Nitrogen Supplementation of Corn Silages. 2. Assessing Rumen Function Using Fatty Acid Profiles of Bovine Milk

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ABSTRACT

The effects of N supplementation strategies on milk fatty acid profiles of dairy cows and their use as a noninvasive technique to diagnose rumen function, and to guide protein feeding decisions on-farm were evaluated in three experiments. Each experiment was designed according to three 3×3 Latin squares with 9 Holstein cows receiving total mixed rations based on corn silage. Experiment 1 was designed to study effects of diets with different ratios of effective rumen-degradable protein (ERDP; g) to fermentable metabolizable energy (FME; j) providing, respectively, a large deficiency, a slight deficiency, and a slight excess in relation to the target level of 11 g of ERDP/MJ FME for lactating cows. Experiment 2 evaluated effects of different proportions of quickly and slowly rumen-degradable protein achieved by replacing soybean meal with urea in the concentrates (0, 0.5, and 1% urea for U0, U5, and U10, respectively). Experiment 3 investigated effects of synchronizing the availability of FME and ERDP in rumen by offering the protein-rich concentrate once or twice per day before the meal (corn silage, ryegrass hay, and energy-rich concentrate), or included in the total mixed ration. Milk fatty acid profiles were significantly affected by dietary N and carbohydrate supply. Principal component factor analysis provided a reasonable description of the data, clearly discriminating between fatty acids that are synthesized by different metabolic pathways. Several sources/pathways were distinguished: de novo synthesis in the mammary gland (short- and medium-chain fatty acids), Δ^9 -desaturase activity (monoenoic fatty

acids), direct absorption from the blood stream (long-chain fatty acids), and de novo synthesis by the rumen microbial populations (odd-chain fatty acids). Discriminant canonical analysis showed that milk odd-chain fatty acids had a higher ability to discriminate between diets than even-chain fatty acids. The *anteiso* C_{15:0} increased in line with increasing sugar supply, and C_{17:0} appears to be a marker of protein deficiency. Additionally, *iso* C_{17:0} and *anteiso* C_{17:0} were associated with the NDF and CP contents of diets. The results suggests that milk odd-chain fatty acids have the potential to be used as a noninvasive technique to assess rumen function in terms of microbial populations, substrates and interactions.

(**Key words:** bovine milk fatty acid profile, nitrogen supplementation, rumen function)

Abbreviation key: D1 = protein-rich concentrate fed once a day before the a.m. meal, D2 = protein-rich concentrate fed twice a day before both meals, DU = protein-rich concentrate given with the basal diet, ERDP = effective rumen-degradable protein, FME = fermentable metabolizable energy, QDP = quickly rumen-degradable protein, RH = diet with a slight excess of ERDP in relation to FME, RL = diet with a large deficiency of ERDP in relation to FME, RM = diet with a slight deficiency of ERDP in relation to FME, SDP = slowly rumen-degradable protein, U0 = concentrate with 0% urea, U5 = concentrate with 0.5% urea, U10 = concentrate with 1% urea.

INTRODUCTION

Earlier work on rumen lipid metabolism focused on fat supplements designed to avoid deleterious effects on rumen function, and maximize the delivery of beneficial polyunsaturated fatty acids to the duodenum (Jenkins, 1993). Although the effects of rumen VFA production on de novo synthesis of shorter even-chain

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fatty acids (C_4 to C_{14}) in milk are well recognized (Grummer, 1991), other links between rumen function and milk fatty acids have not been elucidated. The companion paper (Cabrita et al., 2003) showed that urinary allantoin/creatinine ratios in spot urine samples were of no value in describing rumen function, whereas other studies (Shingfield and Offer, 1998) showed that milk allantoin excretion was similarly ineffective. Consequently, there is a need for further work to define a milk-based diagnostic test for rumen function. We are interested in using milk fatty acid profiles to provide a noninvasive description of rumen function. A reliable test would be of great practical value alongside model-based approaches to feeding the rumen (Dewhurst et al., 2000a).

The focus for this work was the odd-chain fatty acids in milk. Bovine milk contains measurable quantities of pentadecanoic acid ($C_{15:0}$), heptadecanoic acid ($C_{17:0}$), and heptadecenoic acid ($C_{17:1}$), as well as branched-chain isomers *iso* $C_{15:0}$ (13-methyl-tetradecanoic acid), *anteiso* $C_{15:0}$ (12-methyl-tetradecanoic acid), *iso* $C_{17:0}$ (15-methyl-hexadecanoic acid), and *anteiso* $C_{17:0}$ (14-methyl-hexadecanoic acid) (Dewhurst et al., 2000b). The odd-chain fatty acids are major components of the fatty acids of rumen microorganisms (O'Kelly and Spiers, 1991; Jenkins, 1993; Lee et al., 1999), although $C_{17:1}$ has not been detected in pure cultures of rumen bacteria (Bae et al., 2000). These facts, along with the reported absence of odd-chain fatty acids in plant material (Diedrich and Henschel, 1990), led Dewhurst et al. (2000a) to propose that their determination in milk could provide, at least, a qualitative description of microbial synthesis in the rumen. Sauvant and Bas (2001) speculated that higher levels of rumen-degradable true protein might lead to increased levels of branched odd-chain fatty acids (*iso* $C_{15:0}$, *anteiso* $C_{15:0}$, *iso* $C_{17:0}$, and *anteiso* $C_{17:0}$), by supplying increased amounts of branched-chain precursors (i.e., AA).

