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Relative hierarchy of farming practices affecting the fatty acid composition of permanent grasslands and of the derived bulk milk

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ABSTRACT

This study aimed (i) to assess the relative weight of different predicting variables, related to site and grassland characteristics, in determining the fatty acid (FA) composition of permanent grasslands and (ii) to verify if and at which extent the same variables have a significant predicting role for the FA composition of the derived bulk cow milk.

Available data collected from 2003 to 2016 by three European research institutions were used. A dataset (n = 144) was built up including: (i) the proximate and FA compositions of herbage, (ii) site altitude and climatic conditions, (iii) grasslands botanical composition (main family groups), herbage phenology and growing cycle, (iv) herd characteristics and (v) milk gross and FA compositions.

Prediction models for herbage FA profile were highly reliable (R^2 adjusted ≥ 0.80) for C18:3 n3, total monounsaturated FA and total polyunsaturated FA (Σ PUFA). Reliable predictions (R^2 adjusted ≥ 0.60) were obtained for the other main herbage FA. The relative predicting weight was higher for phenology and proximate composition than for botanical composition. Among proximate composition variables, dry matter showed the highest relative weight in determining the majority of the predicting models.

Prediction models for milk FA profile were reliable (R^2 adjusted ≥ 0.60) for Σ *de novo* FA, C16:0, C18:3 n3, total even-chain saturated FA (Σ ECFA), Σ PUFA and the C18:1 *c*9/C16:0 ratio, while they were moderately reliable (R^2 adjusted ≥ 0.50) for C18:1 *t*10 + *t*11, C18:2 n6, C18:2 *c*9*t*11 and the C18:2 n6/C18:3 n3 ratio. Diet composition, site altitude and grazed grassland features concurred to predict milk FA. Fresh herbage proportion in the diet played a relevant role in determining the milk proportion of several FA. Phenology was the main driver for the C18:2 *c*9*t*11 and Σ PUFA proportions, negatively affecting them. Herbage phenology fitted milk FA better than proximate and botanical compositions.

Abbreviations: ADFom, acid detergent fibre; BCFA, branched-chain fatty acids; *c*, *cis*; CP, crude protein; DM, dry matter; ECFA, even-chain saturated fatty acids; F, forbs; FA, fatty acids; G, grasses; GDD, growing degree days; L, legumes; MUFA, monounsaturated fatty acids; aNDFom, neutral detergent fibre; OCFA, odd-chain saturated fatty acids; OMD, organic matter digestibility; PUFA, polyunsaturated fatty acids; SE, standard error; RMSE, root mean square error; SFA, saturated fatty acids; *t*, *trans*; TFA, total fatty acids

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1. Introduction

In the European Union, permanent grasslands cover around 59.5 million hectares, accounting for the 34.2 % of the Utilised Agricultural Area (Dillon, 2018). These areas represent the basic forage resource for about 78 million Livestock Units of grazing livestock, 31 % of which are dairy cows (Peeters, 2015). Feeding fresh forage has proven to be a major driver for dairy farms profitability allowing permanent grassland farms to be competitive with fodder-crop farms (Kellermann and Salhofer, 2014). Counterbalancing low milk productivity, the use of permanent grasslands is associated to the production of dairy products with high nutritional and sensory quality (Leiber et al., 2014). For this reason, grassland-based dairy systems are often linked to the production of typical or labelled (e.g., Protected Designation of Origin and Protected Geographical Indication) dairy products, commonly perceived as healthy, sustainable (e.g., by lowering food-feed competition) and respectful towards animal welfare (Zuliani et al., 2018). These products are also characterised by high market price, in response to the increased consumers' preference and willingness to pay (Bernués et al., 2019).

The variability of European permanent grasslands is substantial in terms of geographical location (e.g., altitude, topography), ecological conditions (e.g., climate and soil), botanical composition, productivity, nutritive value and management system (Michaud et al., 2012), with potential implications on herbage composition (Schaub et al., 2020). In particular, the fatty acid (FA) composition of herbage directly affects the lipid metabolism of ruminants (Buccioni et al., 2012), significantly modifying the FA composition of dairy products (Leiber et al., 2005) with consequences on their sensory profile (Giaccone et al., 2016).

Many studies, mainly in the last 20 years, attempted to investigate which factors are able to affect the FA composition of herbage and to which extent. However, the majority of these studies considered single botanical species or simplified mixtures of few species, with a single factor approach (e.g., botanical composition, phenological stage, nitrogen fertilisation, growing degree days (GDD), etc.) tested under strictly defined experimental conditions (reviewed by Elgersma, 2015). Only a few studies focused on permanent grasslands. Among them, the older ones investigated single sites or single pasture types (Ferlay et al., 2006;Revello-Chion et al., 2011). In a meta-analysis, Glasser et al. (2013) were able to estimate the influence of several individual factors such as forage conservation, cultivation and harvest conditions on lipid content and FA composition of forages from permanent grasslands. Only recently, some authors attempted to face the problem with a multivariate approach (Peiretti et al., 2017; Ravetto Enri et al., 2017). However, these studies highlighted the relationships existing between the botanical, proximate and FA composition of fresh forages without any estimation of the hierarchy of the involved factors and without analysing the derived bulk milk.

Likewise, literature exists on the individual effects of site or herbage characteristics (e.g., altitude, phenology, botanical composition) on milk FA composition (Collomb et al., 2002a, 2002b; Coppa et al., 2015b). However, no information is currently available on the relative weight of these factors in determining the FA composition of bulk milk fat in on-farm conditions.

To fill in this gap of knowledge, we built up a common database using data spread along several years and obtained from dairy farms grazing permanent grasslands from different European geographical areas. Data were analysed using a multifactorial approach. Our purposes were (i) to understand and hierarchize the factors that are the main drivers for the total FA concentration and the FA composition of semi-natural permanent grasslands on farm and (ii) to verify how and with which hierarchy these factors influence the FA composition of the bulk milk obtained from herds grazing those areas.

2. Materials and methods

2.1. Data collection

A dataset was built up including data collected in the years from 2003 to 2016 by three research institutions located in three European countries: (i) Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (France), (ii) University of Turin (Italy) and (iii) Agroscope (Switzerland).

Data concerned 144 herbage samples from permanent grasslands and the same number of bulk milk samples obtained from dairy herds grazing those areas. Data were included in the dataset when the following information for each bulk milk sample was available: FA composition of the milk samples, herd characteristics and management, proximate and FA compositions of the herbage grazed by the cows corresponding to milk sampling. The number of herbage (and as many milk samples) collected in the different countries was equal to 99, 33 and 12 for France, Italy and Switzerland, respectively.

2.2. Sites and grasslands characterization

The studied permanent grasslands were enclosed within the 44 °N and 49 °N latitude, and between the 1 °E and 8 °E longitude. They were located in the western Alps, in east, central and western France, and in central-western Switzerland. For each grassland, altitude was recorded.

Minimum, maximum and average daily air temperature (°C), as well as rainfall and snow precipitations (mm) were obtained by the nearest meteorological stations. Average air temperature above 0 °C for at least five consecutive days after the snow cover disappeared was considered as the beginning of the growing season (Niqueux and Arnaud, 1981; Jouglet et al., 1982). The cumulated rainfall (mm) from the beginning of the growing season was calculated. Cumulative GDD, over a base temperature of 0 °C, were calculated from the beginning of the growing season as the average of the daily maximum and minimum temperatures, considering only positive values (Revello-Chion et al., 2011).

