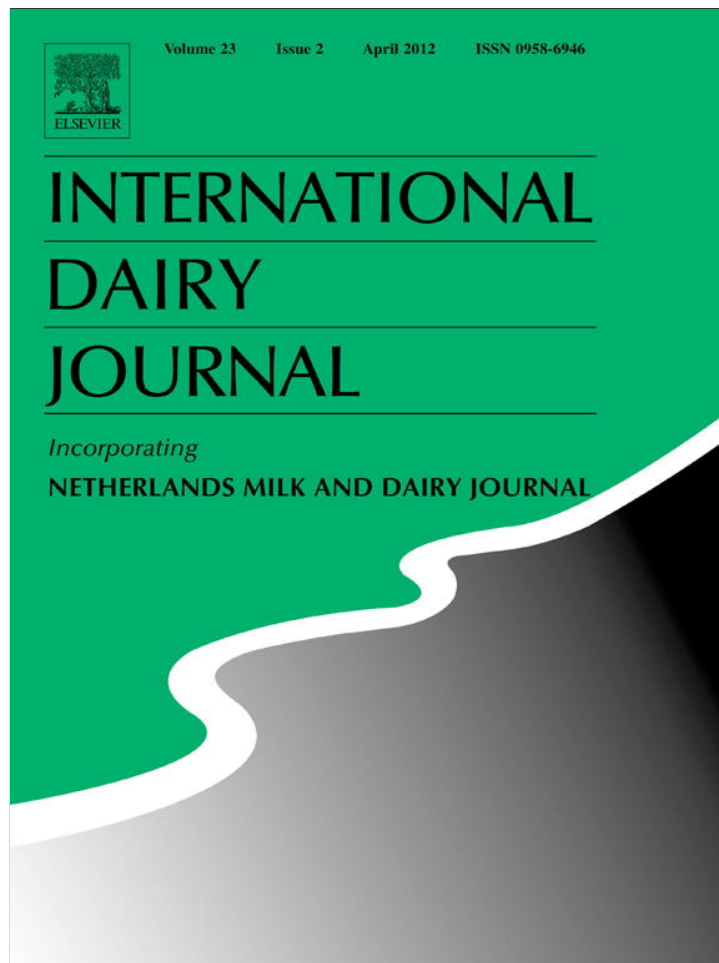


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H, C, N and O stable isotope characteristics of alpine forage, milk and cheese

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ABSTRACT

H, C, N, O stable isotope ratios of forage, milk and the corresponding cheese were analysed to define the isotopic characteristics of two typical mountain cheeses produced in two different terroirs with different types of vegetation. $\delta^{13}\text{C}$ was shown to be mainly influenced by the presence of C4 plants in animal diet and, in the case of C3 plant-based forage it was below -23‰ , varying according to the site and herbage type. $\delta^{15}\text{N}$ is more related to vegetation type and confirmed to be lower than $+4\text{‰}$ for alpine products. The altitude of the origin area influences $\delta^{18}\text{O}$ and $\delta^2\text{H}$. The isotopic values of milk and cheese casein were not significantly different, except $\delta^{18}\text{O}$, while they were both significantly different from the values of forage, except $\delta^2\text{H}$. Using the combined stable isotope ratios a good discrimination level between the products from two different types of pasture at two mountain sites was achieved.

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1. Introduction

The stable isotope ratios of H, C, N, and O in animals and animal products such as milk and cheese reflect those in the diet and drinking water (Camin, Perini, Colombari, Bontempo, & Versini, 2008; Chesson, Valenzuela, O'Grady, Cerling, & Ehleringer, 2010; DeNiro & Epstein, 1978, 1981; Perini, Camin, Bontempo, Rossmann, & Piasentier, 2009), changing according to the tissue or class of compounds considered (e.g., lipids) (Knobbe et al., 2006; McCutchan, Lewis, Kendall, & McGrath, 2003; Metges, Kempe, & Schmidt, 1990). As the isotopic ratios of diet constituents and water are related to local environment, isotope ratio analysis has recently been used to discriminate between milk and cheese produced in different areas, with different dietary regimes and production systems (organic or conventional) (Brescia, Monfreda, Buccolieri, & Carrino, 2005; Bontempo et al., 2011; Camin et al., 2004; Chesson et al., 2010; Crittenden et al., 2007; Engel et al., 2007; Kornexl, Werner, Rossmann, & Schmidt, 1997; Manca et al., 2001, 2006; Molkentin, 2009; Molkentin & Giesemann, 2007; Pillonel et al., 2003; Renou et al., 2004).

The $^{13}\text{C}/^{12}\text{C}$ isotope ratio (expressed as $\delta^{13}\text{C}$) of milk and cheese components is related to the quantity of maize, which is an important constituent of cows' diet in many countries, especially in intensive breeding systems, but is often also used for energy supplementation in other dairy systems. Maize is a C4 plant with higher ^{13}C content

($\delta^{13}\text{C}$: $-12 \div -14\text{‰}$) than C3 plants ($\delta^{13}\text{C}$: $-23 \div -30\text{‰}$) (Camin et al., 2004, 2008; Crittenden et al., 2007; Knobbe et al., 2006; Kornexl et al., 1997; Masud, Vallet, & Martin, 1999; Metges et al., 1990; Minson, Ludlow, & Troughton, 1975; Molkentin, 2009; Molkentin & Giesemann, 2007; 2010; Sacco et al., 2009). Each 10% increase in maize content in the diet corresponds to a 0.7–1.0‰ increase in the $\delta^{13}\text{C}$ of casein and a $\delta^{13}\text{C}$ maximum threshold value of -23.5‰ is suggested for milk casein, above which the presence of maize in the diet cannot be excluded (Camin et al., 2008).

