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Manuela Renna[1*], Paolo Cornale[1], Carola Lussiana[1], Luca Maria Battaglini[1], Germano Turille[2], Antonio Mimosi[1]

[1] Dipartimento di Scienze Agrarie, Forestali e Alimentari; University of Torino; via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy

[2] Institut Agricole Régional, Settore di Zootecnia e Industria Lattiero-Casearia; Regione La Rochére 1/A, 11100 Aosta, Italy

* Corresponding author: Dr. Manuela Renna, Ph.D.; Dipartimento di Scienze Agrarie, Forestali e Alimentari; University of Torino; via L. da Vinci 44, 10095 Grugliasco (TO), Italy. Tel.: +39 011 6708732. Fax: +39 011 6708563. Email: manuela.renna@unito.it

Running title: Feeding strategy and bovine milk quality

ABSTRACT

This study was designed to evaluate the effects of two feeding methods on milk yield, composition and fatty acid (FA) profile obtained from dual-purpose cattle. Twenty-four Aosta Red Pied cows beyond peak of lactation were assigned to two groups and fed hay and concentrates in the proportions 0.69 and 0.31 on a dry matter basis for ten weeks. Concentrates were offered separately from forages six times a day (separate ration, SR) or as a total mixed ration (TMR). The feeding method did not significantly influence dry matter intake (16.8 *vs* 16.9 kg $head^{-1}$ day^{-1} for SR- and TMR-fed cows, respectively), milk yield (17.4 *vs* 17.5 kg $head^{-1}$ day^{-1}), milk fat, protein and lactose contents (36.4 *vs* 35.2, 33.5 *vs* 32.8, and 47.3 *vs* 47.4 g kg^{-1}) and yields (607.9 *vs* 613.4, 567.4 *vs* 572.7, and 805.5 *vs* 829.7 g $head^{-1}$ day^{-1}). The overall milk FA profile was very similar between groups. Milk concentrations of FA used as indirect markers of rumen function (C18:2 *t*10*c*12, odd- and branched-chain FA) and the extent of ruminal biohydrogenation were comparable ($P>0.05$) between SR- and TMR-fed cows, suggesting that ruminal pH did not vary considerably as a consequence of the feeding strategy applied.

Key words: feeding method; Aosta Red Pied cow; production performance; milk composition; fatty acids

INTRODUCTION

In the dairy sector, total mixed rations (TMR) have been used for feeding cows since the 1970s (Eastridge 2006). This feeding method has received great attention mainly because of the related possibilities to increase control over feeding programs and farm

mechanization levels (Rakes 1969), to improve animal performance by means of the synchronous supply of dietary nitrogen and fermentable energy, and consequently to raise income from milk production (Nocek *et al*. 1985; Yan *et al*. 1998). For these reasons, TMR have also aroused interest by the producers' associations of some European Protected Designation of Origin (PDO) cheeses. The Manufacturing Rules of some of these cheeses (e.g., Comté cheese in France, and Parmigiano Reggiano and Fontina cheeses in Italy) prohibit the feeding of dairy cows with silages due to possible contamination by *Clostridium tyrobutyricum*, whose spores can cause blowing problems during cheese ripening (Bertoni *et al*. 2001). When the use of silages is forbidden, TMR are prepared as blends of dried fodders, concentrates and supplements. Water can be added as binding agent to reduce selective consumption by the cows (Leonardi *et al*. 2005).

In the early 1990s the use of 'dry' TMR in the geographical area of Parmigiano Reggiano cheese production (northern Italy) determined improvements of animal performance and allowed significant reductions of costs at farm level (Salghetti & Manghi 2004). Attracted by these results, the producers' associations of Fontina PDO cheese directed their attention towards the TMR feeding method. Differently from Parmigiano Reggiano, which is mainly produced using cow breeds of high genetic merit (e.g., Italian Friesian), Fontina cheese is made with milk exclusively obtained from dual-purpose medium-producing autochthonous Aosta cows. These cattle are traditionally fed with local hay (provided *ad libitum*) and concentrate, separately administered. Until few years ago, concentrate used to be administered twice a day. More recently, many producers supply concentrate with higher frequency (four to six administrations per day).

The majority of the studies dealing with the effect of TMR on dairy cows' performance have been conducted on high-yielding animals. Therefore, there is interest in deepening the effects of TMR, as compared with the traditional system of feeding forages and concentrates in a discrete format, on milk production performance of dual purpose and/or low-medium producing cows, whose milk is often at the basis of the manufacturing of typical cheeses.

Besides that, increasing concern should be addressed to the effects of TMR on milk fatty acid (FA) profile, as no information is currently available on this topic. It is known that one of the advantages related to TMR is the opportunity to prevent detrimental effects due to dramatic fluctuations of ruminal pH. When ruminal pH declines specific *trans*-FA (e.g., C18:2 *t*10*c*12 and C18:1 *t*10) are produced at the expense of the main biohydrogenation intermediates (C18:2 *c*9*t*11 and C18:1 *t*11) of dietary linoleic acid (C18:2 *c*9*c*12) (Troegeler-Meynadier *et al*. 2003; Fuentes *et al*. 2009; Bauman & Griinari 2003). A drop in ruminal pH, can also lead to changes in milk concentrations of odd- and branched-chain FA (OBCFA) (Fievez *et al*. 2012). Milk FA profile should therefore be used as a useful and non-invasive indicator of alterations of rumen fermentations determined by different feeding techniques applied in the dairy sector.