The botanical composition of the grasslands was determined through botanical surveys using the vertical point-quadrat method

(Daget and Poissonet, 1971). The percentages on ground cover of botanical family groups, namely grasses (*Gramineae*, G), legumes (*Leguminosae*, L) and forbs (non-legume dicotyledons, F), were then calculated.

The phenological stage of the plants was determined using the BBCH scale (FBRCAF (Federal Biological Research Centre for Agriculture and Forestry), 2001) at each sampling date.

For each herbage sample, the following parameters were thus included in the dataset:

(i) FA composition;
(ii) proximate composition (dry matter, DM; ash; crude protein, CP; neutral detergent fibre, aNDFom; acid detergent fibre, ADFom);
(iii) site characteristics at the sampling date [altitude; climatic conditions (cumulated rainfall and GDD from the beginning of the year and from the beginning of the growing season)];
(iv) grassland characteristics at the sampling date [botanical composition (G, L and F, expressed as a percentage on ground cover); phenological stage of the dominant grasses; number of the growing cycle (1st or followings)].

2.3. Herds characterization

At each milk sampling, on-farm surveys were carried out to collect information related to the characteristics and management of the herds, following the protocol detailed by Coppa et al. (2013).

For each milk sample, the following parameters concerning the farming practices and the milk chemical composition were then included in the dataset:

(i) FA composition;
(ii) fat and protein contents;
(iii) herd characteristics (cow breeds; number of cows; average individual milk yield; days in milk);
(iv) diet composition of the lactating cows (intake and proportion of different feedstuffs in the diet, on a DM basis).

Fresh herbage intake at pasture was calculated by difference to the potential intake capacity, as detailed by Coppa et al. (2013). Only milk samples deriving from herds having at least 50 % (on a DM basis) of their diet composed of fresh herbage from direct grazing were retained in the dataset.

2.4. Chemical analysis of herbage samples

The proximate and FA compositions of the herbage samples were analysed by three laboratories over the 2003–2017 period.

The detailed procedures of analysis of proximate composition for the French, Italian and Swiss samples are reported in Coppa et al. (2015a), Ravetto Enri et al. (2017) and Renna et al. (2010), respectively. In the dataset, the results of the proximate composition analysis were expressed as g/kg DM, with the exception of the DM that was expressed as g/kg fresh matter.

The FA composition of the herbage samples was determined by direct methylation on ground lyophilized samples, according to the method of Sukhija and Palmquist (1988). Methyl esters were separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil88) and determined by a gas-chromatograph equipped with a flame ionization detector. The TFA content (g/kg DM) was obtained using an internal standard. The details of the applied methods are described in Coppa et al. (2015a), Ravetto Enri et al. (2017) and Renna et al. (2010) for the French, Italian and Swiss samples, respectively. The individual and groups of fatty acid results were expressed as g/kg of total FA (TFA).

2.5. Chemical analysis of milk samples

Bulk milk samples were directly collected from the farm tanks.

The fat, protein, lactose and urea contents of the milk samples were analysed by Fourier transform infrared spectroscopy following the International Dairy Federation (2000).

The FA profile of the milk samples was performed by the same laboratories and with the same instruments used for the analysis of the FA composition of the herbage samples.

The differences in the applied analytical methods among the laboratories - detailed in Coppa et al. (2019), Iussig et al. (2015) and Renna et al. (2010) for the French, Italian and Swiss samples, respectively - were taken into account by adding the not co-eluting individual FA that were common in all the chromatograms, only. The results were expressed as g/kg of total selected FA.

2.6. Statistical analyses

The statistical analysis of data was performed using Minitab v. 14.1 (Minitab Inc., State College, PA, USA). The herbage and milk FA profiles were initially subjected to univariate descriptive statistics, graphically inspected and examined for outliers, according to the procedure followed by Coppa et al. (2013).

General linear models were used to obtain prediction equations for the estimation of variables related to the FA composition of herbage and milk.

Concerning the herbage FA composition, the analytical method applied and the plant growing cycle were used as fixed factors, while the variables describing the site and grassland characteristics (i.e., altitude, climatic conditions, botanical composition, herbage phenology, and herbage proximate composition) were tested as covariates.

Concerning the milk FA composition, the analytical method and the plant growing cycle were used as fixed factors, while the variables describing the site, grassland and herd characteristics (i.e., altitude, climatic conditions, botanical composition, herbage phenology, herbage proximate composition, proportion of different feedstuffs in the diet and days in milk) were tested as covariates.

The linear, quadratic, and logarithmic effects of all the covariates were tested as well. For both herbage and milk, significant covariates were identified using a stepwise reduction procedure. As proposed by Coppa et al. (2013), the root mean square error (RMSE) and the coefficient of determination (R^2 adjusted) were used to describe model fitting. The Fisher's F value of each variable included in a model was used as an indicator of the relative weight of the variable in determining the model itself aiming at hierarchize the variables. Only equations with parameters contributing significantly ($P \leq 0.05$) to the explanation of the FA composition were considered. Model fits were considered moderately reliable when $0.50 \leq R^2$ adjusted < 0.60, reliable when $0.60 \leq R^2$ adjusted < 0.80, and highly reliable when R^2 adjusted ≥ 0.80.

3. Results

3.1. Dataset variability

3.1.1. Sites and grasslands characteristics

Descriptive statistics of the sites and grasslands characteristics, including the proximate and FA compositions of the herbage samples are presented in Table 1.

The studied grasslands were located between 15 and 2500 m a.s.l. The cumulated rainfall and GDD from the beginning of the growing season to the sampling date ranged from 10 to 1496 mm and from 96 °C to 4396 °C, respectively. Permanent grasslands varied largely in dominant species and botanical composition: from the permanent grasslands rich in grasses of the lowland areas to the species-rich semi-natural pastures of the upland regions of the Alps that were dominated by legumes or forbs. The phenological stage ranged from the vegetative stage to the full gain ripening of the main grasses (BBCH: 15 and 95, respectively).

Both the proximate and FA compositions of the herbage samples showed high variability. Maximum values of DM, CP, aNDFom

Table 1
Site characteristics, grassland botanical composition, proximate composition and fatty acid profile of herbage.

Item	Average	Median	SD	Min	Max
GDD (°C)	1415	1184	920	96	4396
Rainfall (mm) [a]	378	292	238	10	1496
Altitude (m a.s.l.)	1087	1000	635	15	2500
Phenology (BBCH scale)[b]	49	50	19	15	95
Botanical composition (% on ground cover)					
Grasses	56	56	18	6	96
Legumes	14	11	12	0	72
Forbs	29	27	18	0	69
Chemical composition (g/kg DM, unless otherwise stated)					
DM (g/kg)	269	266	81	119	523
CP	139	133	38	71	289
aNDFom	513	509	74	339	689
ADFom	301	298	50	148	434
Fatty acid composition (g/kg TFA)					
C16:0	184.9	180.0	35.9	104.2	301.6
C18:0	21.3	19.6	8.2	9.3	50.9
C18:1 *c*9	47.8	41.3	28.3	9.8	176.7
C18:2 n6	175.7	169.8	41.1	107.0	340.2
C18:3 n3	454.0	449.0	116.2	158.0	729.8
Σ SFA[c]	234.1	226.6	53.5	119.7	435.7
Σ MUFA[d]	67.4	64.6	33.3	12.8	201.3
Σ PUFA[e]	631.0	635.6	105.2	327.9	860.1
C18:2 n6/C18:3 n3	0.43	0.39	0.23	0.17	2.05
TFA (g/kg DM)	17.33	16.80	5.52	7.49	38.50

Abbreviations: ADFom = acid detergent fibre; aNDFom = neutral detergent fibre; CP = crude protein; DM = dry matter; GDD = growing degree days; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SD, standard deviation; SFA, saturated fatty acids; TFA, total fatty acids.