The $^{15}\text{N}/^{14}\text{N}$ ratio (expressed as $\delta^{15}\text{N}$) of milk and cheese reflects agricultural conditions, e.g., different fertilisation practices, which vary between regions, closeness to the sea, drought in the area and the presence of leguminous species in the diet (Camin et al., 2004; Crittenden et al., 2007; Knobbe et al., 2006; Kornexl et al., 1997; Manca et al., 2001; Molkentin & Giesemann, 2010; Pillonel et al., 2003). Mountain products from grass-fed cows show $\delta^{15}\text{N}$ values lower than 4‰ (Bontempo et al., 2011; Camin et al., 2004; Pillonel et al., 2003).

The $\delta^{18}\text{O}$ value of milk water records the isotopic composition of drinking water and water intake from fresh forage, with minor deviations due to the contribution of food and atmosphere oxygen to body water (Chesson et al., 2010). As the ^{18}O content of local water is related to the climatic and geographical features of an area (temperature, humidity, rainfall, distance from the sea, altitude, latitude), milk water $\delta^{18}\text{O}$ can distinguish between milk produced in different areas (Chesson et al., 2010; Crittenden et al., 2007; Engel et al., 2007). When cows eat fresh herbage containing water enriched in ^{18}O due to evapo-transpiration, milk $\delta^{18}\text{O}$ increases considerably (Kornexl et al., 1997; Renou et al., 2004).

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The $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of dairy casein are related to the $\delta^{18}\text{O}$ of milk water (Camin et al., 2008) and they can therefore be used to discriminate between regions (Pillonel et al., 2003), as also observed for meat proteins (Camin et al., 2007; Heaton, Kelly, Hoogewerff, & Woolfe, 2008; Perini et al., 2009).

In recent years the knowledge about production chain processes has assumed a growing importance as a working basis to increase the value of local products both in terms of their qualification and traceability. Nevertheless, concerning mountain dairy products in particular, scientific research has often focused on specific segments of the production chain. This study offers an analysis of all the components involved in the whole production chain (from vegetation to milk and the relevant cheese), to investigate the relationship between the alpine environment, available vegetation types and the isotopic ratios of milk and dairy products. With this scope, two typical mountain cheeses (“Nostrale d’alpeggio” and “Asiago d’alvevo”) produced in two terroirs in the south-west and south-east of the Alps were investigated, considering milk and the cheese from cows grazing on different vegetation types (a terroir is a mixture of the natural environment – mainly expressed through the vegetation – farming practices and human know-how, which may lead to the production of typical cheese: Barham, 2003). This paper focuses on H, C, N, and O isotope ratios in animal forage and in milk and cheese casein and on the $^{18}\text{O}/^{16}\text{O}$ of milk water. To our knowledge, neither modification of the stable isotope ratios taking place in the process from milk to the corresponding cheese nor the impact of vegetation type in a narrow area on stable isotope ratios has been thoroughly investigated.

2. Material and methods

2.1. Sampling

The experiment was carried out at two sites: (i) Alpe Valcavera (AV, Cuneo district; latitude $44^{\circ}22'54''$ and longitude $7^{\circ}6'43''$, altitude: 1900–2500 m asl), located in the south-west of the Italian Alps; (ii) Malga Dosso di Sotto (MDS, Vicenza district; latitude $45^{\circ}57'44''$ and longitude $11^{\circ}24'10''$, altitude: 1647 m asl), located in the south-east of the Italian Alps. At each site two herds of 6–8 dairy cows (H1, H2) at late lactation adapted to alpine grazing environments grazed on two vegetation types. The breed of the cows was Italian Red Pied and Tarantaise in AV and Brown Swiss in MDS. Studies of the literature on beef, lamb and pig (as examples: Camin et al., 2007; Harrison et al., 2011; Horacek & Min, 2010; Knobbe et al., 2006; Molkentin, 2009; Molkentin & Giesemann, 2007, 2010; Tuross, Warinner, Kirsanow, & Kester, 2008) did not highlight a great influence of breed on the variability of stable isotope ratios of bioelements. At the AV site (details on AV vegetation are reported in Falchero et al., 2010) they fed on an oligotrophic type mainly made up of *Trifolium alpinum*, *Nardus stricta*, and *Carex sempervirens* (AV-T), and a mesophilous type with *Festuca gr. rubra*, *Alchemilla xanthochlora*, and *Phleum alpinum* (AV-F). At the MDS site (details on MDS vegetation are reported in Lombardi, Cavallero, & Bassignana, 2011) they fed on a mesophilous mesotrophic type with *Agrostis tenuis*, *Achillea millefolium*, *Trifolium repens* (MDS-A), and a mesophilous oligotrophic type with *Festuca gr. rubra*, *A. tenuis*, *Potentilla crantzii* (MDS-F). Two homogeneous areas for each vegetation type were fenced in and used by H1 and H2 following an AB/BA cross-over experimental design at each site (Kenward & Jones, 2003). The vegetation samples were collected on the first day of the adaptation (day 1 and 13) and test (day 7 and 19) periods. The herds grazed in one vegetation area (H1 in AV-T and MDS-A; H2 in AV-F and MDS-F), milk being collected for 6 days (days 7–12) after an adaptation period of 6 days (days 1–6). Subsequently the two herds were swapped over (H1 in AV-F and

MDS-F and H2 in AV-T and MDS-A) and milk was again collected (days 19–24) after 6 days of adaptation (days 13–18). The cows grazed for full days and received no supplements except mineral supplements in AV, whereas in MDS they received supplements based on maize (around 25% of dry feed matter), which were not collected and analysed in this study. Milk samples were taken daily by combining the milk from evening and morning milkings.

For each treatment, 32 forage samples, 96 milk samples and the corresponding cheese following 3-month ripening (96 samples) were collected during the summers of 2007 and 2008. In AV, the location selected for vegetation T in 2008 was at a lower altitude than in 2007, due to late vegetation growth at higher altitude.

The two cheeses were cooked curd cheeses: “Nostrale d’alpeggio” from Alpe Valcavera and “Asiago d’alvevo” from Malga Dosso di Sotto.