On the basis of the above-mentioned considerations, the goal of this experimental trial was to evaluate the effects of feeding frequent supply of concentrate separately from forages or as a TMR on productive performance (dry matter intake and milk production levels) and milk composition, with particular emphasis on FA profile of milk fat, in dual-purpose medium-producing Aosta Red Pied cattle whose milk is used for Fontina PDO cheese manufacturing.

MATERIALS AND METHODS

Animals, experimental design and dietary treatments

The trial was carried out at the Montfleury research farm (Aosta Valley, NW Italy; latitude: 45°43'59" N; longitude: 7°18'2" E; altitude: 560 m a.s.l.). On January 13, 2010, twenty-four multiparous Aosta Red Pied cows whose milk is used for Fontina PDO cheese manufacturing were selected from a herd of 60 lactating cows. They were blocked in two homogeneous groups, according to their stage of lactation (mean and standard deviation: 48 ± 16 and 46 ± 18 days in milk), parity (3.8 ± 1.8 and 3.8 ± 1.9), milk yield (18.2 ± 2.6 and 18.3 ± 2.7 kg $head^{-1}$ day^{-1}), milk gross composition (fat, protein, and lactose contents and yields), and FA profile of milk fat. The groups were then randomly assigned to one of the following treatments (12 cows per treatment): i) SR (separate ration), hay (first and second cuts) and concentrate fed separately at proportionally 0.69 (first cut: 0.40, second cut: 0.29) and 0.31 of dietary dry matter (DM), respectively, and ii) TMR, the same diet as SR group, but with the dietary ingredients mechanically mixed in a mixer wagon prior to feeding. In the pre-trial period (before January 13, 2010), all cows involved in the trial were fed the SR diet. A three-week period (January 13 to February 2, 2010) of adaptation to the experimental diets was provided, while the experimental period covered a total of 70 days, from February 3 to April 13, 2010.

The administered hays were prepared from local swards composed of about 70% grasses and 30% legumes. The concentrate contained 33% maize grain, dry milled; 30% barley grain, dry milled; 20% bran; 12% soybean meal; 5% linseed meal flax (expeller), on a DM basis.

The cows belonging to the SR group received the roughage part of the diet twice a day (at 05:00 and 15:30 h) in a conventional trough-feeding arrangement. In addition, computerized self feeders offered the concentrate as six equal meals starting at 05:00, 07:30, 10:00, 15:30, 19:00, and 22:00 h.

The TMR was prepared fresh daily every morning at 08:00 h. Chopped hay (length: 3 to 5 cm) was uniformly mixed with the concentrate in a mixer wagon (mixing time: 15 to 20 min). During mixing, water (about 10 L $head^{-1}$ day^{-1}) was added to the TMR as binding agent (Leonardi *et al*. 2005). The TMR was provided once a day immediately after preparation.

The amount of feed offered was calculated to fulfill the nutritional requirements of the cows (INRA 2007) and subsequently adjusted to obtain approximately 5 to 10% daily refusals (on as-fed basis).

All the cows involved in the trial were housed in tie-stalls and had *ad libitum* access to mineral blocks and fresh water.

Sampling and laboratory analyses

Feed

Hays and concentrate to be analyzed for their chemical composition and FA profile were collected at the beginning of the trial.

The samples were ground (cutting mill Pulverisette 15 - Fritsch GmbH, Idar-Oberstein, Germany) to pass a 1-mm screen. They were analyzed for DM, crude protein (CP), ether extract (EE), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) according to recommended standard procedures (AOAC 2000). Starch was analyzed by

using a POLAX-2L polarimeter (ATAGO CO., LTD. Japan) according to "Gazzetta Ufficiale della Repubblica Italiana" (2000).

For FA analysis, total lipids were extracted according to Folch *et al*. (1957). Fatty acids were then determined as previously reported by Renna *et al*. (2012). Peaks were identified by comparison of retention times with FAME standards (Matreya Inc., Pleasant Gap, PA, USA). Results were expressed as a percentage of each individual FAME per total FAME detected.

Uneaten feed was daily monitored during the trial for each group of cows. If refusals were present, they were removed and weighed prior to subsequent feed administration in order to estimate feed intake. Refusals were sampled biweekly to be analyzed for their DM content (AOAC 2000).

The proximate composition and FA profile of the experimental feedstuffs are presented in Table 1.

Milk

The cows were machine milked twice a day at 05.30 and 16.30 h. Before starting the milk yield recording and samples collection, a three-week period (from January 13 to February 2, pre-experimental period) of adaptation to the diets was provided. Individual daily milk yields were recorded by automatic meters (Afimilk, TDM, Brescia, Italy) once a week during the trial. Milk samples (50 mL) from each individual cow were collected at the morning milking once a week as well, following the same time schedule as for milk yield recording (for totally 264 samples). These samples were immediately stored at 4°C with azidiol as preservative and transported to the laboratory for the analysis of fat, protein, and lactose (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark).

