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Original article

Influence of the nature of alpine pastures on plasmin activity, fatty acid and volatile compound composition of milk

Christophe BUGAUD[a], Solange BUCHIN[a*], Jean-Baptiste COULON[b], Agnès HAUWUY[c] , Didier DUPONT[a]

[a] Technologie et Analyses Laitières, INRA, BP 89, 39801 Poligny Cedex, France
[b] Unité de Recherches sur les Herbivores, INRA, Theix, 63122 Saint-Genès-Champanelle, France
[c] Groupement d'Intérêt Scientifique des Alpes du Nord, 11 rue Métropole, 73000 Chambéry, France

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Abstract — The influence of pastures on milk characteristics was studied in natura in the Abondance cheese area (Haute-Savoie, France) on 10 diversified pastures exploited by 3 dairy farmers. The effects of the herd were controlled and measured by feeding an identical hay to herds. The plasmin activity and the concentration of long-chain poly-unsaturated fatty acids in milks produced in mountain pastures (n = 5, 1 500 and 1 850 m) were significantly higher ($P < 0.001$ and $P < 0.05$ respectively) than in milks from valley pastures (n = 5, 850 and 1 100 m). The terpene composition of milk was linked to the terpene composition of pastures. Milks from the pastures rich in dicotyledons, in particular *Apiaceae*, contained a greater quantity and a wider variety of terpenes, than milks from the pastures rich in *Gramineae*. The other volatile compounds, casein composition, calcium and phosphorus contents did not seem to be influenced by the type of pasture.

pasture / milk / plasmin / fatty acid composition / volatile compound

Résumé — **Influence de la nature de pâturages alpins sur l'activité de la plasmine, la composition en acides gras et en composés volatils du lait.** L'influence de la nature des pâturages sur les caractéristiques des laits a été étudiée in natura dans la zone du fromage d'Abondance (Haute-Savoie, France) sur 10 types de pâturages divers exploités par 3 producteurs. Les effets liés au troupeau ont été contrôlés et mesurés sur une période où les 3 troupeaux recevaient la même alimentation. L'activité de la plasmine et la teneur en acides gras poly-insaturés à 18 carbones étaient plus élevées dans les laits des pâturages de montagne (n = 5, 1 500–1 850 m) que celles des laits de pâturages de vallée (n = 5, 850–1100 m) (respectivement $P < 0{,}001$, $P < 0{,}05$). La composition en terpènes des laits est

* Correspondence and reprints
Tel.: (33) 3 84 73 63 03; fax: (33) 3 84 37 37 81; e-mail: buchin@poligny.inra.fr

apparue dépendre de celle des pâturages. Les laits issus des pâturages les plus riches en dicotylédones, notamment en ombellifères, contenaient plus de terpènes, tant en quantité qu'en variété, que les laits issus des pâturages riches en graminées. Les compositions en molécules volatiles non terpéniques et en caséines, les teneurs en calcium et phosphore n'ont pas semblé être influencées par le type de pâturage.

pâturage / lait / plasmine / composition en acides gras / composé volatil

1. INTRODUCTION

An essential particularity of cheeses of "protected denomination of origin" (PDO) is the maintenance of the original milk characteristics, which allows the assertion of the specificity or originality of cheeses [17]. Thus, the milk production conditions, which present a high variability in such production systems, are of great importance for the quality of cheese. Among them, animal feeding and in particular the nature of the pastures are considered as a predominant factor by cheesemakers in PDO areas.

The characteristics of the pasture may influence the sensory quality of cheese mainly by way of modifications of milk characteristics. Firstly, there could be a direct transfer of volatile compounds from the forage to the cheese, via the milk, as has been suspected for terpenes [5, 34]. Recently, Viallon et al. [35] have shown that the terpene composition of cream is related to the presence of terpenes in dry forages. The presence of these components in the milk could be involved in milk and cheese flavour [24]. Secondly, the particularity of pastures could modify the composition of the milk, like fatty acid composition [10, 15], casein composition [15], plasmin activity [7] or salt content [15]. Then, these modifications in milk could influence the characteristics of cheese [14, 16, 23, 33].

Nevertheless, in most of these studies, the number of type of pastures was limited or the botanical composition was relatively monospecific. In addition, for some studies realised in natural conditions, no attempt was made to segregate the effect of feed from the effect of herd (breed, stage of lactation) or to distinguish which characteristics of the pastures (botanical composition, altitude, walking conditions, nutritional level) affect the milk composition.

The aim of this study was to establish the relationships between the characteristics of pastures and the milk components, in particular the components which are likely to affect the cheese quality. We have chosen to work under real herd management conditions with pastures in order to have a wide range of types of pastures, as diversified and different as possible, and located at various altitudes in the PDO Abondance cheese area (Haute-Savoie, France). Differences attributed to herds were estimated by feeding an identical hay to herds before the grazing period. The current work is part of a study which aims to understand how pastures influence the sensory and rheological properties of cheese.