[a] Cumulated from the beginning of the growing season to the sampling date.

[b] BBCH scale (FBRCAF (Federal Biological Research Centre for Agriculture and Forestry), 2001): 20 = vegetative stage, beginning of tillering; 30 = beginning of stem elongation; 40 = beginning of booting; 50 = beginning of heading; 60 = beginning of flowering; 70 = beginning of fruit development; 80 = beginning of ripening; 90 = end of ripening.

[c] C12:0 + C14:0 + C16:0 + C18:0 + C20:0 + C22:0.

[d] C16:1 *t*9 + C16:1 *c*9 + C18:1 *c*9 + C18:1 *c*11.

[e] C18:2 n6 + C18:3 n6 + C18:3 n3.

and ADFom were 4.4-, 4.1-, 2.0- and 2.9-fold higher than the corresponding minimum values. The TFA content of the plants ranged from 7.49 to 38.50 g/kg DM. All the herbage FA showed a large range: i.e. C18:3 n3 from 158.0 to 729.8 g/kg TFA and C18:2 n6 from 107.0 to 340.2 g/kg TFA.

3.1.2. Farms characteristics and milk composition

Descriptive statistics of the herd characteristics and milk composition, as well as diet composition of the herds are summarised in Table 2. Twelve different cow breeds were identified in the herds, ranging from dual-purpose autochthonous breeds (i.e., Abondance, Alpine Grey, Barà-Pustertaler, Italian Red Pied, Normande, Piemontese, Swiss-Brown, Tarantaise, Valdostana Red Pied, and crossbreeds) to high-yielding and specialised dairy breeds (i.e., Holstein Friesian, Italian Brown, Montbéliarde). The herd size ranged from a very low number of lactating cows in family farms, especially on upland areas, to larger farms located both in the lowland and upland (up to 97 cows per farm).

The average proportion of fresh herbage from pasture in the diet was equal to 87 % of diet DM. When full grazing was not adopted, the other main dietary ingredients fed to the cows were hay and concentrates (in both cases on average 6 % of diet DM of the total ration). As forage integration to fresh herbage, a limited number of farms (7 out of 144 samples, only) used very low inclusion levels of corn silage (on average, 1% of diet DM of the total ration).

The daily individual milk yield ranged from 4.0 to 33.9 kg. Milk gross composition was very variable, with a range i.e. of 30.2 to 53.5 g/kg for fat content and 27.9 to 43.3 g/kg for protein content. The FA composition of milk fat was also highly variable. As examples, the maximum values for Σ *de novo* FA, C18:0, C18:1 *t*10 + *t*11, C18:2 *c*9*t*11, C18:3 n3 and Σ PUFA proportion were 2.0-,

Table 2
Characteristics, diet composition, dairy performance, milk gross composition and fatty acid profile of the herds.

Item	Average	Median	SD	Min	Max
Dairy cows[a] (n°/farm)	23	16	20	5	97
Diet composition (% on diet DM)					
Pasture	87	90	16	51	100
Hay	6	0	13	0	44
Corn silage	1	0	5	0	35
Concentrates	6	0	8	0	27
Milk yield (kg/cow*day)	15.4	15.0	7.2	4.0	33.9
Milk gross composition (g/kg milk, unless otherwise stated)					
Fat	38.7	38.2	3.8	30.2	53.5
Protein	33.0	32.9	2.5	27.9	43.3
Lactose	47.3	47.3	1.5	43.8	51.0
Urea (mg/dL milk)	22.4	22.2	6.8	9.4	37.3
Milk fatty acid profile (g/kg TFA)					
Σ *de novo* FA[b]	211.0	210.7	29.2	142.6	287.7
C16:0	252.4	249.0	30.5	199.6	374.7
C18:0	104.4	104.6	13.9	63.8	147.6
C18:1 *t*10 + *t*11	36.6	37.1	9.2	16.1	62.7
C18:1 *c*9	219.0	217.9	26.2	160.8	308.9
C18:2 n6	13.6	13.6	3.0	7.7	23.2
C18:3 n3	9.7	8.8	3.6	3.4	20.4
C18:2 *c*9*t*11	14.9	14.0	4.5	6.2	25.6
Σ ECFA[c]	569.9	569.0	43.8	476.2	687.0
Σ OCFA[d]	29.0	28.9	3.1	22.4	37.3
Σ BCFA[e]	28.5	28.5	3.4	20.6	38.8
Σ MUFA[f]	296.7	300.0	28.9	215.2	389.1
Σ PUFA[g]	53.3	51.2	10.8	28.8	83.1
C18:2 n6/C18:3 n3	1.53	1.41	0.51	0.76	3.62
Σ PUFA n6[h]/Σ PUFA n3[i]	1.01	0.96	0.32	0.50	2.31
C18:1 *c*9/C16:0	0.89	0.89	0.18	0.45	1.29

Abbreviations: BCFA, branched-chain fatty acids; DM, dry matter; ECFA, even-chain saturated fatty acids; FA, fatty acids; OCFA, odd-chain saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SD, standard deviation; TFA, total fatty acids.

[a] Cow breeds: Holstein Friesian, Italian Brown, Montbéliarde, Abondance, Alpine Grey, Barà-Pustertaler, Italian Red Pied, Normande, Piemontese, Swiss-Brown, Tarantaise, Valdostana Red Pied, and crossbreeds.

[b] C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C14:0.

[c] C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C14:0 + C16:0 + C18:0 + C20:0 + C22:0.

[d] C5:0 + C7:0 + (C15:0 + C14:1 *c*9) + C17:0.

[e] C13:0 *iso* + C14:0 *iso* + C15:0 *iso* + C15:0 *anteiso* + C16:0 *iso* + (C17:0 *iso* + C16:1 *t*9) + C17:0 *anteiso* + C18:0 *iso*.

[f] C10:1 *c*9 + C16:1 *c*9 + (C17:1 *c*9 + C18:0 *anteiso*) + C18:1 *t*5 + (C18:1 *t*6 + *t*7 + *t*8 + *t*9) + (C18:1 *t*10 + *t*11) + (C18:1 *t*12 + *t*13 + *t*14 + *c*6 + *c*7 + *c*8) + C18:1 *c*9 + C18:1 *c*11 + C18:1 *c*12 + (C18:1 *c*14 + *t*16) + C20:1 *c*9.

[g] (C18:2 *c*9*t*13 + *t*8*c*12) + (C18:2 *c*9*t*12 + *t*8*c*13 + *c*9*t*12) + (C18:2 *c*11*t*15 + *t*9*c*12) + C18:2 n6 + C18:3 n3 + (C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10) + (C18:2 *t*11*c*13 + *c*9*c*11 + C21:0) + C18:2 *t*9*t*11 + C20:2 n6 + C20:3 n6 + C20:4 n6 + C20:5 n3 + C22:5 n3.

