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Effect of a Total Mixed Ration diet on the concentration of amino acid-derived volatiles in milk

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ABSTRACT

The flavour chemistry of milk from Holstein-Friesian cows of two genotypes fed total mixed rations (TMR) or pasture has been compared over three years. Mean concentrations of selected milk aroma compounds, namely skatole, indole, dimethylsulphide, toluene, and of total *p*-cresol (determined after hydrolysis of conjugates) were significantly lower (10% to 39% of pasture treatment, $P < 0.01$) in the milk from the TMR-fed cows. Mean milk non-protein nitrogen (NPN) and dietary crude protein content (CP) were lower (93% and 67% of pasture treatment respectively) for the TMR diet. These results are consistent with an origin for these aroma compounds in the degradation of dietary protein amino acids in the rumen. However, concentrations of total *m*-cresol, which is not thought to derive from dietary protein, were also lower in the milk from TMR-fed cows (19% of pasture treatment). In addition, for the pasture treatment, the patterns of variation of concentrations of skatole, indole and *p*-cresol and NPN in milk and of dietary CP differed considerably between different periods within a dairy season. These findings indicate that factors additional to CP supply are important in the metabolism of these flavour compounds.

Keywords: diet; dairy cows; milk flavour; indoles; cresols; dimethylsulphide; toluene.

INTRODUCTION

The synthesis and accumulation of flavour metabolites in milk from dairy cows is dependent on diet (e.g., Urbach, 1990). An improved understanding of the effects of different dietary regimes on the formation of milk flavour volatiles in the cow could enable management of dairy product flavour to be extended back to the farm.

"Total mixed ration" (TMR) diets for dairy cows are designed to provide an optimised supply of nutrients enabling cows fed TMR to achieve levels of milk production which cannot be matched by cows grazing pasture (Kolver & Muller, 1998). Bendall (2001) established in a gas chromatography – olfactometry study that although sensory responses to aroma extracts of milk from TMR-fed cows and pasture-fed cows differed, the extracts contained a very similar array of aroma-active compounds. He concluded that flavour differences were primarily due to different concentrations in the milk of a common set of flavour compounds. For many of these compounds, these differences may arise from differences in rumen metabolism on the two diets.

Skatole and indole are flavour compounds which occur in higher concentrations in the milk of cattle fed pasture than those on a reduced protein diet (Urbach, 1990). They have been identified as products of ruminal degradation of tryptophan (Kemmer *et al*., 1997 and references therein). On pasture diets, the crude protein content is often relatively high, and amino acids from the excess protein are used as an energy source by rumen microbes (Mackle *et al*., 1999). Bendall (2001) has proposed that under these conditions, amino acids such as tryptophan undergo microbial degradation in the rumen, and that this accounts for the higher skatole and indole in milk from pasture-fed cows. Other milk aroma compounds may similarly be derived from rumen degradation of amino acids. Thus *p*-cresol (4-methylphenol) may derive from tyrosine (Martin, 1982), toluene from phenylalanine and dimethylsulphide from methionine (Yvon & Rijnen, 2001).

In the present study, the concentrations of skatole, indole, *p*-cresol, dimethylsulphide and toluene in milk of cows grazing pasture or fed TMR have been compared over three dairy seasons. These data have been examined in relation to dietary parameters, particularly crude protein content (CP), and the non-protein nitrogen (NPN) content of the milk (48% urea; DePeters & Ferguson, 1992).

If these aroma compounds derive primarily from ruminal degradation of amino acids due to excess dietary protein, they should show a positive association with CP, including variations in CP within a dairy season. A positive association with milk NPN would also be expected as amino acid degradation in the rumen is associated with the generation of excess ammonia, which is metabolised to urea in the animal.

For comparison we have also monitored *m*-cresol (3-methylphenol), a co-analyte of *p*-cresol for which there is no corresponding protein amino acid progenitor. Treatment effects on *p*- and *m*-cresol concentrations were determined by analysis of the phenols released after hydrolysis of conjugates (Lopez & Lindsay, 1993). Milk from two Holstein-Friesian (HF) genotypes of cows was examined, an "Overseas" (OS) and a "New Zealand" (NZ) genotype.

