Condensed tannin expression in white clover leaves for enhanced animal health benefits

Marissa B. ROLDAN[1*], Derek R. WOODFIELD[2], John R. CARADUS[3], Greig R. COUSINS[2], Elizabeth BARR[4], Rupinder KAUR[2] and Christine R. VOISEY[1]

[1]*AgResearch Ltd, PB 11008, Palmerston North 4442, New Zealand*
[2]*PGG Wrightson Seeds Ltd, C/- PB 11008, Palmerston North 4442, New Zealand*
[3]*Grasslanz Technology Ltd, PB 11008, Palmerston North, 4442, New Zealand*
[4]*Grasslanz Technology Australia Pty Ltd, 500 Barrs Road, Mount Bute, VIC 3324, Australia*
*Corresponding author: marissa.roldan@agresearch.co.nz

Abstract

Rapid consumption of lush white clover foliage can cause bloat which in severe cases leads to animal death. One way of mitigating this is to provide a moderate level of condensed tannins (CTs) in the diet. Unfortunately, CTs are largely absent in white clover leaves. Insertion of a transcription factor gene that encodes a CT 'master switch', from a distantly related clover species, enabled stable production of biologically significant levels of CTs in white clover leaves. Leaf CTs reduced methane emissions in rumen fluid *in vitro*. However, in high CT producing genotypes, a yield penalty of up to 50% was incurred in early generations. To alleviate this issue, genotypes with good herbage yield and CT levels >1.5% of DM were backcrossed to the commercial cultivar Mainstay over several generations. Recurrent selection, both in the glasshouse and the field, have delivered white clover with commercially acceptable herbage yield combined with a biologically significant level of leaf CTs. Under laboratory conditions clover leaves containing CTs >2.0% DM effectively reduced frothy foam and biofilm formation. Since these are indicators of bloat incidence, this development has the potential to enhance animal health if implemented in new white clover cultivars.

Keywords: bloat, ethanol film, frothy foam, protein binding, transcription factor gene

Introduction

White clover (*Trifolium repens* L.) is an important legume component of grazed pasture for ruminant livestock production. Having the ability to fix atmospheric N, it is considered a useful soil fertility resource. It supplies part of the N requirement of adjacent companion grasses, improves sward forage quality and reduces the requirement for synthetic N application (Egan et al. 2018). In intensive grazing systems based on perennial ryegrass (*Lolium perenne* L.), white clover plays a crucial role in enhancing animal performance due to its nutritional superiority (Schils et al. 2000; Ulyatt et al. 1988). White clover provides highly palatable forage with low structural fibre and excellent levels of soluble protein for animals (Jones and Lyttleton 1971). However, intake of pasture with high soluble protein promotes rapid protein fermentation by rumen microbiota which traps gas in the rumen (Mangan 1959), a condition known as bloat. Bloat adversely affects both digestive and respiratory functions, and in severe cases, causes suffocation and animal death (Waghorn 1991). Other factors implicated in pasture bloat include the fine particles dispersed from the rupture and disintegration of chloroplasts which enhance coalescence of gas bubbles (Howarth et al. 1986), and conditions that favour excessive production of slimy biofilm in the rumen that restricts the release of fermentation gases (Majak et al. 2003).

Pasture bloat is responsible for 3-5% of ruminant mortality (Allworth et al. 2023; Wang et al. 2023). In the New Zealand dairy industry alone, the annual losses due to bloat was estimated to cost $50M (Hurd et al. 2000; Morris et al. 1997). In a recent study, the weighted average of bloat reduction associated costs has been calculated to be 2% of the total animal health cost (Kerslake et al. 2018). In Australia, bloat is considered the second most costly disorder of beef cattle with estimated annual costs of $84.4 M (Lane et al. 2015). Hence, development of bloat-safe pasture would be a viable approach to improve animal health and productivity and boost the animal industry.

Some pasture legumes such as sainfoin (*Onobrychis* spp.) and birdsfoot trefoil (*Lotus corniculatus*) are considered bloat safe as they produce minimal amounts of foam compared to white clover and lucerne (*Medicago sativa*), (Kendall 1964; McArthur et al. 1964; Pressey et al. 1963). This is attributed to the presence of polymeric flavanols in leaves known as condensed tannins (CTs) which function as protein precipitants (Jones et al. 1973). The tannins form insoluble complexes with major dietary proteins (Jones and Mangan 1977) reducing protein degradation in the rumen (Sayd et al. 2022) and increasing rumen bypass protein to the intestine (Patra and Saxena 2011), thereby improving overall protein utilisation (Brown et al.

ISSN 2463-2872 (Print) ISSN 2463-2880 (Online) https://doi.org/10.33584/jnzg.2023.85.3686

2011). Further, minimising protein degradation in the rumen reduces N losses in urine that lead to increases in soil ammonia (NH_3) volatilization and nitrate leaching, which are potent environmental issues (Calsamiglia et al. 2010; Patra and Saxena 2011).

Development of white clover that stably expresses at least 1.5 % CTs in leaves is reported to be a meaningful target in improving environmental, animal health and productivity benefits (Waghorn et al. 1987). For more than five decades plant breeders and geneticists have been striving for expression of CTs in white clover leaves. By using gene modification techniques, combined with selective plant breeding, the current work has achieved that target. Under laboratory conditions, white clover leaves with soluble CTs of 1.6-2.4% of DM have been shown to be effective in protein binding at ruminal pH (pH 6.5) with the complex dissociating when exposed at the physiological pH (pH 2.5) found in the abomasum (Woodfield et al. 2019). Moreover, at this CT level, ammonia and methane production were reduced by 60% and 16%, respectively in *in vitro* studies (Caradus et al. 2022; Roldan et al. 2022). However, a yield reduction was noted in plants producing high level of CTs, which has been addressed through cycles of recurrent selection for genotypes with high herbage yield (Woodfield et al. 2019).

To assess further benefits of high CT clover on delivering the desired animal health and productivity benefits with negligible yield depression, this study aims to 1) evaluate the ability of selective breeding for herbage yield improvement while maintaining effective levels of CTs, and 2) determine the capacity of CT clover in reducing frothy foam and biofilm complex that results in causing pasture bloat.

