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Plantain-mixed pasture collected in different climatic seasons produced less methane and ammonia than ryegrass–white clover pasture *in vitro*

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ABSTRACT

Context. Plantain (PL) is recognised for reducing nitrate leaching and nitrous oxide emissions in pastoral systems. Evidence has shown that cows fed pure PL produced less methane (CH_4) than cows fed ryegrass. However, it is unclear if the CH_4 reduction can be achieved with PL in mixed pasture. **Aim.** The study evaluated the *in vitro* rumen fermentation profiles of ryegrass–white clover (RWC) and medium-level PL (PLM, containing ~40% PL) pasture collected during different climatic seasons, to determine whether this inclusion level influences CH_4 and rumen ammonia (NH_3) production. **Methods.** Substrates were selected from samples with various proportions of PL. Samples were categorised into three climatic seasons (i.e. spring, summer and autumn) and two pasture types (PLM and RWC). Representative samples for these scenarios were tested in an automated *in vitro* rumen batch culture system for gas, CH_4 (mL/g DM) and NH_3 (mM/g DM) production. **Key results.** In summer samples, PLM produced approximately 8%, 14% and 19% less CH_4 at 12 h, 24 h and potential CH_4 production (PCH_4), respectively. Although gas production (GP) was similar at 12 and 24 h, PLM had 13% lower potential GP than RWC ($P < 0.05$). In spring samples, PLM had approximately 11% greater GP and CH_4 production at 12 h. For the autumn samples, GP and CH_4 production were similar between PLM and RWC ($P > 0.05$). Net NH_3 production from PLM substrates was significantly lower in spring (27%) and autumn (17%) samples, with no differences in summer, despite higher crude protein levels in the selected PLM. **Conclusions.** Compared with RWC, PLM changed rumen fermentation parameters that could translate to potential environmental benefits: PLM produced less net NH_3 in spring and autumn samples (27% and 17%, respectively), and up to 19% less CH_4 production in summer samples. **Implications.** Incorporating ~40% PL into RWC pasture showed a promising reduction of CH_4 emissions and nitrogen losses *in vitro*. If the *in vitro* results translate to cows grazing pasture, this could offer greater environmental benefits with minimal input costs. *In vitro* results suggest that PLM's potential to mitigate CH_4 emissions can be influenced by seasonal variations in pasture quality compared with RWC. However, further animal studies are needed to fully comprehend the CH_4 mitigation potential of this forage.

Keywords: forage quality, greenhouse gas, methane mitigation, mixed pasture, nitrogen losses, plant secondary metabolites, *Plantago lanceolata*, rumen fermentation.

Introduction

Based on grazed pasture systems, New Zealand's dairy industry makes a significant contribution to the national economy by efficient milk production at comparatively lower costs than indoor feeding systems (Ozawa *et al.* 2005). However, this benefit comes with trade-offs of elevated nitrogen (N) losses (Pinxterhuis *et al.* 2024) and methane (CH_4) emissions from enteric fermentation (Leahy *et al.* 2019). In 2022, the agriculture sector

Collection: Australasian Dairy Science Symposium 2024 contributed half (53%) of total emissions, with CH_4 from enteric fermentation accounting for 78.2% of sector's emissions, followed by nitrous oxide (N_2O) emissions from agricultural soils (MfE 2024).

Feeding pastures to cows can provide N in excess of the animals' nutritional requirements (Pacheco and Waghorn 2008). High N intake may increase rumen ammonia (NH_3) concentration, thereby potentially raising urinary N excretion (Beltran *et al.* 2019). This could lead to high N leaching and N_2O emissions (Selbie *et al.* 2015) from the urine patch. Ruminants grazing on low-quality feeds produce around 75% of CH_4 emissions globally (Leng 1993) and offering high-quality pasture can mitigate CH_4 emission per unit of DM intake (DMI; Arndt *et al.* 2022). High-quality pasture is generally characterised by greater concentrations of non-structural carbohydrates (NSC), greater digestibility and crude protein (CP) and lower fibre, particularly neutral detergent fibre (NDF; Waghorn and Clark 2004).

In recent years, incorporating plantain (PL; *Plantago lanceolata* L.) into a permanent pasture mix (RWC) of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) has become increasingly popular in the New Zealand dairy system for its environmental benefits (DairyNZ 2023*a*). Plantain is a mineral-rich forage herb that is easy to establish, adaptable to various agricultural soils and drought tolerant (Stewart 1996). Numerous studies have reported the benefits of PL pasture in the diet of dairy cattle. These benefits include lowering urinary N concentration (Box *et al.* 2017; Minnee *et al.* 2020) and lowering CH_4 emissions (Della Rosa *et al.* 2022).

According to the New Zealand's greenhouse gas inventory, dairy cows produce 21–22 g of CH_4 per kg of DMI (Pickering *et al.* 2024). Methane production from ruminants is influenced by various factors, such as pasture quality, digestibility, DMI, plant bioactive compounds and grazing management (Hristov *et al.* 2013; Danielsson *et al.* 2017). Among these, pasture quality, digestibility and availability of plant bioactive compounds can be influenced by climatic season (Roche *et al.* 2009; Navarrete *et al.* 2016; Lahart *et al.* 2024). When comparing PL and RWC pasture across seasons, PL mixed pasture is usually superior in nutritional value (i.e. greater digestibility, greater NSC:N content, greater mineral content and lower NDF) compared with RWC pasture (Nguyen *et al.* 2022; Herath *et al.* 2023), and PL pasture contains bioactive compounds, such as aucubin and acteoside (Tamura and Nishibe 2002). Both bioactive compounds influence the rumen fermentation profile by lowering net NH_3 production, and modifying either the maximum gas production (acteoside) or the rate at which the gas is produced (aucubin; Navarrete *et al.* 2016).