2. MATERIALS AND METHODS

2.1. Experimental conditions

The experiment was conducted during spring and summer 1998 in three farms (X, Y, Z) where both milk and cheeses were produced. The producers used valley pastures (V), growing between 850 m and 1 100 m, or mountain pastures (M), located between 1 550 m and 1 850 m, in Abondance PDO area (Haute-Savoie, France). Before the grazing period, the three herds were fed with the same hay (H), harvested on a native mountain grassland

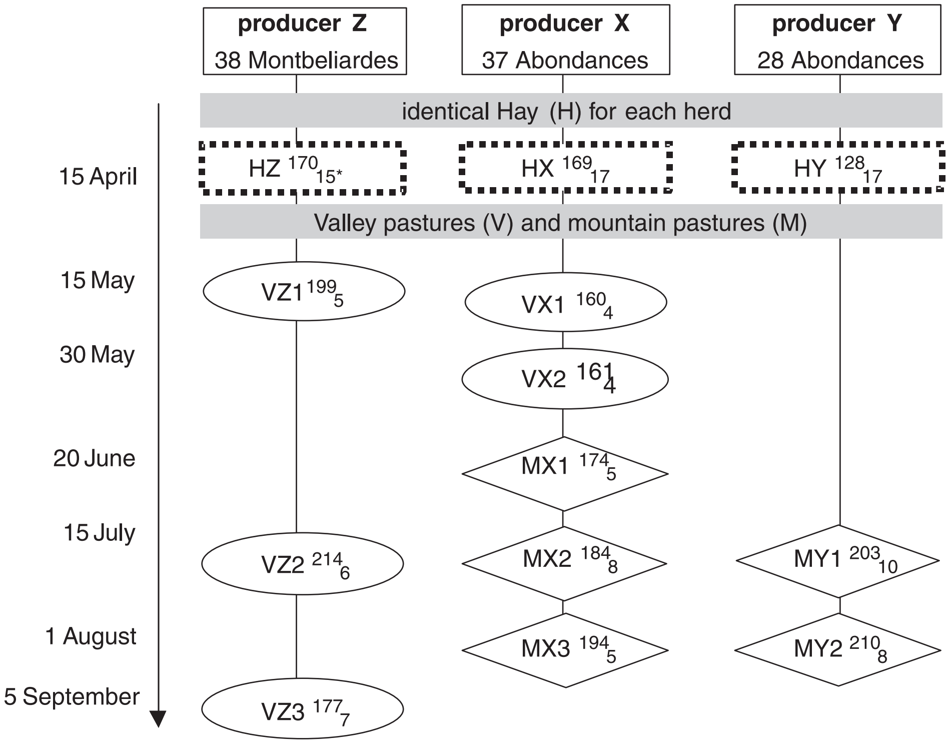

Figure 1. Experimental design. * HZ^a_b where ^{a} was the average stage of lactation and $_b$ the duration of grazing on pasture or feeding the hay.

located at 800 m in the Northern Alps (Evires, Haute-Savoie). Figure 1 presents the experimental design with the planning of grazing.

The botanical composition of the pastures (detailed in Bugaud et al. [8]) was determined by linear surveys, and the contributions of principal plant families in each pasture are given in Table I. The VZ pastures were the richest in *Gramineae* (50–68%), the VX pastures were rich in dicotyledons, in particular in *Apiaceae* and *Geraniaceae* (13–28%). M pastures presented the highest proportions of plants which were not grazed by the cows (*Ranunculaceae*, *Rosaceae*, *Polygonaceae*, *Ericaceae*, *Campanulaceae*). For the VZ3 diet, the cows had grazed a pasture, described in Table I, representing 40% of their total diet. The rest of the diet was composed of fresh grass (35%) from VZ2 pasture (third vegetable cycle) and fresh corn (25%) mixed in the barn.

The conditions of feeding were similar from one herd to another: the forage represented the main part of the diet and the supplementation varied from 0 to 3 kg of concentrate, for which the composition was similar (rolled cereals mix and cakes).

The breeds were Abondance for herds X and Y and Montbéliarde for herd Z. The size of the herds did not vary a lot during the experiment. There were small fluctuations of animals, due to calvings, dryings and mastitic infections.

Table 1. Contribution of principal plant families in the pastures.