Individual milk samples (150 mL) to be used for FA determination were instead collected every two weeks during the morning milking (for totally 144 samples) and frozen at −20°C until analyzed. Fatty acids analysis was performed as previously reported by Renna *et al*. (2012). Peaks were identified by injecting pure FAME standards (Sigma-Aldrich, Milano, Italy; Matreya Inc., Pleasant Gap, PA, USA and Restek Corporation, Bellefonte, PA, USA) and by comparison with the chromatogram published by Collomb and Bühler (2000). Quantification was assessed by using nonanoic acid as internal standard. The results are expressed as absolute values as g $100g^{-1}$ fat.

Statistical analysis

Dry matter intake results were submitted to an independent sample Student's *t* test using the PROC TTEST procedure of Statistical Analysis System (SAS 2006).

The changes in milk yield, milk main constituents and FA were analyzed using the PROC MIXED procedure of SAS (2006) for repeated measures over time. The cow was considered as the experimental unit. Compound symmetry, first-order autoregressive or unstructured covariance structure, according to the smallest Schwarz Bayesian information criterion, was applied (Littell *et al*. 1998). The following model was used:

$$Y_{ijk} = \mu + FT_i + C_{(i)j} + SD_k + (FT \times SD)_{ik} + \varepsilon_{ijk,}$$

where Y_{ijk} = mean of response variable, μ = population mean, FT_i = fixed effect of treatment (feeding technique), $C_{(i)j}$ = random effect of cow within the treatments, SD_k = fixed effect of sampling date, $(FT \times SD)_{ik}$ = fixed effect of interaction between feeding technique and sampling date, and ε_{ijk} = experimental error.

Significance was declared at $P \leq 0.05$. Results of statistical analysis are reported as means (DM intake) and estimate least-squares means (all other investigated parameters).

RESULTS AND DISCUSSION

Effect of feeding technique on dry matter intake, milk yield and milk gross composition

Estimated total DM intakes were very similar between groups, namely 16.8 and 16.9 kg $head^{-1}$ day^{-1} for SR- and TMR-fed cows, respectively. Nocek *et al*. (1985) reported significantly higher DM intake in dairy cows fed TMR if compared to cows fed the roughage and concentrate components of the diet separately. Such an increase in intake could have been referable to a reduction in fermentation fluctuations within the rumen as an effect of more steady daily distributions of concentrate intake (Istasse *et al*. 1986). However, no significant differences in total DM intake were instead observed in other studies (Gordon *et al*. 1995; Yrjänen *et al*. 2003; Ferris *et al*. 2006). The lack of positive DM intake response to the TMR feeding technique in the current trial could be the consequence of both the low inclusion rate (31%) of concentrate in the offered diets and the relatively high frequency of concentrate administration applied with the SR feeding method. In fact, higher feed intakes have been previously associated with TMR feeding if the offered diets were characterized by high (≥0.60) concentrate proportions only (Phipps *et al*. 1984; Istasse *et al*. 1986; Gordon *et al*. 1995). Moreover, high frequencies (four or more times daily) of concentrate administration in case of diet components fed separately, being able to resemble the typical TMR synchrony of fermentable energy and nitrogen supplies to the ruminal microflora (Yan *et al*. 1998), resulted in

comparable DM intakes in cows fed TMR or unmixed diets in other studies (Gordon *et al*. 1995; Yrjänen *et al*. 2003).

The sampling date did not significantly affect DM intake. Such result was expected. In fact, at the beginning of the trial the cows were at their 10th week postpartum and usually only slight changes occur in DM intake of low-medium producing dairy cows approximately between the 8th (maximum DM intake) and the 20th week postpartum (NRC 2001).

Results concerning milk production levels and milk main constituents are shown in Table 2. The mean daily milk yield was not significantly affected by the feeding system (17.4 and 17.5 kg $head^{-1}$ day^{-1} for SR- and TMR-fed cows, respectively). Results previously shown by other authors on the effect of feeding ingredients as complete diets or administered separately on milk production performance are quite contrasting. Lactating cows fed TMR were reported to yield more milk if compared to cows offered the same feedstuffs separately (Gordon *et al*. 1995; Yan *et al*. 1998), sometimes as the consequence of higher DM intake and higher digestibility values of the TMR diet (Istasse *et al*. 1986). Nevertheless, no significant effects on milk production levels have been found in other studies (Phipps *et al*. 1984; Nocek *et al*. 1985; Ferris *et al*. 2006). It is not clear if the moderate milk yield potential of the cows used in the current trial should or should not be considered *per se* a cause of the lack of feeding system influence on milk production levels. Gordon *et al*. (1995) showed that the feeding method affects the production responses of dairy cows independently of their genetic merit. However, on the basis of the results reported by other authors, the milk production potential seems to be one key factor in the regulation of yield response of dairy cows to different concentrate feeding strategies (Agnew *et al*. 1996). As