Materials and Methods

Production of white clover plants expressing condensed tannins in leaves

The production of CT white clover has been described in detail in Roldan et al. (2022), where the TaMYB14-1 transcription factor from *Trifolium arvense,* 'Hares foot clover', first described in Hancock et al. (2012) was used. The primary transgenic (CTG-T_0) event used in their study had a single *TaMYB14-1* gene insertion and produced soluble CTs of 0.77% of DM (Roldan et al. 2022). This was backcrossed to white clover cultivar 'Mainstay' genotypes selected for good general combining ability and subsequent crosses with selected progeny were performed as previously described (Roldan et al. 2022; Woodfield et al. 2019). Pairwise crosses were also performed on selected genotypes. Over several generations, the segregation of progenies from backcrosses and pairwise crosses showed stable CT expression and predictable segregation (data not shown).

Experimental plants and growing condition

The plants used for CT and fresh herbage yield assessment from 2019 to 2021, and for foaming studies, were grown and maintained in a Physical Containment (PC2) glasshouse at AgResearch Ltd, Palmerston North, NZ. White clover seeds were scarified and placed in Petri dishes lined with moistened 3MM paper, incubated at 4ºC for 48 h, and then at 25ºC overnight, prior to sowing in seed trays containing potting mix. When the first trifoliate leaves opened, CT expression was assessed by staining a leaflet with a solution containing *p*-Dimethylaminocinnamaldehyde (DMACA; Sigma-Aldrich) (Li et al. 1996), and the stained tissues were scored for intensity as previously described (Roldan et al. 2022). Seedlings with moderate to high CT expression were selected and grown in PB3 bags for assessment of plant fresh weight, soluble CT quantity, and zygosity. In each generation, the zygosity of progeny was determined as described in (Roldan et al. 2022; Woodfield et al. 2019). Throughout the growing period, the temperature in the glasshouse ranged between 15ºC and 22ºC, and the relative humidity was between 75% to 85%. The leaf tissues used for foaming studies were from homozygous white clover plants (progeny that had inherited 2 alleles of the *TaMYB14-1* gene) and null segregant controls (progeny that had not inherited a *TaMYB14-1* allele) grown in the PC2 glasshouse as described above. Perennial ryegrass (PRG) plants (Accession no. 30634, Margot Forde Germplasm Centre, Palmerston North) were also grown under the glasshouse conditions described above and included in the foaming experiment.

The field trial was conducted in Lismore, Victoria, Australia, under License DIR 176 issued by the Office of the Gene Technology Regulator (OGTR), Australia. The soil type at the site was Lismore grey clay. A turf perennial ryegrass cv. Colosseum was first sown at the experimental site to serve as a companion grass. Progeny from 25 transgenic families (10 heterozygous and 15 homozygous) plus control cv. Mainstay check plants were included. Seeds were germinated in nursery trays in an enclosed bee-proof tent. After initial screening for CT levels as described above, the seedlings were planted under a bee-proof cage in July 2023 as spaced plants at 50 × 50 cm spacings in a randomised complete block design with eight replications. Forty-eight seedlings were planted (6 plants × 8 replicates) for each family. Samples for quantifying leaf soluble CTs were collected in September 2023, and plant growth was evaluated in September and November 2023.

Quantification of soluble condensed tannins in white clover leaves

Soluble CTs were extracted from approximately 10 mg of milled freeze-dried leaves and assayed

spectrophotometrically as described (Peel and Dixon 2007; Roldan et al. 2022; Terrill et al. 1992). Briefly, phenolics were extracted in 750 µL of aqueous acetone buffer composed of 70% (v/v) acetone and 0.5% (v/v) acetic acid by vortexing for 30 s, then sonicating in a water bath at ambient temperature for 1h. After centrifugation at 2500 × g for 10 min, the supernatant was collected and re-extracted with a 1.25 volume of chloroform. The upper aqueous supernatant containing the soluble CTs was collected and assayed in two technical replicates by determining the absorbance at 640 nm using a Tunable Versamax Microplate Reader equipped with a Softmax Pro 7.1 Software (Molecular Devices). Soluble CTs were quantified using epigallocatechin (Indofine Chemical Company, Hillsborough, NJ) as the standard as described (Roldan et al. 2022).

Preparation of leaf extracts, foam stability and biofilm analyses

Aqueous leaf extracts for foam and biofilm measurements were prepared using a protocol modified from Jonker et al. (2012). Briefly, 10 g of frozen leaf tissues, with and without 10% (w/w) polyethylene glycol (PEG), were homogenised in 50 mL of deionized water for 2 × 1 min using a Magic Bullet blender (MBR-1107A). The homogenates were filtered through 4 layers of muslin cloth then centrifuged at 400 × g for 10 min and supernatants were used in the studies. For each treatment, 3 × 8 mL aliquots of the supernatant were transferred to 15-mL screw cap Falcon tubes and then shaken at 1000 rpm for 1 min using a Geno/Grinder (SPEX Sample Prep 2010, Cole -Parmer, Metuchen, NJ, USA). Samples were then incubated at 39ºC, the physiological temperature of the rumen. Foam volume was determined immediately after shaking (0 min) and at 30 min intervals until 150 min by measuring the thickness from the top of the foam to the top of the liquid interface using a digital vernier calliper. There were 3 technical replicates per experiment, and the experiment was repeated 3 times using leaves from different white clover genotypes. The independent experiments served as the biological replicates.

The efficacy of CT clover in reducing foam under laboratory conditions was evaluated using aqueous leaf extracts of white clover leaves expressing CTs of 2.5% DM, plus white clover controls that did not contain leaf CTs, herein termed nulls. To determine the effect of CTs, PEG, which binds to CTs rendering them ineffective in protein-binding, was added to serve as an additional control. With this additive, the treatments in the first set of experiments were named CT-PEG, CT+PEG, Null-PEG and Null+PEG. Following this, the second experiment was conducted to include a 50/50 mixture of CT clover and PRG, with and without PEG.

Biofilm potential in the extract was determined by adding an equal volume of absolute ethanol, followed by gentle mixing, and incubation at 4ºC for 24 h to allow the formation of a viscous biofilm on the surface. The film was harvested by centrifugation at 16,000 × g for 15 min, and after discarding the supernatant, the pellet (film) was dried at 55ºC for 24 h then weighed.