Pastures	**VZ1**	**VZ2**	**VZ3**	**VX1**	**VX2**	**MX1**	**MX2**	**MX3**	**MY1**	**MY2**
Altitude (m)	850	850	850	1060	1020	1600	1550–1600	1600–1800	1700–1850	1700–1750
Number of vegetal species[1]	16	12	14	29	21	23	23	25	26	25
Contribution of principal plant families (expressed in % of the total counted vegetal species)										
Gramineae	61	50	68	26	31	42	40	32	33	27
Fabaceae	18	32	18	21	19	3	19	2	1	8
Apiaceae	0	0	0	21	8	2	3	3	3	13
Geraniaceae	0	0	0	7	5	2	3	2	0	2
Asteraceae	14	15	11	9	8	6	9	9	19	9
Plantaginaceae	1	0	0	0	0	0	3	1	7	0
Ranunculaceae[2 3]	5	2	2	5	8	4	2	8	2	10
Rosaceae[2]	1	1	1	3	9	6	11	12	7	9
Other plant families[2]	0	0	0	8	12	35[4]	10	31[5]	28[6]	22

[1] Mean by linear survey. [2] Plant family for which the most vegetal species are not grazed by cows. [3] Plant family for which the most vegetal species are toxic for cows. [4] *Polygonaceae* represented 20% of total vegetal species. [5] *Cyperaceae* and *Ericaceae* represented together 21% of total vegetal species. [6] *Ericaceae* and *Campanulaceae* represented together 16 % of total vegetal species.

The average stage of lactation of herds during grazing periods varied between 160 and 214 days (Fig. 1). During the mountain grazing periods and HY period, the cows were milked with a milking bucket, otherwise with a pipeline.

2.2. Milk sampling and storage

During the last three days of each period, milk samples were collected from the cheese vat before the addition of the starters. X and Z milk samples were composed of equal quantities of the evening milk stored for 12 h at 10–15 °C and of the morning milk. The Y milk sample contained exclusively the morning milk. For the measure of the plasmin (PLM) activity and plasminogen derived (PLG) activity, 50 $mL\cdot L^{-1}$ of thimerosal (Sigma) were added to the milk samples. For the determination of fatty acid, casein, volatile compound compositions, PLM and PLG activities, samples were stored for 9–10 h at 4 °C before freezing at –18 °C. For the total bacterial count (TBC), samples were stored between 0 and 12 h overtime at 4 °C. For the other analyses, a food preservative (bronopol, DF control System, 0.6 $mg\cdot mL^{-1}$) was added to milk samples, which were stored between 1 and 2 days at 4 °C.

2.3. Milk analyses

Protein and fat contents were assessed by infrared spectroscopy (Milkoscan; Foss Electric, DK-3400 Hilleröd, Denmark). Urea content was determined by the dimethylamino-4-benzaldehyde colorimetric method [32] and somatic cell count (SCC) using an automatic cell counter (Fossomatic 360; Foss Electric). TBC were conducted as recommended by the AFNOR [2]. Total calcium (Ca) content was measured with a complexometric method [27] and phosphorus (P) content by a colorimetric method [1]. PLM and PLG activities were determined using a modification of the method of Rollema et al. [30]. Milk samples (3 mL) were diluted with 1 mL 0.4 $mol\cdot L^{-1}$ sodium citrate solution, centrifuged for 20 min at 27 000 × g and 4 °C. Twenty microlitres of supernatant were transferred into two separate wells of a 96-wells microplate (Nunc F96 Maxisorp, Nunc Kamstrup, Roskilde, Denmark). Five microlitres of urokinase (Sanofi Winthrop, Gentilly, France), a plasminogen activator, was added to one of the two wells and the microplate was incubated for 15 min at 37 °C. Finally, 200 μL of a solution of D-valyl-L-leucyl-L-arginine-4-nitroanilide 0.6 $mmol\cdot L^{-1}$ (Serva, Coger, Paris, France) in a 160 $mmol\cdot L^{-1}$ Tris-HCl pH 7 buffer containing 40 $mmol\cdot L^{-1}$ KCl, 100 $mmol\cdot L^{-1}$ EDTA and 25 $mmol\cdot L^{-1}$ ε-amino caproic acid were added to each well. Plates were incubated at 37 °C and absorbance at 405 nm was determined every 30 min for 3 h. The rate of p-nitroaniline formation was calculated from the linear parts of the absorbance versus time curves. PLM activity was calculated directly from the well without urokinase whereas PLG activity was obtained by subtracting PLM activity from the total PLM + PLG activity of the well with urokinase. Results were expressed in arbitrary units ($dA_{405}\cdot dt^{-1} \times 10^4$). The casein composition was analysed by polyacrylamide gel electrophoresis using urea [3]. The surface of each peak was expressed as percentage of the total area. The fatty acid (FA) composition was analysed by gas chromatography [6]. Volatile compounds were extracted by a dynamic headspace technique (Purge and Trap LSC 3000; Tekmar, Cincinnati, OH) and analysed by gas chromatography (6890 Hewlett Packard, Les Ulis, France) coupled with mass spectrometry (MSD 5973 Hewlett Packard), as described by Buchin et al. [7] with the following modifications: 10 mL of milk sample were introduced into a 25 mL glass U container (5182-0849 Hewlett Packard) connected to the Purge and Trap system, the purge was operated at 40 ºC with helium as purge gas at 46 mL·L^{-1} flow rate for 40 min. The quantity of each volatile compound was recorded in arbitrary units of peak area of specific ions. The quantities of overall terpenes (mono- or sesqui-) were calculated as the sum of the quantities of each terpenes (mono- or sesqui-).

