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Effect of a Hay-Based Diet or Different Upland Grazing Systems on Milk Volatile Compounds

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ABSTRACT: The effect of animal feeding on milk volatile organic compounds (VOCs) of metabolic origin was tested on a hay-based diet (H), a highly diversified pasture under continuous grazing (CG), or a less diversified pasture under rotational grazing (RG). Individual milk of 24 Montbéliarde cows (8 per treatment) were sampled after 2 weeks. Pasture-derived milk was richer ($p < 0.05$) in camphene, sabinene, β-caryophyllene, and skatole than H milk. Neither milk yield nor fat content affected the majority of VOCs measured. Skatole increased slightly with milk yield, while indole and cineole decreased slightly with milk fat content but with poor regression ($R^2 < 0.54$). Multivariate analysis showed that, on the basis of those VOCs of metabolic origin whose concentration differed between treatment (dimethyl-sulfone, skatole, toluene, undecanoic acid, 1-octadecene, benzeneacetaldehyde, octanoic acid, and 2-pentanone-4-hydroxy-4-methyl), it was possible to obtain good discriminations among feeding systems. This study is promising for a future use of VOCs of metabolic origin to trace animal feeding systems.

KEYWORDS: Milk, volatile organic compounds, cow diet, grazing management, traceability

INTRODUCTION

It has been demonstrated that cow feeding strongly affects many aspects of milk quality, such as sensory properties,[1] fatty acid composition,[2−4] and micronutrient content.[4] These effects of animal feeding contribute to the "*terroir*" conception that is determinant for typical and quality-labeled products (e.g., protected designation of origin; protected geographical indication). The higher prices of these products prompts consumers to ask for guarantees of their authenticity. Consequently, efforts have been made to trace cow-feeding systems via direct analysis of animal products.

Cow feeding affects volatile organic compounds (VOCs) in milk,[3−5] notably terpenes. Terpenes are plant secondary metabolites that can be directly and promptly transferred from feed to milk and that undergo only minimal modifications by rumen bacteria.[6] Milk obtained from animals grazing in a pasture contains a much greater diversity of terpenes than milk from cows given preserved forages and/or concentrates.[7,8] Differences in the terpene profile have been shown between milk from cows grazing in lowland or highland pastures and among dairy products derived from cows grazing in different mountain pastures.[7,9] Nevertheless, the wide qualitative variability in the appearance of terpenes in pastures, because of botanical diversity, phenological stage, and geographical location, and in grassland management that determines plant selection in diversified grasslands[9−11] means that terpenes are too dependent upon specific production conditions to be considered as generic indicators of animal feeding.[12] Moreover, many of them are currently available on the European market and commonly used as additives in cow feeds as flavoring or anti-inflammatory agents.

Nonterpenoid VOCs, mainly of metabolic origin, have been studied for their impact on milk flavor.[13−15] The effect of animal feeding on nonterpenoid VOCs has been extensively investigated in meats[3,16] but is hardly studied in milk. Toso et al.[5] found different concentrations of acetone, 2,3-butanedione, 2-butanone, ethanol, acetaldehyde, ethylacetate, ethyl isovalerate, and dimethyl-sulfone between milk from pasture-fed cows and total mixed ration-fed cows in a farm-scale study led under ordinary farming conditions.

To our knowledge, the effect of animal feeding systems on nonterpenoid milk VOCs has not yet been studied under experimental conditions and no research has been conducted to test their changes according to grazing systems applied on upland pastures under different levels of intensification of agronomical practices. Moreover, because previous research was conducted mainly on bulk milk, little is known about the effect of animal-related factors, such as milk yield and fat content, which could have dilution/concentration effects on milk VOCs.

The aim of this study was to investigate the effect of a control diet (hay and concentrate) and two grazing systems typically found in upland areas: continuous grazing on an extensively managed pasture with high biodiversity or rotational grazing on an intensively managed and less biodiversified grassland, on milk VOCs, particularly nonterpenoids. The effects of individual milk yield and milk fat content on milk VOC concentrations were also tested.

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Table 1. Feed Composition and Intakes for Cows Kept Indoors, Measured Daily for All Cows during the Pre-experimental Period and during the Experimental Period for Hay- and Concentrate-Fed Cows

	intake (kg of DM/day)					
items[a]	pre-exp	H	SEM	DM (%)	CP (g/kg of DM)	MFU/kg of DM
hay, first cut	13.1	7.2	0.35	85	65	0.70
hay, second cut		5.3	0.37	85	116	0.80
maize/barley flakes (50:50)	5.0	6.4	0.03	86.4	588	1.02
soybean cake	0.6	1.2	0.00	87.8	3225	1.21

[a] DM, dry matter; pre-exp, pre-experimental period; H, hay and concentrate cow feeding system; SEM, standard error of the mean; CP, crude protein; MFU, milk forage unit.