Since pastures are usually composed of clover and grasses in a mixed sward, a further foaming study was conducted that included a 50/50 mixture of CT clover and PRG. Soluble CTs in leaves were first quantified as described above. Leaf crude soluble protein was also extracted and quantified, with and without a 10% (w/w) PEG control to sequester the CTs. The leaf tissues were powdered in liquid N, then homogenised in an aqueous buffer containing 50 mM of 2-[N-morpholino] ethane sulfonic acid (MES) adjusted to pH 6.5 with 10 N NaOH (Zeller et al. 2015). Homogenates were centrifuged at 4ºC for 10 min at 16,000 × g and the supernatant was transferred to a 1.5 mL fresh tube. The concentration of soluble protein in the leaf extract was assayed using a Qubit fluorometer according to manufacturer's instructions (Molecular probes, Thermo Fisher Scientific, USA). Polyethylene glycol was added to the CT-containing tissues only. Foaming and biofilm formation was determined as described above, except the pH of the extract was determined prior to the start of the foaming study.

Statistical analysis

The mean CT levels and herbage yield of heterozygotes, homozygotes and null control plants were determined for each generation. The mean yield reduction/gain per year for the CT producing progeny plants was expressed relative to the null controls. For foam thickness and biofilm measurement, the means of the three technical replicates per treatment per experiment were computed first and the three independent experiments served as biological replicates. Before performing analysis of variance, data were confirmed for normality of distribution using the Kolmogorov-Smirnov test and for homogeneity of variances. Data were subjected to a one-way analysis of variance using the Minitab Statistical software (https://www.minitab.com/software/cloud), and a mean comparison was completed using the LSD (α=0.05).

Results

White clover leaf condensed tannins and herbage yield improvement

The primary transgenic event (CTG-T_0) used in these experiments had a mean soluble CT level of 0.77% of DM. Backcrosses and pairwise crosses of selected plants in 2019 and 2020 produced progenies with slightly elevated mean CTs of 1.03% and 1.25% of

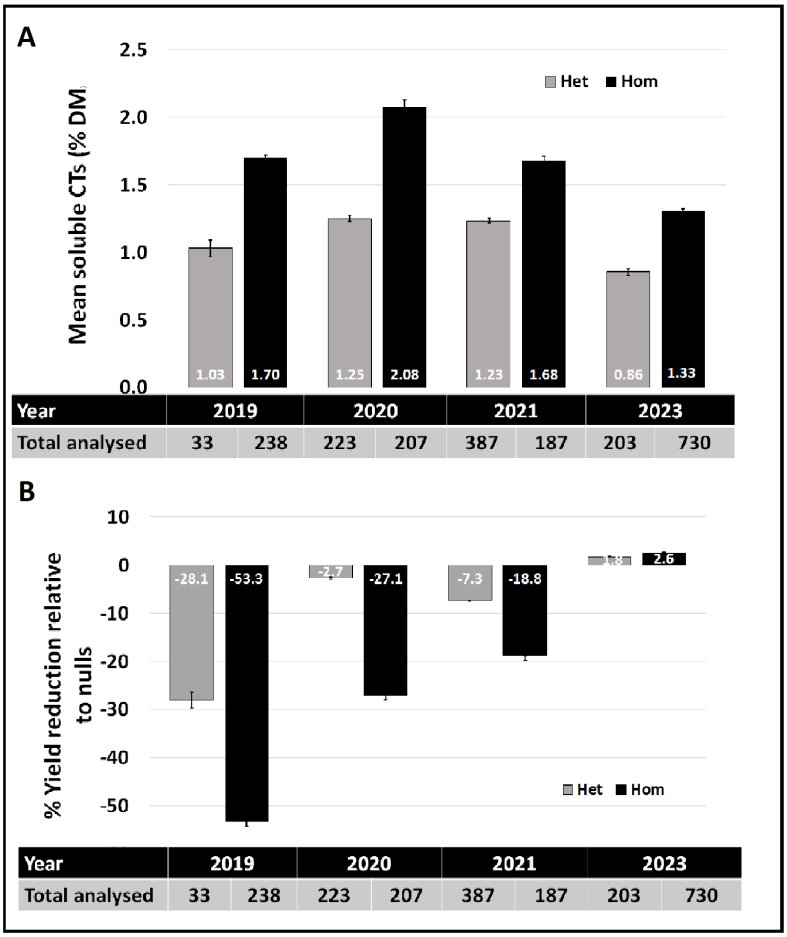

Figure 1 Mean soluble CTs as a percentage of DM (A) and percent yield reduction (B) of heterozygous (Het) and homozygous (Hom) progeny relative to null controls from the same generation in glasshouse-grown plants from 2019 – 2021, and in field grown plants in 2023. The number of plants analysed are shown below each year.

DM for heterozygotes and 1.7% and 2.08% of DM for homozygous plants in both years (Figure 1A). There was a slight decrease in mean soluble CTs in 2021 and 2023 for both heterozygotes and homozygotes as the main breeding focus was on enhanced plant growth and yield.

Throughout this selective breeding strategy, the fresh herbage yield of CT-producing white clover improved in each selection cycle. In 2019, the mean herbage yield of homozygotes was -53.3% relative to the null controls and was -27% and -18.8% in 2020 and 2021, respectively. Yield reduction in heterozygous progenies relative to the null segregants in 2019 and 2021 were -28.1% and -7.3%, respectively (Figure 1B). Plant evaluations in 2023 in the field showed considerable improvement in growth performance, with the heterozygous and homozygous progenies showing a gain of 1.8% and 2.6% when compared to the control null counterparts (Figure 1B). This had been achieved while maintaining an overall mean foliar soluble CTs of >1.33% DM for the homozygotes.

Foam stability and biofilm formation

Foam production was significantly reduced ($P<0.01$) in CT-PEG extracts relative to CT+PEG and Null+PEG and Null-PEG extracts (Figure 2A). The initial foam (Time = 0 min) in the CT-PEG extract was 36% lower relative to the foam in CT+PEG, and 33% lower when compared to null +/-PEG ($P<0.01$). Throughout the observation period, a downward trend for foam disappearance was maintained in CT-PEG, and foam was almost non-existent after 120 min of incubation (Figure 2A). On the contrary, foam disappearance was considerably slower in null and CT+PEG extracts. The CT-PEG extracts also had the lowest ($P<0.01$) ethanol precipitated biofilm (Figure 2B).

