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EFFECT OF WHITE CLOVER (*TRIFOLIUM REPENS*), PERENNIAL RYEGRASS (*LOLIUM PERENNE*) AND *LOTUS CORNICULATUS* ON *IN VITRO* SKATOLE AND INDOLE FORMATION

N.M. SCHREURS[AB], *M.H. TAVENDALE*[A], *G.A. LANE*[A], *T.N. BARRY*[B] *and W.C. MCNABB*[A]

[A] AgResearch Ltd, Grassland Research Centre, Private Bag 11008, Palmerston North, New Zealand
[B] Institute of Veterinary Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand

SUMMARY
Consumers accustomed to eating meat and dairy products from animals raised on grain often describe products from New Zealand pasture-raised animals as having an undesirable flavour. This could adversely affect the acceptability and returns for meat and dairy products in certain oversea markets. This flavour, often described as pastoral, grassy or faecal, has been attributed in part to the high concentration of indole and skatole present in the meat of animals raised on fresh perennial ryegrass/white clover pasture. Skatole and indole are formed in the rumen from the microbial degradation of tryptophan. A pasture diet is high in protein that is both soluble and rapidly degraded, resulting in an abundance of free peptides and amino acids in the rumen. Condensed tannins (CT) in *Lotus corniculatus* have been shown to reduce the solubility and degradability of plant protein in the rumen. This study investigated the *in vitro* rumen fermentation characteristics of white clover (*Trifolium repens*; WC), perennial ryegrass (*Lolium perenne*; PRG) and *Lotus corniculatus* (LC) to determine their effects on skatole and indole formation. All incubations were carried out in the presence and absence of polyethylene glycol (PEG), which inactivates CT. In the absence of PEG, maximal skatole and ammonia concentrations were greater when incubating WC compared with PRG and LC ($P<0.05$). A similar trend was evident for indole concentrations, but statistical significance was not reached. Adding PEG to the incubations with LC increased the concentration of indole, skatole and ammonia ($P<0.05$), but did not affect those incubations with WC or PRG. Differences in peak concentrations by the *in vitro* method were comparable with those found in an *in vivo* study using the same forages. Thus, the *in vitro* method can be used to screen a range of forages to determine their effect on skatole and indole formation in ruminants, and to identify candidate forages for managing the flavour of products from forage-fed ruminants.

Keywords: skatole, indole, *in vitro*, condensed tannin, flavour

INTRODUCTION
For meat and dairy product consumers in overseas markets that are used to products from intensive systems where grain and concentrate diets are fed, the meat from ruminants raised on fresh perennial ryegrass/white clover pasture is often said to have undesirable flavours. These flavours are described as being grassy-, faecal- or animal-like, and affect the acceptability of, and returns from, meat and dairy products in certain markets in Asia, Europe and America. Skatole and indole have been implicated as causing these undesirable flavours (Rousset-Akrim *et al.* 1997; Keen 1998). However, perennial ryegrass (*Lolium perenne*)/white clover (*Trifolium repens*) pasture allows for cost-effective production for both meat and dairy products in New Zealand. Skatole and indole are derived in the rumen from the microbial fermentation of the amino acid, tryptophan (Deslandes *et al*. 2001). Excessive protein solubility and rapid degradability in the rumen, associated with feeding fresh forages, results in abundant tryptophan relative to microbial utilisation, absorption and outflow, leading to elevated skatole and indole formation. Reducing protein solubility and degradability is likely to reduce the amount of skatole and indole formed in the rumen. Condensed tannins (CT) are naturally occurring secondary metabolites found in some plants, and studies have shown that they reduce protein degradability in the rumen (McNabb *et al*. 1996).

An *in vivo* study feeding fresh forages to sheep found that skatole and indole formation was greater when feeding white clover (WC), compared with perennial ryegrass (PRG) or *Lotus corniculatus* (LC) (Schreurs *et al*. 2003). The low skatole and indole concentrations when feeding *Lotus corniculatus* were attributed to the CT content of this forage slowing the degradation of protein in the rumen. Condensed tannins from different plants have been shown to exert different effects on the solubility and degradability of protein (Aerts *et al*. 1999). Therefore, different CT-containing plants could have different effects on the formation of indole and skatole. The objective of this study was to compare

the formation of skatole and indole *in vitro* with that of previous *in vivo* work. If successful, then this technique could be applied to the evaluation of a much wider range of plant material than could be practically handled in an *in vivo* trial. Polyethylene glycol (PEG) is known to inactivate CT (Jones and Mangan 1977). Polyethylene glycol was used in this study to test the activity of CT in the forage on the formation of skatole, indole and ammonia.