2.4. Statistical analyses

We have evaluated the differences of the proteolytic activity and FA composition between valley and mountain milks by an analysis of variance. The hay given to the three herds was the same, and we can thus suppose that the differences between the hay milks correspond to the differences between the three herds. To remove this herd effect, we have considered the ratios between mountain or valley values and hay values for each herd (M/H and V/H). The effect of the localisation of the pasture on the proteolytic activity and FA composition was studied on these ratios. The model of the analysis of variance (Version 6.12, SAS Institute Inc., 1996) was built with 1 factor with 2 levels: mountain vs. valley. For each level, 5 replicates were considered.

Correlation coefficients were calculated between: (1) the quantities of volatile compounds in milk and in pastures, (2) PLM activity in milk from pastures and the altitude, the stage of lactation, SCC and the proportions of casein, and (3) SCC and PLG activity or the pH.

3. RESULTS

3.1. General composition of the milks

Protein content of milk was similar from one herd to another and increased globally during the experiment (Fig. 2). At the same time, the milk yield had decreased. Fat content was lower in Y milks (32–38 g·kg^{-1}) and was higher in Z milks (38–42 g·kg^{-1}). Urea content was highest for VZ1 milks (0.52 g·L^{-1}), which provided from the youngest pasture. SCC was always below 300 000 mL^{-1} except for MX3 milks where it was about 400 000 mL^{-1}. The pH of milks varied between 6.64 and 6.78 and was positively correlated to SCC (R^2 = 0.38, $P < 0.001$). TBC remained low all over the experiment (generally below 10^4 cfu·L^{-1}). Ca and P contents were similar from one grazing period to another (respectively 1.20–1.25 g·kg^{-1}, 0.80–0.95 g·kg^{-1}) except for VZ3 milks (P content was 1.02 g·kg^{-1}).

3.2. Proteolytic activities and casein composition

PLM and PLG activities were 1.4 and 1.5 times higher in HZ milks (from the Montbéliarde breed) than in HX or HY milks (from the Abondance breed) (Fig. 3).

The PLM activity was between 4.0 and 5.2 $dA_{405}·dt^{-1} \times 10^4$ in M milks, while it was between 1.6 and 4.1 $dA_{405}·dt^{-1} \times 10^4$ in H and V milks. The analysis of variance performed on M and V milks data, corrected by the effect of the herd (ratios M/H and V/H calculated with the hay milk data of the same producer), showed significant differences ($P < 0.001$). The PLM activity was significantly correlated with altitude ($R^2 = 0.91$, $P < 0.001$) but not with the stage of lactation and SCC ($P > 0.05$).

The PLG activity did not vary as the PLM activity did (Fig. 3). There was no significant difference between the ratios V/H and M/H. The greatest differences in PLG activity were observed for Z milks. The PLG activity was not significantly correlated to SCC.

The casein composition in milk did not differ from one pasture to another (Fig. 4). Neither β-casein nor γ+κ-caseins were significantly correlated to PLM activity. The Y milks differed from the others by a high frequency of the C variant for β-casein.

