An evaluation of greenhouse gas emissions reduction potential of plantain (*Plantago lanceolata* L.) in pastoral dairy production systems

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ABSTRACT

There is increasing interest in the ability of plantain (PL) to reduce nitrogen (N) leaching losses and mitigate nitrous oxide (N_2O) emissions, while maintaining milk and pasture production. While PL's role in lowering urinary N concentration is well established, the results regarding the effect of PL on N_2O emissions have been inconsistent. Furthermore, evidence has shown that cows fed pure PL produce less methane (CH_4) emissions compared to those fed ryegrass. However, whether this CH_4 reduction can be achieved with PL in mixed pasture, along with a clear understanding of the mechanism(s) behind those reductions, are still to be determined. This thesis evaluates PL's potential to mitigate CH_4 and N_2O emissions through a series of *in vitro* and a field experiment, focusing on mixed pastures with moderate PL levels.

When pastures, either a conventional ryegrass-white clover (RWC) or an RWC mix containing ~40% of PL (PLM), were collected during different seasons and tested in an *in vitro* rumen batch culture system, differences in their chemical composition led to significant differences in CH_4 and rumen ammonia (NH_3) production. Compared to RWC, PLM had lower fibre (neutral detergent fibre and acid detergent fibre), higher lignin, more fermentable carbohydrates (non-structural carbohydrates), and plant secondary metabolites (PSM, acteoside and aucubin) detected only in PLM, while maintaining similar digestibility and crude protein (CP) levels. Consequently, PLM produced up to 27% less net NH_3 in spring, up to 19% less CH_4 in summer, and 17% less net NH_3 in autumn compared to RWC ($p<0.05$) *in vitro*.

Plant secondary metabolites found in PL, have been associated with reducing N losses from grazed pastures. However, their influence on enteric CH_4 emissions remains unexplored. Additionally, the dose-response relationship between CH_4 and NH_3 production at different concentrations of PSM needs to be established. To address this, purified compounds (>99%

purity) of acteoside and aucubin were incubated with perennial ryegrass (RG) as a basal substrate, and gas and CH_4 production were measured *in vitro*.

The addition of acteoside to RG increased gas production (GP) by up to 12%, with a similar quantity of CH_4 production, but a 5–15% lower proportion of CH_4 in gas (%CH_4), compared to the control. Aucubin addition resulted in a longer lag phase for GP and CH_4 production. On addition of aucubin, it took up to 15% more time to reach the halftime ($T^{1/2}$) GP and up to 20% longer to reach the $T^{1/2}$ CH_4 production. The combined treatments of acteoside and aucubin produced up to 13% greater GP with similar CH_4 production and reduced %CH_4 by around 9%. These reductions are attributed to the modification of the hydrogen utilisation pathway (less hydrogen to produce CH_4) affected by acteoside. Aucubin reduced rumen net NH_3 production by up to 46%, with a similar reduction observed when acteoside was combined with aucubin. These reductions are attributed to the possible antimicrobial activity of aucubin.

These results suggest that PL influences rumen fermentation *in vitro*, resulting in lower CH_4 and NH_3 production. Since higher rumen NH_3 correlates with greater urinary N excretion into the environment, reducing NH_3 levels in the rumen is advantageous.

Previous studies have shown that N_2O emissions from PL pastures may be reduced due to smaller N concentrations in urine and/or biological nitrification inhibition (BNI) activity. In this study, urine collected from cows fed diets containing 0% PL, ~20% PL, and diluted urine from PL-fed cows, was applied to pastures containing 0% PL, 30% PL, and 40% PL during spring. The N_2O emissions were measured over 55 days. Results indicated a trend toward lower N_2O emissions as assessed using the emission factor (EF_3) metric, with increasing PL content ($p<0.09$), with an average reduction of around 28% for pastures containing 30–40% PL compared to RWC pastures ($p=0.03$). This reduction in N_2O emissions from PL pastures was attributed to BNI activity rather than differences in urine-N concentrations *per se*.

These results enhance our understanding of PL's role in mitigating environmental impacts from grazing ruminants in temperate systems. This thesis concludes that medium PL pastures (30–40% PL) exhibit significant environmental benefits compared to RWC pastures *in vitro*, with reductions in CH_4 and rumen NH_3 influenced by PSM in PL and the seasonal variability in chemical composition. Moreover, under conditions conducive to higher N_2O emissions (in spring), maintaining 30–40% PL in the pasture could reduce N_2O emissions more effectively than excluding PL.

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Abbreviations are defined at first mention and used consistently throughout the thesis; similarly, they are defined at the first mention of each chapter.

$\%CH_4$	Methane proportion in gas production
A:P	Acetate to propionate ratio
AB:PV	Acetate+butyrate to propionate+valerate ratio
ADF	Acid detergent fibre
ATP	Adenosine triphosphate
BNI	Biological nitrification inhibition
CH_3X	Methoxy compounds
CH_4	Methane
CHO_2^-	Formate
CO_2	Carbon dioxide
CP	Crude protein
CT	Condensed tannins
cv.	Cultivar
DCD	Dicyandiamide
dil_UPL	Diluted urine from the cows fed with plantain diet
DM	Dry matter
DMF	Dimethylformamide
DMI	Dry matter intake
DMPP	3,4-dimethylpyrazole phosphate
DOMD	Digestibility of organic matter in dry matter
E1	Experiment 1

E2	Experiment 2
E3	Experiment 3
EF_3	Emission factor
FCE	Feed conversion efficiency
GHG	Greenhouse gas
GP	Gas production
h	Hours
H_2	Hydrogen
ha	Hectare
H-UN	High urine concentration
L-UN	Lower urine concentration
NDC	Nationally determined contributions
ME	Metabolizable energy
MP	Metabolizable protein
N	Nitrogen
N_2	Di-nitrogen gas
N_2O	Nitrous oxide
N_2O-N	Nitrous oxide nitrogen
NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrates
NH_3	Ammonia
NH_4^+	Ammonium ion
NH_4-N	Soil ammonia-nitrogen ion
NO_3^-	Nitrate ion
NO_3-N	Soil nitrate-nitrogen ion

NSC	Non-structural carbohydrate
OM	Organic matter
OMD	Organic matter digestibility
PCH_4	Potential methane production
PGP	Potential gas production
PL	Plantain
PL0	Pasture without plantain
PLL	Low-level (30%) plantain
PLM	Medium-level (40%) plantain
PLP	Plantain pasture of 30–40% plantain
PSM	Plant secondary metabolites
$R^{1/2}$ GP	Rate of GP at half-time
RDP	Rumen degradable protein
RG	Ryegrass
RUP	Rumen undegradable protein
RWC	Ryegrass-white clover
SCFA	Short-chain fatty acids
$T^{1/2}$ GP	Time at which the incubation reached ½ of PGP
UN	Urinary nitrogen
UPL	Urine collected from cows fed with plantain diet
URWC	Urine collected from cows fed with ryegrass-white clover diet
WFPS	Water filled pore space

CHAPTER 1

General introduction

1.1 Introduction

Based on grazed pastures, New Zealand's dairy industry makes a significant contribution to the national economy of over 11 billion New Zealand dollars (DCANZ, 2023) by producing milk at comparatively lower costs than indoor feeding systems found in other countries (Ozawa et al., 2005). However, this success comes with the collateral cost of elevated nitrogen (N) losses to the environment via large greenhouse gas (GHG) emissions (Clark et al., 2001; Pinxterhuis et al., 2024). According to the recent New Zealand GHG inventory, the agriculture sector contributes half (53%) of total emissions, with methane (CH_4) from enteric fermentation accounting for 78.2% of the sector's emissions, followed by nitrous oxide (N_2O) emissions from agricultural soils (MfE, 2024). Reducing these emissions while maintaining economic viability, is crucial for the sustainability of the dairy sector. However, mitigating CH_4 and N_2O emissions from grazed pasture requires a holistic management approach, as the production and mitigation of both gases involve distinct biochemical processes in the animal and soil (Eckard et al., 2010; Hristov et al., 2013; Venterea et al., 2012).

Feeding pastures to cows often results in providing N in excess of the animal's nutritional requirements (Pacheco & Waghorn, 2008). High N intake may increase rumen ammonia (NH_3) concentration, thereby potentially increasing urinary N (UN) excretion (Beltran et al., 2019). Urine patches have a large N load, which often exceeds the pasture's uptake capacity (Di & Cameron, 2007) and the excess N (in the form of nitrate, NO_3^-) is susceptible to loss to the environment via leaching (Selbie et al., 2015), and as also via N_2O to the atmosphere (de Klein et al., 2020). Furthermore, ruminants grazing on low-quality feeds produce around 75% of CH_4 emissions globally (Knapp et al., 2014). 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). Offering high-quality pasture can mitigate CH_4 emission per unit of dry matter intake (DMI) (Arndt et al., 2022). This approach, in turn, may also be beneficial in lowering UN excretion if a greater ratio of non-structural carbohydrate to dietary N was found in pasture which could lower UN excretion in grazing animals (Minnee et al., 2019; Pinxterhuis et al., 2024).

In the past decade in New Zealand, several projects, such as Forages for Reduced Nitrate Leaching (DairyNZ, 2023a), Pastoral 21 (DairyNZ, 2022), Greener Pastures (Agricom, na), The Plantain Potency and Practice Programme (DairyNZ, 2023b) and Low Nitrogen Systems (DairyNZ, 2024) have explored options to minimise N leaching losses and GHG emissions to the environment. Accordingly, incorporating plantain (PL, *Plantago lanceolata* L.) into a pasture mix, containing perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) (RWC) has become increasingly popular on New Zealand dairy farms (Navarrete et al., 2023; Pinxterhuis et al., 2024).

Plantain is a mineral-rich forage herb that is easy to establish, adaptable to various agricultural soils, and drought-tolerant (Stewart, 1996). Several studies have reported similar pasture and milk production (with minor compositional changes in milk), while maintaining the profitability of the farm compared to standard RWC pastures (Al-Marashdeh et al., 2021; Herath et al., 2023; Nguyen et al., 2022a, 2024). Additionally, PL pastures mitigate N-leaching losses (Pinxterhuis et al., 2024), and lower N_2O emissions to the atmosphere (Simon et al., 2019), while pure PL lowers CH_4 emissions compared to those from ryegrass-fed cows (Della Rosa et al., 2022).

Although these reductions were positive, maintaining PL in RWC pasture remains a challenge, especially two years after establishment (Dodd et al., 2017; Nguyen et al., 2022b). The reduction of N_2O emissions positively correlates with an increasing level of PL and the inclusion of 30-50% PL in the sward can reduce N_2O emissions, by up to 39% (Simon et al., (2019); Vi et al. (2023), however, other researchers have found its effectiveness to be variable (Earl-Goulet et al., 2021; Podolyan et al., 2020). Several factors, beyond the proportion of PL in the pasture, such as climate and soil properties, may influence the ability of PL to reduce N_2O emissions (de Klein et al., 2020; López-Aizpún et al., 2020; Luo et al., 2019). Therefore, the potential of PL to reduce N_2O emissions should be evaluated in pastures where PL content is typically maintained at around 30–40%, as this reflects the commonly observed levels under different climatic conditions and on different soils (MPI, 2024; Nguyen et al., 2022b).

Similarly, when PL was tested *in vitro* (Durmic et al., 2016) and *in vivo* (Della Rosa et al., 2022), lower CH_4 emissions were observed, but the measurements of the effect of PL on CH_4 production in a mixed pasture are variable. A decrease in CH_4 emissions from cows fed mixed pasture, which included PL, was observed by Hammond et al. (2014) and Wilson et al. (2020). In contrast, Jonker et al. (2019) reported an increase in CH_4 yield from animals fed diverse pastures including PL. In a recent study by Koning et al. (2024), they reported higher CH_4 yield for low-PL content pasture (<25% PL with ryegrass) compared to ryegrass pasture. Therefore, it is essential to identify the proportion of PL in the diet that is required to lower CH_4 production.

Several studies suggest that the plant secondary metabolites (PSM) found in PL could modulate N-cycling mechanisms in animals and/or soil in a manner that reduces losses to the environment. For example, PSM have been shown to alter the rumen fermentation profile and lower rumen ammonia (NH_3) levels (Navarrete et al., 2016). Furthermore, PSM in urine from PL-fed cows (Judson et al., 2019; Peterson et al., 2022) and root exudates from PL pastures, reduced soil nitrification rates (Dietz et al., 2013), thereby lowering N leaching and N_2O emissions (Pinxterhuis et al., 2024). Plantain has three major PSM*, viz.* acteoside, aucubin and catalpol, and their production is influenced by genotype and environmental factors such as air temperature, solar radiation, and nutrient availability (Tamura & Nishibe, 2002). Acteoside and aucubin are known to have antimicrobial properties (Navarrete et al., 2016); however, the impact of PSM in PL on enteric CH_4 emissions is unknown.

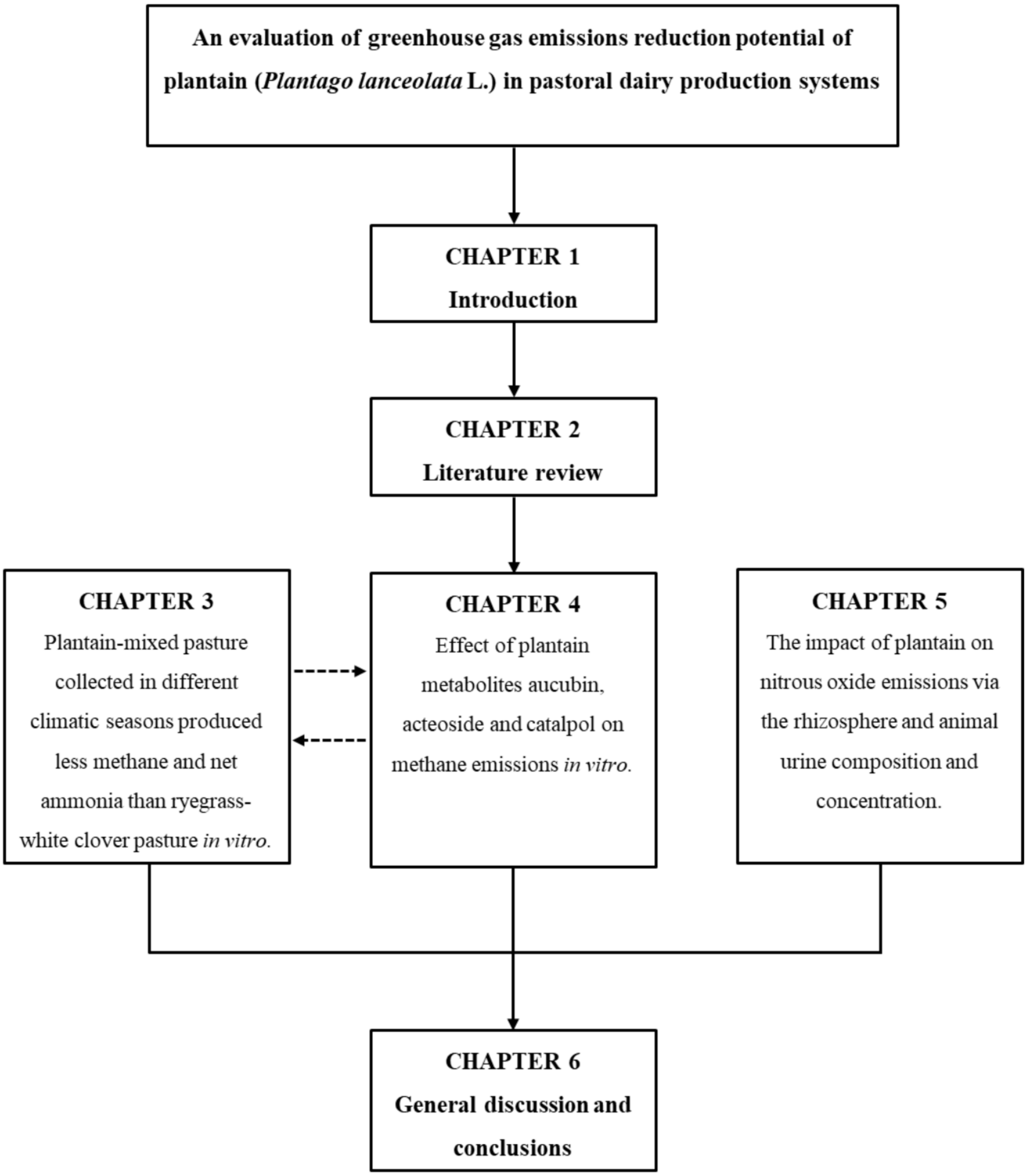
As reports of PL's potential to reduce CH_4 and N_2O emissions vary widely (Earl-Goulet et al., 2021; Koning et al., 2024), it is important to understand the sources of this variability. One possible reason for the discrepancies between the reported results is that the concentrations of PSM varied greatly across studies. This thesis aims to explore the impact of PL and its PSM on mitigating GHG emissions and to understand the mechanism behind such reductions.

1.2 Objectives

This thesis aims to:

1. investigate the impact of PL mixed pasture on reducing *in vitro* CH_4 emissions and rumen ammonia production;
2. investigate the impact of PSM found in PL pasture on *in vitro* CH_4 emissions and ammonia production; and
3. investigate the impact of PL mixed pastures on N_2O emissions from urine applied to a fine-textured soil in spring when wetter conditions coincide with warmer temperatures.

1.3 Thesis structure



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CHAPTER 2

Literature review

2.1 Overview of GHG emissions

Greenhouse gas (GHG) emissions have raised global surface temperature by 1.1°C above 1850–1900 levels between 2011 and 2020, drawing increased public attention to global warming in recent years (Lee et al., 2023; Venghaus et al., 2022). To address this challenge, the Paris Agreement, a multilateral treaty under the United Nations Framework Convention on Climate Change, was signed by all member nations (New Zealand Parliament, 2024). The agreement requires each country to set their own targets, known as nationally determined contributions (NDC), to meet the goals of the Paris Agreement (Himics et al., 2018).

Although New Zealand's overall contribution to global GHG emissions is relatively low, it ranked fifth in per capita emissions among Annex 1 countries (a group of industrialised nations) due to its large agricultural sector (Hopkins et al., 2015). In response, the New Zealand government initially committed to reducing GHG emissions by 30% below 2005 levels by 2030 (MfE, 2020). In January 2025, a revised second NDC was submitted, committing to a 51–55% reduction below 2005 levels by 2035 (MfE, 2025). The Zero Carbon Amendment Act further targets net-zero, except biogenic methane, emissions by 2050. Although biogenic methane has a separate reduction target of 10% below 2017 levels by 2030, and 24–47% by 2050 (Climate Change Response (Zero Carbon) Amendment Act 2019), achieving these targets will require substantial efforts across all sectors to reduce emissions.

Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) are three major anthropogenic gaseous emissions (Tian et al., 2015) and in 2024, atmospheric concentrations of GHG reached a high of 420 ppm of CO_2, 1.921 ppm of CH_4, and 0.337 ppm of N_2O, respectively (NOAA, 2024). Although reducing CO_2 emissions is the main focus, reducing CH_4 and N_2O emissions is crucial due to their significant global warming potentials (GWPs), with CH_4 and N_2O being approximately 28 and 273 times more potent than CO_2, respectively (Lee et al., 2023).

New Zealand was the first country to attempt regulation of agricultural GHG emissions through an emissions market (Cooper & Rosin, 2014). At present, according to New Zealand's GHG inventory, emissions from the agriculture sector contributed half of (53.2%) New Zealand's gross emissions in 2022, enteric fermentation was the main source of agriculture emissions, contributing 78.2% of the sector's emissions. Agricultural soils (15.2%) were the second largest source of the emission (MfE, 2024). The success of policy regulations depends on their compatibility with pastoral farming, and understanding the major drivers and mechanisms of GHG production is essential to manage such emissions.

2.2 Rumen fermentation and enteric methane production

Enteric methane from livestock is the largest source of anthropogenic CH_4 emissions, with cattle contributing the most (Knapp et al., 2014). Methane is produced within the gastrointestinal tract (Figure. 2.1), primarily in the rumen and to a small extent in the large intestine (Hailemariam et al., 2021). Methanogenic Archaea in the rumen utilise CO_2 and hydrogen (H_2) to form CH_4, thereby reducing the metabolic H_2 generated during microbial fermentation (McAllister & Newbold, 2008). As H_2 accumulation can inhibit microbial growth and the formation of short-chain fatty acids (SCFA), any effective mitigation strategies must also provide an alternative H_2 removal pathway (Eckard et al., 2010). Methane production represents a significant energy loss to the host animal, with 6%–10% of consumed energy lost, primarily (95%) through the mouth and nostrils (Eckard et al., 2010; Johnson & Johnson, 1995). Reducing these emissions is challenging, as it involves complex microbial interactions in the cow's rumen (Figure 2.1) that are critical to the animal's basic function (Aguirre-Villegas, 2017).

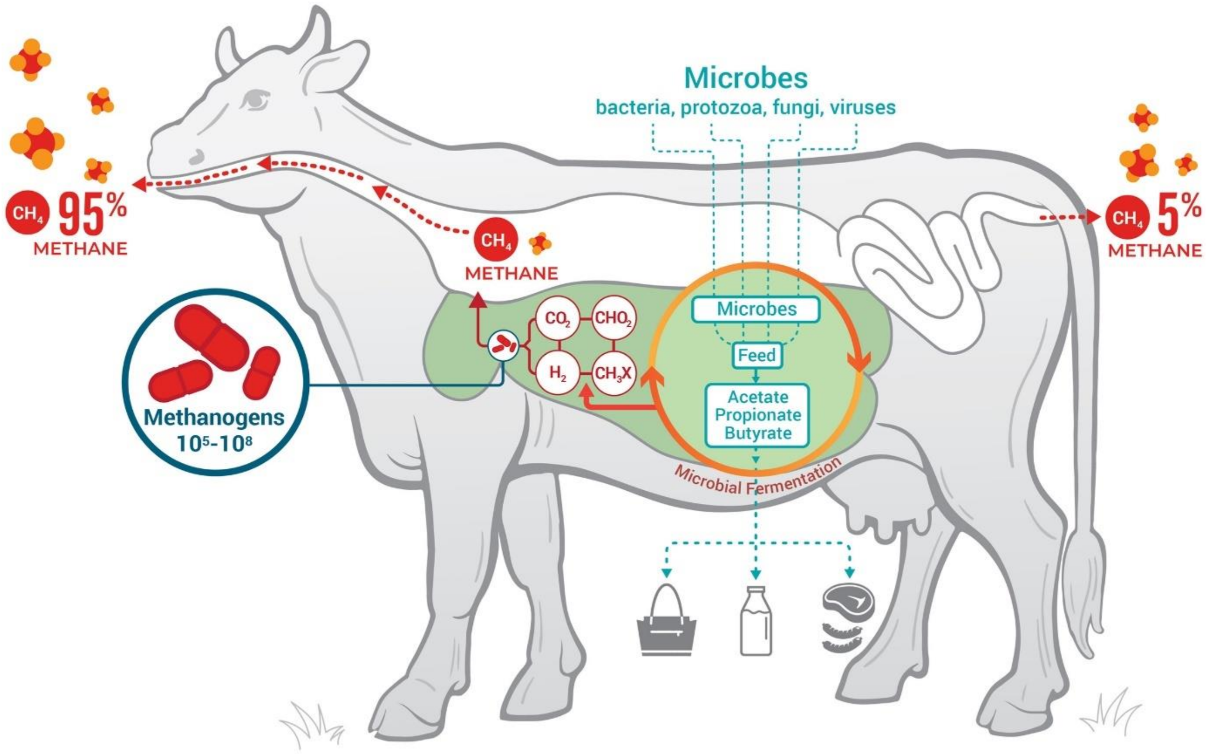

Figure 2.1. Ruminant fermentation processes and products and microbial contributors. Reproduced from Glasson et al. (2022), licensed under CC BY-NC-ND 4.0. Methanogen counts are listed per mL of ruminal content, CO_2: carbon dioxide, H_2; hydrogen, CHO_2^-; formate, CH_3X; methoxy compounds, CH_4; methane.

As a result of ruminal fermentation of feedstuff, three major SCFA; acetate, propionate, and butyrate, along with minor fatty acids such as valerate, caproate, and branched-chain fatty acids (iso-butyrate and iso-valerate), are produced (Della Rosa et al., 2021; Janssen, 2010). The acetate to propionate ratio (A:P) in the SCFA production significantly impacts CH_4 production (Sejian et al., 2011). Another important output of rumen fermentation, aside from the gases produced, is ammonia (NH_3), which is a key product of crude protein (CP) degradation in the rumen. This is discussed in Section 2.3.

When predicting CH_4 production in cattle, dry matter intake (DMI) was the strongest predictor, explaining about 90% of the variation in the data (Jonker et al., 2017). However, DMI can be influenced by feed factors such as forage quality, and digestibility and animal factors such as the live weight of the cattle, cattle class, physiological state and their interactions (Moran, 2005). For national inventory purposes, predictions based on DMI are important, but understanding the mechanisms behind CH_4 emissions is essential to identify influencing factors accurately (Hristov, 2023).

Methane emissions can be expressed using different metrics. Methane production refers to the total amount of CH_4 emitted by an animal per day (g/day). Methane intensity denotes the amount of CH_4 emitted per unit of product output, such as milk or meat (g/kg). Methane yield expresses CH_4 emissions per kilogram of dry matter intake (g/kg DMI). The use of each parameter depends on the specific goals and context, as they highlight different traits of CH_4 emissions (Fresco et al. 2023). In the New Zealand context, reducing total CH_4 production is more important than the other two metrics for meeting its climate change commitments (Climate Change Response (Zero Carbon) Amendment Act 2019).

2.3 Factors affecting methane mitigation strategies

An abundance of CH_4 mitigation strategies have been reviewed over time addressing general overviews (Broucek, 2014; Eckard et al., 2010; Hristov et al., 2013; Knapp et al., 2014), feeding and nutritional management approaches (Beauchemin et al., 2008; Eugène et al., 2021; Palangi & Lackner, 2022), manipulation of microbial communities (Beauchemin et al., 2020; Morgavi et al., 2010) and specific functional interventions, such as the use of plant secondary metabolites (PSM) (Bodas et al., 2012; Janssen, 2010; Rivera & Chará, 2021). Most of these mitigation options can be classified into 3 main categories, (i) animal manipulation, (ii) diet manipulation and (iii) rumen manipulation (Christie, 2019; Eckard et al., 2010).

2.3.1 Animal manipulation

Researchers have reported variations between animals in CH_4 emission since individual CH_4 production varies among animals within the same herd (Beauchemin et al., 2022; Danielsson et al., 2012; Pinares-Patiño et al., 2013). The variations in CH_4 emissions may also arise from heritable factors such as rumen digesta retention rates and host-microbe interactions, suggesting the potential for genetic selection for lower CH_4 emissions (Knapp et al., 2014). Additionally, feed conversion efficiency (FCE) is a heritable trait in dairy cows (Palangi et al., 2022; Waghorn & Hegarty, 2011) and it may be possible for dairy farms to improve profitability and reduce environmental impacts using cows selected and bred for higher FCE and lower CH_4 emission traits (Løvendahl et al., 2018). It was estimated that selective breeding could decrease CH_4 emission by 10–20% during digestion, achieving permanent reductions in CH_4 emissions, however, breeding specifically to lower CH_4 production may conflict with other breeding goals, such as improving economically important traits (Eckard et al., 2010). Reducing the number of unproductive animals on a farm can increase profitability and lower CH_4 emissions (Clark et al., 2001). However, modifying herd composition or shifting to an alternative dairying system may entail higher costs in the short run, and farmers may resist lowering stocking rates to meet emission targets due to the slower adoption of new technologies (Rojas-Downing et al., 2017).

2.3.2 Diet manipulation

Feed plays a major role in rumen fermentation as it directly influences nutrition composition, DMI, amount of digestible nutrients, and pH of the rumen (Danielsson et al., 2017; Johnson & Johnson, 1995; Sejian et al., 2011). Potential reduction of CH_4 emissions per unit of milk through feeding and nutrition can contribute to mitigating CH_4 emissions by around 15% (Aguirre-Villegas, 2017; Knapp et al., 2014). It was estimated that 75% of global ruminant CH_4

emissions came from ruminants grazing low-quality feeds, increasing the quality of the feed is a key concept related to feed efficiency and animal productivity, which can decrease CH_4 emissions per unit of product (Knapp et al., 2014).

The significant components of ruminant feedstuffs (forages, concentrates and supplements) are cellulose, hemicelluloses, pectin, starch, proteins and lipids (Millen et al., 2016). Forages usually contain 12–30% DM, with varying concentrations of CP, fibre, and non-structural carbohydrates (NSC), which include soluble sugars, fructans, organic acids, or starch (Waghorn & Clark, 2004). Plant fibre, often called cell wall or neutral detergent fibre (NDF), provides structural integrity to plants (Waghorn & Clark, 2004). Methane production is highly correlated with fibre digestion in the rumen, and decreasing the fibre content of forages would reduce CH_4 emissions since fibre content and digestibility of forages are negatively correlated (Clark et al., 2011). Neutral detergent fibre is heterogeneous with respect to chemical composition, digestibility, and potential to produce CH_4 (Johnson & Johnson, 1995). The reason for the changes in NDF digestion is lignin found in the cell wall, which acts as a physical barrier and restricts the digestion of cell wall polysaccharides (Jung & Allen, 1995). Lignin serves as an indicator of potential NDF digestibility (Van Soest et al., 2020) and it correlates negatively with CH_4 emissions (Hindrichsen et al., 2004; Moore & Jung, 2001). Except for lignin, the above-mentioned feed components are metabolised to various fermentation products (Millen et al., 2016).

Feeding more digestible carbohydrates often results in higher DMI, increased milk yields, and lower CH_4 emissions. The molar proportion of SCFAs (i.e., A:P) is influenced by the ratio of NDF to NFC in the diet, with forage-based diets generally promoting acetate and butyrate production, while starch-based diets favour propionate production (Knapp et al., 2014). Plant breeding offers the potential for greater digestibility and CH_4 reduction by targeting desired traits in pasture (Beauchemin et al., 2008; Ulyatt et al., 2002).

Dietary fat and essential oil supplements can reduce CH_4 emissions in dairy cows (Pinares-Patiño et al., 2013; Wu et al., 2018) by decreasing ruminal organic matter fermentation (Johnson & Johnson, 1995), thus reducing the CH_4 production. It is recommended that total fat should not exceed 6–7% of dietary DM to prevent reductions in DMI (Beauchemin et al., 2008). Therefore, the cost of supplements and potential digestibility issues should be considered when planning this diet for cows.

2.3.2.1 Use of alternative feedstuff and PSM

The increasing popularity of alternative forages such as forage rape (*Brassica napus* L.) (Della Rosa et al., 2022; Sun et al., 2016), and plantain (*Plantago lanceolata* L.) (M. Della Rosa et al., 2022) has highlighted reductions in CH_4 emissions in recent publications. Studies have shown that diets higher in legumes or containing tannin-rich species can reduce CH_4 emissions, expressed as CH_4 per kg DMI (Pinares-Patiño et al., 2013; Roldan et al., 2021). Wilson et al. (2020), observed a tendency for lower CH_4 production in cows fed forb pastures (chicory; *Cichorium intybus*, plantain, and white clover; *Trifolium repens*) compared to grass-fed cows, and Carmona-Flores et al. (2020) found that diverse pastures with six species produced lower CH_4 emissions per DM consumed compared to simpler pastures with two species. However, more research is needed to better understand the mechanisms underlying these reductions.

The inclusion of seaweeds in cattle diets has shown potential for reduced CH_4 production. *Asparagopsis taxiformis* and *Asparagopsis armata* can inhibit methanogenesis by up to 98% at low inclusion levels, also improving feed efficiency (Glasson et al., 2022). In New Zealand, *Bonnemaisonia hamifera* reduced CH_4 production by 17.1%, 95.4%, and 98.8% at inclusion levels of 2%, 6%, and 10% OM, respectively, with increased hydrogen production. Likewise, *Euptilota formisissima* and *Plocamium cirrhosum* also reduced the production of CH_4 by around 50% and 40%, respectively, at an inclusion level of 10%, with minimal effects on measured fermentation parameters (Mihaila et al., 2022).

