = cows received 100% hay; SIL = cows received 44% grass silage, 43% corn silage, and 13% hay.

[2]Basal diet: HD = 100% hay; SIL = 44% grass silage, 43% corn silage, and 13% hay.

[3]Volatile organic compounds identified using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17). All match factors >80% = tentatively identified (indicated by bold text; match factor >80%; reference RI not defined or greater than ± 15 from the calculated RI).

[4]RI calc = calculated retention index using the formula for the temperature-programmed Kovats index (Girard, 1996).

[5]RI ref = reference retention index after comparison with the NIST chemistry web book; ND = no retention index available in the literature with respect to a comparable analytical method (polar column FFAP, ramp temperature).

[6]TIC = Total ion count area.

[7]*P*-value obtained from nonparametric longitudinal model.

[8]For experimental wk 4 and 8.

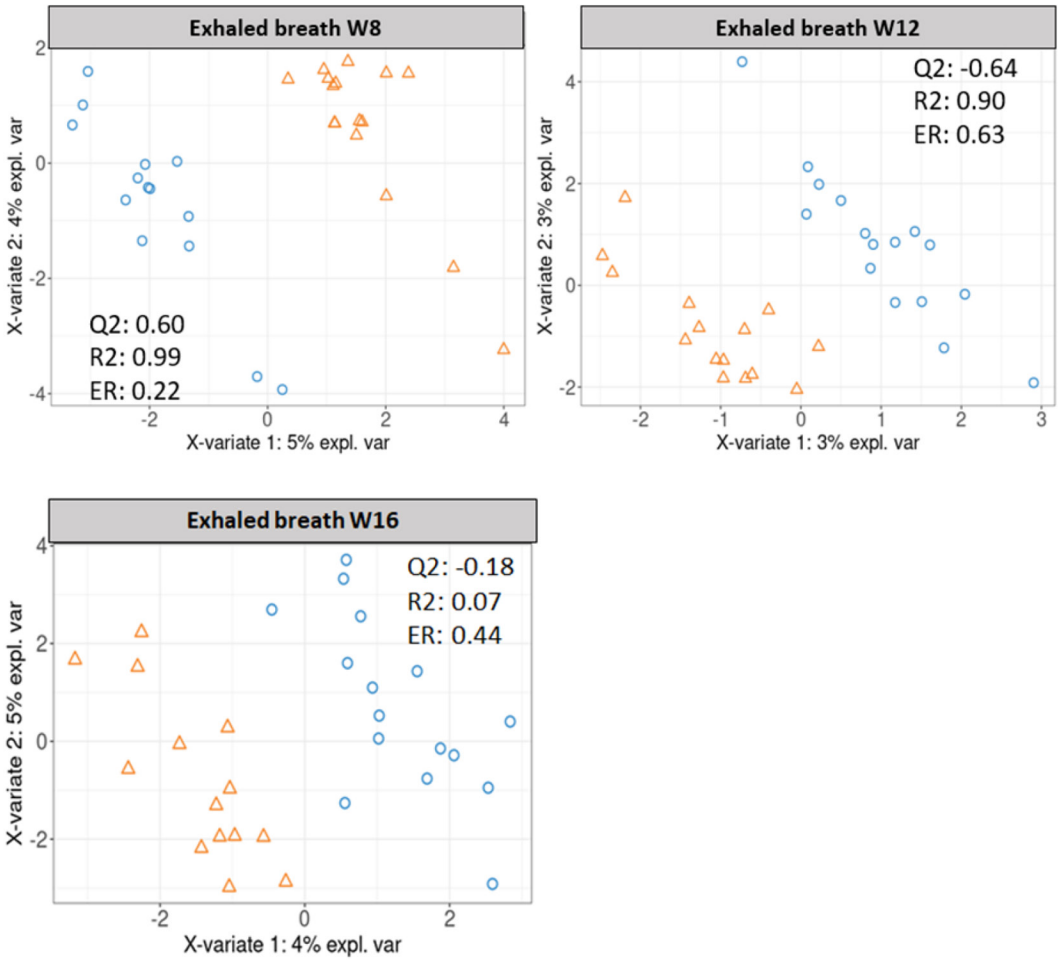

Figure 3. Partial least squares discriminant analysis (PLS-DA) individual plots of axes 1 and 2, presenting samples of exhaled breath from cows fed an experimental (EXP, orange triangles) and control (CON, blue circles) concentrate in sampling wk 8 (W8), 12 (W12) and 16 (W16). Q2 = predictive ability parameter of the PLS-DA models, R2 = the goodness-of-fit value of the PLS-DA models, ER = error rate of the sparse PLS-DA (sPLS-DA) models, and expl. var = explained variance.

Table 5. Discriminatory VOC of exhaled breath of EXP-fed (n = 16) or CON-fed (n = 16) cows in experimental wk 8 and of EXP (n = 1) compared with CON (n = 1) feed concentrate samples

				Exhaled breath[1]				Feed[2]	
				CON	EXP			CON	EXP
Chemical group	Volatile organic compound[3]	RI calc[4]	RI ref[5]	TIC[6]	TIC[6]	SEM	*P*-value[7]	TIC[6]	TIC[6]
Aldehyde	Furfural	1,507	1,493	3,828,756	4,028,470	362,315	<0.01	4,506,057	7,144,718
Alcohol	2-Ethyl-hexanol	1,498	1,504	1,968,357	2,921,655	353,409	<0.01	—	—

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

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

[3]Volatile organic compounds identified using the National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17).