[h] C18:2 n6 + C20:2 n6 + C20:3 n6 + C20:4 n6.

[i] (C18:2 *c*11*t*15 + *t*9*c*12) + C18:3 n3 + C20:5 n3 + C22:5 n3.

2.3-, 3.9-, 4.1-, 6.0- and 2.9-fold higher than their minimum values. The Σ PUFA n6/Σ PUFA n3 ratio ranged from 0.50 to 2.31.

3.2. Prediction models for herbage fatty acid composition

The prediction models for the studied dependent variables related to the FA composition of herbage from permanent grasslands are shown in Table 3.

For all the variables, the fit was highly reliable (R^2 adjusted ≥ 0.80 for C18:3 n3, Σ MUFA and Σ PUFA) or reliable (R^2 adjusted ≥ 0.60 for C16:0, C18:0, C18:1 *c*9, C18:2 n6, Σ SFA, C18:2 n6/C18:3 n3 and TFA). The RMSE was also quite low for all the studied individual FA and groups of FA.

The herbage phenological stage was a significant predicting variable for all the considered FA, with the exception of C16:0.

Table 3
Prediction models for herbage fatty acid composition.

Fatty acid (g/kg TFA)	Variable	Coefficient (± SE)	Fisher's F	Intercept (± SE)	Lab error Fishers' F	RMSE	R^2
C16:0	DM (g/kg)	1.44 (± 0.20)	50.8	141.7 (± 6.0)	39.53	19.6	0.65
	Legumes (%)	0.0250 (± 0.0126)	3.9				
	1 st growing cycle[a]		20.1	−0.78 (± 0.174)			
C18:0	DM (g/kg)	0.400 (± 0.060)	45.1	20.8 (± 4.8)	15.49	3.7	0.72
	Phenology[b]	0.00905 (± 0.00219)	17.1				
	Legumes (%)	0.0126 (± 0.0032)	16.0				
	aNDFom (g/kg DM)	−0.213 (± 0.058)	13.4				
	CP (g/kg DM)	−0.404 (± 0.160)	6.4				
	1 st growing cycle[a]		45.6	−0.229 (± 0.034)			
C18:1 *c*9	Phenology^2[b]	0.000472 (± 0.000064)	55.1	32.2 (± 9.9)	16.21	12.0	0.71
	DM (g/kg)	0.714 (± 0.190)	14.1				
	CP (g/kg DM)	−1.28 (± 0.43)	8.8				
	1 st growing cycle[a]		25.9	−0.549 (± 0.108)			
C18:2 n6	Altitude (m a.s.l.)	−0.00204 (± 0.00033)	37.9	220.0 (± 15.8)	27.54	20.8	0.63
	Phenology[b]	0.055 (± 0.0107)	26.7				
	Grasses (%)	−0.0468 (± 0.0122)	14.8				
	CP (g/kg DM)	−2.08 (± 0.63)	10.8				
C18:3 n3	Phenology[b]	−0.156 (± 0.027)	34.7	619.7 (± 33.1)	53.32	49.6	0.81
	DM (g/kg)	−4.28 (± 0.74)	33.9				
	Grasses (%)	0.135 (± 0.029)	20.9				
	Altitude (m a.s.l.)	0.00322 (± 0.00085)	14.3				
	aNDFom (g/kg DM)	−1.74 (± 0.72)	5.8				
	1 st growing cycle[a]		40.6	3.02 (± 0.47)			
Σ SFA	DM (g/kg)	2.36 (± 0.33)	49.9	149.2 (± 8.0)	4.94	25.4	0.74
	Legumes (%)	0.041 (± 0.0184)	45.9				
	Phenology[b]	0.0303 (± 0.0142)	4.5				
	1 st growing cycle[a]		29.1	−1.22 (± 0.23)			
Σ MUFA	Phenology^2[b]	0.000484 (± 0.000068)	50.1	47.4 (± 10.6)	48.52	13.0	0.80
	DM (g/kg)	0.661 (± 0.205)	10.4				
	CP (g/kg DM)	−0.998 (± 0.464)	4.6				
	1 st growing cycle[a]		32.6	−0.663 (± 0.116)			
Σ PUFA	DM (g/kg)	−4.7 (± 0.58)	64.9	740.1 (± 20.8)	91.85	44.2	0.82
	Phenology[b]	−0.0832 (± 0.0237)	12.4				
	Grasses (%)	0.0623 (± 0.0259)	5.8				
	Altitude (m a.s.l.)	0.00155 (± 0.00076)	4.1				
	1 st growing cycle[a]		32.3	2.40 (± 0.42)			
C18:2 n6/C18:3 n3	Phenology[b]	0.00297 (± 0.00039)	56.9	2.83 (± 0.49)	15.33	0.72	0.70
	Grasses (%)	−0.00240 (± 0.00044)	30.0				
	Altitude (m a.s.l.)	−0.000067 (± 0.000012)	29.0				
	DM (g/kg)	0.0289 (± 0.011)	6.8				
	aNDFom (g/kg DM)	0.0215 (± 0.0105)	4.2				
	1 st growing cycle[a]		16.0	−0.029 (± 0.007)			
TFA (g/kg DM)	CP (g/kg DM)	6.59 (± 0.85)	60.0	106.9 (± 28.4)	32.62	24.8	0.72
	aNDFom (g/kg DM)	−1.24 (± 0.37)	11.4				
	Phenology[b]	−0.0337 (± 0.0121)	7.8				
	1 st growing cycle[a]		7.6	0.603 (± 0.218)			

Abbreviations: aNDFom, neutral detergent fibre; CP, crude protein; DM, dry matter; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; R^2 adjusted, coefficient of determination; RMSE, root mean square error; SE, standard error; SFA, saturated fatty acids; TFA, total fatty acids.

[a] Correction of intercept according to the growing cycle; if growing cycle > 1st: the related additive constant coefficient changes from + to – or vice versa.

[b] Phenology BBCH scale (FBRCAF (Federal Biological Research Centre for Agriculture and Forestry), 2001): 20 = vegetative stage, beginning of tillering; 30 = beginning of stem elongation; 40 = beginning of booting; 50 = beginning of heading; 60 = beginning of flowering; 70 = beginning of fruit development; 80 = beginning of ripening; 90 = end of ripening.

Phenology positively affected the herbage proportions of C18:0, C18:1 *c*9, C18:2 n6, Σ SFA, Σ MUFA and the C18:2 n6/C18:3 n3 ratio, while it negatively affected the proportions of C18:3 n3, Σ PUFA and the TFA concentration. Phenology had the highest relative weight (highest Fisher's F coefficient) in determining the prediction models for C18:1 *c*9, Σ MUFA and C18:2 n6/C18:3 n3 ratio.

Among the proximate composition variables, DM showed a significant predicting role for all the considered FA, with the exception of C18:2 n6 and TFA. The DM content of herbage was positively related to the herbage proportions of C16:0, C18:0, C18:1 *c*9, Σ SFA, Σ MUFA and to the C18:2 n6/C18:3 n3 ratio, while it was negatively related to the herbage C18:3 n3 and Σ PUFA proportions. The DM content showed the highest Fisher's F coefficient in the models for C16:0, Σ SFA and Σ PUFA. The CP and aNDFom contents of herbage were significant in determining the models of five and four out of ten considered dependent variables, respectively. A negative relationship was found between CP and C18:0, C18:1 *c*9, C18:2 n6 and Σ MUFA, while CP positively affected the herbage TFA concentration. The aNDFom content of herbage increased with the increase of the C18:2 n6/C18:3 ratio, while the inverse was observed with proportions of C18:0, C18:3 n3 and TFA in the herbage samples. The ADFom content of herbage was not significant in any models.