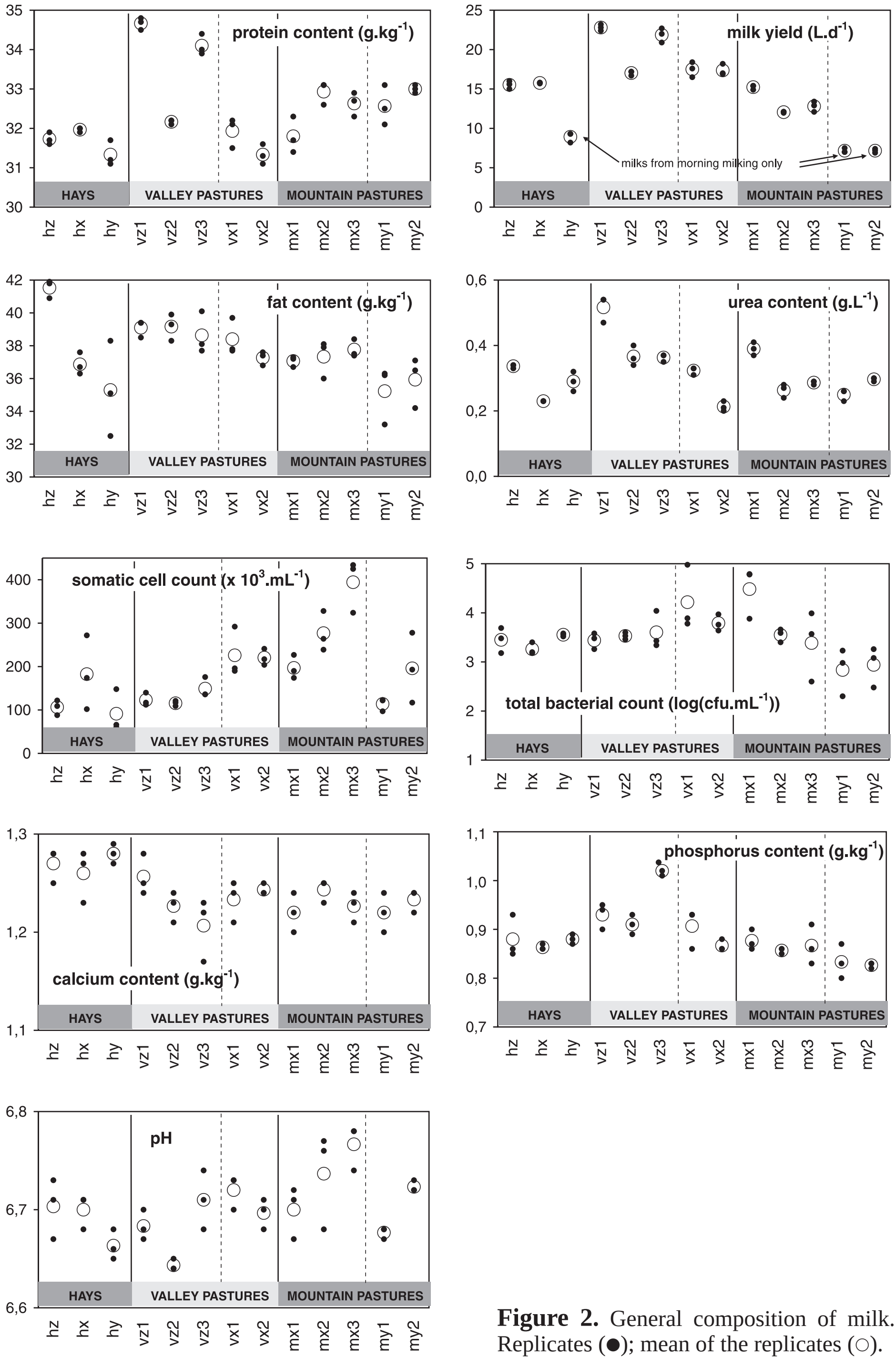

Figure 2. General composition of milk. Replicates (●); mean of the replicates (○).

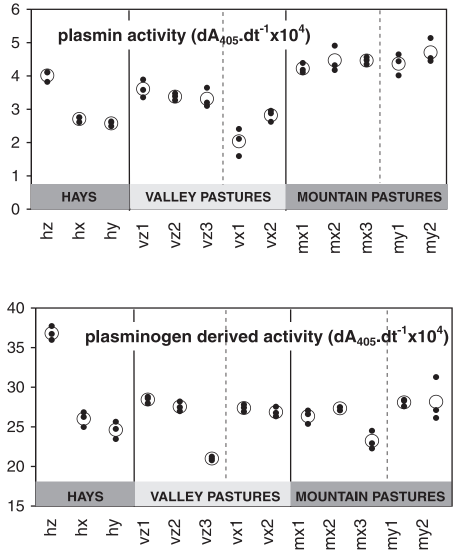

Figure 3. Plasmin and plasminogen-derived activities of milk. Replicates (●); mean of the replicates (○).

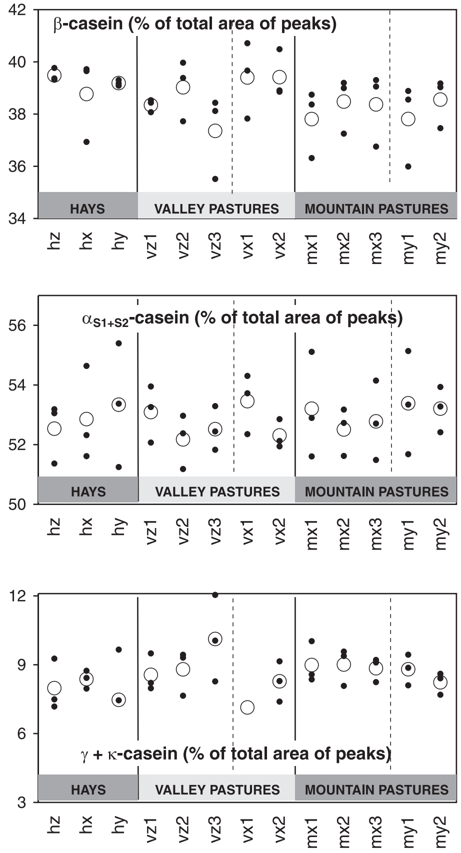

Figure 4. Casein composition of milk. Replicates (●); mean of the replicates (○).