previously mentioned for DM intake, Istasse *et al*. (1986) showed the notable influence of diet's F:C ratios on milk production performance of lactating cows offered complete diets or diet ingredients separately. The proportion of concentrate used in the current study is the one typically applied in winter rations destined to Aosta cows within the Fontina PDO cheese production chain. Such proportion is considerably lower than those used in previous trials with dairy cows of high genetic merit, where higher milk yields by the animals were associated to TMR-feeding (Gordon *et al*. 1995; Yan *et al*. 1998; Salghetti & Manghi 2004). The low proportion of concentrate, associated to the medium genetic merit of the cows, could be consequently considered one of the reasons for the observed lack of positive milk yield response in TMR-fed if compared to SR-fed Aosta Red Pied cows. In addition, it is worth mentioning that significantly lower milk yields were found when concentrate, in case of feeding separate rations, was offered in a limited number of meals per day (Istasse *et al*. 1986), while no differences were observed in case of daily allowance of concentrates in four × 6 hours time windows (Yan *et al*. 1998).

As expected, the sampling date significantly ($P \leq 0.001$) affected the milk production levels, which declined in both groups of cows during the trial (that is from week 10 to week 20 postpartum) following the advance of the stage of lactation. Milk yield was not significantly affected by the interaction between feeding technique and sampling date.

Considering milk gross composition, the concentrations and yields of fat, protein and lactose were not significantly different in TMR- and SR-fed cows as previously published by other authors (Gordon *et al*. 1995; Yrjänen *et al*. 2003; Ferris *et al*. 2006). Feeding a complete diet in place of concentrate separately from grass silage determined, instead, a significant decline in milk fat concentration and yield in high-producing early

lactating Holstein cows in the study conducted by Yan *et al*. (1998). These authors attributed such a difference to a variation in forage intake, and consequently to an unbalanced F:C ratio, between their experimental groups. Concerning protein, it is known that its content and yield can be mainly influenced by the F:C ratio of the diet (enhancements can be obtained by increasing cows' concentrate intake) and by the amount and source of dietary protein and fat (Jenkins & McGuire 2006). In the present trial, the amount and source of dietary protein and fat were identical between the administered diets. Moreover, even if forage and concentrate DM intakes were not separately evaluated, it is plausible to hypothesize that they were comparable between TMR- and SR-fed cows. In fact, the substitution rate between roughage and concentrates is known to be of scarce importance in cases of low concentrate supplementation levels and when using hay instead of silage as roughage component of the diet (Faverdin *et al*. 1991). The lack of significant differences in fat and protein concentrations and yields between TMR- and SR-fed Aosta cows can be therefore attributed to analogous forage and concentrate consumption achievable with the two feeding strategies. Finally, obtained results regarding lactose content and yield were expected as these parameters are usually not significantly modified by nutritional manipulation, particularly if considering normal range diets for dairy cows (Jenkins & McGuire 2006).

The statistical analysis showed that the sampling date significantly affected milk gross composition. Particularly, milk protein content showed higher values in the period from the 15th to the 20th week of lactation if compared to the previous weeks (from the 10th to the 14th), while milk lactose content decreased during the whole experimental period. Milk fat content only showed a tendency towards slightly higher values at the end of the

trial. Such variations in the concentrations of fat, protein and lactose were expected as they usually occur with the advance of lactation stage (Auldist *et al*. 1998). The interaction between feeding technique and sampling date did not significantly influence the content and yield of milk main constituents.

Effect of feeding technique on milk fatty acid profile

To the best of our knowledge, this trial reports for the first time the FA profile of milk fat from cows fed concentrate and forage separately distributed or as a mixed ration. Results concerning groups of FA and individual FA in milk are shown in Tables 3 and 4, respectively.

Absolute values for the detected FA were comparable to those reported by other authors (Collomb *et al*. 2008) with dairy cows reared in the Alps and fed similarly (high-roughage - more than 80% - and low-concentrate diets) to Aosta cows. Milk concentrations of C18:2 *t*10*c*12, *iso*- and *aiso*-branched-chain FA, and linear odd-chain FA whose synthesis is highly dependent on rumen environment (Bauman & Griinari 2003; Fievez *et al*. 2012) were not significantly affected by the feeding technique. Under the chromatographic conditions applied in this trial, C18:1 *t*10, which is also usually associated to a lowering in ruminal pH values (Bauman & Griinari 2003), coeluted with other *trans*-octadecenoic isomers (C18:1 *t*6-11). Therefore, it was not possible to assess whether or not the feeding technique had a significant effect on its concentration in milk fat. However, it is known that C18:1 *t*10 is produced within the rumen by means of a reduction of C18:2 *t*10*c*12 (Bauman & Griinari 2003). Therefore, the lack of significant differences in the C18:2 *t*10*c*12 concentration in milk fat between groups leads to reasonably hypothesize that C18:1 *t*10 as well did not differ considerably according to the feeding method applied. It is worth mentioning, in

addition, that C18:2 *t*10*c*12 was detected in milk from both TMR- and SR-fed cows only in traces (<0.01 g 100 g^{-1} fat), while alterations of ruminal environment are usually associated with higher concentrations in milk fat (Peterson *et al*. 2003). The obtained results are also consistent with the lack of significant differences in milk fat concentrations and yields, which suggests that no milk fat depression occurred during the trial.