2.2. Methods

The forage was ground with 1093 Cyclotec Sample Mill (screen 1 mm, Foss, Padua, Italy). Milk and cheese were stored at -20°C until analyses. Fat was removed from 100 mL of milk by centrifugation (ALC PK 131R, Thermo Electron Corporation, Karlsruhe, Germany; $3712 \times g$, 10 min) and casein was precipitated by acidification at pH 4.3 using 2 M HCl. After centrifugation, the precipitate was rinsed with water and extracted once with acetone and three times with a mixture of petrol ether:ethyl ether (2:1) using a homogeniser (Ultraturrax[®], model X-620, Staufen, Germany; $14,635 \times g$, 3 min) for extraction and a centrifuge ($3082 \times g$, 6 min) for separation of the solvent. The residue made of casein was lyophilised and conserved at room temperature until analysis. Four grams of cheese were extracted three times with 30 mL of the petrol ether:ethyl ether (2:1) mixture, by homogenising with the Ultraturrax device, using the centrifuge to separate the ether from the residue. The defatted cheese was washed with 20 mL of deionised water using a centrifuge ($3082 \times g$, 3 min) to separate the water. The residue made of casein was lyophilised and conserved at room temperature until analysis.

The $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios in forage (≈ 1.5 mg) and casein (≈ 0.5 mg) were measured simultaneously using an Isotope Ratio Mass Spectrometer (IRMS) (DELTA V, Thermo Scientific, Bremen, Germany) following total combustion in an Elemental Analyser (EA Flash 1112, Thermo Scientific), according to the procedures described in previous publications (Camin et al., 2004, 2008). The $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ ratios in both forage (≈ 0.8 mg) and casein (≈ 0.5 mg) were measured using an IRMS (DELTA XP, Thermo Scientific) coupled with a Pyrolyser (high Temperature Conversion Elemental Analyzer, TC/EA, Thermo Scientific), following the method described elsewhere for meat protein (Perini et al., 2009). The $^{18}\text{O}/^{16}\text{O}$ in milk water was measured in 2 mL of bulk milk after equilibration with CO_2 using an ISOPREP 18 (VG Isotech, Middlewich, UK) on-line preparation system that allows $\text{CO}_2/\text{H}_2\text{O}$ equilibration, interfaced to an IRMS (SIRA II, VG Isogas, Middlewich, UK) according to the water equilibration method described for wine (OIV, 2009).

The values were expressed in $\delta_{\text{‰}}$ ($=[(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 1000$, where R is the ratio between the heavier isotope and the lighter one) against international standards (Vienna-Pee Dee Belemnite (V-PDB) for $\delta^{13}\text{C}$, Air for $\delta^{15}\text{N}$, Vienna-Standard Mean Ocean Water (V-SMOW) for $\delta^2\text{H}$ and $\delta^{18}\text{O}$). For the calculation of $\delta_{\text{‰}}$, casein and water working in-house standards were used to calibrate against international reference materials: L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA) for $^{13}\text{C}/^{12}\text{C}$; L-glutamic acid USGS 40 for $^{15}\text{N}/^{14}\text{N}$; V-SMOW (IAEA) for $^{18}\text{O}/^{16}\text{O}$ in water and benzoic acid (IAEA-601) for casein. The $^2\text{H}/^1\text{H}$ values were corrected against the same casein standard with an assigned

value of $\delta^2\text{H}$, according to the 'comparative equilibration technique' (Wassenaar & Hobson, 2003).

The uncertainty (2σ) of measurements was $<0.3\text{‰}$ for the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ analysis, $<3\text{‰}$ for $\delta^2\text{H}$, $<0.2\text{‰}$ and 0.6‰ for $\delta^{18}\text{O}$ in milk water and casein respectively.

2.3. Statistical analysis

The data were analysed using Statistica v 8 (StatSoft Italia srl, Padua, Italy) and SPSS for Windows, Rel. 17.0.0.2008 (SPSS Inc, Chicago, US).

Within the group, normal distribution of data was checked using the Kolmogorov–Smirnov test. Both parametric and non-parametric tests were used: General Linear Models (GLM) and Unequal N Tukey HSD (Honestly Significantly Different) to identify statistical differences between the groups, Pearson test to check the significance of correlations; Kruskal–Wallis and multiple bilateral tests, Spearman test. To limit redundancy the results of non-parametric tests are not reported as they are not different from the ones of the parametric tests.

To check if the combined isotopic parameters could be used to distinguish between milk and cheese from the four different pasture types and the two mountain areas, we carried out a multivariate canonical discriminant analysis. Discrimination robustness was tested using the following cross-validation procedure: a subset of the analysed samples marked as 'unknown' was classified using a model built on the basis of the remaining cases (Camin et al., 2010). Specifically, eight different sets of samples

(three in each group) were removed from the dataset and subsequently the model was calculated each time using the remaining cases to check whether excluded samples were assigned to the correct group.

3. Results and discussion

3.1. Stable isotope composition of forage, milk and cheese

In Table 1 the mean and standard deviation of the isotopic parameters of forage, milk and cheese samples are shown, grouped by site and vegetation type.

The Kolmogorov–Smirnov test found that the data were, in most cases, normally distributed within the groups. As parametric and non-parametric tests generally gave the same results, only the results of the parametric tests are given.

Analysis of variance performed on the dataset using the General Linear Model (GLM) identified the type of sample (forage, milk, cheese) and vegetation type (AV-T, AV-F, MDS-F, MDS-A) as significant variability factors, but not the year.

Unequal N Tukey HSD test and GLM found that the values of milk and cheese casein were not significantly ($p < 0.001$) different, except for $\delta^{18}\text{O}$, and were significantly correlated with each other ($r = 0.89$ for $\delta^{13}\text{C}$; 0.87 for $\delta^{15}\text{N}$; 0.84 for $\delta^2\text{H}$; 0.67 for $\delta^{18}\text{O}$). Moreover, they were significantly ($p < 0.001$) different from those of forage, except for $\delta^2\text{H}$. This means that in general, there is significant isotope fractionation in the animal, in comparison with the feed but there is no isotopic fractionation during cheese-

Table 1

Mean and standard deviation of the isotope ratios of bulk forage, milk and cheese casein and milk water.

