Effect of three species of herbage (*Medicago sativa, Lolium multiflorum, Avena sativa*) on *in vitro* ruminal production of conjugated linoleic and vaccenic acids

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Received September 17, 2008; accepted December 5, 2008

ABSTRACT

Little information is available about the effect of different forage species on the rumen biohydrogenation process. The aim of the present work is to compare the *in vitro* production of CLA and C18:1 isomers after incubation of three different herbage species in rumen liquor from sheep. Pasture herbage samples of lucerne (*Medicago sativa*; MS), ryegrass (*Lolium multiflorum*; LM) and oats (*Avena sativa*; AS) were submitted to *in vitro* fermentation with sheep rumen inoculum. Samples were collected at 2, 4, 6 and 8 hours of fermentation. The fatty acid profile of MS was characterised by 11.62 (g/100 g of lipid extract) of linoleic acid (LA) and 27.08 (g/100 g of lipid extract) of α-linolenic acid (LNA), whereas LA in the other two herbages was 6.60 (g/100 g of lipid extract) and 6.95 (g/100 g of lipid extract) in AS and LM, respectively; LNA was 52.20 (g/100 g of lipid extract) and 54.49 (g/100 g of lipid extract) in AS and LM, respectively. The crude fat content of botanical species was respectively 11.90 (g/100g DM) for AS, and 15.77 (g/100g DM) for LM and 26.17 (g/100g DM) for MS. Rumenic acid (RA, *cis*-9, *trans*-11 CLA) was the predominant CLA isomer and the maximum yield was attained with AS after 6 hours of fermentation (0.81 g/100 g of lipid extract); RA concentration remained quite low with the other two herbages. The concentration of the other isomer (trans-10, cis-12 CLA) was always very low; the maximum yield (0.09 g/100 g of lipid extract) was reached after 6 hours with AS. The maximum yield of vaccenic acid (VA, trans-11 C18:1) was reached after 8 hours with MS (2.64 g/100 g of lipid extract). This herbage also produced the highest amount of trans-10 C18:1 at 6 and 8 hours (0.17 g/100 g of lipid extract). AS appeared to have induced the highest amounts of RA relative to the other two forages. The differences in conjugated dienes and C18:1 isomers content during fermentation could be due not only to different amounts of LA or LNA in the herbage, but also to different releasing times of FA from the plant substrate.

Key words: Forage herbage species, Conjugated linoleic acid, *In vitro* rumen fermentation.

RIASSUNTO

EFFETTI DI TRE SPECIE FORAGGERE (*MEDICAGO SATIVA, LOLIUM MULTIFLORUM, AVENA SATIVA*) SULLA PRODUZIONE RUMINALE *IN VITRO* DELL'ACIDO LINOLEICO CONIUGATO E DELL'ACIDO VACCENICO

*Le specie botaniche presenti nel pascolo sono caratterizzate da una composizione chimica differente compreso il contenuto in acido linoleico (LA) e linolenico (LNA), importanti precursori ruminali dell'acido rumenico (RA, cis-9, trans-11 C18:2) e vaccenico (VA, trans-11 C18:1). In letteratura sono poche le informazioni disponibili sul comportamento delle singole specie foraggiere a livello delle bioidrogenazioni degli acidi grassi polinsaturi. Campioni freschi di Erba Medica (*Medicago sativa*; MS), di Lolium (*Lolium multiflorum*; LM) e di Avena (*Avena sativa*; AS) sono stati fermentati* in vitro *con liquido ruminale ovino. I campionamenti sono stati effettuati a 2, 4, 6 ed 8 h dall'inizio della fermentazione. Il profilo in acidi grassi di MS era caratterizzato da 11,62 (g/100 g di estratto lipidico) di LA e da 27,08 (g/100 g di estratto lipidico) di LNA mentre il contenuto di LA in AS e LM era rispettivamente 6,60 (g/100 g di estratto lipidico) e 6,95 (g/100 g di estratto lipidico). Il contenuto di LNA era invece 52,20 (g/100 g di estratto lipidico) per AS e 54,49 (g/100 g di estratto lipidico) per LM. Le tre specie botaniche erano, inoltre, caratterizzate da un tenore lipidico grezzo pari a 11,90 (g/100g SS) per AS, 15,77 (g/100g SS) per LM e 26,17 (g/100g SS) per MS. Il processo fermentativo delle tre specie botaniche è stato caratterizzato da un andamento crescente del contenuto in grasso. Durante la fermentazione di tutte e tre le essenze foraggere, l'isomero preminente dei CLA è stato RA che ha raggiunto la massima concentrazione dopo 6 h. La percentuale più elevata di RA è stata raggiunta con AS (0,81 g/100g di estratto lipidico), mentre con gli altri due foraggi è rimasta bassa. La fermentazione di MS, invece, è stata caratterizzata da una maggior produzione di VA (2,64 g/100 g di estratto lipidico, 8 h) e di trans-10 C18:1 (0,17 g/100 g di estratto lipidico, 6 h e 8 h). AS, pertanto, risulta favorire la produzione ruminale di RA rispetto agli altri due foraggi. L'efficienza delle bioidrogenazioni ruminali sembra dipendere dalla composizione chimica del foraggio, con particolare riferimento al contenuto in NDF, NSC. Le differenze nel contenuto degli isomeri del C18:1 e del CLA sembrano essere influenzate dal differente contenuto in LA o LNA delle singole specie botaniche, ma si può supporre che anche la differente capacità di rilascio degli acidi grassi dalla matrice possa avere una notevole importanza.*

Parole chiave: *Specie foraggere, Acido linoleico coniugato, Fermentazioni ruminali* in vitro.

Introduction

In the rumen, biohydrogenation reduces unsaturated fatty acids (UFA) and contributes to an accumulation of *cis* and *trans* isomers, including conjugated linoleic acid (CLA), in milk and meat. Rumenic acid (RA, cis-9, trans-11 C18:2) is the main CLA isomer in milk and meat from ruminants and originates partly from ruminal biohydrogenation of linoleic acid (LA, *cis*-9, *cis*-12 C18:2) and mostly (more than 80%) by enzymatic desaturation of vaccenic acid (VA, *trans*-11 C18:1) in the animal tissues (Corl *et al.*, 2001). Moreover, VA is produced by biohydrogenation of RA and of both α and γ linolenic acids (α-LNA, *cis*-9, *cis*-12, *cis*-15 C18:3; γ-LNA, *cis*-6, *cis*-9, *cis*-12 C18:3) during rumen fermentation of feed rich in LNA or in LA (Griinari and Bauman, 1999). Since the extent and type of rumen biohydrogenation determines the amount and the type of fatty acids (FA) leaving the rumen, there is growing interest in studying the process of biohydrogenation. Several *in vitro* experiments have evaluated the effect of different diets on the biohydrogenation process in dairy cows, including the effect of lipid source, of forage:concentrate ratio and of the inclusion of fresh forage (Kelly *et al.*, 1998; Loor *et al.*, 2004). Moreover, the effect of dried relative to fresh forage on the *in vitro* appearance of VA and CLA during incubation was reported by Ribeiro *et al.* (2005) and Buccioni *et al.* (2007).