Table 2. Botanical Composition of the RG and CG Paddocks

RG			CG		
species	SC[a] (%)	SEM	species	SC[a] (%)	SEM
Dactylis glomerata	22.5	2.13	*Agrostis capillaris*	18.4	1.50
Trifolum repens	20.5	2.50	*Trifolium repens*	13.4	2.07
Taraxacum officinale	20.1	2.34	*Festuca* gr. *rubra*	11.2	0.71
Agropyron repens	6.6	1.65	*Plantago lanceolata*	5.5	0.84
Lolium perenne	6.4	1.98	*Achillea* gr. *millefolium*	5.3	0.90
Holcus mollis	5.4	1.03	*Carex caryophyllea*	3.6	0.58
Agrostis tenuis	4.5	1.39	*Helianthemum nummularium*	3.3	0.96
Poa pratensis	4.4	1.03	*Veronica chamaedrys*	3.0	0.59
Poa annua	2.5	0.54	*Thymus pulegioides*	2.6	0.55
Poa trivializ	2.0	0.78	*Galium verum*	2.5	0.39
Plantago major	1.5	0.49	*Dactylis glomerata*	2.1	0.88
			Holcus mollis	1.7	0.52
			Hieracium pilosella	1.6	0.52
			Viola lutea	1.6	0.38
			Poa pratensis	1.5	0.44
			Meum athamanticum	1.3	0.38
			Anthoxanthum odoratum	1.2	0.34
			Trifolium pratense	1.2	0.22
			Avenula pubescens	1.1	0.32
			Chamaespartium sagittale	1.1	0.46
			Cytisus scoparius	1.0	0.40
			Briza media	1.0	0.39
Poaceae, total	56.5	2.44	Poaceae, total	43.1	2.01
dicotyledons, total	45.6	2.15	dicotyledons, total	63.3	3.17
total *n* of species	38		total *n* of species	94	

[a] SC = specific contribution.

MATERIALS AND METHODS

Experimental Design and Animal Management. The experiment was carried out at the Institut National de la Recherche Agronomique (INRA) farm of Marcenat in an upland area of central France (45° 15′ N, 2° 55′ E; altitude, 1135−1215 m; annual rainfall, 1100 mm) in 2009. During a 2 week pre-experimental period (from May 1−15), 24 multiparous Montbéliarde cows were fed a commercial concentrate- and hay-based diet (Table 1). Individual milk yield, milk protein and fat content, and somatic cell count (SCC) were recorded daily during this period, as well as calving date and parity to allocate the cows into three balanced 8 cow groups. At the end of the pre-experimental period, one group was kept indoors (H cows) and fed a hay- and concentrate-based diet, similar to the pre-experimental one, throughout the trial (Table 1). The other two groups were turned out to pasture during the whole day (except during the milking) in two different grazing systems: rotational grazing (RG cows) and continuous grazing (CG cows). The RG and CG cows did not receive any concentrate supplementation, neither at pasture nor during milking. The botanical composition of the grazing plots is reported in Table 2. The RG system was set up on a 7.7 ha medium-biodiversified (38 species) old temporary spontaneously renaturalized *Dactylis glomerata* and *Trifolium repens* hay grassland, at a high stocking density (1.56 LU ha^{-1}; 1 LU = 600 kg of live weight) designed to offer leafy edible biomass throughout the grazing season. This plot was divided into several paddocks, whose number and size varied during the season to ensure similar herbage availability between paddocks. The CG system was set up on a 12.5 ha permanent mesotrophic *Festuca* gr. *rubra* and *Agrostis tenuis* pasture at a low stocking density (0.96 LU ha^{-1})

Table 3. Effect of the Feeding System on Milk VOC Profiles[a]