[4]RI calc = calculated retention index using the formula for the temperature-programmed Kovats index (Girard, 1996).

[5]RI ref = reference retention index after comparison with the NIST chemistry web book; ND = no retention index available in the literature with respect to a comparable analytical method (polar column FFAP, ramp temperature).

[6]TIC = Total ion count area.

[7]*P*-value obtained from nonparametric longitudinal model.

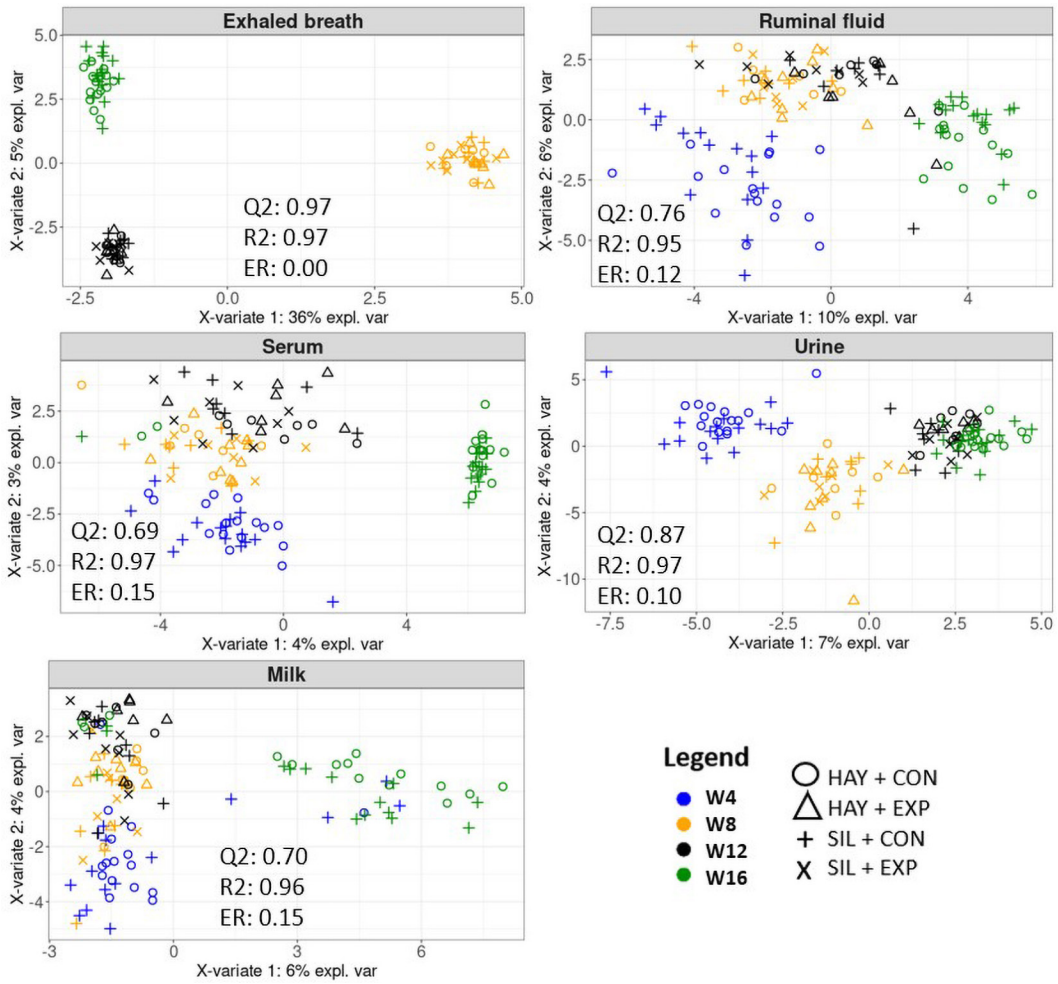

Figure 4. Sparse partial least squares discriminant analysis (sPLS-DA) individual plots of axes 1 and 2, presenting samples of exhaled breath, ruminal fluid, serum, urine, and milk collected in the sampling weeks W4 (blue), W8 (orange), W12 (black), and W16 (green); for exhaled breath from W8 onward. Circles, triangles, plusses, and crosses represent samples of cows fed hay and the control concentrate (HD + CON, n = 8), HD and the experimental concentrate (HD + EXP, n = 8), silage and CON (SIL + CON, n = 8), and SIL and EXP (SIL + EXP, n = 8), respectively. The experimental concentrate contained a blend of essential oils (XtractRuminant, Pancosma, Rolle, Switzerland; 1 g/day per cow). Q2 = predictive ability parameter of the PLS-DA models, R2 = the goodness-of-fit value of the PLS-DA models, ER = error rate of the sparse PLS-DA (sPLS-DA) models, and expl. var = explained variance.