MATERIALS AND METHODS

Dairy cow trial

Groups of HF cows of either NZ or OS genetic origin were managed in two contrasting dietary systems. Management and dietary details have been previously described (Kolver *et al*., 2000; Kolver *et al*., 2002). Cows either grazed generous levels of a ryegrass/white clover pasture (n=26), or were fed a TMR comprising maize silage, grass silage, hay, and concentrates (n=27). In year 1 of the study, all animals were primiparous with a smaller

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but equal proportion of each herd maintained as first lactation animals during later years (Lacy-Hulbert *et al.,* 2002). Cows entering the herds each year were balanced for genotype, breeding worth and calving date and, within genotype, cows were balanced for live weight and sire.

Sampling for aroma metabolite analysis

Milk samples were collected on one day at two-week intervals (with occasional longer intervals) from each of the four treatment herds for chemical and other analyses. Samples were immediately refrigerated at 4°C and transported overnight under refrigeration to Palmerston North for analysis.

Analytical methods

Diet samples were collected weekly and oven-dried at 60°C for near-infrared analysis (Ulyatt *et al*., 1995) of nutrient composition. In year 1, analysis was carried out on a weekly basis. In subsequent years a monthly composite was analysed.

Milk samples were analysed weekly by near-infrared reflectance, and concentrations of milk NPN (as μg g^{-1} milk) were calculated as the difference between crude protein and true protein content.

Concentrations of skatole and indole (ng g^{-1} milk fat) were measured using an adaptation of the method of Dehnhard *et al.,* (1993). Milk fat was separated from whole milk (100 ml) by centrifugation at 10,800 g in a refrigerated centrifuge for 30 minutes. A sub-sample of the milk fat was melted in a water bath (55°C) and a 100 μl aliquot was dissolved in 1 ml of hexane. Internal standard (2-methylindole) was added, and the solution was partitioned with acetonitrile/water (75:25 v/v) and an aliquot (50 μl) of the aqueous fraction was analyzed by HPLC with fluorescence detection (excitation 275 nm, emission 345 nm).

Concentrations of *p*-cresol and *m*-cresol in skim milk were determined as totals after hydrolysis (μg l^{-1}). Hydrolysis of the conjugates in 1 ml of skim milk was carried out by a sequential acid and enzyme treatment procedure adapted from that of Lopez & Lindsay (1993). The hydrolysis products were separated by solid phase extraction on a divinylbenzene cartridge and elution through an alumina cartridge. The released free phenols were analysed by GC-MS (QP-5050A Shimdazu, Kyoto, Japan) utilising 2-ethylphenol as internal standard (Lane & Fraser, 1999). Phenol concentrations have not been reported due to interferences in the analysis.

Concentrations of toluene and dimethylsulphide in milk were determined for a sub-set of samples in the 2000 – 2001 season by solid-phase micro extraction (SPME). Milk (40 ml) containing heptanol (50 ng g^{-1}) as the internal standard was stirred at 50°C in a 50 ml round-bottomed flask fitted with a rubber septum. A Carboxin-coated SPME fibre (Supelco, Castle Hill, NSW, Australia) was used to sample the headspace volatiles for 30 min. Volatiles were desorbed from the SPME fibre in the GC injection port (1 min., 280°C, 5 psi helium head pressure) and refocussed on a short portion of the GC column (Supelco Wax-10, 30m x 0.25 mm ID, 0.25μm film thickness) cooled in liquid nitrogen. After one minute, the coolant was removed and the volatiles were analysed by GC-MS. Relative concentrations were determined as the ratio of peak areas to that of the internal standard (taken as 100). The efficacy of this method for dimethylsulfide was subsequently confirmed by stable isotope dilution analysis.

Statistical analysis

The significance of treatment effects on concentrations of milk constituents was analysed in a general linear model using Minitab statistical software (Minitab Inc., 1999). The flavour compound data was log-transformed for the analysis. Estimated (back-transformed) means and standard errors of means from the analysis of variance are reported in Tables 2 and 3.

