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SKATOLE AND INDOLE FORMATION IN COMPARISON TO RUMEN AMMONIA AND VOLATILE FATTY ACIDS IN SHEEP FED FRESH FORAGES

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SUMMARY

Skatole and indole concentrations in the rumen were compared with the rumen concentrations of ammonia and volatile fatty acids (VFA) in sheep fed fresh white clover (WC; *Trifolium repens*), perennial ryegrass (PRG; *Lolium perenne*) or the condensed tannin-containing forage, *Lotus corniculatus* (LC). Six rumen fistulated Romney wethers were fed the cut forages over two 2-hour meal periods in the morning and afternoon, with rumen contents sampled at intervals after the start of the morning meal. Maximum concentrations of skatole, indole and ammonia were highest when feeding WC ($P<0.05$). Ammonia accumulates in the rumen when there is rapid protein degradation with unsynchronised energy supply. Condensed tannins in LC slowed protein degradation and consequently maximal ammonia, indole and skatole concentration were lower when feeding this forage ($P<0.05$). This indicates that skatole and indole formation will be greater when there is high protein degradability. Ammonia, indole and skatole concentrations in sheep fed PRG were intermediate, and not significantly different from either WC or LC. Maximum concentration of straight-chain volatile fatty acids (VFA) were not significantly different for the forages, but branched-chain VFA concentrations tended to be higher for WC ($P=0.103$) compared with LC. Straight-chain VFAs are a by-product of microbial carbohydrate fermentation in the rumen, but branched-chain VFAs are formed from deamination of amino acids. This suggests that skatole and indole production are not related to carbohydrate digestion in the rumen. In order for meat and milk producers in pastoral systems to ameliorate undesirable flavours and increase demand in certain markets, they will need to consider using alternative forages to reduce protein solubility and degradability.

Keywords: skatole, indole, rumen, ammonia, volatile fatty acids

INTRODUCTION

Consumers of products from systems where concentrate diets are fed often describe the flavour of meat and dairy products from ruminants raised on pasture as being grassy-, faecal- or animal-like (Rousset-Akrim *et al.*1997; Keen 1998). These pastoral flavours are undesirable in certain markets in Asia, Europe and America and, therefore, can affect the returns for meat and dairy products. This can be a limitation for low cost pasture-based agricultural sectors in New Zealand and Australia that export to these markets. The challenge is to develop methods of ameliorating pastoral flavours while maintaining low cost production systems.

The volatile compounds, indole and skatole, have been implicated in pastoral flavours (Young *et al.* 2002; Bendall 2001), and are formed in the rumen from the microbial fermentation of tryptophan (Deslandes *et al.* 2001). High skatole formation in the rumen is dependant on diet, and is associated with increased levels of ammonia and volatile fatty acids (VFA) (Carlson *et al.* 1983). Skatole and indole production is more influenced by rumen fermentation conditions than by tryptophan levels in the rumen (Carlson and Breeze 1984). Recently, wide differences were reported in the formation of skatole and indole in the rumen of sheep fed white clover (WC; *Trifolium repens*), perennial ryegrass (PRG; *Lolium perenne*) and the condensed tannin-containing forage, *Lotus corniculatus* (LC; Schreurs *et al.* 2003). To gain better understanding of factors affecting skatole and indole formation, ammonia and VFA concentrations were compared with skatole and indole in the rumen when feeding the same forages.

MATERIALS AND METHODS

Experimental design, animals and diets

The experimental procedures and protocols were reviewed and approved by the Crown Research Institute Animal Ethics Committee in Palmerston North according to the Animal Protection Act

(1960), Animal Regulations and amendments. Six rumen-cannulated Romney wethers about 12 months of age were housed indoors in metabolism crates. All 6 sheep were fed WC, PRG and LC in succession, each for a period of 3 weeks. The forages were harvested daily and stored at 4°C until required. The sheep were fed twice daily at 0700 and 1700 h, with all animals having been trained to consume as much forage as possible within 2 h. Forage offered and refused was weighed for each sheep to determine intake. Three 200 g samples of the feed offered and refused were taken to determine DM by oven drying at 90°C for 24 h. Samples of the forage offered were frozen, freeze dried and ground through a 1 mm sieve for determination of chemical composition and organic matter digestibility by Near Infra-red Reflectance Spectrometry (NIRS; FeedTech, AgResearch, Palmerston North, New Zealand).