3.3. Fatty acid composition

FA composition in H milks was different from one herd to another (Fig. 5). HY milks had the highest proportion of C18 mono-unsaturated fatty acids (C18:1) and the lowest proportion of palmitic acid (C16:0) and short-chain FA (sum of C4:0 to C14:0). For each herd, the proportions of C18:1 and C18 poly-unsaturated fatty acids (PUFA = C18:2 + C18:3) were higher in milks from pastures (V and M) than H milks. The transition from hay to grazing (HZ to VZ1 and HX to VX1) was accompanied by great increases in the proportions of PUFA (+58% and +31% respectively) and C18:1 (+30% and +25% respectively).

The proportions of C18:1 and PUFA were higher in M milks (respectively 25.7–29.7 $g \cdot g^{-1}$ and 6.3–9.1 $g \cdot g^{-1}$) than in V milks (20.4–25.8 $g \cdot g^{-1}$ and 4.5–5.6 $g \cdot g^{-1}$). In consequence, C16:0 and short chain FA were lower in M milks (respectively 23.6–25.6 $g \cdot g^{-1}$ and 19.2–24.3 $g \cdot g^{-1}$) than V milks (25.6–29.2 $g \cdot g^{-1}$ and 23.2–30.4 $g \cdot g^{-1}$). The analysis of variance showed significant differences between the ratios M/H and V/H for the proportions of PUFA and short-chain FA ($P < 0.05$). On the contrary, no significant differences were observed for the proportions of C18:1 and C16:0.

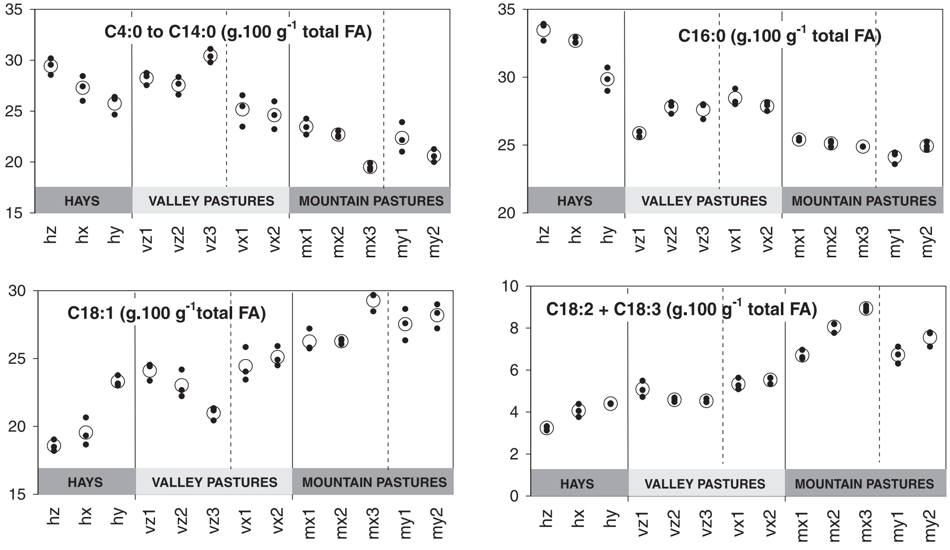

Figure 5. Fatty acid composition of milk. Replicates (●); mean of the replicates (○).

The proportion of C18:1 and PUFA regularly increased in X milks, from valley to mountain pastures. The proportion of PUFA and C18:1 in MX2 and MX3 milks were respectively 50% and 20% higher than in VX milks. The proportions of C18:1 in VZ milks decreased throughout the experiment.

3.4. Volatile compound composition

Overall, 262 volatile compounds were detected in milks. They belonged to 13 chemical families presented in Table II.

Their quantities, excepted for terpenes, were similar in all the milks and not correlated to pastures. VZ milks were approximately 5% less rich in volatile compounds than all the other milks.

The quantities of 12 of the 30 monoterpenes identified in milk (terpinolene, tricyclene, β-pinene, α-fenchene, camphene, limonene, α-terpinene, α-pinene, β-myrcene, α-phellandrene, p-cimene, γ-terpinene) were significantly correlated with those of monoterpenes in pastures ($0.64 < R^2 < 0.95$, $P < 0.01$). As a consequence, the quantity of overall monoterpenes in milk increased linearly with that in forages (Fig. 6). M milks had the highest and H milks the lowest quantities in monoterpenes. V milks presented a wide diversity of quantities: VX

Table II. Number of volatile compounds detected in milk for each chemical family.