Besides ruminal pH, the extent of biohydrogenation occurring within the rumen can be significantly influenced by the amounts of dietary unsaturated FA ingested by the animals (Troegeler-Meynadier *et al*. 2003). However, in the present study the same diet ingredients were fed to the two groups of cows and comparable intakes were observed. Therefore any possible change in the extent of ruminal biohydrogenation should be ascribed to variations in ruminal pH values.

The results obtained in our trial showed that milk concentrations of: i) oleic acid and its detected ruminal biohydrogenation intermediate products (C18:1 *t*6-11 and C18:1 *c*11) (Mosley *et al*. 2002; Proell *et al*. 2002), ii) linoleic acid and its detected ruminal biohydrogenation intermediate products (C18:2 *c*9*t*11, *t*10c12, *t*9*t*11, *t*9*t*12, *t*8*c*12, *c*9*t*12, *t*11*c*15, *t*9*c*12 and C18:1 *c*9, *c*11, *c*12 and C18:1 *t*6 to *t*16) (Collomb *et al*. 2004; Lee & Jenkins 2011a; Honkanen *et al*. 2012), iii) α-linolenic acids and its detected ruminal biohydrogenation intermediate products (C18:2 *c*9*t*11, *t*11*c*13, *c*9*c*11, *t*10*c*12, *t*9*t*11, *t*11*c*15, *c*9*t*13 and C18:1 *c*11, *c*12 and C18:1 *t*10 to *t*16) (Collomb *et al*. 2004; Destaillats *et al*. 2005; Shingfield *et al*. 2010; Lee & Jenkins 2011b), and iv) stearic acid (C18:0) as the final end product of the entire biohydrogenation process (Shingfield *et al*. 2010) did not statistically differ between SR- and TMR-fed cows. Such results suggest

that the feeding technique had no outstanding effects on the extent of biohydrogenation occurring within the rumen.

The absence of significant differences in milk compounds used as indirect non-invasive markers of ruminal pH variations and in the extent of ruminal biohydrogenation indicates that pH values were comparable between the two experimental groups. Explanations could be ascribed to the high F:C ratio and the frequent administration of concentrate applied with the SR feeding strategy. No beneficial effects on rumen function determined by TMR feeding if compared to separate feeding of concentrate as frequent meals during the day were already previously observed by Yrjänen *et al*. (2003). Our results seem also to corroborate the finding by Fan *et al*. (2002) and Cao *et al*. (2010), who showed similar pH values in ruminally fistulated dairy cows fed TMR or diet ingredients separately, with administered diets characterized by a F:C ratio of approximately 50:50 and comparable milk production levels as those achieved in the present study.

The lack of considerable variations in the overall FA profile between SR- and TMR-fed cows in our trial confirm as well earlier findings by Cooke *et al*. (2004), who reported no significant changes in the FA profile of beef intramuscular fat as the consequence of diet ingredients administration as a TMR or in a discrete format. On the basis of the obtained results, it is possible to affirm that the feeding method did not have remarkable effects on the quality of the lipid fraction of milk. The cheese compositional variability in FA is known to depend mainly on the composition of the original milk (Lucas *et al*. 2006; Renna *et al*. 2009). Therefore, both feeding strategies can be conveniently applied within the Fontina PDO cheese production chain without any alteration in the quality of the lipid fraction of the cheese.

The sampling date significantly affected many of the detected FA. The observed changes were quantitatively of negligible importance since the maximum extent of the variation was generally around 10-18%. Moreover, the majority of these changes occurred during the experimental period (weeks 10 to 20 into lactation) without any clear increasing or decreasing trend (data not shown). It is known that milk fat composition changes with lactation stage. However, the main changes in milk FA usually occur in the early lactation period, particularly during the first 10 weeks of lactation (Palmquist *et al*. 1993; Craninx *et al*. 2008). Therefore, the changes observed in our study are likely not to be ascribed to a significant effect of lactation week on milk FA, but probably to slight variations (up to 1.46 kg $head^{-1}$ day^{-1}) in DM effectively ingested by the animals during the trial. The interaction term (FT × SD) did not significantly affect the FA composition of milk.

In conclusion, dual-purpose and of medium genetic merit Aosta cattle in mid-lactation offered the same high forage (61% of total DM) diet either as a TMR or with concentrates offered separately from forages six times daily using computerised self feeders showed comparable DM intake, milk yield, milk gross composition and FA profile of milk fat. Mixing diet ingredients or feeding them separately had no significant influence on milk concentrations of specific FA (C18:2 *t*10*c*12, *iso*- and *aiso*-branched chain FA, and linear odd-chain FA) used as indirect markers of rumen function. Milk concentrations of dietary unsaturated FA, as well as those of their ruminal biohydrogenation intermediate and final products, were not affected by the feeding method applied, suggesting that the latter did not influence the extent of ruminal biohydrogenation. Fatty acids results indirectly indicate that ruminal pH did not vary

considerably between groups. Both tested feeding strategies can be conveniently applied without any alteration in the quality of the lipid fraction of milk.