This study used samples from a series of feeding experiments that investigated responses of dairy cows to different N supplementation strategies of corn silage-based diets (Cabrita et al., 2003). It was conducted with the objective to evaluate the effects of different N supplementation strategies on milk fatty acid profiles. We used principal component factor analysis and discriminant canonical analysis to test the hypothesis that milk fatty acids, particularly odd-chain fatty acids, might be used as a noninvasive technique to diagnose rumen function and to guide protein feeding decisions on-farm. An additional reason for interest in milk odd-chain fatty acids per se is provided by recent work which has suggested possible anti-cancer effects for *iso* $C_{15:0}$ (Yang et al., 2000).

MATERIALS AND METHODS

Nine Holstein cows were used in each of the three experiments designed according to three 3×3 Latin squares reported by Cabrita et al. (2003). Briefly, in experiment 1 the three diets contained (DM basis) about 40% corn silage, 5% coarsely chopped ryegrass hay, and 55% concentrate. The 3 treatments were formulated, according to AFRC (1993), to be isoenergetic, to satisfy the metabolizable protein requirements, and to differ in the ratio of effective RDP (**ERDP**) to fermentable metabolizable energy (**FME**), providing a large deficiency (**RL**), a slight deficiency (**RM**), and a slight excess (**RH**) of ERDP in relation to the AFRC (1993) target level (11 g of ERDP/MJ FME) for lactating cows. The different ERDP:FME ratios were achieved by altering the composition of the concentrates. Diets were fed as TMR for ad libitum intake, with fresh feed offered twice each d (0830 and 1500 h). In experiment 2 the diets were isoenergetic, based on corn silage, and differed in the ratio of quickly rumen-degradable protein (**QDP**) and slowly rumen-degradable protein (**SDP**). The different QDP/SDP ratios were achieved by replacing soybean meal with urea in the concentrates (0, 0.5, and 1.0% urea for treatments **U0**, **U5**, and **U10**, respectively). The TMR consisted of (DM basis) 60% of concentrate, 35% of corn silage, and 5% of ryegrass hay. Fresh feed was offered at 0800 and 1530 h each day. In experiment 3, all treatments were based on a single diet that contained (on a DM basis) corn silage (45%), ryegrass hay (5%), energy-rich concentrate (35%), and protein-rich concentrate [15%; CP: 37.7% (DM); urea: 2% (DM)]. Protein-rich concentrate was either fed once (before the a.m. meal; **D1**) or twice (before both meals; **D2**) a day, or given as a TMR with the basal diet (**DU**).

Milk was sampled at both milkings on 2 consecutive days during the last week of each experimental period (the same day used to collect composite milk samples, feces, urine, and blood samples; Cabrita et al., 2003). Individual milk samples, without preservative, were immediately stored at -15°C until analysis of fatty acid profiles. The lipids and liposoluble compounds of feed and milk samples were extracted as described by Normalisation Française V 03-030 (AFNOR, 1991) and by Standard ISO 14156:2001 (ISO, 2001), respectively. Fatty acid methyl esters prepared according Standard ISO 15884:2002 (ISO, 2002a) and the fatty acid composition determined by GLC according Standard ISO 15885:2002 (ISO, 2002b). Samples ($0.3 \mu\text{l}$ of methyl esters in n-heptane) were injected into a Hewlett-Packard 6890 gas chromatograph (20:1 split ratio) equipped with a flame-ionization detector (Hewlett-Packard, Sunnyvale, CA). Methyl esters of fatty acids

Table 1. Fatty acid profiles (% of total fatty acids) of the feeds used.