Chemical families of volatile compounds	Number
Monoterpenes	30
Sesquiterpenes	35
Saturated hydrocarbons	18
Unsaturated hydrocarbons	31
Benzene derivatives	20
Furanes	13
Ketones	31
Aldehydes	33
Esters	15
Alcohols	17
Ethers	3
Sulphur compounds	3
Chlorinated compounds	13

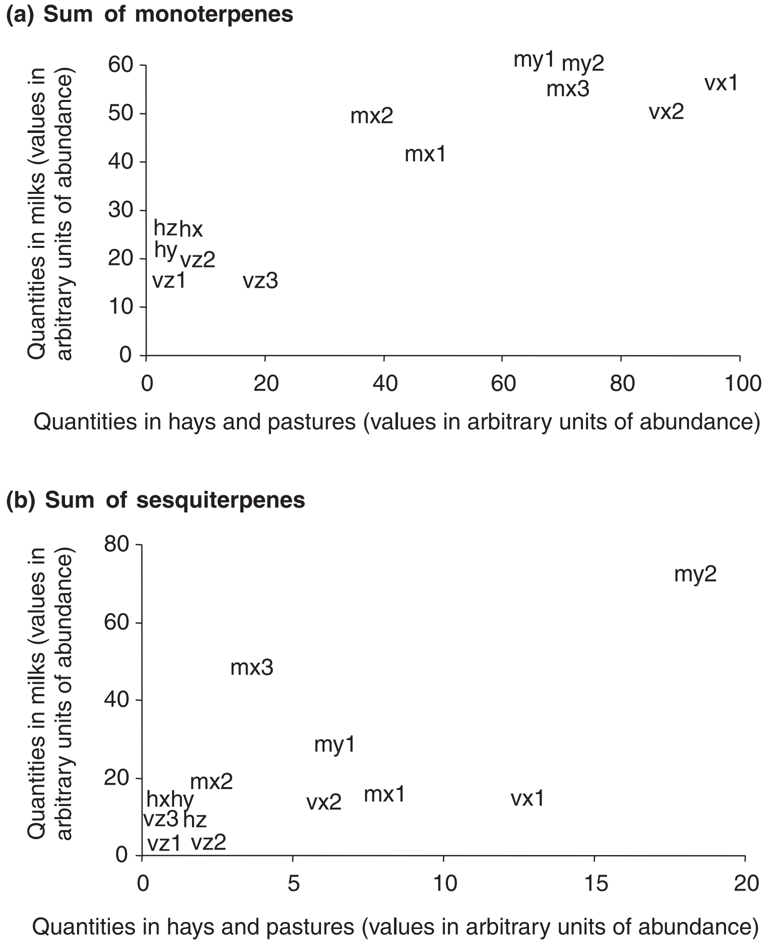

Figure 6. Overall terpene quantity: relationships between milks and forages (hays and pastures). Monoterpenes (a); sesquiterpenes (b).

milks were as highly rich in monoterpenes as M milks and VZ milks were as poor in these compounds as H milks.

Ocimene, isoterpinolene and alloocimene were quantified in pastures but not in milks. Alpha-myrcene was quantified in milks and pastures, but with no significant correlation.

Only 2 of the 35 sesquiterpenes quantified in milks were clearly identified. Their quantity in milks was significantly correlated (for clovene $R^2 = 0.85$, $P < 0.001$; for α-cedrene $R^2 = 0.53$, $P < 0.05$) to their quantity in forages. Nevertheless, the quantity of overall sesquiterpenes in milks was linked to the quantities of sesquiterpenes in the forages (Fig. 6). Neither VZ and H forages nor their corresponding milks contained any sesquiterpenes. MY2 milk, as well as pasture, were the richest in sesquiterpenes. On the contrary, the quantities of sesquiterpenes in MX3 and VX1 milks were not related to those in the corresponding pastures.

4. DISCUSSION

In order to get a wide variety of pastures, we had to experiment in semi-controlled conditions. In particular, the characteristics of the 3 herds (breed, stage of lactation) were different but we have measured the differences of composition of milk between them in a special period when they were fed the same hay. The differences we thus observed were attributed to a herd effect. In particular, the variations of PLM, PLG activities and FA composition were mainly due to breed, as previously observed by Richardson [29], Shaar [31] on PLM activity and by Palmquist and Beaulieu [26] on milk fat. We have assumed that these effects did not interact with feed and remained steady throughout the experiment.

4.1. Effect of valley / mountain pastures on plasmin activity of milk

The greatest effect observed on PLM activity of milk was the localisation of the pasture, in the valley or on the mountain.