Sampling rumen contents
Rumen contents were sampled in the third week of feeding each forage, with each sheep sampled twice, ensuring there was at least 1 day between the first and second sampling. Samples of rumen contents were taken prior to the morning meal and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6.25, 7, and 8 h after the start of the morning meal. Rumen contents from each sample time were gently squeezed through a double layer of cheese cloth to obtain rumen fluid. The pH of the rumen fluid was measured using a pH meter (PHM210 Standard pH meter, Radiometer Copenhagen, France). For skatole and indole analysis, 8 mL of the rumen fluid were frozen at -20°C. Another 5 mL of the rumen fluid were centrifuged at 20000 *g* for 10 min, and the supernatant frozen at -20°C for VFA analysis. A further 1.5 mL of the rumen fluid were added to 15 μL of concentrated hydrochloric acid, centrifuged at 11000 *g* for 15 min, and the supernatant frozen at -20°C for ammonia analysis.

Skatole/indole, VFA and ammonia concentrations in rumen fluids
Analyses were carried out on samples pooled from the 2 days of sampling. Indole and skatole concentrations in rumen fluid were analysed using a method adapted from Mattavi *et al.* (1999). Solid phase extraction was carried out on 0.5 mL of rumen fluid using an Isolute ENV+ cartridge (International Sorbent Technologies, Mid Glamorgan, England, 50 mg), and skatole and indole were eluted with methanol. To 2 mL of the elutant, 50 μL of 2-methylindole were added (internal standard, 0.05 μg/μL) and the indoles determined by high performance liquid chromatography with fluorescence detection (excitation, 285 nm and emission, 350 nm). A reverse-phase platinum C_{18} column (150 x 4.6 mm; Alltech, Auckland, NZ) was used. The mobile phase consisted of 70% acetic acid solution (1.2 mg/mL) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, England) at a flow rate of 1 mL/min. Volatile fatty acid concentrations were determined in the rumen fluid using the gas chromatography method of Attwood *et al.* (1998). Sample preparation involved the addition of 100 μL of 5% orthophosphoric acid and 100 μL of 1 mM caproic acid (internal standard) to 1.2 mL rumen fluid, followed by centrifugation (6000 *g* for 10 min). Ammonia in rumen fluid was analysed by reductive amination of 2-oxoglutarate to give a decrease in absorbance at 340 nm due to oxidation of NADPH proportional to the ammonia concentration (Neeley and Phillipson 1968).

Calculations and statistical analysis
To take account of differences in intakes observed between sheep, the concentrations of skatole, indole, branched-chain VFA (BC-VFA) and ammonia were then adjusted for crude protein intakes at the 2-h morning feed, and straight-chain VFA (*n*-VFA) concentrations adjusted for dry matter intakes at the 2-h morning feed. The maximal concentrations given in Table 2, therefore, represent concentrations per unit of intake. Data for intake and maximum concentrations of ammonia, skatole, indole and VFA were statistically analysed by analysis of variance using the GLM procedure of SAS (SAS 2001).

RESULTS

White clover was highest in crude protein and organic matter digestibility. *Lotus corniculatus* had the highest concentration of condensed tannin, with a protein content similar to that of white clover. Neutral detergent fibre content (NDF) was highest in the perennial ryegrass, and its protein content was much lower compared with the other 2 forages (Table 1). Dry matter intakes (DMI) and crude protein intakes (CPI) were lowest with WC.

Maximal concentrations of indole, skatole and ammonia were higher when feeding WC compared with LC. Ammonia, indole and skatole concentrations in sheep fed PRG were intermediate and not significantly different from either WC or LC. No difference was found between the forages for the maximum concentrations of *n*-VFA or BC-VFA (Table 2), however, maximum BC-VFA concentrations tended to be higher when feeding WC compared with feeding LC (P=0.103).

Table 1. Composition and average intakes of forages offered to sheep for 2 h at the morning feed.

	White clover	Perennial ryegrass	*Lotus corniculatus*	s.e.
Crude protein (g/kgDM)	265	172	248	-
Total condensed tannin (g/kgDM)	3.6	0.2	14.2	-
Neutral detergent fibre (g/kgDM)	242	455	227	-
Organic matter digestibility (%)	83.3	81.7	79.3	-
Dry matter intake (g/2-hour meal)	114[a]	351[b]	278[b]	42
Crude protein intake (g/2-hour meal)	31[a]	61[b]	67[b]	9

Means in the same row with different superscripts are significantly different (P<0.05)

Table 2. Maximum concentrations of ammonia, indole, skatole and volatile fatty acids (VFA) in the rumen for an 8 h postprandial period for sheep fed white clover, perennial ryegrass and *Lotus corniculatus*