The potential of PSM present in forage species to reduce enteric CH_4 production is under exploration. Tannins (condensed tannins, CT and hydrolysable tannin) and saponins have shown promise for CH_4 mitigation through different mechanisms (Naumann et al., 2017; Piñeiro-Vázquez et al., 2015). There is no unanimity on the mechanism by which CT inhibits CH_4; however, CT is assumed to directly inhibit the CH_4 producing archaea in the rumen and attach with compounds in the rumen to decrease fermentation, thus reducing the availability of substrates for use by the methanogens to produce CH_4 (Wilson et al., 2020). Depending on the type and concentration of tannins in the diet, enteric CH_4 emissions can be reduced by up to 50% (Bodas et al., 2012; Naumann et al., 2017), though they may negatively impact plant fibre degradation (Buddle et al., 2011). Plantain contains PSM, including acteoside, aucubin, and catalpol, which can alter the rumen fermentation profile; notably, acteoside increases propionate production *in vitro* (Navarrete et al., 2016), though its effect on CH_4 reduction has not yet been reported (a detailed discussion is provided in section 2.9.5)

2.3.3 Rumen manipulation

Manipulating microbial populations in the rumen through chemical substances by introducing competitive or predatory microbes or with vaccination can reduce CH_4 production (Eckard et al., 2010). The New Zealand Agricultural Greenhouse Gas Research Centre considers vaccine as a highly desirable tool for reducing enteric CH_4 emissions, as it requires no changes to farm systems, is administered occasionally, leaves no residues in products, and can be applied to all ruminant livestock (NZAGRC, 2024). However, Wright et al. (2004) highlight that rumen methanogen populations are influenced by diet and geographic location, posing challenges to the development of a universal methanogen vaccine. Therefore, this technology is not available for wider use yet.

2.4 Nitrogen metabolism in rumen

Protein (CP or N) metabolism in the rumen involves a combination of metabolic processes involving the rumen microbes and the animal (Bach et al., 2005). A simplified diagram is shown in Figure 2.2. Briefly, dietary protein in the ruminant's diet comprises true protein and non-protein nitrogen (NPN), such as ammonia, nitrates and nucleic acids (Pacheco & Waghorn, 2008). Upon ingestion, microbes present in the rumen degrade the true proteins into peptides, amino acids and NH_3. This process provides an N source (mainly NH_3) for the microbes to synthesise microbial protein with SCFA and Adenosine triphosphate (ATP) as energy sources. This microbial protein is the primary source of the amino acids when digesta passes to the small intestine (Bach et al., 2005; Firkins et al., 2007).

Nutritional models for feeding protein to dairy cattle have evolved from basic CP to more complex systems based on rumen degradable protein (RDP) and rumen undegradable protein (RUP). The RDP is broken down in the rumen into NH_3 and peptides, while RUP escapes rumen degradation and is hydrolysed in the small intestine (Bach et al., 2005). Ammonia not utilised by microbes is absorbed into the bloodstream, transported to the liver, and converted to urea and then the urea can be either excreted in urine or recycled back to the rumen through saliva or blood to sustain microbial activity (Annison & Bryden, 1998). Excessive dietary RDP or insufficient energy supply can result in elevated NH_3 production, increasing metabolic costs for detoxification and posing risks of NH_3 toxicity (Bach et al., 2005; Pacheco & Waghorn, 2008).

Microbial protein and RUP are key contributors to the supply of metabolisable protein (MP), which plays a vital role in supporting growth, reproduction, and milk production in animals

(Pacheco & Waghorn, 2008). However, N utilisation in ruminants is often inefficient, with a substantial amount of ingested N being excreted as urea (Bach et al., 2005; Tamminga, 1992). This inefficiency becomes more pronounced when diets are either energy-deficient or contain excessive N intake since NH_3 utilisation by microbes in the rumen is primarily carbohydrate-limited, and increasing carbohydrate availability in the rumen improves the efficiency of microbial utilisation of NH_3 (Kolver et al., 1998; Russell, 1998). Therefore, optimising microbial activity while providing sufficient carbohydrates and lowering protein in the diet can reduce lower N excretion via urine and possibly lower N losses to the environment (Pacheco & Waghorn, 2008; Tamminga, 1992).

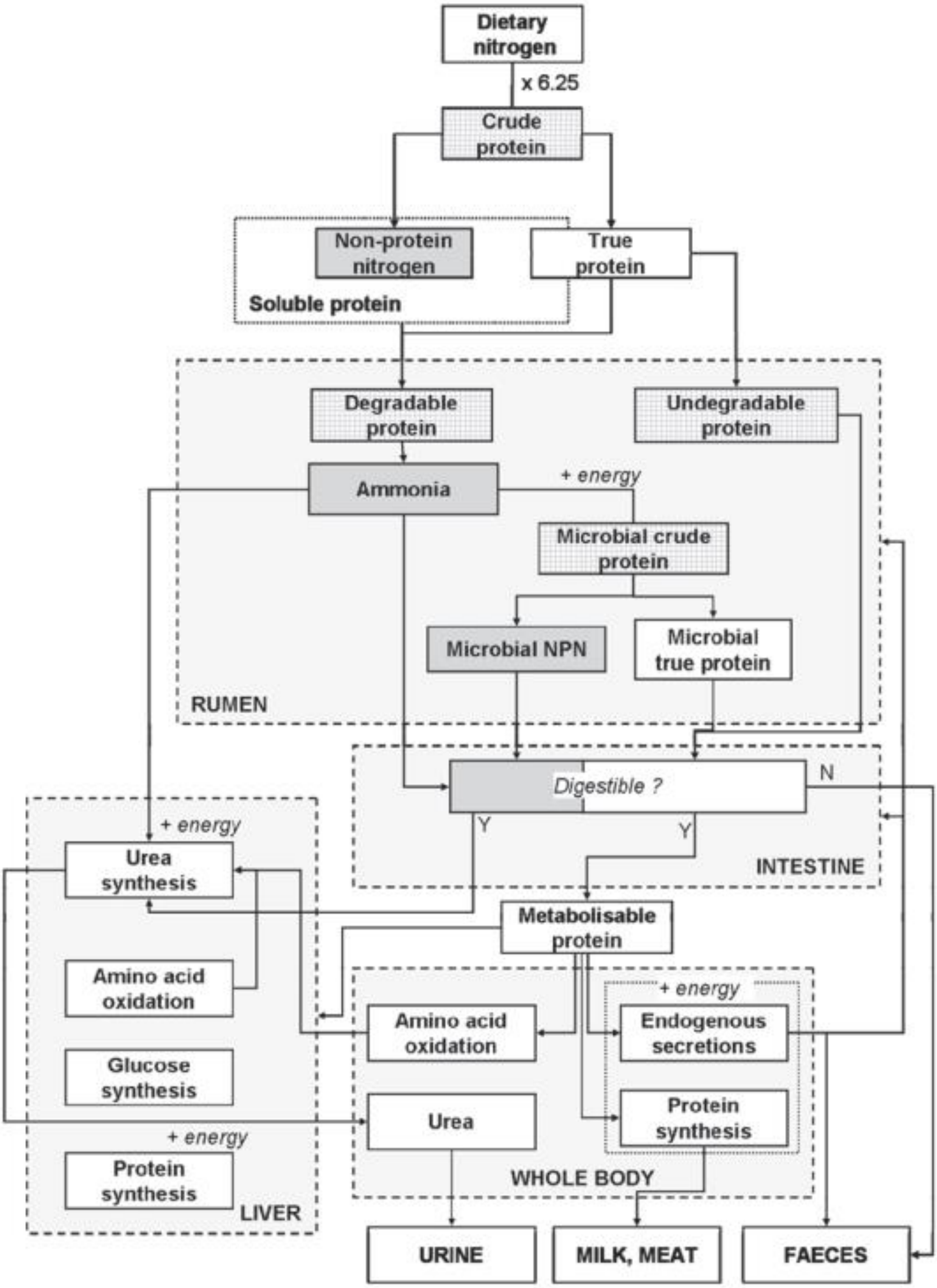

Figure 2.2. Simplified diagram of nitrogen transactions in the ruminant. Reproduced from Pacheco and Waghorn (2008).

2.5 The impact of urine patches on nitrogen losses

From cows feeding on temperate pasture, only about 5–30% of consumed N is converted into products (i.e. milk and meat). The remaining 70–95% of ingested N is excreted in urine and dung (Selbie et al., 2015). The dietary N intake will affect the urinary N (UN) excretion of cows in most cases, which is much more vulnerable to losses than faecal N (Dijkstra et al., 2013). This is attributed to the fact that, as N intake increases, the proportion of N excreted in dung remains relatively constant, while the proportion excreted in urine increases (Selbie et al., 2015).

According to Selbie et al. (2015), on average, cattle urine contains approximately 6.9 g N L^{-1}, with an average urine volume of 2.1 L per event and 10–12 times events per day. The wetted area of a urine patch covers approximately 0.24 m^2, with a range extending up to 0.49 m^2. This creates a huge UN load under a urine patch 613 kg N ha^{-1} for a dairy cow (Selbie et al., 2015). This exceeds the pasture's capacity to uptake N, and the excess N will be lost either via the soil profile (nitrate [NO_3^-] leaching to waterways) or to the atmosphere by NH_3 volatilisation or N_2O emissions (Cameron et al., 2013; Di & Cameron, 2016; Saggar et al., 2005).

2.6 Nitrous oxide gas production in pasture lands

Agricultural practices contribute to 60% of anthropogenic global N_2O emissions, with most emissions from the transformation of N deposited in animal excreta on soil (López-Aizpún et al., 2020). Similarly, half of N_2O emissions that arise from agricultural soils in New Zealand are due to the direct deposition of urine and dung from grazing animals (MfE, 2024).

N_2O production in grazed pastures is largely driven by biochemical processes involving denitrifying and nitrifying microorganisms (Clark et al., 2001) as shown in Figure 2.3. When N enters the soil (in the form of fertiliser or animal excreta), it undergoes transformations, releasing N_2O as an intermediate gaseous product (Cameron et al., 2013). Denitrifiers reduce nitrogen oxides (e.g., NO_3^-) to dinitrogen gas (N_2) in anaerobic soil microsites, provided sufficient NO_3^- and carbon are available. However, incomplete denitrification can result in N_2O emission (de Klein et al., 2003). Similarly, nitrifier denitrification; where nitrite (NO_2^-) is reduced to nitrogen monoxide (NO), then N_2O, and finally to N_2, can also produce obligatory N_2O (Wang et al., 2021). Conversely, nitrifying microbes oxidize ammonium (NH_4^+) to NO_3^- under aerobic conditions, releasing N_2O as a by-product (de Klein et al., 2003). The majority of N_2O emissions from agricultural activities involving animals arise from denitrification, with nitrification contributing to a lesser extent (de Klein & Eckard, 2008; Gillam et al., 2008).

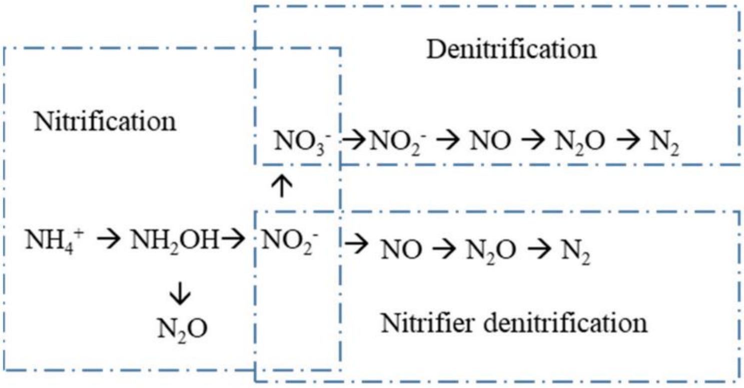

Figure 2.3. Major nitrogen (N) transformations leading to the emission of nitrous oxide (N_2O) in soils. Reproduced from Wang et al. (2021), licensed under CC BY 4.0.

Several reviews have examined the factors influencing N_2O emissions and mitigation strategies in grazed pastures (Dalal et al., 2003; de Klein & Eckard, 2008; De Rosa et al., 2020; Di & Cameron, 2016; Jayasundara et al., 2016; Oenema et al., 1997; van der Weerden et al., 2020). While numerous factors can impact N_2O emissions, these can primarily be categorized into environmental and management factors (Wang et al., 2021). Mitigation strategies to reduce N_2O emissions broadly fall into two categories: improving the efficiency of nitrogen cycling in animal production and improving soil management practices (Christie, 2019; Eckard et al., 2010).

2.6.1 Factors affecting nitrous oxide production in agricultural soils

Environmental factors significantly influence N_2O emissions by affecting nitrification and denitrification processes (de Klein et al., 2001). The abundance of soil microorganisms that drive these processes and the availability of N inputs are some of the key drivers of increased N_2O production (Blackmer & Bremner, 1978; Eckard et al., 2010). Low soil aeration, often linked with increased moisture (or increased water-filled pore space (WFPS)) (Dalal et al., 2003; Gillam et al., 2008; Wang et al., 2021), enhanced soil carbon availability, provides an essential energy source for microbes, also plays a critical role (Dalal et al., 2003). In addition, higher soil temperatures, soil pH and salinity which are conducive to nitrifying or denitrifying microbes further influence the extent of N_2O production (Dalal et al., 2003; de Klein et al., 2001; Wang et al., 2021).

2.6.2 Mitigation strategies to reduce nitrous oxide emissions in grazed pastures

Emissions of N_2O are generally greater from urine than dung because increasing ruminant N intake results in an exponential increase in UN content, with UN dominated by urea (Clough et al., 2020). Increased CP intake and subsequent excretion of N via urine back to the soil is one of the primary sources of N_2O emissions and managing urine patches is the primary factor that should be considered when mitigating N_2O emission (de Klein et al., 2020). Another strategy involves animal breeding which has the potential to improve the N conversion efficiency within the rumen or produce animals that urinate more frequently so a greater spread of urine would reduce N_2O emissions (de Klein & Eckard, 2008). Feeding CT is another effective approach to reduce N_2O emissions, as they form complexes with proteins, thereby slowing the rate and extent of rumen protein degradation. This process redirects a greater proportion of N to dung rather than urine, reduces rumen NH_3 concentrations, and subsequently decreases UN excretion (Christie, 2019; Kapp-Bitter et al., 2023; McMahon et al., 2000).

Similarly, PSM have also been known to lower ruminal NH_3 production (Navarrete et al., 2016); this is explained in more detail in Section 2.9.5.

Farm management practices, such as using standoff pads and restricting grazing on seasonally wet soils, help reduce N input from urine and minimise soil compaction, which can increase anaerobic conditions (Luo et al., 2008). Additionally, effective management of the rate, source, and timing of N fertilizer application improves pasture growth efficiency and minimizes N_2O losses (de Klein et al., 2001; Saggar et al., 2004). Another notable strategy focuses on Nitrification inhibitors. These are chemical compounds that inhibit the oxidation of NH_4^+ to NO_3^- in soils and thus reduce N_2O emissions (Di & Cameron, 2002). Nitrification inhibitors such as DCD (dicyandiamide), DMPP (3,4-dimethylpyrazole phosphate), and nitrapyrin (2-chloro-6-(trichloromethyl) pyridine) are known to reduce N_2O emissions (Adhikari et al., 2021; Di et al., 2016). However, using nitrification inhibitors may pose potential health risks; for instance, DCD has been shown to enter the food chain through animal ingestion, raising concerns about its effects on grazing animals and humans, a risk that may also apply to DMPP and nitrapyrin (Adhikari et al., 2021). Therefore, extensive research is needed to advise the application of any chemical inhibitors.

2.7 Dairy farming systems in New Zealand

New Zealand dairy farming systems are based on year-round grazing of a permanent pasture mix of perennial ryegrass (*Lolium perenne*) and white clover (Kemp, 2002) where cows are rotationally grazed in paddocks. The high utilisation of perennial pasture is a key factor in the low cost of production of Australian and New Zealand dairy systems and, hence, in their ability to maintain international competitiveness (Pembleton, 2015). The stocking rate is generally determined by the production of perennial pastures (Luo & Ledgard, 2021). The major roles of supplements are to provide farmers with the security to increase stocking rates and, to overcome seasonal shortages in pasture growth (feed gaps) and quality (Donaghy & Fulkerson, 2001).

The dairy system practice in most seasonal calving dairy farms in New Zealand involves cows calving from late July to mid-September in expectation of increased spring growth of pasture that will match the cows' requirement for dry matter or metabolisable energy (ME) as closely as possible (Waghorn & Clark, 2004). Pasture growth begins to accelerate in August and generally reaches maximum rates near the end of September or early October. Most cows are synchronised to be calved by this time to maximise the efficiency of the use of grown pasture by grazing, and those cows are dried off in April/May to help conserve feed for dairy cows in winter and save pasture for the following spring (Moller, 1997).

2.8 Feed supply and use for alternative forages

Ryegrass is the most widely used temperate grass in New Zealand as it grows well in a variety of soil types and is easy to establish and manage (Waghorn & Clark, 2004). It usually forms a compatible mixture with white clover and several other pasture species (Charlton & Stewart, 1999) and has efficient responses to irrigation (Rawnsley et al., 2009). Around 70% of annual growth occurs during spring which is associated with the transition from high-nutritive value vegetative growth to low-nutritive value reproductive development (Pembleton et al., 2016).

The annual production and seasonal supply of feed directly influence farm milk production and profitability. The herbage production and quality of this ryegrass-white clover mix is often limited over the summer months, leading to feeding deficits and lower animal production when soil moisture is low, and air and soil temperatures are high (Powell et al., 2007; Sanderson et al., 2003). Maintaining an adequate feed supply throughout the year is an important consideration in dairy systems therefore, utilising alternative forage species which are better adapted to these conditions may provide options for maintaining feed supply (Minnee et al., 2013). As a result, farmers are becoming interested in alternative forages that have deeper root systems and/or greater drought tolerance (Lee et al., 2015). Compared with ryegrass swards, diverse swards that contain both legumes and herbs (e.g., chicory and plantain) can supply equivalent or greater DM production and a greater metabolisable energy production per hectare per year and can increase animal production (Garrett et al., 2021).

2.9 Utilisation of plantain in the mixed sward of the conventional feed base

2.9.1 Inclusion of plantain in the pasture

Plantain is a herb species that provides a mineral-rich forage that is palatable to grazing animals, which is easy to establish, grows on a wide range of agricultural soils, and is tolerant of drought and many common diseases and pests (Stewart, 1996). Wild plantain, a native perennial plant commonly found across the British Isles, thrives on neutral to basic soil types (Foster, 1988). As European civilisations expanded, they introduced their pasture species, such as plantain, to various parts of the world (Eady et al., 2024). Earlier wild plantain varieties were typically dormant in winter and grew as prostrate flat weeds, making them unsuitable for grazing due to their lack of erect leaves and low DM production (Stewart, 1996). At present, through breeding, cultivated forms are available, ranging from late-heading winter-dormant types to upright winter-active forage plants (Stewart, 1996). Two key cultivars were developed in New Zealand, Ceres Tonic and Grasslands Lanceolate, which exhibit an erect (and/or semi-erect) growth habit, larger leaves, medium to high tiller density, and improved summer growth, making them better suited to modern grazing systems (Rumball et al., 1997; Stewart, 1996). Later, plantain was successfully commercialised over almost three decades and became a successful forage for livestock (Judson et al., 2019).

2.9.2 Dry matter production

Plantain-based pasture can yield annually from 18.5–22.3 t DM/ha/year in the first year to 10.6–20.6 t DM/ha/year in the third, showing a clear advantage to plantain-based pastures over ryegrass-white clover (Moorhead & Piggot, 2009). Some studies show that pure swards of Ceres Tonic have produced 14.9–19.1 t DM/ha/year (Minnee et al., 2013; Powell et al., 2007). Plantain, when incorporated into ryegrass-white clover, the mixed pasture produces a DM yield of 13.3–14.5 t DM/ha/year, comparable to parallel sown ryegrass-white clover pastures (Herath et al., 2023; Nguyen et al., 2022c). However, there was increasing evidence suggesting that the plantain population declined within the ryegrass-white clover pasture mix, and often required resowing after 3 years of establishment due to its limited persistence (Dodd et al., 2019; Nguyen et al., 2022c; Taylor et al., 2021).

Plantain as pure pasture or as a component within ryegrass-white clover pasture mix produced superior nutritive value compared to conventional ryegrass-white clover pasture (Cranston et al., 2015; Herath et al., 2023; Nguyen et al., 2022c). Pastures containing plantain typically had lesser herbage DM, lower structural fibre but higher non-structural fibre content, similar herbage N concentration and less soluble and degradable N compared with ryegrass pastures (Minnee et al., 2019). Additionally, they typically have similar to or increase the organic matter digestibility, ash, starch, and minerals such as phosphorus, sulphate, calcium, magnesium, sodium, chloride, zinc, boron, and cobalt (Nguyen et al., 2022c).

2.9.3 Dry matter intake and milk yield

Incorporating plantain either as pure pasture or as a proportion in ryegrass-white clover diet to cows maintained (Al-Marashdeh et al., 2021; Mangwe et al., 2020; Nguyen, 2023) or increased DMI (Box et al., 2022; Mangwe et al., 2020; Minnee et al., 2020; Nkomboni et al., 2021; Wilson et al., 2020) and no significant negative impact on DMI was reported.

Feeding plantain to dairy cows does not significantly alter milk composition compared to traditional ryegrass-white clover pastures; however, there is a small reduction in milk fat content, but this response is inconsistent (Pinxterhuis et al., 2024). A meta-analysis by Nguyen et al. (2022b), found that feeding plantain can maintain or enhance milk and milk protein production, particularly in late lactation. While no significant impact on milk protein content was observed overall, a small increase was noted in some cases (Nguyen et al., 2022b).

2.9.4 Nutrition uptake

Plantain has a deep and strong root system and a symbiosis with mycorrhiza, which gives it a very good appropriation of water and nutrients, especially from the subsoil (Pol et al., 2021). In water-deficit conditions, the roots of the plantain can reach up to almost 190 cm deep in the soil profile after one year of cultivation and have the ability to take up immobile and mobile nutrients from the soil (Pol et al., 2024). Even though plantain is accepted as a summer-growing forage species, its greater tolerance to variations in growing temperature meant that plantain content in the mixed pasture was more seasonally stable than other alternative forages, either chicory or lucerne (*Medicago sativa*) (Woodward et al., 2013).

2.9.5 Plant secondary metabolites in plantain pastures

Plant secondary metabolites, or bioactive compounds, are substances produced by plants that do not directly contribute to their primary functions of plant growth, reproduction, or development (Bourgaud et al., 2001). Instead, PSM contribute to a plant’s adaptation and survival, serving as a defence against pathogens, herbivores, and environmental stressors (Bennett & Wallsgrove, 1994). Additionally, these compounds can have antimicrobial, allelopathic, UV-protective, and anti-feeding properties (Bourgaud et al., 2001).

In plantain, various functional groups of PSM are present including iridoid glycosides, phenylethanoid glycosides, flavonoids, polyphenols, saponins and tannins (Miehe-Steier et al., 2015; Stewart, 1996; Wurst et al., 2010). Some of these groups exhibited several benefits (Table 2.1). Among these, iridoid glycosides and phenylethanoid glycosides are relatively higher in concentration in narrow-leaf plantain, which is widely used in farming practices.

Table 2.1. Plant secondary metabolites, their functional group and relative abundance in plantain cultivars.

Functional Group	Functional Role	Compound (s)	Relative abundance in cultivars used for farming	References
Iridoid Glycosides	Defence against herbivores, antimicrobial properties, protein denaturation in pathogens, anti-cancer, anti-ageing, anti-inflammatory, antioxidant, hepatoprotective and osteoprotective etc.	Aucubin, Catalpol	Moderate to high	Fuchs and Bowers (2004); Marak et al. (2000); Pankoke et al. (2013); Rahamouz-Haghighi (2023).
Phenylethanoid Glycosides	Antioxidant, anti-inflammatory, antimicrobial, immunomodulating, hepatoprotective effects	Acteoside, Plantamajoside and Isoacteoside,	Moderate to high	Budzianowska et al. (2023); Gonda et al. (2014); Gonda et al. (2013).
Tannins	Antifeedant, binds proteins and reduce protein digestibility	Condensed and hydrolysable tannins	Low	Dietz et al. (2013); Pankoke et al. (2013); Stewart (1996).

The key PSM in plantain include aucubin, catalpol, and acteoside (syn. verbascoside), with plantamajoside detected in low and often trace concentrations (Fajer et al., 1992; Gardiner et al., 2016; Stewart, 1996; Tamura & Nishibe, 2002). Table 2.2 presents the ranges of PSM measured across various studies, which include hydroponic experiments, grazing trials, and both pure plantain pastures and mixed pastures where plantain as one of the species.

Table 2.2. Range of plant secondary compound concentrations reported across studies.

Cultivar/ breeding line	Stand	Study type	Year/ season		Acteoside (mg/g DM)	Aucubin (mg/g DM)	Catalpol (mg/g DM)	Study/ author
Ceres Tonic	Pure	Grazing trial	Year 1		23.6 – 35.4	1.78 – 3.8	<0.1	Navarrete et al. (2016)
			Year 2		0.5 – 41.7	0.44 – 6.87	<0.1	
Grasslands Lancelot	Pure	Field study			34 – 71	21 – 48	10 – 20	Tamura and Nishibe (2002)
Ceres Tonic					15 – 41	10 – 27	10 – 20	
Endurance	Pure	Field study			16.1 – 66.7	5.0 – 18[†]	<6	Box and Judson (2018)
Hercules					14.5 – 55.2	2.5 – 25[†]	<6	
Elite 2					6.0 –50	5.0 – 18[†]	<1	
PG742					6.0 –50	2.0 – 22[†]	<1	
Tonic					6.0 – 50	2.0 – 15[†]	<1	
Ceres Tonic	Pure	Field study	Year 1	Spring	9.1 – 36	3.2 – 5.85	<1	Box et al. (2019)
				Summer	1.7 – 5.86	0.9 – 1.72	<1	
				Autumn	8.1 – 10.2	1.7 – 2.5	<1	
			Year 2	Spring	10.6 – 22.9	1.6 – 2.7	<1	
				Summer	2.2 – 6.4	0.5 – 0.9	<1	
				Autumn	4.4 – 7.5	2.3 – 3.7	<1	
nr	Pure				60 – 94	10.0 – 45	9.0 – 11	Fajer et al. (1992)
Ecotypes[#]	Pure				4.4 – 14.3	9.8 – 41.8	1.2	Al-Mamun et al. (2008)
Grasslands Lancelot					10.6	6.5	<0.1	
Ceres Tonic					3.2	17.8	n.r	
Agritonic	Pure stand - leaves	Hydroponic experiment			n.r	4.0 – 13.1	<1	Rodriguez et al. (2021)
Ceres Tonic					n.r	3.9 – 11	<1	
Tonic	Pure stand and PL-clover mixed pasture	Grazing trial	Year 1	Spring	n.r	6.2 – 6.5	n.r	Navarrete et al. (2022)
				Summer	n.r	4.1 – 7.0	n.r	
				Autumn	n.r	2.1 – 2.8	n.r	
			Year 2	Spring	n.r	2.7 – 4.2	n.r	
				Summer	n.r	0.4 – 6.8	n.r	
				Autumn	n.r	3.3 – 6.1	n.r	
Plantain trial at Massey University								
Agritonic	PL-ryegrass/ white clover mixed pasture	Grazing trial	Year 1&2		4.8 – 8.9	3.8 – 6.6	<1	Nguyen (2023)
			Years 3–4	Spring	1.4 – 13.2	0.1 – 6.0	<1	Present study chapter 3 results[*]
				Summer	1.1 – 8.3	0.8 – 4.2	<1	
				Autumn	0.3 – 10.1	1.3 – 4.2	<1	

[#] an average of 25 ecotypes is presented in this table and more details can be found in the original study, [*] data presented using samples collected by the author during pasture production years 3 and 4, along with archived data from years 1 and 2. n.r: not reported, [†] values were extracted from figures in the original study.

Significant variation in PSM concentrations has been recorded across studies, reflecting differences in genotype and environmental factors and their interactions. Reported levels of acteoside range, up to high as nearly 100 mg/g of DM, and aucubin concentrations up to around 50 mg/g DM, depending on the cultivar and growing conditions (Table 2.2). For instance, Grasslands Lancelot and Ceres Tonic exhibited relatively high concentrations of acteoside and aucubin, with peak values generally observed in spring (Table 2.2). Catalpol availability was generally low and sometimes undetectable in many cultivars (Al-Mamun et al., 2008; Box & Judson, 2018). Aucubin is a precursor of catalpol so low catalpol is presumably genetically determined (Al-Mamun et al., 2008).

Environmental factors, including seasonal changes, temperature, solar radiation, sampling practices (e.g. time of day or season) and plant root interaction with belowground microorganisms, can impact the production of PSM (Navarrete et al., 2016; Pankoke et al., 2013; Tamura & Nishibe, 2002; Wurst et al., 2010). This variation suggests that growing conditions, beyond genetic factors alone, play a substantial role in determining PSM concentrations across studies.

2.10 Importance of plantain pastures in pastoral systems

2.10.1 Impact on nitrate leaching

Plantain is recognised for its role in influencing the N cycle, offering farmers practical solutions to achieve environmental targets. Research spanning lysimeter experiments (Carlton et al., 2019; Welten et al., 2019; Woods et al., 2018) to paddock-scale measurements (Navarrete et al., 2023; Navarrete et al., 2018) has demonstrated its effectiveness. Lysimeter studies revealed 14%–89% NO_3^- leaching reductions when pure plantain and/or in mixtures with ryegrass-white clover were compared to control pastures. Similarly, paddock-scale measurements confirmed these findings, showing 20%–60% lower NO_3^- leaching even when plantain comprised 30%–40% of the DM of plantain in ryegrass-white clover-based pastures (Pinxterhuis et al., 2024).

As shown in Figure 2.4 and discussed below, several mechanisms have been proposed to explain plantain's effects on the N cycle and its role in reducing N losses.

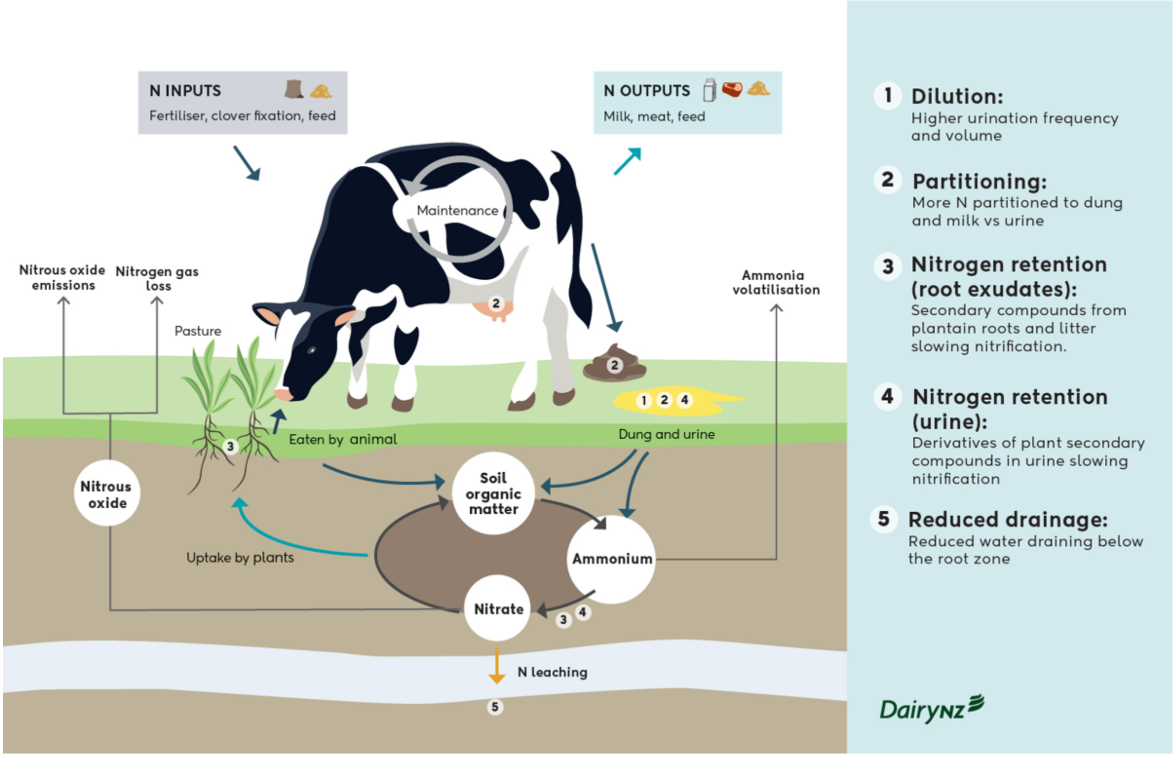

Figure 2.4. Proposed mechanisms of plantain impacting the nitrogen cycle. Reproduced from (DairyNZ, 2025).