The grassland botanical composition (namely the proportion of grasses or legumes on ground cover) was also a significant predictor in four (positive relationship: C18:3 n3 and Σ PUFA; negative relationship: C18:2 n6 and C18:2 n6/C18:3 n3) and three (C16:0, C18:0 and Σ SFA, in all the cases with a positive relationship) FA models, for grasses and legumes respectively. The percentage of forbs on ground cover was not significant in any of the models. The relative weight of the botanical composition of the grasslands in predicting the models was generally lower than the relative weight exerted by phenology or proximate composition.

Concerning variables linked to site characteristics, altitude was a significant predictor for C18:2 n6 (for which it showed the highest effect), C18:3 n3, Σ PUFA, and the C18:2 n6/C18:3 n3 ratio. The C18:3 n3 and PUFA proportions of herbage increased with increasing altitude, while C18:2 n6 and the C18:2 n6/C18:3 n3 ratio decreased. Climatic-related variables (GDD and rainfall) were not significant in any models.

The growing cycle had a significant effect for all the models, with the exception of C18:2 n6. The Fisher's F coefficient of this effect was high in almost all the cases, with particular relevance for C18:0 and C18:3 n3. The 1st growing cycle was associated to higher proportions of C18:3 n3, Σ PUFA and TFA, and to lower proportions of C16:0, C18:0, C18:1 *c*9, Σ SFA and Σ MUFA as well as of the C18:2 n6/C18:3 n3 ratio in the herbage.

3.3. Prediction models for bulk milk fatty acid composition

The prediction models for the studied dependent variables related to the FA composition of bulk milk obtained from dairy cows grazing permanent grasslands are shown in Table 4. Reliable predictions (R^2 adjusted ≥ 0.60) were obtained for Σ *de novo* FA, C16:0, C18:3 n3, Σ ECFA, Σ PUFA and for the C18:1 *c*9/C16:0 ratio (also called spreadability index; (Hurtaud et al., 2009). Moderately reliable predictions (R^2 adjusted ≥ 0.50) were obtained for C18:1 *t*10 + *t*11, C18:2 n6, C18:2 *c*9*t*11 and for the C18:2 n6/C18:3 n3 ratio. For other studied FA variables (C18:0, C18:1 *c*9, Σ OCFA, Σ BCFA, Σ MUFA and Σ PUFA n6/Σ PUFA n3 ratio), even if significant prediction models were obtained, the models were not reliable (R^2 adjusted < 0.50). Consequently, these FA variables will not be further discussed.

The proportion of fresh herbage in the cow diet was a significant predicting variable for all the considered FA. This predicting variable showed the highest Fisher's F coefficient in three out of ten considered FA variables, namely C18:3 n3, C16:0 and the C18:1 *c*9/C16:0 ratio. An increase in the milk proportions of C18:1 *t*10 + *t*11, C18:2 n6, C18:3 n3, C18:2 *c*9*t*11 and Σ PUFA, as well as in the C18:1 *c*9/C16:0 ratio was observed with the increase of the proportion of fresh herbage in the cow diet. Conversely, the proportion of fresh herbage in the diet negatively affected the proportions of Σ *de novo* FA, C16:0, Σ ECFA and the C18:2 n6/C18:3 n3 ratio in milk fat. The proportion of concentrates in the diet exerted a significant predicting role in the models for C18:2 n6 and C18:2 n6/C18:3 n3 (R^2 = 0.54 and 0.51, respectively), with a positive relationship and the highest relative weight in determining the prediction models. Other variables related to the diet composition of the cows (hay and corn silage proportions) were not significant in any models.

Variables related to herbage characteristics were significant and had a relevant role for all the models, except for C18:2 n6 and the C18:2 n6/C18:3 n3 ratio, for which their influence was minor.

The herbage phenology was the most determinant variable in predicting C18:2 *c*9*t*11 and Σ PUFA, in both cases with an inverse relationship. Phenology also exerted a significant role in the prediction models for C18:1 *t*10 + *t*11 (negative relationship), Σ ECFA and the C18:2 n6/C18:3 n3 ratio (positive relationship).

Among the variables related to the proximate composition of the herbage samples, CP was the most influencing in predicting the FA composition of milk fat. The CP content of herbage was positively related to the Σ *de novo* FA, and negatively related to the C16:0, C18:2 n6 and C18:3 n3 proportions of milk. In all the cases, the Fisher's F coefficient of CP was lower than that of the proportion of fresh herbage in the cow diet. The fibre fractions of herbage were significant predictors in two of the considered milk FA models, only. In particular, aNDFom and ADFom showed a significant role in the prediction models for the C18:1 *c*9/C16:0 ratio and Σ PUFA, respectively, with a positive relationship and a low relative predicting weight. The DM content of the plants was not significant in any models.

The botanical composition of the permanent grasslands exerted a significant predicting role in half of the considered milk FA variables. The proportion of grasses on ground cover was a significant predicting variable for C18:1 *t*10 + *t*11, C18:2 *c*9*t*11 and for the C18:1 *c*9/C16:0 ratio, with the lowest Fisher's F coefficient. Grasses were positively related to the C18:1 *t*10 + *t*11 and C18:2 *c*9*t*11 proportions of milk, and negatively related to the C18:1 *c*9/C16:0 ratio. The proportions of both legumes and forbs on ground cover were less influential, being significant only in the predictions of C18:3 n3 and Σ *de novo* FA, respectively. In both cases, a negative relationship was found.

Concerning variables linked to site characteristics, altitude was the most influencing predicting variable in the models for C18:1 *t*10 + *t*11 and Σ ECFA, exerting a positive and a negative effect, respectively. Altitude also exerted a significant predicting role for C16:0 (negative relationship), C18:2 *c*9*t*11 and Σ PUFA (positive relationship) in the former and latter models with the lowest relative weight. Climatic-related variables (GDD and rainfall) were not significant in any models.

The fixed effect of growing cycle was significant for Σ *de novo* FA, C18:1 *t*10 + *t*11, C18:2 *c*9*t*11 and Σ ECFA. The Fisher's F coefficient of this effect was the highest in the model for Σ *de novo* FA and ranked second for C18:2 *c*9*t*11 and Σ ECFA models. The relative weight of growing cycle was lower when compared to that of other predicting variables (such as altitude, phenology and proportion of fresh herbage in the cow diet) in the model for C18:1 *t*10 + *t*11. The 1st growing cycle was associated to higher Σ *de novo*

Table 4
Prediction models for milk fatty acid composition.