the ingestion of rye grass with higher protein solubility. An alternative explanation for the differences in p-cresol and indole concentrations may relate to the differences in feed conservation between hay and silage. It seems that p-cresol is a degradation product of β-carotene and the isoflavone formononetin, which are both present in plants. Dairy cows fed fresh grass and clover had higher p-cresol concentrations in the milk compared with TMR-fed cows (Faulkner et al., 2018), possibly due to a greater abundance and breakdown of β-carotene and formononetin in fresh-fed herbage (Faulkner et al., 2018). These 2 plant components can be broken down during the drying process of hay by heat and UV radiation into p-cresol (Müller et al., 2007; Kalač et al., 2013), resulting in higher concentrations of p-cresol in HD cows compared with SIL cows. Furthermore, Islam et al. (2024) indicated a relationship of exhaled VOC concentration and composition with the type and solubility of ingested feed. Increased p-cresol levels also appeared to be responsible for the barnyard aroma of milk (Faulkner et al., 2018). The higher CP intake of SIL cows in W4 and W8 may also explain the higher concentrations of dimethyl sulfone in ruminal fluid (W4, W8) and exhaled breath (W8). Villeneuve et al. (2013) compared the VOC profile of milk from cows fed timothy grass as hay, pasture, or silage and found the highest dimethyl sulfone concentration when cows were grazing. Taylor and Kiene (1989) explained increased dimethyl sulfone formation in the rumen from the microbial degradation of methionine, especially from substrates with higher

Table 6. Discriminatory VOC of exhaled breath, ruminal fluid, serum, urine, and milk that differed between the sampling weeks

Biological matrix	Chemical group	Volatile organic compound[2]	RI calc[3]	RI ref[4]	TIC[1]				SEM	*P*-value[6]
					W4[5]	W8	W12	W16		
Exhaled breath	Aldehyde	Furfural	1,507	1,493		6,432,259	1,424,967	1,218,768	80,070	<0.01
	Alcohol	1-Pentanol	1,262	1,256		520,589	970,061	391,184	31,321	<0.01
		Phenol	2,053	2,039		9,587,557	4,084,648	3,526,250	49,857	<0.01
	Alkane	Hexadecane	1,600	1,600		10,822,106	112,889,537	103,948,379	1,564,955	<0.01
	Alkene	**1-H-Indene**	1,827	ND		56,606	1,289,798	1,994,899	45,208	<0.01
		3-Tetradecene	1,433	1,432		1,811,582	469,007	346,724	17,296	<0.01
	Anhydride	Acetic anhydride	1,254	1,240		660,645	3,176,301	827,310	85,914	<0.01
	Ester	Dimethyl succinate	1,552	1,558		530,259	103,424	1,026,847	42,153	<0.01
	Ketone	**1,6-Dioxacyclododecane-7,12-dione**	2,370	ND		22,730	24,975	251,422	4,330	<0.01
		1-(4-Methylphenyl)-ethanone	1,832	1,815		543,926	955,986	290,453	35,614	<0.01
		4-Hydroxy-4-methyl-pentan-2-one	1,399	1,396		3,671,240	8,768,045	2,151,465	310,901	<0.01
	Nitrile	**Fumaronitrile**	1,765	ND		43,785,039	9,376,508	8,983,315	539,406	<0.01
Ruminal fluid	Aldehyde	Tetradecanal	1,948	1,940	945,337	763,862	685,879	394,178	42,556	0.03
	Alcohol	1-Dodecanol	1,981	1,981	777,708	656,600	625,012	518,531	16,305	0.01
	Alkane	Pentadecane	1,590	1,500	211,247	79,525	78,137	66,859	9,307	0.04
		Undecane	1,097	1,100	129,309	38,808	30,834	132,839	8,277	<0.01
	Alkene	1,3-bis(1,1-Dimethylethyl)-benzene	1,443	1,444	591,207	529,454	522,789	2,210,268	86,231	<0.01
		α-Farnesene	1,684	1,696	92,236	178,373	204,415	229,557	9,230	<0.01
		(2E)-3,7,11,15-Tetramethylhexadec-2-ene	1,815	1,802	442,480	248,423	215,415	250,373	21,557	<0.01
	Azole	Pyrrole	1,544	1,547	505,843	545,763	340,987	205,820	18,031	<0.01
	Ketone	**cis-4,6-Dimethylcyclohexane-1,3-dione**	2,146	ND	138,085	212,040	241,641	316,793	14,067	<0.01
	Nitrile	Benzonitrile	1,644	1,629	1,571,954	1,605,522	1,067,929	126,297	72,137	<0.01
Serum	Aldehyde	Benzaldehyde	1,585	1,569	350,322	383,091	381,211	551,871	15,470	<0.01
	Alkane	Dodecane	1,195	1,200	674,469	1,079,822	1,101,759	1,085,128	38,322	<0.01
		2,2,4,6,6-Pentamethyl-heptane	951	956	1,614,540	3,273,190	4,043,794	3,905,991	177,950	<0.01
	Nitrile	Benzonitrile	1,644	1,629	292,200	266,784	216,254	277,611	5,157	<0.01
Urine	Aldehyde	2,6,6-Trimethyl-1,3-cyclohexadiene-1-carboxaldehyde	1,661	1,648	26,568	64,168	257,792	199,143	15,447	<0.01
		Lilac aldehyde	1,759	ND	172,914	38,573	0.00	1,511	5,999	<0.01
		2,6,6-Trimethylcyclohexa-1,4-dienecarbaldehyde	1,548	ND	180,859	113,960	78,832	50,940	6,666	<0.01
	Alkene	Benzene	971	967	4,665,998	3,141,014	3,845,699	3,916,002	119,525	<0.01
	Azine	1,3-Diazine	1,289	1,276	1,102,113	1,766,643	1,085,831	1,102,334	80,255	<0.01
	Azole	1-Methyl-pyrrole	1,167	1,170	1,377,589	2,153,082	1,337,600	1,383,740	109,917	<0.01
	Ester	**2,2,4-Trimethyl-1,3-pentanediol diisobutyrate**	1,904	ND	677,569	974,664	1,538,786	2,175,172	99,219	<0.01
	Nitrile	Benzonitrile	1,644	1,629	555,603	463,110	330,172	172,806	27,587	<0.01
		1-Piperidineacetonitrile	1,718	ND	153,101	92,287	63,726	57,967	5,357	<0.01
		3-Pyridinecarbonitrile	1,860	1,875	1,190,123	2,055,923	1,409,581	1,414,292	76,154	0.02
		1-Pyrrolidinylacetonitrile	1,611	ND	33,503	81,612	600,681	721,711	55,517	0.02
	Pyridine	4-Methyl-pyridine	1,275	1,289	261,380	495,197	287,722	371,199	27,468	<0.01
	Ketones	Cyclohexanone	1,341	1,333	1,258,126	644,108	357,427	219,481	18,433	<0.01
		5-Ethyldihydro-furanone	1,760	1,745	544,178	684,977	536,927	603,377	25,982	<0.01
	Sulfones	Dimethyl sulfone	1,882	1,890	148,380	341,711	28,582	62,692	25,593	<0.01
Milk	Alcohols	**2-Methyl-1-hexadecanol**	1,721	ND	123,771	60,577	5,522	32,200	9,280	<0.01
		Phenol	2,053	2,039	1,146,916	1,718,115	690,443	1,107,090	34,120	<0.01
		1-Pentanol	1,262	1,256	1,149,530	874,846	584,182	684,554	60,105	<0.01
	Alkanes	2,2,4,6,6-Pentamethyl-heptane	951	956	2,571,733	3,463,683	4,651,735	4,176,227	164,238	<0.01
		2,4-Dimethyl-heptane	805	797	13,743	0.00	0.00	2,391,050	157,698	<0.01
		4-Methyl-octane	831	823	0.00	0.00	0.00	1,090,949	128,282	<0.01