RESULTS

The mean crude protein content provided in the total mixed ration formulation was much lower that of the pasture samples (Table 1) (67% of pasture). Both diets had similar metabolisable energy and acid detergent fibre content, but the soluble carbohydrate content was very much higher, and the neutral detergent fibre content was lower in the TMR than in the pasture (275% and 79% of pasture respectively).

TABLE 1: Mean composition (SEM) of the pasture and TMR diets fed to lactating dairy cows during the 1998-1999, 1999-2000, and 2000-2001 dairy seasons. Units are g $100g^{-1}$ DM unless otherwise stated.

	Pasture	TMR
Crude protein (CP)	26.4 (0.3)	17.8 (0.3)
Lipid	4.5 (0.1)	6.2 (0.1)
Ash	10.4 (0.3)	9.3 (0.3)
Acid detergent fibre (ADF)	20.3 (0.5)	20.6 (0.5)
Neutral detergent fibre (NDF)	39.7 (0.7)	31.4 (0.7)
Soluble carbohydrates	11.0 (0.7)	30.2 (0.7)
Metabolisable Energy (MJ kg^{-1} DM)	11.6 (0.2)	11.8 (0.2)

TABLE 2: Mean (SEM) concentration of metabolites in milk from lactating dairy cows of two genotypes fed pasture or TMR during the 1998-1999, 1999-2000, and 2000-2001 dairy seasons.

Diet:				
Metabolite	**Units**	**Pasture**	**TMR**	**Significance**
NPN[a]	μg g^{-1}	353 (7)	327 (8)	*
Skatole[b]	ng g^{-1}	102 (7)	14 (1)	***
Indole[b]	ng g^{-1}	67 (4)	14 (1)	***
p-Cresol[c]	μg l^{-1}	2112 (59)	808 (22)	***
m-Cresol[c]	μg l^{-1}	421 (25)	92 (8)	***
Toluene[d]	Rel.	1015 (127)	95 (12)	***
DMS[de]	Rel.	60 (13)	14 (3)	**

[a] NPN = non-protein nitrogen in whole milk.
[b] Concentration in milk-fat.
[c] Concentration (free and conjugated) in skim milk.
[d] Relative concentration in headspace above milk in arbitrary units. Data for 2000–2001 only
[e] DMS = dimethylsulphide.

Mean concentrations of milk metabolites for the pasture and TMR diets are shown in Table 2. Concentrations of all of the selected aroma metabolites were significantly lower in milk from the TMR-fed cows than in the milk from pasture-fed cows (10% to 39% of pasture-fed). The difference was least marked for the milk NPN concentration (93% of pasture-fed). The data

presented are pooled measurements across the two genotypes. Minor but significant genotype and genotype x diet effects were also observed (data not shown). On the pasture treatment, concentrations of skatole and indole were higher in the milk of OS cows than that of NZ cows. Concentrations of *p*-cresol were higher in the milk of OS than NZ cows on both diets.

For several of the metabolites, concentrations in milk varied within a dairy season. To analyse these variations, the dairy season was subdivided in arbitrary periods of approximately two months (Early, Mid, Summer, Late; Table 3) and the mean concentrations of dietary CP and milk metabolites for these periods were compared. The CP content of the pasture diet and mean milk NPN concentration for pasture-fed cows was higher in the Early and Late periods. Concentrations of skatole and indole were highest in the Early period, and lowest in the Late periods. Concentrations of *m*-cresol were higher in the Mid and Summer periods. Concentrations of toluene were highest in the Late period. Variations in concentrations of *p*-cresol and dimethyl sulphide in milk were not significant (data not shown).

DISCUSSION

This study establishes that concentrations of skatole, indole, dimethylsulphide, toluene and total *p*-cresol and *m*-cresol were in each case lower in the milk from cows fed a TMR diet than in milk from cows fed a pasture diet (Table 2). The NPN concentration in the milk of the TMR-fed cows was also lower (Table 2), although by a much smaller differential, indicating that on the TMR diet with a much lower CP content, there was less excess ammonia generated in the rumen due to a lower degree of ruminal degradation of amino acids. The reduced degradation of the parent amino acids may account for the lower concentrations of skatole, indole, *p*-cresol, dimethylsulphide and toluene in milk from TMR-fed cows compared to pasture-fed cows. However, this hypothesis does not account for the lower concentrations on TMR of *m*-cresol, for which (as noted) there is no corresponding progenitor amino acid.