	compound name	retention Index	method of identification	presence (*n* of samples)	treatment[b] H	RG	CG	SEM	treatment effect
alcohols	1-pentanol	765	L + KI	22	3.32	3.75	3.55	0.25	ns[c]
	2-furanmethanol	854	L + KI	6	0.46	2.35	0.52	0.40	ns
	2-octanol	1009	L + KI	23	4.60	4.30	4.78	0.23	ns
	1-dodecanol	1475	L + KI	18	2.31	2.86	3.48	0.35	ns
aldehydes	2-heptenal, (*E*)-	958	L + KI	24	3.60	3.85	3.75	0.09	ns
	2,4-heptdienal, (*E*,*E*)-	1018	L + KI	23	3.60	3.98	3.47	0.19	ns
	4-pentenal, 2-ethyl-	1028	L + KI	23	4.98	4.10	4.84	0.22	ns
	benzeneacetaldehyde	1050	L + KI	24	3.47 b	3.78 a	3.41 b	0.09	*d*
	benzaldehyde, 4-ethyl-	1174	L + KI	23	3.86	3.94	3.50	0.17	ns
	propenal, 3-phenyl-2-(*Z*)-	1201	L	24	4.17	4.10	4.22	0.04	ns
	6-decenal	1209	L + KI	24	4.22	4.44	4.34	0.06	ns
	2,4-nonadienal, (*E*,*E*)-	1222	L + KI	21	3.43	3.06	3.05	0.27	ns
	2-decenal	1266	L + KI	24	4.35	4.61	4.43	0.11	ns
heterocyclic compounds	furan, 2-methyl-	604	L + KI	24	4.19	4.28	4.20	0.10	ns
	benzofuran, 2,3-dihydro-2-methyl-	1303	L	24	3.91	4.00	3.92	0.07	ns
	butane, 2,3-epoxy-(*Z*)-		L	23	4.18	4.80	4.77	0.21	ns
hydrocarbons	benzene	662	L + KI	24	4.01	4.04	3.98	0.05	ns
	toluene	769	L + KI	24	4.45 b	5.41 a	5.34 a	0.15	*e*
	benzene, ethyl-	867	L + KI	8	1.18	1.47	0.81	0.35	ns
	xylene, (*m*)-	874	L + KI	24	3.63	3.78	3.42	0.12	ns
	benzene, ethenyl-	896	L + KI	24	4.28	4.29	4.38	0.05	ns
	decane, 2,2,8-trimethyl-	1026	L + KI	23	3.77	3.88	3.44	0.19	ns
	heptane, 2,2,4,6,6-pentamethyl-	1034	L + KI	18	1.80	3.12	3.20	0.33	ns
	decane, 4-methyl-	1061	L + KI	24	3.99	4.20	4.20	0.09	ns
	decane, 2-methyl-	1067	L + KI	23	3.41	3.87	4.16	0.19	ns
	1-undecene	1086	L + KI	9	1.94	1.12	1.49	0.41	ns
	benzene, 4-ethyl-1,2-dimethyl-	1097	L + KI	22	3.30	3.92	3.23	0.23	ns
	1-decyne	1105	L	24	4.81	4.93	4.74	0.08	ns
	1-dodecene	1292	L + KI	24	3.79	3.69	3.81	0.05	ns
	nonene, 2,6,8-trimethyl-(*E*)-	1328	L	10	1.37	1.82	1.40	0.38	ns
	1-octadecene	1790	L + KI	24	5.35	5.55	5.78	0.07	*f*
	hexadecane, 2,6,10,14-tetramethyl-	1808	L + KI	24	4.32	4.36	4.35	0.07	ns
	1-nonadecene	1875	L + KI	24	5.34	5.50	5.40	0.04	ns
ketones	cyclopentanone	791	L + KI	16	1.95	3.35	2.46	0.38	ns
	2-pentanone-4-hydroxy-4-methyl	842	L + KI	24	4.40 a	4.16 b	4.45 a	0.05	*d*
	2,3-octanedione	980	L + KI	20	1.97	2.94	2.36	0.24	ns
	8-nonen-2-one	1088	L + KI	22	3.24	3.16	3.45	0.23	ns
	2-nonanone	1096	L + KI	24	4.62	4.94	4.82	0.07	ns
	2-undecanone	1295	L + KI	24	4.65	4.78	4.61	0.06	ns
	benzoquinone, 2,6-ditert-buthyl-(*p*)-	1486	L	17	2.44	3.38	2.18	0.37	ns
organic acids	δ-decalactone	1510	L + KI	24	5.10	5.24	5.27	0.06	ns
	δ-dodecalactone	1720	L + KI	24	4.98	5.23	5.03	0.07	ns
	butanoic acid	775	L + KI	21	3.51	4.37	5.32	0.37	ns
	hexanoic acid	967	L + KI	24	5.37	5.71	5.82	0.01	ns
	benzoic acid	1156	L + KI	24	3.95	3.73	3.98	0.07	ns
	octanoic acid	1161	L + KI	24	5.93	6.07	6.12	0.08	ns
	nonanoic acid	1262	L + KI	24	4.15	4.40	4.44	0.07	ns
	decanoic acid	1358	L + KI	24	6.34	6.22	6.47	0.13	ns
	undecanoic acid	1458	L + KI	24	3.35	3.85	4.20	0.14	*f*
	dodecanoic acid	1556	L + KI	24	5.90	6.08	5.77	0.12	ns
	tetradecanoic acid	1768	L + KI	24	5.19	5.58	5.15	0.12	ns
esters	butanoic acid ethenyl ester	629	L	24	4.53	4.88	4.65	0.08	ns

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Table 3. Continued

	compound name	retention Index	method of identification	presence (*n* of samples)	treatment[b] H	RG	CG	SEM	treatment effect
	hexanoic acid methyl ester	923	L + KI	22	2.95	4.12	3.32	0.24	ns
	octanoic acid methyl ester	1129	L + KI	24	3.70 b	4.53 a	4.24 a	0.10	*g*
	dodecanoic acid methyl ester	1324	L + KI	23	3.28 b	4.67 a	4.33 a	0.22	*d*
	hexanoic acid heptyl ester	1480	L + KI	6	0.48	1.04	1.56	0.37	ns
	phthalic acid diisobutyl ester	1873	L + KI	23	3.41	3.74	3.84	0.19	ns
phenolic compounds	phenol	977	L + KI	24	3.54	3.61	3.64	0.07	ns
	phenol, 2,4,6-trimethyl-	1202	L + KI	24	4.02	3.89	4.07	0.04	ns
	phenol, 2,3,6-trimethyl-	1233	L + KI	21	2.92	3.81	3.44	0.27	ns
	phenol, 2,4-bis(1,1-dimethylethtyl)-	1517	L + KI	24	3.97	4.11	4.18	0.06	ns
indoles	indole	1307	L + KI	24	3.68	3.90	3.83	0.09	ns
	skatole	1404	L + KI	24	4.03 c	4.77 a	4.59 b	0.10	*e*
monoterpenes	cumene	930	L + KI	24	3.34	3.61	3.52	0.09	ns
	camphene	942	L + KI	19	1.22 b	3.63 a	3.92 a	0.32	*g*
	sabinene	978	L + KI	18	0.97 b	2.29 a	3.06 a	0.26	*e*
	β-pinene	986	L + KI	7	0.00 b	0.00 b	3.70 a	0.40	*g*
	cymene-(*p*)	1031	L + KI	12	0.49 b	1.51 b	4.07 a	0.42	*g*
	cineole	1041	L + KI	24	4.14	4.22	4.30	0.06	ns
	cresol-(*p*)	1073	L + KI	24	4.44	4.69	4.54	0.06	ns
sesquiterpenes	β-caryophyllene	1449	L + KI	24	3.48 c	3.98 b	5.06 a	0.16	*g*
	alloaromadendrene	1484	L + KI	8	0.00 b	0.00 b	3.77 a	0.37	*g*
	germacrene-D	1504	L + KI	8	0.00 b	0.78 b	2.59 a	0.33	*e*
	γ-cadinene	1523	L + KI	14	0.97 b	1.56 b	4.77 a	0.45	*g*
sulfur compounds	dimethyl-sulfone	914	L + KI	24	4.14 b	4.51 a	4.66 a	0.07	*e*