Site/vegetation ^a	Feed/product ^b	Isotope ratios ^c				
		$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	$\delta^2\text{H}$	$\delta^{18}\text{O}$	$\delta^{18}\text{O}_{\text{water}}$
AV-F 07	Forage	-26.9 ± 0.1	-0.2 ± 0.7	-122 ± 4	20.6 ± 0.4	-5.1 ± 0.2
	Milk	-24.2 ± 0.1	3.4 ± 0.4	-118 ± 5	10.8 ± 1.1	
	Cheese	-24.0 ± 0.2	3.2 ± 0.4	-119 ± 2	9.9 ± 0.9	
AV-T 07	Forage	-26.5 ± 0.1	-1.3 ± 0.5	-116 ± 3	21.4 ± 0.8	-3.1 ± 2.1
	Milk	-23.9 ± 0.3	3.0 ± 0.3	-118 ± 4	11.1 ± 1.2	
	Cheese	-23.9 ± 0.2	3.0 ± 0.5	-118 ± 2	10.0 ± 0.9	
MDS-F 07	Forage	-26.9 ± 0.1	-2.3 ± 0.5	-108 ± 2	21.6 ± 0.6	-2.3 ± 0.9
	Milk	-23.1 ± 0.2	3.4 ± 0.1	-109 ± 4	12.6 ± 0.9	
	Cheese	-23.0 ± 0.1	3.4 ± 0.1	-110 ± 2	11.7 ± 1.3	
MDS-A 07	Forage	-27.1 ± 0.1	-0.8 ± 0.7	-111 ± 2	21.9 ± 0.1	-0.5 ± 1.4
	Milk	-23.3 ± 0.2	3.8 ± 0.2	-105 ± 7	13.7 ± 1.3	
	Cheese	-23.2 ± 0.3	3.7 ± 0.4	-109 ± 4	12.1 ± 1.3	
AV-F 08	Forage	-26.6 ± 0.6	-1.8 ± 0.5	-109 ± 1	21.9 ± 0.7	-4.4 ± 0.3
	Milk	-23.9 ± 0.2	3.4 ± 0.3	-116 ± 1	9.9 ± 0.6	
	Cheese	-23.2 ± 0.3	3.4 ± 0.3	-115 ± 1	9.4 ± 0.4	
AV-T 08	Forage	-26.7 ± 0.4	-0.6 ± 1.3	-99 ± 5	22.4 ± 0.3	-4.8 ± 0.5
	Milk	-23.3 ± 0.2	2.9 ± 0.2	-115 ± 2	9.8 ± 0.7	
	Cheese	-23.4 ± 0.2	2.9 ± 0.1	-115 ± 3	9.3 ± 0.6	
MDS-F 08	Forage	-27.7 ± 0.4	-2.0 ± 0.7	-107 ± 4	22.1 ± 0.4	-2.2 ± 0.5
	Milk	-23.0 ± 0.2	3.5 ± 0.3	-104 ± 2	11.9 ± 0.7	
	Cheese	-23.1 ± 0.2	3.5 ± 0.3	-104 ± 2	10.6 ± 0.8	
MDS-A 08	Forage	-27.6 ± 0.2	-0.9 ± 0.5	-109 ± 5	22.0 ± 0.5	-1.3 ± 0.3
	Milk	-23.1 ± 0.1	3.8 ± 0.2	-104 ± 2	12.4 ± 0.4	
	Cheese	-23.1 ± 0.2	3.9 ± 0.2	-103 ± 2	11.5 ± 0.6	

^a Site/vegetation key: AV, Alpe Valcavera; MDS, Malga Dosso di Sotto; F, mesophilous vegetation type; T, oligotrophic vegetation type; A, mesophilous mesotrophic vegetation type; 07, 2007 summer; 08, 2008 summer.

^b Number of samples per item were: forage, 4; milk and cheese, 12.

^c Values are: ‰ versus V-PDB for $\delta^{13}\text{C}$; ‰ versus AIR for $\delta^{15}\text{N}$; ‰ versus V-SMOW for $\delta^2\text{H}$, $\delta^{18}\text{O}$ and $\delta^{18}\text{O}_{\text{water}}$.

making, as previously hypothesized but never verified experimentally to our knowledge. There did not seem to be a reasonable explanation for the fact that cheese and milk $\delta^{18}\text{O}$ were shown to be statistically different (mean value: 10.5‰ vs 11.5‰). Further studies are necessary to confirm and explain these findings.

On the basis of these findings, we grouped the milk and cheese samples and the two years together for further consideration.

In Fig. 1 the Box Plot Whisker graphs and the results of the multiple comparison Unequal *N* Tukey HSD test for each variable are shown.

The $\delta^{13}\text{C}$ values of milk and cheese, but not those of forage, were significantly ($p < 0.001$) influenced by vegetation type (Fig. 1a). The Tukey test showed no differences between MDS samples that were grouped together, probably due to smaller differences between MDS-A and MDS-F vegetation compared to AV-T and AV-F. The $\delta^{13}\text{C}$ values in casein were 2.9 (± 0.3)‰ and 4.2 (± 0.4)‰ higher than in forage for AV and MDS respectively. In the literature, an increase in

the value of $\delta^{13}\text{C}$ passing from the diet to the animal product in the range of 1–4‰ is reported for milk from grass-fed cows, while maize feeding results in a decrease of up to -4‰ (Camin et al., 2004, 2008; Knobbe et al., 2006; Masud et al., 1999; Metges et al., 1990; Minson et al., 1975). The increase in MDS $\delta^{13}\text{C}$ could be related both to isotope fractionation during feed protein conversion into animal and milk protein, and to maize supplementation, which is known to have a significantly higher ^{13}C content (-12 to -14‰). Maize supplementation also justifies the higher $\delta^{13}\text{C}$ values in MDS milk and cheese casein, as compared with AV milk and cheese casein, reversing the trend observed in the forage. In AV, where the diet did not include maize, $\delta^{13}\text{C}$ values in casein ranged between -24.6 and -23.0‰, thus often below the $\delta^{13}\text{C}$ maximum threshold value of -23.5‰ for casein from animals not fed with maize (Camin et al., 2008). Only in AV-T 2008 the milk and cheese samples collected at the beginning of exploitation had values above the threshold (data not shown), probably because of the influence of