When pure PL was tested both *in vitro* (Durmic *et al.* 2016) and *in vivo* (Della Rosa *et al.* 2022), it reduced CH_4 emissions, but the effect of PL in a mixed pasture remains unclear. Hammond *et al.* (2014) observed a decrease in CH_4 yield (g of CH_4 per unit of DMI) from heifers grazing a mixture of ryegrass with five species, including wild PL. Wilson *et al.* (2020) reported a tendency for lower CH_4 emissions from cows fed mixed pasture, which included PL, chicory and white clover, compared with those fed with a mixture of grass species and white clover. In contrast, Jonker *et al.* (2019) reported an increase in CH_4 yield from animals fed diverse pastures including PL, but the total CH_4 emissions were similar to those of RWC. In these studies, the reduction in CH_4 emissions cannot be solely attributed to the PL, as other species were included in varying proportions. Additionally, in a more recent study, Koning *et al.* (2024) observed greater CH_4 yield for pasture containing <25% PL compared with ryegrass pasture. As the responses reported vary widely, it is important to understand the sources of the variation of these results. This study examined the seasonal variability of PL mixed pasture *in vitro* to test its ability to mitigate CH_4.

In the mixed pasture, maintaining higher levels of PL is challenging, as it declines naturally over time (Dodd *et al.* 2019; Nguyen *et al.* 2022). The results from the study site of the Massey University PL trial, which includes measurements in four production seasons, suggest that approximately 20–50% PL is achievable in practice using different sowing rates (MPI 2024). Thus, it is also crucial to assess how such proportions of PL in RWC mix affect CH_4 emissions.

Accordingly, the present study aimed to assess the *in vitro* rumen fermentation profiles of RWC and PL mixed pasture, consisting of a medium-level of PL, ~40% PL (PLM), collected during different climatic seasons. The objectives of this study were to evaluate the chemical compositions of PLM and RWC substrates to determine their influence on CH_4 emissions and NH_3 production, with a particular focus on the potential of PL, at ~40% in the pasture, to reduce CH_4 emissions, as it would be a more practicable PL percentage to have in a mixed pasture.

Methods

Experimental site description

Substrates assessed in the present study were selected from historical pasture samples collected from the Plantain Potency and Practice program's (DairyNZ 2023*b*) Massey University trial site at Dairy 4, Palmerston North, New Zealand (40°23′27″S, 175°36′44″E) containing four pasture treatments with a targeted proportion of PL in a RWC pasture mix of 0% (PL0), 30% (PL30), 50% (PL50) and 70% (PL70). Pasture establishment and general management were reported previously by Nguyen *et al.* (2022). In brief, over four production seasons (September to May), 32 grazings were conducted with lactating dairy cows, and pasture quality was analysed for 23 events. Eighteen of those events were specifically analysed for the presence of bioactive compounds.

Botanical composition was measured by manually separating pasture components, oven drying at 75°C until they reached constant weight and were then reported as a percentage of DM. For pasture quality and bioactive compounds analysis, samples were oven-dried at 60°C until a constant weight was achieved. The dried samples were ground through a 1-mm sieve, and one subsample was taken and analysed for chemical composition of pasture quality at a commercial laboratory (RJ Hill Laboratories, Hamilton, New Zealand), whereas another subsample was taken to test the bioactive compounds, aucubin, acteoside and catalpol, using high-performance liquid chromatography, as described by Navarrete *et al*. (2016). Pasture samples were submitted for quality analysis after each grazing event, whereas bioactive compound concentrations were assessed collectively as one batch at the end of each production season. Once dried, the samples were stored in airtight, opaque containers at room temperature in a low-light environment until incubation.

Chemical composition analysis for pasture samples was conducted by using near-infrared spectroscopy (Hill-Labs 2022). The samples were tested for the following parameters: organic matter digestibility (OMD) in DM (determined using Australian Fodder Industry Association pepsin-cellulase procedure and derived as *in vivo* using a linear regression based on calibration samples from Lincoln University), NDF, acid detergent fibre (ADF), lignin (calibration based on acid detergent extraction followed by treatment with 72% sulfuric acid in the Ankom Daisy Incubator), ash (calibration based on weight loss after ashing at 600°C for 2 h), soluble sugars (calibration based on an 80:20 ethanol:water extraction and colorimetric determination), crude fat (calibration based on petroleum spirit extraction by Ankom auto analyser, AOCS official procedure AM-5-04), CP (N multiplied by 6.25, with N calibration based on total N by Dumas combustion), OMD in DM (DOMD; solubilised organic matter as a portion of the DM) and NSC = 100 − (CP + ash + crude fat + NDF).

Substrate selection for *in vitro*

A collated dataset was created using variables from the pasture quality and bioactive compound analysis to categorise the samples. These variables included PL percentage in the pasture, CP, NDF, ADF, lignin, (lignin/NDF), ash, DOMD, soluble sugar, NSC, catalpol, aucubin and acteoside.

The PL percentage varied across years, with the lowest annual production of 18% and the highest of 50% (MPI 2024). The targeted annual population for PL70 was not achieved in any year (Supplementary Table S1). Therefore, regardless of the pasture treatments (PL0, PL30, PL50 and PL70), 0–5% PL was defined as RWC, and 30–50% PL as PLM. Data were grouped by these categories (RWC, PLM) and by season (spring, summer, autumn), and based on the mean, a representative sample for each season and pasture type (n = 6) was selected for the *in vitro* incubation.

In vitro study experimental design and treatments

The *in vitro* fermentation was conducted in a fully automated batch culture system, adhering to the protocols outlined by Muetzel *et al*. (2014).

Each treatment (shown in Table 1) was incubated in two sets. The first set continuously measured CH_4 and GP over 48 h, and the second set was used to obtain sub-samples at 3, 6, 9, 12, 24 and 48 h for NH_3 plus one end-point sample for analysis of volatile fatty acids (VFA). At each time point, bottles from the second set were briefly removed from incubation one at a time and returned to the incubator after sampling. Using separate sets kept gas measurements and sample collection independent yet aligned to the same fermentation timeline. Each of these incubations was repeated three times (replicates) for statistical evaluation, and each replicate had two duplicate bottles (analytical replicates). Each replicate consisted of a mixture of rumen fluid from two cows.