[a] VOC concentrations are expressed as $\log_{10}$ of the peak area. [b] H, hay and concentrate diet; RG, rotational grazing system on a low-biodiversity pasture; CG, continuous grazing system on a high-biodiversity pasture; L, library; KI, Kovatz indices. [c] ns = not significant. [d] $p < 0.05$. [e] $p < 0.01$. [f] $p < 0.1$. [g] $p < 0.001$.

designed to offer high botanical diversity (94 species) and marked structural heterogeneity of vegetation over the season. After 2 weeks of grazing (May 29), 24 individual milk samples were collected from cows from all three treatments. The morning milk was pooled in a 60:40% proportion with the previous evening milk (stored at 4 °C overnight), then sampled in Pyrex bottles with a Teflon cup, and stored at −30 °C until VOC analysis.

Sample Preparation and Volatile Compound Analysis. Milk VOC analysis was conducted by the Dipartimento di Scienze Agronomiche, Agrochimiche e delle Produzioni Animali (DACPA) laboratory at the University of Catania (Italy). Milk samples were handled as follows: thawed milk was thoroughly mixed to obtain a homogeneous matrix, and 40 mL was centrifuged at 20000*g* for 30 min at 4 °C to separate the cream from the aqueous milk fraction. The recovered fat (3.5 ± 0.05 g) was immediately placed in a 10 mL glass vial, capped with a polytetrafluoroethylene (PTFE) septum, and stored at −80 °C. Within 5 days of storage, the volatile compounds were extracted from the milk fat and analyzed. Headspace (HS) volatile compounds were extracted by solid-phase microextraction (SPME). The vial containing the still-frozen sample was placed in a water bath set at 60 °C (±2 °C) for 20 min, and then a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA) was exposed to the HS over the sample at 60 °C (±2 °C) for 20 min. Once the adsorption time was finished, the fiber was removed from the vial and immediately inserted into the gas chromatography (GC, TRACE 2000, Thermo-Finnigan, San Jose, CA) injector set at 250 °C. Desorption time was 4 min. For the GC analysis, the injector was operated in splitless mode at a temperature of 250 °C and fitted with a 0.75 mm inlet linear (Supelco, Bellefonte, PA). Helium was used as a carrier gas at a flow rate of 1.0 mL min^{-1}. Volatile compounds were separated using a Supelco SPB 5 column (60 m × 0.32 mm × 1 μm). The initial oven temperature of 40 °C was held for 5 min, ramped up at a rate of 3 °C min^{-1} to 230 °C, and then held at 230 °C for 5 min, given a total acquisition program of 73 min. The GC−mass spectrometry (MS) interface was heated at 280 °C. VOC mass spectra were generated by ion-trap MS (Polaris Q, Thermo-Finnigan, San Jose, CA). Acquisition was performed in electron impact (EI) mode (70 eV) at 10 microscans/s, scanning the mass range of 33−230 atomic mass units (amu). Between each sample injection, the inlet was cleaned by increasing the temperature to 270 °C and holding this temperature for 40 min and the SPME fiber was cleaned by injection at 270 °C for 40 min. Compounds were identified by a comparison against mass spectra from the National Institute of Standards and Technology (NIST) 7 Mass Spectral Library and against linear retention indices (LRIs).[17,18] The LRIs were calculated by previous injection of standard *n*-alkanes from 5 to 17 carbon atoms. To avoid memory effects because of the SPME fiber and the GC column, four samples from different treatments were analyzed each day of analysis. Furthermore, the injection sequence of samples from different dietary treatments was changed over the 6 days of analysis.

Statistics. Statistical analyses were performed using SPSS for Windows (version 16.0; SPSS, Inc., Chicago, IL). The individual milk sample was used as a statistical unit. The Anderson−Darling test was used to test whether data followed a normal distribution. Where not normally distributed ($p < 0.05$), a $\log_{10}$ transformation of the data was performed before analysis of variance (ANOVA). A first GLM ANOVA was performed on the VOC peak area using milk yield and fat content as covariates and treatment and the interaction treatment × covariates as

fixed factors. Because these latter interactions were never significant, the interactions were removed from the model for subsequent data treatment steps. For VOCs unaffected by the covariates, milk yield and milk fat were removed from the model and the data were treated again with a GLM ANOVA using animal feeding systems as fixed factors. The REGWQ test was used as a post-hoc test. For VOCs that were significantly affected by the covariates, a linear regression was performed using milk yield or milk fat content as the independent variable and milk VOC content as the dependent variable. Regressions were split by treatment for the VOCs, for which both covariates and treatment effect were significant. Those nonterpene VOCs that were significantly affected by treatment in the GLM ANOVA ($p < 0.10$) were treated by linear discriminant analysis (LDA) with variables enter method. The resulting discrimination functions were validated by the "leave-one-out" method (full cross-validation).