Fatty acid (g/kg TFA)	Variable	Coefficient (± SE)	Fisher's F	Intercept (± SE)	Lab error Fishers' F	RMSE	R^2
Σ *de novo* FA[c]	Pasture (% diet DM)	−0.49 (± 0.115)	18.3	243.4 (± 14.9)	16.35	17.4	0.62
	Forbs (%)	−0.0372 (± 0.0113)	10.8				
	CP (g/kg DM)	1.42 (± 0.70)	4.1				
	1 st growing cycle[a]		22.9	1.01 (± 0.21)			
C16:0	Pasture (% diet DM)	−0.638 (± 0.115)	30.9	352.7 (± 12.4)	39.6	14.3	0.70
	CP (g/kg DM)	−2.16 (± 0.57)	14.2				
	Altitude (m a.s.l.)	−0.00118 (± 0.00033)	12.9				
C18:0	aNDFom (g/kg DM)	0.561 (± 0.171)	10.7	65.1 (± 10.9)	5.47	10.0	0.35
	Phenology[c]	0.0145 (± 0.0061)	5.8				
	Pasture (% diet DM)	0.176 (± 0.079)	5.0				
	Altitude (m a.s.l.)	−0.000507 (± 0.000228)	5.0				
	Grasses (%)	−0.0126 (± 0.0064)	3.9				
	1 st growing cycle[a]		4.2	0.257 (± 0.125)			
C18:1 *t*10 + *t*11	Altitude (m a.s.l.)	0.000835 (± 0.000135)	38.5	21.0 (± 3.4)	6.92	06.0	0.56
	Phenology[b]	−0.0205 (± 0.0036)	31.9				
	Pasture^2 (% diet DM)^2	0.00155 (± 0.00029)	28.5				
	Grasses (%)	0.00805 (± 0.00376)	4.6				
	1 st growing cycle[a]		10.8	−0.232 (± 0.071)			
C18:1 *c*9	Grasses (%)	−0.0379 (± 0.0127)	8.9	191.7 (± 17.9)	2.8	11.4	0.26
	aNDFom (g/kg DM)	0.929 (± 0.334)	7.7				
C18:2 n6	Concentrates (% diet DM)	0.176 (± 0.027)	41.4	9.5 (± 1.9)	20.6	1.9	0.54
	Pasture (% diet DM)	0.0643 (± 0.014)	21.1				
	CP (g/kg DM)	−0.205 (± 0.070)	8.7				
C18:3 n3	Pasture (% diet DM)	0.0627 (± 0.0101)	38.3	6.9 (± 1.4)	59.6	1.7	0.74
	Legumes (%)	−0.00346 (± 0.00141)	6.0				
	CP (g/kg DM)	−0.163 (± 0.069)	5.6				
C18:2 *c*9*t*11[d]	Phenology[b]	−0.0127 (± 0.0017)	53.9	8.5 (± 1.6)	8.9	2.8	0.59
	Altitude (m a.s.l.)	0.000377 (± 0.000063)	35.9				
	Pasture^2 (% diet DM)^2	0.00062 (± 0.00014)	20.7				
	Grasses (%)	0.00719 (± 0.00178)	16.4				
	1 st growing cycle[a]		40.8	−0.223 (± 0.035)			
Σ ECFA[e]	Altitude (m a.s.l.)	−0.00236 (± 0.00058)	16.4	639.7 (± 16.2)	31.2	26.9	0.62
	Pasture (% diet DM)	−0.723 (± 0.209)	12.0				
	Phenology[b]	0.0403 (± 0.0159)	6.4				
	1 st growing cycle[a]		16.1	1.27 (± 0.32)[d]			
Σ OCFA[f]	Grasses (%)	0.00584 (± 0.00163)	12.8	31.1 (± 2.3)	4.3	2.7	0.23
	aNDFom (g/kg DM)	−0.105 (± 0.044)	5.8				
	1 st growing cycle[a]		8.2	−0.0821 (± 0.0287)			
Σ BCFA[g]	Pasture (% diet DM)	0.0582 (± 0.0162)	13.0	24.6 (± 4.3)	4.1	2.6	0.31
	CP (g/kg DM)	−0.416 (± 0.125)	11.1				
	Forbs (%)	−0.0039 (± 0.0017)	5.2				
	ADFom (g/kg DM)	0.183 (± 0.087)	4.5				
Σ MUFA[h]	Pasture (% diet DM)	0.634 (± 0.143)	19.5	222.8 (± 23.1)	2.6	22.1	0.33
	Grasses (%)	−0.0393 (± 0.0137)	8.3				
	aNDFom (g/kg DM)	1.078 (± 0.376)	8.2				
	Phenology[b]	−0.0251 (± 0.0127)	3.9				
	1 st growing cycle[a]		12.7	−0.936 (± 0.262)			
Σ PUFA[i]	Phenology[b]	−0.0192 (± 0.0039)	24.1	18.5 (± 6.2)	29.0	6.2	0.63
	Pasture (% diet DM)	0.216 (± 0.049)	19.2				
	ADFom (g/kg DM)	0.63 (± 0.177)	12.7				
	Altitude (m a.s.l.)	0.00045 (± 0.000137)	10.7				
C18:2 n6/C18:3 n3	Concentrates (% diet DM)	0.136 (± 0.037)	13.6	16.8 (± 1.8)	17.8	2.6	0.51
	Pasture (% diet DM)	−0.0551 (± 0.0187)	8.7				
	Phenology[b]	0.00299 (± 0.0015)	4.0				
Σ PUFA n6[j]/Σ PUFA n3[k]	Concentrates (% diet DM)	0.084 (± 0.0242)	12.0	13.6 (± 1.7)	12.1	1.7	0.44
	Pasture (% diet DM)	−0.0376 (± 0.0122)	9.4				
	CP (g/kg DM)	−0.0950 (± 0.0626)	2.3				

(*continued on next page*)

Table 4 (*continued*)

Fatty acid (g/kg TFA)	Variable	Coefficient (± SE)	Fisher's F	Intercept (± SE)	Lab error Fishers' F	RMSE	R^2
C18:1 *c*9/C16:0	Pasture (% diet DM)	0.0383 (± 0.0065)	34.3	3.55 (± 1.05)	18.3	1.1	0.61
	aNDFom (g/kg DM)	0.0523 (± 0.0172)	9.2				
	Grasses (%)	−0.00138 (± 0.00067)	4.2				

Abbreviations: ADFom, acid detergent fibre; aNDFom, neutral detergent fibre; BCFA, branched-chain fatty acids; CP, crude protein; DM, dry matter; ECFA, even-chain saturated fatty acids; FA, fatty acids; MUFA, monounsaturated fatty acids; OCFA, odd-chain saturated fatty acids; PUFA, polyunsaturated fatty acids; R^2 adjusted, coefficient of determination; RMSE, root mean square error; SE, standard error; TFA, total fatty acids.

[a] Correction of intercept according to the growing cycle; if growing cycle > 1st: the related additive constant coefficient changes from + to – or vice versa.

[b] Phenology BBCH scale (FBRCAF (Federal Biological Research Centre for Agriculture and Forestry), 2001): 20 = vegetative stage, beginning of tillering; 30 = beginning of stem elongation; 40 = beginning of booting; 50 = beginning of heading; 60 = beginning of flowering; 70 = beginning of fruit development; 80 = beginning of ripening; 90 = end of ripening.

[c] C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C14:0.

[d] Coeluted with C18:2 *t*7*c*9 and C18:2 *t*8*c*10.

[e] C4:0 + C6:0 + C8:0 + C10:0 + C12:0 + C14:0 + C16:0 + C18:0 + C20:0 + C22:0.

