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## Postprandial indole and skatole formation in the rumen when feeding white clover, perennial ryegrass and *Lotus corniculatus*

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### ABSTRACT

In some international markets, meat and milk products from pasture-fed animals is associated with an undesirable pastoral flavour and odour. Sensory evaluation and chemical analysis of products has associated these flavours and odours with the presence of skatole and indole. Skatole and indole are formed in the rumen from the degradation of dietary protein. Condensed tannins (CT) have been shown to reduce protein degradation in the rumen and could reduce the formation of skatole and indole. This study investigated the concentrations of skatole and indole in the rumen of sheep after feeding white clover (WC), perennial ryegrass (PRG) and the CT forage, *Lotus corniculatus* (LC). Six rumen-fistulated Romney wethers were fed the cut forages and rumen contents sampled at intervals after the start of feeding. Feeding WC resulted in higher ( $P<0.05$ ) peak concentrations of indole and skatole in the rumen per kilogram of crude protein eaten (CPI) compared to PRG and LC. There was a higher peak concentration of indole, but not skatole, in the rumen of sheep fed PRG compared to sheep fed LC ( $P<0.05$ ). Feeding LC resulted in lower peak concentrations of skatole compared to feeding WC and lower peak concentrations of indole than when feeding WC or PRG. White clover in pastures may be a key factor of the high skatole and indole contents in meat and milk products obtained from pasture-based grazing systems. CT forages seem a likely solution to reducing ruminal skatole and indole formation.

**Keywords:** skatole; indole; meat flavour; condensed tannins; rumen; forages.

### INTRODUCTION

Pasture is an inexpensive source of nutrients and much effort has gone into maximising ruminant production from it in New Zealand (Boutonnet, 1999). However, in some international markets, feeding fresh pasture diets has been associated with undesirable pastoral flavours in dairy (Keen, 1998) and meat products (Larick *et al.*, 1987). As a result, in those markets there is a preference for products from grain-raised animals (Melton, 1983). As New Zealand is the world's largest exporter of sheep meat, acceptance of sheep meat in international markets is an important economic issue (Prescott *et al.*, 2001). Therefore, ameliorating undesirable flavours in meat and milk products, while maintaining New Zealand's low-cost production system, would offer additional opportunities to cater for specific market preferences.

Sensory and chemical analysis of meat has shown that pastoral flavour and odours maybe associated with high concentrations of the compounds, skatole and indole (Young *et al.*, 1997). Skatole and indole are formed in the rumen from deamination and decarboxylation of the amino acid, tryptophan (Deslandes *et al.*, 2001). Thus, higher concentrations of skatole and indole present in tissues of pasture-fed ruminants may be a consequence of the high levels of rapidly degradable protein in fresh forages.

It is hypothesised that condensed tannins (CT), known to be present in some forages, may be able to reduce skatole and indole synthesis in the rumen by slowing the degradation of dietary protein or inhibiting the activity of the rumen microbes involved in proteolysis or the subsequent deamination/decarboxylation of tryptophan. This study compared the concentrations of skatole and indole in the rumen of sheep after feeding fresh white clover (WC), perennial ryegrass (PRG) or the CT-

containing forage *Lotus corniculatus* (LC).

### MATERIALS AND METHODS

#### Animals, forages and sampling

Six Romney wethers, fistulated in the rumen (85 mm ID flexible rumen cannula) were housed indoors in metabolism crates at AgResearch Grasslands in Palmerston North. The sheep were fed WC, PRG and LC respectively, over successive periods of three weeks. Two weeks were allowed for adjustment to the diet and the third week was when sampling occurred. In the second week, rumen contents of all sheep were baled out on two separate occasions. The rumen contents of each sheep were weighed and then placed back into the sheep. Duplicate samples of the rumen contents from each sheep were taken to determine dry matter.

The animals were allowed to eat the designated forage for two hours at 08:00 hours and 16:00 hours each day. Animals had access to water at all times. When sampling, rumen contents were taken at -0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6.25, 7, and 8 hours after the start of the morning meal. Rumen contents were squeezed through a double layer of cheesecloth and 10 ml of the fluid frozen in liquid nitrogen and then transferred to a -20°C freezer for later determination of skatole and indole. Each animal was sampled from twice on separate days.