	Experiment 1					Experiment 2					Experiment 3	
	CS ¹	RH ²	RL ³	RM ³	RH ³	CS ^{1,4}	RH ^{2,4}	U0 ⁵	U5 ⁵	U10 ⁵	CEn ⁶	CP ⁶
C _{12:0}	0.08	1.26	0.41	0.53	0.28	0.11	0.96	0.16	0.15	0.14	0.24	0.12
C _{14:0}	0.14	2.17	0.47	0.63	0.62	0.21	1.72	0.42	0.37	0.36	0.53	0.30
C _{14:1}	ND ⁷	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
<i>iso</i> C _{15:0}	ND	ND	ND	ND	ND	ND	0.16	ND	ND	ND	ND	ND
<i>anteiso</i> C _{15:0}	ND	1.17	ND	ND	ND	ND	0.47	ND	ND	ND	ND	ND
C _{15:0}	0.07	0.92	0.14	0.09	0.18	0.05	0.53	0.08	0.10	0.15	0.12	0.08
C _{16:0}	10.31	41.47	15.93	16.83	16.54	9.98	41.43	18.45	18.36	18.32	22.14	9.86
C _{16:1}	0.14	0.92	0.41	0.35	0.43	0.12	1.40	0.16	0.16	0.18	0.27	0.18
<i>iso</i> C _{17:0}	ND	0.46	ND	0.08	ND	ND	0.13	ND	ND	ND	ND	ND
<i>anteiso</i> C _{17:0}	0.07	0.07	0.08	0.07	0.08	ND	0.07	0.05	0.06	0.08	0.07	ND
C _{17:0}	0.18	0.60	0.10	0.09	0.10	0.25	0.33	0.11	0.13	0.12	0.14	0.09
C _{17:1}	ND	ND	0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND
C _{18:0}	1.80	8.31	4.18	7.73	8.79	1.69	11.80	8.85	9.39	8.89	9.24	4.79
C _{18:1}	26.07	11.25	23.41	23.25	21.63	22.61	14.65	18.49	20.27	20.53	20.74	24.23
C _{18:2}	51.89	14.69	42.07	34.21	26.58	58.85	12.08	38.25	40.40	39.57	39.12	54.13
C _{18:3}	4.06	7.40	3.00	2.51	2.21	3.75	8.19	2.82	2.75	2.64	2.51	2.20
C _{20:0}	0.44	1.69	0.42	0.45	0.42	0.43	1.28	0.38	0.42	0.43	0.37	0.73
C _{20:1}	0.33	0.31	0.60	0.90	1.02	0.38	0.21	0.36	0.32	0.33	0.33	0.31
C _{22:0}	0.38	2.75	0.27	0.37	0.56	0.33	1.88	0.56	0.28	0.41	0.19	0.82
C _{24:0}	0.36	2.77	0.28	0.42	0.85	0.65	1.76	0.79	0.34	0.26	0.22	0.49

C_{4:0}, C_{6:0}, C_{8:0}, C_{10:0}, and C_{11:0} fatty acids were not detectable.

¹Corn silage.

²Ryegrass hay.

³Concentrates are named according to the effective RDP:fermentable metabolizable energy ratio of the diet: RL, RM, and RH providing a large deficiency, a slight deficiency, and a slight excess, respectively.

⁴Used in experiments 2 and 3.

⁵Concentrates are named according to their urea content, U0: 0%; U5: 0.5%; and U10: 1.0%.

⁶Concentrates are named according to the main nutrients supplied: CEn: energy-rich nutrients and CP: N-rich nutrients.

⁷Not detectable.

in lipid fractions were separated using a 60-m × 0.25-mm i.d. fused-silica capillary column (SP-2380, Supelco, Inc., Bellefonte, PA). The injector and detector temperatures were maintained at 260 and 290°C, respectively. The initial oven temperature was held at 60°C for 0.10 min, increased at 17°C/min to 168°C (held for 27 min), and then increased at 4°C/min to 235°C (held for 5 min). Helium was the carrier gas. Fatty acids were expressed as percentages (by weight) of the total fatty acids.

The data were subjected to a least squares ANOVA for a three 3 × 3 Latin square design (Steel and Torrie, 1980) using the general linear model procedure of SAS (SAS System for Windows, version 8e, 1999-2001, SAS Institute Inc., Cary, NC). The model included square, cow within square, period within square, dietary treatment, milking, and residual error. The milking time × dietary treatment interaction was never significant ($P > 0.05$) and so was removed from the model. When the overall dietary treatment effect was significant, the least significant difference test was used to compare means. Mean milk fatty acid profiles, calculated per cow and dietary treatment, were subjected to principal component factor analysis and to canonical dis-

criminant analysis using the factor and discriminant procedures of SAS, respectively (SAS version 8e, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The mean chemical composition of the diets is given in the companion paper (Cabrita et al., 2003; Table 3). The main differences in composition between experiments were the higher level of CP in experiment 2, and the lower level of starch in experiment 1. Within experiment 1, diets varied in CP content from 14.0 to 17.5% of DM, whereas diet RL had a high total sugar content as a result of the high inclusion of citrus pulp. The true protein content of diets decreased through diets U0, U5, and U10 in experiment 2, while other nutrients remained similar. In experiment 3, the diet composition was the same across treatments, with the only difference being the diurnal pattern of distribution of the protein-rich concentrate. The fatty acid composition of diet ingredients is shown in Table 1. The major difference in fatty acid composition of the diets was the increase in C_{18:0} and decrease in C_{18:2} and C_{18:3} content between diets RL and RH in experiment 1;

Table 2. Experiment 1: least square means for milk fatty acid profiles (% of total fatty acids) from the different dietary treatment.