2.10.1.1 Effect of plantain on urinary nitrogen concentration and excretion

There is unanimity across studies regarding the effect of feeding plantain on the N concentration in cow urine. A consistent trend of decreasing UN concentration with increasing plantain content in the diet has been observed in multiple studies (Box et al., 2022; Cheng et al., 2017b; Minnee et al., 2020; Navarrete et al., 2022; Nguyen et al., 2024).

Pinxterhuis et al. (2024) summarised a reduction in UN concentration ranging from 13% to 75% as plantain inclusion increased in cow diets.

Another common response of feeding plantain is an increase in urine volume of the cows fed plantain (Box et al., 2022; Box et al., 2017; Marshall et al., 2021; Minnee et al., 2020; Navarrete et al., 2022). This is largely attributed to the increased water content of plantain thus increased water intake and the impact of PSM found in plantain, which have been proposed to influence this to a certain extent (Judson et al., 2018; Minnee et al., 2020; Nguyen et al., 2022a). The increase in urine volume, often referred to as a "dilution effect", results in more frequent urinations per day, which contributes to lower UN concentrations (Pinxterhuis et al., 2024).

Several studies have reported that increasing the proportion of plantain in the diet leads to reduced total UN excretion (Cheng et al., 2017a; Marshall et al., 2021; Minnee et al., 2020). However, some studies have reported lower UN concentrations without significant changes in total UN excretion at lower plantain inclusion levels (Box et al., 2022; Nguyen et al., 2023).

Minnee et al. (2020) tested various plantain inclusion levels (15%, 30%, and 45%) and observed a significant reduction in UN excretion only at the highest proportion (45%). Similarly, Box et al. (2017) found that UN excretion decreased when 50% of the pasture consisted of plantain. These findings are supported by a meta-analysis by Nguyen et al. (2022a), which showed a strong negative relationship ($R^2=0.69$) between increasing plantain content in the diet and UN concentration. However, the relationship between plantain content and total N excretion was weaker but still significant ($R^2=0.22$), suggesting that higher plantain content may be needed to have a greater effect on UN excretion.

2.10.1.2 Animal nitrogen partitioning

There is growing evidence that when plantain is included in diets with similar N intake, a higher proportion of N is directed into dung and milk rather than urine (Cheng et al., 2017a; Marshall et al., 2022; Minnée et al., 2020; Nguyen et al., 2024; Wilson et al., 2020). This effect is influenced by three main mechanisms: i) increased soluble carbohydrate (NSC) content, ii) enhanced N digestibility, and iii) the presence of PSM that reduce rumen N metabolism (Pinxterhuis et al., 2024).

Lower urinary N excretion is a typical response to increasing NSC content in herbage (Edwards et al., 2007; Moorby, 2014). The utilisation of dietary N by microbes to produce microbial protein is limited by available carbohydrate (Russell et al., 1992). Enhanced energy availability with increased NSC in the rumen promotes microbial growth, increases microbial protein synthesis, and improves N use efficiency (Bach et al., 2005; Firkins et al., 2007). Since plantain herbage typically has a similar or lower N content than ryegrass (Minnee et al., 2019; Nguyen et al., 2022c), its higher NSC to N ratio likely shifts dietary N from urine to other sinks (Box et al. 2017; Hutton et al. 2011; Moorhead et al. 2002).

In general, plantain and ryegrass contain a similar amount of total N, but plantain has less soluble protein (SP) (on average 12 vs. 38% of total N) and more RUP (on average 64% vs. 31% of total N) (Minnée et al., 2019) than ryegrass. Minnée et al. (2020) reported a strong positive relationship between plantain content in the diet and partitioning of N to faeces ($R^2 = 0.8$, $p < 0.001$) from lactating dairy cows, with no negative effect on animal production. In agreement, a study by Marshall et al. (2021) showed that lactating dairy cows fed diets of 100% plantain partitioned 30% more dietary N to faeces than cows fed ryegrass diets.

According to Navarrete et al. (2016), PSM found in plantain reduced the net NH_3 production especially, acteoside acted as an additional energy source to the rumen microbiome and reduced net NH_3 production possibly due to increased microbial protein further, aucubin also reduced net NH_3 production possibly due to its antimicrobial activity. This evidence suggests lower ruminal NH_3, which could subsequently be utilised more for the production of milk, meat, and dung rather than excreted in urine.

2.10.2 Impact of plantain on nitrous oxide emissions

Another significant environmental benefit of incorporating plantain into swards is the reduction in N_2O emissions compared to ryegrass-white clover pastures. Increasing evidence shows that higher plantain content in both the sward and the diet leads to reduced N_2O emissions (Simon et al., 2019; Vi et al., 2023). This reduction is primarily attributed to two main effects. First, the lower UN load from cows fed plantain results from increased urine volume, reduced UN concentrations, and more dispersed urine patches (as detailed in Section 2.10.1). Since UN loading rates in urine patches have a positive correlation with N_2O emissions, lower UN loads result in reduced cumulative N_2O emissions (Marsden et al., 2016; Singh et al., 2021). Second, the N_2O emission factor (EF_3), which represents the percentage of N applied to soil that is emitted as N_2O (de Klein et al., 2003), is influenced by factors such as agricultural management, soil properties, and climate conditions (Shang et al., 2020). Arguably, EF_3 values are generally expected to be similar under comparable conditions for the same UN load. However, when similar UN loads were applied, lower EF_3 values were observed with increasing proportions of plantain in the pasture, suggesting a potential sward effect (Simon et al., 2019; Vi et al., 2023).

2.10.2.1 Biological nitrification inhibition activity of plantain and its impact on nitrous oxide emissions

Biological nitrification inhibition (BNI) is the release of root compounds by plants that suppress nitrifying microbes (ammonia-oxidizing bacteria) and limit the initial step of nitrification, which is the conversion of NH_4^+ to NO_3^- (Simon et al., 2021; Subbarao et al., 2007). Plantain has demonstrated BNI activity in several studies, potentially through the release of PSM via root exudates and decomposition of plant litter (roots and leaves) (de Klein et al., 2020; Dietz et al., 2013; Luo et al., 2018; Pijlman et al., 2020; Simon et al., 2019). When plantain leaf extracts or commercially sourced aucubin were applied to urine patches, they delayed soil nitrification and reduced N_2O emissions (Gardiner et al., 2020a, 2020b; Rodriguez et al., 2021). Similarly, urine collected from plantain-fed cows showed moderate BNI activity, likely due to the release of PSM or their derivatives, resulting in short-term nitrification inhibition (Judson et al., 2018; Peterson et al., 2022). The reduction in N_2O emissions observed in plantain has been linked to its biological BNI activity (Ding et al., 2024; Simon et al., 2019). A pot experiment comparing rhizosphere and bulk soils of plantain and ryegrass revealed a lower abundance of the ammonia-oxidizing bacteria gene (amoA) in the rhizosphere soil of plantain after urine application, compared to ryegrass. However, no differences were observed in bulk soil, suggesting that plantain may exert a BNI effect specifically in its rhizosphere (Simon et al., 2021). However, according to the current knowledge, the BNI activity in plantain is genotype-specific (Judson et al., 2018), and it varies across different soil types (Ding et al., 2024). These findings highlight the need for further research to fully understand the underlying mechanisms and optimise the use of plantain for BNI in various agricultural systems.

2.10.3 Impact of plantain on methane emissions

When plantain substrates were tested *in vitro* alongside alternative forages, they showed a reduction in CH_4 production and a lower A:P and lower NH_3 production (Durmic et al., 2016). Similarly, an *in vitro* study using the commercially available PSM acteoside showed a lower A:P and reduced NH_3 production; however, CH_4 emissions were not measured in that study (Navarrete et al., 2016). In a recent study by (Della Rosa et al., 2022), CH_4 emissions were measured using respiration chambers with non-lactating dairy cows. Methane emissions per unit of dry matter intake were 15% to 28% lower in cows fed pure plantain compared to those fed ryegrass. The authors attributed this reduction to the lower digestibility of plantain but did not account for the potential effects of PSM.

The implications of excess NH_3 production, discussed in Section 2.3, suggest no direct relationship with CH_4 emissions; however, it correlates with increased UN excretion and, subsequently, N_2O emissions.

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CHAPTER 3

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

This chapter has been under review.

Sivanandarajah, K., Donaghy, D., Molano, G., Horne, D., Kemp, P., Navarrete, S., Ramilan, T., & Pacheco, D. (2025). Plantain-mixed pasture collected in different climatic seasons produced less methane and ammonia than ryegrass-white clover pasture *in vitro. Animal Production Science*

An abstract from this chapter has been presented at the Australasian Dairy Science Symposium 2024.

Abstract

Plantain (PL, *Plantago lanceolata*) is recognised for reducing nitrate leaching and nitrous oxide emissions in pastoral systems. Some evidence has shown that cows fed pure PL produced less methane (CH_4) than cows fed ryegrass (*Lolium perenne*). However, it is unclear if lower CH_4 emission can be achieved with PL in mixed pasture. This study evaluated the *in vitro* rumen fermentation profiles of ryegrass-white clover (*Trifolium repens*) and medium-level PL (~40%) pasture collected during different climatic seasons, to determine whether this PL level influences CH_4 and rumen ammonia (NH_3) production. 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 (i.e., containing 30–50% PL [PLM] and 0–5% PL [RWC]). Representative samples for these scenarios were incubated in an automated *in vitro* rumen batch culture system for 48 hours (h). In summer, PLM produced around 8%, 14%, and 19% less CH_4 at 12 h, 24 h, and the potential CH_4 production (PCH_4), respectively. Although gas production (GP) was similar at 12 and 24 h, PLM had 13% lower potential GP (PGP) than RWC. In spring, PLM had around 11% greater GP and CH_4 production at 12 h. During autumn, 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%), with no differences in summer despite higher crude protein levels in PLM. Compared to RWC, substrates with ~40% PL changed rumen fermentation parameters that could translate to potential environmental benefits: PLM produced less net NH_3 in spring and autumn (27% and 17%, respectively) and up to 19% less CH_4 production in summer. Incorporating ~40% PL into a dairy pasture could lower CH_4 emissions and reduce nitrogen losses with minimal input costs if the *in vitro* results translate to cows grazing pasture. *In vitro* results suggest that

PLM’s potential to mitigate CH_4 emissions can be influenced by seasonal variations in pasture quality compared to RWC. However, further animal studies are needed to fully comprehend the CH_4 mitigation potential of this forage.

3.1 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 (Knapp et al., 2014). In 2022, the agriculture sector 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 often results in providing N in excess of the animals' nutritional requirements (Pacheco & 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). Additionally, ruminants grazing on low-quality feeds produce around 75% of CH_4 emissions globally (Knapp et al., 2014) and offering high-quality pasture can mitigate CH_4 emission per unit of DM intake (Arndt et al., 2022).

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, 2023a). 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 DairyNZ (2024), New Zealand cows produce 21 to 22 g of CH_4 per kg of dry matter (DM). Methane production from ruminants is influenced by various factors such as pasture quality, digestibility, DM intake (DMI), plant secondary metabolites (PSM), and grazing management (Danielsson et al., 2017; Hristov et al., 2013). Among these, pasture quality, digestibility and availability of PSM can be influenced by the season (Navarrete et al., 2016; Roche et al., 2009). When comparing PL and RWC pasture across seasons, PL mixed pasture is usually superior in nutritional value compared to RWC pasture (Herath et al., 2023; Nguyen et al., 2022) and PL pasture contains PSM such as aucubin and acteoside (Tamura & Nishibe, 2002). Both compounds influence the rumen fermentation profile by lowering net NH_3 production, increasing potential gas production (PGP) (as seen with acteoside) and reducing the rate of gas production (GP) with increasing dose (as seen with aucubin) (Navarrete et al., 2016).

When tested *in vitro* (Durmic et al., 2016) and *in vivo*, (Della Rosa et al., 2022) PL 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 to 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. Additionally, in a more recent study, Koning et al. (2024) observed higher CH_4 yield for low PL content pasture (<25% PL with ryegrass) compared to 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 and 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 (DairyNZ, 2023b), which includes measurements in four production seasons, suggest that around 20–50% PL is achievable using different sowing rates in farm conditions.

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, approximately 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 approximately 40% in the pasture, to reduce CH_4 emissions as it would be a more practicable PL percentage to have in a mixed pasture.

3.2 Methods

3.2.1 Experimental site description

Substrates assessed in the present study were selected from historical pasture samples collected from the Plantain Potency and Practice programme’s (DairyNZ, 2023b) Massey University trial site at Dairy 4, Palmerston North, New Zealand (40º23′27″ S 175º36′44″ E) containing four pasture treatments: 0% (PL0), 30% (PL30), 50% (PL50), and 70% (PL70) PL in a RWC pasture mix. Pasture establishment and general management were reported previously by Nguyen et al. (2022). Over four production seasons, 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 PSM.

Botanical composition was measured by manually separating pasture components, oven drying at 75ºC until they reached constant weight, and were then reported as a percentage of DM. For pasture quality and PSM analysis, samples were oven-dried at 60ºC until achieving a constant weight. 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 (Hill-Labs, 2022), while another subsample was taken to test the PSM aucubin, acteoside and catalpol using high-performance liquid chromatography as described in Navarrete et al. (2016).

Chemical composition analysis for pasture samples was carried out by using near-infrared spectroscopy. 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), neutral detergent fibre (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 two hours [h]), soluble sugars (calibration based on an 80:20 ethanol:water extraction and colorimetric determination), crude protein (CP, N multiplied by 6.25), digestibility of organic matter in DM (DOMD, solubilised organic matter as a portion of the DM) and non-structural carbohydrates (NSC, = 100 - (CP + Ash + CFat + NDF).

3.2.2 Substrate selection for *in vitro*

A data matrix was created using variables from the pasture quality and PSM 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 at 18% and the highest at 47% (Table 3.1). The targeted annual population for PL70 was not achieved in any year. 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.

Table 3.1. Botanical composition, expressed as a ratio of plantain:ryegrass:white clover in dry matter percentage (DM%), of perennial ryegrass-white clover (PL0), 30% (PL30), 50% (PL50), and 70% (PL70) plantain treatments over four production seasons.

Production season/year	Treatments			
	Plantain:ryegrass:white clover in %DM[a]			
	PL0[b]	PL30[c]	PL50[d]	PL70[e]
Production season 1/ 2019/20	4:66:10	26:47:6	35:36:9	43:31:7
Production season 2/ 2020/21	3:65:19	32:37:21	47:25:21	42:26:19
Production season 3/ 2021/22	2:79:13	18:64:13	24:57:13	44:14:34
Production season 4/ 2022/23	3:72:17	24:51:18	27:53:12	32:41:22
Plantain % range[f]	2–4	18–32	24–47	32–44

[a]Ratio of the yearly averages of botanical composition expressed in %DM, [b]ryegrass-white clover control treatment, [c]treatment aimed to have 30% plantain pasture, [d]treatment aimed to have 50% plantain pasture, [e]treatment aimed to have 70% plantain pasture, [f]range of yearly averages of plantain percentage in each treatment.

3.2.3 *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 3.2) was incubated in two sets. One set 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 short-chain fatty acids (SCFA). 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.

Table 3.2. 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

3.2.4 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 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 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.

3.2.5 Incubation preparation

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 shaker inside the incubator. The bottles were connected to the automated gas measurement system via a 23-gauge needle, shaken horizontally at 120 rpm, and gas composition from each bottle was recorded automatically for 48 h.

A sample of the medium was collected for analysis of NH_3 and SCFA (0-hour 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 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 SCFA within the following three weeks.

3.2.6 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 SCFA analysis. Short-chain fatty acids were analysed using gas chromatography described by Attwood et al. (1998). Approximately 100 µL of the remaining supernatant for NH_3 concentration analysis using the colorimetric method described by Weatherburn (1967) scaled down to run in 96-well microplate format.

3.2.7 Model fitting and data analysis

Gas and CH_4 production from each bottle were fitted to a logistic exponential model (France et al., 2000) to estimate the *in vitro* GP using the following formulae:

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

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

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

Where:

V: cumulative volume of gas or CH_4 produced up to time t (mL/g DM)

a: asymptotic GP (potential gas production [PGP], 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)

3.2.8 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 (mmol/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 and CH_4 production parameters and endpoint SCFA, were analysed using linear mixed models in the R package ‘lme4’ (Bates et al., 2014). Residuals were checked for normality using the Shapiro-Wilk test, and log transformations were performed where necessary to meet the assumptions of ANOVA. The R package ‘emmeans’ (Lenth R,

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., 2015) 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 SCFA concentrations, were analysed using a one-way ANOVA with treatment as a fixed effect and biological replicates 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.

3.3 Results

3.3.1 Chemical composition of pasture types

Season is used, for brevity, to describe the results of samples collected on 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 3.3). 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 greater in autumn (477 g/kg DM) compared to spring (430 g/kg DM) and summer (446 g/kg DM), while PLM pasture contained similar NDF content across seasons (386 to 398 g/kg DM) and was around 13% lower in each season compared to 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 ($p<0.01$), while in summer and autumn, both had similar soluble sugar content. On average, PLM pasture contained about 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 to 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 high in spring compared to other seasons in both pasture types ($p<0.001$).

The NSC to N ratio was significantly higher in PLM compared to 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 greater 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 to RWC ($p<0.001$), with acteoside levels being greater than aucubin. An interaction between seasons and pasture was observed for the PSM (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).

Minor differences in the analysed mean values and absolute values of selected pasture samples for *in vitro* were observed in a few variables, such as increased CP in autumn RWC and increased soluble sugar in summer RWC (Table 3.3); however, these values remained within the typical range for the given season.

Table 3.3. 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.

			Chemical composition (g/kg DM)											
	Season	Substrates^{A}	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	430 b	244 abc	220 bc	80 b	210 b	7.1 ab	701 a	786 a	54 e	12.7 e	0.1 c	1.4 c
		PLM	386 c	234 c	260 a	95 a	212 b	8.2 a	691 a	778 a	72 cd	18.9 bc	6.0 a	13.2 a
	Summer	RWC	446 b	255 a	194 c	65c	220 ab	6.1 b	660 b	741 b	70 d	15.7 d	0.8 c	1.1 c
		PLM	398 c	244 bc	236 ab	65 c	226 ab	6.9 b	662 b	746 b	83b	21.2 b	4.2 b	8.3 b
	Autumn	RWC	477 a	251 ab	141 d	59 c	229 ab	4.5 c	657 b	742 b	79 bc	16.5 cd	1.3 c	0.3 c
		PLM	393 c	236 c	216 bc	69 bc	237 a	6.2 b	664 b	757 ab	96 a	25.1 a	4.2 ab	10.1 ab
		SEM	19.1	10.9	11.8	7.2	22.3	10.9	18	19.6	4.3	1.2	0.7	1.1
	p values	Pasture Type (T)	<0.001	<0.001	<0.001	<0.01	ns	<0.001	ns	ns	<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	ns	ns
		T x S	<0.01	ns	<0.05	<0.05	ns	ns	ns	ns	ns	ns	<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	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. ^{A} RWC: ryegrass-white clover pasture and PLM: medium-level plantain pasture (30-50%), nd: not detected, ns: not significant, T x S: interaction between season and pasture type. 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,* SEM: standard error of means of the pasture types.

3.3.2 Gas and methane production parameters

Assessment of the CH_4 effects requires measurement of both CH_4 and gas production. To distinguish between results that indicate low fermentability of a substrate (i.e., low gas and CH_4 production) and an inhibition of methanogenesis (i.e., lower CH_4 proportion in the gas produced). Table 3.4 and Figure 3.1 show total gas and CH_4 production parameters measured *in vitro* over 48 h from selected pastures listed in Table 3.3. 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 to 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 about 8% and 9% at 12 and 24 h; however, the %CH_4 in PGP remained similar (Table 3.4).

In spring, PLM had around 11% greater GP and 12% greater CH_4 production at 12 h. As time progressed, both PLM and RWC produced similar GP and CH_4 levels. Despite a general trend for a reduction in the %CH_4, no significant difference was found at any given time point for spring pastures (Table 3.4).

During autumn, GP and CH_4 production were similar between PLM and RWC at both 12 and 24 h. Medium-level PL pasture (PLM) showed a numerically lower CH_4 production at 12 and 24 h (2% and 6% reductions, respectively), along with a 3% increase in GP, though these differences were not statistically significant ($p>0.05$). 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 around 24% faster than RWC. In autumn, both pastures reached their both $T^{1/2}$ gas and $T^{1/2}$ CH_4 in similar times. However, while 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 to RWC (Table 3.4).

3.3.3 Short-chain fatty acids analysis

End-point SCFA concentrations were similar between substrates collected in each season. A high acetate-to-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 to RWC in both summer and autumn. Additionally, a high molar proportion of butyrate was observed in the PLM pasture compared to RWC during spring and autumn (Table 3.5).

Table 3.4. Estimated marginal means and standard error of the means (SEM) of gas and methane (CH_4) production parameters.

Seasons	**Substrates**	**%of CH_4/GP 12h**	**% of CH_4/GP 24h**	**% of potential CH_4/GP**	**GP at 12h (mL/g DM)**	**GP at 24h (mL/g DM)**	**PGP (mL/g DM)**	**CH_4 at 12h (mL/g DM)**	**CH_4 at 24h (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[a]	11.72 ab	13.82 a	14.38 a	179.36 b	253.03 a	285.22 ab	21.04 bc	35 ab	41.17 a	8.73 a	11.67 a	12.64 b	1.72 a
	PLM[b]	11.83 ab	13.47 a	13.65 ab	198.94 a	255.03 a	282.29 ab	23.55 a	34.31 ab	38.53 ab	6.62 bc	9.34 b	14.25 a	1.75 a
Summer	RWC[a]	12.12 ab	13.95 a	13.64 ab	191.25 a	252.66 a	298.7 a	23.18 a	35.25 a	40.89 a	7.89 a	10.25 ab	12.21 bc	1.70 a
	PLM[b]	11.14 c	12.66 b	12.68 b	192.12 a	240.43 ab	261.32 bc	21.41 b	30.45 c	33.1 b	6.03 c	8.79 b	14.4 a	1.65 a
Autumn	RWC[a]	12.19 a	13.96 a	13.93 ab	168.13 c	231.08 b	254.97 c	20.52 bc	32.26 bc	35.52 ab	8.13 a	10.37 ab	12.23 bc	1.74 a
	PLM[b]	11.64 bc	13.38 a	13.28 ab	172.95 bc	227.99 b	268.42 bc	20.14 c	30.46 c	35.55 ab	7.7 ab	10.34 ab	11.19 c	1.44 b
	SEM	0.43	0.74	0.85	5.31	4.24	6.49	1.2	1.86	2.82	0.49	0.57	0.55	0.08

[a]ryegrass-white clover pasture, [b]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 $T^{1/2}$, values marked with the same letters within columns are not statistically different at the 5% significance level, SEM: standard error of means.

Table 3.5. Total short-chain fatty acid (SCFA) production and molar proportions of individual SCFAs of medium-level plantain pasture (PLM) and ryegrass-white clover pasture (RWC) across different seasons.

Seasons	Substrates	SCFA (mmol)	Acetate %	Propionate %	Butyrate %	Caproate %	Valerate %	Isobutyrate %	Isovalerate %	Minor %	[A:P]	[AcBu:PrVa]
Spring	RWC[a]	83.61 a	65.89 bc	17.89 ab	9.89 cd	0.09 c	1.72 b	1.73 b	2.79 b	6.33 b	3.68 bcd	3.86 c
	PLM[b]	83.74 a	65.95 bc	17.56 b	10.29 b	0.16 a	1.59 c	1.67 c	2.77 bc	6.2 bc	3.75 bc	3.98 b
Summer	RWC[a]	83.91 a	66.02 b	17.47 b	10.86 a	0.08 c	1.55 c	1.49 e	2.53 e	5.65 d	3.78 b	4.04 b
	PLM[b]	80.68 ab	67.08 a	16.75 c	10.1 bc	0.11 b	1.69 b	1.6 d	2.67 d	6.08 c	4.01 a	4.19 a
Autumn	RWC[a]	81.87 ab	65.72 bc	18.02 a	9.55 d	0.09 c	1.91 a	1.83 a	2.88 a	6.71 a	3.65 cd	3.78 c
	PLM[b]	76.65 b	65.51 c	18.08 a	10.27 b	0.12 b	1.66 b	1.66 c	2.7 cd	6.13 c	3.62 d	3.84 c
	SEM	2.71	0.35	0.1	0.1	0.01	0.07	0.1	0.19	0.36	0.03	0.04

[a]ryegrass-white clover pasture. [b]medium-level plantain pasture (30-50%), % denotes the molar proportions of respective SCFA to total SCFA production, [A:P]: acetate to propionate ratio, [AcBu:PrVa]: acetate + butyrate to propionate + valerate ratio, values marked with the same letters within columns are not statistically different at the 5% significance level, SEM: standard error of means.

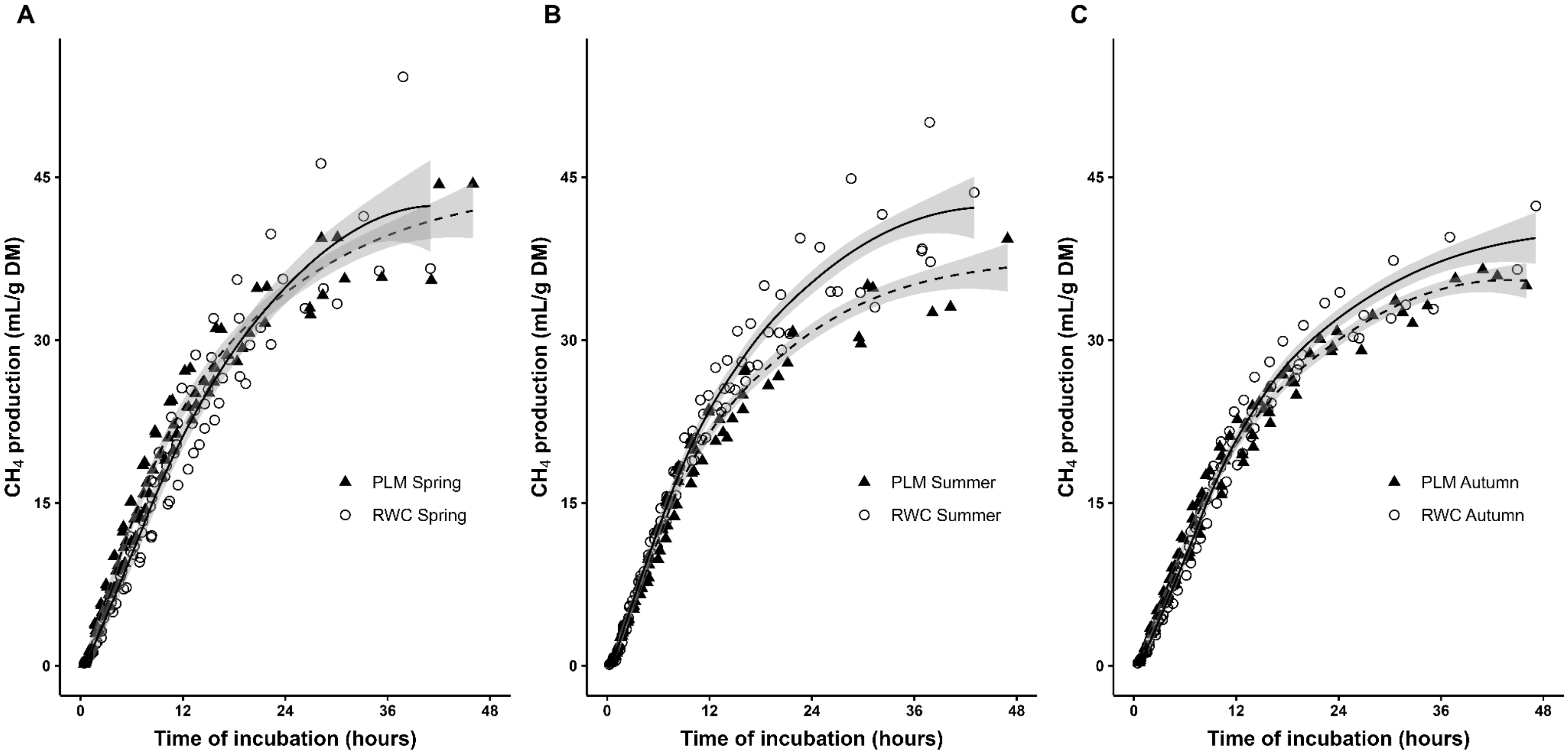

Figure 3.1. Real-time observations of methane production (CH_4, mL/g DM) for medium-level plantain pasture (PLM, ▲, solid line) and ryegrass-white clover pasture (RWC, ○, dashed line) collected in (A) spring, (B) summer and (C) autumn, over 48 hours of *in vitro* batch culture incubation.

3.3.4 Net ammonia production

The net NH_3 production over time was less for PLM compared to RWC from the spring and autumn samples. In spring, the net NH_3 production was 27% lower for PLM pasture (Table 3.6). Specifically, PLM pasture collected during spring consistently showed lower net NH_3 production at 6, 12, and 24 h, while during autumn, it was lower at 9 and 12 h (Figure 3.2).

Table 3.6. Total and net ammonia (NH_3) production over time throughout the 48 h of *in vitro* incubation.

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

[a]ryegrass-white clover pasture, [b]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, values marked with the same letters within columns are not statistically different at the 5% significance level, n.s: not significant, –: not applicable, SEM: standard error of means

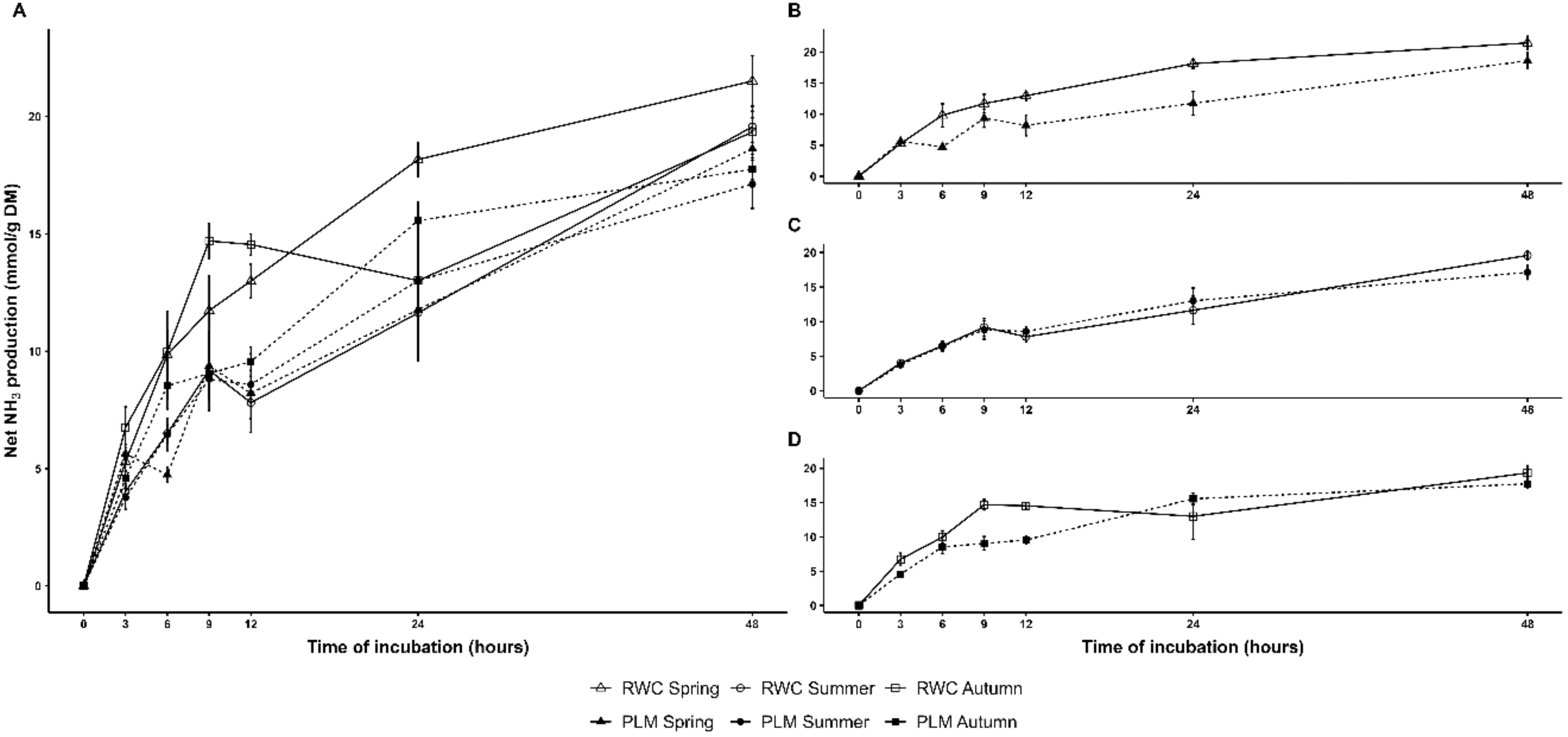

Figure 3.2. *In vitro* net ammonia (NH_3) production (mmol/g DM) over time of medium-level plantain pasture (PLM, dashed line) and ryegrass-white clover (RWC, solid line) collected (A) across seasons with comparison of (B) spring, (C) summer and (D) autumn, at each timepoint of *in vitro* batch culture incubation. Bars denoting standard error of the mean (SEM) at each time point.