MATERIALS AND METHODS

Forage preparation and analysis

Prior to running the incubations, frozen samples of WC, PRG and LC were chopped to 2-3 cm lengths, and minced using a meat mincer (Kreft Compact R70) to obtain a particle size similar to that of chewed forages (Barrell *et al*. 2000). Dry matter (DM) was determined on triplicate samples of 20 g of the minced forages by oven drying at 90°C for 24 h. Sub-samples of the minced forages were frozen, freeze dried and ground (to particles size <1 mm) to determine chemical composition by Near Infra-red Reflectance Spectrometry (NIRS; Feedtech, AgResearch Limited, Palmerston North, New Zealand). The CT concentration in freeze-dried and ground sub-samples of the minced forages were determined using the butanol-HCl method (Terrill *et al.* 1992).

Experimental design and in vitro procedure

Minced fresh forage equivalent to 0.5 g of DM was weighed into 50 mL vented sealed bottles. The bottles containing the fresh minced plant material were warmed in an incubator before 10 mg cellubiose, 12 mL of carbon dioxide saturated buffer (McDougall 1948), 0.5 mL cysteine sulphide reducing agent and 3 mL rumen fluid were added (Barrell *et al*. 2000). Ten bottles were prepared for each plant species and half had 80 mg polyethylene glycol (PEG; MW 3350) added. The incubation medium for each treatment was sampled from sequential bottles prior to incubation, and after 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12 h of incubation. This was repeated 4 times for each forage.

Indole, skatole and ammonia concentration in rumen fluid media

Skatole and indole concentrations in the rumen fluid were analysed using a modified method of Mattavi *et al.* (1999). Incubation medium (0.5 g) was added to 0.5 mL of methanol (MeOH), mixed well and centrifuged (2000 *g* for 5 min). The supernatant was removed and the pellet washed twice by resuspending in 1 mL phosphate-buffered solution (PBS) containing potassium dihydrogen orthophosphate (2.4 mg/mL) and disodium hydrogen phosphate (3.9 mg/mL), and centrifuged (2000 *g* for 5 min). The supernatants were combined and loaded onto an Isolute ENV+ column (International Sorbent Technologies, Mid Glamorgan, England, 50 mg). The column was then sequentially eluted with 1 mL 20% MeOH in PBS, 1 mL 55% MeOH in PBS, and then 2 mL MeOH. The MeOH elutant had 50 µL of internal standard added (2-methylindole; 0.05 µg/µL) and was analysed by high performance liquid chromatography (HPLC). Chromatography was carried out with a reverse-phase column (150 x 4.6 mm; Platinum C18, Alltech, Auckland, NZ) with fluorescence detection (excitation, 285 nm and emission, 350 nm). 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. A calibration 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. Ammonia in rumen fluid was analysed by reductive amination of 2-oxoglutarate giving 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

Peak processing for indole and skatole was performed using Class VP software (version 5.032, Shimadzu, Oceania, Henderson, New Zealand) with quantitation calculated relative to the internal standard. Maximal concentrations in the 4 replications were compared as the *in vitro* method is a closed system, and products of fermentation build up over time. Maximal concentrations of indole, skatole and ammonia were adjusted for the crude protein in the minced forages added and volume in the incubation bottles. The maximal concentration was statistically analysed using a factorial randomised block design. An additive model was applied using PROC MIXED of SAS (SAS 2001), where forage type and PEG status (added or not added) were fixed effects, and replication a random effect.

RESULTS

White clover and LC had similar crude protein concentrations while the crude protein concentration of PRG was lowest (Table 1). Neutral detergent fibre concentrations in the forages were higher in PRG than in WC and LC. Organic matter digestibility was high for both WC and LC, but slightly lower for PRG. The WC and PRG herbage contained only trace amounts of CT whereas *Lotus corniculatus* contained 35 g total CT/kg DM (Table 1).

Table 1. Composition of minced plants used in *in vitro* incubations.

	White clover (WC)	Perennial ryegrass (PRG)	*Lotus corniculatus* (LC)
Crude protein (g/kgDM)	276	122	267
Neutral detergent fibre (g/kgDM)	210	411	186
Organic matter digestibility (%)	>87.0	82.7	86.4
Condensed tannin (g/kgDM):			
- Free	Not detected	Not detected	11.6
- Protein-bound	1.2	0.2	23.1
- Fibre-bound	0.2	0.3	0.7
- Total	1.4	0.5	35.4

In the absence of PEG, maximal indole concentrations did not differ significantly between the forage treatments, but tended to be higher for WC. Maximal skatole and ammonia concentrations were higher when incubating WC compared with PRG and LC in the absence of PEG (Table 2). When PEG was added to the incubations with LC, there was a significant increase in indole, skatole and ammonia production, with similar maximal concentrations to those incubations with WC. The addition of PEG to incubations with PRG and WC did not influence indole, skatole or ammonia concentration.