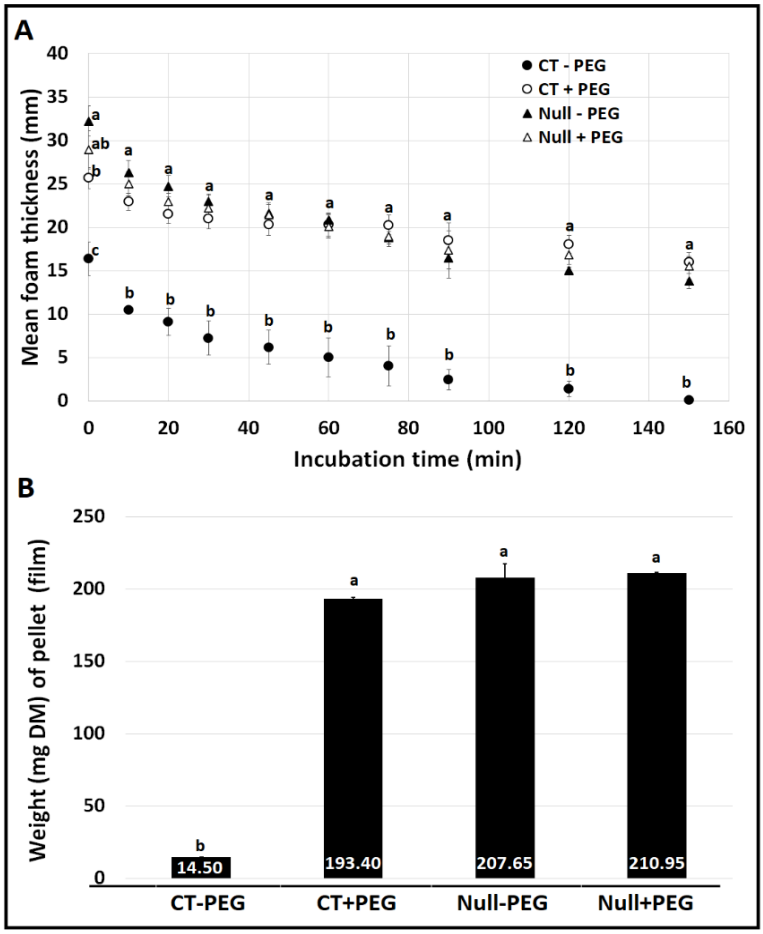

Figure 2 Mean foam thickness in CT and null leaf extracts (A) incubated at rumen temperature (39ºC), with and without polyethylene glycol (PEG), and dry weight of ethanol precipitated biofilm (B) from the leaf extracts used in the foaming studies. Different letters above each time point indicate significant differences among means by LSD ($\alpha \leq 0.05$).

In addition to 100% CT clover with or without PEG, the effects of a 50/50 mixture of CT white clover and non-transformed PRG (also with or without PEG) on foaming was also compared. Table 1 shows that the CT clover -PEG samples yielded the lowest levels of soluble protein in the extract ($P<0.01$), representing <25% of the protein obtained from the same tissue extracts where PEG was added (CT clover +PEG). Similarly, the soluble protein in CT clover/PRG -PEG was 54% lower compared to CT clover/PRG +PEG. Comparing the null clover and PRG controls, the PRG-PEG leaf extracts had lower soluble protein ($P<0.01$) relative to the null clover (Table 1). The soluble CTs in these tissues were 2.25% and 1.13% of DM for the CT clover and CT clover/PRG mixture, respectively. As expected, the null clover control and the PRG contained no CTs in the leaves. The mean pH of the homogenised extracts used for foaming ranged from pH 6.2 - 6.6 (Table 1) and no significant differences were detected ($P>0.05$).

The mean thickness of foam in the CT clover extracts -PEG was lower than CT clover +PEG and the null white clover controls (Figure 3A). Foam thickness in the null white clover control extracts (-PEG) were all statistically greater ($P<0.01$) than the PRG-PEG control. Foam thickness in the CT clover/PRG-PEG 50:50 mixture initially started out significantly lower than all the other samples (at 0 and 30 min). However, by 30 minutes of incubation it was the same as that of the CT clover-PEG extract. Beyond that, the foam level stabilised such that at 60 min of incubation it was greater than that of CT clover-PEG ($P=0.01$), and by 120 min of incubation it had the same foam thickness as the PRG-PEG control, which had continued to decline over time.

As in the previous experiment, the ethanol-precipitated biofilm in CT clover -PG was lowest (15.1 mg, $P<0.01$), followed by the CT clover/PRG (50/50)-PEG (Figure 3B). In contrast, the null clover

Table 1 Quantities of soluble protein and soluble CTs in leaf tissue extracts with and without added polyethylene glycol (PEG), and mean pH of homogenised leaf extracts used in the foaming studies.

Plant tissue	Crude Protein (μg/mL) * (n=3)	Soluble CTs (% DM) * (n=3)	Mean pH of leaf extracts (n=3)
CT clover -PEG	371.3 f	2.25 a	6.6
CT clover +PEG	1660.0 b	2.25 a	6.2
Null clover -PEG	1856.7 a	0.00 c	6.2
CT clover/PRG (50/50) -PEG	744.0 e	1.13 b	6.3
CT clover/PRG (50/50) +PEG	1388.3 c	1.13 b	6.3
PRG -PEG	1116.6 d	0.00 c	6.6
ANOVA P-value	< 0.01	< 0.01	> 0.05

*Within each column, means that do not share a letter are significantly different by LSD at α=0.05.

-PEG treatment had the greatest weight of biofilm (74.5 mg, P<0.01). Calculation of the Pearson coefficient of correlation (r) showed that the amount of crude protein is highly significantly correlated to the weight of ethanol precipitated biofilm (r=0.97, P<0.01).

Discussion

Genetic modification has successfully developed white clover genotypes able to produce leaf CTs at a biologically significant level (Caradus et al. 2022; Hancock et al. 2012; Roldan et al. 2022). Initially the breeding focus was to elevate leaf CTs to optimal levels, rather than enhancing plant growth and yield. However, progeny with high CT levels often showed a significant reduction in yield, especially those that were homozygous (Woodfield et al. 2019). Inbreeding depression could be a contributory factor due to the dominating contribution of the transgenic T_0 parent (Cousins and Woodfield 2006), although a possible trade-off between growth and CT production cannot be ruled out. Similar growth and/or defence trade-offs have also been reported in plants with modified lignin content (Ha et al. 2021), which have been attributed to production of metabolites that affect plant growth (Bonawitz and Chapple 2013) or diversion of resources towards defence (Züst and Agrawal 2017) at the expense of growth traits. An inhibitory effect of condensed tannin on gibberellin-induced growth has been reported (Corcoran et al. 1972) but since then little research has been conducted to further elucidate this issue.