3.4 Discussion

A key finding of the present study was that a medium-level (around 40%) of PL in pasture has the potential to reduce CH_4 production by up to 19% *in vitro* compared to RWC. This aligns with previous studies that reported lower CH_4 production from pure PL pasture *in vitro* when compared to other forage species (Durmic et al., 2016) and *in vivo* when compared to 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 pasture across different climatic seasons were the primary drivers of these changes.

3.4.1 The effect of pasture quality on gas and methane emissions

Plantain pasture generally produces high levels of NSC, lignin and improved or similar digestibility compared to ryegrass pasture (Minnee et al., 2019; Stewart, 1996). In the present study, greater levels of NSC, lignin content, and similar DOMD were observed in PLM substrates across different seasons. Also, the reported values of digestibility for PL mixed pastures were in a similar range of previous reports (Herath et al., 2023; Nkomboni et al., 2021). Others have reported that pure PL, particularly at its mature stage, has lower total tract DM digestibility (Della Rosa et al., 2022). The similar DOMD observed between pasture types in the present study could be due to the composition of the pastures, with the PLM mix containing only about 40% PL.

Lignin serves as an indicator of potential NDF digestibility (Jung & Allen, 1995; Van Soest et al., 2020), and it correlates negatively with digestibility and CH_4 emissions (Hindrichsen et al., 2004; Moore & Jung, 2001) while NSC correlate positively with GP (Getachew et al., 2004). Despite the higher lignin content in PLM than in RWC, the total GP at 24 h was similar in both

PLM and RWC across substrates. This suggests that the antagonistic effects of lignin and NSC on digestibility may have counterbalanced one another and led the forages to yield similar GP. Nevertheless, the limited sample size in the present study restricts the ability to construct a relationship between those 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 to RWC *in sacco.* These authors report that more PL DM had disappeared than RWC from 6 to 24 h. 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 more than 24 h due to the rumen outflow rate (Keim et al., 2013). Consequently, the results from the first few h 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) would not be a significant concern for animal productivity. For example, milk production data collected from the trial site remained consistent across all pasture types, and no negative effects on milk production were reported at any PL feeding level (Nguyen, 2023).

A promising reduction *in vitro* CH_4 production, especially in summer, and a trend toward 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.

3.4.2 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. The present study confirms that these reductions can be achieved even with a medium-level of PL in the pasture.

Protein degradation occurs in the rumen, where around 75–90% of the ingested CP in forage is degraded, with the remainder being ruminant undegradable protein (Waghorn & Clark, 2004). The protein degradation produces NH_3, which is either utilised by rumen microbiota for their growth, forming microbial CP (MCP), and excessive NH_3 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 & Waghorn, 2008). Hence, ruminal NH_3 N not utilised for microbial protein synthesis will likely be excreted in urine. This represents a net loss to the animal and contributes to environmental pollution (Tamminga, 1992).

Lower ruminal NH_3 concentrations and greater MCP production have been shown to be a typical response to available carbohydrate (Kolver et al., 1998). A positive relationship between CP intake and urinary N losses has been well established (Box et al., 2018). Therefore, the ratio between NSC:N is crucial in reducing NH_3 pool in the rumen. Table 3.5 indicates that PLM pasture had significantly lower net NH_3 production in both spring and autumn, which could be attributed to the higher NSC:N ratio in those seasons compared to RWC. During the summer, a similar NSC:N ratio was observed in both RWC and PLM pastures, resulting in similar net NH_3 production for the two forages.

3.4.3 The effect of plant secondary metabolites on methane and net ammonia production

Plantain contained various PSM groups such as iridoid glycosides (aucubin), 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 (Box & Judson, 2018; Navarrete Quijada, 2015), likely due to the PLM containing only around 40% PL, leading to a proportional decrease in the concentrations of these PSM.

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 PSM. On the other hand, despite a 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 PSM on this variable.

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 draw definitive conclusions about the specific impact of these PSM on NH_3 and CH_4 production.

3.5 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 spring produced up to 27% less net NH_3; in summer, up to 19% less CH_4; and in autumn, 17% less net NH_3 production. The reduction of net NH_3 production could lead to lower urinary N output from cows, which is a critical source of nitrate leaching and nitrous oxide emissions. While the result on CH_4 production by PL at approximately 40% in the substrate is promising, further animal studies at different seasons and inclusion levels of PL are required to confirm a CH_4 mitigation potential for this forage.

3.6 References

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CHAPTER 4

Effect of plantain (*Plantago lanceolata* L.*)* metabolites aucubin, acteoside and catalpol on methane emissions *in vitro*

This chapter has been published.

Sivanandarajah, K., Donaghy, D., Kemp, P., Navarrete, S., Horne, D., Ramilan, T., Molano, G., & Pacheco, D. (2025). Effects of Plantain (*Plantago lanceolata* L.) Metabolites Aucubin, Acteoside, and Catalpol on Methane Emissions *In Vitro*. *Journal of Agricultural and Food Chemistry*. https://doi.org/10.1021/acs.jafc.4c12140

Abstract

Interest in utilising plantain (PL) in temperate pasture-based dairy systems is increasing due to its environmental benefits, as it reduces nitrogen (N) losses in farmlands and has the potential to lower methane (CH_4) emissions compared to ryegrass-based pastures. Plant secondary metabolites (PSM), acteoside (syn. verbascoside), aucubin and catalpol in PL are associated with various bioactive properties. Both acteoside and aucubin have been associated with reducing N losses from grazed pastures, but the influence of these PSM on enteric CH_4 emissions has not yet been studied. Furthermore, the dose-response relationship of CH_4 and ammonia (NH_3) production at different concentrations of PSM needs to be established. The current study aimed to fill these research gaps by conducting a set of three separate experiments that assess the influence of these PSM on *in vitro* fermentation profiles of substrates. Experiment 1 (E1) evaluated PL mixed pastures (on average 50% PL in pasture DM) with varying levels of PSM (expressed as mg PSM/g pasture DM): PL1 (acteoside 10, aucubin 9), PL2 (acteoside 18, aucubin 6), PL3 (acteoside 6, aucubin 9), and ryegrass (RG) (0 PSM, as control). In experiment 2 (E2), individual PSM concentrations from E1 were tested using purified compounds, along with additional concentrations (acteoside 36, aucubin 24, catalpol 10). Experiment 3 (E3) examined similar PSM levels (50, 75, and 100 for both acteoside and aucubin) and in combinations (acteoside 75+aucubin 50 and acteoside 50+aucubin 75); additionally, catalpol 50 was tested. All experiments were conducted using an automated batch culture system for gas and CH_4 production, and samples were taken for short-chain fatty acids (SCFA) and NH_3 analysis. Data were analysed using mixed-effects models. Acteoside addition to RG increased gas production (GP) by up to 12%, with similar CH_4 production, and around 5 to 15% lesser proportion of CH_4 in gas (%CH_4) compared to control RG. Aucubin addition to RG resulted in a longer lag phase in GP and CH_4, taking up to 15% more time to reach the halftime ($T^{1/2}$) GP and up to 20% longer to reach the $T^{1/2}$ CH_4 production. Aucubin addition resulted in 12% lower CH_4 production at 12 hours in E2. However, this reduction was not observed in E3. The combined treatments of acteoside and aucubin produced up to 13% greater GP, similar CH_4 production and less %CH_4. Aucubin reduced NH_3 production by up to 46%, with a similar reduction (46%) in net NH_3 observed due to the interaction of acteoside and aucubin. Catalpol produced less CH_4 in E2; however, it was not consistent in E3 and ineffective in altering the SCFA profile and reducing %CH_4 or NH_3 production. This study provides evidence that PSM influenced aspects of rumen fermentation *in vitro*, leading to lower production of CH_4 and NH_3. These results add to our understanding of the role of plantain as a tool to mitigate environmental impacts from grazing ruminants in temperate grazing systems.

4.1 Introduction

The New Zealand dairy sector is one of the largest export-earning sectors in the country, contributing over 11 billion New Zealand dollars to the economy (DCANZ, 2023). Developing strategies that mitigate the environmental impact, particularly greenhouse gas (GHG) emissions from dairy systems, is a growing concern (Scott & Blanchard, 2021). Reducing these emissions while maintaining the economic viability is crucial for the sustainability of the dairy sector. Key processes contributing to GHG emissions are enteric fermentation, manure decomposition, and denitrification in soil (Kebreab et al., 2016). Among these, enteric fermentation accounts for approximately 42% of New Zealand's total GHG emissions, with dairy cattle responsible for half of the CH_4 produced through this process (MfE, 2024). In addition to CH_4 emissions, managing nitrogen (N) excretion from livestock is also crucial. High urinary N excretion can lead to pollution of waterways and groundwater, as well as increased nitrous oxide (N_2O) emissions, both of which are significant environmental challenges (Waghorn et al., 2019). Research has identified the potential of plantain (PL, *Plantago lanceolata* L.) to reduce N leaching and N_2O emissions from grazed pastures via lowering urine N concentration of cows (Box et al., 2017; Navarrete et al., 2022; Simon et al., 2019). The presence of plant secondary metabolites (PSM) in PL has been identified as one of the key factors driving these environmental benefits (DairyNZ, 2023a). A recent study on pure PL pasture reported lower CH_4 emissions when cows fed with PL than ryegrass (Della Rosa et al., 2022). However, that study did not explore the impact of PSM on CH_4 emissions.

Plantain has three major PSM*, viz.* acteoside, aucubin and catalpol and their production is influenced by genotype and environmental factors such as air temperature, solar radiation, nutrient availability, etc. (Tamura & Nishibe, 2002). While the concentration of PSM varies widely across studies, acteoside and aucubin are generally found at higher levels than catalpol (Box et al., 2019; Box & Judson, 2018; Navarrete et al., 2016). The highest concentrations of acteoside in PL, ranging from approximately 60 to 94 mg/g DM, were reported by Fajer et al. (1992) with no specific cultivar mentioned, and Tamura and Nishibe (2002) reported the highest aucubin concentrations, ranging from approximately 21 to 48 mg/g DM, in the cultivar Grasslands Lancelot. Some PL cultivars only produce trace quantities of catalpol (Tamura & Nishibe, 2002).

Acteoside and aucubin are known to have antimicrobial properties, and they positively influence the *in vitro* fermentation profile and reduce ammonia (NH_3) production (Navarrete et al., 2016). However, the impact of PSM in PL on enteric CH_4 emissions is unknown. A study on *Paulownia* leaf extract, which is rich in phenolic compounds (including acteoside, its derivatives, iridoid glycosides like aucubin and catalpol, and other phenolic compounds at 84.4 mg/g DM), found that it significantly reduced NH_3 concentration, and decreased CH_4 production during *in vitro* rumen fermentation (Nowak et al., 2022). This suggests that PSM in PL could also reduce enteric CH_4 emissions. Therefore, investigating whether PSM in PL influence enteric CH_4 emissions is crucial for understanding the impact of PL pasture on CH_4 emissions.

The current study evaluated how PSM; acteoside, aucubin, and catalpol in PL affect CH_4 emissions. It also investigated how naturally available concentrations of PSM found in a typical PL-mixed pasture affect *in vitro* rumen fermentation profiles. We hypothesised that PSM found in PL, either individually or in combination, enhance rumen fermentation profiles and reduce CH_4 and NH_3 production *in vitro*, with the reduction by individual PSM being dose-dependent.

4.2 Methods

4.2.1 *In vitro* study design and treatments.

Three *in vitro* experiments were conducted to evaluate the rumen fermentation profiles of PSM when incubated with substrates in an automated batch culture system, adhering to the protocols of Muetzel et al. (2014).

In experiment 1 (E1), selected PL substrates were sourced from archived samples collected at Massey University PL trial site (DairyNZ, 2023b) at Dairy 4 farm, Palmerston North, New Zealand (40°23′27″ S 175°36′44″ E). The PL-mixed pastures were established with PL (cv. Agritonic), perennial ryegrass (*Lolium perenne* cv. ONE^{50}) and white clover (*Trifolium repens* cv. Tribute). A detailed description of field trial establishment and grazing management is provided by Nguyen et al. (2022).

The selected PL substrates, containing about 50% PL in the DM for E1, had similar chemical compositions but varying intrinsic concentrations of their PSM, specifically acteoside and aucubin. These variations reflect the observed differences in PSM concentrations in the field across PL-mixed pastures, where PL1 had 10 acteoside and 9 aucubin, PL2 had 18 acteoside and 6 aucubin, and PL3 had 6 acteoside and 9 aucubin (values expressed as mg/g DM). These substrates were incubated along with ryegrass (RG) for comparison.

Experiment 2 (E2) assessed similar concentrations of PSM to those in E1, by testing each compound individually added to RG using commercially available PSM (Extrasynthese S.A, France, purity >99%). It also examined concentrations of 36 mg acteoside/g pasture DM and 24 mg aucubin/g pasture DM aimed to represent 100% PL pasture, and additionally, 10 mg catalpol/g pasture DM was tested in E2. Dimethylformamide (DMF) was used to dissolve the PSM in E2; therefore, an additional control treatment was included (RG added with DMF, RG-

DMF). The RG used in E1 also served as a run control of the experiments (E1 and E2), as this was used previously in this batch culture system.

Experiment 3 (E3) was implemented based on the results of E1 and E2, to assess the fermentation profile of high concentrations of previously reported PSM and the interaction of acteoside and aucubin in particular. Three levels were chosen for aucubin and acteoside (50, 75 and 100 mg PSM/pasture DM) to compare the dose response at high concentrations, and two combinations (50:75 and 75:50 mg of acteoside:aucubin/pasture DM) were tested to explore the interaction between acteoside and aucubin. Unlike E2, the PSM in E3 were weighed and added directly along with RG to the incubation bottles prior to the experiment to avoid any possible confounding effect of DMF in fermentation profiles. Additionally, 50 mg catalpol/g of pasture DM was tested in E3. The RG substrate (used in E2 and E3) was collected from AgResearch Aorangi farm, located near Palmerston North, New Zealand.

The treatments in each experiment (Table 4.1) were incubated in two sets. Each set included duplicate bottles (analytical replicates) containing a mixture of rumen fluid from two donor cows per run (biological replicate). One set continuously measured gas and CH_4 production over 48 hours (h), while subsamples were taken from the second set. Each incubation was repeated in three runs (replicates) for statistical evaluation.

Stock solutions were prepared for E2 using DMF to ensure complete dissolution of the PSM and accurate dosing. Acteoside (274 mg), aucubin (180 mg), and catalpol (50 mg) were dissolved in 9 mL, 9 mL, and 3 mL of DMF, respectively, resulting in final concentrations of 30.44 mg/mL for acteoside, 20 mg/mL for aucubin, and 16.67 mg/mL for catalpol (Table 4.1).

Table 4.1. Treatments, substrates, concentrations of plant secondary metabolites (PSM) and their equivalent amount to mg/g of pasture dry matter (DM) incubated in experiments 1, 2 and 3.

Experiments	Treatments	Substrate	Acteoside (mg/g pasture DM)	Aucubin (mg/g pasture DM)	Catalpol (mg/g pasture DM)	Concentration of PSM per bottle (mg/bottle)	Volume added from the stock solution with DMF per bottle (μl)	Concentration of PSM (mg/g pasture DM)
Experiment 1 (E1)	RG[a]	Ryegrass	n.d	n.d	n.d	—	—	—
	PL1[b]	Plantain	9.9	9.0	n.d	—	—	18.9
	PL2[c]		18.3	5.8	n.d	—	—	24.1
	PL3[d]		6.6	9.9	n.d	—	—	16.5
Experiment 2 (E2)	RG[e]	Ryegrass	n.d	n.d	n.d	—	—	—
	ACT-18		18.3	—	—	9.2	300.0	18.3
	ACT-10		9.9	—	—	4.8	160.0	9.7
	ACT-6		6.6	—	—	3.3	110.0	6.7
	AUC-12		—	12.0	—	6.0	300.0	12.0
	AUC-9		—	8.9	—	4.5	225.0	9.0
	AUC-6		—	5.8	—	3.0	150.0	6.0
	ACT-36		36.5	—	—	18.3	600.0	36.5
	AUC-24		—	24.0	—	12.0	600.0	24.0
	CAT-10		—	—	10.0	5.0	300.0	10.0
	RG-DMF[f]		—	—	—	—	300.0	—
Experiment 3 (E3)	RG[g]	Ryegrass	n.d	n.d	n.d	—	—	—
	ACT-50		50.0	—	—	25.0	—	50.0
	ACT-75		75.0	—	—	37.5	—	75.0
	AUC-100		100.0	—	—	50.0	—	100.0
	AUC-50		—	50.0	—	25.0	—	50.0
	AUC-75		—	75.0	—	37.5	—	75.0
	AUC-100		—	100.0	—	50.0	—	100.0
	CAT-50		—	—	50.0	25.0	—	50.0
	ACT75+AUC50		75.0	50.0	—	62.5	—	125.0
	ACT50+AUC75		50.0	75.0	—	(62.5	—	125.0

[a]ryegrass (RG) substrate and control for E1, [b]PL1: plantain with ~10 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, [c]PL2: plantain with ~ 18 mg acteoside/g pasture DM and 6mg aucubin/g pasture DM, [d]PL3: plantain with ~ 6 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, [e]ryegrass used as a substrate to incubate plant secondary metabolites and control for E2, [f]ryegrass substrate added with dimethylformamide and additional control treatments for E2, [g]ryegrass used as a substrate to include plant secondary metabolites and a control treatment for E3, n.d: not detected, — : not applicable.

4.3 Rumen sample collection and *in vitro* medium preparation.

A total of 3.2 L of buffer solution was prepared according to Mould et al. (2005). The buffer was heated in a water bath to 39°C and saturated with CO_2 for about 30 minutes, and a reducing agent (NaOH 2.5 mmol and cysteine-HCl 2.5 mmol) was added prior to the rumen fluid collection.

Rumen fluid was collected before the morning feeding, and donor animals were maintained according to the guidelines approved by the AgResearch Animal Ethics Committee (application AE 699). Rumen fluid was collected from two fistulated cows, and an equal volume (400 mL from each cow) was added to make up 4 L of *in vitro* medium by continuously gassing with CO_2. A sample of the medium was collected to analyse short-chain fatty acids (SCFA) and NH_3 (0 h sample).

Before the incubation, substrates were weighed to 500 ± 10 mg, added to 125 mL pre-labelled bottles, and warmed to 39°C in the incubator. Individual compounds (PSM) were pipetted to the bottles from the respective stock solutions described in Table 4.1 for E2, and PSM were added directly into bottles for E3. A 50 mL aliquot of medium was dispensed under a stream of CO_2 into each bottle, capped with a butyl rubber stopper, mixed well and randomly placed on a rack in a reciprocal shaker inside the incubator. Each bottle was connected to a gas measurement system via a 23-gauge needle and shaken at 120 rpm horizontally. Samplings were done at 3,6,9,12,24, and 48 h using a 3 mL syringe via a needle connected to a manual valve. At each sampling, the bottle was shaken manually, and then 1.8 mL of medium was pipetted to 2 mL Eppendorf tubes and centrifuged (21,000 × g for 10 min at 4˚C). An aliquot of 900 µl supernatant and 100 µl of internal standard (19 mmol ethyl butyrate in 20% (v/v) phosphoric acid) was transferred into 1.5 mL micro-tube and stored at -20˚C until further analysis of NH_3 and SCFA.

4.4 Laboratory analysis for samples from *in vitro* incubation.

The samples were thawed and centrifuged at 21,000 × *g* for 10 min at 4ºC. An aliquot of 800 μL supernatant was transferred into a 2 mL crimp cap vial for SCFA analysis. Short-chain fatty acids were analysed using gas chromatography as described by Attwood et al. (1998). Approximately 200 μL of the remaining supernatant was for NH_3 concentration analysis using the colourimetric method described by Weatherburn (1967), scaled down to run in 96-well plates.

Chemical composition analysis for substrates was carried out by using near-infrared spectroscopy. 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), neutral detergent fibre (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 two h), soluble sugars (calibration based on an 80:20 ethanol: water extraction and colorimetric determination), starch (calibration based on enzymic hydrolysis of starch), crude fat (calibration based on petroleum spirit extraction by Ankom auto analyser, AOCS official procedure AM-5-04), crude protein (CP) (N multiplied by 6.25).

4.5 Model fitting and data analysis.

Gas and CH_4 production from each bottle was fitted to a logistic exponential model to estimate the *in vitro* GP kinetics described by France et al. (2000) using the following formulae:

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

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

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

Where:

V: cumulative volume of gas or CH_4 produced by fermentation up to time t (mL g^{-1} DM)

a: potential GP [PGP] (mL g^{-1} DM)

b: fermentation rate constant (h^{-1})

c: parameter determining curve steepness and lag phase.

t: time (h)

$T^{\frac{1}{2}}a$: time at which the incubation reached ½ of PGP (h)

$R^{\frac{1}{2}}a$: rate of GP at half-time (mL g DM.h^{-1})

Gas and CH_4 production parameters were analysed in statistical software R version 4.3.0 (R Core Team, 2024) using linear mixed models in packages ‘lme4’ (Bates et al., 2014) and ‘emmeans’ (Lenth R, 2023). Each treatment was treated as a fixed effect, and the incubation run was treated as a random effect. A multiple comparison of predicted means was performed, and the p-values were adjusted using the Tukey *post-hoc* test to evaluate the significance of differences between group means. Treatment effects were considered significant at an adjusted p-value threshold of <0.05.

Data for combined treatments (ACT75+AUC50 or ACT50+AUC75) were tested separately by planned contrasts using the ‘emmeans’ package to assess treatment interactions and efficacy. Specifically, combined treatments were compared against the averages of their individual components (e.g., ACT75+AUC50 vs. (ACT-75 + AUC-50)/2, and direct comparisons between individual treatments were also evaluated. Statistical significance was determined using contrasts derived from estimated marginal means within a linear mixed-effects model framework.

For net NH_3 production, NH_3 values were corrected for the incubated substrate's DM and the time 0 NH_3 concentration (mmol NH_3/g DM). Additionally, in E1, net NH_3 concentrations were corrected for the CP incubated per bottle (mmol NH_3/g CP). Repeated-measures analyses were performed on the net NH_3 production over time (3, 6, 9, 12, and 24 h) for each experiment. The model included treatment and time as fixed effects, biological replicates as a random effect, and the incubation bottle as the subject of analysis. Similarly, for total SCFA production, individual SCFAs (e.g., acetate, propionate, butyrate, etc.) and their molar proportion were analysed over time (12 and 24 h).

4.6 Results

4.6.1 Compositional analysis of substrates.

Ryegrass had lower levels of ADF (24% lower), lignin (35% lower), and NSC (93% lower), but higher levels of OMD (17% higher) and CP (37% higher) compared to the selected PL substrates. These values were more than two interquartile ranges of the average of the PL substrates. The NDF was similar across all substrates, including RG. Plant secondary metabolites were present only in PL substrates and catalpol was detected at low concentrations (<1 mg/g of pasture DM) in the PL substrates (Table 4.2).

4.6.2 *In vitro* gas and methane production in experiment 1.

After 12 h of incubation, PL3 produced, on average, 7% less GP than RG, PL1 and PL2. The PL2 and PL3, on average, had 8% and 4% less CH_4 production compared to RG and PL1, respectively, at 12 h. At 24 h of incubation, PL1, PL2, and PL3 produced on average 9%, 8%, and 13% less CH_4 and 6%, 5%, and 10% less GP, respectively, compared to RG ($p<0.05$). The PGP, PCH_4 and PCH_4/PGP were similar between PL and RG substrates. The PL2 produced, on average, 5% less $\%CH_4$ in GP at 12 h ($p<0.05$) compared to the other PL substrates and RG. A trend ($p=0.09$) for a lower $\%CH_4$ in GP in all PL substrates (PL1, PL2, PL3) compared to RG was observed at 24 h of incubation. The three PL substrates showed different $T^{1/2}$ GP (PL3> PL2 = RG > PL1), but the $R^{1/2}$ GP was, on average, 18% slower in PL substrates compared to RG. There were no differences in the $T^{1/2}$ CH_4 between the PL substrates and RG, but the $R^{1/2}$ CH_4 was slower (30%) in all PL substrates compared to RG.

Table 4.2. Compositional analysis of ryegrass (RG) and plantain (PL) substrates incubated in experiment 1.

Substrates		**OMD (%DM)**	**NDF (%DM)**	**ADF (%DM)**	**Lignin (%DM)**	**Lignin /NDF %**	**Ash (%DM)**	**SSS (%DM)**	**CP (%DM)**	**Crude fat (%DM)**	**NSC (%DM)**	**NSC: CP**	**Plantain[a] %**	**Acteoside (mg/g pasture DM)**	**Aucubin (mg/g pasture DM)**	**Catalpol (mg/g pasture DM)**
Ryegrass	**(RG)**	83.2[b]	42.8	21.3[b]	6.3[b]	14.7[b]	12.6	5.8	27.2[b]	3.8[b]	13.6[b]	0.5	n.d	n.d	n.d	n.d
Plantain (PL) substrates	**PL1[c]**	70.0	41.5	25.5	8.7	21.0	11.9	6.2	16.8	2.5	27.2	1.6	45.3	9.9	9	0.9
	PL2[d]	68.5	42.7	27.5	8.3	19.4	11.5	7.5	16.6	2.5	26.8	1.6	48.0	18.3	5.8	0.5
	PL3[e]	67.3	43.8	26.3	8.4	19.2	11.8	6.3	17.7	2.2	24.6	1.4	56.5	6.6	9.9	0.6

[a]plantain percentage in the substrate, [b]RG value was over two interquartile ranges compared to the averages of PL1, PL2 and PL3 within columns, n.d: not detected, [c]plantain substrate (PL1) containing 10 mg of acteoside/g pasture DM and 9 mg aucubin/g pasture DM, [d]plantain substrate (PL2) containing 18 mg acteoside/g pasture DM and 6 mg aucubin/g pasture DM, [e]plantain substrate (PL3) containing 6 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, DM: dry matter, OMD: organic matter digestibility *in vivo*, NDF: neutral detergent fibre, ADF: acid detergent fibre, SSS: soluble sugars and starch.

Table 4.3. Gas and methane (CH_4) production parameters of plantain (PL) substrates compared to ryegrass (RG) substrate *in vitro* in experiment 1.

Substrates		**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)**	**CH_4/GP at 12 h (%)**	**CH_4 /GP at 24 h (%)**	**PCH_4/ PGP (%)**	**$T^{1/2}$ GP (h)**	**$T^{1/2}$ CH_4 (h)**	**$R^{1/2}$ GP (mL/h)**	**$R^{1/2}$ CH_4 (mL/h)**
Ryegrass	**(RG)**	201.8^{a}	266.3^{a}	295.6	25.3^{a}	38.4^{a}	41.7	12.5^{a}	14.4	14.1	7.4^{b}	9.7	14.5^{a}	2.1^{a}
Plantain (PL) substrates	**PL1**[a]	200.5^{a}	249.5^{b}	307.5	25.1^{ab}	35.0^{b}	44.2	12.5^{a}	14.0	14.2	6.9^{c}	10.1	12.3 ^{b}	1.5^{b}
	PL2[b]	204.2^{a}	254.2^{b}	311.6	24.4^{b}	35.4^{b}	42.6	11.9^{b}	13.9	13.6	6.8^{b}	10.0	12.6^{b}	1.6^{b}
	PL3[c]	188.3^{b}	240.3^{c}	305.2	23.3^{c}	33.4^{c}	45.1	12.3^{ab}	13.8	14.6	7.9^{a}	12.0	10.7^{c}	1.3^{b}
	SEM	9.52	7.25	21.01	2.4	3.19	5.56	0.72	0.93	1.0	0.15	1.13	0.23	0.15
	p–value	<0.01	<0.01	0.18	<0.01	<0.01	0.5	0.02	0.09	0.59	<0.01	0.17	<0.01	<0.01

[a]PL1: plantain substrate containing 10 mg of acteoside/g pasture DM and 9 mg aucubin/g pasture DM, [b]PL2: plantain substrate containing 18 mg acteoside/g pasture DM and 6 mg aucubin/g pasture DM, [c]PL3: plantain substrate containing 6 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, PGP: potential gas production, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, T ½: the half time required to reach the potential gas or methane production, $R^{1/2}$: the rate to produce half of potential gas or methane production, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means.

4.6.3 Short-chain fatty acids and net ammonia production of substrates in experiment 1.

The total SCFA production (mmol) was, on average, 11% lower in PL substrates compared to RG ($p<0.05$). Greater molar proportions of acetate, butyrate (except PL2=RG), valerate, and caproate were observed in PL substrates compared to RG, in contrast, propionate, iso-butyrate and iso-valerate molar proportions were greater in RG compared to PL substrates. PL substrates had greater ratios of acetate to propionate (A:P) and [acetate+butyrate] to [propionate+valerate] (AB:PV) compared to RG ($p<0.05$).

When compared only within PL substrates, PL2 produced lower A:P and AB:PV ratios compared to PL1 and PL3. Especially, PL2 produced more propionate and valerate, less butyrate, and an equal amount of acetate compared to PL1 and PL3 ($p<0.05$). Plantain substrates PL1, PL2, and PL3 produced 46%, 30%, and 32% less net NH_3, respectively, compared to RG ($p<0.05$). However, the net NH_3 production per g CP incubated in each bottle was similar across substrates ($p=0.08$) (Table 4.4).

Table 4.4. Comparison of total short-chain fatty acids (SCFA), molar proportion of SCFA and net ammonia (NH_3) production *in vitro* of plantain (PL) substrates and ryegrass (RG) substrate in experiment 1 over time.

		Absolute concentrations			Molar proportions (mol/100 mol total SCFA)							Ratios	
Items		**SCFA**^{a}	**Net** NH_3^{b}	**Net** NH_3 **per CP**^{c}	**Acetate**	**Butyrate**	**Propionate**	**Valerate**	**Caproate**	**Iso-butyrate**	**Iso-valerate**	**A:P**^{d}	**AB:PV**^{e}
Ryegrass	(RG)	74.2^{a}	11.5^{a}	42.2	67.5^{b}	10.7^{c}	16.9^{a}	1.30^{d}	0.10^{c}	1.43^{a}	2.14^{a}	4.02^{c}	4.32^{d}
Plantain substrates	PL1^{f}	68.0^{bc}	6.2^{b}	37.5	68.6^{a}	11.0^{b}	16.0^{c}	1.44^{b}	0.18^{a}	1.15^{b}	1.67^{c}	4.29^{a}	4.56^{b}
	PL2^{g}	68.7^{b}	8.1^{b}	48.1	68.6^{a}	10.5^{c}	16.5^{b}	1.50^{a}	0.16^{b}	1.16^{b}	1.56^{d}	4.18^{b}	4.42^{c}
	PL3^{h}	65.9^{c}	7.8^{b}	43.9	68.3^{a}	11.4^{a}	15.8^{c}	1.37^{c}	0.17^{a}	1.21^{b}	1.78^{b}	4.34^{a}	4.66^{a}
	SEM	4.02	1.84	9.71	1.26	1.28	0.87	0.18	0.02	0.23	0.40	0.16	0.18
	Substrates	<0.01	<0.01	0.08	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	Time	<0.01	<0.01	<0.01	<0.01	0.23	0.09	<0.01	<0.01	<0.01	<0.01	0.09	0.01
	Substrates × Time	0.40	0.17	0.27	0.05	0.11	0.9	<0.01	0.35	0.02	<0.01	0.94	0.8

^{a}total short-chain fatty acid (mmol), ^{b}net ammonia production in mmol NH_3 per g of DM incubated, ^{c}net ammonia production in mmol NH_3 per g of CP incubated, ^{d}acetate to propionate ratio (A:P), ^{e}[acetate+butyrate] to [propionate+valerate] ratio (AB:PV), ^{f}PL1: plantain substrate containing 10 mg of acteoside/g pasture DM and 9 mg aucubin/g pasture DM, ^{g}PL2: plantain substrate containing 18 mg acteoside/g pasture DM and 6 mg aucubin/g pasture DM, ^{h}PL3: plantain substrate containing 6 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, values with the same letter within the same rows are not significant ($p>0.05$), SEM: standard error of means.