Since pasture is the main feed source in several extensive rearing systems, such as dairy sheep in the Mediterranean or dairy cows in the Alpine area, some recent papers have focused on the effect of individual forage species on FA composition of ruminant products. Cabiddu *et al.* (2005) found that the polyunsaturated FA (PUFA) level in milk was higher in ewes grazing pure legumes and a grass legume mixture than in milk of ewes grazing pure grass pastures. Collomb *et al.* (2002) and Mele *et al.* (2007) found a correlation between the botanical composition of pasture and the FA content in bovine and ovine milk, respectively. Nevertheless, little or no information are available about the effect of different forage species on the rumen biohydrogenation process. Since individual forage species are characterised by different chemical compositions, including varied levels of LA and LNA, the rumen degradation of individual herbage species could lead to differences in the appearance and accumulation of biohydrogenation products in the rumen liquor, especially for C18:1 or CLA isomers content.

The aim of the present work is to compare the *in vitro* appearance of CLA and C18:1 isomers after incubation of three different herbage species in rumen liquor from sheep.

Material and methods

Artificial rumen

A gas production apparatus was used (Buccioni *et al.*, 2001; Antongiovanni *et al.*, 2002) consisting of a thermostatic chamber (39 °C) equipped with forty 250 ml glass fermentation vessels, each stirred continuously and connected to an electronic pressure transducer (pre-set at 65 kPa) and an electronic gas valve. When the inside gas pressure reached a pre-set value, the valve opened to release about 2 ml of gas. The fermentation pattern was monitored by PC software (Labview 5.0, National Instr., Austin, TX).

Rumen inoculum

A whole rumen from a ewe fed a diet of tall fescue hay and maize meal (70/30 DM) was collected at the slaughter house and transferred to the laboratory in a thermostatic box. The rumen was cut open and some of the content was squeezed out (Mauricio *et al.*, 2001) and strained through four layers of cheese cloth into a flask under a flux of CO_2, as described by Cone *et al.* (1996). An aliquot of 250 ml of the liquor was buffered by adding 750 ml of an artificial saliva solution (McDougall, 1948). Another sample of fresh rumen fluid was collected as the blank, and was analysed for initial fatty acid composition in order to test for the presence of possible artefacts (Table 1).

To avoid unsaturated FA oxidation, the fresh herbages (*Medicago sativa,* MS; *Avena sativa*, AS and *Lolium multiflorum*, LM) were immersed in liquid nitrogen immediately after being cut and were transported to the laboratory to be freeze dried (Ribeiro *et al.*, 2005). Herbage samples (1 g) were incubated with 100 ml inoculum, with 3 replicates per sample. Fermentation times were 2, 4, 6, and 8 h and pH was monitored throughout the fermentation.

Fresh plant sample analysis

Samples of the botanical species (MS, AS and LM) were oven dried at 60°C for 24 h. The dried samples were analysed for crude protein, ash and ether extracts that were determined according to procedures 954.01, 954.05 and 920.39 of AOAC (1990), respectively. Neutral detergent fibre (aNDF), acid detergent fibre (ADF) and acid detergent

Table 1. Fatty acid profile of fresh rumen fluid collected for the blank (g/100 g lipid extract).

Fatty acid	
C12:0	0.01
C14:0	0.04
C15:0 anteiso	0.01
C15:0	0.02
C16:0	0.21
C16:1	0.01
C17:0	0.01
C18:0	0.05
C18:1 cis-9	0.02
C18:1 cis-11	0.00
C18:1 cis-12	0.01
C18:1 trans- 9	0.00
C18:1 trans-10	0.00
C18:1 trans-11	0.01
C18:1 trans-12	0.02
C18:1 trans-13 +14	0.00
C18:2 cis-9, cis-12	0.02
C18:2 cis-9, trans- 11	0.00
C18:2 trans-10, cis-12	0.00
C18:3 cis-9, cis-12, cis-15	0.01

lignin (ADL) were assayed by sequential analysis with sodium sulfite and heat stable amylase, and were expressed inclusive of residual ash (Van Soest *et al.*, 1991). Non fibre carbohydrates (NSC) were calculated according to Cornell Net Carbohydrate and protein System (CNCPS; Licitra *et al.*, 1996). Moreover, the FA profile of the three botanical species was determined after the fat was extracted from the fresh herbage samples (Folch *et al.*, 1957), and after a two-step FA methylation process (Christie, 1982; Roach *et al.,* 2002): 1) methylation of free FA with diazomethane; and 2) trans-esterification of glycerides and phospholipids with sodium methylate in methanol (MeO^{-} Na^{+}/MeOH, 0.5 M).

Extraction and methylation of rumen fluid FA

At the end of the fermentation time, centrifugation (15 min at 500 x g) was used to separate residual feed from the rumen liquor (Ribeiro *et al.*, 2005). The rumen liquor was immediately freeze-dried and stored at -20°C. The samples were analysed for crude fat content according to procedure 920.39 of AOAC (1990). To determine the FA profile, sub-samples of the lyophilised material (0.5 g) were methylated with 2 ml of 0.5 mol/L sodium methoxide (10 min at 50°C) followed by 3 ml of 5% methanolic HCl (10 min at 50 °C) and FA methylesters (FAME) extracted using n-hexane (with C19:0 as the internal standard), as described by Park *et al.* (2001) and by Kramer *et al.* (1997). The FAME were separated on a GC equipped with a capillary column (CP-select CB for FAME Varian, Middelburg, the Netherlands: 100 m x 0.25 mm i.d.; film thickness 0.20 µm) and quantified using nonadecanoic acid (C19:0) methyl ester (Sigma Chemical Co., St. Louis, MO) as the internal standard. The injector and flame ionisation detector temperatures were 270 °C and 300 °C, respectively. The programmed temperature was 40 °C for 4 min, increased to 120 °C at a rate of 10 °C/min, maintained at 120 °C for 1 min, increased to 180 °C at a rate of 5 °C/min, maintained at 180 °C for 18 min, increased to 200 °C at a rate of 2 °C/min, maintained at 200 °C for 1 min, increased to 230 °C at a rate of 2 °C/min and maintained at this last temperature for 19 min. The split ratio was 1:100 and helium was the carrier gas with a flux of 1 ml/min. Individual FAME were identified by comparison of the relative retention times of FAME peaks from samples with those of the standard mixture 37 Component FAME Mix (Supelco, Bellefonte, PA). Individual *trans*-9 C18:1, *trans*-11 C18:1, *trans*-12 C18:1, *trans*-13 C18:1 (Supelco), individual *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Matreya Inc.), a CLA mix standard (Sigma Chemical Co) and published isomeric profiles (Griinari *et al.*, 1998; Kramer *et al.*, 2004) were used to identify *trans*-C18:1 and CLA isomers of interest. Nonadecanoic acid was used as an internal standard to avoid overestimation biases that can arise when results are expressed as a relative percentage of the area of analysed peaks (since areas of small peaks are not included). All FA composition results are expressed as g/100g of lipid extract.