Meanwhile, a plausible approach to resolving yield reduction is through recurrent phenotypic selection. This strategy involves intentional selection of desirable traits to improve plant performance and enhance the productivity of crops (Bailey-Serres et al. 2019). For high CT white clover, plants producing leaf CTs >1.5% of DM and vigour corresponding to the top 5% of ranked individuals, were selected and either crossed to elite non-transformed genotypes with good general combining ability or pairwise crossed to selected high CT plants to produce the next generation. Although yield depression was greatest in 2019 (Figure 1B), several cycles of recurrent selection appear to have overcome the yield depression noted previously for high CT white clover. Evaluation of plants in 2023 in the field in Australia showed that the yield gap between high CT clover and its null counterpart has been overcome, indicating the possibility of delivering farmers bloat-safe white clover with growth traits comparable to existing commercial cultivars.

Foam stability in leaf extracts

Formation of stable foam associated with rapid fermentation of high soluble protein levels in white clover (Jones et al. 1970; Tan et al. 2024) and lucerne (McArthur et al. 1964; Stifel et al. 1968) has been identified as a major contributor causing pasture bloat, a serious disorder of dairy cattle in New Zealand (Clarke and Reid 1974) and in other dairy producing countries (Brennan 2023; Fay et al. 1980). White clover is considered a serious bloat risk due to high levels of soluble proteins, (Hungate et al. 1955) whereas pastures consisting of plants with appreciable levels of CTs in leaves are regarded as bloat safe (Lees 1992; Min et al. 2003; Waghorn et al. 1998; Waghorn and Jones 1989). This is because CTs have a high affinity for protein, causing less frothy foam associated with bloat incidence (Waghorn and Jones 1989; Wang et al. 2012). In the current study, the level of soluble protein in extracts from white clover leaves containing soluble CTs >2.0% DM, was lowest when polyethylene glycol was not added (CT clover-PEG). In the absence of PEG, the CTs bind to protein in the extracts (Makkar et al. 1995), and as the CT-protein complex precipitates upon centrifugation, this reduces the concentration of soluble protein remaining in the supernatant. The reduction in foam thickness and stability observed in the CT-PEG may be attributable to a reduction in soluble protein in

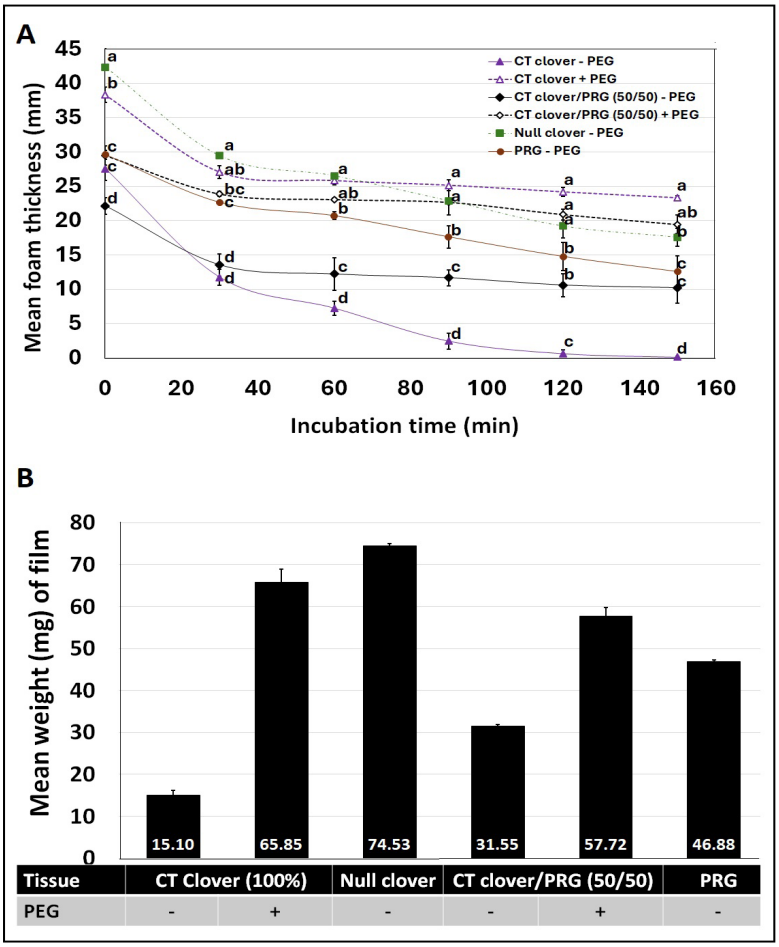

Figure 3 Mean foam thickness (A) and dry weight of ethanol precipitated biofilm (B) from the leaf extracts used in the foaming studies in high CT clover and CT clover/PRG 50:50 mixture with or without PEG, and null clover and PRG without PEG controls. Different letters above each time point indicate significant differences among means by LSD ($\alpha \leq 0.01$).

the extract, as the bulk of the proteins complex with CTs, rendering them insoluble.

Correspondingly, the 50/50 mixture of high CT clover/PRG -PEG also reduced foam thickness relative to the null clover extract and the 50/50 CT clover/PRG mixture where PEG was added (Figure 3). The foam in this 50/50 CT clover/PRG mixture-PEG, although lowest of all treatments at the start of the incubation, within 60 min was greater relative to the 100% CT clover -PEG extracts. This is consistent with reports of dose-dependent foam formation and stability when different levels of CTs from high CT-producing plants such as *Onobrychis viciifolia*, *Hedysarum coronarium*, and *Lotus pedunculatus* were mixed with red clover leaf protein at different CT-protein ratios (Tanner et al. 1995). That study showed that as the ratio of CT to protein increased, the foam formed decreased in a dose dependent manner.

Results of the current study show that the soluble protein extracted from CT-containing tissues was lower in the absence of PEG, indicating that CTs form complexes with protein that precipitated upon centrifugation, hence less amount was detected from the supernatant. When the same CT-containing tissues were homogenised without PEG, the foam was reduced compared to homogenised CT-containing tissues where PEG was added. The results are consistent with previous findings that, in the absence of PEG, the white clover CTs effectively form complexes with protein at ruminal pH levels (Roldan et al. 2022; Woodfield et al. 2019). This has been attributed to the white clover leaf CT chemistry having a mean degree of polymerisation and a prodelphinidin:procyanidin ratio which was favourable for CT-protein complexation (Roldan et al. 2022), as has also been reported in studies with other CT expressing legumes (McMahon et al. 2000;

Mueller-Harvey et al. 2019; Waghorn and Jones 1989).

