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Effects of Plantain (*Plantago lanceolata* L.) Metabolites Aucubin, Acteoside, and Catalpol on Methane Emissions *In Vitro*

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ABSTRACT: Plantain (PL) contains plant secondary metabolites (PSM), such as acteoside, aucubin, and catalpol, known for their bioactive properties. While acteoside and aucubin have been linked to reducing nitrogen losses in grazed pastures, their effects on enteric methane (CH_4) emissions remain unexplored. Three *in vitro* batch culture experiments were conducted to assess the effects of PSM on rumen fermentation, using PL pastures with varying PSM concentrations, purified PSM compounds, and/or their combinations added to ryegrass (*Lolium perenne*, RG), which does not contain these PSM. Aucubin addition to RG extended the time to reach halftime for gas production (GP) and CH_4 by 15−20% due to its antimicrobial effects. Acteoside, alone or with aucubin, promoted propionate production, an alternative hydrogen sink, which reduced the acetate to propionate ratio, increased GP by up to 13%, and decreased CH_4 proportion in gas by 5−15%. Aucubin reduced ruminal net ammonia (NH_3) production by up to 46%, with a similar reduction observed when combined with acteoside. This study highlights the potential of PSM to mitigate CH_4 emissions and reduce nitrogen losses from dairy cows, warranting *in vivo* evaluation of PSM and targeted breeding of PL pastures with increased PSM content.

KEYWORDS: *bioactive compounds, verbascoside, dairy, pasture, rumen fluid, ammonia, nitrogen losses*

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.[1] Developing strategies that mitigate the environmental impact, particularly greenhouse gas (GHG) emissions from dairy systems, is a growing concern.[2] 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.[3] 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.[4] 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.[5] Research has identified the potential of plantain (PL, *Plantago lanceolata* L.) to reduce N leaching and N_2O emissions from grazed pastures *via* lowering the urine N concentration of cows.[6−8] The presence of plant secondary metabolites (PSM) in PL has been identified as one of the key factors driving these environmental benefits.[9,10] A study on pure PL pasture reported lower CH_4 emissions when cows fed with PL than ryegrass (RG),[11] and a recent *in vitro* study reported that PL-red clover (PL-RC) pasture blend reduced CH_4 emissions compared to RG−RC mix.[12] The authors attributed this reduction to the presence of PSM in PL-RC on methanogen count,[12] although the impact of individual compounds on CH_4 emission and their dose responses are yet to be established.

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.[13] While the concentration of PSM varies widely across studies, acteoside and aucubin are generally found at higher concentrations than catalpol.[10,14,15] The highest concentrations of acteoside in PL, ranging from approximately 60 to 94 mg/g dry matter (DM), were reported by Fajer et al.,[16] with no specific cultivar mentioned, and Tamura and Nishibe[13] reported the highest aucubin concentrations, ranging from approximately 21−48 mg/g DM, in the cultivar Grasslands Lancelot. Some PL cultivars only produce trace quantities of catalpol.[13]

Acteoside and aucubin are known to have antimicrobial properties, and they positively influence the *in vitro* fermentation profile and reduce ammonia (NH_3) production.[10] 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

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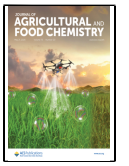

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Table 1. Treatments, Substrates, Concentrations of PSM, and Their Equivalent Amount to mg/g of Pasture DM Incubated in Experiments 1, 2, and 3 (E1, E2, and E3)

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 6 mg 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 PSM and control for E2. [f]Ryegrass substrate added with dimethylformamide and additional control treatments for E2. [g]Ryegrass used as a substrate to include PSM and a control treatment for E3, n.d: not detected, —: not applicable.

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.[17] 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 hypothesized 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.

2. MATERIALS AND METHODS

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.[18]

In experiment 1 (E1), PL substrates were sourced from archived samples collected at Massey University PL trial site[19] 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 (WC, *Trifolium repens* cv. Tribute). A detailed description of field trial establishment, sample collection, and grazing management is provided by Nguyen et al.[20] These pasture samples were oven-dried at 60 °C and stored in an airtight plastic container for about 15 months until the time of incubation, with PSM were assessed as one batch after sample collection. The PSM concentrations were determined by high-performance liquid chromatography, as described by Navarrete et al.[10]

Three pasture samples were selected, namely, PL1, PL2, and PL3 for E1, containing about 50% PL in the DM, that 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 and 9 mg/g pasture DM, PL2 had 18 and 6 mg/g pasture DM, and PL3 had 6 and 9 mg/g pasture DM of acteoside and aucubin, respectively. 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 at representing 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

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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 concentrations 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 all experiments was collected from AgResearch Aorangi farm, located near Palmerston North, New Zealand.