Statistical analysis

Data were processed by GML of SAS (1999) using a linear model with two factors, herbage and fermentation time, with interaction:

$y_{ijk} = \mu + H_i + T_j + H_i \cdot T_j + e_{ijk}$

where y_{ij} is the observed value; µ is the overall mean; H_i the herbage (i = 1, 2, 3); T_j the fermentation time (j = 1 to 4); H_i T_j the interaction between herbage and fermentation time and e_{ij} the residual error. For simplicity, only one level of probability (*P< 0.05) was adopted for the significance of differences between means.

Results and discussion

For the duration of fermentation, pH was monitored and the values were stable at about 6.7 ± 0.1 for all samples. Martin and Jenkins (2002) suggested that culture pH significantly affects the production of *trans*- C18:1 and CLA isomers by mixed rumen bacteria, with the highest production occurring at pH 6.0. In our experiment, pH remained constant (near neutral) for the duration of fermentation, therefore changes in FA composition should be related to differences in FA release by forage species. Forage residual was removed by centrifugation at each sample time before the FA methylation, in order to analyse only the FA released by forage samples in the rumen liquor and microbial FA. In this way, FA composition of rumen liquor lipids only referred to FA released by the forages and to microbial FA and not to FA remaining in vegetable particles. Fatty acid profile of blank was poor in fatty acids, as consequence of treatment for inoculum preparation (Table 1). In fact, the rumen was obtained from a sheep slaughtered after 24 h of fasting and, after, the liquor was squeezed out and strained through four layers of cheese cloth to eliminate feed residues. This procedure makes it possible to obtain a rumen liquor with a low content of fatty acids because feed particles and bacteria associated to solid phase (SAB) are removed.

Chemical analysis of the herbage samples showed that AS was richer in ether extract and NFC (Table 2), whereas MS and LM were characterised by a similar content of total FA, twofold higher than that of AS (Table 3).

During the whole fermentation time, there was a progressive appearance of crude fat and FA in the rumen liquor for all three herbages. The increase of crude fat in rumen liquor was due to FA increase because the unsaponifiable fraction remained almost unvaried. The initial FA content in the lipid extract from the rumen liquor was less than 8% for all herbages, with the highest percentage for LM. After 8 h of fermentation, FA content was 98%, 76% and 31% of lipid extract for MS, AS and LM, respectively (Table 4). Release of FA from LM appeared faster relative to AS and MS, as reflected by the higher level of FA content in rumen liquor lipids after 2 h (Table 4). Nevertheless, after 4h the FA release from LM reached the highest level and the amount of FA in LM rumen liquor lipids did not vary until the end of fermentation. On the contrary, in the AS and MS rumen liquor, the FA pro-

Table 2. Chemical composition of the three fresh herbages (g/100 g DM).

		Avena sativa	*Lolium multiflorum*	*Medicago sativa*
DM	%	11.90	15.77	26.17
CP	"	9.80	15.94	15.50
EE	"	2.50	1.79	1.70
aNDF	"	33.10	43.50	56.00
ADF	"	19.00	26.75	36.59
ADL	"	4.50	1.80	6.32
Ash	"	7.30	11.96	9.10
NFC	"	55.10	26.81	28.58

Legend: DM, dry matter; CP, crude protein; EE, ether extract; aNDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NFC, non fibre carbohydrates.

Table 3. Fatty acid composition of the three fresh herbages (g/100 g fatty acids).

Fatty acid	*Avena sativa*	*Lolium multiflorum*	*Medicago sativa*
C14:0	0.25	0.12	0.28
C16:0	9.26	10.36	22.95
C18:0	0.30	0.64	3.29
C18:1 cis-9	0.69	0.83	1.19
C18:2 cis-9, cis-12	6.60	6.95	11.62
C18:3 cis-9, cis-12, cis-15	52.20	54.49	27.08
others	30.70	26.60	33.60
fatty acid/fat*	23.95	56.87	52.43

**total fatty acid concentration in crude fat.*

Table 4. Total fatty acid (g/100 g lipid extract) and lipid extract appearance (g/100 g DM) in the rumen liquor during fermentation of the three herbages. Data shown are the means of 3 replicates.

Herbage	2h	4h	6h	8h	SEM
		Fatty acid appearance (g/100 g lipid extract)			
Avena sativa	$5.83^{\alpha a}$	$19.18^{\beta a}$	$75.08^{\gamma a}$	$76.40^{\gamma a}$	0.64
Lolium multiflorum	$7.63^{\alpha b}$	$28.64^{\beta b}$	$29.89^{\beta b}$	$30.89^{\beta b}$	0.64
Medicago sativa	$5.72^{\alpha a}$	$12.31^{\beta c}$	$79.43^{\gamma c}$	$97.93^{\delta c}$	0.64
		Crude fat appearance (g/100 g DM)			
Avena sativa	1.16^{α}	1.31^{α}	1.56^{β}	1.82^{β}	0.10
Lolium multiflorum	0.96^{α}	1.26^{β}	$1.45^{\beta\gamma}$	1.72^{γ}	0.10
Medicago sativa	1.12^{α}	1.46^{β}	1.67^{β}	1.64^{β}	0.10

*Within a column, means with different Latin superscripts are significantly different (*P< 0.05); within a row, means with different Greek superscripts are significantly different (*P< 0.05).*

gressively accumulated until the maximum level was reached at 6 h and 8 h, respectively. The different behaviour of FA release may affect the FA profile of rumen liquor because only the fatty acids really accessible to microorganisms can be biohydrogenated; after the fat release from feed matrix, glycerides and phospholipids are hydrolyzed to free fatty acids and glycerol. So, lipolysis is the prerequisite of rumen biohydrogenation because the unsaturated fatty acid can be hydrogenated only if the carbossilic group is free; (Bickerstaffe *et al.*, 1972; Jenkins, 1993).