Continued

Table 6 (Continued). Discriminatory VOC of exhaled breath, ruminal fluid, serum, urine, and milk that differed between the sampling weeks

Biological matrix	Chemical group	Volatile organic compound[2]	RI calc[3]	RI ref[4]	TIC[1]				SEM	*P*-value[6]
					W4[5]	W8	W12	W16		
	Alkenes	1,2,4-Trimethyl-benzene	1,318	1,308	38,343	45,962	27,720	2,007,695	36,671	<0.01
	Carboxylic acid	Decanoic acid	2,331	2,316	9,593	4,836	0.00	91,561	7,578	<0.01
	Ketones	Cyclohexanone	1,341	1,333	610,637	482,639	61,870	234,200	32,048	<0.01
		1,4-Cyclohex-2-enedione	1,808	ND	105,509	130,953	186,788	209,177	16,133	<0.01
		2-Undecanone	1,628	1,622	238,883	172,464	4,690	8,359	10,395	<0.01

[1]TIC = Total ion count area.

[2]Volatile organic compounds identified using National Institute of Standards and Technology NIST/EPA/NIH mass spectral library (NIST17). All match factors >80%. Bold text indicates tentatively identified compounds (match factor >80%; reference RI not defined or > ± 15 from calculated RI).

[3]RI calc = calculated retention index using the formula for the temperature-programmed Kovats index (Girard, 1996).

[4]RI ref = reference retention index after comparison with the NIST chemistry web book; ND = no retention index available in the literature with respect to a comparable analytical method (polar column FFAP, ramp temperature).

[5]W4, 8, 12, 16 = experimental week 4, 8, 12, 16 (for exhaled breath from W8 on).

[6]*P*-value obtained from nonparametric longitudinal model.