Fatty acids (%)	RL ¹	RM ¹	RH ¹	SEM	P
n	16	16	16		
C _{4:0}	0.70	0.58	0.62	0.071	NS
C _{6:0}	0.68	0.52	0.57	0.050	NS
C _{8:0}	0.65 ^a	0.49 ^b	0.51 ^b	0.033	**
C _{10:0}	2.15	1.51	1.89	0.177	†
C _{11:0}	0.24 ^a	0.19 ^b	0.20 ^b	0.011	*
C _{12:0}	2.90 ^a	2.43 ^b	2.37 ^b	0.119	**
C _{14:0}	10.81 ^a	9.66 ^b	9.28 ^b	0.188	***
C _{14:1}	0.95	0.96	0.97	0.048	NS
C _{15:0}	1.10	1.04	1.13	0.044	NS
<i>anteiso</i> C _{15:0}	0.62 ^a	0.48 ^b	0.48 ^b	0.040	*
<i>iso</i> C _{15:0}	0.27	0.31	0.29	0.050	NS
C _{16:0}	32.03 ^a	33.44 ^b	34.34 ^c	0.281	***
C _{16:1}	2.13	2.23	2.22	0.045	NS
C _{17:0}	0.69 ^a	0.60 ^b	0.55 ^c	0.019	***
<i>anteiso</i> C _{17:0}	0.21	0.21	0.22	0.009	NS
<i>iso</i> C _{17:0}	0.48	0.47	0.45	0.016	NS
C _{17:1}	0.52	0.21	0.38	0.179	NS
C _{18:0}	11.13	10.87	11.76	0.405	NS
C _{18:1}	25.27 ^a	29.01 ^b	27.66 ^b	0.483	***
C _{18:2}	3.71 ^a	2.51 ^b	2.10 ^c	0.103	***
C _{18:3}	<0.05	<0.05	<0.05		
C _{14:1} /C _{14:0}	0.09	0.10	0.11	0.005	†
C _{16:1} /C _{16:0}	0.07	0.07	0.07	0.003	NS
C _{17:1} /C _{17:0}	0.89	0.34	0.70	0.330	NS

^{a,b,c}Values in the same row that share a common superscript are not statistically different ($P > 0.05$).

¹Diets are named according to the effective RDP:fermentable metabolizable energy ratio of the diet: RL, RM, and RH providing a large deficiency, a slight deficiency, and a slight excess, respectively.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† $P < 0.10$.

this was associated with the use of an hydrogenated fat supplement. Levels of four of the odd-chain fatty acids (*iso* C_{15:0}, *anteiso* C_{15:0}, *iso* C_{17:0}, and C_{17:1}) were generally below the detection limit (0.05% of total fatty acids). Feeds contained traces of C_{15:0}, *anteiso* C_{17:0}, and C_{17:0}. There were no relationships between levels of these fatty acids in feeds and in milk.

The effects of the different N supplementation strategies on milk fatty acid profiles are presented in Tables 2, 3, and 4 for experiment 1, 2, and 3, respectively. Overall milk fat from these 3 experiments was rich in C_{14:0} (9.28 to 11.19%), C_{16:0} (32.0 to 37.8%), C_{18:0} (7.75 to 11.76%), and C_{18:1} acids (24.4 to 29.0%), with only low levels of C_{18:2} (2.1 to 3.1%) and C_{18:3} (0 to 0.25%); this is typical of milk produced from diets with a high content of corn silage and only low levels of grass-based forage (Chilliard et al., 2001).

Diet RL in experiment 1 led to a significant increase in the de novo synthesized fatty acids C_{8:0}, C_{12:0}, and C_{14:0}, in comparison with diets RM and RH. It is likely that this effect results both from the high levels of sugars and pectins (from citrus pulp), and the low level of fat in this diet. Citrus pulp leads to a high acetate

+ butyrate rumen fermentation (Wing, 1975; Sutton et al., 1987), whereas supplementary long-chain fatty acids can inhibit de novo synthesis of fatty acids in the mammary gland (Palmquist and Jenkins, 1980; Grummer, 1991). Levels of C_{16:0} in milk from both experiments 1 and 2 changed in line with dietary supply-effects of increased inclusion of hydrogenated fat and the substitution of soybean meal by urea, respectively. Similarly, levels of C_{18:2} in milk changed in line with the substantial differences in dietary supply of C_{18:2} in experiment 1. Higher levels of C_{18:2} with diet RL may have been further promoted by the effect of low dietary N in diminishing rates of biohydrogenation (Gerson et al., 1983). In experiment 2, the levels of C_{18:2} in milk were reduced with the increase in urea inclusion. The lack of agreement between levels of C_{18:1} in diets and milk from experiment 1 reflect the fact that there are other sources of this fatty acid in milk, including biohydrogenation of C_{18:2} and C_{18:3} and desaturation of C_{18:0}.