Rumen sampling and *in vitro* medium preparation

A reduced carbonate-based buffer solution (6.0 mM Na_2HPO_4, 9.6 mM KH_2PO_4, 0.5 mM $MgCl_2$, 64.5 mM $NaHCO_3$ and 17.8 mM NH_4HCO_3) was prepared, as described by Mould *et al*. (2005). Rumen fluid was collected separately from two fistulated, non-lactating cows before the morning feeding to reduce variability between incubations. The donor cows grazed a RWC pasture year-round. The collection of rumen fluid from fistulated cows and the management of these animals were approved by the AgResearch Grasslands Animal Ethics Committee, Palmerston North, New Zealand (AE699), in accordance with the Animal Welfare Act of 1999 and its amendments in New Zealand. The collected rumen fluid was filled to the top of pre-warmed insulated flasks, maintaining both temperature and anaerobic conditions during transport to the laboratory. Equal volumes of rumen fluid from each donor cow were combined and added to the buffer solution, making up 20% of the total volume (v/v) of the *in vitro* rumen-buffer mixture (medium), which was continuously flushed with CO_2 to help maintain anaerobic conditions.

Incubation preparation and sub-sample collection

The substrates were weighed to 500 ± 10 mg and added to 125-mL serum bottles, which were labelled and pre-warmed to 39°C in an incubator. A 50-mL aliquot of the medium was dispensed into each incubation bottle under CO_2 flushing. The bottles were capped with butyl rubber stoppers, shaken manually and then randomly placed on a rack in a reciprocal

Table 1. Treatments or substrates used in the *in vitro* incubation.

Season	Pasture type	Substrate or treatment
Spring	Ryegrass	RWC spring
	Plantain mixed pasture	PLM spring
Summer	Ryegrass	RWC summer
	Plantain mixed pasture	PLM summer
Autumn	Ryegrass	RWC autumn
	Plantain mixed pasture	PLM autumn

shaker inside the incubator. The bottles were connected to the automated gas measurement system via a 23-gauge needle, shaken horizontally at 120 rpm. Gas pressure within each incubation bottle was automatically monitored at 1-min intervals throughout the incubation period, with gas released and sampled for gas chromatograph analysis whenever the internal pressure exceeded a threshold of 9 kPa (Muetzel *et al.* 2014).

A sample of the medium was collected for analysis of NH_3 and VFA (0-h sample). Samples of the fermented material were collected using a 3-mL syringe connected to a manual valve via a needle. At each sampling point, the bottle was shaken manually and then a 1.8 mL of sample was pipetted into 2-mL Eppendorf tubes and centrifuged (21,000*g* for 10 min at 4°C). An aliquot of 900 μL of supernatant was mixed with 100 μL of internal standard (19 mM ethyl butyrate in 20% (v/v) phosphoric acid) in a 1.5-mL micro-tube, and stored at −20°C until further analysis of NH_3 and VFA within the next 3 weeks.

Laboratory analysis for samples from *in vitro* incubation

The frozen samples were thawed and centrifuged at 21,000*g* for 10 min at 4°C. An aliquot of 800 μL supernatant was transferred into a 2-mL crimp cap vial for VFA analysis. Volatile fatty acids were analysed using gas chromatography, as described by Attwood *et al.* (1998). Approximately 100 μL of the remaining supernatant was collected for NH_3 concentration analysis using the colorimetric method described by Weatherburn (1967) scaled down to run in 96-well microplate format.

Model fitting and data analysis

Gas and CH_4 production from each bottle were separately fitted to a logistic model (France *et al.* 2000) as a function of time to estimate the *in vitro* gas and CH_4 production parameters using the following formulae:

$$V(t) = \frac{a \times (1 - \exp(-b \times t))}{(1 + c \times \exp\ (-b \times t))}$$

$$T^{\frac{1}{2}}a = \frac{\ln(c+2)}{b}$$

$$R^{\frac{1}{2}}a = \frac{a \times (c+1) \times b \times \exp(b \times T^{\frac{1}{2}a})}{(\exp(b \times (T^{\frac{1}{2}a})) + c)}$$

Where:

- V: cumulative volume of gas or CH_4 produced up to time *t* (mL/g DM)
- *a*: potential gas or CH_4 production, which is the maximum volume that can be produced per unit of DM (mL/g DM)
- *b*: fermentation rate constant (/h)
- *c*: constant determining curve steepness and lag phase
- *t*: time (h)
- $T^{\frac{1}{2}}\ a$: half-time (h)
- $R^{\frac{1}{2}}a$: rate of GP at half-time (mL/h DM)

Statistical analysis

Ammonia values were corrected for the amount of incubated substrate and the time 0 NH_3 concentration in the medium, and reported as net NH_3 production (mM/g DM). All statistical analyses were performed using the statistical software R version 4.4.0 (R Core Team 2024). The chemical composition (NDF, ADF, lignin, % of lignin in NDF, DOMD, OMD, CP, NSC, acteoside, aucubin), gas, CH_4 production parameters and endpoint VFA, were analysed using linear mixed models in the R package ‘lme4’ (Bates *et al.* 2015). Residuals were checked for normality using the Shapiro–Wilk test, and log transformations were performed where necessary (for acteoside and aucubin) to meet the assumptions of ANOVA. The R package ‘emmeans’ (Lenth 2023) was used for multiple comparisons using estimated marginal means, and *P*-values were adjusted using the Tukey *post hoc* test. Treatment effects were declared significant at adjusted $P < 0.05$. The ‘multcompView’ package (Graves *et al.* 2019) was used to convert the *P*-values into a character-based display in which characters identify groups that are significantly different/not different ($P < 0.05$). Back-transformed values were used to display the means in the respective log-transformed analyses.