CP content, such as fresh grass, compared with silage and hay. Dimethyl sulfone was further found in exhaled breath, for example, by its transport through the ruminal wall into the bloodstream, its exhalation from the lungs, or its release from the rumen by the ructus. Therefore, the VOC profiles of SIL cows were strongly influenced by their diet, as also shown by the several correlating VOC among the biological matrices, which showed higher concentrations in SIL cows. The higher concentrations of dimethyl sulfoxide, p-cymene, and diethyl butandioate in exhaled breath, and higher concentrations of α, 4-dimethyl-benzenemethanol in the urine of SIL cows compared with HD cows may concern the intake of corn silage. Squara et al. (2022) detected these VOC in corn before and after 229 d of ensiling. Terpenes, such as p-cymene, originate from plants' biomass and are not altered during ruminal fermentation; therefore, they are detectable in biological matrices and dairy products after the ingestion of such plants. Consequently, these VOC can be considered exogenous markers in exhaled breath and urine for corn ingestion. The higher concentration of ethyl acetate in the exhaled breath of SIL cows can be explained by the metabolism of certain yeast species that degrade glucose by alcohol acetyltransferase enzymes into such esters (Kruis et al., 2017). Other VOC with higher levels in SIL cows compared with HD cows, namely, cyclopropane, hexanal, and 2-tridecanone in rumen fluid and 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one, 4-hexene-2-one, and 2-acetylfuran in urine, are probably related to the silage fermentation process. Cyclopropane is a cellular component of some *Lactobacillus* strains and is produced during fermentative activity. Accordingly, cyclopropane indicates the presence and activity of *Lactobacillus* spp. in ensiled feed, biological matrices, and dairy products (Lolli et al., 2018). Therefore, the absence of cyclic fatty acids has already been used as a biomarker of quality and authenticity in the meat sector to confirm nonsilage feeding (Lolli et al., 2020). Hexanal, 2-tridecanone and 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-buten-1-one, also called β-damascenone, originate from the degradation of linoleic acid, tridecanoic acid, and lactic acid, respectively (Delcarte et al., 2001; Squara et al., 2022) during the ensiling process. Squara et al. (2022) found higher hexanal, other C6 derivatives, and β-damascenone in ensiled corn compared with fresh corn. Moreover, 2-tridecanone can be produced by *Paenibacillus* during the ensiling process (Grady et al., 2016). Johanningsmeier and McFeeters (2015) found that the anaerobic bacterial strain *Lactobacillus buchneri* produced 2-acetylfuran from lactic acid during biomass fermentation. The higher concentration of p-(1-propenyl)-toluene in the exhaled breath of SIL cows, as well as its presence in silage but not in hay samples,