Biofilm formation

Increased biofilm production is another factor that contributes to pasture bloat (Min et al. 2006) and is associated with highly digestible proteins that enhance fermentation activities in the rumen (Jonker et al. 2012; Wang et al. 2012). Legumes with high nutritive quality provide an ideal substrate for the production of slimy biofilm (Gutierrez et al. 1963) which is composed of intracellular complexes that enhance the viscosity of rumen contents and entrap fermentation gases in a stable foam (Wang et al. 2012). The biofilm produced from steers bloating on clover pastures is reportedly composed of 61-64% protein (Gutierrez et al. 1963).

In this study, the protein extracted was low in CT-producing tissues with no PEG added, and within the tissues used in foaming, there was a very strong correlation between protein content and weight of ethanol-precipitated biofilm. This confirms previous reports that proteins promote biofilm formation and bloat incidence (Gutierrez et al. 1963; Min et al. 2006). High CT clovers are therefore a viable option to boost the health of ruminants by reducing bloat susceptibility, alongside their potential for providing environmental benefits through reduction in biogenic methane and soil nitrogen emissions to air and water (Roldan et al. 2022; Woodfield et al. 2019).

Conclusions

Recurrent phenotypic selection has delivered white clover with commercially acceptable herbage yield combined with biologically significantly CT levels. In addition to the potential mitigation of greenhouse gas emissions reported previously, consumption of white clover expressing CTs in leaves has the potential to deliver additional animal health benefits. This can occur through a reduction in both foam production and biofilm formation in the rumen with CT-expressing white clover, thereby also reducing the incidence of bloat. However, the potential of these advancements needs to be confirmed in animal trials. Changes in the New Zealand genetic modification regulatory system is needed to enable this technology to be implemented in new cultivar releases for the benefit of the livestock sector.

ACKNOWLEDGEMENTS

This research was funded by PGG Wrightson Seeds Ltd, Grasslands Innovation Ltd and Grasslanz Technology Ltd. We thank Ashleigh Paap (PGG Wrightson Seeds Ltd NZ), Tony Stratton, (Grasslands Technology Australia Pty Ltd), and Nazanin Noorifar (AgResearch Ltd) for the technical assistance.

REFERENCES

Allworth MB, McQuillan M, McGrath SR, Wilson CS, Hernandez-Jover M. 2023. A survey on bloat in southern Australian beef production systems. *Australian Veterinary Journal 101(3)*: 121-126. https://doi.org/10.1111/avj.13226

Bailey-Serres J, Parker JE, Ainsworth EA, Oldroyd GED, Schroeder JI. 2018. Genetic strategies for improving crop yields. *Nature 575(7781)*: 109-118. https://doi.org/10.1038/s41586-019-1679-0

Bonawitz ND, Chapple C. 2013. Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Current Opinion in Biotechnology 24(2)*: 336-343. https://doi.org/https://doi.org/10.1016/j.copbio.2012.11.004

Brennan A. 2023. Clover management to avoid bloat. *Irish Farmers Journal*. https://doi.org/https://www.farmersjournal.ie/dairy/grass-and-feeding/clover-management-to-avoid-bloat-780018

Brown TA, Hayot Carbonero C, Mueller-Harvey I, Smith L. 2011. Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume. *Plant Genetic Resources*, *9(1)*: 70-85. https://doi.org/10.1017/S1479262110000328

Calsamiglia S, Ferret A, Reynolds CK, Kristensen NB, van Vuuren AM. 2010. Strategies for optimizing nitrogen use by ruminants. *Animal 4(7)*: 1184-1196. https://doi.org/10.1017/s1751731110000911

Caradus JR, Voisey CR, Cousin GR, Kaur R, Woodfield DR, Blanc A, Roldan MB. 2022. The hunt for the "holy grail": Condensed tannins in the perennial forage legume white clover (*Trifolium repens* L.). *Grass and Forage Science 77(2)*: 111-123. https://doi.org/https://doi.org/10.1111/gfs.12567

Clarke RTJ, Reid CSW. 1974. Foamy Bloat of Cattle. A Review. *Journal of Dairy Science*, *57(7)*: 753-785. https://doi.org/10.3168/jds.S0022-0302(74)84964-7

Corcoran MR, Geissman TA, Phinney BO. 1972. Tannins as gibberellin antagonists. *Plant Physiology 49(3)*: 323-330. https://doi.org/10.1104/pp.49.3.323

Cousins GR, Woodfield DR. 2006. Effect of inbreeding on growth of white clover. *Advances in Pasture Plant Breeding: Grassland Research and Practice Series 12*: 131-134. https://doi.org/https://doi.org/10.33584/rps.12.2006.3026

Egan M, Galvin N, Hennessy D. 2018. Incorporating white clover (Trifolium repens L.) into perennial ryegrass (Lolium perenne L.) swards receiving varying levels of nitrogen fertilizer: Effects on milk and herbage production. *Journal of Dairy Science 101(4)*: 3412-3427. https://doi.org/https://doi.org/10.3168/jds.2017-13233

Fay JP, Cheng KJ, Hanna MR, Howarth RE, Costerton JW. 1980. In Vitro Digestion of Bloat-Safe and

Bloat-Causing Legumes by Rumen Microorganisms: Gas and Foam Production. *Journal of Dairy Science 63(8)*: 1273-1281. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(80)83078-5

Gutierrez J, Essig HW, Williams PP, Davis RE. 1963. Properties of a Slime Isolated from the Rumen Fluid of Cattle Bloating on Clover Pasture. *Journal of Animal Science 22(2)*: 506-509. https://doi.org/https://doi.org/10.2527/jas1963.222506x

Ha CM, Rao X, Saxena G, Dixon RA. 2021. Growth–defense trade-offs and yield loss in plants with engineered cell walls. *New Phytologist 231(1)*: 60-74. https://doi.org/https://doi.org/10.1111/nph.17383

Hancock KR, Collette V, Fraser K, Greig M, Xue H, Richardson K, Jones C, Rasmussen S. (2012). Expression of the R2R3-MYB transcription factor TaMYB14 from T*rifolium arvense* activates proanthocyanidin biosynthesis in the legumes *Trifolium repens* and *Medicago sativa*. *Plant Physiology 159(3)*: 1204-1220. https://doi.org/https://doi.org/10.1104/pp.112.195420