The chemical composition was analysed using a two-way ANOVA, considering treatment and season of the year, as well as their interactions, as fixed effects. Replicates of the treatment and production year were included as random effects. Gas and CH_4 production parameters, along with endpoint VFA concentrations, were analysed using a one-way ANOVA with treatment as a fixed effect, and biological replicates ($n = 3$; each consisting of a pooled rumen fluid mixture from two donor cows per run to capture natural biological variability) of the experiment as random effects. For the analysis of net NH_3 production, a repeated measurement model including the fixed effects of treatment and time, the random effect of biological replicates, the incubation bottle as the subject of repeated measurement, and the interaction between treatment and time of sampling was used.

Results

Chemical composition of pasture types

Season is used, for brevity, to describe the results of samples collected at a particular time of the year, and does not mean that the experiments were conducted in different seasons.

The chemical composition of the pastures (PLM and RWC) varied seasonally and between pasture types (Table 2).

Table 2. Chemical composition (g/kg DM) of medium-level plantain pasture (PLM) and ryegrass–white clover pasture (RWC) and chemical composition of selected samples for *in vitro* incubation, across seasons.

	Season	Substrates	Chemical composition (g/kg DM)											
			NDF	ADF	NSC	Soluble sugar	CP	NSC/N (ratio)	DOMD	OMD *in vivo*	Lignin	Lignin in NDF (%)	Aucubin	Acteoside
Average nutritive values across seasons	Spring	RWC	430b	244 abc	220bc	80b	210b	7.1ab	701a	786a	54e	12.7e	0.1c	1.4c
		PLM	386c	234c	260a	95a	212b	8.2a	691a	778a	72cd	18.9bc	6.0a	13.2a
	Summer	RWC	446b	255a	194c	65c	220ab	6.1b	660b	741b	70d	15.7d	0.8c	1.1c
		PLM	398c	244bc	236ab	65c	226ab	6.9b	662b	746b	83b	21.2b	4.2b	8.3b
	Autumn	RWC	477a	251ab	141d	59c	229ab	4.5c	657b	742b	79bc	16.5cd	1.3c	0.3c
		PLM	393c	236c	216bc	69bc	237a	6.2b	664b	757ab	96a	25.1a	4.2ab	10.1ab
	P-values	s.e.m.	19.1	10.9	11.8	7.2	22.3	10.9	18.0	19.6	4.3	1.2	0.7	1.1
		Pasture type (T)	<0.001	<0.001	<0.001	<0.01	n.s.	<0.001	n.s.	n.s.	<0.001	<0.001	<0.001	<0.001
		Season (S)	<0.001	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.	n.s.
		T × S	<0.01	n.s.	<0.05	<0.05	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.001	<0.001
Samples selected for incubation	Spring	RWC	451	242	179	64	240	4.7	688	761	49	10.9	0.2	0.6
		PLM	366	215	245	73	250	6.1	708	793	72	19. 7	5.0	11.1
	Summer	RWC	458	261	213	94	201	6.7	669	738	64	13.6	nd	nd
		PLM	377	226	241	84	234	6.4	694	785	74	19.6	2.6	9.5
	Autumn	RWC	441	234	129	56	278	2.9	668	755	61	13.8	0.8	0.5
		PLM	400	235	231	64	229	6.2	673	754	92	23	5	21

Values marked with the same letters within columns are not statistically different at the 5% significance level.
RWC, ryegrass–white clover pasture; PLM, medium-level plantain pasture (30–50%); nd, not detected; n.s., not significant; T × S, interaction between pasture type and season; NDF, neutral detergent fibre; ADF, acid detergent fibre; NSC, non-structural carbohydrate; CP, crude protein; N, nitrogen; DOMD, digestibility of organic matter in dry matter; OMD *in vivo*, organic matter digestibility *in vivo*; s.e.m., standard error of means of the pasture types.

A significant interaction between pasture type and season was found in NDF ($P < 0.01$), NSC ($P < 0.05$) and soluble sugars ($P < 0.05$). The NDF content of RWC pasture was significantly greater (~13%) than that of PLM in each season. For the PLM pasture, similar NDF content was observed across seasons, ranging from 386 to 398 g/kg DM. Additionally, the NDF content in autumn RWC was significantly greater than spring and/or summer RWC. A general trend of declining NSC content from spring to summer in both pasture types was observed. However, NSC content for PLM in each season was greater than that of RWC ($P < 0.001$), with differences of 18%, 22% and 53% in spring, summer and autumn, respectively. Soluble sugar content was greater in spring for both pasture types, with PLM significantly greater than RWC ($P < 0.01$); whereas in summer and autumn, both had similar soluble sugar content. On average, PLM pasture contained approximately 24% greater lignin content than RWC pasture across seasons, with seasonal effects also observed on lignin content ($P < 0.001$). Lignin content increased from spring to autumn for both pasture types, but the content in PLM was greater in each season compared with RWC. Pasture CP and DOMD were significantly affected by season ($P < 0.001$), and were similar between pasture types in each season. Digestibility (DOMD) was greater in spring compared with other seasons in both pasture types ($P < 0.001$).

The NSC:N ratio was significantly greater in PLM compared with RWC during autumn, and was statistically similar in spring and summer. A declining trend in NSC:N was observed from spring to autumn in both pastures; however, PLM had grater values in each season, with high absolute values in spring and summer, and greater values in autumn ($P < 0.001$). Both aucubin and acteoside concentrations were greater in PLM compared with RWC ($P < 0.001$), with acteoside levels being greater than aucubin. An interaction between seasons and pasture was observed for acteoside and aucubin, with PLM pasture having the greatest levels of these compounds in spring, the lowest levels in summer and levels in the autumn pasture being intermediate. Catalpol was detected at low levels in PLM (<1 g/kg DM in general, with a maximum value of 2.41 g/kg DM); therefore, it was excluded from the analysis (data not shown).

Numerical differences in the analysed mean values and absolute values of selected pasture samples for *in vitro* were observed (Table 2); however, these values remained within the typical range for the given season.

Gas and methane production parameters

Assessment of the methane effects requires measurement of both methane and gas production, to distinguish between results that indicate low fermentability of a substrate (i.e. low gas and methane production) and an inhibition of methanogenesis (i.e. lower methane proportion in the gas produced). Table 3 and Fig. 1 show total gas and CH_4 production parameters measured *in vitro* over 48 h from selected pastures listed in Table 2.