The treatments in each experiment (Table 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 h (h), while subsamples were taken from the second set. Each incubation was repeated in three runs (replicates = 3), while analytical replicates were averaged per run for statistical evaluation.

Stock solutions were prepared for E2 using DMF to ensure complete dissolution of the PSM and accurate dosing since it was not interfered with rumen fermentation up to a level of 50 mL/L.[21] Acteoside (274 mg), aucubin (180 mg), and catalpol (50 mg) were dissolved in 9, 9, 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 1).

2.2. Rumen Sample Collection and *In Vitro* Medium Preparation. A total of 3.2 L of buffer solution was prepared according to Mould et al.[22] The buffer was heated in a water bath to 39 °C and saturated with CO_2 for about 30 min, 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 grazed in the standard pasture (RG-WC) year around and 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 analyze short-chain fatty acids (SCFA) and NH_3 (0 h sample).

Before the incubation, the substrates were weighed to 500 ± 10 mg, added to 125 mL prelabeled 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 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 microtube and stored at −20 °C until further analysis of NH_3 and SCFA.

2.3. Laboratory Analysis for Samples from *In Vitro* Incubation. The 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. The SCFA were analyzed using gas chromatography as described by Attwood et al.[23] Approximately 200 μL of the remaining supernatant was for NH_3 concentration analysis using the colorimetric method described by Weatherburn,[24] scaled down to run in 96-well plates.

Chemical composition analysis for substrates was carried out by using near-infrared spectroscopy at an accredited commercial laboratory (RJ Hill Laboratories Ltd., Hamilton, New Zealand).[25] The samples were tested for the following parameters and reported on DM basis: organic matter digestibility (OMD) *in vivo* (determined using Australian Fodder Industry Association *in vitro* Pepsin-Cellulase procedure and derived as *in vivo* using a linear regression based on calibration samples from Lincoln University), neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (calibration based on acid detergent extraction followed by treatment with 72% sulfuric acid in the Ankom Daisy Incubator), ash (calibration based on weight loss after ashing at 600 °C for 2 h), soluble sugars (calibration based on an 80:20 ethanol: water extraction and colorimetric determination), starch (calibration based on enzymic hydrolysis of starch), crude fat (calibration based on petroleum spirit extraction by an Ankom auto analyzer, AOCS official procedure AM-5-04), crude protein (CP) (N multiplied by 6.25, whereas N calibration based on total N by Dumas combustion).

2.4. Model Fitting and Data Analysis. Gas and CH_4 production from each bottle was fitted to a logistic model to estimate the *in vitro* gas production (GP) kinetics described by France et al.,[26] using the following formulas

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

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

$$R^{1/2}a = \frac{a \times (c + 1) \times b \times \exp(b \times T^{1/2a})}{\exp(b \times (T^{1/2a})) + c} \quad (3)$$

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), c: parameter determining curve steepness and lag phase. t: time (h), and $T^{1/2}a$: time at which the incubation reached 1/2 of PGP (h)$R^{1/2}a$: rate of GP at half-time (mL g DM·h^{-1}).

Gas and CH_4 production parameters were analyzed in statistical software R version 4.3.0[27] using linear mixed models in packages "lme4"[28] and "emmeans".[29] 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 *posthoc* 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-measure 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 analyzed over time (12 and 24 h).

3. RESULTS

3.1. Compositional Analysis of Substrates. Plantain substrates had greater concentrations of ADF (24% higher),

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lignin (35% higher), and NSC (93% higher) but less OMD (17% lower) and CP (37% lower) compared to the RG control. These values were more than two interquartile ranges of the average of the PL substrates. The NDF was similar across all substrates, including RG. PSM were present only in PL substrates, and catalpol was detected at low concentrations (<1 mg/g of pasture DM) in the PL substrates (Table 2).