4.6.4 *In vitro* gas and methane production in experiment 2.

In E2, around 12% and 8% lower CH_4 production by AUC-24 was observed at 12 and 24 h, respectively, and it required 9% more time to reach $T^{1/2}$ GP compared to RG. Additionally, AUC-24 produced 6% and 8% less %CH_4 at 24 h and PGP. The CH_4 production was similar in ACT-36 compared to RG, although a 7% reduction in %CH_4 at 24 h was observed. Gas and CH_4 productions were not affected by PSM added individually to incubation in E2 at similar concentrations to those observed in E1 (ACT-6, ACT-10, ACT-18, AUC-6, AUC-9, and AUC-12) at any given time points ($p<0.05$). About 10% and 7% lower CH_4 production was observed in CAT-10, with a similar %CH_4 to RG controls ($p<0.05$).

Ryegrass and RG-DMF were statistically similar in all measured parameters. However, a possible confounding effect of DMF in RG was observed. For instance, differences in a few parameters listed above (e.g., CH_4 production at 24 h, %CH4 at 24 h, %PCH4 at PGP) compared to RG were not significant from RG-DMF, except for CH_4 production at 12 h and $T^{1/2}$ GP, which had remained lower in AUC-24 compared to RG and RG-DMF (Table 4.5).

Table 4.5. *In vitro* gas and methane (CH_4) production parameters of different concentrations of plant secondary metabolites (PSM) tested in experiment 2.

Substrates		**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)**	**CH4/GP at 12 h (%)**	**CH_4/GP at 24 h (%)**	**PCH_4/ PGP (%)**	**$T^{1/2}$ GP (h)**	**$T^{1/2}$ CH_4 (h)**	**$R^{1/2}$ GP (mL/h)**	**$R^{1/2}$ CH_4 (mL/h)**
Control treatments	RG^{a}	202.9	267.3	294.1	25.5 ^{a}	38.6 ^{a}	42.1	12.6	14.4 ^{a}	14.3 ^{a}	7.27 ^{b}	9.7	14.7 ^{ab}	2.09
	RG-DMF^{b}	206.7	269.5	296.4	25.2 ^{ab}	37.6 ^{abc}	40.4	12.1	13.9 ^{abc}	13.6 ^{ab}	7.06 ^{b}	9.6	15.0 ^{ab}	2.13
Acteoside treatments^{c}	ACT-6	206.8	272.5	299.1	25.1 ^{ab}	38.6 ^{a}	42.4	12.0	14.1 ^{ab}	14.1 ^{a}	7.25 ^{b}	10.1	15.0 ^{ab}	2.08
	ACT-10	201.3	268.4	298.1	24.5 ^{abc}	37.8 ^{abc}	41.1	12.1	14.0 ^{abc}	13.7 ^{ab}	7.55 ^{ab}	10.0	14.3 ^{ab}	2.07
	ACT-18	204.6	270.7	300.0	24.6 ^{abc}	37.8 ^{abc}	41.3	12.0	13. 9 ^{abc}	13.7 ^{ab}	7.39 ^{ab}	10.0	14.5 ^{ab}	2.05
	ACT-36	203.5	269.6	295.8	23.5 ^{abc}	36.3 ^{abc}	39.7	11.5	13.4 ^{c}	13.4 ^{ab}	7.37 ^{ab}	10.1	14.9 ^{ab}	1.98
Aucubin Treatments^{d}	AUC-6	204.5	272.1	300.7	24.8 ^{abc}	38.2 ^{ab}	41.9	12.1	14.0 ^{abc}	13.9 ^{ab}	7.48 ^{ab}	10.1	14.7 ^{ab}	2.06
	AUC-9	204.1	271.4	299.4	24.9 ^{abc}	38.1 ^{ab}	41.2	12.1	14.0 ^{abc}	13.7 ^{ab}	7.46 ^{ab}	9.9	14.7 ^{ab}	2.12
	AUC-12	207.0	272.4	298.2	25.0 ^{ab}	37.8 ^{abc}	41.1	12.0	13.8 ^{bc}	13.7 ^{ab}	7.21 ^{b}	9.8	15.1 ^{a}	2.1
	AUC-24	192.4	261.1	293.1	22.5 ^{c}	35.5 ^{c}	38.5	11.6	13.5 ^{bc}	13.1 ^{b}	7.93 ^{a}	10.3	13.5 ^{b}	1.95
Catalpol	CAT-10^{e}	192.7	256.4	287.6	23.0 ^{bc}	35.7 ^{bc}	39.9	11.8	13.9 ^{abc}	13.8 ^{ab}	7.58 ^{ab}	10.4	13.6 ^{b}	1.89
	SEM	10.01	8.98	13.5	2.98	3.78	4.37	0.91	0.98	0.91	0.34	0.35	0.49	0.17
	p-value	0.02†	0.08	0.62	<0.01	<0.01	0.07	0.07	0.01	0.01	<0.01	0.55	0.01	0.26

^{a}ryegrass control, ^{b}dimethylformamide added ryegrass control, ^{c}treatments within the columns correspond to varying doses of acteoside (denoted as ACT) and the numeric values separated by a hyphen indicating the amount of acteoside in mg/ g of pasture DM, ^{d}treatments within the columns correspond to varying doses of aucubin (denoted as AUC) and the numeric values separated by a hyphen indicating the amount of aucubin in mg/ g of pasture DM, ^{e}10 mg catalpol/ g pasture DM, †the model was significant for gas production at 12 hours ($p<0.05$), PGP: potential gas production, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, T ^{½}: the half time required to reach the potential gas or methane production, $R^{1/2}$: the rate to produce half of potential gas or methane production, but the mean comparisons were similar across all treatments, making them incomparable, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means.

4.6.5 Short-chain fatty acids production, net NH_3 production of substrates added with plant secondary metabolites in experiment 2.

The AUC-24 and ACT-36 produced approximately 20% and 30% greater SCFA, respectively, compared to RG. Catalpol (CAT-10) produced similar SCFAs to RG and RG-DMF ($p>0.05$). Both AUC-24 and AUC-36 showed increased molar proportions of minor fatty acids and lower butyrate production, although butyrate production in AUC-24 was similar to RG-DMF. Similar ratios of A:P and AB:PV across treatments were observed. Caproate production was greater in AUC-36, and both ACT-36 and AUC-24 produced greater molar proportions of iso-butyrate than the controls.

Significant reductions (up to 33%) in net NH_3 production were observed in AUC-12, AUC-24, and ACT-36 compared to RG, with a negative trend correlating to increasing concentrations of PSM (Figure 3.1). However, these reductions were not significant compared to RG-DMF (Table 4.7).

Table 4.6. Comparison of short-chain fatty acids (SCFAs) and molar proportion of SCFA production *in vitro* of ryegrass (RG) substrate added with aucubin (AUC-24), acteoside (ACT-36), or catalpol (CAT-10) in experiment 2 over time.

		Molar proportion (mol per 100 mol of total SCFA)[a]							Ratios	
Substrates	**SCFA**[b]	**Acetate**	**Butyrate**	**Propionate**	**Valerate**	**Caproate**	**Iso-butyrate**	**Iso-valerate**	**A:P**[c]	**AB:PV**[d]
RG[e]	74.2^{b}	67.5	10.7^{a}	16.9	1.34^{a}	0.10^{b}	1.43^{b}	2.14	4.02	4.32
RG–DMF[f]	70.6^{b}	66.5	10.6^{ab}	17.6	1.35^{a}	0.12^{b}	1.55^{b}	2.20	3.78	4.08
ACT–36[g]	91.5^{a}	66.6	9.5^{c}	17.0	1.22^{ab}	0.18^{a}	3.47^{a}	2.29	3.95	4.22
AUC–24[h]	84.6^{a}	66.6	9.8^{bc}	17.5	1.11^{b}	0.10^{b}	3.07^{a}	2.14	3.87	4.17
CAT–10[i]	66.6^{b}	66.3	10.7^{a}	18.1	1.34^{a}	0.14^{ab}	1.43^{b}	2.16	3.68	3.99
SEM	3.43	1.22	1.17	0.96	0.20	0.02	0.26	0.42	0.17	0.18
Treatments (Tr)	<0.01	0.99	<0.01	0.42	<0.01	<0.01	<0.01	0.75	0.51	0.58
Sampling Time (Ti)	<0.01	0.53	0.95	0.12	<0.01	<0.01	0.85	<0.01	0.42	0.66
Tr × Ti	0.25	0.42	0.44	0.76	0.96	0.47	0.32	0.75	0.7	0.74

[a]molar proportion of individual SCFA's, [b]total short-chain fatty acids (mmol), [c]acetate to propionate ratio (A:P), [d][acetate+butyrate] to [propionate+valerate] ratio (AB:PV), [e]ryegrass control, [f]dimethylformamide added ryegrass control, [g]ryegrass added with 36 mg acteoside/g pasture DM, [h]ryegrass added with 24 mg aucubin/g pasture DM, [i]ryegrass added with 10 mg catalpol/g pasture DM, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means.

Table 4.7. Comparison of net ammonia (NH_3) production *in vitro* at different concentrations of plant secondary metabolites (PSM) compared to ryegrass (RG) in experiment 2 over time.

Items	Treatments	Net ammonia (NH_3) production
Controls	RG[a]	11.47 ^{a}
	RG-DMF[b]	9.73 ^{ab}
Acteoside[c]	ACT-6	10.03 ^{ab}
	ACT-10	9.84 ^{ab}
	ACT-18	9.64 ^{ab}
	ACT-36	7.67 ^{b}
Aucubin[d]	AUC-6	10.22 ^{ab}
	AUC-9	9.55 ^{ab}
	AUC-12	8.78 ^{b}
	AUC-24	7.71 ^{b}
Catalpol	CAT-10[e]	9.07 ^{ab}
	SEM	1.95
	p–values	
	Treatment	<0.001
	Time	<0.001
	Treatments × Time	n.s

[a]ryegrass control, [b]dimethylformamide added ryegrass control, [c]treatments within the rows correspond to varying doses of acteoside (denoted as ACT) and the numeric values separated by a hyphen indicating the amount of acteoside in mg/ g of pasture DM, [d]treatments within the rows correspond to varying doses of aucubin (denoted as AUC) and the numeric values separated by a hyphen indicating the amount of aucubin in mg/g of pasture DM, [e]10 mg catalpol/g pasture DM, values with the same letter within the same column are not significant ($p>0.05$), SEM: standard error of means, n.s: not significant at 5% significant level.

4.7 Interaction effects and individual impact of plant secondary metabolites added to ryegrass incubation in experiment 3.

Methane production was similar to RG across PSM in E3; however, increased GP and lower %CH_4 were observed in some treatments ($p<0.05$). Around 13% more gas (PGP) and a 9% reduction in %CH_4 (12 h) were observed due to the interaction of ACT-50 and AUC-75 when added to RG in treatment ACT50+AUC75 ($p<0.008$), compared to control RG. The interaction effect between ACT-50 and AUC-75 was observed at 12 h GP, 24 h GP, PGP, and their respective %CH_4 productions. Another interaction was found when both ACT-75 and AUC-50 were included in RG in treatment ACT75+AUC50, resulting in a lower %CH_4 at 12 h ($p<0.005$) and a higher $R^{1/2}$ GP ($p<0.029$) than RG (Table 4.8).

An increase of 12% in GP was observed in ACT-100 at both 12 h and 24 h and a decrease in %CH_4 at 12 h and 24 h, as well as in potential %CH_4 compared to RG. A 7% increase in GP was observed in ACT-75 at 12 h compared to RG. Additionally, a 10% higher rate in ACT-100 and a 9% lower $R^{1/2}$ GP in AUC-100 were observed compared to RG. The ACT-100 treatment had an $R^{1/2}$ GP that was also equal to the combined treatment ACT75+AUC50.

Aucubin inclusion (AUC-100) in RG resulted in a longer lag phase in GP. Compared to RG, it took 15% more time to reach its $T^{1/2}$ GP and about 20% more time to reach its $T^{1/2}$ CH_4, while other treatments required similar time as the control. Catalpol did not significantly affect any gas or CH_4 production parameters and did not contribute to any reduction in *in vitro* CH_4 emissions.

Table 4.8. *In vitro* gas and methane (CH_4) production parameters of treatments containing different concentrations of plant secondary metabolites (PSM) added to ryegrass (RG) tested in experiment 3.

Treatments		**GP at 12 h (mL/g DM)**	**GP at 24 h (mL/g DM)**	**PGP (mL/g DM)**	**CH4 at 12 h (mL/g DM)**	**CH_4 at 24 h (mL/g DM)**	**PCH_4 (mL/g DM)**	**CH4/GP at 12 h (%)**	**CH_4/GP at 24 h (%)**	**PCH_4/ PGP (%)**	**$T^{1/2}$ GP (h)**	**$T^{1/2}$ CH_4 (h)**	**$R^{1/2}$ GP (mL/h)**	**$R^{1/2}$ CH_4 (mL/h)**
Control	RG[a]	190.5 ^{cd}	282.7 ^{c}	328.7 ^{b}	21.6 ^{ab}	36.2	44.7 ^{ab}	11.4 ^{ab}	12.8 ^{a}	13.6 ^{a}	9.9 ^{bc}	12.4 ^{bc}	13.3 ^{b}	1.6 ^{ab}
Acteoside treatments[b]	ACT-50	204.4 ^{abc}	297.5 ^{abc}	342.7 ^{ab}	22.2 ^{ab}	37.5	46.8 ^{ab}	10.8 ^{abc}	12.6 ^{a}	13.7 ^{a}	9.5 ^{bc}	12.7 ^{bc}	14.3 ^{ab}	1.7 ^{a}
	ACT-75	205.3 ^{ab}	296.3 ^{abc}	344.6 ^{ab}	21.8 ^{ab}	36.6	46.1 ^{ab}	10.6 ^{bcd}	12.4 ^{ab}	13.3 ^{a}	9.4 ^{bc}	12.6 ^{bc}	14.0 ^{ab}	1.6 ^{ab}
	ACT-100	213.5 ^{a}	306.6 ^{ab}	354.5 ^{ab}	21.3 ^{ab}	34.7	40.6 ^{b}	10.0 ^{d}	11.3 ^{b}	11.4 ^{b}	9.3 ^{bc}	11.4 ^{c}	14.6 ^{a}	1.7 ^{a}
Aucubin treatments[c]	AUC-50	200.2 ^{abcd}	292.5 ^{abc}	340.1 ^{ab}	23.0 ^{a}	38.4	47.9 ^{ab}	11.5 ^{a}	13.1 ^{a}	14.0 ^{a}	9.7 ^{bc}	12.4 ^{bc}	13.9 ^{ab}	1.7 ^{a}
	AUC-75	198.0 ^{bcd}	294.9 ^{abc}	350.2 ^{ab}	22.2 ^{ab}	37.7	47.3 ^{ab}	11.2 ^{ab}	12.8 ^{a}	13.4 ^{a}	10.2 ^{b}	12.7 ^{bc}	13.5 ^{b}	1.7 ^{a}
	AUC-100	187.3 ^{d}	286.8 ^{bc}	360.5 ^{ab}	20.4 ^{b}	36.4	50.4 ^{a}	10.9 ^{abc}	12.7 ^{a}	13.9 ^{a}	11.5 ^{a}	14.8 ^{a}	12.2 ^{c}	1.5 ^{b}
Combined treatment	ACT50+ AUC75[d]	211.4 ^{ab}	310.4 ^{a}	372.0 ^{a}	21.8 ^{ab}	37.7	48.8 ^{ab}	10.3 ^{cd}	12.1 ^{ab}	13.0 ^{ab}	10.1 ^{bc}	13.3 ^{ab}	14.0 ^{ab}	1.7 ^{a}
	ACT75+ AUC50[e]	205.9 ^{ab}	294.2 ^{abc}	336.7 ^{ab}	21.6 ^{ab}	36.6	45.1 ^{ab}	10.5 ^{cd}	12.5 ^{a}	13.5 ^{a}	9.2 ^{c}	12.6 ^{bc}	14.5 ^{a}	1.7 ^{ab}
Catalpol	CAT-50[f]	200.0 ^{abcd}	298.94 ^{abc}	353.7 ^{ab}	21.8 ^{ab}	38.0	48.7 ^{ab}	10.9 ^{abc}	12.7 ^{a}	13.6 ^{a}	10.23 ^{b}	13.2 ^{abc}	13.8 ^{ab}	1.7 ^{a}
SEM		4.72	5.23	11.75	1.05	2.88	5.13	0.53	0.9	1.06	0.53	0.89	0.55	0.12
p-value		<0.001	<0.001	<0.001	0.004	0.025	0.042	0.01	0.03	0.021	0.005	0.007	0.004	0.035
Contrasts, p-values[g]														
ACT50+AUC75 vs. (ACT-50 + AUC-75)/2		0.008	0.011	0.021	0.432	0.855	0.420	<.0001	<.0001	0.043	0.267	0.068	0.428	0.214
ACT-50 vs AUC-75		0.170	0.702	0.516	0.707	0.767	0.789	0.002	0.149	0.528	0.011	0.900	0.001	0.996
ACT75+AUC50 vs. (ACT-75 + AUC-50)/2		0.364	0.999	0.619	0.092	0.241	0.314	0.005	0.174	0.528	0.266	0.920	0.029	0.624
ACT-75 vs AUC-50		0.231	0.623	0.862	0.057	0.072	0.403	0.001	0.005	0.119	0.588	0.781	0.688	0.016

[a]ryegrass control, [b]treatments within the rows correspond to varying doses of acteoside (denoted as ACT) and the numeric values separated by a hyphen indicating the amount of acteoside in mg/ g of pasture DM, [c]treatments within the rows correspond to varying doses of aucubin (denoted as AUC) and the numeric values separated by a hyphen indicating the amount of aucubin in mg/g of pasture DM, [d]50 mg acteoside/g pasture DM added with 75 mg aucubin/g pasture DM, [e]75 mg acteoside/g pasture DM added with 50 mg aucubin/g pasture DM, [f]50 mg catalpol/g pasture DM, [g]p-value of planned contrasts in selected treatments, PGP: potential gas production, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, $T^{½}$: the half time required to reach the potential gas or methane production, $R^{1/2}$: the rate to produce half of potential gas or methane production, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means.

4.7.1 Short-chain fatty acid production of treatments containing different concentrations of plant secondary metabolites added to ryegrass tested in experiment 3.

Total SCFA concentrations compared to RG were similar for all treatments. Significant interactions in combined treatments were observed in molar proportions of SCFAs and the A:P and AB:PV ratios. The combined treatments produced greater proportions of propionate, butyrate, and caproate, along with lower proportions of acetate, iso-butyrate, and iso-valerate compared to RG, resulting in lower A:P, AB:PV ratios (Table 4.9).

A linear increase in the molar proportions of propionate was observed with increasing concentrations of acteoside (RG [ACT-0], ACT-50, ACT-75, and ACT-100), while butyrate and valerate molar proportions remained similar to RG, even at higher concentrations. Conversely, these treatments showed lower molar proportions and a linear decrease in acetate, leading to a linear decrease in A:P and AB:PV (Figure 4.2). Additionally, increased caproate, iso-butyrate and iso-valerate production was observed. A linear decrease in the molar proportions of acetate and propionate was observed with increasing concentrations of aucubin (RG [AUC-0], AUC-50, AUC-75, AUC-100). In contrast, the molar proportion of butyrate showed a linear increase, while the molar proportion of valerate and the A:P ratio remain similar. A higher AB:PV ratio was observed in AUC-100 compared to RG in E3 (Figure 4.2). Additionally, increased caproate production compared to RG was observed (Table 4.9).

Significant interaction was found between ACT-75 and AUC-50 in ACT75+AUC50 combined treatment, resulting in a 46% reduction in net NH_3 compared to the RG ($p<0.004$). This reduction was 6% greater than the expected additive reduction of the individual treatments, ACT-75 and AUC-50. For instance, ACT-75 and AUC-50, when applied individually, were statistically similar to RG ($p>0.05$), showing numerical reductions of approximately 16% and 24%, respectively. Another combined treatment, ACT50+AUC75, produced 35% lower net NH_3 than RG, but no interaction was found ($p=0.148$). This reduction was similar to that of AUC-75 (36%), and while the ACT-50 treatment was statistically similar to RG (with a numerical reduction of 11%), combining both treatments did not increase the magnitude of the reduction (Table 4.9). Net NH_3 production decreased linearly as the concentration of the PSM increased in acteoside and aucubin. Aucubin significantly reduced net NH_3 production at AUC-75 and AUC-100 by 34% and 46%, respectively. Acteoside did not significantly reduce net NH_3 production compared to RG, but a trend was observed in E3 (Figure 4.1).

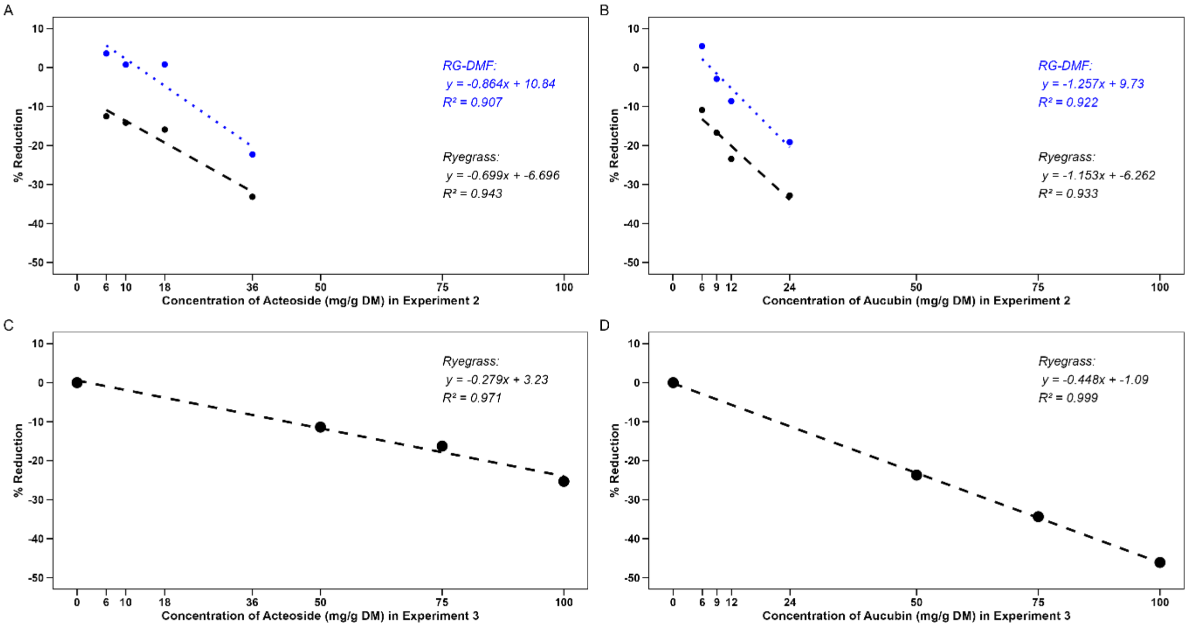

Figure 4.1. Percentage change in net NH_3 production at different types and concentrations of plant secondary metabolites (PSM) compared to the control ryegrass (RG and RG-DMF) in experiments 2 and 3.

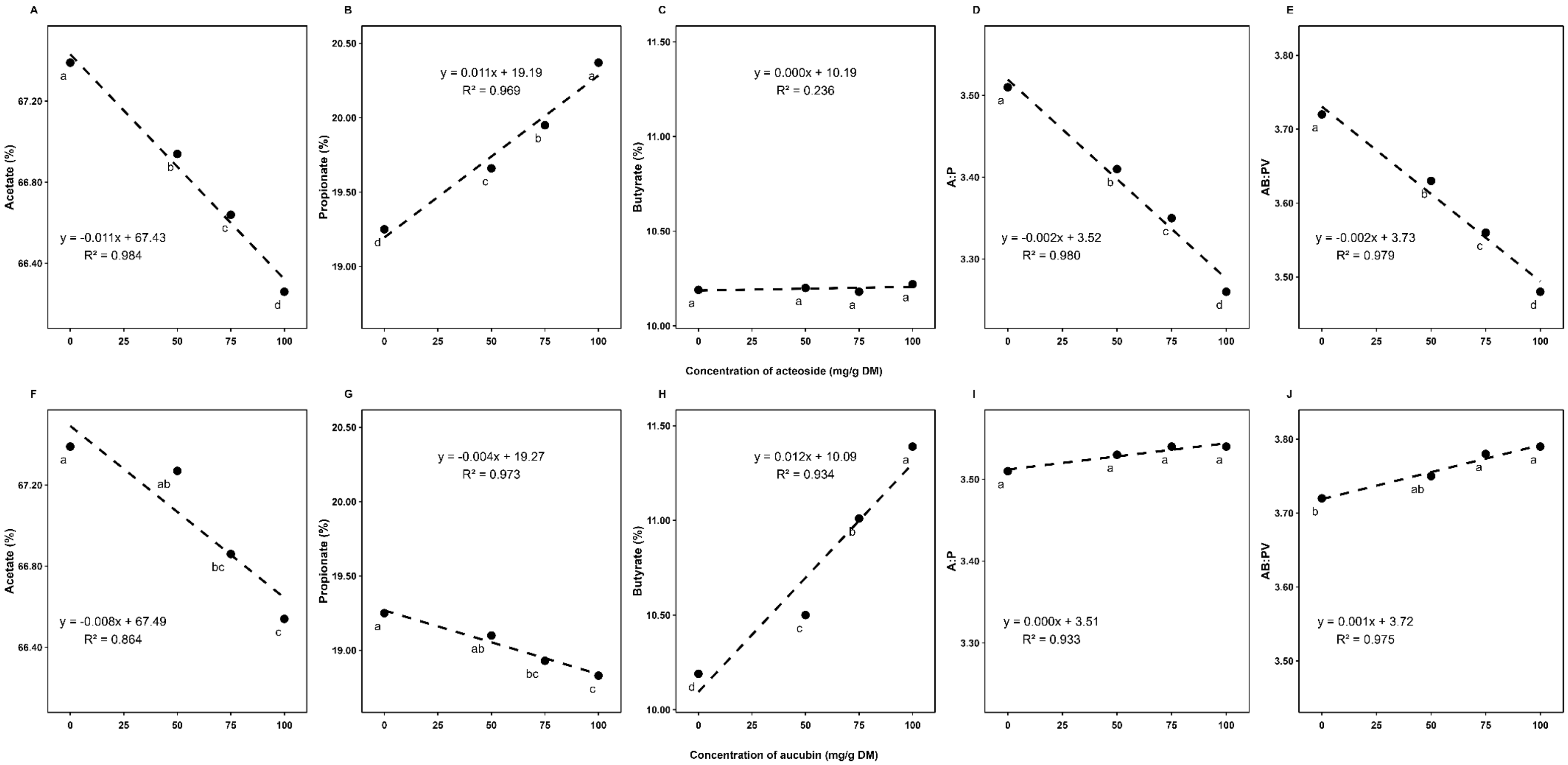

Figure 4.2. Effect of acteoside on molar proportion of short-chain fatty acids (SCFA) (mol per 100 mol of total SCFA, (A) to (C)), effect of aucubin on molar proportion of SCFA (mol per 100 mol of total SCFA, (F) to (H)), A:P; acetate to propionate ratio, AB:PV; acetate+butyrate to propionate+valerate ratio, effect of acteoside on A:P (D), AB:PV (E) and effect of aucubin on A:P (I), AB:PV (J) when incubated with ryegrass (RG) in experiment 3, values marked with the same letter within the same parameter (within individual plots) are not statistically different, the 0 concentration refers to control RG (0 PSM).

Table 4.9. Comparison of total short-chain fatty acids (SCFA), the molar proportion of SCFA and net ammonia (NH_3) production *in vitro* of ryegrass substrate (RG) added with different concentrations of plant secondary metabolites (PSM) in experiment 3 over time.

		Absolute concentration		Molar proportion (mol per 100 mol of total SCFA)							Ratios	
Treatments		**SCFA**[a]	**Net NH_3**[b]	**Acetate**	**Butyrate**	**Propionate**	**Valerate**	**Caproate**	**Iso-butyrate**	**Iso-valerate**	**A:P**[c]	**AB:PV**[d]
Control	RG[e]	68.6 ^{ab}	5.5 ^{a}	67.4 ^{a}	10.2 ^{d}	19.3 ^{d}	1.64	0.06 ^{d}	0.71 ^{a}	0.76 ^{a}	3.51 ^{ab}	3.72 ^{bc}
Acteoside Treatments[f]	ACT-50	68.8 ^{ab}	4.9 ^{ab}	67.0 ^{abc}	10.2 ^{d}	19.7 ^{c}	1.62	0.19 ^{b}	0.70 ^{ab}	0.70 ^{abc}	3.41 ^{cd}	3.63 ^{de}
	ACT-75	69.6 ^{ab}	4.6 ^{abc}	66.6 ^{cd}	10.2 ^{d}	20.0 ^{bc}	1.64	0.23 ^{ab}	0.69 ^{abc}	0.67 ^{bcd}	3.35 ^{de}	3.56 ^{e}
	ACT-100	68.8 ^{ab}	4.1 ^{abc}	66.3 ^{de}	10.2 ^{d}	20.4 ^{a}	1.62	0.26 ^{a}	0.64 ^{cd}	0.63 ^{d}	3.26 ^{g}	3.48 ^{f}
Aucubin Treatments[g]	AUC-50	69.2 ^{ab}	4.2 ^{abc}	67.3 ^{ab}	10.5 ^{c}	19.1 ^{de}	1.65	0.09 ^{cd}	0.67 ^{abcd}	0.71 ^{abc}	3.53 ^{ab}	3.75 ^{abc}
	AUC-75	68.5 ^{ab}	3.6 ^{bc}	66.9 ^{bc}	11.0 ^{b}	18.9 ^{e}	1.73	0.09 ^{cd}	0.67 ^{abcd}	0.70 ^{abc}	3.54 ^{ab}	3.78 ^{ab}
	AUC-100	65.6 ^{b}	3.0 ^{c}	66.5 ^{cd}	11.4 ^{a}	18.8 ^{e}	1.76	0.11 ^{c}	0.67 ^{abcd}	0.70 ^{abc}	3.54 ^{a}	3.79 ^{a}
Combined treatment	ACT50+ AUC75[h]	70.5 ^{a}	3.6 ^{bc}	65.9 ^{e}	11.0 ^{b}	19.8 ^{c}	1.76	0.24 ^{a}	0.66 ^{bcd}	0.65 ^{cd}	3.33 ^{ef}	3.57 ^{e}
	ACT75+ AUC50[i]	72.3 ^{a}	3.0 ^{c}	65.8 ^{e}	10.7 ^{c}	20.2 ^{ab}	1.73	0.27 ^{a}	0.62 ^{d}	0.67 ^{bcd}	3.26 ^{fg}	3.49 ^{f}
Catalpol	CAT-50[j]	70.4 ^{a}	4.2 ^{abc}	67.0 ^{abc}	10.5 ^{cd}	19.3 ^{d}	1.71	0.08 ^{cd}	0.69 ^{abc}	0.73 ^{ab}	3.47 ^{bc}	3.68 ^{cd}
SEM		1.81	0.92	0.34	0.31	0.53	0.13	0.03	0.01	0.16	0.09	0.08
Treatments		<0.01	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Time		<0.01	<0.001	<0.01	<0.01	<0.01	0.88	<0.01	<0.01	<0.01	<0.01	<0.01
Treatment × Time		0.38	<0.001	0.05	0.07	0.07	<0.01	<0.01	0.01	<0.01	0.12	0.51
Contrasts, p-value[k]												
ACT50+AUC75 vs. (ACT-50 + AUC-75)/2		0.146	0.148	<.0001	0.003	<.0001	0.042	<.0001	0.048	0.021	<.0001	<.0001
ACT-50 vs AUC-75		0.842	0.029	0.586	0.005	<.0001	0.019	<.0001	0.113	0.833	<.0001	<.0001
ACT75+AUC50 vs. (ACT-75 + AUC-50)/2		0.015	0.004	<.0001	<.0001	<.0001	0.04	<.0001	<.0001	0.03	<.0001	<.0001
ACT-75 vs AUC-50		0.792	0.478	<.0001	0.177	<.0001	0.744	<.0001	0.285	0.002	<.0001	<.0001

[a] total short-chain fatty acids (mmol), [b] net ammonia production (mmol NH_3/g DM incubated), [c] acetate to propionate ratio (A:P), [d] [acetate+butyrate] to [propionate+valerate] (AB:PV), [e] ryegrass control, [f] treatments within the rows correspond to varying doses of acteoside (denoted as ACT) and the numeric values separated by a hyphen indicating the amount of acteoside in mg/g of pasture DM, [g] treatments within the rows correspond to varying doses of aucubin (denoted as AUC) and the numeric values separated by a hyphen indicating the amount of aucubin in mg/g of pasture DM, [h] 50 mg acteoside/g pasture DM added with 75 mg aucubin/g pasture DM, [i] 75 mg acteoside/g pasture DM added with 50 mg aucubin/ g pasture DM, [j] 50 mg catalpol/g pasture DM, [k] p-value of planned contrasts in selected treatments, values with the same letter within the same columns are not significant (p>0.05), SEM: standard error of means.