The weight of feed offered and refused was recorded for each sheep to determine the intake of each diet. Triplicate forage samples (200 g) of the feed offered and refused were taken at each meal to determine dry matter (DM) content following oven drying at 90°C for 24 hours. Samples of the forage offered and refused were collected throughout the trial, frozen and freeze dried to determine chemical composition by Near Infra-red Reflectance Spectrometry (NIRS; Feedtech, AgResearch, Palmerston

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North, New Zealand). Condensed tannin content of the forages was determined on freeze dried and ground (to pass through a 0.5 mm sieve) samples of the forage offered using the butanol-HCl method (Terrill *et al.*, 1992).

**Rumen fluid preparation and HPLC analysis**

Skatole and indole determination in the rumen fluid was performed using a modified method of Mattavi *et al.* (1999). Rumen fluid (0.5 ml) was added to 0.5 ml of methanol (MeOH), mixed well and then centrifuged (Hermle Z233M at 2000 g for 5 minutes). The supernatant was removed and the pellet washed twice by resuspending in 1 ml buffer solution (BS) containing potassium dihydrogen orthophosphate (2.4 mg/ml) and disodium hydrogen phosphate (anhydrous; 3.9 mg/ml), and then centrifuging. The supernatants were removed between washings, combined and loaded onto an Isolute ENV+ column (International Sorbent Technologies, Mid Glamorgan, England, 50 mg) that had been conditioned by eluting with 1 ml MeOH and then 1 ml 20% MeOH in BS. The column was then sequentially eluted with 1 ml 20% MeOH in BS, 1 ml 55% MeOH in BS and then 2 ml MeOH. The MeOH elutant had 50 µl of internal standard added (2-methylindole; 0.05µg/µl) and was analysed by high performance liquid chromatography (HPLC). An external standard containing indole (0.025 µg/µl), 2-methylindole (0.05 µg/µl) and skatole (0.1 µg/µl) was also analysed by HPLC to calculate the concentration of these compounds in the rumen fluid samples.

The HPLC system consisted of a Shimadzu pump (LC10ADvp), auto-injector (SIL-10ADvp) and detector (RF-10Ax1; Shimadzu Oceania, Henderson, NZ). The chromatography was performed with a mobile phase consisting of 70% acetic acid solution (1.2 mg/ml) and 30% isopropanol (Hypersolv, BDH Laboratory Supplies, England) isocratic at 1 ml/min. Injection volume was 5 µl with chromatographic separation carried out on a reverse-phase platinum C18 column (150 x 4.6 mm; Alltech, Auckland, NZ). The fluorescence excitation was set to 285 nm and the emission to 350 nm for the detection of skatole, indole and 2-methylindole. Data acquisition and peak processing were performed using Shimadzu, Class-VP software (version 5.032, Shimadzu Oceania)

**Calculations and statistical analysis**

Skatole and indole concentrations of each sheep were adjusted for rumen volume and crude protein intake. All data was statistically analysed using Genstat, version 6.1.0.20 (Lawes Educational Trust, Oxford, UK). Intake was analysed using ANOVA. Skatole and indole data were analysed by fitting quadratic x quadratic curves for each sheep to obtain summary statistics for parameters such as peak concentration. These statistics were then analysed using ANOVA.

**RESULTS**

**Feed composition and intakes**

Table 1 shows the differences in mean DM, crude protein (CP) and CT concentrations in the three forages. White clover had the lowest DM concentration but the highest CP content. Perennial ryegrass was the opposite

**TABLE 1:** Average dry matter (g/kg) and crude protein content (g/kgDM) of the forages offered to sheep (n=6) and mean dry matter and crude protein intakes for the morning feeds. Means in rows with different superscripts are significantly different (P<0.05).