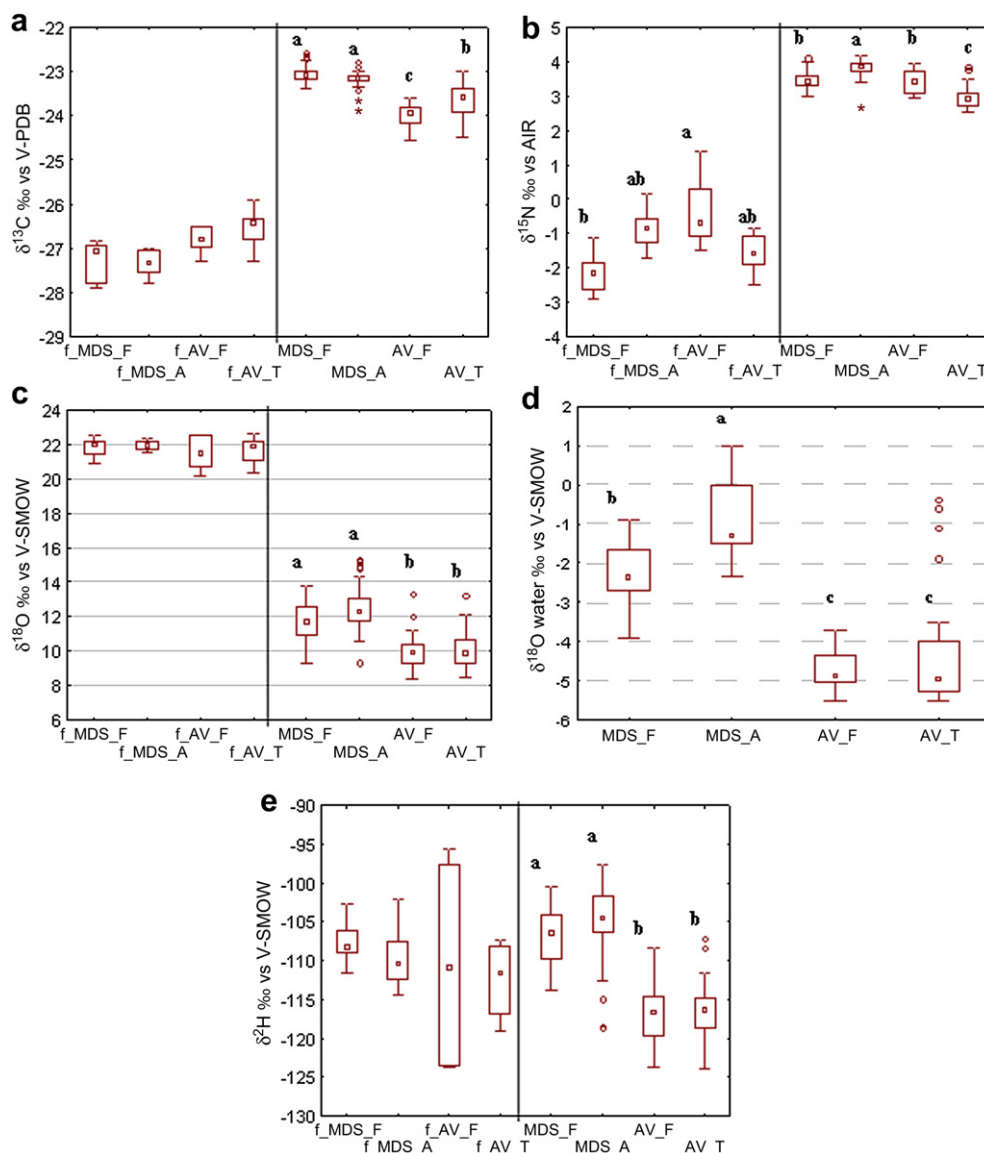


Fig. 1. Box Plot Whisker graphs and results of the Unequal *N* Tukey HSD test on the isotopic values (a, $\delta^{13}\text{C}$; b, $\delta^{15}\text{N}$; c, $\delta^{18}\text{O}_{\text{casein}}$; d, $\delta^{18}\text{O}_{\text{water}}$; e, $\delta^2\text{H}$) of forage (indicated by the prefix "f") and milk and cheese casein (no prefix) collected in Alpe Valcavera (AV) and Malga Dosso di Sotto (MDS) and produced on different vegetation types, mesophilous (F), oligotrophic (T), and mesophilous mesotrophic (A). Mean values with different letters are significantly different ($p < 0.001$): □ median; □, 25%–75%; ⊥, sp. not-outlier; ○, outlier; ★ extreme.

dietary composition during the previous period. Indeed, an adaptation period of 6 days is not sufficient for a complete turnover of C, as already observed by other authors (Camin et al., 2008; Wilson, MacKenzie, & Brookes, 1988).

The $\delta^{15}\text{N}$ values of forage, milk and cheese were mainly influenced by vegetation type (Fig. 1b). The vegetation of AV-T and MDS-F contained a higher percentage of leguminous plants (mainly *Trifolium* species) resulting in lower $\delta^{15}\text{N}$ values for forage, milk and cheese, as leguminous plants fix air-N, which has a $\delta^{15}\text{N}$ equal to 0‰ (Martinelli, Victoria, Trivellin, Devol, & Richey, 1992; Yoneyama, Yamada, Kojima, & Yazaki, 1984), in agreement with values reported in the literature (Knobbe et al., 2006; Martinelli et al., 1992; Steele, Bonish, Daniel, & Ohara, 1983). The $\delta^{15}\text{N}$ values of casein were 4.6‰ \pm 0.7 higher than those of forage, mainly in MDS (5.1‰ as compared with 4.1‰ in AV). The differences between $\delta^{15}\text{N}$ values were slightly greater than reported in the literature (1–4‰; Camin et al., 2004, 2008; Knobbe et al., 2006; Masud et al., 1999; Steele & Daniel, 1978). As observed for $\delta^{13}\text{C}$, maize in the MDS diet could result in higher $\delta^{15}\text{N}$ values, because the content of ^{15}N in maize (not measured here) is normally higher than in grazing vegetation (Camin et al., 2008; Knobbe et al., 2006). Casein $\delta^{15}\text{N}$ between 2.5‰ and 4.2‰ confirmed the maximum limit of 4‰ for mountain dairy products.