Table 2. Mean maximum indole, skatole and ammonia concentrations (adjusted for crude protein) in total rumen incubation media for 3 forages in the presence and absence of polyethylene glycol (+/-PEG).

	White clover (WC)		Perennial ryegrass (PRG)		*Lotus corniculatus* (LC)		s.e.m.
	- PEG	+ PEG	- PEG	+ PEG	- PEG	+ PEG	
Indole (µg/gCP)	330^{ab}	342^{ab}	200^{b}	282^{ab}	179^{b}	415^{a}	68.6
Skatole (µg /gCP)	584^{a}	437^{a}	167^{b}	382^{ab}	356^{b}	566^{a}	88.2
Ammonia (mg/gCP)	40.5^{ab}	45.6^{b}	12.2^{c}	15.0^{c}	36.9^{a}	43.8^{b}	2.74

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

DISCUSSION

In vivo trials are expensive to run, require a high input of human resources, and the use of fistulated animals that need to be housed indoors. To investigate a range of forages *in vivo* would not be practical, and imposes a huge demand on resources. In contrast, *in vitro* methods are relatively inexpensive. Although the *in vitro* method is a closed system compared with measurement directly from the rumen, the maximal concentrations of indole, skatole and ammonia showed the same trends as those found *in vivo* between forages. The correspondence between the results found for the *in vivo* (Schreurs *et al* 2004) and *in vitro* studies suggests that the *in vitro* method could provide a practical tool for screening a range of CT-containing forages to evaluate which most effectively reduces skatole and indole formation.

High rumen concentrations of skatole and indole when feeding WC compared with PRG and LC have been observed *in vivo* (Schreurs *et al*. 2003), and this has been associated with a higher ammonia concentrations in the rumen (Schreurs *et al.* 2004). The results of this study are consistent with the *in vivo* findings. The degradation of dietary protein in the rumen by microbial fermentation is preceded by solubilisation of the protein, the process whereby protein is released from plant cells into the rumen environment. Degradation is the catabolism of those proteins by microbes to form peptides and amino acids, and further degradation of the amino acids to form ammonia and products of the amino acid side chain, such as indole and skatole. The protein in WC is both more soluble and more degradable than that of other forages (Min *et al.* 2000). Thus, the higher concentration of skatole and indole that occurred when incubating WC rather than PRG and LC is likely to be due to a greater degree of degradation of dietary protein, and subsequent fermentation of tryptophan from the plant material.

Lotus corniculatus contained significant levels of CT, while PRG and WC only contained trace amounts. Adding PEG to incubations with LC increased the concentration of indole, skatole and

ammonia compared with those incubations without PEG. The lower indole, skatole and ammonia concentrations with LC are likely to be due to the inhibitory effect of the CT in this forage on protein degradation since including PEG in the incubations increased indole, skatole and ammonia concentrations to levels similar to those incubations with WC. McNabb *et al.* (1996) demonstrated that CT reduced the degradability of the major leaf protein, ribulose-1,5-bisphosphate carboxylase (Rubisco), which accounts for about 40% of the total protein in leaves. Condensed tannins may exert their effects on the degradation of Rubisco by interacting directly with Rubisco and sterically interfering with the binding of proteases. Alternatively, CT may interact with proteolytic enzymes of microbial origin. Thus, the CT in LC reduces protein solubility and degradability, limiting the availability of precursors (peptides and amino acids) for indole, skatole and ammonia formation. The relatively low maximal concentrations when incubating PRG is likely to be a consequence of the higher fibre content slowing the degradability of this forage.

The effectiveness of CT in reducing protein degradation suggests that CT-containing forages may be a viable option for reducing the formation of skatole and indole in the rumen within grazing systems, providing a practical means of improving meat and dairy product flavour, to cater for preferences in specific markets. Forage plants can contain a range of CT concentrations and, furthermore, the chemical structure and reactivity of the CT varies markedly between forages (Aerts *et al*. 1999). Further investigation is required into the effects of different CT forages and the influence of concentration and CT type on ruminal indole and skatole formation. This study demonstrates that *in vitro* methods will provide an efficient way to carry out further investigations.

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Email: nicola.schreurs@agresearch.co.nz