[f] C5:0 + C7:0 + (C15:0 + C14:1 *c*9) + C17:0.

[g] C13:0 *iso* + C14:0 *iso* + C15:0 *iso* + C15:0 *anteiso* + C16:0 *iso* + (C17:0 *iso* + C16:1 *t*9) + C17:0 *anteiso* + C18:0 *iso*.

[h] C10:1 *c*9 + C16:1 *c*9 + (C17:1 *c*9 + C18:0 *anteiso*) + C18:1 *t*5 + (C18:1 *t*6 + *t*7 + *t*8 + *t*9) + (C18:1 *t*10 + *t*11) + (C18:1 *t*12 + *t*13 + *t*14 + *c*6 + *c*7 + *c*8) + C18:1 *c*9 + C18:1 *c*11 + C18:1 *c*12 + (C18:1 *c*14 + *t*16) + C20:1 *c*9.

[i] (C18:2 *c*9*t*13 + *t*8*c*12) + (C18:2 *c*9*t*12 + *t*8*c*13 + *c*9*t*12) + (C18:2 *c*11*t*15 + *t*9*c*12) + C18:2 n6 + C18:3 n3 + (C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10) + (C18:2 *t*11*c*13 + *c*9*c*11 + C21:0) + C18:2 *t*9*t*11 + C20:2 n6 + C20:3 n6 + C20:4 n6 + C20:5 n3 + C22:5 n3.

j C18:2 n6 + C20:2 n6 + C20:3 n6 + C20:4 n6.

[k] (C18:2 *c*11*t*15 + *t*9*c*12) + C18:3 n3 + C20:5 n3 + C22:5 n3.

FA and Σ ECFA proportions, and to lower C18:1 *t*10 + *t*11 and C18:2 *c*9*t*11 proportions in milk.

4. Discussion

4.1. Dataset variability

4.1.1. Sites and grasslands characteristics

The studied grasslands were very diverse and covered a homogeneous gradient in terms of climatic conditions, altitude and botanical composition. The areas where the grasslands were located are characterised by different climates. They ranged from the continental climate typical of central France and the endalpic Alpine valleys to the sub-oceanic climate typical of the esalpic Alpine valleys up to the oceanic climate typical of the North Eastern regions of France. The grasslands at low altitude were dominated by grasses or much less frequently by legumes, while the increase of the altitude was related to the increase of contribution of forbs, in accordance with Collomb et al. (2002b) and Michaud et al. (2012). The wide range in phenology reflected the herbage sampling dates (May to November, with annual variations) and the different growing cycles at which the herbage was sampled. As a consequence, the herbage proximate composition and the total FA concentration were also very variable, and in both cases in agreement with absolute values and ranges reported in literature (Revello-Chion et al., 2011; Glasser et al., 2013; Elgersma, 2015). The sum of the five considered individual FA accounted for the 88 % of TFA, and the relative proportion of the individual FA was in line with values previously reported for fresh grass (Elgersma, 2015).

4.1.2. Farms characteristics and milk composition

The farming management practices were very diverse and covered a homogeneous gradient. Comparing the characteristics of our dataset to the average EU data describing dairy farming systems, it appears that both the dairy herd size and the milk yield per cow are lower [EU-28: 33 livestock units (European Commission, 2018) and about 18.5 kg/cow*day (Coppa et al., 2013)]. However, such a relatively small herd size and low milk yield per cow can be typically found in extensive farming systems (Mele et al., 2016; Coppa et al., 2019), in agreement also with a large variability of the reared breeds.

The milk gross composition was in line with values previously reported by other authors for the same breeds involved in our study (among others: Renna et al., 2014; Coppa et al., 2015b; Mele et al., 2016). The FA composition of bulk milk fat was in line with previous data from dairy cows fed high proportions of fresh forages (Ferlay et al., 2006; Coppa et al., 2015b).

4.2. Prediction models for herbage fatty acid composition

Our models demonstrated that, under real management conditions, herbage phenological stage, DM content and growing cycle had the highest effect in determining the FA profile of semi-natural and species-rich grasslands followed by botanical composition, altitude, CP and aNDFom.

We found a decrease of the TFA concentration with increasing the herbage phenological stage, in agreement with controlled studies (Wyss and Collomb, 2010; Glasser et al., 2013). Alpha-linolenic acid was also strongly reduced when phenology progressed (Clapham et al., 2005; Cabiddu et al., 2009; Glasser et al., 2013). When the herbage grew older, we found an increase in the proportions of C18:0, C18:1 *c*9 and C18:2 n6, also confirming the results obtained by other authors for C18:2 n6 (Cabiddu et al., 2009; Wyss and Collomb, 2010; Glasser et al., 2013). The lack of the effect of phenology on the concentration of C16:0 is in agreement with the findings of Cabiddu et al. (2009).

The herbage DM content also exerted an important role as predicting variable for the herbage FA composition. At a given phenological stage, the DM content of herbage is affected by the leaf-to-stem ratio (that can also vary depending on species; (Cabiddu et al., 2017). The stems are characterised by higher contents of DM and lower proportions of C18:3 n3 (Elgersma, 2015; Cabbidu et al., 2017), that is in agreement with the same effects exerted by DM on the FA considered in our study. Our models demonstrated that the herbage aNDFom content negatively affected the TFA content and the C18:3 n3 proportion of the grasslands, as previously reported for species-rich grasslands in the Western Italian Alps (Ravetto Enri et al., 2017). Our study also demonstrated that the TFA concentration of herbage was primarily related to the CP content. The explanatory role of CP for forage FA may be a consequence of their shared location in the photosynthetic organs of the plants (Glasser et al., 2013). However, the marginal or inconsistent predicting role of CP for most of the studied herbage FA, is in contrast with literature (Wyss and Collomb, 2010; Revello-Chion et al., 2011; Khan et al., 2012).

In our study, the botanical composition of the grasslands did not significantly affect the TFA concentration of herbage, in accordance with Glasser et al. (2013) and in disagreement with other studies comparing single species (Boufaïed et al., 2003; Clapham et al., 2005; Elgersma et al., 2013). However, when natural grasslands instead of single species are studied, the results are more contrasting. Gorlier et al. (2012) reported a significant negative correlation between the proportion of grasses and the C18:2 n6 proportion of pastures. Conversely, Peiretti et al. (2017) found that C18:0 positively correlated with grasses, while legumes were uncorrelated to the FA composition of pastures.

(Revello-Chion et al. 2011) reported a significant positive effect of altitude on the TFA concentration of grasslands, but when applying a multivariate approach, we did not find such a significant contribution. However, our study highlighted the relevant effect of the growing cycle on the herbage TFA content (low relative weight) and on the FA profile (medium to high relative weight). Our findings are in agreement with Dewhurst et al. (2001) who found that, in temperate regions, the TFA content and C18:3 n3 proportion are higher in first growing cycle than during the summer regrowths, because of the more favourable summer climate for the development of the C4 cycle annual grasses, which are known to be poorer in C18:3 n3 than the C3 grasses (Costa et al., 2019).

4.3. Prediction models for bulk milk fatty acid composition

The percentage of fresh herbage in the diet had an important effect on the proportion of almost all considered milk FA variables, as already reported by Coppa et al. (2013). Concentrate supplementation is able to affect the FA profile of milk from grazing cows significantly (Ferlay et al., 2006; Renna et al., 2010). However, we showed that when the concentrate supplementation is low, the milk proportion of main biohydrogenation intermediates of dietary PUFA (and in particular of the C18:2n-6 intermediates), such as C18:1 *t*10 + *t*11 and C18:2 *c*9*t*11, is not driven by the supplementation.