Branched and odd chain fatty acid

Branched and odd chain FA (BOCFA) concentration increased during fermentation, regardless of the herbage incubated (Table 5 and 6), but *iso*-FA content in lipid extract was always lower than that of *anteiso*-FA. There were some differences among the herbages revealed during fermentation. LM seemed to accumulate a higher amount of total *iso*-FA at the beginning of the fermentation time relative to MS and AS. As fermentation proceeded, *iso*-FA content slightly increased in rumen liquor incubated with LM, whereas for MS and AS the *iso*-FA content increased more than fourfold (Table 5). At the end of fermentation, the highest content of *iso*-FA was in the rumen liquor incubated with MS, with *iso*-C16:0 the main FA.

The content of *iso*-C15:0 significantly increased after 6 h of fermentation for all forage species (Table 5). Unlike *iso*-C15:0, *iso*-C17:0 content increased with fermentation time only for AS and MS, although *iso*-C17:0 content did not differ among herbages at the end of fermentation (Table 5). The content of *iso*-C14:0 increased with fermentation time in all samples, but at the end of fermentation time the rumen liquor incubated with AS showed the highest content.

Table 5. Total iso fatty acid appearance in the rumen liquor at different fermentation times (g/100 g lipid extract). Data shown are the means of 3 replicates.

Fatty acid		Fermentation time, h				SEM
		2	4	6	8	
C14:0 *iso*	*Avena sativa*	$0.09^{\alpha a}$	$0.15^{\alpha\beta a}$	0.17^{β}	$0.31^{\gamma a}$	0.02
	Lolium multiflorum	$0.16^{\alpha b}$	$0.21^{\alpha\beta b}$	$0.21^{\alpha\beta}$	$0.24^{\beta b}$	
	Medicago sativa	$0.14^{\alpha b}$	$0.20^{\alpha\beta b}$	$0.20^{\alpha\beta}$	$0.24^{\beta b}$	
C15:0 *iso*	*Avena sativa*	0.05^{α}	0.09^{α}	0.40^{β}	0.40^{β}	0.03
	Lolium multiflorum	0.10^{α}	0.11^{α}	0.37^{β}	0.45^{β}	
	Medicago sativa	0.10^{α}	0.14^{α}	0.38^{β}	0.46^{β}	
C16:0 *iso*	*Avena sativa*	$0.16^{\alpha a}$	$0.39^{\beta a}$	$0.52^{\gamma a}$	$0.67^{\delta a}$	0.04
	Lolium multiflorum	$0.47^{\alpha b}$	$0.56^{\alpha\beta b}$	$0.59^{\alpha\beta a}$	$0.62^{\beta a}$	
	Medicago sativa	$0.00^{\alpha c}$	$0.61^{\beta b}$	$0.75^{\gamma b}$	$0.79^{\gamma b}$	
C17:0 *iso*	*Avena sativa*	$0.06^{\alpha a}$	$0.12^{\alpha a}$	$0.13^{\alpha a}$	0.23^{β}	0.02
	Lolium multiflorum	0.19^{b}	$0.18^{\alpha b}$	0.19^{ab}	0.22	
	Medicago sativa	$0.09^{\alpha a}$	$0.19^{\beta b}$	$0.22^{\beta b}$	0.22^{β}	
Total	*Avena sativa*	$0.36^{\alpha a}$	$0.75^{\beta a}$	$1.22^{\gamma a}$	$1.61^{\delta a}$	0.03
	Lolium multiflorum	$0.92^{\alpha b}$	$1.06^{\beta b}$	$1.36^{\gamma b}$	$1.53^{\delta a}$	
	Medicago sativa	$0.33^{\alpha a}$	$1.14^{\beta b}$	$1.55^{\gamma c}$	$1.71^{\delta b}$	

*Within a column, means with different Latin superscripts are significantly different (*P< 0.05); within a row, means with different Greek superscripts are significantly different (*P< 0.05).*

Similar to *iso*-FA, anteiso- and *odd*-chain FA content increased with fermentation time, but the forage samples showed different profiles. When fermentation was initiated, lipid extract of rumen liquor incubated with AS had the lowest content of *anteiso*- and *odd*-chain FA, but by the end of the fermentation time it had the highest content (Table 6). At the end of fermentation, LM showed the lowest content of anteiso- and odd-chain FA, while MS was intermediate between LM and AS. In particular, AS had significantly higher *anteiso*-C15:0, *anteiso*-C17:0 and C17:0 content as compared to LM and MS at 8 h of fermentation. LM had the highest content of C15:0 at the beginning of the fermentation time, but the lowest content at the end of fermentation as a consequence of a high increase of C15:0 in AS and MS. C13:0 was detected at trace levels in the lipid extract of rumen liquor and its content did not differ among herbages (Table 6).

Chemical composition of the herbages may concur to affect the FA composition of rumen liquor. Indeed, the chemical composition of the diet can affect the distribution of rumen bacteria species and their efficiency (Archimede *et al.*, 1995); in particular, the number of cellulosolytic bacteria tends to increase with a diet rich in NDF or forage (Weimer *et al.*, 1999). Moreover, Shingfield *et al.* (2005) and Nielsen *et al.* (2004) showed that changes in dietary starch and NDF affect the ratio of *odd*-chain, *iso*- and *anteiso*-FA in milk, as a consequence of changes in rumen microbial strains. In this study, although only individual herbages were incubated with rumen liquor, changes in chemical composition of the herbages seemed to significantly affect the accumulation of BOCFA. The fermentation of all three herbage species showed a progressive increase of BOCFA in liquid rumen lipids; *anteiso*-FA and *odd*-chain FA reached higher values than *iso*-FA (Table 5 and 6). Nevertheless, anteiso-C15:0 and *anteiso*-C17:0 accumulated to a higher extent in the AS rumen liquor lipids (Table 6). The higher content of *anteiso*-C15:0 in AS rumen fluid could be related to the higher content of NFC and a lower percentage of NDF in this herbage (Table 2). Vlaeminck *et al.* (2006) studied the effect of forage:concentrate ratio on fatty acid composition of rumen bacteria isolated from ruminal and duodenal digesta and found a strong negative correlation between dietary forage proportion and the percentage of *anteiso*-C15:0 in total BOCFA of the bacteria (r_{pearson} =-0.771). In our study, the higher level of NSC and the lower level of NDF content in AS could have led to an increase in *anteiso*-C15:0 accumulation.

Even linear fatty acids

At 2 h of fermentation, the even linear chain fatty acids (ELFA) profile was quite similar among treatments with the exception of a higher content of C14:0 for LM and the presence of C16:0 only in rumen liquor incubated with AS (Table 7). During fermentation, the content in lipid extract of all FA tended to increase regardless of the type of herbage, but AS and MS showed higher increases than LM (Table 7). Consequently, at the end of fermentation, LM rumen liquor had the lowest content of all FA measured. The content of C18:0 (stearic acid, SA) increased after 4 h for AS and LM and after 6 h for MS (Table 7). The maximum accumulation was reached at 8h for MS.