In summer pastures, PLM produced approximately 8%, 14% and 19% less CH_4 at 12 h, 24 h and potential CH_4 production (PCH_4), respectively, compared with RWC. Gas production was similar at 12 h and 24 h, but a 13% lower PGP was observed in PLM. This led to reductions in CH_4 proportion in GP (%CH_4) of approximately 8% and 9% at 12 and 24 h; however, the %CH_4 in PGP remained similar (Table 3).

In spring, PLM had approximately 11% greater GP and 12% greater CH_4 production at 12 h. As time progressed beyond 12 h, both PLM and RWC produced similar GP and CH_4 levels. Despite a general trend for a numerical reduction in the %CH_4, no significant difference was found at any given time point for spring pastures (Table 3).

During autumn, PLM was not significantly different from RWC in GP and CH_4 production at 12 h ($P > 0.05$). However, the PLM resulted in lower %CH_4/GP at 12 h ($P < 0.05$), but was not different at 24 h ($P > 0.05$). Additionally, PGP and PCH_4 remained similar between PLM and RWC.

The fermentation rate ($R^{1/2}$) at half-time of PGP ($T^{1/2}$ gas) was faster in PLM during spring and summer (13 and 18% greater, respectively). Similarly, in spring and summer, PLM reached its $T^{1/2}$ gas approximately 24% faster than RWC. In autumn, both pastures reached their $T^{1/2}$ gas and $T^{1/2}$ CH_4 at similar times. However, although the $R^{1/2}$ gas was similar for both pastures in autumn, the $R^{1/2}$ CH_4 was significantly lower (17%) in PLM compared with RWC (Table 3).

Volatile fatty acids analysis

End-point VFA concentrations were similar between substrates collected in each season. A high acetate:propionate ratio was observed in PLM for the summer substrates ($P < 0.05$). Notably, the PLM substrate produced higher molar proportions of minor fatty acids compared with RWC in both summer and autumn. Additionally, a high molar proportion of butyrate was observed in the spring and autumn PLM pasture (Table 4).

Net ammonia production

The net NH_3 production over time was less for PLM compared with RWC from the spring and autumn samples. In spring, the net NH_3 production was 27% lower for PLM pasture (Table 5). Specifically, PLM pasture collected during spring consistently showed lower net NH_3 production at 6, 12 and 24 h, whereas during autumn, it was lower at 9 and 12 h (Fig. 2).

Table 3. Estimated marginal means of gas and methane (CH_4) production parameters.

Seasons	Substrates	% of CH_4/GP 12 h	% of CH_4/GP 24 h	% of potential CH_4/GP	GP at 12 h (mL/g DM)	GP at 24 h (mL/g DM)	PGP (mL/g DM)	CH_4 at 12 h (mL/g DM)	CH_4 at 24 h (mL/g DM)	PCH_4 (mL/g DM)	$T^{1/2}$ PGP (h)	$T^{1/2}$ CH_4 (h)	$R^{1/2}$ PGP (mL/h DM)	$R^{1/2}$ PCH4 (mL/h DM)
Spring	RWC	11.72ab	13.82a	14.38a	179.36b	253.03a	285.22ab	21.04bc	35.00ab	41.17a	8.73a	11.67a	12.64b	1.72a
	PLM	11.83ab	13.47a	13.65ab	198.94a	255.03a	282.29ab	23.55a	34.31ab	38.53ab	6.62bc	9.34b	14.25a	1.75a
Summer	RWC	12.12ab	13.95a	13.64ab	191.25a	252.66a	298.70a	23.18a	35.25a	40.89a	7.89a	10.25ab	12.21bc	1.70a
	PLM	11.14c	12.66b	12.68b	192.12a	240.43ab	261.32bc	21.41b	30.45c	33.10b	6.03c	8.79b	14.40a	1.65a
Autumn	RWC	12.19a	13.96a	13.93ab	168.13c	231.08b	254.97c	20.52bc	32.26bc	35.52ab	8.13a	10.37ab	12.23bc	1.74a
	PLM	11.64bc	13.38a	13.28ab	172.95bc	227.99b	268.42bc	20.14c	30.46c	35.55ab	7.70ab	10.34ab	11.19c	1.44b
	s.e.m.	0.43	0.74	0.85	5.31	4.24	6.49	1.20	1.86	2.82	0.49	0.57	0.55	0.08

Values marked with the same lowercase letters within columns are not statistically different at the 5% significance level.

RWC, ryegrass-white clover pasture; PLM, medium-level plantain pasture (30–50%); GP, gas production; h, hours; PGP, potential gas production; CH_4, methane production; PCH_4, potential methane production; $T^{1/2}$ PGP, half-time of potential gas production (h); $T^{1/2}$ PCH_4, half-time of potential CH_4 production (h); $R^{1/2}$ PGP, rate of PGP at $T^{1/2}$; $R^{1/2}$ PCH_4, rate of PCH_4 production at $T^{1/2}$; s.e.m., standard error of the means.