Alpha-linolenic acid (C_{18:3}) was only detected in milk from experiment 3 and this may reflect reduced biohydrogenation capacity with these low-protein, high-

Table 3. Experiment 2: least square means for milk fatty acid profiles (% of total fatty acids) from the different dietary treatment.

Fatty acids (%)	U0 ¹	U5 ¹	U10 ¹	SEM	P
n	18	18	14		
C _{4:0}	0.99	0.91	0.89	0.072	NS
C _{6:0}	0.88	0.77	0.78	0.052	NS
C _{8:0}	0.71	0.63	0.65	0.033	NS
C _{10:0}	2.24	2.03	2.15	0.081	NS
C _{11:0}	0.28	0.26	0.27	0.011	NS
C _{12:0}	3.19	2.92	3.10	0.084	†
C _{14:0}	11.19	10.88	11.12	0.121	NS
C _{14:1}	1.03 ^a	0.96 ^b	0.96 ^b	0.017	**
C _{15:0}	1.38	1.37	1.43	0.035	NS
<i>anteiso</i> C _{15:0}	0.43	0.41	0.38	0.013	NS
<i>iso</i> C _{15:0}	0.18	0.18	0.16	0.009	NS
C _{16:0}	36.81 ^a	37.42 ^{ab}	37.81 ^b	0.232	*
C _{16:1}	2.11	2.08	2.09	0.030	NS
C _{17:0}	0.60	0.60	0.62	0.013	NS
<i>anteiso</i> C _{17:0}	0.16	0.17	0.17	0.007	NS
<i>iso</i> C _{17:0}	0.31 ^a	0.31 ^a	0.28 ^b	0.009	*
C _{17:1}	0.17	0.17	0.18	0.008	NS
C _{18:0}	9.00	9.39	8.89	0.159	†
C _{18:1}	24.43	25.24	24.74	0.363	NS
C _{18:2}	2.46 ^a	2.32 ^{ab}	2.20 ^b	0.067	*
C _{18:3}	<0.05	<0.05	<0.05		
C _{14:1} /C _{14:0}	0.09	0.09	0.09	0.003	NS
C _{16:1} /C _{16:0}	0.06	0.06	0.06	0.001	NS
C _{17:1} /C _{17:0}	0.28	0.27	0.27	0.008	NS

^{a,b,c}Values in the same row that share a common superscript are not statistically different ($P > 0.05$).

²Diets are named according to the urea content of the concentrates U0: 0%; U5: 0.5%; and U10: 1.0%.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† $P < 0.10$.

starch diets (Gerson et al., 1983). Sauvant and Bas (2001) stated that the efficiency of hydrogenation is significant and positively related with acetate:propionate ratio in the rumen and negatively related with the efficiency of microbial growth and the proportion of concentrate in diets. A reduced amount of lipolytic bacteria from grain feeding (low pH) appears to explain the diminished capacity for biohydrogenation, since a free carboxyl group on fatty acids is a prerequisite for the initial isomerization step in biohydrogenation (Jenkins, 1993, 1994).

There were significant treatment effects on levels of odd-chain fatty acids in milk, particularly in experiments 1 and 3. Most notable were higher levels of *anteiso* C_{15:0} with diet RL, and a progressive reduction in levels of C_{17:0} with increasing dietary CP content in experiment 1. The highest levels of many of the odd-chain fatty acids (significantly so in the case of C_{15:0}, *anteiso* C_{15:0}, C_{17:0}, and *anteiso* C_{17:0}) were associated with the asynchronous single protein meal of diet D1 in experiment 3. As these fatty acids are thought to derive from rumen microbes, the differences found can reflect differences in substrates and/or the rumen environment.

Table 5 presents a correlation matrix for the entire dataset, including milk odd-chain fatty acid profiles, dietary composition, and predicted microbial CP supply. These results show the significant relationships between dietary supply of N and carbohydrate and milk odd-chain fatty acid profiles. The *anteiso* C_{15:0} was significantly and positively correlated with dietary sugars content ($r = 0.596$), with the highest levels of this fatty acid in diet RL probably explained by the high inclusion of citrus pulp. This effect is consistent with the higher content of this fatty acid in liquid-associated bacteria (Lee et al., 1999). Levels of *anteiso* C_{15:0} in all of these experiments were similar to values obtained with diets based on pea-wheat bi-crop silages or mature silage with a low content of water soluble carbohydrates in the work reported by Salawu et al. (2002).

Dietary CP content was significantly and negatively correlated with *iso* C_{17:0}, *anteiso* C_{17:0}, and C_{17:0} ($r = -0.669$; $r = -0.578$; and $r = -0.559$, respectively), suggesting that these fatty acids might be markers of protein deficiency. Levels of C_{17:0} ranged from 0.80 for the 14.5% CP (in the DM) asynchronous diet D1 down to 0.55% of milk fatty acids for the 17.5% CP (in the

Table 4. Experiment 3: least square means for milk fatty acid profiles (% of total fatty acids) from the different dietary treatment.