Trans- and cis-C18:1 isomers

At the beginning of fermentation, *trans*- and *cis*-C18:1 isomers were also detected at similar levels in all samples (Table 8). During fermentation, some isomers tended to accumulate in rumen liquor lipids, while other isomers reached a maximum and then decreased, with different patterns detected among herbages.

VA and t*rans*-13-14 C18:1 progressively accumulated in MS rumen liquor and

Table 6. Total anteiso and linear odd fatty acid appearance in the rumen liquor at different fermentation times (g/100 g lipid extract). Data shown are the means of 3 replicates.

Fatty acid		Fermentation time, h 2	4	6	8	SEM
C15:0 *anteiso*	*Avena sativa*	0.29^{α}	$0.53^{\beta a}$	0.79^{γ}	$1.21^{\delta a}$	0.04
	Lolium multiflorum	0.31^{α}	$0.81^{\beta b}$	0.80^{β}	$0.80^{\beta b}$	
	Medicago sativa	0.34^{α}	$0.79^{\beta b}$	0.83^{β}	$0.98^{\gamma c}$	
C17:0 *anteiso*	*Avena sativa*	0.12^{α}	0.20^{β}	0.36^{γ}	$0.69^{\delta a}$	0.06
	Lolium multiflorum	0.10^{α}	0.24^{β}	0.40^{γ}	$0.40^{\gamma b}$	
	Medicago sativa	0.05^{α}	0.15^{β}	0.40^{β}	$0.40^{\beta b}$	
C13:0	*Avena sativa*	0.00^{α}	0.00^{α}	0.03^{α}	0.06^{β}	0.01
	Lolium multiflorum	0.00^{α}	0.00^{α}	0.06^{β}	0.06^{β}	
	Medicago sativa	0.00^{α}	0.00^{α}	0.04^{β}	0.05^{β}	
C15:0	*Avena sativa*	$0.18^{\alpha a}$	$0.44^{\beta a}$	$0.55^{\gamma a}$	$0.88^{\delta a}$	0.02
	Lolium multiflorum	$0.53^{\alpha b}$	$0.53^{\alpha b}$	$0.55^{\alpha\beta a}$	$0.60^{\beta b}$	
	Medicago sativa	$0.33^{\alpha c}$	$0.73^{\beta c}$	$0.73^{\beta b}$	$0.82^{\gamma a}$	
C17:0	*Avena sativa*	$0.04^{\alpha a}$	0.15^{β}	$0.19^{\beta a}$	$0.40^{\gamma a}$	0.03
	Lolium multiflorum	$0.06^{\alpha ab}$	0.16^{β}	$0.19^{\beta a}$	$0.22^{\beta b}$	
	Medicago sativa	$0.12^{\alpha b}$	0.22^{β}	$0.26^{\beta b}$	$0.35^{\gamma c}$	
Total	*Avena sativa*	$0.63^{\alpha a}$	$1.32^{\beta a}$	$1.92^{\gamma a}$	$3.24^{\delta a}$	0.07
	Lolium multiflorum	$1.00^{\alpha b}$	$1.74^{\beta b}$	$2.00^{\gamma a}$	$2.08^{\beta b}$	
	Medicago sativa	$0.94^{\alpha b}$	$2.22^{\beta c}$	$2.26^{\beta b}$	$2.60^{\gamma c}$	

*Within a column, means with different Latin superscripts are significantly different (*P< 0.05); within a row, means with different Greek superscripts are significantly different (*P< 0.05).*

reached a maximum at the end of fermentation (2.64 and 0.71g/100 g of lipid extract, respectively). In AS rumen liquor, the highest levels of these FA were detected at 6 h, with a significant decrease observed at 8 h.

Trans-10 and *trans*-12 C18:1 reached the highest level at 6 h for AS and for MS.

For all samples, VA was the main *trans*-FA (TFA), followed by *trans*-13-14 C18:1. At the end of fermentation, LM rumen liquor showed the lowest accumulation of all *cis*- and *trans*-C18:1, with the exception of *cis*-9 C18:1 (oleic acid, OA). In LM rumen liquor, OA reached a maximum at 4 h and then its concentration in lipid extract did not change until the end of fermentation.

For AS, OA reached the highest level at 6 h, whereas for MS the maximum accumulation was not reached until the end of fermentation. Moreover, after 8h of fermentation the content of OA was higher in MS rumen fluid than in AS and LM (Table 8).

CLA isomers

Only two isomers of CLA were detected above trace level. RA was the predominant CLA isomer and the maximum was detected at 6 h with AS (0.81 g/100 g of lipid extract), as shown in Table 9. RA concentration remained quite low with the other two herbages. The concentration of the other isomer (*trans*-10, *cis*-12 CLA) was always very low. Again, the maximum yield (0.09 g/100 g of lipid extract) was reached after 6 hours with AS.

RA was the predominant CLA isomer and the maximum yield was attained with AS at the sixth hour of fermentation. These data agree with Buccioni *et al.* (2007) who,

Table 7. Even linear fatty acid profile of rumen liquor at different fermentation times (g/100 g lipid extract). Data shown are the means of 3 replicates.