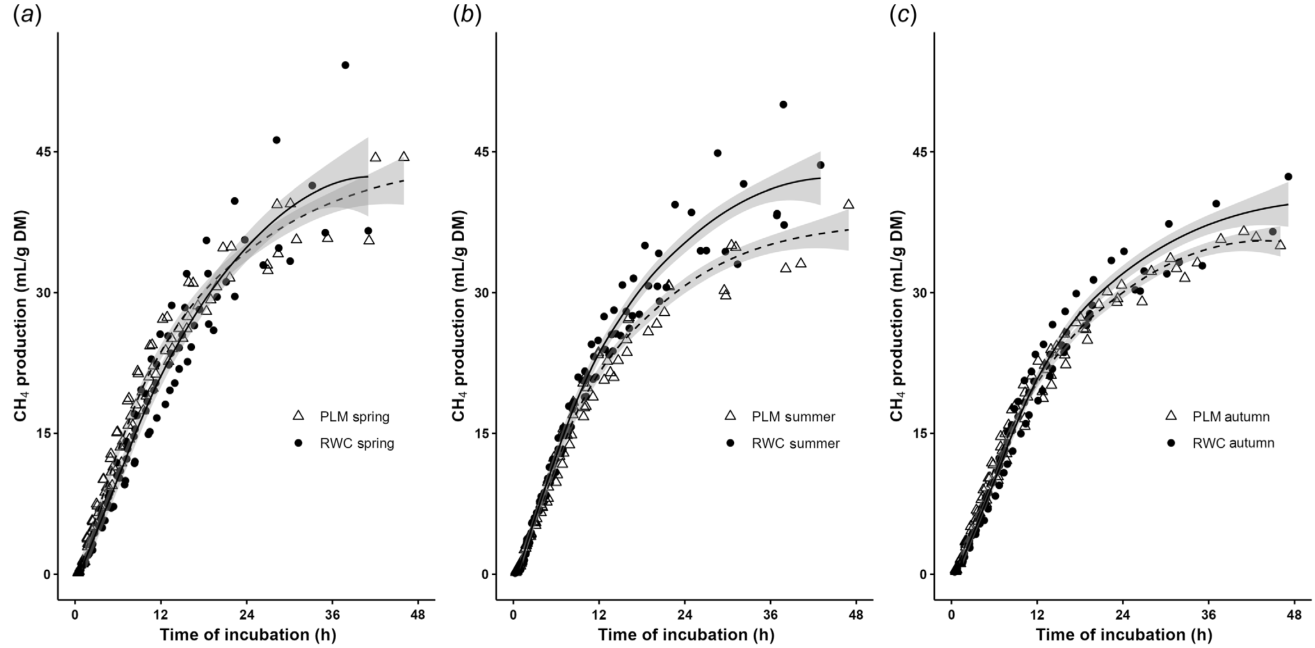

Fig. 1. Methane production (CH_4, mL/g DM) for medium-level plantain pasture (PLM, Δ, dotted line) and ryegrass–white clover pasture (RWC, ●, solid line) collected in (*a*) spring, (*b*) summer and (*c*) autumn, over 48 h of *in vitro* batch culture incubation.

Table 4. Total volatile fatty acid (VFA) production and molar proportions of individual volatile fatty acids (VFAs) of medium-level plantain pasture (PLM) and ryegrass–white clover pasture (RWC) across different seasons.

Seasons	Substrates	VFA (mM)	Acetate (%)	Propionate (%)	Butyrate (%)	Caproate (%)	Valerate (%)	Isobutyrate (%)	Isovalerate (%)	Minor (%)	(A:P)	(AcBu:PrVa)
Spring	RWC	83.61a	65.89bc	17.89ab	9.89cd	0.09c	1.72b	1.73b	2.79b	6.33b	3.68bcd	3.86c
	PLM	83.74a	65.95bc	17.56b	10.29b	0.16a	1.59c	1.67c	2.77bc	6.20bc	3.75bc	3.98b
Summer	RWC	83.91a	66.02b	17.47b	10.86a	0.08c	1.55c	1.49e	2.53e	5.65d	3.78b	4.04b
	PLM	80.68ab	67.08a	16.75c	10.10bc	0.11b	1.69b	1.60d	2.67d	6.08c	4.01a	4.19a
Autumn	RWC	81.87ab	65.72bc	18.02a	9.55d	0.09c	1.91a	1.83a	2.88a	6.71a	3.65cd	3.78c
	PLM	76.65b	65.51c	18.08a	10.27b	0.12b	1.66b	1.66c	2.70cd	6.13c	3.62d	3.84c
	s.e.m.	2.71	0.35	0.10	0.10	0.01	0.07	0.10	0.19	0.36	0.03	0.04

Values marked with the same lowercase letters within columns are not statistically different at the 5% significance level.
RWC, ryegrass–white clover pasture; PLM, medium-level plantain pasture (30–50%); (%), denotes the molar proportions of respective VFA to total VFA production; (A:P), acetate to propionate ratio; (AcBu:PrVa), acetate + butyrate to propionate + valerate ratio; s.e.m., standard error of the means.

Discussion

A key finding of the present study was that PLM pasture has shown the potential to reduce CH_4 production by up to 19% *in vitro* compared with RWC. This aligns with previous studies that reported lower CH_4 production from pure PL pasture *in vitro* when compared with other forage species (Durmic *et al.* 2016), and *in vivo* when compared with ryegrass (Della Rosa *et al.* 2022). However, the measured reductions were not consistent across seasons for all parameters (e.g. CH_4 production, GP and %CH_4). This suggests that variations in the chemical composition of PLM, RWC or both, across different climatic seasons likely influenced the observed outcomes.

The effect of pasture quality on gas and methane emissions

Plantain generally has lower NDF, greater levels of NSC, lignin and improved or similar digestibility compared with ryegrass pasture (Stewart 1996; Minnee *et al.* 2019). In the present study, PLM substrates also had lower NDF, and greater NSC and lignin content compared with RWC, whereas digestibility, as measured by DOMD, remained similar between the two pasture types. The reported values of digestibility for PL mixed pastures were in a similar range of previous reports (Nkomboni *et al.* 2021; Herath *et al.* 2023). Others have reported that pure PL, particularly at its mature stage, has lower total tract DM

Table 5. Total and net ammonia (NH_3) production over time throughout the 48 h of *in vitro* incubation.

Season	Substrates	Total ammonia (mM)	Net ammonia production (mM/g DM)	% difference in net ammonia compared with RWC
Spring	RWC	17.72a	13.26a	–
	PLM	16.12b	9.73b	−26.63
Summer	RWC	16.13b	9.79b	–
	PLM	16.06b	9.62b	−1.7 (n.s.)
Autumn	RWC	17.61a	13.05a	–
	PLM	16.61b	10.84b	−16.96
s.e.m.		1.54	0.61	
Source of variation				
Treatment		***	***	
Time		***	***	
Treatment × time		0.06	0.06	

Values marked with the same lowercase letters within columns are not statistically different at the 5% significance level.
RWC, ryegrass-white clover pasture; PLM, medium-level plantain pasture (30–50%). ***indicates significance at $P < 0.001$. Net ammonia = (total ammonia production – time 0 ammonia in the medium) per DM incubated.

digestibility (Della Rosa *et al.* 2022). In contrast to the latter study, the similar DOMD observed between pasture types in the present study could be due to the botanical and chemical composition of the pastures, as our PLM mix contained only approximately 40% PL.