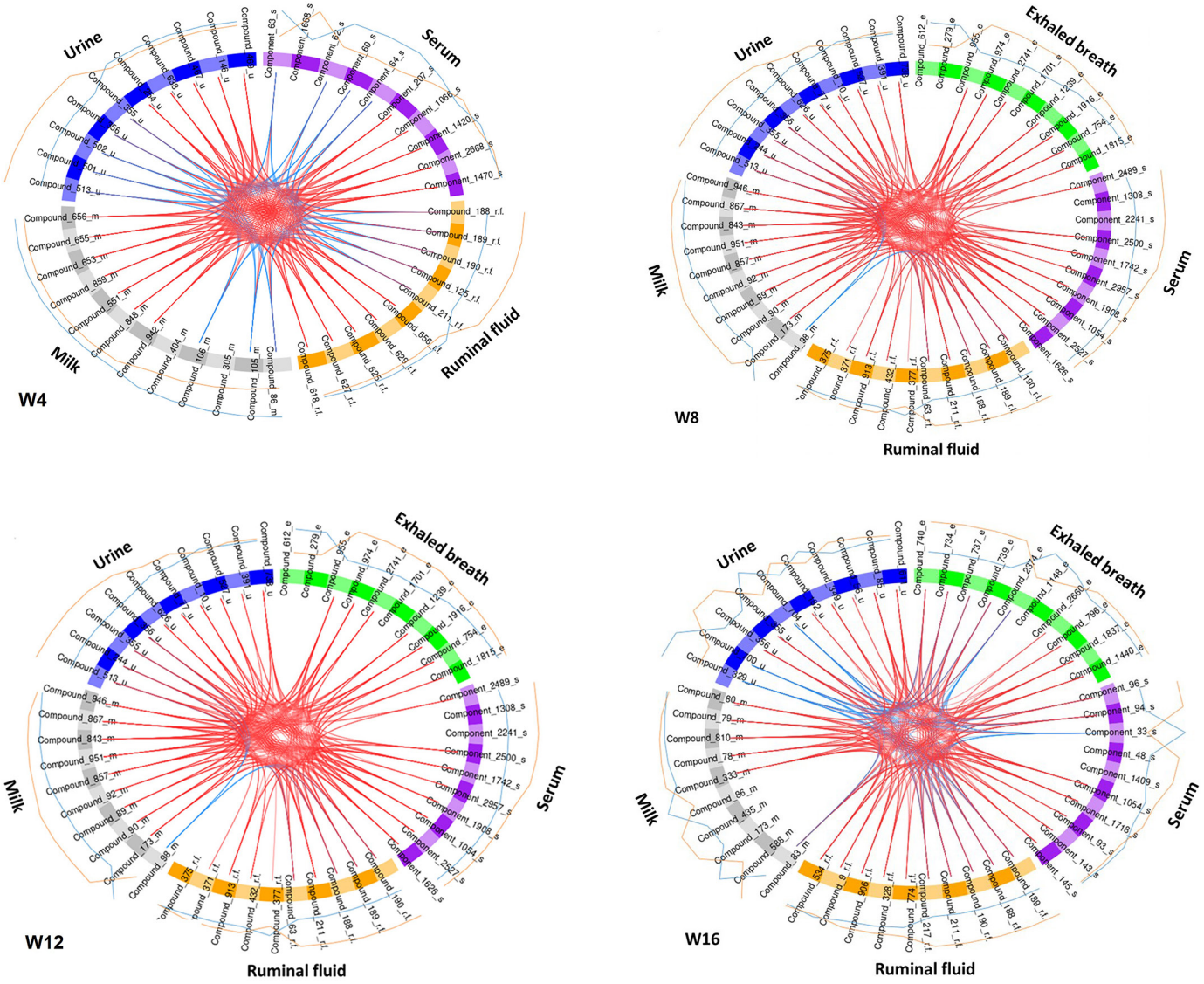

Figure 5. Circos plots indicating Pearson correlations ($r \geq 0.7$ and $r \leq -0.7$) among VOC from exhaled breath, milk, urine, serum, and ruminal fluid across the 4 sampling weeks (W4, W8, W12, and W16; for exhaled breath from W8 on). Compounds assigned to blue, green, violet, orange, and gray bands were found in urine, exhaled breath, serum, ruminal fluid, and milk, respectively. The red and blue lines in the inner circle represent positive and negative correlations, respectively. The blue and orange lines outside the circle represent the total peak area of the corresponding VOC in the corresponding biological matrices of cows fed hay (n = 16, blue lines) and silage (n = 16 orange lines).

could be explained by its formation during the ensiling process. Toluene derivatives have been shown to be microbially synthesized under anaerobic conditions from phenylalanine (Srain and Pantoja-Gutiérrez, 2022) and are therefore also found during green waste composting (Kumar et al., 2011). p-(1-Propenyl)-toluene was further found in the exhaled breath of SIL cows, for example, by its transport through the ruminal wall into the bloodstream and its exhalation from the lungs, or its release from the rumen by the ructus. In HD cows, the higher concentration of 6,10,14-trimethyl-pentadecan-2-one, 2-undecanone, and 2-butanone in their ruminal fluid and isophorone in their urine may be due to plants' drying processes. Tava (2001) found increased concentrations of 6,10,14-trimethyl-pentadecan-2-one in hay due to degradative reactions, mainly due to chlorophyll degradation, during feed dehydration (Figueiredo et al., 2007) compared with non-dried herbage. Oliveira-Alves et al. (2021) detected high levels of 2-undecanone in sea asparagus (*Salicornia ramosissima*) after oven drying, compared with freeze-dried or fresh plants, where very low levels or no 2-undecanone was found. 2-Butanone