Fatty acid		Fermentation time, h				SEM
		2	4	6	8	
C12:0	*Avena sativa*	0.04^{α}	0.09^{α}	$0.44^{\beta a}$	$0.44^{\beta a}$	0.04
	Lolium multiflorum	0.09	0.09	0.08^{b}	0.08^{b}	
	Medicago sativa	0.10^{α}	0.15^{α}	$0.36^{\beta a}$	$0.30^{\beta c}$	
C14:0	*Avena sativa*	$0.10^{\alpha a}$	0.21^{α}	$0.89^{\beta a}$	$0.85^{\beta a}$	0.09
	Lolium multiflorum	$0.46^{\alpha b}$	$0.31^{\alpha\beta}$	$0.31^{\alpha\beta b}$	$0.23^{\beta b}$	
	Medicago sativa	$0.13^{\alpha a}$	0.21^{α}	$0.73^{\beta a}$	$0.83^{\beta a}$	
C16:0	*Avena sativa*	$0.83^{\alpha a}$	$5.03^{\beta a}$	$23.62^{\gamma a}$	$23.00^{\gamma a}$	0.57
	Lolium multiflorum	$0.00^{\alpha b}$	$7.24^{\beta b}$	$7.50^{\beta b}$	$7.99^{\beta b}$	
	Medicago sativa	$0.00^{\alpha b}$	$3.97^{\beta a}$	$19.30^{\gamma c}$	$22.79^{\delta a}$	
C17:1	*Avena sativa*	0.00^{α}	0.02^{α}	$0.07^{\beta a}$	$0.03^{\alpha\beta a}$	0.02
	Lolium multiflorum	0.00	0.00	0.00^{b}	0.00^{a}	
	Medicago sativa	0.00^{α}	0.02^{α}	$0.05^{\alpha a}$	$0.11^{\beta b}$	
C18:0	*Avena sativa*	2.79^{α}	$10.16^{\beta a}$	$31.32^{\gamma a}$	$34.17^{\delta a}$	0.56
	Lolium multiflorum	3.89^{α}	$14.64^{\beta b}$	$16.67^{\gamma b}$	$16.41^{\gamma b}$	
	Medicago sativa	3.64^{α}	$3.24^{\alpha c}$	$41.78^{\beta c}$	$52.01^{\gamma c}$	
C20:0	*Avena sativa*	0.04^{α}	0.12^{α}	$0.62^{\beta a}$	$0.61^{\beta a}$	0.06
	Lolium multiflorum	0.01^{α}	0.13^{β}	$0.13^{\beta b}$	$0.14^{\beta b}$	
	Medicago sativa	0.04^{α}	0.09^{α}	$0.50^{\beta a}$	$0.63^{\beta a}$	
C20:5 n-3	*Avena sativa*	0.00	0.00	0.00	0.01^{a}	0.01
	Lolium multiflorum	0.00	0.00	0.00	0.00^{a}	
	Medicago sativa	0.00^{α}	0.00^{α}	0.00^{α}	$0.04^{\beta b}$	
C22:0	*Avena sativa*	0.00^{α}	0.00^{α}	$0.33^{\beta a}$	$0.30^{\beta a}$	0.03
	Lolium multiflorum	0.00	0.00	0.01^{b}	0.02^{b}	
	Medicago sativa	0.00^{α}	0.03^{α}	$0.19^{\beta c}$	$0.30^{\gamma a}$	
C24:0	*Avena sativa*	0.00^{α}	0.00^{α}	$0.33^{\beta a}$	$0.16^{\gamma a}$	0.03
	Lolium multiflorum	0.00	0.00	0.01^{b}	0.04^{b}	
	Medicago sativa	0.00^{α}	0.05^{α}	$0.25^{\beta c}$	$0.33^{\gamma c}$	

*Within a column, means with different Latin superscripts are significantly different (*P< 0.05); within a row, means with different Greek superscripts are significantly different (*P< 0.05).*

in a previous trial, showed that the maximum yield of CLA was obtained around the sixth hour of fermentation with fresh forage and after 18 hours with dried forage; then RA was remarkably hydrogenated.

For all samples, the accumulation of VA in the rumen fluid after the second and fourth hours of fermentation could be mainly due to α-LNA biohydrogenation, because RA was not detected before the sixth hour. Indeed, α-LNA is hydrogenated to VA after initial isomerisation of the *cis*-12 double bond (Griinari and Bauman, 1999). An additional contributor to VA accumulation could be C18:1 isomerisation. Indeed, a previous paper reported that OA may be isomerised by mixed ruminal bacteria to form several *trans*-C18:1 isomers, including VA (Mosley *et al.*, 2002). Moreover, the

Table 8. C_{18} fatty acids in rumen liquor at different fermentation times (g/100 g lipid extract). Data shown are the means of 3 replicates.

Fatty acid		Fermentation time, h				SEM
		2	4	6	8	
C18:1 *cis*-9	*Avena sativa*	0.43^{α}	$0.35^{\alpha a}$	$1.20^{\beta a}$	$1.20^{\beta a}$	0.25
	Lolium multiflorum	0.64^{α}	$1.31^{\beta b}$	$1.30^{\beta a}$	$1.29^{\beta a}$	
	Medicago sativa	0.38^{α}	$1.11^{\beta b}$	$3.86^{\gamma b}$	$4.80^{\delta b}$	
C18:1 *cis*-11	*Avena sativa*	0.00^{α}	0.08^{α}	$0.12^{\alpha a}$	$0.43^{\beta a}$	0.06
	Lolium multiflorum	0.00^{α}	0.12^{α}	$0.36^{\beta b}$	$0.15^{\alpha b}$	
	Medicago sativa	0.01^{α}	0.10^{α}	$0.39^{\beta b}$	$0.51^{\gamma a}$	
C18:1 *cis*-12	*Avena sativa*	0.04^{α}	0.05^{α}	0.03^{α}	$0.15^{\beta a}$	0.04
	Lolium multiflorum	0.02	0.02	0.05	0.06^{b}	
	Medicago sativa	0.04^{α}	0.03^{α}	$0.12^{\alpha\beta}$	$0.16^{\beta c}$	
C18:1 *trans*-9	*Avena sativa*	0.00^{α}	0.00^{α}	$0.15^{\beta a}$	$0.12^{\beta a}$	0.02
	Lolium multiflorum	0.00	0.00	0.01^{b}	0.03^{b}	
	Medicago sativa	0.00^{α}	0.01^{α}	$0.14^{\beta a}$	$0.18^{\beta a}$	
C18:1 *trans*-10	*Avena sativa*	0.00^{α}	0.00^{α}	$0.13^{\beta a}$	$0.09^{\beta a}$	0.02
	Lolium multiflorum	0.00	0.00	0.02^{b}	0.05^{a}	
	Medicago sativa	0.00^{α}	0.01^{α}	$0.17^{\beta a}$	$0.16^{\beta b}$	
C18:1 *trans*-11	*Avena sativa*	0.09^{α}	0.38^{β}	$2.07^{\gamma a}$	$1.72^{\delta a}$	0.12
	Lolium multiflorum	0.15^{α}	0.49^{β}	$0.35^{\beta b}$	$0.45^{\beta b}$	
	Medicago sativa	0.08^{α}	0.41^{β}	$1.76^{\gamma c}$	$2.64^{\delta c}$	
C18:1 *trans*-12	*Avena sativa*	0.13^{α}	$0.22^{\alpha\beta}$	$0.79^{\gamma a}$	$0.33^{\beta a}$	0.09
	Lolium multiflorum	0.23	0.16	0.11^{b}	0.18^{a}	
	Medicago sativa	0.12^{α}	0.18^{α}	$0.60^{\beta c}$	$0.51^{\beta b}$	
C18:1 *trans*-13 + 14	*Avena sativa*	0.02^{α}	0.11^{α}	$0.71^{\beta a}$	$0.44^{\gamma a}$	0.08
	Lolium multiflorum	0.01	0.14	0.08^{b}	0.16^{b}	
	Medicago sativa	0.02^{α}	0.12^{α}	$0.35^{\beta c}$	$0.71^{\gamma c}$	

*Within a column, means with different Latin superscripts are significantly different (*P< 0.05); within a row, means with different Greek superscripts are significantly different (*P< 0.05).*

rate determining step of the whole LA or LNA biohydrogenation process is the reduction of VA to SA, with a consequent accumulation of VA (Harfoot and Hazelwood, 1997).