	White clover	Perennial ryegrass	Lotus corniculatus
<b>Nutrient Composition:</b>			
Dry matter (g/kg)	122	233	148
Crude protein (g/kgDM)	272	175	240
Total condensed tannin (g/kgDM)	3.6	0.2	14.0
<b>Intake:</b>			
Dry matter (g ± SEM)	114 ± 27 <sup>a</sup>	351 ± 51 <sup>b</sup>	278 ± 45 <sup>b</sup>
Crude protein (g ± SEM)	31 ± 7 <sup>a</sup>	62 ± 9 <sup>b</sup>	67 ± 11 <sup>b</sup>

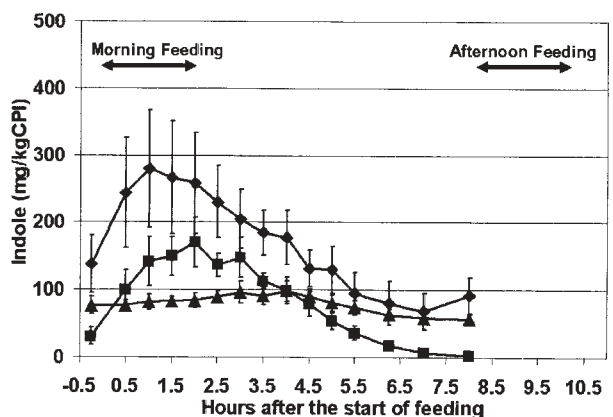
of the white clover with the highest DM and lowest CP content and LC had intermediate DM and CP concentrations. All forages contained condensed tannin, LC having the highest concentration at 14 g/kgDM. LC was the only forage to have free condensed tannin at 11 g/kgDM.

Dry matter intake was significantly lower when the sheep were fed white clover compared to when they were fed PRG or LC (P<0.05). However, the dry matter intakes were not different between LC and PRG (Table 1). Correspondingly, the same statistical differences were observed for crude protein intake (CPI; Table 1). Table 1 contains DMI and CPI for only the two-hour feeding period in the morning. Average daily DMI was 261 ± 90 g for WC, 856 ± 158 g for PRG and 512 ± 75 g for LC. Average daily CPI was 71 ± 25 g for WC, 150 ± 28 g for PRG and 123 ± 18 g for LC. These daily DM intakes were all significantly different for all the forages (P<0.05) and daily CPI intakes were different when comparing WC to PRG (P<0.01) and LC (P<0.05) but not statistically different between PRG and LC.

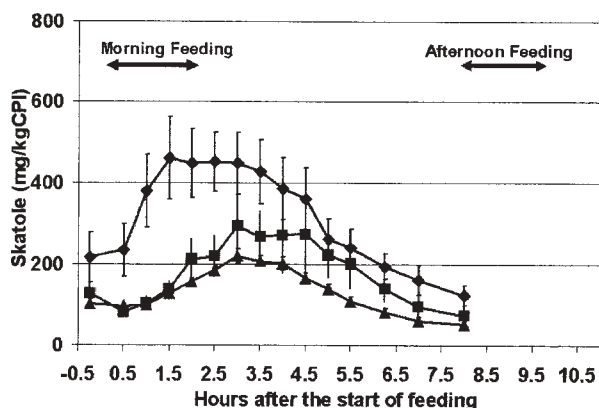
**Rumen skatole and indole**

Peak concentration of indole was 279 ± 87, 171 ± 37, 98 ± 16 mg/kg CPI when feeding WC, PRG and LC, respectively. Peak concentration of indole was significantly lower for LC than WC (P<0.01) or PRG

**FIGURE 1:** Mean concentration of indole adjusted for crude protein intake (CPI) in the rumen of sheep (n=6) fed white clover (WC,◆), perennial ryegrass (PRG, ■) and Lotus corniculatus (LC,▲). Error bars shown are the SEM.



**FIGURE 2:** Mean concentration of skatole adjusted for crude protein intakes (CPI) in the rumen of sheep (n=6) fed white clover (WC, ◆), perennial ryegrass (PRG, ■) and *Lotus corniculatus* (LC, ▲). Error bars shown are the SEM.



( $P < 0.05$ ) and no statistical difference was observed for peak indole concentration when comparing WC to PRG (Figure 1). Indole concentration peaked during feeding for WC and PRG while LC peaked about two hours after feeding had ended (Figure 1).

Peak concentration of skatole was  $452 \pm 74$ ,  $272 \pm 96$ ,  $208 \pm 31$  mg/kg CPI when feeding WC, PRG and LC, respectively. Peak concentration of skatole was significantly higher when animals were fed WC compared to PRG ( $P < 0.05$ ) and LC ( $P < 0.001$ ). There was no statistical difference in peak skatole concentration between PRG and LC (Figure 2). When animals were fed on WC, the formation of skatole was more rapid and peak concentration was reached within 30 minutes after the end of feeding while PRG and LC peaked about 1.5-2 hours after the end of feeding.