Howarth R, Cheng K, Majak W, Costerton J. 1986. *Ruminant bloat.* Control of digestion and metabolism in ruminants. Proceedings of the Sixth International Symposium on Ruminant Physiology Banff, Canada. https://www.cabidigitallibrary.org/doi/full/10.5555/19861486529

Hungate RE, Fletcher DW, Dougherty RW, Barrentine BF. 1955. Microbial activity in the bovine rumen: its measurement and relation to bloat. *Applied Microbiology 3(3)*: 161-173. https://doi.org/10.1128/am.3.3.161-173.1955

Hurd S, Wheeler T, Morris C. 2000. Rumen foam stability in cattle selected for low or high bloat susceptibility. *Proceedings of the New Zealand Society of Animal Production*, Hamilton. https://www.nzsap.org/proceedings/2000/rumen-foam-stability-cattle-selected-low-or-high-bloat-susceptibility

Jones WT, Anderson LB, Ross MD. 1973. Bloat in cattle. *New Zealand Journal of Agricultural Research 16(3)*: 441-446. https://doi.org/10.1080/00288233.1973.10421128

Jones WT, Lyttleton JW. 1971. Bloat in cattle: XXXIV A survey of legume forages that do and do not produce bloat. *New Zealand Journal of Agricultural Research 14(1)*: 101-107. https://doi.org/10.1080/00288233.1971.10421306

Jones WT, Lyttleton JW, Clarke RTJ. 1970. Bloat in cattle: XXXIII. The soluble proteins of legume forages in New Zealand, and their relationship to bloat. *New Zealand Journal of Agricultural Research 13(1)*: 149-156. https://doi.org/10.1080/00288233.1970.10421204

Jones WT, Mangan JL. 1977. Complexes of the condensed tannins of sainfoin (Onobrychis viciifolia scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *Journal of the Science of Food and Agriculture 28(2)*: 126-136. https://doi.org/10.1002/jsfa.2740280204

Jonker A, Gruber MY, Wang Y, Coulman B, McKinnon JJ, Christensen DA, Yu P. 2012. Foam stability of leaves from anthocyanidin-accumulating Lc-alfalfa and relation to molecular structures detected by fourier-transformed infrared-vibration spectroscopy. *Grass and Forage Science 67(3)*: 369-381. https://doi.org/10.1111/j.1365-2494.2012.00853.x

Kendall WA. 1964. Foam Formation From Forages in Vitro1. *Crop Science 4(4)*: 391-393. https://doi.org/https://doi.org/10.2135/cropsci1964.0011183X000400040018x

Kerslake JI, Amer PR, O'Neill PL, Wong SL, Roche JR, Phyn CVC. 2018. Economic costs of recorded reasons for cow mortality and culling in a pasture-based dairy industry. *Journal of Dairy Science 101(2)*: 1795-1803. https://doi.org/https://doi.org/10.3168/jds.2017-13124

Lane J, Jubb T, Shephard R, Webb-Ware J, Fordyce G. 2015. *Priority list of endemic diseases for the red meat industries*. MLA. Limited. https://www.semanticscholar.org/paper/Priority-list-of-endemic-diseases-for-the-red-meat-Lane-Jubb/97088b22145fad9f6a20c8ef7f975424e064c87a

Lees GL. 1992. Condensed tannins in some forage legumes: their role in the prevention of ruminant pasture bloat. *Basic Life Science 59*: 915-934. https://doi.org/https://doi.org/10.1007/978-1-4615-3476-1_55

Li YG, Tanner G, Larkin P. (1996). The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture 70(1)*: 89-101. https://www.scopus.com/inward/record.uri?eid=2-s2.0-0003015963&partnerID=40&md5=31cd1461dadbbf4d8904a26307decbeb

Majak W, McAllister T, McCartney D, Stanford K, Cheng K. 2003. Bloat in cattle. *Agriculture and Agri-Food Canada*. https://doi.org/https://www1.agric.gov.ab.ca/$department/deptdocs.nsf/ba3468a2a8681f69872569d60073fde1/c147cb84919a6fd4872256d430057b2cd/$FILE/420_0-1.pdf

Makkar HPS, Blümmel M, Becker K. 1995. Formatin of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in in vitro techniques. *British Journal of Nutrition 73(6)*: 897-913. https://doi.org/10.1079/BJN19950095

Mangan JL. 1959. Bloat in cattle: XI. The foaming properties of proteins, saponins, and rumen liquor. *New Zealand Journal of Agricultural Research 2(1)*: 47-61. https://doi.org/10.1080/00288233.1959.10427123

McArthur JM, Miltimore JE, Pratt MJ. 1964. Bloat Investigations: The Foam Stabilizing Protein of Alfalfa. *Canadian Journal of Animal Science 44(2)*: 200-206. https://doi.org/10.4141/cjas64-031

McMahon LR, McAllister TA, Berg BP, Majak W, Acharya SN, Popp JD, Coulman BE, Wang Y, Cheng KJ. 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Canadian Journal of Plant Science 80(3)*: 469-485. https://www.scopus.com/inward/record.uri?eid=2-s2.0-0033808497&partnerID=40&md5=37065502a3cbdfb07800e3c21c1438aa

Min BR, Barry TN, Attwood GT, McNabb WC. 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: A review. *Animal Feed Science and Technology 106(1)*: 3-19. https://doi.org/https://doi.org/10.1016/S0377-8401(03)00041-5

Min BR, Pinchak WE, Anderson RC, Fulford JD, Puchala R. 2006. Effects of condensed tannins supplementation level on weight gain and *in vitro* and *in vivo* bloat precursors in steers grazing winter wheat. *Journal of Animal Science 84(9)*: 2546-2554. https://doi.org/10.2527/jas.2005-590

Morris CA, Cullen NG, Geertsema HG. 1997. Genetic studies of bloat susceptibility in cattle. *Proceedings of the New Zealand Society of Animal Production* : 19-21. https://www.nzsap.org/system/files/proceedings/1997/ab97006.pdf

Mueller-Harvey I, Bee G, Dohme-Meier F, Hoste H, Karonen M, Kölliker R, Lüscher A, Niderkorn V, Pellikaan WF, Salminen JP, Skøt L, Smith LMJ, Thamsborg SM, Totterdell P, Wilkinson I, Williams AR, Azuhnwi BN, Baert N, Brinkhaus AG, Waghorn GC. 2019. Benefits of condensed tannins in forage legumes fed to ruminants: Importance of structure, concentration, and diet composition. *Crop Science 59(3)*: 861-885. https://doi.org/https://doi.org/10.2135/cropsci2017.06.0369