ACKNOWLEDGMENTS

The authors wish to thank their colleague Vanda Malfatto, Dr. Andrea Barmaz, Dr. Luca Vernetti-Prot, and Dr. Patrick Celesia for their careful technical assistance. Financial support of the Institut Agricole Régionale, Settore di Zootecnia e Industria Lattiero-Casearia (Aosta, Italy) is also gratefully acknowledged.

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Table 1. Proximate composition (g kg^{-1} DM, unless otherwise stated) and fatty acid profile (% of total FAME) of the experimental feedstuffs.

	Experimental feedstuffs		
	Hay (first cut)	Hay (second cut)	Concentrate
Main nutrients			
DM (g kg^{-1})	915	913	893
Ash	89	124	78
CP	124	136	151
EE	19	22	35
NDF	627	604	226
ADF	336	300	166
Starch	15	15	387
NSC†	141	114	510
NE_L (MJ kg^{-1} DM)	4.56	4.84	7.24
Fatty acids			
C14	8.30	17.64	3.00
C14:1	0.30	0.40	0.05
C16	22.74	20.91	10.71
C16:1 *c*9	1.08	0.73	0.12
C18	8.91	4.18	3.99
C18:1 *c*9	7.13	4.27	21.27
C18:1 *c*11	0.61	0.55	1.46
C18:2 *c*9*c*12	18.89	15.40	31.59
C20	2.88	0.94	0.17
C18:3 *c*9*c*12*c*15	29.19	34.98	27.65
Σ SFA	42.82	43.66	17.87
Σ MUFA	9.11	5.96	22.89
Σ PUFA	48.07	50.38	59.24

Abbreviations: DM, dry matter; FAME, fatty acid methyl ester; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; NSC, nonstructural carbohydrates; NE_L, net energy for lactation; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

† Calculated as 1000 - (NDF + CP + EE + ash).

Table 2. Milk yield and gross composition of Aosta Red Pied cows fed hay and concentrate separately (SR) or as a total mixed ration (TMR).

	Feeding technique		P†	
	SR (n = 132)‡	TMR (n = 132) ‡	FT	SD
Milk yield (kg head^{-1} day^{-1})	17.4	17.5	ns	***
Milk composition (g kg^{-1})				
Fat	36.4	35.2	ns	0.08
Protein	33.5	32.8	ns	***
Lactose	47.3	47.4	ns	***
Component yield (g head^{-1} day^{-1})				
Fat	607.9	613.4	ns	***
Protein	567.4	572.7	ns	***
Lactose	805.5	829.7	ns	***

Abbreviations: n, number of milk samples; FT, feeding technique; SD, sampling date.

† Probability: *** P≤0.001; ns, not significant (P>0.05). The P-value is shown if, thus being not significant, it shows a tendency (0.05<P<0.10). The effect of interaction between feeding technique and sampling date (FT × SD) was not significant; therefore significance are only presented for feeding technique (FT) and sampling date (SD).

‡ Total number of milk samples equal to 132 (12 cows × 11 sampling days).

Table 3. Mean contents (g 100 g^{-1} fat) of groups of fatty acids in milk fat of Aosta Red Pied cows fed hay and concentrate separately (SR) or as a total mixed ration (TMR).

	Feeding technique		P[†]	
	SR (n = 72)[‡]	TMR (n = 72)[‡]	FT	SD
Σ short chain[a]	9.78	9.61	ns	***
Σ medium chain[b]	48.24	47.00	ns	**
Σ long chain[c]	28.20	26.80	ns	ns
Σ saturated[d]	63.05	61.53	ns	ns
Σ branched chain[e]	2.71	2.63	ns	**
Σ *iso* branched chain[f]	1.23	1.20	ns	**
Σ *aiso* branched chain[g]	1.48	1.43	ns	**
Σ monounsaturated[h]	19.84	18.82	ns	ns
Σ C18:1[i]	16.84	16.08	ns	ns
Σ C18:1 *trans*[j]	1.74	1.58	ns	*
Σ polyunsaturated[k]	3.35	3.10	ns	ns
Σ C18:2[l]	2.48	2.28	ns	ns
Σ C18:2 *trans*[m]	1.05	0.99	ns	*
Σ *trans* without CLA[n]	4.33	3.98	ns	ns
Σ n3 FA[o]	0.80	0.75	ns	**
Σ n6 FA[p]	2.35	2.17	ns	*
n6/n3	2.99	2.94	ns	***
Σ CLA[q]	0.54	0.50	ns	**
Σ unsaturated[r]	23.20	21.91	ns	ns
HSFA[§]	42.16	41.22	ns	*

Abbreviations: n, number of milk samples; FT, feeding technique; SD, sampling date; CLA, conjugated linoleic acid; FA, fatty acids; HSFA, hypercholesterolemic saturated fatty acids.