4.8 Discussion

A key finding of this study was the potential of acteoside and aucubin to reduce CH_4 production from PL pastures. Plantain substrates (PL1, PL2, and PL3) selected with similar chemical composition but varying intrinsic PSM concentrations showed no clear effect of PSM on reducing CH_4 production compared to RG in E1. In E2, to avoid the confounding effects of chemical composition differences, the addition of PSM at levels naturally found in PL mixed pastures (acteoside: ACT-6, ACT-10, ACT-18, ACT-36; aucubin: AUC-6, AUC-9, AUC-12, AUC-24; and additionally catalpol: CAT-10) to RG resulted in similar GP at 12 h, at 24 h and PGP compared to RG. However, a lower %PCH_4 and %CH_4 at 24 h was observed in the AUC-24 and ACT-36 treatments compared to RG, respectively. In E3, the inclusion of a higher level (>50 mg/g of PSM) of acteoside increased GP and reduced %CH_4 by up to 15%. Furthermore, acteoside interaction with aucubin (ACT50+AUC75) produced around 13% greater GP and reduced %CH_4 by up to 9%. Both in E2 and E3, the inclusion of aucubin reduced the $R^{1/2}$GP and increased $T^{1/2}$GP compared to RG and acteoside. The higher aucubin concentrations in E2 (AUC-24) and in E3 (AUC-100), resulting in more time to produce $T^{1/2}$ GP were in agreement with research by Navarrete et al. (2016).

A greater GP with acteoside did not lead to proportionally greater production of CH_4 in treatments. Rather, it reduced the %CH_4 in the gas, suggesting its ability to reduce CH_4 production. The formation of CH_4 in rumen fermentation is closely associated with the profile of SCFA formed (Ungerfeld, 2020). Acetate and butyrate formation promote CH_4 production, while propionate and valerate formation can be considered a competitive pathway for hydrogen use in the rumen, decreasing CH_4 production (Moss et al., 2000; Wilkinson, 2012). A lower A:P or AB:PV ratio can be used as an indication of lower CH_4 production in the rumen (Della Rosa et al., 2021). The treatments which produced less %CH_4 (ACT-100, ACT50+AUC75 in E3 and PL2 when only compared within PL substrates in E1) had lower A:P or AB:PV ratios and considerably higher acteoside concentration. However, an inconsistent result was observed with ACT-36 in E2. Around 7% reduction of %CH_4 at 24 h was observed in ACT-36 treatment in E2, but the A:P or AB:PV ratios were similar to that of RG.

Acteoside, a phenylethanoid glycoside (Rossi et al., 2024), is composed of four moieties: caffeic acid, phenylethyl alcohol and sugars such as glucose and rhamnose (Zhou et al., 2020). The increased GP observed in E3 with acteoside treatments could be due to the increased fermentation of liberated sugars (Navarrete et al., 2016) during non-specific glycoside breakdown in the rumen (Getachew et al., 2002). The fermentation of rhamnose sugar in the rumen might be one of the critical factors in the reduction of CH_4 production (%CH_4), as rhamnose is the only monosaccharide known to produce low CH_4 emissions among available carbohydrates (Südekum, 1994; Sun et al., 2022). A study by Czerkawski and Breckenridge (1972) reported that rhamnose fermentation led to reduced CH_4 production and no significant accumulation of hydrogen gas, suggesting that the hydrogen generated is likely utilised in other metabolic processes rather than CH_4 production. The authors also reported that acetate production initially increased rapidly with rhamnose but then decreased while propionate continued to rise (Czerkawski & Breckenridge, 1972). Similarly, no hydrogen gas production was detected in the batch culture system in the present study. However, a lower A:P ratio and a decrease in %CH_4 production suggested that rhamnose sugar hydrolysis might be one of the main drivers of %CH_4 reduction observed with acteoside.

Aucubin (AUC-24) and catalpol (CAT-10) treatments produced lower CH_4 production but similar gas, %CH_4 and A:P or AB:PV ratios compared to controls in E2. The presence of aucubin in the incubation led to a longer lag in GP in E2, E3 and a longer lag in CH_4 production in E3. This may be due to the antimicrobial properties of aucubin (Navarrete et al., 2016). Aucubin belongs to the secondary metabolite family of iridoid glycosides, and according to Radu et al. (2010), iridoids have a significant effect on different bacterial strain types. Aucubin presence led to a decrease in acetate and propionate production but an increase in butyrate production in E3 (Figure 4.2), suggesting that aucubin may have selectively inhibited the growth of acetate and propionate-producing microbes while having little to no effect on butyrate-producing microbes. A study testing *in vitro* fermentation of *Paulownia* leaf extract containing phenolic compounds, including acteoside and aucubin, reported a reduction in the microbial population that feeds on fibrous plant material. This led to a decrease in acetate producing bacteria (*Fibrobacter succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens)* and an increase in butyrate-producing bacteria (*Butyrivibrio proteoclasticus*) (Nowak et al., 2022). Accordingly, the observed drop in GP rate in the present study with aucubin inclusion could be attributed to the reduction in the microbial community due to the inhibition of specific microbes. Additionally, Nowak et al. (2022) observed a reduction in methanogen counts alongside increased propionate-producing microbes (*Prevotella spp*. and *Megasphaera elsdenii*), resulting in higher propionate production when incubated with *Paulownia* leaf extract. The possible decrease in methanogens might explain the observed reduction in CH_4 production of a few aucubin treatments and %CH_4 in the combined treatments of the present study. However, further research is needed to determine whether acteoside, aucubin, or both are responsible for affecting methanogens in the rumen. The similar increase in propionate production observed in both studies may be attributed to the presence of acteoside. The present study confirms that combined treatments (ACT50+AUC75 or ACT75+AUC50) produced higher propionate levels when acteoside was present. In contrast, individual treatments with aucubin (AUC-24 in E2 or AUC-50, AUC-75 or AUC-100 in E3) did not increase propionate production (Figure 4.2).

Another important finding of the study was that interaction between acteoside and aucubin in treatment ACT75+AUC50 or AUC-100 alone reduced the net NH_3 production in the rumen by nearly half. According to Navarrete et al. (2016), both acteoside and aucubin reduced net NH_3 concentration *in vitro* through two different mechanisms, i.e., acteoside increases microbial protein synthesis by providing additional energy, leading to lower NH_3 in the rumen and in contrast, aucubin reduces NH_3 production due to its antimicrobial activity. In the present study, a lower NH_3 production was observed in ACT50+AUC75 (36% reduction) and ACT75 +AUC50 (46% reduction) compared to RG, suggesting that both mechanisms operate synergistically, providing a greater net reduction in net NH_3 production. It also suggests that greater acteoside concentrations could lead to greater efficiency of microbial protein production rather than antimicrobial inhibition. Similarly, Nowak et al. (2022) observed that acteoside and aucubin together reduced NH_3 levels *in vitro* rumen fermentation by decreasing protozoa, inhibiting proteolytic bacteria (*Butyrivibrio fibrisolvens*) and leading to a shift toward microbes like *Anaerovibrio lipolytica* which are less involved in NH_3 production. Based on that report, we can speculate that in the present study, aucubin may have inhibited certain microbial types that are relevant to NH_3 production;

resulting in lower net NH_3 production in treatments such as AUC-24, AUC-75 and AUC-100. On the other hand, in combined treatments where both acteoside and aucubin were present, while aucubin inhibited ammonia-producing microbes, acteoside may have increased energy availability for other microbes, potentially increasing protein synthesis, leading to a greater reduction in net NH_3 production. However, further study is needed to isolate the effects of acteoside and aucubin, as rumen microbial communities appeared to be influenced by both compounds. Microbial DNA analysis is needed to gain a better understanding of the mechanisms behind the reduction in CH_4, and NH_3 observed in this study; however, it was beyond the scope of this study, so we recommend further research in this area.

4.8.1 Implication of plantain's plant secondary metabolites on dairy greenhouse gas emission.

Plantain substrates (PL1, PL2 and PL3) produced up to 13% less CH_4 production in E1 compared to RG. Similarly, up to 10% less GP production was observed in PL substrates. At 24 h, all PL substrates produced less SCFA than RG. These lower CH_4, GP and SCFA productions may likely be a result of the lower digestibility of the PL substrates used in E1 (OMD of RG (83.2% DM), PL substrates (67.3–70.0 % DM)). Similar findings have been reported where pure PL fed with RG resulted in lower CH_4 and GP, suggesting that RG is of higher quality in those experiments (Della Rosa et al., 2022). The lower digestibility of the PL substrates can negatively affect cows' voluntary intake and reduce productivity (Mertens & Grant, 2020). However, the milk production data from the trial site showed no reduction in yield (Nguyen, 2023), which aligns with several other reports supporting the effectiveness of PL in dairy systems (Box et al., 2017; Nkomboni et al., 2021), suggesting that grazing on PL-mixed pastures provides cows with sufficient energy without negatively impacting their production. However, the RG used in E1 provided a general comparison with PL, as it served more as an experimental control rather than a true comparison because these pastures were harvested from different soil types and under different management practices, both of which can affect pasture quality (Sun et al., 2008). Further research is needed to compare PL pastures with commonly sown ryegrass-white clover pastures grown under similar conditions to make a valid comparison and to explore the potential of plantain in reducing CH_4 emissions.

A 5% reduction in %CH_4 was observed in the PL2 at 12 h compared to other PL substrates. This reduction may be attributed to the higher acteoside content in PL1 (18 mg acteoside/g of DM), as indicated by its lower A:P ratio compared to other PL substrates. Also, in E1, PL substrates produced less net NH_3 in the *in vitro* rumen environment by up to 45% compared to RG. The NH_3 production is influenced by the CP content of pasture (Chaves et al., 2006), and the RG used in this study had around 37% higher CP values than PL substrates, resulting in similar net NH_3 production per g CP incubated among treatments ($p=0.08$). This fact suggested that the lower CP concentration in PL could be the main cause of the net NH_3 reduction in E1. However, in E2, a trend of decreasing net NH_3 production was observed with increasing concentrations of PSM (both acteoside and aucubin) for substrates with similar CP concentrations when naturally occurring PSM levels were tested (Figure 4.1). The results from experiment 3 suggest that aucubin alone is particularly effective in reducing net NH_3 production, and when combined with acteoside, it also contributes significantly to the reduction. The interaction of PSM observed in this study did not contribute to the reduced net NH_3 production in E1, as it was overshadowed by the influence of CP on net NH_3 production.

4.9 Conclusions

The management of CH_4 emissions and N losses from pasture based dairy sector continues to be essential for mitigation of negative environmental impacts. The present study evaluated the potential of PL-mixed pasture and PSM found in PL to reduce CH_4 and NH_3 production *in vitro* in a dose-dependent manner. There was sufficient *in vitro* evidence that acteoside and aucubin decreased CH_4 production, warranting further research on dairy cows grazing PL-mixed pastures. The inclusion of aucubin in the basal feed (RG) led to a longer lag phase in CH_4 production, likely due to anti-microbial activities. Acteoside reduced CH_4 production and altered SCFA molar proportions, by promoting alternative hydrogen utilisation pathways. Additionally, the role of aucubin and acteoside in decreasing NH_3 production *in vitro* was also confirmed in the present study. Although PL substrates tested in this study produced lower CH_4 (up to 13%) and net NH_3 (up to 46%), these reductions were largely attributed to the lower OMD and CP content of PL pasture. When higher concentrations of PSM were tested, they demonstrated significant anti-methanogenic activity and reduced net NH_3 production, highlighting the potential of PSM to reduce CH_4 and N losses. The PSM concentration used in this study was near the maximum reported in the literature, suggesting that targeted plant breeding aimed at increasing PSM content may help reduce CH_4 emissions from PL pastures.

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CHAPTER 5

The impact of plantain (*Plantago Lanceolata* L.) on nitrous oxide emissions via the rhizosphere and animal urine composition and concentration

This chapter has been under review.

Sivanandarajah, K., Horne, D., Saggar, S., Navarrete, S., Kemp, P., Ramilan, T., & Donaghy, D. (2025). The impact of plantain (*Plantago Lanceolata* L.) on nitrous oxide emissions via the rhizosphere and animal urine composition and concentration.

Abstract

Urine patches are a significant source of nitrous oxide (N_2O) emissions due to their concentrated nitrogen (N) content. Plantain (PL, cv. Agritonic) in the dairy cattle diet has been shown to reduce urinary nitrogen (UN) concentration, thereby lowering the UN load in urine patches. Additionally, PL root exudates and urine from ruminants grazing PL can lead to biological nitrification inhibition (BNI), both of which may contribute to reducing N_2O emissions. However, the effectiveness of PL as a mitigation for N_2O emission has not been consistent across all studies. This large-scale field study evaluated the impact of different proportions of PL within ryegrass-white clover (RWC) pasture on N_2O emissions from a poorly drained Tokomaru silt loam soil during spring with different UN sources. Urine was collected separately from cows fed 0% PL in a RWC diet (URWC, 5.5 g N L^{-1}), and ≤20% PL in a RWC based diet (UPL, 5.8 g N L^{-1}). A third treatment was created by diluting some of the UPL (dil_UPL, 4.1 g N L^{-1}), with all values given as N concentration. The urine was applied to pastures of 0% PL (RWC), 30% PL (PLL), and 40% PL (PLM) using a device which simulates a cattle urination event, allowing natural spread of urine into the soil. Each pasture type received water (NoU) as a control, with each treatment replicated five times (n=60). The N_2O fluxes were measured using the static chamber method. A tendency for lower cumulative N_2O emissions was observed as PL content increased in pasture compared to RWC ($p<0.06$), along with a lower emission factor (EF_3, % N_2O emitted per unit of N applied) in PLM (28% lower) compared to RWC ($p<0.09$). Urine types significantly affected N_2O emissions ($p=0.03$), with dil_UPL producing 36% lower cumulative N_2O emissions than URWC and urine type did not significantly affect EF_3. However, PL content tended to lower EF_3 values. On average, the EF_3 value while combining both 30% and 40% PL in the pasture was 28% lower compared to RWC ($p<0.03$), indicating possible BNI activity associated with PL pasture. Under conditions conducive to greater N_2O emissions, maintaining 30–40% PL in the pasture could reduce N_2O

emissions up to 27%. This reduction was attributed to BNI activity from the PL rhizosphere rather than differences in urine-N concentrations.

5.1 Introduction

Over the past 150 years, rising atmospheric nitrous oxide (N_2O) concentrations have contributed to stratospheric ozone depletion and climate change (Ravishankara et al., 2009), with the present concentration in the atmosphere of 336 parts per billion and a rate of increase of 2% per decade (Tian et al., 2024; Tian et al., 2020). Nitrous oxide emissions (~8%) and enteric methane emissions (~42%) from agriculture make up half of New Zealand's total greenhouse gas (GHG) emissions in 2022 (MfE, 2024). In accordance with the Paris Agreement, the New Zealand Government committed to reducing the country's GHG emissions by 30% below 2005 levels by 2030 (MfE, 2020). However, mitigating N_2O emissions in agricultural soils requires a combination of approaches involving soil biochemical processes and management practices (Venterea et al., 2012).

In grazed dairy pastures, cattle urine patches are the main source of nitrogen (N), which is lost to the environment (Selbie et al., 2015). Urine patches have a large N load, which often exceeds the pasture's uptake capacity (Di & Cameron, 2007). Some of the excess N in urine patches is lost as N_2O emissions to the atmosphere (de Klein et al., 2020). Urine and dung patches from dairy cattle contribute 32.4% of agricultural soil GHG or around 5% of total agricultural emissions in New Zealand (MfE, 2024). The emission factor (% N_2O emitted as a proportion of urinary N applied) for urine patches is 0.98% which is significantly higher than for dung patches (0.12%) (van der Weerden et al., 2020), indicating the need for practices that reduce the quantities of N in urine patches that is at risk of being lost via gaseous and leaching pathways. Nitrous oxide emissions from soils are primarily driven by two microbial processes: nitrification and denitrification (de Klein et al., 2003). Nitrification is an aerobic process in which ammonium (NH_4^+) is oxidised to nitrate (NO_3^-), with N_2O generated as a byproduct (Simon et al., 2019). In contrast, denitrification occurs under anaerobic conditions, where nitrate (NO_3^-) is reduced to various gaseous forms of N, including N_2O (Simon et al., 2019) and the only known pathway for N_2O consumption is complete denitrification which produces N_2 from N_2O (Ding et al., 2024). However, multiple factors, including soil properties, climatic conditions, plant type, plant uptake, soil microorganisms, animal diet and urine patch size regulate the N_2O production processes in field environments (de Klein et al., 2020; López-Aizpún et al., 2020; Luo et al., 2019).

Plantain (PL) has been recognised for its ability to offer environmental benefits such as reduced N leaching at paddock level, reduction of GHG emissions in grazed pastureland (Navarrete et al., 2023; Pinxterhuis et al., 2024). There are a few known mechanisms proposed for PL pastures that can influence N losses to the environment when compared with cows fed a ryegrass/clover (RWC) based diet, such as i. urinary N dilution, ii. partitioning more N into dung and iii. biological nitrification inhibition (BNI) (Pinxterhuis et al., 2024). When ruminants ingest PL, it results in urinary N dilution and low urinary N excretion, which can result in lower urinary N (UN) loading rates in the localised urine patches (Box et al., 2017; Mangwe et al., 2019; Minnee et al., 2020; Navarrete et al., 2022). The higher non-structural carbohydrate to structural carbohydrate ratio and the presence of plant secondary metabolites (PSM) in PL, such as acteoside and aucubin, have been shown to reduce the ammonia ruminal production (Navarrete et al., 2016). This may be contributing to the influence PSM in PL in changing the N partitioning, and directing more N into dung rather than urine (Pinxterhuis et al., 2024). The BNI activity by PL has been modulated by the release of PSM in PL via root exudates (Peterson et al., 2023). Another input of PSM in soils is possibly decaying of leaf and root material, which also contributes to inhibiting soil nitrification (Gardiner et al., 2020; Rodriguez et al., 2021; Simon et al., 2021), These PSM found in PL and their derivatives can be also excreted in the urine of animals, which may also play a role in influencing soil nitrification and reducing N_2O emissions (Judson et al., 2019; Peterson et al., 2022).

Although PL has shown advantages as a natural mitigation tool for N_2O emissions in a number of studies (Simon et al., 2019; Vi et al., 2023), other researchers have found its effectiveness to be variable (Earl-Goulet et al., 2021; Podolyan et al., 2020). Several factors, beyond the proportion of PL in pasture, influence its ability to reduce N_2O emissions. For example, Luo et al. (2018), Simon et al. (2019) and Vi et al. (2023) reported reductions in N_2O emissions attributed to BNI activity and low UN load in PL pasture, based on experiments conducted in well-drained, moderately well-drained, and poorly-drained soils, respectively, during autumn. In contrast, Podolyan et al. (2020) did not observe BNI activity of PL in free-draining Balmoral stony silt loam, but a reduction in N_2O emissions was attributed to low UN load, irrespective of pasture type. Similarly, Earl-Goulet et al. (2021) did not observe N_2O reductions for similar UN load in the same soil type during late summer-late autumn suggesting the soil type may have influenced the results in BNI expression. Ding et al. (2024) observed a reduction in N_2O emissions in well-drained Allophanic soil but not in poorly drained Gley soil during the autumn-winter period, attributing the reduction to BNI expression, which was specific to soil type and influenced by soil moisture levels.

In general, soil type and environmental conditions may significantly influence plantain’s BNI effect. To fully gauge the potential of plantain’s rhizosphere to express BNI activity, additional research is needed to quantify N_2O emissions from urine patches in various climates and soil types. This knowledge, along with an improved understanding of the factors influencing N loss pathways, will help the development of potential strategies to mitigate N_2O emissions.

Therefore, the present study evaluated the effect of PL during spring under warmer soil temperatures coinciding with wet conditions, which is likely to increase N_2O emissions in the fine-textured, artificially drained Tokomaru silt loam soil. It was hypothesised that a plantain-mixed pasture would emit less N_2O compared to a ryegrass-white clover sward because of diet and the BNI effect.

5.2 Materials and methods

5.2.1 Experimental site description and pasture composition

The experimental site was located at Massey University's PL trial site (DairyNZ, 2023) on Dairy 4 farm, Palmerston North, New Zealand (40º23′27″ S 175º36′44″ E). The site was maintained under a rain-fed irrigation system and had been rotationally grazed with dairy cows for four consecutive years, receiving (at least) 65% of the diet from the pasture treatment and 35% of the diet as supplements. Massey Dairy 4 can be classified as a high input system five farm according to DairyNZ farm systems classification (Eastwood et al., 2017). The soil type of the study site is classified as fine textured artificially drained Tokomaru silt loam, an Argillic-fragic Perch-gley Pallic (Hewitt, 2010).

Three pasture treatments selected from those established by direct drilling in autumn 2019. These consisted of variable sowing rates of perennial ryegrass (cv. ONE50) and PL (cv. Agritonic) along with white clover (cv. Tribute). Sowing rates (kg ha^{-1}) were adjusted based on PL and ryegrass (RG) seeds for each treatment *viz*. RWC (0PL+20RG), PLL (4PL+15RG), PLM (7PL+10RG), with clover sown at a constant rate of 3 kg ha^{-1}. Over time, PL populations declined, requiring additional seeding to maintain treatment effects. In autumn 2020, 3 and 6 kg ha^{-1} of PL were undersown on PLL and PLM. In spring 2021, 4 and 6 kg ha^{-1} of PL were undersown on PLL and PLM. Finally, in autumn 2023, 4 and 6 kg ha^{-1} of PL were undersown on PLL and PLM, respectively. The pastures received urea fertiliser at 30 kg N ha^{-1} in October 2021, February, and April 2022. The grazing and pasture management are described in detail by Nguyen (2023).

For each of the five replicates of the pasture treatments (RWC, PLL and PLM), a representative area of 2 m × 20 m in each plot was fenced off to exclude grazing for the seven months prior to the experiment to prevent excreta deposition from grazing cows and to minimise the effect of previous dung and urine patches (de Klein et al., 2014). To ensure representative sampling, sections were identified within the excluded area, with approximately 20 plants per m^2 for the PLL treatment and 30 plants per m^2 for the PLM treatment. These areas were marked, and later, chambers were placed over the designated sections.

5.2.2 Cow diet and urine collection

Cows were grazed in either conventional RWC or PL-mixed pasture (PLL or PLM). The PL mixed pastures contained about 20% of the DM in the diet due to the supplement offered was 6.33 kg DM $cow.day^{-1}$ (Table 5.1). Cows were transitioned to their respective diets over 6 days, followed by 2 days of urine collection (Massey University Animal Ethics Approval No. 22/40). Urine collection occurred during morning and afternoon milking at the milking shed. The urine samples were bulked for each treatment and stored at 4ºC to prevent urea hydrolysis until application. An aliquot of each urine type was taken for immediate total N analysis at the Nutrition Laboratory, Massey University. The similarity in PL content in the diet of the PLL and PLM (on average 17% and 21% PL, respectively) resulted in urine samples of similar UN concentrations. Therefore, a diluted PL urine sample, with UN concentrations approximating a PL content in the diet of 27-30% DM, was prepared by adding RO water to the urine of PL-fed cows at a 3:1 v/v ratio based on concentrations reported at such level of inclusion to diet in previous reports (Nguyen et al., 2024; Vi et al., 2023). As a result, the total N concentration of urine from RWC-fed cows (URWC) and urine from PL-fed cows (UPL) were 5.5 g L^{-1} and 5.8 g L^{-1}, respectively, while the diluted urine of PL-fed cows (dil_UPL) urine had a concentration of 4.1 g L^{-1} (Table 5.2).

Table 5.1. Species composition of ryegrass-white clover pasture (RWC) and the plantain (PL) in both the pasture and the cows' diet during grazing for urine collection.

Treatment	RWC[a]	PL-mixed pasture[b]
Grass (% DM)	91.3 ± 2.0	57.5 ± 3.3
White clover (% DM)	3.9 ± 1.2	6.0 ± 1.3
Plantain in the pasture (% DM)	0	32.7 ± 3.6
Plantain in the diet[c] (% DM)	0	19.1 ± 2
Dead materials (% DM)	4.1 ± 0.7	2.4 ± 0.5
UN concentration[d] (g N L^{-1})	5.5	5.8

[a]botanical and diet composition of cows fed with conventional ryegrass-white clover pasture, [b]botanical and diet composition of plantain mixed pasture [c]plantain in the diet after 6.33 kg DM cow.day^{-1} supplement was offered, [d]urinary nitrogen concentration of bulk urine samples, ± standard error of means.

5.2.3 Experimental design

The experimental design was a 3 × 3 factorial, with three sward treatments and three urine treatments, each replicated five times. The sward treatments were RWC, ~30% PL in the sward (PLL), and ~40% PL in the sward (PLM). The urine treatments included URWC, UPL, and dil_UPL. Additionally, a control treatment (NoU, water treatment) was included in the trial design (Table 5.2).

The pasture was cut 5 cm above ground level; residuals were removed from the selected areas, and a galvanised metal ring (~80 cm diameter) was inserted 10 cm into the ground before the urine application for N_2O measurements. Similarly, soil sampling areas for each replicate were selected adjacent to the gas sampling spots, with the pasture cut at the same height before urine application.

On October 5th, 2023, urine patches were created using a frame with a container and tap positioned 1.2 m above the ground to simulate a typical dairy cow urination. The flow rate was set to approximately 0.2 L per second (Haynes & Williams, 1993), mimicking natural cow urination flow. At the centre of the ring, 2 L urine was voided and allowed to spread naturally, corresponding to each treatment (Table 5.2). Control treatments received the same volume of water. Similarly, urine (or NoU) was applied with a 0.5 m buffer between patches, and a 0.5 m diameter plastic ring was placed and labelled to mark the area for soil sample collection.

Table 5.2. Treatments, urinary nitrogen contents, and application rates.

Pasture	Plantain % in the pasture	Urine Source	Urinary nitrogen concentration $(g\ N\ L^{-1})$	Treatment name
Ryegrass-white clover (RWC)	-	Ryegrass-white clover (URWC)	5.5	RWC + URWC
		Plantain diet (UPL)	5.8	RWC + UPL
		Diluted urine from a plantain diet (dil_UPL)	4.1	RWC + dil_UPL
		Water / No urine (NoU)	-	RWC + NoU
Low Plantain (PLL)	30	Ryegrass White clover (URWC)	5.5	PLL + URWC
		Plantain diet (UPL)	5.8	PLL + UPL
		Diluted urine from a plantain diet (dil_UPL)	4.1	PLL + dil_UPL
		Water / No urine (NoU)	-	PLL + NoU
Medium Plantain (PLM)	40	Ryegrass White clover (URWC)	5.5	PLM + URWC
		Plantain diet (UPL)	5.8	PLM + UPL
		Diluted urine from a plantain diet (dil_UPL)	4.1	PLM + dil_UPL
		Water / No urine (NoU)	-	PLM + NoU

5.2.4 Nitrous oxide sampling

Nitrous oxide gas samples were collected using the static chamber method. Each chamber had a 0.80 m internal diameter, a height of 0.30 m, and a volume of 151 L. After urine application, N_2O fluxes were measured 13 times over 54 days. On each sampling day, the chambers were placed inside galvanised metal rings and sealed using inflated bicycle tubes to ensure a gas-tight seal between the metal ring and the chamber. The chambers were covered with white wet towels to help maintain a constant temperature inside. Due to the spatial distribution of chamber arrangements and the large volume of the chambers, they were closed for 2 h, and gas samples were taken at 0, 60, and 120 minutes. Time 0 sample was taken as soon as each chamber was closed. Gas samples (25 mL) were collected from the port fitted to the chamber lid using a three-way tap connected to a plastic syringe and injected through a septum into pre-eventuated vials.

Nitrous oxide analysis was conducted using Shimadzu GC-17a and Shimadzu GC2010 gas chromatographs equipped with a ^{63}Ni-electron capture detector with oxygen-free N as a carrier gas (Shimadzu Oceania Pty Ltd, Nelson, New Zealand) (Saggar et al., 2007).

Samples were collected between 10:00 a.m. and 1:00 p.m. on each sampling day (van der Weerden et al., 2013). For the first four weeks, N_2O sampling was done twice a week, then weekly until background levels were reached. Three background air samples were also taken on each sampling day.

Hourly N_2O emissions (mg N m^{-2} h^{-1}) were calculated for each chamber according to (de Klein et al., 2003), from the following equation.

$$N_2O\ \text{flux} = \frac{\delta N_2O}{\delta T} \times \frac{M}{Vm} \times \frac{V}{A}$$

Where:

N_2O flux : Hourly N_2O emission (mg $m^{-2}h^{-1}$),

δN_2O : The increase in headspace N_2O over time (µL L^{-1}),

δT : Enclosure period (hours),

M : Molar weight of N in N_2O (g mol^{-1}),

Vm : Molar volume of gas at the sampling temperature (L mol^{-1}),

V : Headspace volume (m^3),

A : Area covered (m^2).

During each sampling, the hourly emissions were converted to daily estimates for each chamber and integrated over time to calculate the cumulative emissions over the measurement period. Net N_2O emission from a urine patch was calculated by subtracting the respective cumulative background emissions from the non-urine application chambers (NoU chambers) from the cumulative emissions of the urine patch.

Net emission from a urine patch = Cumulative emission from a chamber - background emission from NoU treatment

The N_2O emission factors (EF_3) for each urine patch were calculated using the equation below.

$$\mathrm{EF_3} = \frac{\text{Net N}_2\text{O total (per chamber)}}{\text{Urine-N applied per chamber}}$$

Where:

EF_3 : N_2O-N emitted as % of N applied,

Net N_2O-N total (chamber) : Net N_2O-N emission inside the chamber (emission from urine patch).

Daily total rainfall and mean 10 cm soil and ambient air temperatures were obtained from the nearest meteorological station located ~1 km from the experimental site (NZ Meteorological Service based at AgResearch Ltd, Palmerston North).

5.2.5 Soil sampling and measurements

Soil samples were collected in the designated areas on day 0 and on days 1, 7, 14, 21, and 42 after urine application. At each sampling, two soil cores (25 mm in diameter) were taken from depths of 0–50 mm and 50–100 mm. After collecting the cores, the soil holes were filled with sealed plastic tubes in a similar dimension to minimise any effects on soil aeration. These cores were bulked by depth, and plant roots were removed before the samples were analysed for pH, moisture content, and mineral N.

To assess ammonium (NH_4-N) and nitrate (NO_3-N) content, a 5 g sub-sample of the field-moist soil was extracted with 30 mL of 2M KCl at a 1:6 soil-to-extractant ratio. This mixture was shaken on an end-over-end shaker for one hour, then the extract was filtered through Whatman no. 41 filter paper for colourimetric determination using a Technicon AutoAnalyser. Another 5 g portion of the field moist soil was oven-dried at 105°C for 24 h to measure gravimetric soil water content. Water-filled pore space (WFPS %) was calculated from gravimetric soil moisture content, bulk density of the soil in each plot and an assumed particle density of 2.65 Mg m^{-3} (Simon et al., 2019). Soil pH was measured in a ratio of soil: deionised water =1:2.5 (w/v).

5.2.6 Pasture analysis

Pasture inside the chamber was harvested at 5 cm height twice during the experimental periods (at day 30 and 55 after treatment applications). The net pasture accumulated dry matter (DM) yield (DM ha^{-1}) was calculated for each chamber, and the botanical composition was reported as DM% from a subsample of the cut pasture, with components manually separated. The herbage was oven-dried at 65ºC for 48 h and weighed. The dried herbage was finely ground and analysed for total N concentration for each species component of the pasture. Total N was determined by Dumas combustion (AOAC, 2020; method 968.06).