Out of the expected effect of fresh herbage proportion in cow diet, our models showed that the FA profile of milk fat was mainly driven by herbage phenology. Even if a predicting role of herbage phenology was expected in our farming conditions, its position as the main factor related to the herbage characteristics able to affect both herbage and milk FA is a novelty of our study. Coppa et al. (2015b) demonstrated that the higher is the proportion of fresh herbage in the diet, the higher is the number of milk FA significantly affected by phenology, but most of the published researches addressed herbage botanical composition as a driver for milk FA profile (Collomb et al., 2002b; Leiber et al., 2005; Gorlier et al., 2012). In accordance with literature data (Coppa et al., 2015b; Radonjic et al., 2019), milk Σ ECFA and the C18:2 n6/C18:3 n3 ratio increased while C18:1 *t*10 + *t*11, C18:2 *c*9*t*11 and Σ PUFA decreased at the increase of herbage phenology, the latest three dependent FA variables being the most sensitive to the effect of herbage phenology.

The increase of Σ PUFA and of biohydrogenation intermediates of C18:3 n3 in milk with increasing altitude, associated with the contemporary retention of altitude and herbage botanical composition in the models, could suggest a synergic effect of the C18:3 n3 increase in herbage and of changes in plant secondary metabolites (Collomb et al., 2002a; Leiber et al., 2005) due to variations in botanical composition along the altitudinal gradient (Gorlier et al., 2012; Manzocchi et al., 2019). Rumen microbiota can be impaired when cows ingest high concentrations of plant secondary metabolites (Leiber et al., 2005). However, the low relative weight of botanical composition (notably, the absence of forbs proportion as explanatory variable in all the models of biohydrogenation intermediates) suggests that, when operating at a large scale with an extended variability in semi-natural grasslands, the botanical composition only exerts a minor influence on the FA composition of milk if compared to herbage phenology and site altitude.

Among herbage proximate constituents, CP was found to be the most influencing one. However, in all cases, the relative predicting weight of this covariate was much lower than that of other predicting factors.

The effect of growing cycle on milk FA composition was in contrast with the expectation based on the effect on herbage FA profile. Higher herbage proportions of C18:3 n3 and Σ PUFA at the first grazing cycle did not correspond to higher C18:3 n3, Σ PUFA or their biohydrogenation intermediate proportions in milk. This result is not easy to interpret, but may depend on grazing selection by cows, that could be facilitated in avoiding stems on regrowth (Koczura et al., 2019), when the not reflowering grasses stay leafy.

4.4. Complementary role of the predicting variables

Even if trials conducted under real management conditions on commercial farms are not designed to investigate biological mechanistic functions, some hypotheses concerning the relevance of both herbage phenology and proximate composition in our herbage and milk FA prediction models can be put forward. An evolution in herbage phenology is known to consistently affect the herbage proximate composition (mainly CP and aNDFom) (Gorlier et al., 2012; Coppa et al., 2015b; Radonjic et al., 2019). This explains the relevance of proximate composition in driving the herbage FA profile found in literature (Gorlier et al., 2012; Radonjic et al., 2019). When considering several growing cycles, regrowth of not reflowering grasses can stay vegetative along the rest of the season, but their aNDFom and CP can evolve during time, resulting in different proximate compositions. Thus, when herbage phenology is measured and considered as a separate explanatory variable in the statistical models, as in our study, proximate composition noticeably lowers its relative predicting weight. Furthermore, the phenological stage measured on the main dominant grasses can be considered a valuable indicator (as shown by its relevance in our models), but it could not be exhaustive in describing the whole vegetal community in natural grasslands, as it is for monospecific grasslands. At a given phenological stage of dominant grasses, proximate composition can differ according to the exposure, slope and other site characteristics, or again to the leaf-to-stem ratio of the associated plants, which is known to be a relevant factor driving herbage FA composition (Elgersma et al., 2013; Elgersma, 2015; Cabiddu et al., 2017).

Altitude is known to affect the botanical composition of pastures (Cavallero et al., 2007). However, the most diffused vegetation communities (i.e. dominated by *Brachypodium rupestre, Nardus stricta, Festuca* gr. *rubra* or *Dactylis glomerata*) can be found in large altitudinal gradients (Cavallero et al., 2007). Altitude can also have an indirect influence on the FA content of forages, being related to the climate and the length of the growing season (Revello-Chion et al., 2011). Within the same vegetation community, altitude-related differences in light radiation and length of photoperiod at a similar phenological stage can increase plant growth rate, cell wall lignification and oxidative processes and can also change the content of plant secondary metabolites within the same species (Sangwan et al., 2001; Bovolenta et al., 2008; Khan et al., 2012).

Similar considerations can be done for bulk milk models, but with an added component that can in turn play a role: the ruminant. Several studies showed that cows' grazing selection of preferred species or plant parts (like leaves) or the exploitation of upper leafy of lower stem-rich layers can affect milk FA composition (Coppa et al., 2011, 2015a; Koczura et al., 2019). The grazing selection can thus increase the independence of apparently related herbage- or site-linked factors, like phenological stage and proximate or botanical composition and altitude. Additionally, altitude can also induce different responses in animal metabolism, i.e. inducing metabolic energy deficiency (Leiber et al., 2005). Consequently, phenology, proximate composition, botanical composition and altitude can be complementary in predicting herbage and milk FA proportions.

4.5. Practical implications for herd management for the improvement of milk FA composition

Out of the still known maximisation of fresh herbage proportion in cows' diet, the priority has to be given to an early grassland exploitation to improve both herbage and milk FA compositions. Herbage phenology, which is easy to measure on field by farmers can be the best indicator to promptly allow the cows to graze. The transhumant or upland farms appear to be advantaged compared to the lowland farms, as the utilisation of grasslands at an upper altitude also contributes to improve milk FA composition. Animal metabolism and grazing selection can concur to make herbage botanical and proximate compositions less relevant in determining milk FA composition.

5. Conclusions

This work demonstrated that it is possible to predict reliably the FA composition of semi-natural and species-rich permanent grasslands under real management conditions based on site altitude and grassland characteristics. Herbage phenology and proximate composition (mainly DM) weigh more than botanical composition in predicting herbage FA. Herbage phenology and site altitude play also a significant role in predicting the FA composition of grassland-derived bulk cow milk fat, while herbage proximate and botanical compositions only show a minor influence.

The provided predicting models could help stakeholders and farmers designing management strategies to improve the nutritional quality of the milk from their grazing herds.

CRediT authorship contribution statement

Manuela Renna: Writing - original draft, Conceptualization, Validation, Investigation. **Anne Ferlay:** Investigation, Resources, Project administration, Funding acquisition. **Carola Lussiana:** Investigation, Resources, Data curation, Conceptualization. **Didier Bany:** Resources, Data curation. **Benoit Graulet:** Investigation, Resources, Project administration, Funding acquisition. **Ueli Wyss:** Investigation, Resources. **Simone Ravetto Enri:** Investigation, Ressources, Data curation. **Luca Maria Battaglini:** Project administration, Funding acquisition. **Mauro Coppa:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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