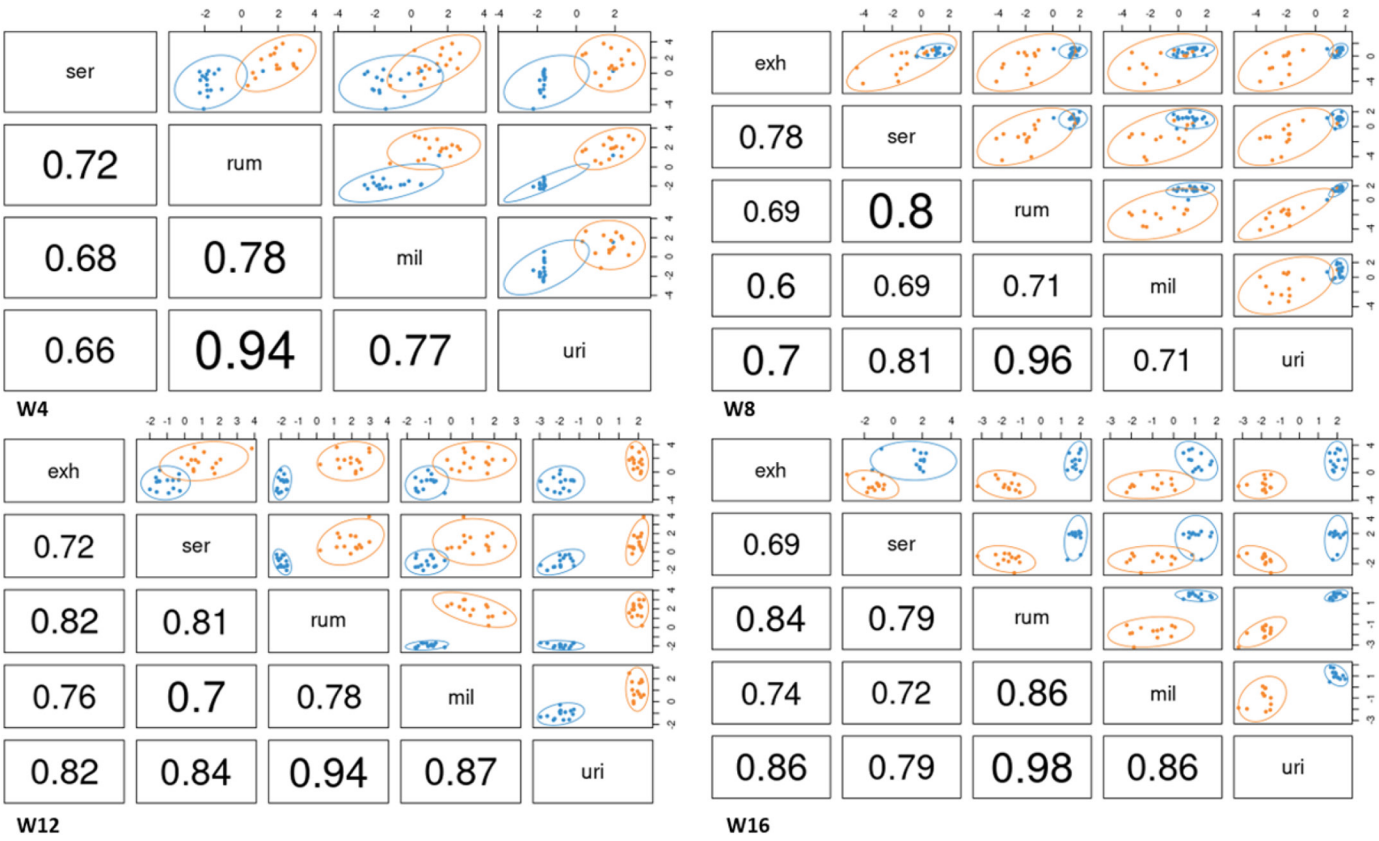

Figure 6. Sample scatterplot displaying the correlations between the first axes of the PLS-DA models discriminating between the cows fed hay (n = 16, blue dots) and silage (n = 16 orange dots) of each biological matrix (upper diagonal plot) and corresponding Pearson correlation coefficients (lower diagonal plot; larger size indicates stronger correlation) within each of the 4 sampling weeks (W4, W8, W12, and W16; for exhaled breath from W8 on). Ellipses represent 95% CI. exh = exhaled breath, rum = ruminal fluid, ser = serum, uri = urine, mil = milk.

was detected by Andreae (2019) due to biomass burning and by de Gouw et al. (1999) as a cutting- and drying-induced VOC. Due to the drying process of plant biomass, increased β-carotene degradation leads to a higher isophorone concentration in hay compared with silage (Carmona et al., 2006).

Essential Oils Versus Control Concentrate-Fed Cows. No effects were found regarding the concentrate type on feed intake or milk production, neither during the experimental weeks W5 through W12, nor after (W13–W16). Therefore, we can reject both the hypothesis that essential oils affect feed intake and milk production (hypothesis 1) and the hypothesis that essential oils have a time-dependent effect on these variables (hypothesis 2). The concentrate type did not affect the VOC profile of ruminal fluid, serum, urine, or milk, but did affect that of exhaled breath in W8. One discriminatory VOC identified was furfural, which was more abundant in EXP cows and the EXP concentrate compared with CON cows and CON concentrate. The presence of furfural in the CON concentrate may be due to naturally occurring essential oils in plant constituents of the basal diet. The detection of furfural as a discriminatory VOC only in W8 could be because some (n = 20) cows in this sampling week consumed concentrate feed within 2 h before the collection of exhaled breath. In W12, this happened only for 14 cows. Besides furfural, 2-ethyl-hexanol was detected in the exhaled breath of EXP and CON cows. Similar to furfural, 2-ethyl-hexanol is a component of chili pepper (*Capsicum annuum*; Wesołowska et al., 2015), which was part of the essential oils mixture in the EXP concentrate but can obviously originate from other sources, as suggested by the detection in CON cows. However, 2-ethyl-hexanol was not detected in the concentrates, which could be due to low concentrations (below the detection limit). Chewing concentrates (How et al., 2021) and VOC preconcentration during exhaled breath sampling using SPE cartridges (Majors, 2008) could also cause the higher concentrations of this VOC in exhaled breath samples compared with feed or other biological matrices. In conclusion, we can confirm the assumption that essential oils influence the VOC profile only for exhaled breath (hypothesis 1), and likely only when sampled shortly after ingestion of essential oils.