5.2.7 Statistical analyses

Data were analysed in the statistical software R version 4.3.0 (R Core Team, 2024) using mixed models in packages 'lme4' (Bates et al., 2014) and treatment means were compared using 'emmeans' (Lenth R, 2023). The normality of data was assessed using the Shapiro-Wilk test. Cumulative N_2O emissions, EF_3, soil mineral N, total N and pasture DM were analysed pasture and urine sources as fixed effects. Significant and marginal effects were determined at $p<0.05$ and $0.05<p< 0.10$, respectively, followed by *Tukey's* test.

5.3 Results

5.3.1 Pasture production during nitrous oxide trial

Despite some variability among replicates, the PL pasture composition in PLL and PLM aligned with the targets of 30% and 40%, respectively. In response to urine application, the net accumulated pasture yield of the three pasture types increased compared to the respective control treatments (NoU). On average around a 14% increase in cumulative pasture yield was observed in PLM and PLL, respectively, compared to RWC pasture ($p<0.01$), while urine sources had no significant effect on cumulative pasture yield (Table 5.3). Cumulative N uptake by each pasture type was statistically similar, but a significant effect of urine source on cumulative N uptake was observed, where the dil_UPL application yielded lower N uptake by around 14% compared to the URWC application ($p=0.01$). Approximately 31% higher DM production and 30% greater N uptake were observed in the PLM+URWC and/or PLL+URWC compared to RWC+dil_UPL, which had the lowest values among all urine-applied treatments and exhibited the most significant differences ($p<0.05$).

Table 5.3. Pasture accumulation during the experiment, cumulative DM production (kg DM ha^{-1}) and nitrogen (N) uptake by pasture (kg N ha^{-1}) in simulated urine patches when different sources of urine were applied.

Treatments	Urine source	Pasture type	Average grass %	Average clover %	Average plantain %	Average weed %	Average dead material %	Cumulative N uptake by grass (kg N ha^{-1})	Cumulative N uptake by clover (kg N ha^{-1})	Cumulative N uptake by plantain (kg N ha^{-1})†	Net cumulative DM (kg ha^{-1})	N uptake (kg ha^{-1})
RWC+URWC	URWC	RWC	77.2 a	12.9	0.9 b	1.9	7.1	134.3 ab	31.2	–	4900 abcd	181 ab
RWC+UPL	UPL	RWC	81.2 a	13.0	0.1 b	1.7	4.1	152.8 a	30.0	–	5389 abc	194 ab
RWC+dil_UPL	dil_UPL	RWC	78.4 a	12.2	0.2 b	1.9	7.4	115.1 bc	26.1	–	4405 bcde	155 bc
RWC+NoU	NoU	RWC	70.9 ab	19.0	1.3 b	1.3	6.2	72.8 de	29.9	–	3321 e	111 c
PLL+URWC	URWC	PLL	49.8 c	10.8	31.0 a	4.6	3.8	100.7 bcd	26.3	59.7 abc	5721 a	203 a
PLL+UPL	UPL	PLL	56.6 bc	4.1	32.2 a	2.3	4.9	97.9 cd	9.1	57.7 abc	5414 ab	177 ab
PLL+dil_UPL	dil_UPL	PLL	54.2 bc	8.6	31.9 a	1.7	3.6	90 cd	19.9	58 abc	5549 ab	177 ab
PLL+NoU	NoU	PLL	48.5 c	9.7	33.6 a	3.4	4.8	51.2 e	15.4	38.5 c	4020 de	114 c
PLM+URWC	URWC	PLM	44.2 c	6.8	43.5 a	1.2	4.3	89.2 cd	17.6	87.8 a	5872 a	205 a
PLM+UPL	UPL	PLM	46.2 c	8.1	40.2 a	1.4	4.2	82.4 cde	18.8	72.8 ab	5421 ab	183 ab
PLM+dil_UPL	dil_UPL	PLM	41.9 c	8.7	41.2 a	4.6	3.6	75.1 de	21.0	73.1 ab	5600 ab	184 ab
PLM+NoU	NoU	PLM	42.2 c	9.6	41.6 a	2.0	4.6	49.7 e	15.5	47.1 bc	4142 cde	120 c
		SEM	3.88	3.00	2.98	0.98	1.08	7.27	7.26	7.43	257	9.30
		p-values*										
		Pasture type (P)	<0.01	0.11	<0.01	0.41	0.04	<0.01	0.20	<0.01	<0.01	0.21
		Urine source (U)	0.43	0.76	0.92	0.45	0.74	0.02	0.68	0.53	0.37	0.01
		Interaction (P×U)	0.95	0.72	0.97	0.06	0.24	0.23	0.75	0.68	0.21	0.13

RWC: standard ryegrass-white clover pasture; PLL: pasture containing ~30% plantain (PL); PLM: pasture containing ~40% PL; URWC: urine collected from RWC-fed cows; UPL: urine collected from PL-fed cows; dil_UPL: diluted urine of PL-fed cows; NoU: control (water) treatments. *except for the cumulative N uptake by PL (kg N ha^{-1}), p-values were presented for the (3 × 3) factorial ANOVA of three pasture types (PLL, PLM, and RWC) and three urine sources (UPL, dil_UPL, and URWC), excluding control treatments (NoU) from the analysis. †p-values and letters were presented for two types of plantain pastures (PLL and PLM) and three urine sources (UPL, dil_UPL, and URWC), excluding control treatments (NoU) from the analysis, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means.

5.3.2 Nitrous oxide emissions and differences in EF_3

Daily N_2O fluxes showed temporal variations, with emissions from all treatments steadily increasing following urine application and peaking at 7 and again at 14 days after application (DAA). There were some significant differences at 4 DAA. At 4 DAA, the highest daily emission was observed in the RWC+ URWC treatment, while the lowest was recorded in the PLM+ dil_UPL treatment, and all urine treatments in the PLM pasture emitted lower daily N_2O emissions compared to RWC+URWC. By day 18, despite some minor fluctuations, N_2O emissions reached the background levels, and no further changes were observed till the termination of measurements at day 52 (Figure 5.1.A).

Marginal and significant differences were observed between pasture type ($p=0.06$) and urine type ($p=0.03$), respectively, in cumulative N_2O emissions per urine patch; however, no interactions were detected. On average, urine patches (UPL, URWC or dil_UPL) deposited in RWC pasture emitted higher cumulative N_2O-N emissions (0.23 g) compared to PLM pasture (0.16 g), while emissions from patches in PLL (0.18 g) did not differ significantly from either RWC or PLM. Cumulative N_2O emissions were significantly affected by urine type, with dil_UPL emitting 37% lower emissions than URWC ($p<0.03$). The lowest cumulative and net emissions from a urine patch were recorded in the PLL+ dil_UPL treatment (0.11 g N_2O-N urine patch^{-1}), which was approximately 55% lower than the highest-emitting treatment, RWC+URWC (Table 5.4).

Emission factors ranged from 1.22 (PLL+UPL) to 2.24 (RWC+URWC), with marginal significance observed between PLM and RWC pastures ($p<0.09$). Urine type did not have a significant effect and there were no interactions. Compared to the RWC+URWC (highest EF_3) treatment, EF_3 values were numerically, if not significantly, lower by 46% and 38% in the PLM+UPL (lowest) and PLL+UPL (second lowest) treatments, respectively.

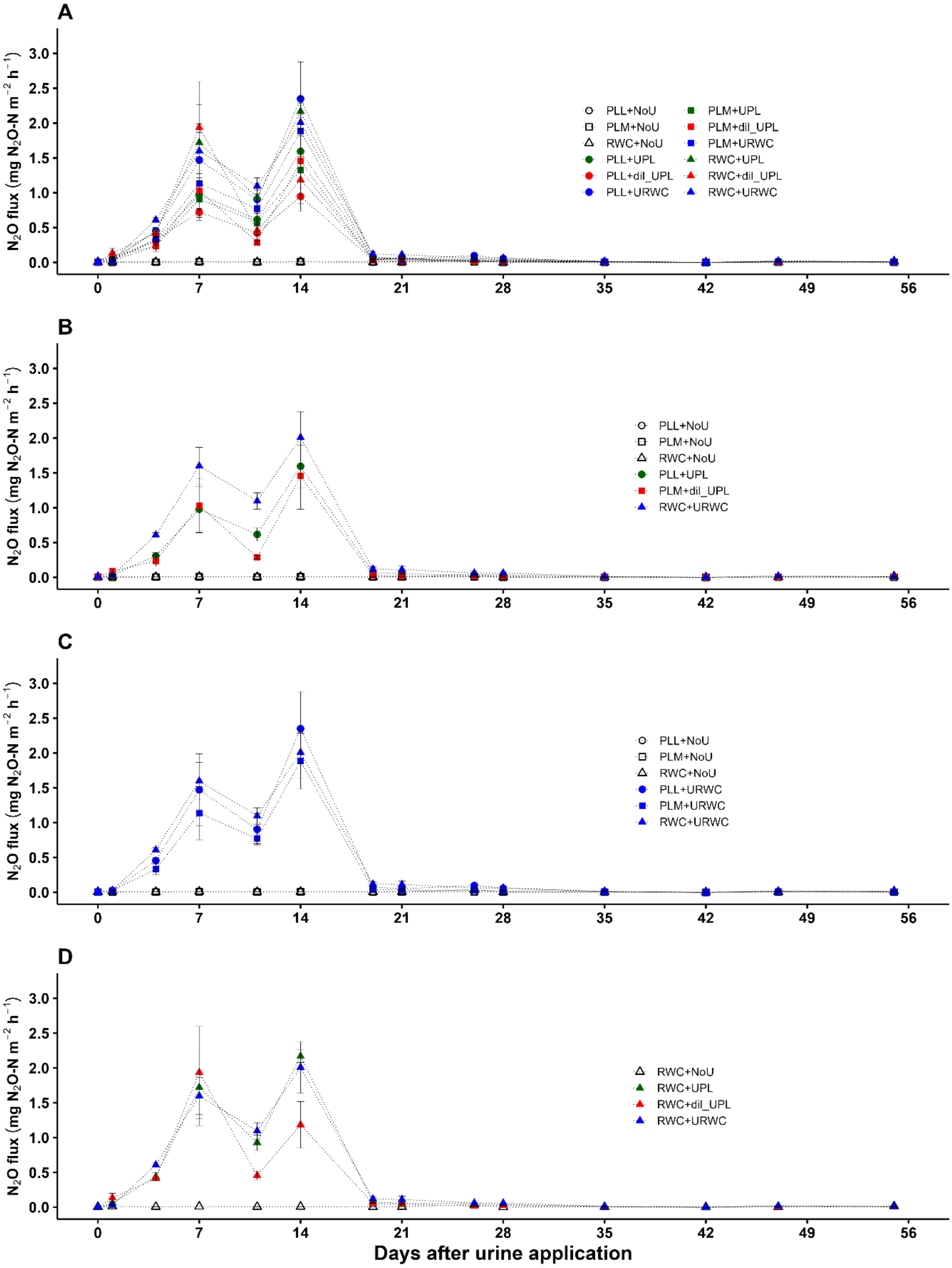

Figure 5.1. Nitrous oxide fluxes from urine patches in swards of standard ryegrass-white clover (RWC) or containing proportions of plantain of ~30% (PLL), ~40% (PLM); and treated with urine from cows fed on diets with standard ryegrass-white clover pasture (URWC), ~20%

plantain (UPL) or diluted urine of ~20% plantain fed cows (dil_UPL). All pasture treatments included a non-urine control (NoU, water). (A), comparison of all 12 treatments in the experiment. (B), effect of plantain in the pasture and plantain in the diet. (C), effect of plantain in the pasture treated with URWC. (D), comparison of different urine sources (URWC, UPL and dil_UPL) applied to RWC pasture. Error bars denote the standard error of the mean (n=5).

Table 5.4. Cumulative nitrous oxide (N_2O) emissions, net N_2O emissions (g N_2O-N urine patch^{-1}) and emission factors (EF_3) from low plantain (PLL), medium plantain (PLM) and ryegrass-white clover (RWC) pastures when urine collected from cows fed with RWC, PLL and diluted PLL were applied.

Treatment	Urine type	Pasture type	Urinary N concentration (g N L^{-1})	Cumulative N_2O (mg N_2O-N chamber^{-1})[a]	EF_3[b]
RWC+URWC	URWC	RWC	5.5	252.4 a	2.24
RWC+UPL	UPL	RWC	5.8	246.7 a	2.08
RWC+dil_UPL	dil_UPL	RWC	4.1	184.5 a	2.18
RWC+NoU	NoU	RWC	–	5.5 b	–
PLL+URWC	URWC	PLL	5.5	246.5 a	2.21
PLL+UPL	UPL	PLL	5.8	166.4 a	1.4
PLL+dil_UPL	dil_UPL	PLL	4.1	114.4 ab	1.35
PLL+NoU	NoU	PLL	–	3.5 b	–
PLM+URWC	URWC	PLM	5.5	191.3 a	1.72
PLM+UPL	UPL	PLM	5.8	143.7 ab	1.22
PLM+dil_UPL	dil_UPL	PLM	4.1	141.9 ab	1.7
PLM+NoU	NoU	PLM	–	2.4 b	–
			SEM	31.5	0.36
			p-value*		
			Pasture type	0.06	0.09
			Urine type	0.03	0.24
			Pasture × urine	0.70	0.71

[a]total N_2O emissions over the experimental period from each treatment, [b]emission factor (N_2O-N emitted as a percentage of N applied), RWC: standard ryegrass-white-clover pasture, PLL: pasture containing ~30% plantain (PL), PLM: pasture containing ~40% PL, URWC: urine collected from RWC-fed cows, UPL: urine collected from PL-fed cows, dil_UPL: diluted urine of PL-fed cows, NoU: control (water) treatments, *control (NoU) treatments were not included in the 3 × 3 factorial ANOVA analysis, values with the same letter within the same columns are not significant ($p>0.05$), SEM: standard error of means, –: not applicable.

Due to the similarity in PL% among replicates between treatments (Table 5.3), the two PL treatments (PLL and PLM) were combined into a PL group (PLP), while PL-free pastures were grouped as PL0. Likewise, given the similar UN concentrations in UPL and URWC (Table 5.3), these were combined as high UN (H-UN), with dil_UPL categorised as low UN (L-UN). When this scenario was tested to understand the effect of pasture with and without PL and high and low UN concentration using a 2 × 2 factorial design, significant differences were observed in N_2O emissions and EF_3 (Table 5.5).

The presence of PL in the pasture (PLP, on average 30–40% PL) reduced N_2O emissions by 26% ($p<0.05$) and EF_3 values by 27% ($p<0.05$) compared to PL-free pastures (PL0, i.e. RWC). Lower UN concentration decreased N_2O emissions by 27% ($p<0.05$) relative to higher UN concentration, while EF_3 values were unaffected.

Table 5.5. Effects of plantain pasture (with and without plantain) under different urinary nitrogen concentrations (high and low) on N_2O emissions from cattle urine patches.

Pasture type	**Urine type**	**Average UN concentration ($g\ N\ L^{-1}$)**	**Net N_2O ($g\ N2O\text{-}N$ urine $patch^{-1}$)[a]**	**EF_3[b]**
PLP (PLL+PLM)	H-UN (URWC+UPL)	5.65	0.18 ± 0.02	1.64 ± 0.19
PLP (PLL+PLM)	L-UN (dil_UPL)	4.10	0.13 ± 0.02	1.53 ± 0.27
PL0 (RWC)	H-UN (URWC+UPL)	5.65	0.24 ± 0.02	2.16 ± 0.17
PL0 (RWC)	L-UN (dil_UPL)	4.10	0.18 ± 0.03	2.18 ± 0.39
		p-value		
		Pasture type (P)	0.03	0.03
		Urine type (U)	0.02	0.80
		P × U	0.91	0.81

[a]net N_2O emission = cumulative N_2O emission from a chamber – background N_2O emissions, [b]emission factor (N_2O-N emitted as a percentage of N applied), PLP: plantain (PL) within ryegrass-white clover pasture containing 30–40% of PL (n=30), PL0: ryegrass-white clover pasture (n=15), H-UN: higher urinary nitrogen concentration (n=30), L-UN: lower urinary nitrogen concentration (n=15), PLL: pasture containing ~30% PL, PLM: pasture containing ~40% PL, URWC: urine collected from RWC-fed cows, UPL: urine collected from PL-fed cows, dil_UPL: diluted urine of PL-fed cows, values marked ± standard error of means, statistical significance declared at $p<0.05$.

5.3.3 Weather and soil moisture condition

During the spring of 2023 (1st October–29th November, where urine was applied on 5th October), the total rainfall was 151 mm. Cumulative rainfall during the first 14 days after urine application, where the higher peaks of N_2O were measured, was 52 mm, and during the first 4 weeks, it was 90 mm (Figure 5.2). The average air and soil temperature during the first 14 days was 12.9, 11.9°C and during the first 4 weeks, it was 13.6 and 13°C, respectively. Both soil and air temperatures were lowest at the beginning of the trial and increased as the trial progressed. The average air and soil temperatures were 14.1 and 14.3°C during the whole experimental period.

The WFPS% did not differ significantly among treatments on any sampling day. The highest values, around or above 75%, were observed during the first three weeks of the experiment across all treatments. During the first two weeks, when the highest N_2O emissions were observed, all urine treatments had WFPS levels exceeding 80%. The WFPS measured on 21 DAA did not reflect the climate data around this period (Figure 5.2). Presumably, this is explained by the differences in rainfall at the site and at the weather station. A considerably lower WFPS% (ranging from 26–42%) was observed among treatments 42 days after urine application.

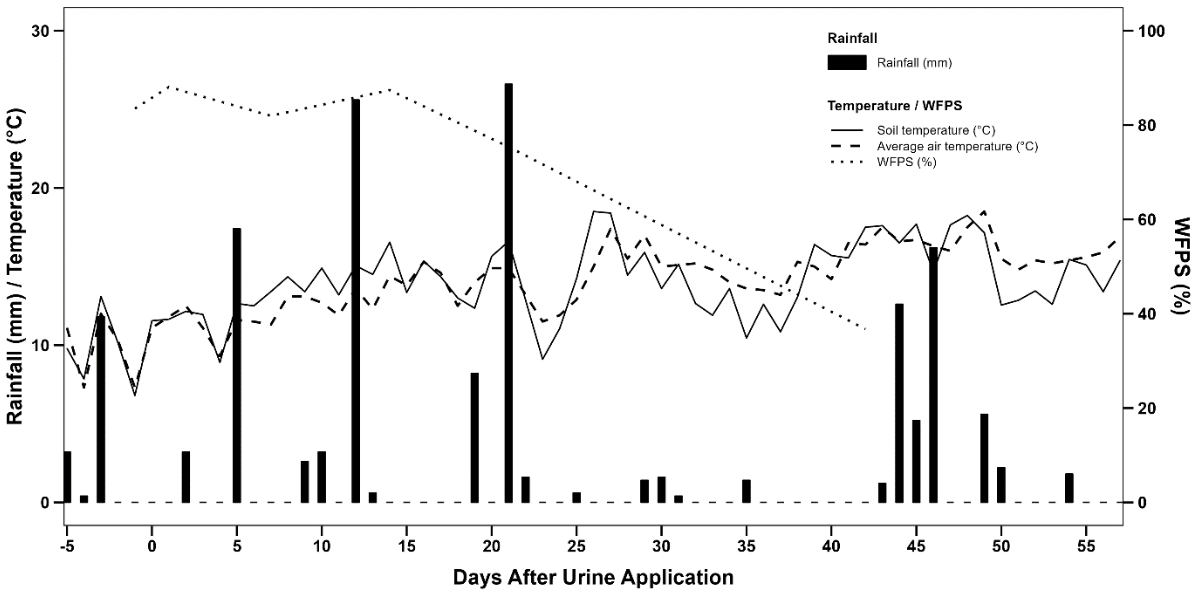

Figure 5.2. Daily precipitation (mm), soil (100 mm depth) and average air temperatures (°C) over 60 days during the field trial (weather station data) and average soil water-filled pore space (WFPS%) measured in the field at 0–100 mm depth.

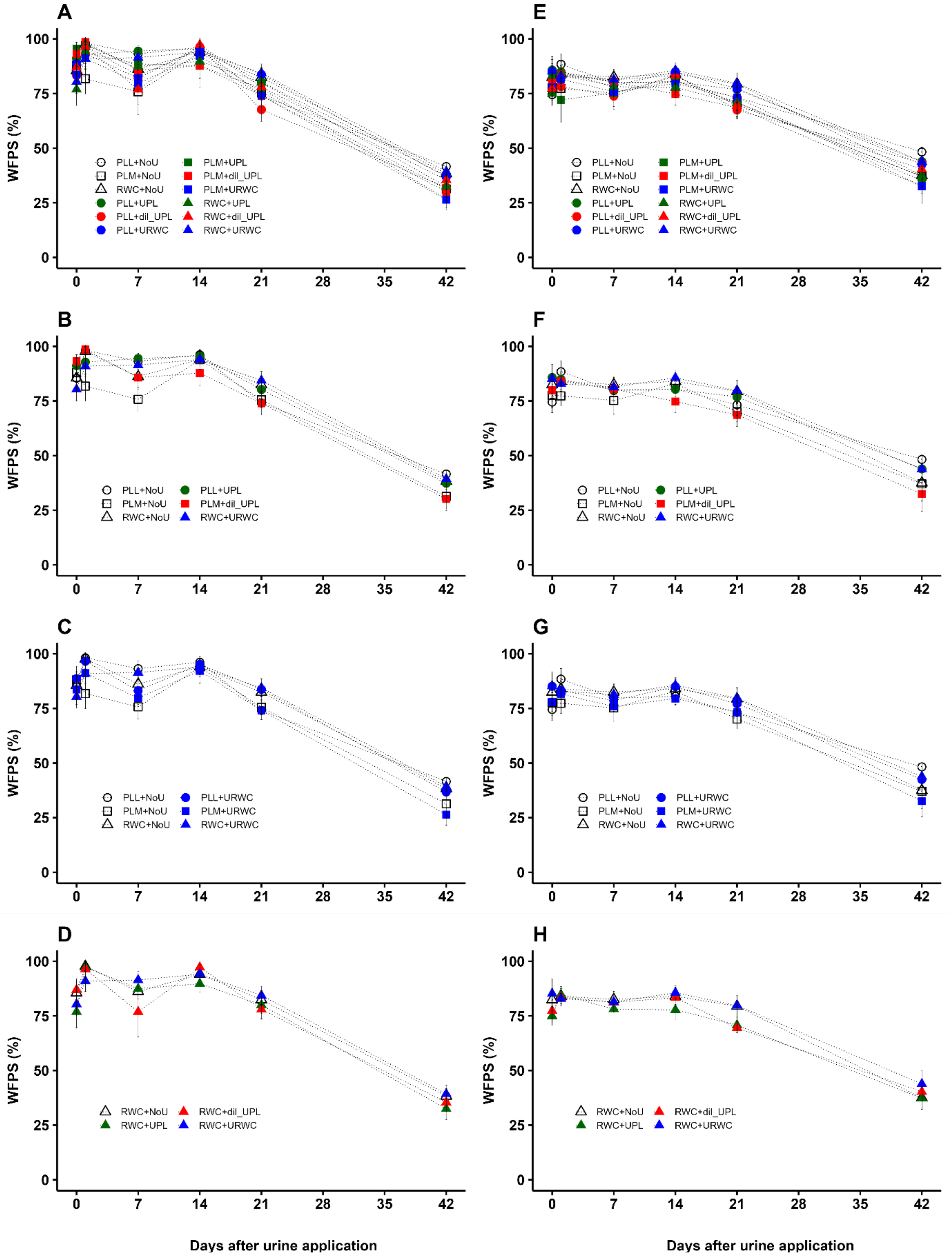

Figure 5.3. Soil water filled pore space (WFPS%) in 0–50 mm (A) and 50–100 mm (E) soil depths from urine patches in ryegrass-white clover pasture (RWC) mixed with plantain (PL) at ~30% (PLL) and ~40% (PLM); which were treated with urine from cows fed on diets with

RWC pasture, ~20% PL, or diluted urine of ~20% PL (URWC, UPL or dil_UPL). All pasture treatments included a non-urine control (NoU, water). The effect of plantain in the pasture and plantain in the diet (B and F), the effect of pasture treatments in urine from conventional RWC pasture-fed cows (C and G), the effect of plantain in the pasture treated with URWC (D and H) on WFPS% at 0–50 mm and 50–100 mm depths shown respectively. Data points are mean values within treatment, error bars denoted by the standard error of means (n=5). There were no significant differences among treatments ($p<0.05$) at the experimental site from 04th October 2023 (day–0) to 29th November 2023 (day–42).

5.3.4 Soil mineral N concentrations during the experiment

In the present study, soil ammonium (NH_4-N) concentrations peaked one day after urine application and then gradually decreased in the 0–5 cm soil layer. Pasture types showed similar soil NH_4-N, while urine type significantly affected soil NH_4-N concentrations on 1, 7 and 14 DAA (dil_UPL< UPL=URWC). The highest value was observed in the PLL+URWC treatment (516 mg N kg^{-1}), while the lowest value was recorded in PLL+dil_UPL (304 mg N kg^{-1}). By 7 DAA, a higher concentration was found in the PLL+UPL treatment (313 mg N kg^{-1}), with the lowest concentration observed in RWC+dil_UPL (86 mg N kg^{-1}). Thereafter, all treatments exhibited similar NH_4-N levels. Soil NH_4-N concentrations were lower in the 5–10 cm soil layer (ranging from 0 to 81 mg N kg^{-1}) than in the 0–5 cm soil layer. At 14 DAA, PLL+URWC and PLM+URWC had higher NH_4-N concentrations compared to RWC+dil_UPL and PLM+dil_UPL, though concentrations were otherwise similar between treatments on other sampling days (Figure 5.4.A).

When comparing the combined effect of pasture type and its corresponding urine type, PLL+UPL showed higher NH_4-N concentrations in the soil compared to RWC+URWC at 1, 7, and 14 DAA, though the difference was not statistically significant (Figure 5.4.B).

When RWC urine was applied to different pasture types, soil NH_4-N concentrations were statistically similar across treatments on each sampling day (Figure 5.4.C). One day after application, RWC+dil_UPL produced lower NH_4-N concentrations than RWC+UPL, but by 7 DAA, RWC+URWC had higher NH_4-N concentrations than RWC+dil_UPL (Figure 5.4.D).

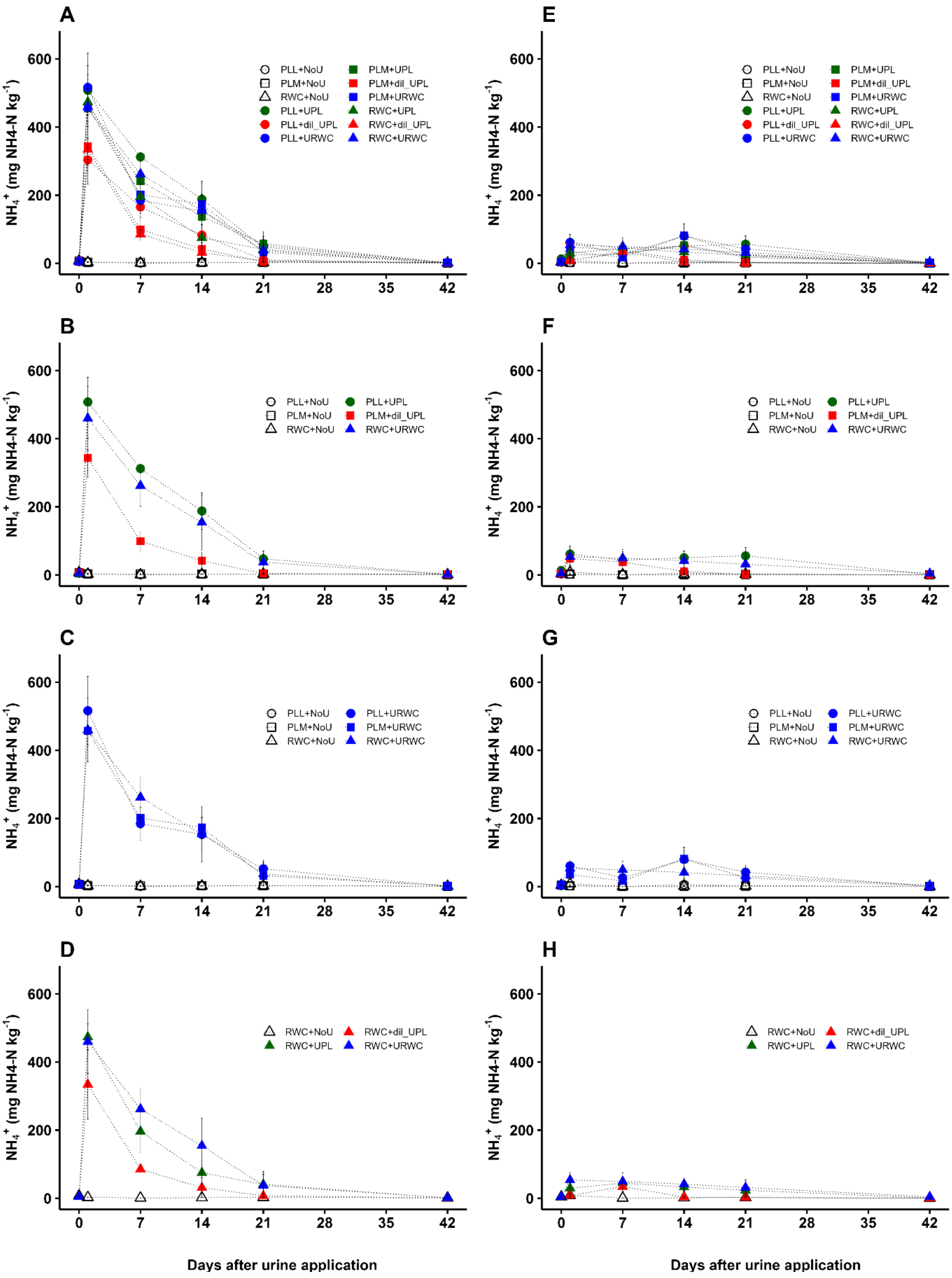

Figure 5.4. Soil ammonium N concentration (mg NH$_4$-N kg^{-1}) in 0–50 mm (A) and 50–100 mm (E) soil depths from urine patches in ryegrass-white clover pasture (RWC) mixed with plantain (PL) at ~30% (PLL) and ~40% (PLM); which were treated with urine from cows fed

on diets with RWC pasture, ~20% PL, or diluted urine of ~20% PL (URWC, UPL or dil_UPL). All treatments included water control treatments (NoU). The effect of plantain in the pasture and plantain in the diet (B and F), the effect of pasture treatments in urine from conventional RWC pasture-fed cows (C and G), the effect of plantain in the pasture treated with URWC (D and H) on WFPS% at 0–50 mm and 50–100 mm depths shown respectively. Data points are mean values within treatment, error bars denoted by the standard error of means (n=5).

Figure 5.5 (A-H) shows the soil NO_3-N concentrations at both the 0–50 mm and 50–100 mm soil depths throughout the experiment. Pasture types showed similar soil NO_3-N concentrations, while urine type significantly affected NO_3-N concentrations at 0–50 mm depth on 21 and 42 DAA (dil_UPL< UPL=URWC). In the 0–50 mm soil layer, the soil NO_3-N concentration in some treatments peaked at 7 days after application (PLM+dil_UPL, RWC+dil_UPL), while other treatments showed a gradual increase, peaking at 21 DAA.

At 42 DAA, in the 0–50 mm depth, soil NO_3-N concentrations in PLL+UPL and RWC+URWC were 45 mg N kg^{-1} and 39 mg N kg^{-1}, respectively. These treatments had significantly higher NO_3-N content compared to RWC+dil_UPL and PLM+dil_UPL, which had returned to background levels by 42 DAA.

5.3.5 Soil pH

The background soil pH was approximately 6, with plantain pastures (PLL and PLM) having lower pH values than the RWC pasture at the 5–10 cm depth (6.0 and 6.1, respectively). Urine application significantly increased the pH at 1 DAA, reaching up to 7.9 in the 0–5 cm soil layer. In the 5–10 cm soil layer, the pH remained around 6.0, but a drop below the background level was observed at 21 DAA in the PLM+dil_UPL and all URWC treatments (Figure 5.6).