RESULTS AND DISCUSSION

SPME Analysis. A total of 75 VOCs were identified in this study (Table 3), 37 of which, to the best of our knowledge, have never previously been found in milk. The SPME GC−MS technique that we applied has already been successfully used for milk VOC extraction.[19,20] Most of the milk VOCs are present in very low concentrations, and aggressive extraction protocols (high temperatures, solvent extraction, or derivatization) can lose some VOCs or form artifacts.[12] The use of a triphasic fiber, such as the DVB/CAR/PDMS chosen for the present study, gives the advantage of recovering VOC with both high polarity (thanks to the DVB phase) and low polarity (thanks to the PDMS phase).[21,22] The CAR phase possesses a great adsorptive property,[23] thus increasing the retention capacity of the fiber.[21] This type of fiber has been successfully used in other studies aiming at discriminating animal feeding systems through the use of the VOCs extracted from animal tissues.[24]

Terpene VOCs. Milk terpene VOC composition is reported in Table 3. Feeding systems affected the concentration of four monoterpenes [camphene, sabinene, β-pinene, and cymene-(p)] of the seven found and all the sesquiterpenes (β-caryophyllene, alloaromadendrene, germacrene-D, and γ-cadinene), as expected on the basis of previously reported changes in terpene concentrations in milk according to animal feeding.[3,4,7] The concentrations of β-pinene and cymene-(p) and all sesquiterpenes were higher in CG milk ($p < 0.01$), which is consistent with numerous studies on the concentrations of major terpenes in pasture with a high botanical diversity and rich in dicotyledons.[3,7] In general, terpene variability was stronger than nonterpene variability. In fact, the number of samples within treatments where a specific VOC was undetected was generally higher for terpenes, particularly monoterpenes, than for other classes of VOCs. Consequently, differences among treatments were stronger for sesquiterpenes than for monoterpenes, probably because the higher volatility of monoterpenes could induce partial loss during sampling preparation for VOC analysis.[12] To our knowledge, alloaromadendrene, a VOC belonging to the sesquiterpenes family, has never previously been reported in milk. Its presence was found by Cornu et al.[25] in *Achillea* gr. *millefolium*. This species was abundant in the CG pasture but absent in the RG pasture. We only found alloaromadendrene in CG milk, suggesting its origin from this botanical species.

Nonterpene VOCs. Milk nonterpene VOC composition is reported in Table 3. Feeding system affected the concentration of 9 of 64 nonterpene VOCs found in our trial: undecanoic acid, octanoic acid methyl ester, dodecanoic acid methyl ester, 1-octadecene, dimethyl-sulfone, 2-pentanone-4-hydroxy-4-methyl, toluene, skatole, and benzeneacetaldehyde.

We found lower amounts of dimethyl-sulfone in H milk than in pasture-derived milks (−9.6%; $p = 0.006$). Toso et al.,[5] comparing milk from cows fed hay or maize silage or grass silage, found similar results. Nevertheless, the origin of this compound is still unclear. It has been proposed that dimethyl-sulfone could derive from the heat-induced oxidation of sulfide-dimethyl, which arises from the amino acid methionine,[19] and therefore, this compound could be an artifact originated during the SPME extraction, while some authors have reported that dimethyl-sulfone appeared in milk treated at 60 °C.[19] In the present study, this would not explain the differences observed in the appearance of dimethyl-sulfone in milk from the three feeding systems. Nevertheless, also other authors have found this compound in raw milk,[14,5] suggesting that it might be naturally occurring in milk. Fresh grass appears richer than hay in methionine,[26] and in general, herbage-based diets present a high protein/readily digestible carbohydrate ratio, explaining the higher concentration of dimethyl-sulfone in the pasture-derived milk in our trial. 1-Octadecene, which have never been previously reported in milk, was found in higher amounts in pasture-derived milk (+7.4%; $p = 0.055$). The origin of hydrocarbons in milk is not yet well-understood. Buchin et al.[27] found that hydrocarbon content was higher in cheese produced from pasture-fed cows than milk cows fed a hay-based diet, hypothesizing their transfer from feed to cheese. Octanoic acid methyl ester and dodecanoic acid methyl ester, which were identified in milk for the first time in this study, were found at higher concentrations in pasture-derived milk (+15.5%; $p = 0.001$ and +27.1%; $p = 0.012$, respectively). Total esters were also higher in pasture-derived milk. These compounds can be formed in the mammary gland from the enzymatic esterification of short-chain alcohols and free fatty acids (FFAs) after milking.[28] The effect of the animal diet on milk FFA content was previously reported by Ferlay et al.,[2] who found a higher FFA content in pasture-derived milk than in milk from a hay-based diet after 24 h of cold storage. Thus, in our trial, the higher ester amounts in pasture-derived milk may have been produced by a high availability of FFAs as a substrate for enzymatic esterification activity. This hypothesis could also explain the higher concentration of undecanoic acid found in CG and RG than in H milk (+20.3%; $p = 0.064$). However, the lack of differences for shorter chain organic acids, also expected according to the previous hypothesis, makes these results still difficult to understand.

We found that the toluene concentration was higher in pasture-derived milk than milk from cows fed indoors (+20.7%; $p = 0.010$), which is in agreement with the work by Croissant et al.[1] Toluene in raw milk has been indicated as responsible for rancid notes.[20] Toluene is a product of β-carotene light-induced oxidation.[28] β-Carotene, an essential plant pigment with an antiphotooxidation function, is highly sensitive to light oxidation. Consuming fresh grass results in high β-carotene content in milk, while grass preservation (i.e., hay) leads to marked β-carotene losses,[7] explaining the lower toluene content in H milk in our trial.