3.2. *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 3).

3.2.1. 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).

3.3. *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 ($p < 0.05$) (Table 5).

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, % CH_4

Table 2. Compositional Analysis of Ryegrass (RG) Used in all Three Experiments and Plantain (PL) 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 fiber, ADF: acid detergent fiber, SSS: soluble sugars and starch.

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Table 3. Gas and Methane (CH_4) Production Parameters of Plantain (PL) Substrates Compared to Ryegrass (RG) Substrate *In Vitro* in Experiment 1[a]

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[b]	266.3[b]	295.6	25.3[b]	38.4[b]	41.7	12.5[b]	14.4	14.1	7.4[b]	9.7	14.5[a]	2.1[a]
plantain (PL) substrates	PL1[b]	200.5[b]	249.5[c]	307.5	25.1[b]	35.0[c]	44.2	12.5[b]	14.0	14.2	6.9[c]	10.1	12.3[b]	1.5[b]
	PL2[c]	204.2[b]	254.2[c]	311.6	24.4[c]	35.4[c]	42.6	11.9[c]	13.9	13.6	6.8[b]	10.0	12.6[b]	1.6[b]
	PL3[d]	188.3[c]	240.3[d]	305.2	23.3[d]	33.4[d]	45.1	12.3[b]	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]PGP: potential GP, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, $T^{1/2}$: 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 ($p > 0.05$). [b]PL1: plantain substrate containing 10 mg of acteoside/g pasture DM and 9 mg aucubin/g pasture DM. [c]PL2: plantain substrate containing 18 mg acteoside/g pasture DM and 6 mg aucubin/g pasture DM. [d]PL3: plantain substrate containing 6 mg acteoside/g pasture DM and 9 mg aucubin/g pasture DM, SEM: standard error of means.

at 24 h, % PCH_4 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 5).

3.3.1. Short-chain Fatty Acids and Net NH_3 Production of Substrates Added with PSM 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 SCFA 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 (Table 6).

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 1). However, these reductions were not significant compared to RG-DMF (Table 7).

3.4. Interaction Effects and Individual Impact of PSM 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 8).

An increase of 12% in GP was observed in ACT-100 at both 12 and 24 h, and a decrease in % CH_4 at 12 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.

3.4.1. Short-chain Fatty Acid Production of Treatments Containing Different Concentrations of PSM 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 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

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Table 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) Substrates 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[b]	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 acids (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.

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 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 2). Additionally, increased caproate production compared to RG was observed (Table 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 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 1).

4. DISCUSSION

The key finding of this study was the potential of acteoside and aucubin to reduce CH_4 production from PL pastures when present in high concentrations. 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 concentrations 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.[10]

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.[30] 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.[31,32] A lower A/P or AB/PV ratio can be used as an indication of lower CH_4 production in the rumen.[33] 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,[10] is composed of four moieties: caffeic acid, phenylethyl alcohol, and sugars such as glucose and rhamnose.[34] The increased GP observed in E3 with acteoside treatments could be due to the increased fermentation of liberated sugars[10] during nonspecific glycoside breakdown in the rumen.[35] 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

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Table 5. *In Vitro* gas and Methane (CH_4) Production Parameters of Different Concentrations of 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)	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)
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[a]	2.09
	RG-DMF[b]	206.7	269.5	296.4	25.2[a]	37.6[a]	40.4	12.1	13.9[a]	13.6[a]	7.06[b]	9.6	15.0[a]	2.13
acteoside treatments[c]	ACT-6	206.8	272.5	299.1	25.1[a]	38.6[a]	42.4	12.0	14.1[a]	14.1[a]	7.25[b]	10.1	15.0[a]	2.08
	ACT-10	201.3	268.4	298.1	24.5[a]	37.8[a]	41.1	12.1	14.0[a]	13.7[a]	7.55[a]	10.0	14.3[a]	2.07
	ACT-18	204.6	270.7	300.0	24.6[a]	37.8[a]	41.3	12.0	13. Nine[a]	13.7[a]	7.39[a]	10.0	14.5[a]	2.05
	ACT-36	203.5	269.6	295.8	23.5[a]	36.3[a]	39.7	11.5	13.4[c]	13.4[a]	7.37[a]	10.1	14.9[a]	1.98
aucubin treatments[d]	AUC-6	204.5	272.1	300.7	24.8[a]	38.2[a]	41.9	12.1	14.0[a]	13.9[a]	7.48[a]	10.1	14.7[a]	2.06
	AUC-9	204.1	271.4	299.4	24.9[a]	38.1[a]	41.2	12.1	14.0[a]	13.7[a]	7.46[a]	9.9	14.7[a]	2.12
	AUC-12	207.0	272.4	298.2	25.0[a]	37.8[a]	41.1	12.0	13.8[b]	13.7[a]	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[b]	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[b]	35.7[b]	39.9	11.8	13.9[a]	13.8[a]	7.58[a]	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[f]	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 corresponding 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 corresponding 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. [f]The model was significant for GP at 12 h ($p < 0.05$), PGP: potential GP, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, $T^{1/2}$: the half time required to reach the potential gas or methane production, and $R^{1/2}$: 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.