Fatty acids (%)	DU ¹	D2 ¹	D1 ¹	SEM	P
n	14	14	14		
C _{4:0}	0.57 ^{ab}	0.66 ^a	0.42 ^b	0.054	*
C _{6:0}	0.47 ^{ab}	0.55 ^a	0.40 ^b	0.039	*
C _{8:0}	0.41	0.48	0.38	0.028	†
C _{10:0}	1.41	1.53	1.36	0.074	NS
C _{11:0}	0.20	0.21	0.21	0.012	NS
C _{12:0}	2.39	2.47	2.37	0.092	NS
C _{14:0}	10.08	10.16	9.96	0.205	NS
C _{14:1}	1.25	1.20	1.21	0.034	NS
C _{15:0}	1.30 ^a	1.24 ^a	1.47 ^b	0.051	*
<i>anteiso</i> C _{15:0}	0.41 ^a	0.46 ^b	0.45 ^b	0.010	*
<i>iso</i> C _{15:0}	0.22	0.23	0.22	0.005	NS
C _{16:0}	35.26	35.99	35.54	0.405	NS
C _{16:1}	2.76	2.68	2.91	0.067	†
C _{17:0}	0.72 ^a	0.71 ^a	0.80 ^b	0.017	**
<i>anteiso</i> C _{17:0}	0.25 ^a	0.26 ^{ab}	0.28 ^b	0.007	*
<i>iso</i> C _{17:0}	0.53	0.49	0.54	0.018	NS
C _{17:1}	0.27 ^{ab}	0.28 ^a	0.28 ^b	0.009	*
C _{18:0}	7.94	8.42	7.75	0.184	†
C _{18:1}	27.74	26.39	27.49	0.575	NS
C _{18:2}	2.99 ^a	2.70 ^b	3.07 ^a	0.074	**
C _{18:3}	0.24	0.24	0.25	0.008	NS
C _{14:1} /C _{14:0}	0.13	0.12	0.12	0.010	NS
C _{16:1} /C _{16:0}	0.08	0.07	0.08	0.005	NS
C _{17:1} /C _{17:0}	0.38	0.35	0.36	0.020	NS

^{a,b,c}Values in the same row that share a common superscript are not statistically different ($P > 0.05$).

¹Treatments are named according to the mode of distribution of the protein-rich concentrate: DU: as a TMR with the basal diet; D2: in two meals, immediately before the distribution of the basal diet; and D1: in one meal, immediately before the distribution of the morning meal.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

† $P < 0.10$.

DM) diet RH. The concentration of C_{17:0} in milk fatty acids was similarly low with the high-protein diets (18.2 to 20.2% CP in the DM) used by Salawu et al. (2002). It is not clear why levels of C_{17:0} should increase with protein deficiency, though one explanation is a change in the microbial population. For example, it is known that there are significant differences between rumen bacteria in their content of C_{17:0} (Bae et al., 2000). The significant negative correlation between dietary NDF and dietary CP ($r = -0.445$) and the fact that *iso* C_{17:0} and *anteiso* C_{17:0} were also significantly and positively correlated with dietary NDF content ($r = 0.567$ and $r = 0.463$, respectively) leaves open the possibility that these fatty acids are related to NDF supply. The high positive correlation ($r = 0.789$) found between *iso* C_{17:0} and *anteiso* C_{17:0} might also suggest that both fatty acids are associated with the same rumen microbial population.

The decrease in the dietary true protein content in experiment 2 tended to reduce levels of *iso* C_{17:0} and *anteiso* C_{15:0}, possibly reflecting a reduction in the supply of branched-chain precursors (i.e., AA) as sug-

gested by Sauvant and Bas (2001). The effect on levels of branched odd-chain fatty acids noted in experiment 3 could be explained by an increased supply of precursors as a result of rumen protein inefficiency with the asynchronous diet (D1). However, this hypothesis is not supported by the results of experiment 1, which involved large differences in CP content and showed either no effect or a negative effect of additional protein on branched-odd-chain fatty acids in milk, with the exception of *iso* C_{15:0}. It is difficult to interpret the correlations between dietary starch content and milk odd-chain fatty acid profiles, due the fact that starch content within experiment and between experiments 2 and 3 was very similar, and due to the high significant correlation ($r = -0.805$) between dietary NDF and starch contents.