Indole and skatole have been associated with animal notes typical of pasture-derived milk flavor.[14] The higher concentration of skatole in RG and CG than in H milk (+18.4 and +13.9%; $p = 0.001$, respectively) is consistent with literature data, citing skatole as a tracer of the pasture-based diet in both milk[1,13] and meat fat.[16] Skatole is produced by the deamination and

Table 4. Milk Yield and Gross Milk Composition in Relation to the Animal Feeding System

items[a]	pre exp	SEM	H	RG	CG	SEM	significance
milk yield (L cow^{-1} day^{-1})	22.9	1.17	22.2	24.1	25.1	0.84	ns[b]
fat (g/L)	37.4	0.73	39.4	39.7	37.4	0.89	ns
protein (g/L)	30.8	0.51	31.8	32.8	32.2	0.51	ns
lactose (g/L)			47.4	47.1	46.6	0.25	ns
SCC (10^3/mL)	107.6	8.85	35.5	93.4	78.6	20.62	ns

[a] pre exp, pre-experimental period; H, hay and concentrate diet; RG, rotational grazing system on a low-biodiversity grassland; CG, continuous grazing system on a high-biodiversity pasture. [b] ns = not significant.

decarboxylation of the free amino acid tryptophan by bacteria in the rumen.[29] The high protein content and the high protein/readily digestible carbohydrate ratio in herbages increase the amount of rumen tryptophan available to bacteria, consequently increasing skatole production.[16] The higher skatole concentration in RG than CG milk (+3.9%; $p = 0.001$) could be related to the presence in the RG pasture of Fabaceae species, which have a higher protein/readily digestible carbohydrate ratio than Poaceae species.

Among ketones, 2-pentanone-4-hydroxy-4-methyl was lower in RG milk than in H and CG milk (−6.6%; $p = 0.050$). 2-Pentanone-4-hydroxy-4-methyl had previously been found in milk by Toso et al.[5] Most ketones are derived from the oxidation of polyunsaturated fatty acids (PUFAs).[29] Given that grass is richer in PUFA than concentrates,[3] we expected to find higher levels of ketones in milk from grass-fed cows, but this was not the case. Indeed, a pasture is richer in natural antioxidants (such as carotenoids and vitamins A and E) than hay-based diets, and this could have prevented lipid oxidation in the milk from CG and RG groups. A similar explanation may be relevant for the milk lactone concentration, which we found to be unaffected by dietary treatments. Urbach[15] suggested that δ-lactones arise from the PUFA oxidative pathway.

2,3-Octaendione is a VOC derived from linoleic acid and linolenic acid degradation by lipoxygenase enzymes present in leafy herbage. This VOC has also been cited as a meat marker of pasture feeding.[3,16] However, we found that feeding systems had no significant effect on the 2,3-octanedione concentration in milk, although it tended to be higher in pasture-derived milk.

Benzeneacethaldehyde was previously detected in milk by Mounchili et al.,[20] but its origin remains unclear. In general, aldehydes are produced by the degradation of amino acids (branched-chain aldehydes) or lipids (straight-chain aldehydes).[28] This second mechanism is supported by other authors who indicated PUFA (in particular linoleic and linolenic acids) oxidation as the main source of aldehydes in milk[13] and meat fat,[16] but it is not the case of benzeneacetaldehyde. Moreover, Moio et al.[14] found higher amounts of benzeneacetaldehyde in milk from sheep grazing a natural pasture than sheep grazing a grass meadow or fed a total mixed ratio, while we found higher benzeneacetaldehyde concentrations in milk from the RG cows than milk from H and CG cows (+9.9%; $p = 0.033$).

Milk Yield and Fat Content and VOCs. Milk yield gross composition (fat, protein, and lactose content) and SCC were not affected ($p > 0.05$) by the feeding system (Table 4). Milk yield ranged from 17.5 to 27.1, from 15.9 to 26.7, and from 20.0 to 31.8 L cow^{-1} day^{-1} for H, RG, and CG cows, respectively. Milk fat ranged from 32.9 to 46.7, from 33.5 to 51.4, and from

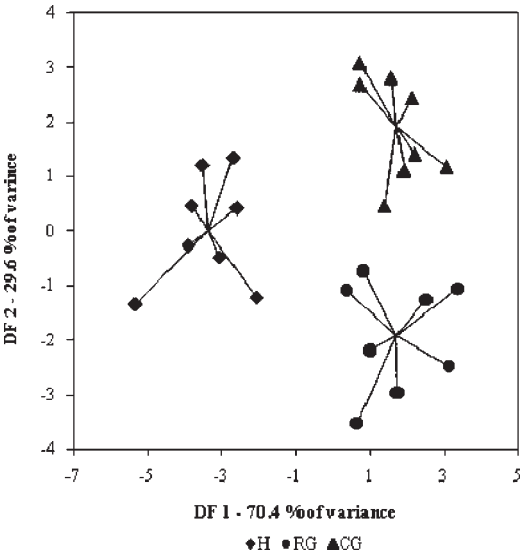

Figure 1. LDA performed on nonterpene VOCs, for which ANOVA showed significant effects of the animal feeding system. Plot of sample distribution projected onto the two discriminant functions (DF1 and DF2).