Further, analysis of the variations of concentrations in the milk of the aroma compounds and NPN, and of the CP content of the diet within feed treatments during the dairy season (Table 3 and Results), indicates dietary CP supply is not the only important factor affecting the concentrations of these aroma compounds. The much lower milk concentrations of indole and skatole observed in the Late period than in the Early period for pasture-fed cows cannot be explained in terms of dietary CP supply or milk NPN content which were relatively high in both periods.

Also, despite the variations observed in dietary CP and milk NPN content, the concentrations of *p*-cresol in milk did not vary significantly between periods. *p*-Cresol may derive from rumen degradation of both tyrosine in dietary protein and fibre-associated plant phenolics (Lane and Fraser, 1999). Concentrations of the isomer *m*-cresol however, are likely to derive only from fibre-associated plant phenolics (Lane and Fraser, 1999), and were also much lower in the milk from TMR-fed cows (Table 2). Comparing the differing patterns of variation of *m*-cresol and *p*-cresol between periods, the relative constancy of *p*-cresol concentrations may derive from compensating variations in fibre and protein degradation in the rumen.

This study has established that a TMR diet, in addition to having a large effect on milk yield, also results in considerably reduced concentrations in the milk of several flavour metabolites believed to derive from ruminal degradation of amino acids. One factor may be a better balance between protein and other nutrients on a TMR diet compared to the high CP content often found in pasture diets. However the similar treatment effect observed for a metabolite (*m*-cresol) believed to derive primarily from plant phenolics, and the differing pattern of variations in metabolite concentrations in milk from pasture-fed cows between different periods of the dairy season indicates that factors in addition to CP supply are

TABLE 3: Variation within dairy season of dietary crude protein and concentrations of metabolites in milk from lactating dairy cows groups fed pasture or TMR. Mean (SEM) of concentrations for seasonal periods during 1998-1999, 1999-2000, and 2000-2001.

		CP [a]	**NPN** [b]	**Skatole** [c]	**Indole** [c]	***m*-Cresol** [d]	**Toluene** [e]
	Units:	g/100 g DM	μg g^{-1}	ng g^{-1}	ng g^{-1}	μg l^{-1}	Rel.
Diet	**Period** [f]						
Pasture	Early	27.7 (0.4)	365 (2)	212 (20)	91 (9)	381 (38)	901 (201)
	Mid	25.4 (0.4)	338 (2)	99 (10)	62 (7)	540 (58)	727 (147)
	Summer	24.7 (0.4)	347 (2)	61 (6)	59 (6)	587 (67)	
	Late	25.9 (0.5)	408 (2)	57 (7)	55 (7)	273 (35)	1602 (404)
TMR	Early	18.3 (0.4)	362 (2)	23 (2)	20 (2)	73 (6)	69 (14)
	Mid	18.2 (0.4)	333 (2)	17 (2)	14 (1)	106 (10)	59 (12)
	Summer	17.9 (0.4)	325 (2)	11 (1)	11 (1)	147 (15)	
	Late	17.0 (0.5)	334 (2)	7 (1)	11 (1)	75 (7)	208 (53)
Significance							
Period		***	*	***	***	***	***
Diet × Period		**	NS	*	NS	NS	NS

[a] CP = Crude protein content of diet.
[b] NPN = non-protein nitrogen in whole milk.
[c] Concentration in milk-fat.
[d] Concentration in skim milk.
[e] Relative concentration in headspace above milk in arbitrary units. Data for 2000 –2001 only.
[f] early: late August – October; mid: November – December; summer: January – February; late: March - early May.

important. The relative importance of these factors might be clarified by studies with a modified TMR formulated to match the composition of pasture diets.

The results suggest that diet manipulation may be a tool for modifying dairy product flavour on the farm. While complete TMR diets, as used in this study, are not currently cost-competitive with pasture in New Zealand, this research points to the possibilities of using TMR, either alone or as a supplement, to produce milks of modified flavour for specialist uses.

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