The amount and the relative percentage of C18:1 isomers in rumen liquor lipids varied with time of fermentation and the kind of herbage (H x T interaction was significant with P≤ 0.05). In all samples, VA remained the main *trans* C18:1 isomer, followed by *trans*-13-14 C18:1. MS seemed to induce the highest accumulation of VA when compared to the other two forages. These data agree with Ribeiro *et al.* (2005) who showed that VA and *trans*-13 C18:1 were the main *trans* C18:1 isomers in rumen liquor when fresh lucerne was incubated. The differences among herbages in conjugated dienes and C18:1 isomers content during the fermentation times could be due to different releasing times of FA from the vegetable substrate. In fact, to make plant glycerides and free FA available for biohydrogenation, microorganisms first have to break the vegetable cell wall. Doreau and Ferlay (1994) showed that

Table 9. Linolenic and Linoleic acids isomers of rumen liquor at different fermentation times (g/100 g lipid extract). Means of 3 replicates.

Fatty acid		Fermentation time, h				SEM
		2	4	6	8	
C18:2 cis9, cis12	*Avena sativa*	0.38^{α}	0.72^{α}	$3.45^{\beta a}$	$3.35^{\beta a}$	0.48
	Lolium multiflorum	0.54	1.02	0.49^{b}	0.18^{b}	
	Medicago sativa	0.44^{α}	0.92^{α}	$3.15^{\beta a}$	$3.56^{\beta a}$	
C18:2 cis9, trans11	*Avena sativa*	0.00^{α}	0.00^{α}	$0.81^{\beta a}$	$0.06^{\gamma a}$	0.01
	Lolium multiflorum	0.00	0.00	0.00^{b}	0.01^{b}	
	Medicago sativa	0.00^{α}	0.00^{α}	$0.05^{\beta c}$	$0.07^{\beta a}$	
C18:2 trans10, cis12	*Avena sativa*	0.00^{α}	0.00^{α}	$0.09^{\beta a}$	0.01^{α}	0.02
	Lolium multiflorum	0.00	0.00	0.00^{b}	0.00	
	Medicago sativa	0.00	0.00	0.01^{b}	0.03	
C18:3 cis9, cis12, cis15	*Avena sativa*	0.54^{α}	$0.43^{\alpha a}$	$1.93^{\beta a}$	$3.12^{\gamma a}$	0.24
	Lolium multiflorum	0.63^{α}	$1.47^{\beta b}$	$1.01^{\alpha\beta b}$	$1.21^{\beta b}$	
	Medicago sativa	0.27^{α}	$0.45^{\alpha a}$	$1.19^{\beta b}$	$1.72^{\gamma b}$	

*Within a column, means with different Latin superscript are significantly different (*P< 0.05); within a row, means with different Greek superscript are significantly different (*P< 0.05).*

the lipolysis rate of plant lipids contained in the cell wall is influenced by the ability of rumen microbes to remove the surrounding cellular matrices. Harfoot and Hazelwood (1997) reported that the hydrolysis of esterified plant lipids is the rate limiting step for the next biohydrogenation. So, the time spent by ruminal microbes to produce free FA may affect the efficiency of the whole biohydrogenation process. However, Lee *et al.* (2002) and Faruque *et al.* (1974), cited by Dewhurst *et al.* (2006), showed that plant lipases present in the leaves of pasture plants may remain active for at least 5 hours in the presence of metabolising rumen microorganisms and they may be responsible for the first stages of lipolysis when ruminants graze fresh pasture.

Conclusions

Herbages with different chemical composition showed different ruminal biohydrogenation behaviour, leading to differences in the FA profile of rumen liquor lipids during *in vitro* fermentation. In particular, NDF and NSC content seemed to play an important role in the amount of FA that accumulated as fermentation progressed. Literature confirms that the efficiency of rumen biohydrogenation seems to depend on the balance of nutritional components offered as the feed. *Avena sativa*, after 6 hours of fermentation, had the highest amounts of RA accumulation in rumen liquor lipids relative to the other two forages. The differences in conjugated dienes and C18:1 isomers content during fermentation could be due not only to different amounts of LA or LNA in the herbage, but also to different releasing times of FA from the plant substrate. Further studies are needed to examine the effect of availability of energy and degradable protein to rumen microbes on the efficiency of the biohydrogenation process.

The authors acknowledge "CeRA" research centre and Dr. N. Staglianò for the technical assistance.

The work has been realised by financial support of the FISR project: "Quality of animal products and human health: improvement of lipid and mineral fraction in cow, sheep and goat milk and dairy products to improve nutraceutical value and safety of these foods" from the Ministry of Agricultural and Forestry Policies (MiPAF) and from the Ministry of University and Research (MiUR) and by financial support of the *Ente Cassa di Risparmio di Firenze,* Italy.

REFERENCES

Antongiovanni, M., Buccioni, A., Rapaccini, S., 2002. The gas production technique for the assessment of forage quality: a promising approach. Proc. 19th Gen. Meet. Eur. Grassland Federation, La Rochelle, France, 7:180-181.

AOAC, 1990. Official Methods of Analysis of the AOAC. 15th ed. Association of Official Analytical Chemists, Arlington, VA, USA.

Archimede, H., Sauvant, D., Hervieu, J., Poncet, C., Dorleans, M., 1995. Digestive interactions in ruminants: relationships between whole tract and stomach evaluation. Anim. Feed Sci. Tech. 54:327-340.

Bickerstaffe, R., Noakes, D.E., Annison, E.F., 1972. Quantitative aspects of fatty acid biohydrogenation, absorbition and transfer into milk fat in the lactating goat, with special reference to the cis and trans isomers of octadecenoate and linoleate. Biochem. J. 130:607-624.

Buccioni, A., Antongiovanni, M., Petacchi, F., Mele, M., Secchiari, S.P., Minieri, S., 2007. Effect of dried or green herbage on vaccenic acid and conjugated linoleic acid production during in vitro rumen fermentation. Anim. Feed Sci. Tech. 140:207-213.

Buccioni, A., Antongiovanni, M., Rapaccini, S., Petacchi, F., 2001. Rumen degradation kinetics of feeds measured by means of in vitro gas production. pp 150-153 in Proc. 14th Nat. Congr. ASPA, Firenze, Italy.

Cabiddu, A., Decandia, M., Addis, M., Piredda, G., Pirisi, A., Molle, G., 2005. Managing Mediterranean pastures in order to enhance level of beneficial fatty acids in sheep milk. Small Ruminant Res. 59:169-180.

Christie, W.W., 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesterol esters. J. Lipid Res. 23:1072-1075.