	White clover	Perennial ryegrass	*Lotus corniculatus*	s.e.
Ammonia (mol/kgCPI)	1.1[a]	0.4[ab]	0.3[b]	0.20
Indole (mg/kgCPI)	94[a]	44[ab]	24[b]	15.2
Skatole (mg/kgCPI)	157[a]	65[ab]	49[b]	21.9
Straight-chain VFA (mol/kgDMI)	0.8	0.4	0.4	0.13
Branch-chain VFA (mol/kgCPI)	0.3	0.1	0.1	0.05

Means in the same row with different superscripts are significantly different (P<0.05)

DISCUSSION

Maximum concentrations of skatole, indole and ammonia were significantly higher per unit of crude protein intake with the WC than the LC diet. The botanical composition of New Zealand pastures usually contains a white clover component of 0-30%. The high concentrations of indole and skatole, even with the low intakes of white clover observed in this study, indicate that, in the grazing situation, even a small component of the grazed pasture as white clover may result in high indole and skatole formation in the rumen. Ammonia is produced in the rumen from the degradation of amino acids released by hydrolysis of dietary protein. Highly soluble and degradable protein results in excessive ammonia formation relative to energy in the form of adenosine triphosphate (ATP) available to convert it to microbial protein. Skatole and indole are likely to be formed when protein solubility and degradability is high since there will be more free amino acids released into the rumen and degraded to form reduction products (e.g., indole and skatole from tryptophan), providing an electron sink for reducing equivalents, allowing the generation of ATP for rumen microbes (Carlson and Breeze 1984).

The WC had a higher protein concentration than PRG and LC, and it is known that protein is more soluble and degradable in legumes that do not contain condensed tannin (CT) (Min *et al*. 2000). These high levels of soluble and degradable protein are likely to cause the high concentrations of rumen ammonia, skatole and indole. The CT present in LC slows protein degradation in the rumen (Min *et al.* 2000), resulting in more plant protein by-passing rumen fermentation, and less protein being broken down to ammonia. Consequently, a low concentration of ammonia in the rumen is often observed when feeding LC. In this study, feeding LC was associated with lower ammonia, skatole and indole concentrations in the rumen. Therefore, the lower protein degradability that results from feeding a CT-containing forge like LC also reduces the availability of tryptophan for conversion to skatole and indole in the rumen. The protein content of the perennial ryegrass diet is much lower than for the legumes, and the concentration of ammonia, skatole and indole are intermediate between WC and LC, consistent with an intermediate level of protein degradation in the rumen.

Straight-chain VFAs are the products of carbohydrate digestion in the rumen. Burke *et al*. (2002) showed that feeding white clover produced greater VFA concentrations than other forages, but in the current study, maximum *n*-VFA concentration in the rumen was not significantly different between the forages. The higher *n*-VFA concentration when feeding WC, although non-significant, complements the results of Hammond *et al*. (1984), who found that lower VFA concentration in the rumen was associated with less skatole, and a higher ammonia concentration was associated with high skatole. However, the non-significant differences for *n*-VFA between forages suggests that skatole and indole formation is not directly associated with carbohydrate digestion.

Branched-chain VFAs in the rumen are formed from amino acid degradation by a similar process to that for indole and skatole formation from tryptophan. Maximal BC-VFA concentrations did not differ significantly between the forages, but tended to be higher when WC was fed. This is consistent with a tendency for BC-VFA to be formed when protein solubility and degradability is high to provide energy to rumen microbes.

High skatole and indole concentrations are associated with faecal odours in fat from pasture-raised sheep. For sheep fed a maize-based concentrate diet, there was lower skatole concentration in the fat, and sensory analysis resulted in no faecal odour being observed (Young *et al.* 2002). Feeding concentrates is impractical and economically unviable for most New Zealand production systems that rely on pasture grazing. Thus, the solution to improving meat and dairy product flavour will need to be forage-based so it can be incorporated into current grazing systems. It would appear that high dietary protein solubility and degradability in the rumen is important for promoting skatole and indole synthesis, and ammonia will be produced concurrently. Therefore, rumen ammonia concentration can be used as an indicator of protein solubility and degradability and to determine potential skatole/indole production with different feeds. This study shows that indole and skatole production is reduced when forage protein is protected from degradation in the rumen by CT, and this is reflected in lower rumen ammonia concentrations. These factors should be taken into consideration when choosing a feed and aiming to reduce skatole and indole formation in the rumen to improve the sensory aspects of meat and dairy products. The high indole and skatole concentrations when feeding WC suggest that traditional pastures that incorporate WC may not be very suitable to improve flavours of meat and dairy products.

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