Time-Dependent Volatile Organic Compound Profiles

The BW of the animals increased continuously over time from one experimental week to another. The intake of CP, ADF, NDF, starch, and WSC, as well as milk production and composition, fluctuated during the experimental weeks. At the metabolite level, VOC from exhaled breath showed the best discrimination concerning the sampling weeks, perhaps due to their high sensitivity to dietary influences. These VOC also reflect their influenceability by the animals' metabolic changes over lactation (aldehydes, alcohols, anhydrides, esters, ketones, and nitriles), such as the disparities in the protein catabolism indicated by 1,6-dioxacyclododecane-7,12-dione (Tong et al., 2017). Furthermore, compounds from the environment (alkanes and alkenes; McDonald et al., 2018) might affect the VOC profile of exhaled breath. Although we assume a very high dietary sensitivity of the VOC profile of exhaled breath, it is necessary to establish methods for exhaled breath sampling with minimized VOC contamination from the environment. Care must also be taken to minimize the variation of factors other than the experimental factors to be studied, such as ambient temperature, to avoid interfering with the effects to be studied. Furthermore, ruminal fluid, urine, serum, and milk were strongly influenced by the sampling weeks, responding differently over time, with particularly distinct profiles for W4 (all 4 matrices), W12 (serum only), and W16 (ruminal fluid and milk). The discriminatory VOC from these matrices were alkanes and alkenes, which could have environmental origins (vehicle exhaust, petrol evaporation, biomass burning), the use of volatile chemical products (solvents, paints, pesticides, and so on), and vegetation emissions. The detected VOC, such as aldehydes, alcohols, azines, azoles, carboxylic acids, ethers ketones, nitriles, pyridines, sulfones, and terpenes, however, indicated animals' metabolic status over time and might be involved in changes in the fatty acid metabolism (tetradecanal, 1-dodecanol, 1-pentanol, and decanoic acid; Rizzo, 2014) and protein metabolism (lilac aldehyde, dimethyl sulfone; Taylor and Kiene, 1989; Nierop Groot and de Bont, 1998; Dötterl et al., 2006; Reynaud et al., 2010; Cortinovis and Caloni, 2015).

Comparison of Volatile Organic Compounds from Biological Matrices

The VOC profiles from ruminal fluid and urine were most suitable for describing diet-specific changes in dairy cows fed silage- or hay-based diets, followed by those from exhaled breath. No discrimination between animal feeding groups was possible using VOC from serum and milk, indicating their poor suitability for this purpose. The decreasing order of discrimination and, therefore, the suitability of the biological matrices to reveal diet-specific VOC profiles, may be due to the direction of the nutrient flow and the metabolism throughout and within the organism. The rumen is the first organ in the nutrient flow hierarchy. Therefore, the feeding effects are most pronounced here. After absorption through the ruminal and intestinal mucosa into the bloodstream and metabolization, particularly by the liver, some VOC in serum may decrease in concentration below detectable levels or be converted to hydrophilic and nonvolatile conjugates (de Lacy Costello et al., 2014). This generally lower total VOC concentration in serum, especially without preconcentration, leads to a lower signal-to-noise ratio, thereby increasing the probability of artifact detection and reducing the sensitivity of VOC detection. Hence, a preconcentration step before their analysis, which was performed for exhaled breath using SPE cartridges during sampling to improve identifiability (Majors, 2008), may be appropriate for serum VOC detection. The VOC in exhaled breath differed from those in serum, probably due to several barriers between these 2 matrices, such as the pulmonary alveolar membrane and the airway epithelium, biotransformation in the lung, for example, via cytochrome P450 enzymes or epoxide hydrolases (Castell et al., 2005), and the possible exhalation of ruminal fermentation gases during the ructus, which influences the composition of VOC in exhaled breath (Islam et al., 2023). The VOC found in ruminal fluid were not detected in exhaled breath, suggesting that these VOC were not transported via the esophagus to exhaled breath or that their concentrations were below the detectable limit because the cows were visually not ruminating during the 3 min of breath sampling. In urine, due to chemical transformations and concentrations in the kidney, different VOC are detectable, as well as in higher concentrations compared with the other biological matrices, especially from serum (de Lacy Costello et al., 2014). Most urinary VOC represent the end products of the metabolic utilization of ingested feed and are therefore strongly related to dietary intervention (Sun et al., 2015). The VOC profile in milk was probably less affected by feeding because the blood–milk barrier makes milk regarding certain metabolites a more filtered fluid compared with blood and ruminal fluid (Wellnitz and Bruckmaier, 2021). Furthermore, in the present study, some VOC from milk may have been lost due to pooling the morning milk with the previous evening's milk sample, which should be avoided in future studies.

The correlations among the VOC of exhaled breath, ruminal fluid, and urine showed strong relationships. Hence, the use of exhaled breath should be developed as a less invasive method to identify potential biomarkers to characterize fermentation processes and their influence on animals' metabolisms. Furthermore, exhaled VOC

might be useful for tracking the feeding regimen or non-allowed feeds.

NOTES

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Nonstandard abbreviations used: APD = absorbable protein at the duodenum; CON = control protein and energy concentrate mixture; DHS-VTT = dynamic headspace vacuum transfer in-tube extraction; DHS-V-ITEX = dynamic headspace vacuum in-tube extraction; ER = error rate; EXP = experimental concentrate including a blend of essential oils; exh = exhaled breath; expl. var = explained variance; HD = hay-based diet; mil = milk; PLS-DA = partial least squares discriminant analysis; Q2 = predictive ability parameter; QC = quality control; R2 = goodness-of-fit; RI = retention index; SIL = silage-based diet; SPE = solid-phase extraction; sPLS-DA = sparse PLS-DA; rum = ruminal fluid; ser = serum; TIC = total ion count; uri = urine; VIP = variable importance in projection; VOC = volatile organic compounds; W4 = week 4; W8 = week 8; W12 = week 12; W16 = week 16; WSC = water-soluble carbohydrates.

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