Microbial CP supply, predicted according AFRC (1993) and presented in the companion paper (Cabrita et al., 2003), was significantly and negatively correlated with all odd-chain fatty acids, with the exception of *iso* C_{15:0} and C_{15:0}. It is difficult to identify whether this effect is real or if it reflects the relationship be-

Table 5. Correlation between milk odd-chain fatty acids (% of total fatty acids), dietary composition (% of DM) and predicted microbial CP supply (MCP; g/d).

	iso C _{15:0}	anteiso C _{15:0}	C _{15:0}	iso C _{17:0}	anteiso C _{17:0}	C _{17:0}	C _{17:1}	%CP	%Starch	%NDF	%Sugars	MCP
iso C _{15:0}	1.000											
anteiso C _{15:0}		1.000										
C _{15:0}			1.000									
iso C _{17:0}				1.000								
anteiso C _{17:0}					1.000							
C _{17:0}						1.000						
C _{17:1}							1.000					
%CP								1.000				
%Starch									1.000			
%NDF										1.000		
%Sugars											1.000	
MCP												1.000

P* < 0.05.*P* < 0.01.****P* < 0.001.

tween dietary composition (mainly CP content) and milk odd-chain fatty acids discussed above, since in most cases predicted microbial CP supply was limited by RDP supply.

Principal component factor analysis, which examines relationships within a single set of variables, was used to gain insight into the patterns of change of different fatty acid groups within the range of samples and diets used in this work. Clusters of fatty acids that are further away from the origin suggest common origins or pathways (Massart-Leën and Massart, 1981), which are important to the overall variation within fatty acid results. Recently, Fievez, Vlaeminck, and Dewhurst (2003, personal communication) used principal component analysis on milk fatty acid profiles from a much wider range of dietary treatments than was used in the current study and showed that it is possible to identify common pathways of digestion and metabolism of fatty acids (including de novo synthesis, dietary supply, microbial synthesis, and the action of Δ^9 -desaturase in the mammary gland).

The principal component factor analysis appeared to provide a reasonable description of our data. Results are presented in Figures 1 (comparison of factors 1 and 2), and 2 (comparison of factors 1 and 3). The first factor, explaining 47.2% of total variation, aggregated short- and medium-chain fatty acids in opposition to *iso* C_{17:0}, *anteiso* C_{17:0}, and C_{18:1}. The second factor explained 21.7% of variation and aggregated C_{14:1}, C_{16:1}, C_{15:0} and C_{17:0} in opposition to C_{18:0} and factor 3 explained 13.3 % of variation and aggregated *anteiso* C_{15:0}, C_{17:0}, *iso* C_{17:0} and C_{18:2}, in opposition to C_{16:0}.

Immediately apparent is the very close association between the short-chain fatty acids (C_{4:0} to C_{10:0}) and, to a lesser extent, the medium-chain fatty acids (C_{12:0} and C_{14:0}), reflecting their common origin in de novo synthesis. The fact that C_{16:0} was not clustered with short- and medium-chain fatty acids might be due to its dual origin, with a proportion dependent on dietary supply (Jenkins, 1993). The fact that C_{11:0} clustered with C_{12:0} and C_{14:0} confirms the suggestion (Diedrich and Henschel, 1990) that it is a chain elongation fatty acid. The loading plots (Figures 1 and 2) also showed a clear association of the monoenoic milk fatty acids (C_{14:1}, C_{16:1}, and to a lesser extent C_{17:1}), which are predominantly produced by mammary Δ^9 -desaturase activity on the corresponding saturated fatty acids. Whereas these results confirm similarity (factors 1 and 3), there is clearly something different (factor 2) about the pathways for production of C_{17:1} in milk. This is also confirmed by the fact that the ratio C_{17:1}/C_{17:0} (0.645, 0.273, and 0.362 for experiments 1, 2, and 3 respectively) was very different to the ratios C_{16:1}/C_{16:0} (0.067, 0.056, and 0.078, respectively) and C_{14:1}/C_{14:0}

(a)

(b)

Figure 1. Plots of factor 1 versus factor 2 from principal component factor analysis based on milk fatty acids (a) unrotated and (b) rotated (orthogonal).

(a)

(b)

Figure 2. Plots of factor 1 versus factor 3 from principal component factor analysis based on milk fatty acids (a) unrotated and (b) rotated (orthogonal).

Figure 3. Plot of diet means for canonical variates based on milk even-chain fatty acids (CAN 1 versus CAN 2). Dietary treatments are named according to the effective RDP:fermentable metabolizable energy ratio of the diet: RL, RM, and RH provided a large deficiency, a slight deficiency, and a slight excess, respectively; to the urea content of the concentrates included, U0: 0%; U5: 0.5%; and U10: 1.0%; and to the mode of distribution of the protein-rich concentrate: DU: as a TMR with the basal diet; D2: in two meals, immediately before the distribution of the basal diet; and D1: in one meal, immediately before the distribution of the morning meal.