Patra AK, Saxena J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *Journal of the Science of Food and Agriculture 91(1)*: 24-37. https://doi.org/10.1002/jsfa.4152

Peel GJ, Dixon RA. (2007). Detection and quantification of engineered proanthocyanidins in transgenic plants. *Natural Products Communications 2(10)*: 1009-1014. http://digital.library.unt.edu/ark:/67531/metadc488160

Pressey R, Synhorst SH, Bertram J, Allen RS, Jacobson NL. 1963. Foaming Properties of Alfalfa and Their Relationship to Bloat. *Journal of Animal Science 22(4)*: 970-978. https://doi.org/10.2527/jas1963.224970x

Roldan MB, Cousins G, Muetzel S, Zeller WE, Fraser K, Salminen J.-P, Blanc A, Kaur R, Richardson K, Maher D, Jahufer Z, Woodfield DR, Caradus JR, Voisey CR. 2022. Condensed Tannins in White Clover (*Trifolium repens*) Foliar Tissues Expressing the Transcription Factor TaMYB14-1 Bind to Forage Protein and Reduce Ammonia and Methane Emissions in vitro. *Frontiers in Plant Science 12(3033)*: https://doi.org/https://doi.org/10.3389/fpls.2021.777354

Sayd T, Chambon C, Popova M, Morgavi DP, Torrent A, Blinet S, Theron L, Niderkorn V. 2022. Impact of Tannin Supplementation on Proteolysis during Post-Ruminal Digestion in Wethers Using a Dynamic In Vitro System: A Plant (Medicago sativa) Digestomic Approach. *Journal of Agricultural and Food Chemistry 70(7)*: 2221-2230. https://doi.org/10.1021/acs.jafc.1c07378

Schils RL, M, Boxem TJ, Jagtenberg CJ, Verboon MC. (2000). The performance of a white clover based dairy system in comparison with a grass/fertiliser-N system. II. Animal production, economics and environment. *NJAS - Wageningen Journal of Life Sciences 48(3)*: 305-318. https://doi.org/https://doi.org/10.1016/S1573-5214(00)80020-2

Stifel FB, Vetter RL, Allen RS, Horner HT. (1968). Chemical and ultrastructural relationships between alfalfa leaf chloroplasts and bloat. *Phytochemistry 7*: 355-364. https://doi.org/DOI:10.1016/S0031-9422(00)90873-2

Tan Z, Liu J, Wang L. 2024. Factors affecting the rumen fluid foaming performance in goat fed high concentrate diet. *Frontiers in Veterinary Science 11*: https://doi.org/10.3389/fvets.2024.1299404

Tanner GJ, Moate PJ, Davis LH, Laby RH, Yuguang L, Larkin PJ, Li YG. 1995. Proanthocyanidins (condensed tannin) destabilise plant protein foams in a dose dependent manner. *Australian Journal of Agricultural Research 46(6)*: 1101-1109. https://doi.org/10.1071/AR9951101

Terrill TH, Rowan AM, Douglas GB, Barry TN. 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *Journal of the Science of Food and Agriculture 58(3)*: 321-329. https://doi.org/10.1002/jsfa.2740580306

Ulyatt MJ, Thomson DJ, Beever DE, Evans RT, Haines MJ. 1988. The digestion of perennial ryegrass (*Lolium perenne* cv. Melle) and white clover (*Trifolium repens* cv. Blanca) by grazing cattle. *British Journal of Nutrition 60(1)*: 137-149. http://www.scopus.com/inward/record.url?eid=2-s2.0-0024040378&partner

ID=40&md5=4fd094cfb1bcd99400a1f08fb67e17df

Waghorn GC. 1991. Bloat in cattle 47 relationships between intra-ruminal pressure, distension, and the volume of gas used to simulate bloat in cows. *New Zealand Journal of Agricultural Research 34(2)*: 213-220. https://doi.org/10.1080/00288233.1991.10423362

Waghorn GC, Douglas GB, Niezen JH, McNabb WC, Foote AG. 1998. Forages with condensed tannins – their management and nutritive value for ruminants. *Proceedings of the New Zealand Grassland Association 60*: 89-98. https://doi.org/https://www.grassland.org.nz/publications/nzgrassland_publication_221.pdf

Waghorn GC, Jones WT. 1989. Bloat in cattle. 46. Potential of dock (*Rumex obtusifolius*) as an antibloat agent for cattle. *New Zealand Journal of Agricultural Research 32*: 227-235. https://doi.org/https://doi.org/10.1080/00288233.1989.10423458

Waghorn GC, Ulyatt MJ, John A, Fisher MT. 1987. The effect of condensed tannins on the site of digestion of amino acids and other nutrients in sheep fed on Lotus corniculatus L. *British Journal of Nutrition 5(1)*: 115-126. https://doi.org/https://doi.org/10.1079/BJN19870015

Wang Y, Majak W, McAllister TA. 2012. Frothy bloat in ruminants: Cause, occurrence, and mitigation strategies. *Animal Feed Science and Technology 172(1-2)*: 103-114. https://doi.org/10.1016/j.anifeedsci.2011.12.012

Wang Y, Wang L, Wang Z, Xue B, Peng Q, Hu R, Yan T. (2023). Recent advances in research in the rumen bloat of ruminant animals fed high-concentrate diets. *Frontiers in Veterinary Science 10* Article 1142965. https://doi.org/10.3389/fvets.2023.1142965

Woodfield DR, Roldan MB, Voisey CR, Cousins GR, Caradus JR. 2019. Improving environmental benefits of white clover through condensed tannin expression. *Journal of New Zealand Grasslands 81*: 195-202. https://doi.org/https://doi.org/10.33584/jnzg.2019.81.382

Zeller WE, Sullivan ML, Mueller-Harvey I, Grabber JH, Ramsay A, Drake C, Brown RH. 2015. Protein precipitation behavior of condensed tannins from Lotus pedunculatus and Trifolium repens with different mean degrees of polymerization . *Journal of Agricultural and Food Chemistry 63(4)*: 1160-1168. https://doi.org/https://doi.org/10.1021/jf504715p

Züst T, Agrawal AA. 2017. Trade-Offs Between Plant Growth and Defense Against Insect Herbivory: An Emerging Mechanistic Synthesis. *Annual Review of Plant Biology 68*: 513-534. https://doi.org/10.1146/annurev-arplant-042916-040856