[†] Probability: *** P≤0.001; ** P≤0.01; * P≤0.05; ns, not significant (P>0.05). The effect of interaction between feeding technique and sampling date (FT × SD) was not significant; therefore significance are only presented for feeding technique (FT) and sampling date (SD).

[‡] Total number of milk samples equal to 72 (12 cows × 6 sampling days).

[§] Calculated as: C12 + C14 + C16.

[a] C4, C5, C6, C7, C8, C10, C10:1.

[b] C12, C13 *iso*, C13 *aiso*, C12:1 *c* + C13, C14 *iso*, C14, C15 *iso*, C14:1 *t*, C15 *aiso*, C14:1 *c*, C15, C16 *iso*, C16, C17 *iso*, C16:1 *t*, C17 *aiso*, C16:1 *c*.

[c] C17, C18 *iso*, C17:1 *t*, C18 *aiso*, C18, Σ C18:1, C19, Σ C18:2, C20, C20:1 *t*, C18:3 *c*6*c*9*c*12, C20:1 *c*5, C20:1 *c*9, C20:1 *c*11, C18:3 *c*9*c*12*c*15, C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10,

C18:2 *t*11*c*13 + *c*9*c*11, C18:2 *t*9*t*11, C20:2 *c,c* n6, C22, C20:3n6, C20:3n3, C20:4n6 (AA), C20:5n3 (EPA), C22:5n3 (DPA), C22:6n3 (DHA).

[d] C4, C5, C6, C7, C8, C10, C12, Σ branched chain, C14, C15, C16, C17, C18, C19, C20, C22.

[e] C13 *iso* + *aiso*, C14 *iso*, C15 *iso* + *aiso*, C16 *iso*, C17 *iso* + *aiso*, C18 *iso* + *aiso*.

[f] C13 *iso*, C14 *iso*, C15 *iso*, C16 *iso*, C17 *iso*, C18 *iso*.

[g] C13 *aiso*, C15 *aiso*, C17 *aiso*, C18 *aiso*.

[h] C10:1, C12:1 *c* + *C*13, C14:1 *ct*, C16:1 *ct*, C17:1 *t*, Σ C18:1, C20:1 *t*, C20:1 *c*5, C20:1 *c*9, C20:1 *c*11.

[i] C18:1 *t*5, *t*6-11, *t*12-14 + *c*6-8, *c*9, *c*11, *c*12, *c*14 + *t*16.

[j] C18:1 *t*5, *t*6-11, *t*12-14 + *c*6-8.

[k] Σ C18:2, C18:3 *c*6*c*9*c*12, C18:3 *c*9*c*12*c*15, C20:2 *c,c* n6, C20:3n3, C20:3n6, C20:4n6 (AA), C20:5n3 (EPA), C22:5n3 (DPA), C22:6n3 (DHA).

[l] C18:2 *t,t*-NMID + *t*9*t*12, *c*9*t*13 + *t*8*c*12, *c*9*t*12, *c,c*-MID + *t*8*c*13, *t*11*c*15, *t*9c12, *c*9*c*12, *c*9*c*15, *c*9*t*11 + *t*7*c*9 + *t*8*c*10, *t*10*c*12, *t*11*c*13 + *c*9*c*11, *t*9*t*11.

[m] C18:2 *t,t*-NMID + *t*9*t*12, *c*9*t*13 + *t*8*c*12, *c*9*t*12, *c,c*-MID + *t*8*c*13, *t*11*c*15, *t*9*c*12, C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10, C18:2 *t*10*c*12, C18:2 *t*11*c*13 + *c*9*c*11, C18:2 *t*9*t*11.

[n] C14:1 *t*, C16:1 *t*, C17:1 *t*, Σ C18:1 *t* , Σ C18:2 *t* (without CLA *trans*), C20:1 *t*.

[o] C18:2 *t*11*c*15 + C18:2 *c*9*c*15, C18:3 *c*9*c*12*c*15, C20:3n3, C20:5n3 (EPA), C22:5n3 (DPA), C22:6n3 (DHA).

[p] C18:1 *t*12, C18:1 *c*12, C18:2 *t,t*-NMID + *t*9*t*12, C18:2 *c*9*t*12, C18:2 *t*9*c*12, C18:2 *c*9*c*12, C18:3 *c*6*c*9*c*12, C20:2 *c,c* n6, C20:3n6, C20:4n6 (AA).

[q] C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10, *t*10*c*12, *t*11*c*13 + *c*9*c*11, *t*9*t*11.

[r] C10:1, C12:1 *c* + C13, C14:1 *ct*, C16:1 *ct*, C17:1 *t*, Σ C18:1, Σ C18:2, C20:1 *t*, C18:3 *c*6*c*9*c*12, C20:1 *c*5, C20:1 *c*9, C20:1 *c*11, C18:3 *c*9*c*12*c*15, C18:2 *c*9*t*11 + *t*7*c*9 + *t*8*c*10, C18:2 *t*11*c*13 + *c*9*c*11, C18:2 *t*10*c*12, C18:2 *t*9*t*11, C20:2 *c,c* n6, C20:3n6, C20:3n3, C20:4n6 (AA), C20:5n3 (EPA), C22:5n3 (DPA), C22:6n3 (DHA).