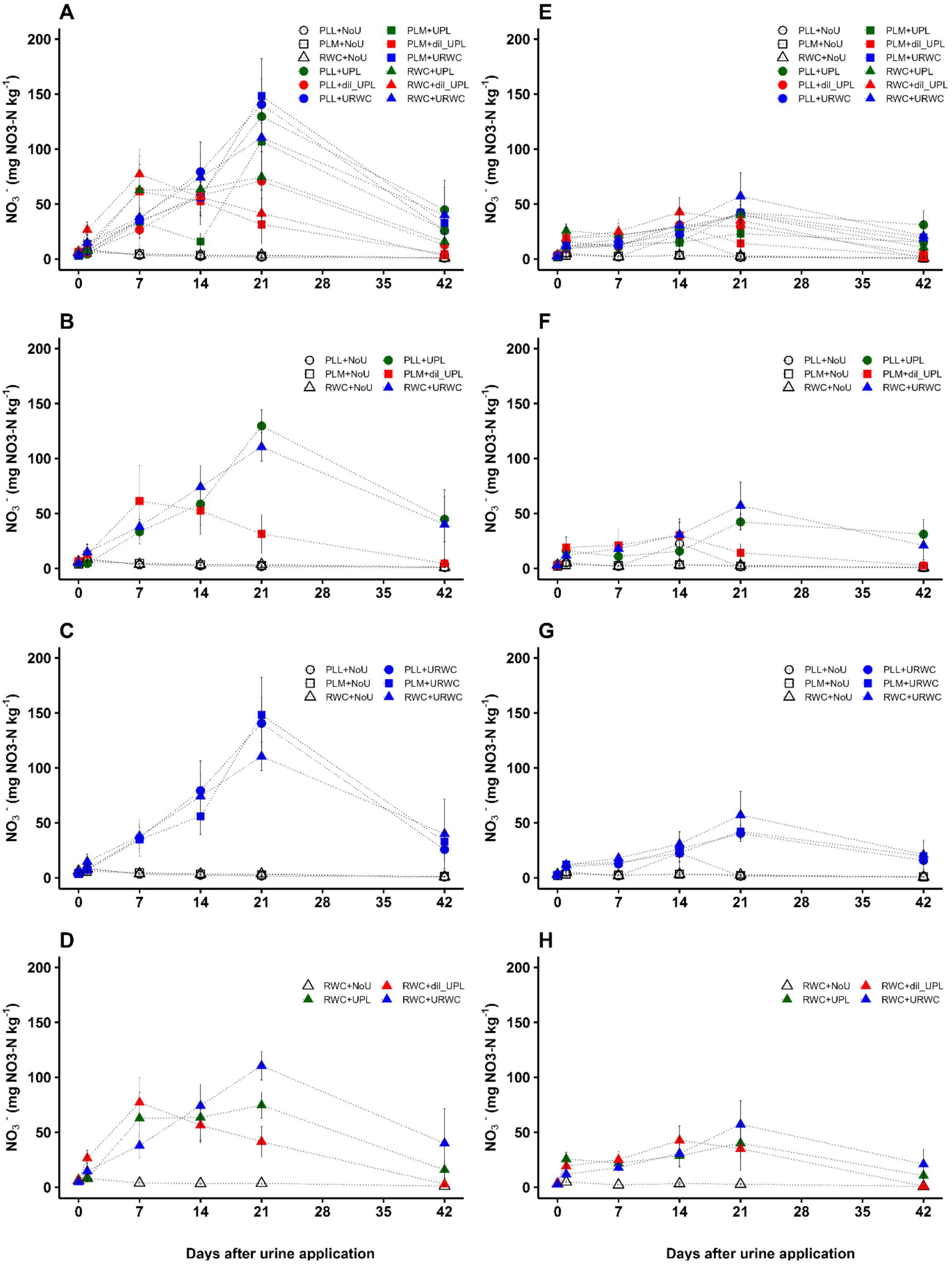

Figure 5.5. Soil nitrate N concentration (mg NO_3-N kg^{-1}) in 0–50 mm (A) and 50–100 mm (E) soil depths from urine patches in ryegrass-white clover pasture (RWC) mixed with plantain (PL) at ~30% (PLL) and ~40% (PLM); which were treated with urine from cows fed on diets

with RWC pasture, ~20% PL, or diluted urine of ~20% PL (URWC, UPL or dil_UPL). All treatments included water control treatments (NoU). The effect of plantain in the pasture and plantain in the diet (B and F), the effect of pasture treatments in urine from conventional RWC pasture-fed cows (C and G), the effect of plantain in the pasture treated with URWC (D and H) on WFPS% at 0–50 mm and 50–100 mm depths shown respectively. Data points are mean values within treatment, error bars denoted by the standard error of means (n=5).

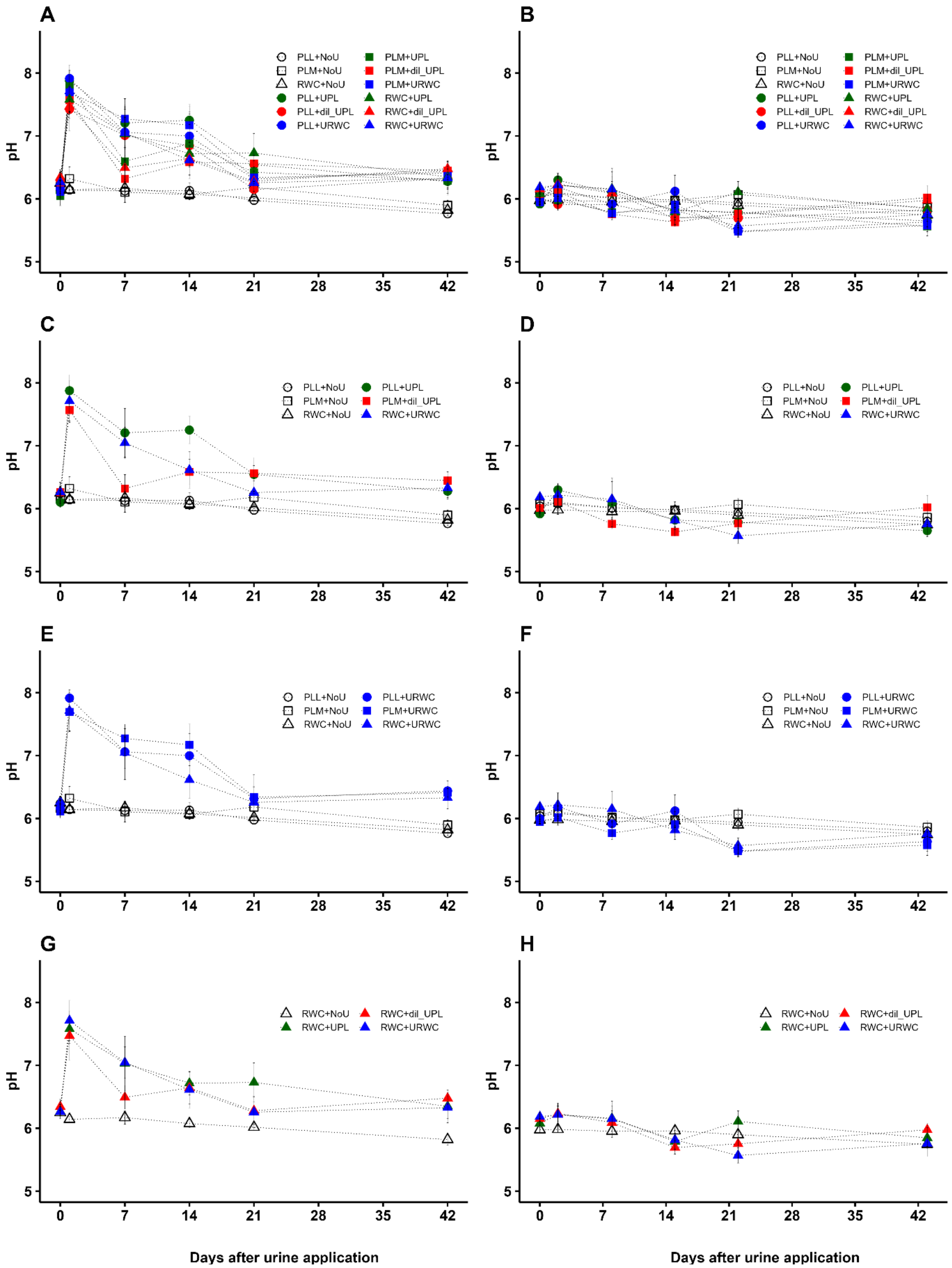

Figure 5.6. Soil pH in 0–50 mm (A) and 50–100 mm (E) soil depths from urine patches in ryegrass-white clover pasture (RWC) mixed with plantain (PL) at ~30% (PLL) and ~40% (PLM); which were treated with urine from cows fed on diets with RWC pasture, ~20% PL, or

diluted urine of ~20% PL (URWC, UPL or dil_UPL). All treatments included water control treatments (NoU). The effect of plantain in the pasture and plantain in the diet (B and F), the effect of pasture treatments in urine from conventional RWC pasture-fed cows (C and G), the effect of plantain in the pasture treated with URWC (D and H) on WFPS% at 0–50 mm and 50–100 mm depths shown respectively. Data points are mean values within treatment, error bars denoted by the standard error of means (n=5).

5.4 Discussion

5.4.1 Nitrogen concentration in urine and its impacts on N_2O emissions

The urinary N loading rate is a critical factor influencing cumulative N_2O emissions from grazed pasture (Di & Cameron, 2016; Selbie et al., 2014). This loading rate is a function of UN concentration, urine volume and spread (Selbie et al., 2015). Adequate PL in the diet has been shown to reduce the N concentration in urine patches (Pinxterhuis et al., 2024) and, on occasions, subsequent N_2O emissions and emission factors (Simon et al., 2019; Vi et al., 2023). In the present study, no reduction in UN concentration was observed in cows fed PL compared to those fed RWC. This is likely to be due to insufficient PL in the diet (on average 19% in the diet). Decreases in UN concentrations have previously been measured only when PL content exceeded 20% in the diet, which agrees with this study (Box et al., 2022; Box et al., 2017; Minnee et al., 2017; Nkomboni et al., 2021).

Diluting the urine, i.e the dil_UPL treatment resulted in approximately 37% less cumulative N_2O emissions than URWC, supporting the hypothesis that lower UN concentration leads to reduced N_2O fluxes (Simon et al., 2019). Interestingly, despite having a similar (or marginally higher) UN concentration, N_2O emissions and emission factors from UPL were numerically lower than for URWC: this could be linked to the potential BNI activity of the metabolites in the UPL (Peterson et al., 2022).

5.4.2 Effect of plantain rhizosphere on biological nitrification inhibition.

Another mechanism that may lower cumulative N_2O emissions in PL pasture is the BNI activity of plantain root exudates, which affects the activity of ammonia-oxidizing bacteria and archaea (Carlton et al., 2019). The PSM of PL, especially aucubin, was found to be particularly effective in delaying soil mineralisation and inhibiting nitrification (Dietz et al., 2013;

Rodriguez et al., 2021). The lower soil nitrification rates in PL pasture can be linked to derivatives of PSM found in the urine excreted by grazing animals (Peterson et al., 2022) and the release of PSM via root exudates (Pinxterhuis et al., 2024). Ideally, this would result in lower N_2O emissions due to lower NO_3-N content in the soil (Ding et al., 2024). However, no differences in soil NO_3-N content were observed in the present study among the pasture types. Similarly, a few studies reported that even though N_2O emissions were lower, no direct soil nitrification inhibition was observed (Camilla et al., 2017; Simon et al., 2019). Simon et al. (2021) found that PL inhibited ammonia-oxidizing bacteria, resulting in a lower gene abundance in the PL rhizosphere soil compared to the control and bulk soil, suggesting that BNI activity may be higher near PL roots. The lack of differences in NO_3-N content in this study may be due to uneven NO_3-N distribution in the simulated urine patches, which was similar to a cow's natural urination. Although soil samples were collected within the pasture, they may not have been from the plantain rhizosphere, potentially missing the effects of mineral N inhibition.

5.4.3 Impact of soil moisture on nitrous oxide emissions

Despite some numerical differences, WFPS values were similar across treatments in the 0–100 mm depth during the experimental period (Figure 5.3), and WFPS remained above 75% until 21 DAA. This similarity in WFPS contrasts with findings from Rodríguez Gelós (2020) and Vi et al. (2023), who both observed greater WFPS under PL than RGWC, possibly due to PL inactivity in winter Rodríguez Gelós, (2020) and different moisture extraction patterns in summer (Vi et al., 2023). At 21 DAA, despite a peak in soil NO_3-N values (Figure 5.5), the N_2O-N emissions remained small and close to background values across all treatments (Figure 5.1). While high NO_3−N concentrations under urine patches are known to favour N_2O production (Weier et al., 1993), this result in the present study could be attributed to complete denitrification (NO_3-N converted to N_2). Complete denitrification is promoted by factors such as high soil water content, neutral to slightly basic pH, elevated soil temperature, low oxygen diffusion, and the presence of labile carbon (Saggar et al., 2013). A recent study by Ding et al. (2024), shows that high soil moisture content (>70% WFPS) promotes N losses via N_2 emissions rather than N_2O, with over 50% of the applied N lost as N_2 during their experiment. The high WFPS and slightly higher soil temperature during the experimental period in the present study may explain the low N_2O levels observed after 21 DAA. Ding et al. (2024) also observed the presence of PL in the pasture enhances N_2 emissions more than N_2O emissions, which may also contribute to the tendency of PL pastures to produce less N_2O compared to RWC in the present study.

5.4.4 Increased pasture production and similar nitrogen uptake of plantain pastures

Urine application increased pasture yield and N uptake compared to the control (NoU treatments). Although there was increased DM production in PLL and PLM, the cumulative N uptake by the PLM and PLL was similar to that of RWC. When PL is included in a RWC pasture mix, it generally produces a similar DM production per ha compared to RWC in spring, summer, and autumn (Al-Marashdeh et al., 2021; Nguyen, 2023). In the present study, the estimated DM yield was measured only within the urine patches but usually in moderately stocked dairy farms, the area covered by urine patches is around 25% of the paddock (Di & Cameron, 2002; Moir et al., 2011). Therefore, measuring pasture DM within the urine patch may not be directly comparable to paddock scale measurements. Increased pasture DM yield but similar pasture N levels in the pasture could be an indication of higher N use efficiency by PL pasture.

5.4.5 Nitrous oxide EF_3

In spring, pastures on the fine-textured Tokomaru silt loam with higher proportions of PL tended to produce lower net N_2O emissions and EF_3 values. The lower UN concentration (4.1 N g L^{-1}) decreased N_2O emissions by 26% compared to H-UN (5.65 N g L^{-1}), but it produced similar EF_3 values, indicating that, in terms of EF_3, there is little advantage to the more dilute UN associated with diets which contain PL.

While significant reductions in N_2O emissions and EF_3 values were not observed for PLL or PLM compared to RWC (Table 5.4), the grouping of PLL and PLM, (i.e. on average 30–40% PL) reduced net N_2O emissions by 26% ($p<0.05$) and EF_3 values by 27% ($p<0.05$). This could be attributed to PL's rhizosphere BNI effect in reducing soil N_2O emissions. These results tend to suggest that PL's BNI effect is the primary factor in reducing spring emissions of N_2O. The results aligned with previous findings suggesting that PL pastures have the ability to produce lower N_2O emissions and EF_3 values (Simon et al., 2019; Vi et al., 2023).

In the present study, EF_3 values ranged from 1.22–2.24% (Table 5.4), exceeding the typical EF_3 value of 0.98% reported for flat and low-sloped agricultural soils in New Zealand (MfE, 2024). Vi et al. (2023) found smaller EF_3 values (0.15–0.31%) in a study undertaken at the present trial site comparing mixed pasture swards under different UN loads during summer and autumn. The authors attributed the reduced EF_3 to the dry conditions during their experiment.

In contrast, Rodríguez Gelós (2020) reported EF_3 values ranging from 0.9–1.4% in spring and 0.3–2.5% in winter in a field trial conducted on the same soil type, comparing a pure plantain sward to a RWC sward. Similarly, de Klein et al. (2003) reported a high EF_3 value of 2.5% for Mottled Fragic Pallic Soil, a poorly drained silt loam. In the present study, the higher emissions were linked to increased WFPS% at the trial site, as the Tokomaru silt loam is prone to surface compaction and remains wet after rainfall for a prolonged period despite being artificially drained. Compacted soils and higher WFPS create anaerobic conditions, promoting denitrification and thus elevating N_2O emissions (Saggar et al., 2013).

It is important to note that the N_2O emission factors in the present study were based on a single urine application in spring. To establish a comparable annual emission factor for inventory purposes, N_2O emissions from applications in winter, summer, and other seasons should also be assessed (de Klein et al., 2003). Total urine N load per hectare from cows grazing swards with and without PL also needs to be considered before urine patch results can be applied at paddock and farm scales. Furthermore, the contrasting results for EF_3 values between the present study and the study of Vi et al. (2023), emphasise the need for additional research across different seasons and soil types to fully evaluate the potential of PL in mixed pastures as a tool for mitigating N_2O emissions.

5.5 Conclusions

The present field study tested the potential of PL to reduce N_2O emissions using three sources of urine-N during spring in a fine textured soil. Results indicated a trend toward lower N_2O emissions, with increasing PL content, with an average EF_3 reduction of around 27% for pastures containing 30–40% PL compared to RWC pastures. This reduction in EF_3 can be attributed to the influence of the PL rhizosphere on the BNI activity in the soil. The UN concentration significantly impacts the cumulative N_2O emission from individual cattle urine patches; however, it was ineffective in reducing EF_3. Future work should consider scaling these findings from the urine patch level to paddock, block and farm levels accounting for differences in urination behaviour between cows fed pastures containing PL and those without.

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CHAPTER 6

General discussion and conclusions

6.1 Background of the thesis

A paradigm shift in utilising plantain (PL) in pastoral dairy systems occurred when research showed that including sufficient amounts of PL in the diet could reduce nitrogen (N) losses to waterways and the atmosphere by lowering N concentrations in urine (UN) excreted by dairy cattle without impacting pasture yield or animal production (Navarrete et al., 2018; Nguyen, 2023; Simon et al., 2019; Totty et al., 2013). Amid growing environmental regulations from the New Zealand government which require farmers to meet specific greenhouse gas (GHG) emission targets (Herath et al., 2023; Minnée et al., 2019), PL has become a viable option for sustainable farming due to its environmental benefits (DairyNZ, 2023a). However, research on PL's effects on GHG emissions, particularly methane (CH_4) and nitrous oxide (N_2O), remains limited, especially in mixed pastures.

A few studies have suggested that feeding cows pure PL diets produced less CH_4 emissions (Della Rosa et al., 2022; Durmic et al., 2016). However, results were variable when cows were fed mixed pasture, as they were influenced by factors such as PL proportion and the presence of other species in the pasture (Jonker et al., 2019; Koning et al., 2024; Wilson et al., 2020). Plantain has plant secondary metabolites (PSM) which can alter the rumen fermentation profile (Navarrete et al., 2016); however, their direct effect on CH_4 has not previously been tested. Similarly, reductions in N_2O emissions have been observed with PL pasture due to biological nitrification inhibition (BNI) and low UN loads (Luo et al., 2007; Simon et al., 2019). However, the N_2O results are inconsistent, with soil type significantly affecting N_2O emissions (Ding et al., 2024). A few studies reported no effect of PL on N_2O emissions (Earl-Goulet et al., 2021; Podolyan et al., 2020), and there remains a limited understanding of PL's BNI activity in the soil (de Klein et al., 2022). This highlights the need to validate PL's ability to reduce N_2O emissions across soil types in different climatic seasons.

The main aim of this thesis was to assess the potential of PL to reduce two major GHG emissions, i.e. CH_4 and N_2O, in pastoral dairy systems. The findings are summarised under the following headings, and are based on a series of *in vitro* experiments carried out to evaluate the ability of PL and its PSM to mitigate CH_4 emissions, and a field study assessing the ability of PL to reduce N_2O emissions in a pasture with low to medium-level of PL (30–40% PL) compared to conventional ryegrass/white clover (RWC) pasture.

6.2 Effect of plantain pasture and their plant secondary metabolites on CH_4 emissions

6.2.1 Key findings of Chapter 3

This chapter evaluated: (i) the seasonal variation in the chemical composition of RWC and PL-mixed pastures collected over four production seasons at the Plantain Potency and Practice Programme study site (DairyNZ, 2023b); and (ii) the *in vitro* rumen fermentation profiles of representative pastures to assess how seasonal variations in chemical composition impact CH_4 and ammonia (NH_3) production.

Based on the data collected over four production seasons in the Plantain Potency and Practice Programme study site at Massey University, around 20–50% PL is an achievable population in a mixed pasture with RWC. When around 40% PL in the pasture (PLM) was tested *in vitro*, up to 19% lower CH_4 production was observed in PL samples collected during the summer period. To my knowledge, this is the first study reporting that a medium level of PL has the potential to lower the CH_4 emission *in vitro* and this aligns with previous studies that reported lower CH_4 production from pure PL pasture *in vitro* when compared to other forage species (Durmic et al., 2016) and *in vivo* when compared to ryegrass (Della Rosa et al., 2022). Additionally, a lower net NH_3 production was observed from PL samples collected during spring (27% lower) and autumn (17% lower), compared to RWC pasture (Table 3.6). This finding is consistent with previous research by Durmic et al. (2016) and Navarrete et al. (2016), who reported similar reductions of *in vitro* NH_3 production from pure PL pastures.

The chemical composition of PLM and RWC pastures varied seasonally and by pasture type, with significant interactions of pasture type and seasons in neutral detergent fibre (NDF), non-structural carbohydrates (NSC), and soluble sugars (Table 3.3). The PLM had higher NSC (28%), NSC:N ratio, soluble sugars (12%), lignin (24%), and PSM, while showing lower NDF (13%) and acid detergent fibre (ADF, 5%) compared with RWC. The crude protein (CP) and digestibility of organic matter in dry matter (DOMD) were similar across both pastures but varied seasonally, with DOMD peaking in spring. Higher production of PSM (exclusive to PLM) was found in spring compared to summer, with autumn showing intermediate levels (Table 3.3). Due to these changes in chemical parameters, PLM had a faster rate ($R^{1/2}$) and shorter time ($T^{1/2}$) to reach its potential gas production (PGP), compared to RWC (Table 3.4).

According to the results from my thesis, to a certain extent, acteoside and aucubin in PLM may have influenced net NH_3 and CH_4 production. Previous studies have shown that acteoside and aucubin are capable of reducing net NH_3 in the rumen (Navarrete et al., 2016), and these two compounds, along with other secondary metabolites (e.g. a phenol extract from *Paulownia* leaves), reduced CH_4 emissions by lowering methanogen counts and decreasing NH_3 production through antimicrobial effects (Nowak et al., 2022). Accordingly, the present study suggests that the reductions in CH_4 production in PLM collected during summer, and the lower $R^{1/2}$ observed in PLM collected during autumn, may be attributed to the antimicrobial effects of PSM. Additionally, despite the higher CP content in the summer PLM sample (~16% higher than RWC), net NH_3 production was similar between these pastures, suggesting an influence of the PSM on net NH_3 production (Table 3.6). However, due to the confounding effects of chemical composition on net NH_3 and CH_4 production, it was challenging to draw definitive conclusions regarding the specific impact of these bioactive compounds on NH_3 and CH_4 production purely based on these results.

This gap was addressed in Chapter 4 through three additional *in vitro* experiments. This chapter investigates the role of PSM (acteoside, aucubin, catalpol) in reducing CH_4 emissions by evaluating: (i) PL substrates with similar chemical composition (to eliminate the confounding effect of chemical composition on CH_4 emissions) but varying intrinsic natural PSM levels, compared with ryegrass; and (ii) the effects of different PSM concentrations, both individually and in combination, by incubating commercially sourced PSM with ryegrass, which naturally does not contain these PSM.

6.2.2 Key findings of Chapter 4.

First, both acteoside and aucubin showed potential inhibition of CH_4 production *in vitro*. According to available literature to date, this is the first study to report a detailed understanding of the CH_4 inhibition potential of PSM and their mechanism to mitigate CH_4 emissions. The inclusion of aucubin in the basal feed (ryegrass) led to a longer lag phase in gas and CH_4 production due to anti-microbial activity. Acteoside reduced CH_4 production and altered short-chain fatty acid molar proportions by lowering the acetate to propionate (A:P) or [acetate+butyrate] to [propionate+valerate] (AB:PV) ratio in a dose-dependent manner. Similarly, when aucubin was incubated with acteoside, the combined treatments also reduced A:P and AB:PV ratios. This shift towards higher propionate (and/or valerate) molar proportions indicated an alternative hydrogen utilisation pathway, consuming available hydrogen in the rumen that would otherwise be available for CH_4 production (Della Rosa et al., 2021). A similar effect of lower A:P or AB:PV ratios was observed at 50% PL in the pasture in experiment 1.

When comparison was made within different PL substrates, PL substrates which had relatively higher acteoside levels resulted in a lower proportion of CH_4 in gas in total gas production (Table 4.3). This suggests that PSM in PL have the potential to reduce CH_4 emissions in mixed pastures (approximately 50% PL in this experiment).

Secondly, the capacity of PSM to reduce net NH_3 production was validated. In addition to that, even in smaller concentrations, which are common in PL mixed pastures, the effectiveness of PSM in reducing net NH_3 production was identified. Aucubin alone, as well as in combination with acteoside, reduced net NH_3 production by up to 46% (Table 4.9). Although the reduction was possible by both acteoside and aucubin individually (Navarrete et al., 2016), the interaction between acteoside and aucubin (Table 4.9) suggests that their mechanisms may operate independently yet synergistically. This is significant because both acteoside and aucubin are commonly present together in PL pasture, offering a natural advantage for using PL as a mitigation option to reduce CH_4 emissions.

The ryegrass substrate reported was collected from a different farm with different management and had higher DOMD than the PL substrates used in this study (Table 4.2). Although a lower CH_4 production (up to 13% lower from PL substrates 24 h after incubation) was reported in experiment 1, the gas production was also lower (up to 8%), suggesting that lower digestibility was one of the key components that could lower CH_4 production but would also be expected to negatively affect cow production (Della Rosa et al., 2022). However, the pastures incubated in Chapter 3 (PLM or RWC) were collected from the same study site and subjected to similar management, and herbage digestibility and milk production did not differ between pasture types (unpublished data from Massey PL trial site and Nguyen et al., 2024); in addition, other studies with PL similarly suggested no observed compromise in milk production or animal performance (Box et al., 2017; Nguyen et al., 2022; Nkomboni et al., 2021). However, more research is needed in this area.

6.2.3 Significance of these findings for CH_4 mitigation

While *in vitro* studies offer insight into the effects of PL pastures and PSM-infused substrates on animals, including a potential CH_4 reduction of up to 19% compared with RWC, it is uncertain whether this level of mitigation can be achieved under farm conditions. The absence of *in vivo* studies is a limitation; therefore, caution is needed when extrapolating these results to farm-scale applications, and future studies should use *in vivo* testing to further explore this.

Lower net NH_3 production can be translated into lower UN excretion from dairy cattle, as ruminal NH_3 not utilised within the body will likely be excreted in urine (Pacheco & Waghorn, 2008). This represents a net loss to the animal and contributes to environmental pollution (Tamminga, 1992). Several studies reporting lower UN concentrations in response to feeding PL (Box et al., 2022; Minnee et al., 2020; Navarrete et al., 2022), along with the *in vitro* results from my thesis, provide strong evidence that this could be one of the possible explanations of observed reductions in those *in vivo* studies.

The concentrations of PSM tested in this study align with the range of PL PSM concentrations reported in previous studies, providing a comprehensive understanding of their potential effectiveness. Achieving higher PSM levels in PL through forage breeding could be a valuable strategy for mitigating both CH_4 emissions and N excretion in ruminants, since greater reductions were observed when PL had higher PSM levels.

Plant secondary metabolites in cattle diets have both benefits and risks. Some PSM can cause toxicity issues (Flythe & Harlow, 2023), since they help protect plants from herbivores, pests and pathogens (Bennett & Wallsgrove, 1994). Based on *in vitro* research by Nowak et al. (2022), low gas production (GP) during compound incubation may suggest a potential negative impact on the rumen microbial population, which could, in turn, affect cow performance.

However, Chapter 4 results showed that GP was unaffected by aucubin and even increased with acteoside (Table 4.8), suggesting a potential for promoting beneficial rumen microbes.

6.3 Effect of PLM (on average 30–40% PL in RWC) on N_2O emissions during spring

6.3.1 Key findings from chapter 5

This chapter evaluated the impact of 30% and 40% PL within RWC pasture on N_2O emissions compared to RWC from a poorly drained Tokomaru silt loam soil during spring. A trend was observed towards lower N_2O emissions with increasing PL content, with an average of 28% lower emission factor EF_3 (% N_2O emitted per unit of N applied) value in PL pasture (30–40%) than RWC ($p=0.03$). This reduction in EF_3 can be attributed to the influence of the PL rhizosphere on the biological nitrification inhibition activity in the soil. The lower UN concentration decreased cumulative N_2O emissions from individual patches by 27% compared to the higher UN concentration; however, it was ineffective in reducing EF_3.

6.3.2 Significance of these findings for mitigating N_2O emissions

During spring, 30–40% PL in the pasture could reduce N_2O emissions by up to 27% compared to having no PL in the pasture. This suggests that maintaining PL in pasture under conditions conducive to greater N_2O emissions could serve as an effective mitigation strategy for N_2O losses.

Achieving a lower UN load through diet, and thereby reducing cumulative N_2O emissions from urine patches, requires sufficient PL content in the diet. During the experimental period, PL content in the diet was below 20%, and urine from PL-fed cows showed no difference in UN concentration compared to urine from RWC-fed cows. In such cases, when cows excrete similar UN concentrations, maintaining adequate PL in the sward for its BNI effect could be an effective option to mitigate N_2O emissions.

6.4 Recommendations for future research

The results of this thesis identified the mechanisms by which PL-mixed pastures and their PSM reduce CH_4 emissions. Additionally, it explored the source of variations in the reduction of CH_4 and N_2O emissions, which will help us understand more about the potential use of PL and how it can be used effectively to achieve environmental benefits. However, this thesis also identified aspects that require attention in future studies to evaluate the capacity of this PL to reduce GHG.

1. We can infer that both acteoside and aucubin have the potential to alter the rumen fermentation profile by altering rumen microbial communities. To isolate the effects of acteoside, aucubin, or their presence in combination, microbial DNA analysis is required to better understand the mechanisms behind the reductions in CH_4 and NH_3 that were observed in the current study.
2. Additionally, both acteoside and aucubin need to be tested *in vivo*, both at lower concentrations that are representative of natural combinations in mixed pasture, and also at higher concentrations to reflect their potential future administration as a supplement to animals for mitigation purposes.
3. The variation in BNI expression of PL pasture in different proportions with different seasons needs to be investigated. Furthermore, evidence from the literature indicates that BNI expression may vary across soil types (Ding et al., 2024). Therefore, additional research is needed to identify the minimum PL threshold required to reduce N_2O emissions on a range of soil types, as well as to determine if this effect varies with different climates.
4. Increased urine volume and higher urination frequency are typical responses of cows fed PL diets (Box et al., 2022). There is a lack of research on increased urination events

and its associated N_2O emissions in PL pastures. Urine distribution across the paddock is likely complex, with some areas receiving single urination events, others multiple overlapping deposits, and some remaining urine-free (Romera et al., 2012). Considering urination frequency and UN excretion is essential to fully understand the impact of PL pasture on N_2O emissions at a paddock scale. A modelling study is needed to scale these findings and compare emissions from PL pastures to those in RWC as a control.

6.5 Main conclusions from this thesis

1. There was sufficient *in vitro* evidence that acteoside and aucubin decreased CH_4 production, warranting further *in vivo* research on dairy cows.
2. Acteoside promoted alternative hydrogen utilisation pathways by lowering A:P or AB:PV ratios and reducing CH_4 production. A similar reduction was observed when aucubin was added to acteoside.
3. Aucubin exhibited antimicrobial activities in addition to inhibiting CH_4 and the role of aucubin and acteoside in decreasing net NH_3 production *in vitro* was confirmed.
4. The incorporation of PL at medium levels (40%) in a mixed RWC pasture exhibited potential environmental benefits compared with RWC pasture *in vitro*, with reductions in CH_4 and NH_3; these results were influenced by seasonal variability in the chemical composition of PL. Further animal studies are required to confirm any role of PL in CH_4 mitigation.
5. The N_2O emissions from urine patches were lower by ~28%, with an average of 30–40% PL in a RWC pasture compared to no PL in the pasture (RWC), potentially due to PL’s BNI activity in the soil.

6. To achieve lower N_2O emissions through a PL-based diet, a sufficient proportion of PL (>20%) may be needed during spring.

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In which chapter is the manuscript/published work?	Chapter 3
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