33.9 to 40.6 g/L for H, RG, and CG cows, respectively. Almost all of the research on milk VOCs has been conducted analyzing bulk milk.[4,5] Our experimental design based on individual milk analysis is the first to investigate the effect of milk yield and milk fat content on VOC concentrations. The vast majority of VOCs were unaffected by both milk yield and milk fat content. Indole and cineole seem to undergo a dilution effect at increasing milk fat contents ($p = 0.007$; $R^2 = 0.298$; $\alpha = 6.363 \pm 1.78$; $\beta = -0.066 \pm 0.047$ and $p = 0.049$; $R^2 = 0.243$; $\alpha = 5.884 \pm 1.33$; $\beta = -0.043 \pm 0.035$, respectively). However, because the fits found were weak, this dilution effect needs to be confirmed with a higher number of samples. Skatole increased with milk yield in the RG treatment ($p = 0.038$; $R^2 = 0.541$; $\alpha = 3.198 \pm 2.06$; $\beta = 0.059 \pm 0.088$). This positive relationship could result from an increased herbage intake with a high milk yield. Higher herbage intake leads to higher relative protein availability in the rumen for skatole production, with consequently higher hematic transfer to milk and adipose tissues.[28,30] However, even though the β coefficient was positive, the confidence intervals also included negative values, meaning that these results need to be interpreted with caution. The absence of the effect of milk yield on skatole in CG could be related to a possible inhibition of rumen skatole formation by tannins,[30] which are known to be found in high quantities in dicotyledons, which were more abundant in the CG plot. No significant interactions between covariates and treatment were found for the VOCs.

Multivariate Analysis on VOCs. To test the effectiveness of VOCs of metabolic origin as potential tracers of the feeding system used, a LDA was performed on those affected by treatment (Figure 1). The LDA correctly classified the three treatments by the *a priori* classification; however, one H sample was classified as RG, and two samples of RG and CG were assigned to the other grazing system by the cross-validation. These results are promising. However, the controlled condition and limited number of milk samples in our trial allow us just to suggest the use of nonterpene VOCs as potential tracers of cow feeding. In addition, little is known about the latency and dynamics of stabilization of milk nonterpene

VOC concentrations in correspondence to diet change, as studied for terpenes.[6] Even so, the interest in nonterpene VOCs highlighted in this study is reinforced by Toso et al.[5] and Croissant et al.,[1] who were able to discriminate pasture-derived milk from total mixed ratio-fed cow commercial milk, showing a significant contribution in discrimination by toluene, skatole, indole, and sulfur compounds.

Adding terpene to nonterpene VOCs allowed us to obtain 100% discrimination in cross-validations among treatments (results not shown). However, the wide variability of terpenes related to geographical origin, pasture botanical composition and phenological stage,[7−11] their high volatility and low concentrations,[12] and their common commercial use as flavor agents or as anti-inflammatory additives for animal feeding makes them too specific to be proposed as potential generic tracers.

In conclusion, our results show that, in addition to milk terpenes, nonterpene VOCs are also strongly affected by the cow feeding system and grazing system on an upland pasture. Their potential as generic tracers could be tested on a larger scale conducted in noncontrolled conditions in a wide variety of production contexts. Further studies will also be necessary to understand variation dynamics in milk nonterpene VOC concentrations during diet changes.

Milk yield and fat content do not appear to be related to the milk VOC concentration, but this needs to be confirmed in more detailed studies, selecting cows covering a wider range of milk yields and milk fat contents.

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ABBREVIATIONS USED

CG, continuous grazing farming system; DM, dry matter; FFA, free fatty acid; GC, gas chromatography; H, indoor-reared cow feeding system (hay and concentrate diet); LDA, linear discriminant analysis; MS, mass spectrometry; PUFA, polyunsaturated fatty acid; RG, rotational grazing farming system; SCC, somatic cell count; SEM, standard error of the mean; SPME, solid-phase microextraction; VOC, volatile organic compound

REFERENCES

(1) Croissant, E. A.; Washburn, S. P.; Dean, L. L.; Drake, D. A. Chemical properties and consumer perception of fluid milk from conventional and pasture-based production systems. *J. Dairy Sci.* **2007**, *90*, 4924–4953.

(2) Ferlay, A.; Martin, B.; Pradel, P.; Coulon, J. B.; Chilliard, Y. Influence of grass-based diets on milk fatty acid composition and milk lipolytic system in Tarantaise and Montbéliarde cow breeds. *J. Dairy Sci.* **2006**, *89*, 4026–4041.

(3) Prache, S.; Cornu, A.; Berdagué, J. L.; Priolo, A. Traceability of animal feeding diet in the meat and milk of small ruminants. *Small Ruminant Res.* **2005**, *59*, 157–168.

(4) Engel, E.; Ferlay, A.; Cornu, A.; Chilliard, Y.; Agabriel, C.; Bielicki, G.; Martin, B. Relevance of isotopic and molecular biomarkers for the authentication of milk according to production zone and type of feeding. *J. Agric. Food Chem.* **2007**, *55*, 9099–9108.

(5) Toso, B.; Procida, G.; Stefanon, B. Determination of volatile compounds in cows' milk using headspace GC−MS. *J. Dairy Res.* **2002**, *69*, 569–577.

(6) Viallon, C.; Martin, B.; Verdier-Metz, I.; Pradel, P.; Garel, G. P.; Coulon, J. B.; Berdagué, J. L. Transfer of monoterpenes and sesquiterpenes from forages into milk fat. *Lait* **2000**, *80*, 12–16.

(7) Martin, B.; Verdier-Metz, I.; Buchin, S.; Hurtaud, C.; Coulon, J. B. How does the nature of forages and pasture diversity influence the sensory quality of dairy livestock products? *Anim. Sci.* **2005**, *81*, 205–212.

(8) Fernandez, C.; Astier, C.; Rock, E.; Coulon, J. B.; Berdagué, J. L. Characterization of milk by analysis of its terpene fraction. *Int. J. Food Sci. Technol.* **2003**, *38*, 445–451.