Table 4. Mean contents (g 100 g^{-1} fat) of individual fatty acids in milk fat of Aosta Red Pied cows fed hay and concentrate separately (SR) or as a total mixed ration (TMR).

	Feeding technique		P†	
	SR (n = 72)‡	TMR (n = 72) ‡	FT	SD
C4	3.08	2.95	ns	***
C5	0.02	0.02	ns	**
C6	2.14	2.10	ns	**
C7	0.02	0.02	ns	**
C8	1.30	1.30	ns	***
C10	2.84	2.86	ns	**
C10:1	0.38	0.35	ns	***
C12	3.35	3.33	ns	**
C13 *iso*	0.04	0.04	ns	***
C13 *aiso*	0.10	0.09	ns	***
C12:1 *c* + C13	0.19	0.18	ns	ns
C14 *iso*	0.19	0.18	ns	**
C14	10.25	10.11	ns	***
C15 *iso*	0.31	0.30	ns	***
C14:1 *t*	0.01	0.01	ns	**
C15 *aiso*	0.54	0.53	ns	***
C14:1 *c*	0.92	0.93	ns	***
C15	1.22	1.22	ns	***
C16 *iso*	0.36	0.35	ns	*
C16	28.53	27.82	ns	**
C17 *iso*	0.33	0.32	ns	ns
C16:1 *t*	0.06	0.05	ns	*
C17 *aiso*	0.64	0.61	ns	ns
C16:1 *c*	1.07	1.08	ns	***
C17	0.56	0.53	ns	***
C18 *iso*	0.01	0.01	ns	***
C17:1 *t*	0.06	0.06	ns	ns
C18 *aiso*	0.21	0.20	ns	**
C18	6.74	6.42	ns	***
C18:1 *t*5	0.01	0.01	ns	*
C18:1 *t*6-11	1.41	1.26	ns	*
C18:1 *t*12-14 + *c*6-8	0.33	0.31	ns	*
C18:1 *c*9	14.23	13.78	ns	*
C18:1 *c*11	0.46	0.44	ns	ns
C18:1 *c*12	0.16	0.16	ns	*
C18:1 *c*14 + *t*16	0.19	0.18	ns	ns
C19	0.09	0.08	ns	*
C18:2 *t*,*t*-NMID + *t*9*t*12	0.07	0.06	ns	ns

C18:2 *c*9*t*13 + *t*8*c*12	0.06	0.06	ns	*
C18:2 *c*9*t*12	0.09	0.09	ns	ns
C18:2 *c*,c-MID + *t*8*c*13	0.09	0.09	ns	ns
C18:2 *t*11*c*15	0.08	0.08	ns	**
C18:2 *t*9*c*12	0.12	0.11	ns	**
C18:2 *c*9*c*12 (LA)	1.41	1.28	ns	***
C18:2 *c*9*c*15	0.01	0.01	ns	***
C20	0.13	0.13	ns	ns
C20:1 *t*	0.03	0.02	ns	ns
C18:3 *c*6*c*9*c*12	0.02	0.02	ns	ns
C20:1 *c*5	<0.01	<0.01	ns	*
C20:1 *c*9	0.14	0.13	ns	ns
C20:1 *c*11	0.04	0.04	ns	***
C18:3 *c*9*c*12*c*15 (ALA)	0.58	0.54	ns	**
CLA *c*9*t*11 + *t*7*c*9 + *t*8*c*10	0.51	0.47	ns	**
CLA *t*10*c*12	<0.01	<0.01	ns	ns
CLA *t*11*c*13 + *c*9*c*11	0.02	0.02	ns	*
CLA *t*9*t*11	0.01	0.01	ns	ns
C20:2 *c*,*c* n6	0.02	0.02	ns	**
C22	0.03	0.03	ns	***
C20:3 n6	0.05	0.05	ns	ns
C20:3 n3	0.01	0.01	ns	*
C20:4 n6 (AA)	0.07	0.07	ns	ns
C20:5 n3 (EPA)	0.06	0.06	ns	**
C22:5 n3 (DPA)	0.06	0.06	ns	ns
C22:6 n3 (DHA)	<0.01	<0.01	ns	ns

Abbreviations: n, number of milk samples; FT, feeding technique; SD, sampling date; *c*, *cis*; *t*, *trans*; NMID, non methylene interrupted diene; MID, methylene interrupted diene; LA, linoleic acid; ALA, α-linolenic acid; CLA, conjugated linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

† Probability: *** P≤0.001; ** P≤0.01; * P≤0.05; ns, not significant (P>0.05). The effect of interaction between feeding technique and sampling date (FT × SD) was not significant; therefore significance are only presented for feeding technique (FT) and sampling date (SD).

‡ Total number of milk samples equal to 72 (12 cows × 6 sampling days).