The $\delta^{18}\text{O}$ values of milk and cheese were affected by pasture type ($p < 0.001$, Fig. 1c), although sample sharing in the groups suggests that the site was more influential than the vegetation. The same difference, i.e., lower AV values in comparison to MDS, was also observed for milk water (Fig. 1d), whose isotopic composition is related to that of the drinking water (Chesson et al., 2010), depending on latitude, altitude, distance from the evaporation source, temperature and the amount of precipitation (Clark & Fritz, 1997; Van der Veer, Voerkelius, Lorentz, Heiss, & Hoogewerff, 2009). As the two sites were at almost the same latitude, the aforementioned differences in all the $\delta^{18}\text{O}$ values were probably due to differences in altitude at the sites, as at higher altitude there is lighter water vapour (hence with lower $\delta^{18}\text{O}$). Conversely vegetation did not affect the $\delta^{18}\text{O}$ of bulk forage (Fig. 1c).

Trends similar to $\delta^{18}\text{O}$ were observed for $\delta^2\text{H}$ (Fig. 1e). In fact we found a significant relation ($p < 0.001$) between $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values for forage ($\delta^2\text{H}: 6.52 \times \delta^{18}\text{O} - 251.69$; $R^2: 0.40$), milk ($\delta^2\text{H}: 3.66 \times \delta^{18}\text{O} - 152.96$; $R^2: 0.55$), and cheese ($\delta^2\text{H}: 2.67 \times \delta^{18}\text{O} - 139.77$; $R^2: 0.32$). Moreover milk casein $\delta^2\text{H}$ could be predicted using the $\delta^{18}\text{O}$ of milk water ($\delta^2\text{H}_{\text{cas}} = 3.19 \times \delta^{18}\text{O}_{\text{milk water}} - 101.16$; $R^2 = 0.60$), which also effectively predicts milk casein $\delta^{18}\text{O}$ ($\delta^{18}\text{O}_{\text{cas}} = 0.67 \times \delta^{18}\text{O}_{\text{milk water}} + 13.56$; $R^2 = 0.65$), as found in previous experiments (Camin et al., 2008).

The experimental results substantiate the hypothesis that both the H and O isotope composition of casein can record geography, as observed in human hair keratin (Ehleringer et al., 2008) and in animal protein (Heaton et al., 2008; Perini et al., 2009).

3.2. Discrimination between production area and vegetation type of alpine milk and cheese

To check if the combined isotopic parameters could be used to distinguish between milk and cheese from the four different pasture types, we carried out a multivariate canonical discriminant analysis. Milk and cheese scores for the first two canonical variables (CAN) are plotted in Fig. 2. The first two CANs account for 98% and 99% of the variability for milk (Fig. 2A) and cheese (Fig. 2B) respectively. For both, CAN1 is mainly loaded negatively by casein $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ and separates the two sites (AV versus MDS), whereas CAN2 is mainly determined positively by $\delta^{13}\text{C}$ and negatively by $\delta^{15}\text{N}$ and divides the two pasture types into each site. After cross-validation, 83% of milk samples (88% before cross-validation) and 80% of cheese samples (82% before cross-validation) were correctly

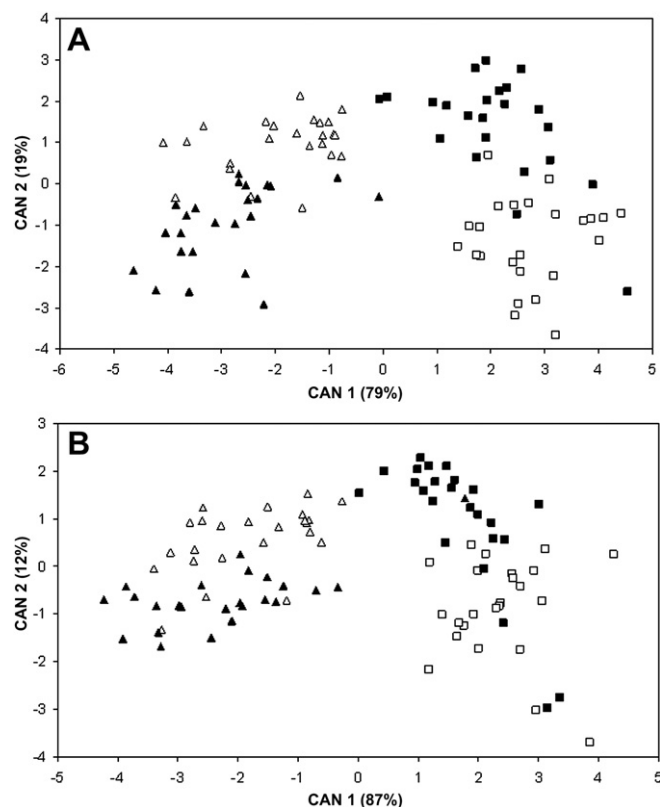


Fig. 2. Canonical Discriminant analysis of (A) milk data and (B) cheese data ($N = 96$): plot of the first two canonical variables (CAN 1 versus CAN 2) with loadings in brackets. The samples were collected in Alpe Valcavera (AV) and Malga Dosso di Sotto (MDS) and produced on different vegetation types, mesophilous (F), oligotrophic (T) and mesophilous mesotrophic (A): □, AV-F; ■, AV-T; △, MDS-F; ▲, MDS-A.

classified. Considering only the site of origin, 96% of the milk and cheese samples were correctly discriminated.