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Table 6. Comparison of Short-chain Fatty Acids (SCFA) and Molar Proportion of SCFA Production *In Vitro* of Ryegrass (RG) Substrates 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[a]	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[a]	0.18[a]	3.47[a]	2.29	3.95	4.22
AUC-24[h]	84.6[a]	66.6	9.8[b]	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[a]	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 SCFAs. [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.

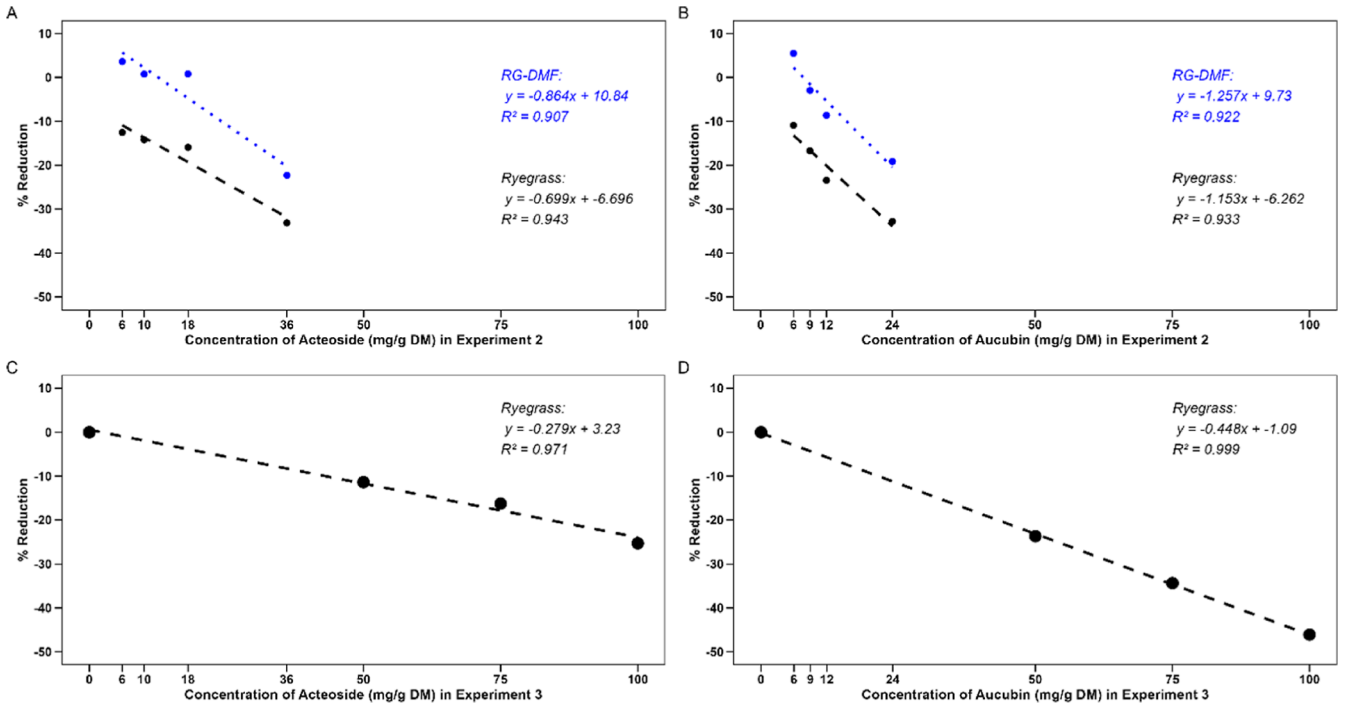

Figure 1. Percentage change in net NH_3 production in experiment 2 (A,B) and experiment 3 (C,D) in response to different types and concentrations of PSM: acteoside (A, C) and aucubin (B,D), compared to the control ryegrass treatments (RG and RG-DMF).