(0.098, 0.089, and 0.123, respectively). Similarly the simple correlation between $C_{14:1}$ and $C_{16:1}$ was 0.74, whereas the correlations between $C_{14:1}$ and $C_{17:1}$, and $C_{16:1}$ and $C_{17:1}$ were 0.05 and 0.11, respectively. The fact that $C_{18:1}$ did not cluster with the other monoenoic fatty acids is expected because of its multiple origins noted above. For this reason we must be cautious in assigning any significance to the clustering of *anteiso* $C_{17:0}$, *iso* $C_{17:0}$, and $C_{18:1}$, although this effect was evident even when performing separate principal component factor analyses for each experiment.

Canonical discriminant analysis, a dimension-reduction technique related to principal component and canonical correlation, was used to provide a summary of univariate ANOVA. Plots of canonical variates for each diet, based on even- and odd-chain fatty acids, are presented in Figures 3 and 4, respectively. The milk odd-chain fatty acids were more sensitive to dietary treatments than even-chain fatty acids, as clearly shown by the greater separation of treatments in experiment 3.

Table 6 provides the raw canonical coefficients for the independent variables based on separate consideration of either even- or odd-chain fatty acids. Comparison of the size of coefficients confirmed the results of univariate analysis in identifying fatty acids that

varied across the dataset. Coefficients based on even-chain fatty acids showed that $C_{6:0}$ and $C_{8:0}$ contributed most to the variates, which illustrates the major importance of ruminal substrates for de novo synthesis in the mammary gland. The most significant odd-chain fatty acids were $C_{17:0}$, *anteiso* $C_{15:0}$, *anteiso* $C_{17:0}$, and *iso* $C_{17:0}$.

CONCLUSIONS

This study demonstrates that milk fatty acid profiles are significantly affected by both N and carbohydrate supply. Principal component factor analysis appeared to provide a reasonable description of the data, clearly discriminating between fatty acids that are synthesized in the mammary gland (short- and medium-chain fatty acids), that depend on Δ^9 -desaturase activity (monoenoic fatty acids), that are mainly absorbed directly from the blood stream (long-chain fatty acids) and those that depend on rumen microbial populations (odd-chain fatty acids). Additionally, milk odd-chain fatty acids provided a greater discriminatory power between diets. The *anteiso* $C_{15:0}$ increased in line with dietary sugar supply, and $C_{17:0}$ seems to be a marker of protein deficiency. Additionally, *iso* $C_{17:0}$ and *anteiso* $C_{17:0}$ were associated with NDF and CP contents of

Figure 4. Plots of diet means for canonical variates based on milk odd-chain fatty acids (a) CAN 1 versus CAN 2 and (b) CAN 1 versus CAN 3. Dietary treatments are named according to the effective RDP:fermentable metabolizable energy ratio of the diet: RL, RM, and RH provided a large deficiency, a slight deficiency, and a slight excess, respectively; to the urea content of the concentrates included, U0: 0%; U5: 0.5%; and U10: 1.0%; and to the mode of distribution of the protein-rich concentrate: DU: as a TMR with the basal diet; D2: in two meals, immediately before the distribution of the basal diet; and D1: in one meal, immediately before the distribution of the morning meal.

Table 6. Raw canonical coefficients for the significant canonical variables for milk even- and odd-chain fatty acids.

Even-chain	CAN 1 ¹	CAN 2 ¹	Odd-chain	CAN 1 ¹	CAN 2 ¹	CAN 3 ²
C _{4:0}	0.8451	5.2316	C _{15:0}	-2.4666	-1.8419	-1.6997
C _{6:0}	10.6579	-4.1669	<i>anteiso</i> C _{15:0}	6.1965	-4.5297	6.5648
C _{8:0}	-16.4190	2.4765	<i>iso</i> C _{15:0}	-0.2785	-5.2112	-3.5447
C _{10:0}	0.4284	-1.3051	C _{17:0}	8.6975	16.7390	6.2894
C _{12:0}	1.5129	0.0984	<i>anteiso</i> C _{17:0}	-6.0185	1.3788	-18.2093
C _{14:0}	0.3699	1.1581	<i>iso</i> C _{17:0}	7.1892	-7.2549	-4.2372
C _{14:1}	-0.2115	-0.0677	C _{17:1}	2.0919	0.1569	2.2970
C _{16:0}	0.4747	0.2281				
C _{16:1}	-0.2368	-0.0955				
C _{18:0}	0.1596	0.1290				
C _{18:1}	0.4517	0.0725				
C _{18:2}	-1.0584	1.7230				
R ^{2,3}	0.754	0.618		0.733	0.542	0.497

¹*P* < 0.001.²*P* < 0.05.³Between CAN_i and the class variables.

diets. The results suggest that milk odd-chain fatty acids have potential to be used as a noninvasive technique to assess rumen function in terms of microbial populations, substrates and interactions, though this hypothesis should be further validated, for example by the use of molecular microbial ecology techniques. Studies are also needed to identify the effects of other factors, such as stage of lactation (mobilization of body fat) and dietary fat sources on these relationships.

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