## DISCUSSION

Much higher concentrations of both indole and skatole were obtained in the rumen of the sheep fed white clover compared to the other forages inspite of lower protein intakes. Peak concentration of skatole and indole occurred sooner after the start of feeding when sheep were fed white clover compared to the other forages. It is known that protein digestion in the rumen is comprised of both solubilisation and degradation. Solubilisation is the process whereby protein is released from plant cells into the rumen environment and degradation is the catabolism of those proteins by microbes to form peptides, amino acids and ammonia (Min *et al.*, 2000). The protein in white clover is both more soluble and more degradable than that of perennial ryegrass and *Lotus corniculatus* (Min *et al.*, 2000). Highly soluble and degradable proteins can release more amino acids, and release those amino acids into the rumen more rapidly. Thus, the higher concentration of skatole and indole that occurred when animals were fed white clover is likely to be due to a greater availability of tryptophan. The earlier peaks of skatole and indole concentrations are also likely to be due to the protein in white clover being solubilised and degraded more quickly. For all forages, the earlier peaks of indole compared to skatole concentrations is likely to be due to the fact that the formation of indole from

tryptophan is a two step chemical process while three steps are required to form skatole.

White clover may play a significant role in causing the pastoral flavour in meat from ruminants that have been grazing pasture. The results of this study have shown that per unit of CPI, white clover produced much higher concentrations of skatole and indole in the rumen compared to higher intakes of perennial ryegrass. This is shown clearly in Figures 1 and 2 where concentrations have been adjusted for crude protein intakes and white clover resulted in higher peak concentrations of skatole and indole per unit of crude protein eaten. In the grazing situation, this means that even low intakes of white clover can attribute to high levels of indolic compounds in the rumen. Pasture composition in New Zealand varies, generally containing 0-30% white clover and 70-100% perennial ryegrass. So although white clover is rarely the dominant species, it may still be largely responsible for the pastoral flavours in meat and milk that are characteristic of grazing this type of pasture.

Peak concentration of indole and skatole in the rumen tended to be lower when LC was fed compared to WC and PRG. While *Lotus corniculatus*, like white clover, is a legume, the CT in this forage may have been responsible for the low concentration of skatole and indole generated in the rumen. Condensed tannins are able to bind to protein in the rumen, forming insoluble complexes that slow and reduce solubilisation and degradation of plant protein in the rumen (Mangan, 1988; Min *et al.*, 2000; Aerts *et al.*, 1999). Condensed tannins also reduce proteolysis by inhibiting rumen bacteria (Jones *et al.*, 1994). Thus, the lower concentration of skatole and indole in the rumen, when feeding LC, is likely to be due to CT present in that forage. By this model, the CT in *Lotus corniculatus* would reduce the availability of tryptophan or inhibit the rumen microbes involved in the formation of skatole and indole.

The areas under the curves given in figures 1 and 2 would give an estimation of the total production of skatole and indole in the rumen for the three forages. It is likely that total skatole and indole production would be the highest for white clover and lowest for *Lotus corniculatus* with perennial ryegrass being intermediate. However, further analysis is required to determine whether these production values would be significantly different considering the variation between animals is quite large.

The CT concentration in the *Lotus corniculatus* tested in this study was low compared to other CT forages (Terrill *et al.* 1992). The CT from different forages has been shown to exert quite different effects on protein degradation (Aerts *et al.*, 1999). Further study is required to determine if other CT-containing forages can reduce indole and skatole production in the rumen to a greater extent than that observed with the *Lotus corniculatus* and to determine if varying the CT concentration in the diet affects the formation of these indolic compounds in the rumen.

## CONCLUSION

Low intakes of white clover were associated with high peak concentrations of skatole and indole in the rumen. Thus, white clover appears to be the key contributor to

the ruminal formation of these undesirable pastoral flavour compounds when animals are fed pasture. *Lotus corniculatus* resulted in lower concentrations of skatole and indole in the rumen and this may have been associated with the presence of CT in that forage. Therefore, the use of CT-containing forages may provide a practical means of ameliorating the pastoral flavour in meat and dairy products destined for markets sensitive to these flavours and odours.

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