Collomb, M., Butikofer, U., Sieber, R., Jeangros, B., Bosset J.O., 2002. Correlation between fatty acids in cows' milk fat produced in the Lowlands, Mountains and Highlands of Switzerland and botanical composition of the fodder. Int. Dairy J. 12:661-666.

Cone, J.W., van Gelder, A.H., Visscher, A.H., Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Anim. Feed Sci. Tech. 61:113-128.

Corl, B.A., Baumgard, L.H., Dwyer, D.A., Griinari, J.M., Phillips, B.S., Bauman, D.E., 2001. The role of D9 desaturase in the production of cis-9, trans-11 CLA. J. Nutr. Biochem. 12:622-630.

Dewhurst, R.J., Shingfield, K.J., Lee, M.R.F., Scollan, N.D., 2006. Increasing the concentrations of beneficial polyunsaturated fatty acid in milk produced by dairy cows in high-forage systems. Anim. Feed Sci. Tech. 131:168-206.

Doreau, M., Ferlay, A., 1994. Digestion and utilization of fatty acids by ruminants. Anim. Feed Sci. Tech. 45:379-396.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissue. J. Biol. Chem. 226:497-509.

Griinari, J.M., Bauman, D.E., 1999. Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza and G.J. Nelson (eds.) Advances in Conjugated Linoleic Acid Research. AOCS Press, Champaign, IL, USA, Vol. 1, pp 180-200.

Griinari, J.M., Dwyer, M.A., McGiure, M.A., Bauman, D.E., Palmquist, D.L., Nurmela, K.V., 1998. Trans-octadecenoic acids and milk fat depression in lactating dairy cows. J. Dairy Sci. 81:1251-1261.

Harfoot, C., Hazlewood, G.P., 1997. Lipid metabolism in the rumen. In: P.N. Hobson (ed.) The Rumen Microbial Ecosystem. Elsevier 2nd ed., London, UK, pp

382-426.

Jenkins, T.C., 1993. Lipid metabolism in the rumen. J. Dairy Sci. 76:3851-3863.

Kelly, M.L., Kolver, E.S., Bauman, D.E., Van Amburgh, M.E., Muller, L.D., 1998. Effect of intake of pasture on concentrations of conjugated linoleic acid in milk of lactating cows. J. Dairy Sci. 81:1630-1636.

Kramer, J.K.G., Cruz-Hernandez, C., Deng, Z.Y., Zhou, J.Q., Jahreis, G., Dugan, M.E.R., 2004. Analysis of conjugated linoleic acid and trans 18:1 isomers in synthetic and animal products. Am. J. Clin. Nutr. 79:1137S-1145S.

Kramer, J.K.G., Fellner, V., Dugan, M.E.R., Sauer, F.D., Mossoba, M.M., Yurawecz, M.P., 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 32:1219-1228.

Lee, M.R.F., Theodorou, M.K., Chow, T.T., Enser, M., Scollan, N.D., 2002. In vitro evidence for plant enzyme mediated lipolysis in the rumen. P. Nutr. Soc. 61:103 (abstr.).

Licitra, G., 1996. Standardization of procedures for nitrogen fractionation of ruminant feed. Anim. Feed Sci. Tech. 57:347-358.

Loor, J.J., Ueda, K., Ferlay, A., Chilliard, Y., Doreau, M., 2004. Biohydrogenation, Duodenal Flow, and Intestinal Digestibility of Trans Fatty Acids and Conjugated Linoleic Acids in Response to Dietary Forage: Concentrate Ratio and Linseed Oil in Dairy Cows. J. Dairy Sci. 87:2472-2485.

Martin, S.A. Jenkins, T.C., 2002. Factors affecting conjugated linoleic acid and trans-C18:1 fatty acid production by mixed ruminal bacteria. J. Anim. Sci. 80:3347-3352.

Mauricio, R.M., Owen, E., Mould, F.L., Givens, I., Theodorou, M.K., France, J., Davies, D.R., Dhanoa, M.S., 2001. Comparison of bovine rumen liquor and bovine faeces as inoculum for an *in vitro* gas production technique for evaluating forages. Anim. Feed Sci. Tech. 89:33-48.

McDougall, E.I., 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. 43:99-109.

Mele, M., Macciotta, N.P.P., Serra, A., Secchiari, P., 2007. Fatty acid composition of milk and cheese from sheep fed rough or cultivated pasture. Page 87 in Proc. 5th Int. Symp. FIL-IDF, Challenge to sheep and goats milk sectors, Alghero (SS), Italy.

Mosley, E.E., Powell, G.L., Riley, M.B., Jenkins, T.C., 2002. Microbial biohydrogenation of oleic acid to *trans* isomers *in vitro*. J. Lipid Res. 43:290-296.

Nielsen, T.S., Sejrsen, K., Andersen, H.R., Lund, P., Straarup, E.M., 2004. Effect of silage type and energy concentration on conjugated linoleic acid (CLA) in milk fat from dairy cows. J. Anim. Feed Sci. 13 (Suppl.1):697-700.

Park, P.K., Albright, K.J., Cai, Z.Y., Pariza, M.W., 2001. Comparison of methylation procedures for conjugated linoleic acid artefact formation by commercial (trime thylsilyl)diazomethane. J. Agr. Food Chem. 49:1158-1164.

Ribeiro, C.V.D.M., Karnati, S.K.R., Eastridge, M., 2005. Biohydrogenation of fatty acids and digestibility of fresh alfalfa of alfalfa hay plus sucrose in continuous culture. J. Dairy Sci. 88: 4407-4017.

Roach, J.A.G., Mossoba, M.M., Yurawecz, M.P., Kramer, J.K.G., 2002. Chromatographic separation and identification of conjugated linoleic acid isomers. Anal. Chim. Acta 464:207-226.

SAS, 1999. User's Guide: Statistics, Version 8.0 Edition. SAS Inst. Inc., Cary, NC, USA.

Shingfield, K.J., Reynols, C.K., Lupoli, B., Toivonen, V., Yurawecz, M.P., Delmonte, P., Griinari, J.M., Grandison, A.S., Beever, D.E., 2005. Effect of forage type and proportion of concentrate in the diet on milk fatty acid composition in cows given sunflower and fish oil. Anim. Sci. 80:225-238.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

Vlaeminck, B., Fievez, V., Demeyer, D., Dewhurst, R.J., 2006. Effect of forage:concentrate ratio on fatty acid composition of rumen bacteria isolated from ruminal and duodenal digesta. J. Dairy Sci. 89:2668-2678.

Weimer, P.J., Waghorn, G.C., Odt, C.L., Mertens, D.R., 1999. Effect of diet on population of three species of ruminal cellulolytic bacteria in lactating dairy cows. J. Dairy Sci. 82:122-134.