4. Conclusions

The results demonstrate that the stable isotope ratios of H, C, N and O of milk and cheese are linked to the terroir, in particular to the type of vegetation and the environment. The combination of the four stable isotope ratios made it possible to separate dairy products from two alpine sites and from different pasture types within each site.

The study moreover confirms that alpine milk and cheese from grazing cows, without maize supplementation for more than two weeks before milk collection, are characterised by $\delta^{15}\text{N}$ lower than +4‰ and $\delta^{13}\text{C}$ lower than -23.5 ‰. In different mountain cheeses, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ can be used to distinguish the altitude of the provenance area, $\delta^{15}\text{N}$ to determine different grazing vegetation types and in the case of dietary supplements $\delta^{13}\text{C}$ can determine different amounts of maize in the animal diet.

In this work, the possibility of differentiating between the two mountain sites is mainly useful in the case of milk, because the two cheeses can also be distinguished visually. On the other hand, the ability of stable isotope analysis to separate different vegetation types is to some extent advantageous for both milk and cheese.

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References

- Barham, E. (2003). Translating terroir: the global challenge of French AOC labelling. *Journal of Rural Studies*, 19, 127–138.
- Bontempo, L., Larcher, R., Camin, F., Hoelzl, S., Rossmann, A., Horn, P., & Nicolini, G. (2011). Elemental and isotopic characterisation of typical Italian alpine cheeses. *International Dairy Journal*, 21, 441–446.
- Brescia, M. A., Monfreda, M., Buccolieri, A., & Carrino, C. (2005). Characterisation of the geographical origin of buffalo milk and mozzarella cheese by means of analytical and spectroscopic determinations. *Food Chemistry*, 89, 139–147.
- Camin, F., Bontempo, L., Heinrich, K., Horacek, M., Kelly, S. D., Schlicht, C., et al. (2007). Multi-element (H, C, N, S) stable isotope characteristics of lamb meat from different European regions. *Analytical and Bioanalytical Chemistry*, 389, 309–320.
- Camin, F., Larcher, R., Nicolini, G., Bontempo, L., Bertoldi, D., Perini, M., et al. (2010). Isotopic and elemental data for tracing the origin of European olive oils. *Journal of Agricultural and Food Chemistry*, 58, 570–577.
- Camin, F., Perini, M., Colombari, G., Bontempo, L., & Versini, G. (2008). Influence of dietary composition on the carbon, nitrogen, oxygen and hydrogen stable isotope ratios of milk. *Rapid Communications in Mass Spectrometry*, 22, 1690–1696.
- Camin, F., Wietzerbin, K., Cortes, A. B., Haberhauer, G., Lees, M., & Versini, G. (2004). Application of multielement stable isotope ratio analysis to the characterization of French, Italian, and Spanish cheeses. *Journal of Agricultural and Food Chemistry*, 52, 6592–6601.
- Chesson, L. A., Valenzuela, L. O., O'Grady, S. P., Cerling, T. E., & Ehleringer, J. R. (2010). Hydrogen and oxygen stable isotope ratios of milk in the United States. *Journal of Agricultural and Food Chemistry*, 58, 2358–2363.
- Clark, I., & Fritz, P. (1997). Tracing the hydrological cycle. In *Environmental isotopes in hydrogeology* (pp. 35–78). Boca Raton, FL, USA: Lewis Publishers.
- Crittenden, R. G., Andrew, A. S., LeFournour, M., Young, M. D., Middleton, H., & Stockmann, R. (2007). Determining the geographic origin of milk in Australasia using multi-element stable isotope ratio analysis. *International Dairy Journal*, 17, 421–428.
- DeNiro, M. J., & Epstein, S. (1978). Influence of diet on the distribution of carbon isotopes in animals. *Geochimica Cosmochimica Acta*, 42, 495–506.
- DeNiro, M. J., & Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica Cosmochimica Acta*, 45, 341–351.
- Ehleringer, J. R., Bowen, G. J., Chesson, L. A., West, A. G., Podlesak, D. W., & Cerling, T. E. (2008). Hydrogen and oxygen isotope ratios in human hair are related to geography. *Proceedings of the National Academy of Sciences of the USA*, 105, 2788–2793.
- Engel, E., Ferlay, A., Cornu, A., Chilliard, Y., Agabriel, C., Bielicki, G., et al. (2007). Relevance of isotopic and molecular biomarkers for the authentication of milk according to production zone and type of feeding of the cow. *Journal of Agricultural and Food Chemistry*, 55, 9099–9108.
- Falchero, L., Lombardi, G., Gorlier, A., Lonati, M., Odoardi, M., & Cavallero, A. (2010). Variation in fatty acid composition of milk and cheese from cows grazed on two alpine pastures. *Dairy Science and Technology*, 90, 657–672.
- Harrison, S. M., Schmidt, O., Moloney, A. P., Kelly, S. D., Rossmann, A., Schellenberg, A., et al. (2011). Tissue turnover in ovine muscles and lipids as recorded by multiple (H, C, O, S) stable isotope ratios. *Food Chemistry*, 124, 291–297.
- Heaton, K., Kelly, S. D., Hoogewerff, J., & Woolfe, M. (2008). Verifying the geographical origin of beef: the application of multi-element isotope and trace element analysis. *Food Chemistry*, 107, 506–515.
- Horacek, M., & Min, J. S. (2010). Discrimination of Korean beef from beef of other origin by stable isotope measurements. *Food Chemistry*, 121, 517–520.
- Kenward, M. G., & Jones, B. (2003). *Design and analysis of cross-over trials*. Boca Raton, FL, USA: Chapman and Hall/CRC.
- Knobbe, N., Vogl, J., Pritzkow, W., Panne, U., Fry, H., Lochotzke, H. M., et al. (2006). C and N stable isotope variation in urine and milk of cattle depending on the diet. *Analytical and Bioanalytical Chemistry*, 386, 104–108.