Additionally, NDF levels remained relatively consistent across seasons for PL samples. However, for RWC, NDF content was greater in autumn compared with spring and summer (autumn > spring = summer). As the season progressed, NSC had a decreasing trend, whereas lignin increased in both pasture types. The DOMD was highest in spring, and declined in summer and autumn for both pastures. This aligned with the previous studies reporting PL pastures and RWC (Ulyatt *et al.* 2002; Navarrete *et al.* 2022; Herath *et al.* 2023).

Lignin serves as an indicator of potential NDF digestibility, and it correlates negatively with digestibility and CH_4 emissions (Moore and Jung 2001; Hindrichsen *et al.* 2004), whereas NSC correlate positively with GP (Getachew *et al.* 2004). In the present study, lignin was greater in PLM than RWC across seasons. Despite the high lignin content, the total GP at 24 h was similar in both PLM and RWC regardless of the season. This suggests that the antagonistic effects of lignin and NSC on digestibility may have counterbalanced one another, and led to the forages to yield similar GP. Nevertheless, the limited sample size in the present study restricts the ability to construct a relationship between chemical composition and CH_4 variables.

The PGP for PLM was lower than RWC in summer; however, in other seasons, PGP was similar between substrates. Notably, the GP at 24 h was similar between both substrates regardless of the season. This can be explained by the faster rate of degradation at half-time ($R^{1/2}$), and the shorter time to reach half of the PGP ($T^{1/2}$ PGP). These parameters suggest a more rapid utilisation of the fermentable substrate by microbes in the early part of the incubation. Box *et al.* (2018) reported a faster DM degradation rate in PL compared with RWC *in sacco*. These authors report that more PL DM had disappeared than RWC from hours 6–24. This suggests that with longer incubation times, once the fermentable carbohydrate pool is depleted, the degradation of substrate is slowed down, leading to the lower PGP in PLM.

In high-producing livestock, most digesta would rarely remain in the rumen for >24 h due to the rumen turnover (Keim *et al.* 2014). Consequently, the results from the first few hours of incubation in this study may be more relevant than PGP to explain what happens *in vivo*. Therefore, lower PGP – an indicator of lower total tract digestibility (Della Rosa *et al.* 2022) – may not be a significant concern for animal productivity. The lack of negative impacts on milk production from cows grazing the different PL pastures used in the current study (Nguyen 2023) suggests that the lower PGP observed *in vitro* may not be a direct indicator of the productivity effects of PL *in vivo*.

Although the spring PLM sample did not show potential for reducing CH_4 emissions *in vitro*, a promising reduction, especially in summer, and a trend towards lower levels in autumn substrates suggest that animal trials are necessary to confirm whether CH_4 reduction can be achieved at the farm level. Such trials would help to fully assess the potential of PLM as a CH_4 mitigation tool.

The effect of pasture quality on net ammonia production

Another key finding of the present study was a reduction of up to 27% of net NH_3 production from PL pasture collected in both spring and autumn. This finding is consistent with previous research by Durmic *et al.* (2016) and Navarrete *et al.* (2016), who reported similar reductions from pure PL pastures in *in vitro* studies. The present study demonstrated that such reductions can be achieved even with a medium-level of PL in the pasture. However, this effect was not seen in summer, indicating the effects on ammonia production may be influenced by seasonal variation in forage composition.

Protein degradation occurs in the rumen, where approximately 75–90% of the ingested CP in forage is degraded, with the remainder being rumen undegradable protein (Waghorn and Clark 2004). The protein degradation produces NH_3, which is either utilised by rumen bacteria for their growth, forming microbial CP, or absorbed into the bloodstream. The absorbed NH_3 can be recycled within the body or converted into urea and excreted primarily in the urine (Pacheco and Waghorn 2008). Hence, ruminal NH_3 N not utilised for microbial protein synthesis will likely be excreted