In the mountain pastures, a higher altitude is associated with a reduction of the *Gramineae* and an increase of other vegetal species, which cows do not particularly graze (*Rosaceae*, *Renonculaceae*, *Cyperaceae*). As hypothesized by Buchin et al. [7], the presence of such plants may contribute to a higher plasmin concentration in milk, by way of modifications during blood transfer. Because of area, topography and botanical heterogeneity of the mountain pastures, cows expend a great deal of energy for finding their food. Extreme walking conditions increase the permeability of the mammary gland membrane for SCC and blood proteins [11]. In our study, the extreme walking conditions on mountain pastures did not induce a great increase of SCC, except probably for MX3 (> 400 000 cells·mL^{-1}). In this case, SCC could be a cause of the higher PLM activity [21]. As with the walking conditions, the altitude (less oxygen, lower atmospheric pressure and lower temperatures) could also be a cause but no work has been carried out to study this effect.

Neither a bad sanitary state, nor a feed restriction in mountain pastures could be suspected of influencing the PLM activity. Firstly, the sanitary state of the herds was good throughout all the experiment. Secondly, the decrease of the concentrate supplementation throughout the experiment, and in particular between valley and mountain pastures, was too slight (< 1 kg·d^{-1}) to affect PLM activity [20, 25]. The stage of lactation between the 5th and 7th month was known not to highly influence PLM activity [4, 28]. In our experiment, it was not correlated to PLM activity, but this factor added to the others previously discussed could amplify differences of PLM activity in milk between valley and mountain pastures.

4.2. Effect of valley / mountain pastures on fatty acid composition of milk

The environmental conditions of grazing, which are less favourable for cows on mountain pastures, could explain the higher proportion of long-chain unsaturated FA in M milks. The decrease in temperature (Chilliard Y., personal communication) or more walking (unpublished results) may induce an increase in the proportion of oleic acid in milk due to a lipid mobilisation. The higher proportion of C18:1 in MX3 milks could be explained by the long walk (more than an hour) required for the cows to reach the pastures. The milk yield in mountain pastures was lower than in the valley, probably due to a decrease in the quantity of feed ingested by cows in mountain pastures. The decrease in feed intake might induce the decrease in precursors of de novo FA synthesis and thus the decrease in short– and medium-chain FA secretion [13]. This could explain the higher proportion of long-chain unsaturated FA in M milks.

Even if the higher proportion of PUFA in M milks confirms the results of Collomb et al. [10], it is difficult to explain this effect only by the botanical composition of pastures. Following analyses of the FA composition in the grass samples, no clear relationship between the proportions of FA in the milks and the pastures was observed [9]. Neither the proportions of linolenic acid, nor of linoleic acid in grass samples were significantly correlated to PUFA in milks. The higher proportion of PUFA in milks from mountain pastures may be more related to a lower rate of ruminal biohydrogenation than the FA composition in the grass. The effect of the stage of lactation is unlikely because its variation between the 5th and the 7th was too small to affect the FA composition [12].

4.3. Effect of botanical composition of pastures on the composition of volatile compounds in milk

The relationship between the terpenes in milks, by both their nature and their quantity, and the terpenes in the pastures confirms the results found by Viallon et al. [35] who demonstrated the direct transfer of terpenes from forage to milk. As observed by Bugaud et al. [8] and Mariaca et al. [22], the quantity of terpenes in the pastures depends on the botanical composition of these pastures. The pastures rich in *Gramineae* were the poorest in terpenes and the pastures rich in dicotyledons such as *Apiaceae*, *Asteraceae* or *Plantaginaceae* were the richest in terpenes. That notably explains the differences of concentrations of terpenes in milks between the valley pastures, X and Z. The poor relationship of the concentrations of terpenes, and especially of sesquiterpenes, between pastures and milks of VX1 and MX3 might be due to the difficulty in collecting and analysing representative samples of the grass eaten by the cows, because of the heterogeneity of the pastures.

None of the oxygenated monoterpenes quantified in pasture samples [8] was found in milks because of their probable degradation by the microorganisms in the rumen of cows [35].

The higher concentrations of terpenes, mainly sesquiterpenes, in milks than in pastures were linked to the analytical method. Because forages were more concentrated in volatile compounds than milks, the extraction and quantification conditions (purge time and temperature, detection threshold) were milder in forages.

The absence of correlations between milks and pastures for the other volatile compounds suggests that these compounds were degraded by the metabolism of cows or produced by milk microorganisms. Among the few studies about the transfer, from feed to milk, of this type of compound [18, 19], only Honkanen et al. [18] found a direct transfer of alcohols and esters from rumen to milk. But, this transfer was not quantitative. According to Keen and Wilson [19], the aldehydes and alcohols found in the milk are produced by the grass enzymes during the mastication.

5. CONCLUSION

This study has shown the effects of the nature and the related environmental parameters of pastures on the proteolytic activity, the fatty acid and volatile compound composition in milk. Some components could be directly provided from pasture, such as terpenes, while other components, such as plasmin activity and fatty acid composition, could be indirectly affected by the pasture and its environment.

In further articles, we will study how the effect of pasture on milk is expressed by the characteristics of cheese.

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