- Kornel, B. E., Werner, T., Rossmann, A., & Schmidt, H. L. (1997). Measurement of stable isotope abundances in milk and milk ingredients as possible tool for origin assignment and quality control. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung A*, 205, 19–24.
- Lombardi, G., Cavallero, A., & Bassignana, M. (2011). *I terroir delle Alpi per la caratterizzazione e la difesa delle produzioni casearie d'alpeggio* (1st ed.). Lodi, Italy: E. Piano.
- Manca, G., Camin, F., Coloru, G. C., Del Caro, A., Depentori, D., Franco, M. A., et al. (2001). Characterization of the geographical origin of Pecorino Sardo cheese by casein stable isotope ($^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$) ratios and free amino acid ratios. *Journal of Agricultural and Food Chemistry*, 49, 1404–1409.
- Manca, G., Franco, M. A., Versini, G., Camin, F., Rossmann, A., & Tola, A. (2006). Correlation between multielement stable isotope ratio and geographical origin in Peretta cows' milk cheese. *Journal of Dairy Science*, 89, 831–839.
- Martinelli, L. A., Victoria, R. L., Trivellin, P. C. O., Devol, A. H., & Richey, J. E. (1992). ^{15}N natural abundance in plants of the Amazon River floodplain and potential atmospheric N_2 fixation. *Oecologia*, 90, 591–596.
- Masud, Z., Vallet, C., & Martin, G. J. (1999). Stable isotope characterization of milk components and whey ethanol. *Journal of Agricultural and Food Chemistry*, 47, 4693–4699.
- McCutchan, J. H., Jr., Lewis, W. M., Jr., Kendall, C., & McGrath, C. C. (2003). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. *Oikos*, 102, 378–390.
- Metges, C., Kempe, K., & Schmidt, H. L. (1990). Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the $\delta^{13}\text{C}$ value of food in dairy cows. *British Journal of Nutrition*, 63, 187–196.
- Minson, D. J., Ludlow, M. M., & Troughton, J. H. (1975). Differences in natural carbon isotope ratios of milk and hair from cattle grazing tropical and temperate pastures. *Nature*, 256, 602.
- Molkentin, J. (2009). Authentication of organic milk using $\delta^{13}\text{C}$ and the α -linolenic acid content of milk fat. *Journal of Agricultural and Food Chemistry*, 57, 785–790.
- Molkentin, J., & Giesemann, A. (2007). Differentiation of organically and conventionally produced milk by stable isotope and fatty acid analysis. *Analytical and Bioanalytical Chemistry*, 388, 297–305.
- Molkentin, J., & Giesemann, A. (2010). Follow-up of stable isotope analysis of organic versus conventional milk. *Analytical and Bioanalytical Chemistry*, 398, 1493–1500.
- OIV. (2009). Organisation Internationale de la Vigne et du Vin method number MA-E-AS2-12-MOUO18. Method for $^{18}\text{O}/^{16}\text{O}$ isotope ratio determination of water in wines and must (Oeno 353/2009). In *Compendium of international methods of analysis of wines and musts*, Vol. 2. Paris, France: OIV.
- Perini, M., Camin, F., Bontempo, L., Rossmann, A., & Piasentier, E. (2009). Multi-element (H, C, N, O, S) stable isotope characteristics of lamb meat from different Italian regions. *Rapid Communications in Mass Spectrometry*, 23, 2573–2585.
- Pillonel, L., Badertscher, R., Froidevaux, P., Haberhauer, G., Hölzl, S., Horn, P., et al. (2003). Stable isotope ratios, major, trace and radioactive elements in Emmental cheeses of different origins. *Lebensmittel-Wissenschaft und -Technologie*, 36, 615–623.
- Renou, J. P., Deponge, C., Gachon, P., Bonnefoy, J. C., Coulon, J. B., Garel, J. C., et al. (2004). Characterization of animal products according to its geographic origin and feeding diet using nuclear magnetic resonance and isotope ratio mass spectrometry Part I: Cow milk. *Food Chemistry*, 85, 63–66.
- Sacco, D., Brescia, M. A., Sgaramella, A., Casiello, G., Buccolieri, A., Ogrinc, N., et al. (2009). Discrimination between Southern Italy and foreign milk samples using spectroscopic and analytical data. *Food Chemistry*, 114, 1559–1563.
- Steele, K. W., Bonish, P. M., Daniel, R. M., & Ohara, G. W. (1983). Effect of rhizobial strain and host plant on nitrogen isotopic fractionation in legumes. *Plant Physiology*, 72, 1001–1004.
- Steele, K. W., & Daniel, R. M. (1978). Fractionation of nitrogen isotopes by animals: a further complication to the use of variations in the natural abundance of ^{15}N for tracer studies. *Journal of Agricultural and Food Chemistry*, 90, 7–9.
- Tuross, N., Warinner, C., Kirsanow, K., & Kester, C. (2008). Organic oxygen and hydrogen isotopes in a porcine controlled dietary study. *Rapid Communications in Mass Spectrometry*, 22, 1741–1745.
- Van der Veer, G., Voerkelius, S., Lorentz, G., Heiss, G., & Hoogewerff, J. A. (2009). Spatial interpolation of the deuterium and oxygen-18 composition of global precipitation using temperature as ancillary variable. *Journal of Geochemical Exploration*, 101, 175–184.
- Wassenaar, L. I., & Hobson, K. A. (2003). Comparative equilibrium and online technique for determination of non-exchangeable hydrogen of keratins for use in animal migration studies. *Isotopes in Environmental and Health Studies*, 39, 211–217.
- Wilson, G. F., MacKenzie, D. D. S., & Brookes, I. M. (1988). Importance of body tissues as sources of nutrients for milk synthesis in the cow, using C as a marker. *British Journal of Nutrition*, 60, 605–617.
- Yoneyama, T., Yamada, N., Kojima, H., & Yazaki, J. (1984). Variations of natural ^{15}N abundances in leguminous plants and nodule fractions. *Plant Cell Physiology*, 25, 1561–1565.