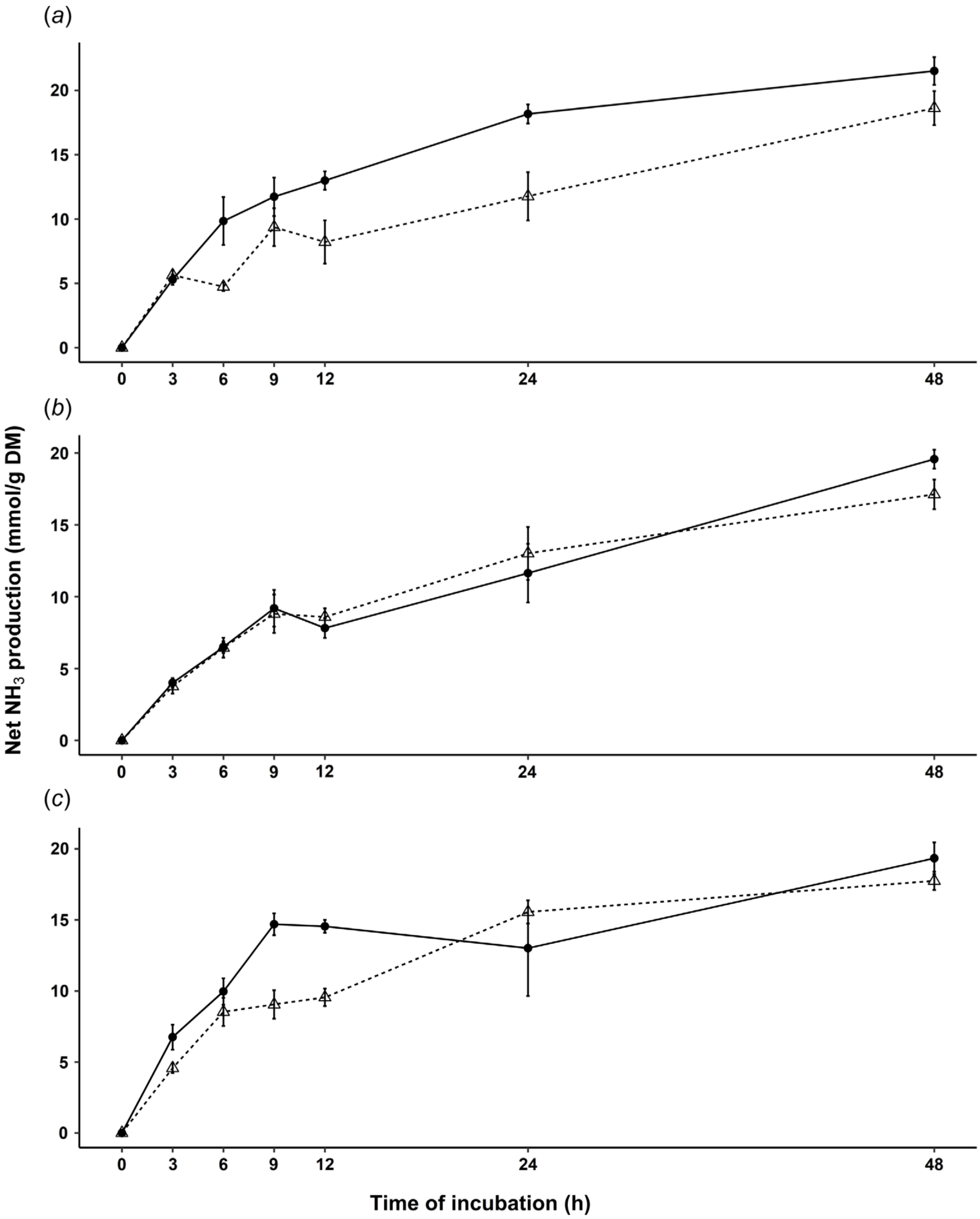

Fig. 2. *In vitro* net ammonia (NH_3) production (mM/g DM) over time of medium-level plantain pasture (PLM, Δ, dotted line) and ryegrass–white clover pasture (RWC, ●, solid line) collected across seasons with comparison of (*a*) spring, (*b*) summer and (*c*) autumn, at each timepoint of *in vitro* batch culture incubation. Bars denoting standard error of the mean (s.e.m.) at each time point.

in urine. This represents a net loss to the animal and contributes to environmental pollution (Tamminga 1992).

A positive relationship between CP intake and urinary N losses has been well established (Box *et al.* 2018; Bougouin *et al.* 2022). Lower ruminal NH_3 concentrations and greater microbial CP production have been shown to be typical responses to increased carbohydrate availability in rumen (Kolver *et al.* 1998), which indicates the importance of the ratio NSC:N in reducing NH_3 in the rumen. The greater NSC:N ratios of PLM pasture compared with RWC in spring and autumn may have contributed to the significantly lower net NH_3 production observed in PLM during these seasons (Table 4). In contrast, during summer, both RWC and PLM pastures had similar NSC:N ratios, resulting in similar net NH_3 production.

The effect of bioactive compounds on methane and net ammonia production

Plantain contains various plant secondary compound groups, such as iridoid glycosides (aucubin) and phenylpropanoid glycoside (acteoside; Stewart 1996). The selected PLM pasture had approximately 11, 10 and 21 g/kg DM of acteoside, and 5, 3 and 5 g/kg DM of aucubin in spring, summer and autumn, respectively. The levels of acteoside and aucubin measured in this study were within the mid-range reported in previous studies (Navarrete Quijada 2015; Box and Judson 2018), likely due to the PLM containing only approximately 40% PL, leading to a proportional decrease in the concentrations of these bioactive compounds.

According to Navarrete *et al*. (2016) acteoside increases GP by serving as an additional energy source for microbes. Additionally, both acteoside and aucubin reduce net NH_3 production, likely due to improved N utilisation by acteoside or the antimicrobial activity of aucubin (Navarrete *et al.* 2016). A study on *Paulownia* leaf extract, rich in acteoside with its derivatives, aucubin and other phenolic compounds, demonstrated a significant decrease in CH_4 and NH_3 production during *in vitro* rumen fermentation by altering microbial population, particularly methanogens and protozoa (Nowak *et al*. 2022).

In the present study, acteoside and aucubin found in PLM substrates may have influenced the NH_3 and CH_4 production to a certain extent. For example, the observed reductions in CH_4 production in summer PLM and the lower $R^{1/2}$ of PCH_4 observed in autumn PLM may be due to the antimicrobial actions of bioactive compounds. In contrast, despite a numerically higher CP content in summer PLM samples (16% higher than RWC), the net NH_3 production was similar between the forages, suggesting an effect of the bioactive compounds on this variable.

However, due to the confounding effects of chemical composition on net NH_3 and CH_4 production, and the lack of studies reporting the interaction between acteoside and aucubin in reducing these emissions, it is challenging to quantify the specific impact of these bioactive compounds on NH_3 and CH_4 production based on this study design.

Conclusions

The present study shows that in different seasons, PLM pasture exhibited evidence of potential environmental benefits compared with RWC pasture. Medium-level PL substrate (~40% PL) collected in summer produced up to 19% less CH_4, suggesting a potential for PLM to reduce enteric emissions. Additionally, reductions in net NH_3, up to 27% in spring and 17% in autumn, indicate the potential for lower urinary N output from cows, a critical source of nitrate leaching and N_2O emissions. These findings are consistent with a broader body of *in vivo* research that shows that dietary inclusion of PL can reduce animal-level N losses to the environment. However, further animal studies at different seasons and inclusion levels of PL are required to confirm a CH_4 mitigation potential for this forage.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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