(9) Bugaud, C.; Buchin, S.; Coulon, J. B.; Hauwuy, A.; Dupont, D. Influence of the nature of alpine pasture on plasmin activity, fatty acid and volatile compound composition of milk. *Lait* **2001**, *81*, 401–414.

(10) Sangwan, N. S.; Faaroqi, A. H. A.; Shabih, F.; Sangwan, R. S. Regulation of essential oil production in plants. *Plant Growth Regul.* **2001**, *34*, 3–21.

(11) Tornambé, G.; Cornu, A.; Pradel, P.; Kondjoyan, N.; Carnat, A. P.; Petit, M.; Martin, B. Changes in terpene content in milk from pasture-fed cows. *J. Dairy Sci.* **2006**, *89*, 2309–2319.

(12) Pillonel, L.; Bosset, J. O.; Tabacchi, R. Rapid preconcentration and enrichment techniques for the analysis of food volatiles: A review. *Food Sci. Technol.* **2002**, *35*, 1–14.

(13) Bendall, J. G. Aroma compounds of fresh milk from New Zealand cows fed different diets. *J. Agric. Food Chem.* **2001**, *49*, 4825–4832.

(14) Moio, L.; Rillo, L.; Ledda, A.; Addeo, F. Odour constituents of ovine milk in relationship to diet. *J. Dairy Sci.* **1996**, *79*, 1322–1331.

(15) Urbach, G. Effect of feed on flavor in dairy foods. *J. Dairy Sci.* **1990**, *73*, 3639–3650.

(16) Vasta, V.; Priolo, A. Ruminant fat volatiles as affected by diet. A review. *Meat Sci.* **2006**, *73*, 218–228.

(17) Kondjoyan, N.; Berdagué, J. L. *Compilation of Relative Retention Indices for the Analysis of Aromatic Compounds*; Laboratoire Flaveur Publisher, Institut National de la Recherche Agronomique (INRA): Clermont-Ferrand, France, 1996.

(18) Mass Spec Data Center, National Institute of Standards and Technology (NIST). *"Retention Indices" in NIST Chemistry WebBook*; Linstrom, P. J., Mallard, W. G., Eds.; NIST: Gaithersburg, MD, 2000; NIST Standard Reference Database Number 69, 20899, http://webbook.nist.gov.

(19) Vazquez-Landaverde, P. A.; Torres, J. A.; Qian, M. C. Quantification of trace volatile sulfur compounds in milk by solid-phase microextraction and gas chromatography-pulsed flame photometric detection. *J. Dairy Sci.* **2006**, *89*, 2919–2927.

(20) Mounchili, A.; Wichtel, J. J.; Bosset, J. O.; Dohoo, I. R.; Imhof, M.; Altieri, D.; Mallia, S.; Stryhn, H. HS-SPME gas chromatographic characterization of volatile compounds in milk tainted with off-flavour. *Int. Dairy J.* **2005**, *15*, 1203–1215.

(21) Kataoka, H.; Lord, H. L.; Pawliszyn, J. Applications of solid-phase microextraction in food analysis. *J. Chromatogr., A* **2000**, *880*, 35–62.

(22) Kanavouras, A.; Kiritsakis, A.; Hernandez, R. J. Comparative study on volatile analysis of extra virgin olive oil by dynamic headspace and solid phase micro-extraction. *Food Chem.* **2005**, *90*, 69–79.

(23) Brunton, N. P.; Cronin, D. A.; Monahan, F. J. The effects of temperature and pressure on the performance of Carboxen/PDMS

fibres during solid phase microextraction (SPME) of headspace volatiles from cooked and raw turkey breast. *Flavour Fragrance J.* **2001**, *16*, 294–302.

(24) Vasta, V.; Luciano, G.; Dimauro, C.; Röhrle, F.; Priolo, A.; Monahan, F. J.; Moloney, A. P. The volatile profile of *longissimus dorsi* muscle of heifers fed pasture, pasture silage or cereal concentrate: implication for dietary discrimination. *Meat Sci.* **2011**, *87*, 282–289.

(25) Cornu, A.; Carnat, A. P.; Martin, B.; Coulon, J. B.; Lamaison, J.-L.; Berdagué, J. L. Solid-phase microextraction of volatile components from natural grassland plants. *J. Agric. Food Chem.* **2001**, *49*, 203–209.

(26) Younge, B. A.; Murphy, J. J.; Rath, M. Nutrient metabolism in the rumen and milk production in cows fed on grass silage and fresh grass-based diets. *Livest. Prod. Sci.* **2004**, *88*, 43–54.

(27) Buchin, S.; Delague, V.; Duboz, G.; Berdagué, J. L.; Beuvier, E.; Pochet, R.; Grappin, R. Influence of pasteurization and fat composition of milk on volatile compounds and flavor characteristics of a semi-hard cheese. *J. Dairy Sci.* **1998**, *81*, 3097–3108.

(28) Moio, L.; Dekimpe, J.; Etievant, P; Addeo, F. Neutral volatile compounds in the raw milks from different species. *J. Dairy. Res.* **1993**, *60*, 199–213.

(29) Priolo, A.; Micol, D.; Agabriel, J. Effect of grass feeding system on ruminant meat color and flavour. A review. *Animal Res.* **2001**, *50*, 185–200.

(30) Priolo, A.; Vasta, V.; Fasone, V.; Lanza, C. M.; Biondi, L.; Bella, M.; Whittington, F. M. Meat odour and flavour and indoles concentration in ruminal fluid and adipose tissue of lambs fed green herbage or concentrates with or without tannins. *Animal* **2009**, *3*, 454–460.