only monosaccharide known to produce low CH_4 emissions among available carbohydrates.[36−38] A study by Czerkawski and Breckenridge[39] reported that rhamnose fermentation led to reduced CH_4 production and no significant accumulation of hydrogen gas, suggesting that the hydrogen generated is likely utilized 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.[39] Similarly, no hydrogen GP 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 production, % 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.[10] Aucubin belongs to the secondary metabolite family of iridoid glycosides, and iridoids have a significant effect on different bacterial strain types.[40] Aucubin presence led to a decrease in acetate and propionate production but an increase in butyrate production in E3 (Figure 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.[17] This led to a decrease in acetate producing bacteria

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Table 7. Comparison of Net Ammonia (NH_3) Production *In Vitro* at Different Concentrations of 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]	09.73[a]
acteoside[c]	ACT-6	10.03[a]
	ACT-10	09.84[a]
	ACT-18	09.64[a]
	ACT-36	07.67[b]
aucubin[d]	AUC-6	10.22[a]
	AUC-9	09.55[a]
	AUC-12	08.78[b]
	AUC-24	07.71[b]
catalpol	CAT-10[e]	09.07[a]
	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 corresponding 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 corresponding 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.

(*Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*) and an increase in butyrate-producing bacteria (*Butyrivibrio proteoclasticus*).[17] 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.,[17] observed a reduction in methanogen counts alongside increased propionate-producing microbes (*Prevotella* spp. and *Megasphaera elsdenii*), resulting in higher propionate production when incubated with acteoside and aucubin abundant *Paulownia* leaf extract. This reduction in methanogens may explain the lower CH_4 production in a few aucubin treatments and lower % CH_4 in the combined treatments of the present study, possibly due to aucubin's antimicrobial actions. 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 production 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 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.,[10] both acteoside and aucubin reduced the net NH_3 concentration *in vitro* through two different mechanisms, that is, 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.,[17] observed that acteoside and aucubin together reduced NH_3 production *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. One limitation of this study is that the *in vitro* digestibility of the PSM was not measured during experiments. Consequently, the duration of their presence in the *in vitro* environment remains unclear. Additionally, 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.

4.1. Implication of Plantain's PSM on Dairy GHG 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. Although using PL-mixed pasture provide a practical application to lower CH_4 emissions *via* rumen fermentation, 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.[11]

The lower digestibility of the PL substrates can negatively affect cows' voluntary intake and reduce productivity.[41] In the current study, the digestibility (measured in the pasture as OMD) of the PL treatments was below the control, which could have negative effects on milk yield *in vivo*. 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.[42] Nevertheless, many studies report similar digestibility and similar or better milk yield in PL-mixed pasture-fed cows compared with RG-WC,[6,43,44] suggesting that grazing on PL-mixed may be a viable option to mitigate CH_4 emissions. Therefore, further research is needed to compare PL pastures with commonly sown RG-WC pastures grown under similar conditions to make a valid

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Table 8. *In Vitro* gas and Methane (CH_4) Production Parameters of Treatments Containing Different Concentrations of 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)	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 (%)	$^5CH_4$/GP at 24 h (%)	$^6PCH_4$/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[c]	282.7[c]	328.7[b]	21.6[a]	36.2	44.7[a]	11.4[a]	12.8[a]	13.6[a]	9.9[b]	12.4[b]	13.3[b]	1.6[a]
acteoside treatments[b]	ACT-50	204.4[a]	297.5[a]	342.7[a]	22.2[a]	37.5	46.8[a]	10.8[a]	12.6[a]	13.7[a]	9.5[b]	12.7[b]	14.3[a]	1.7[a]
	ACT-75	205.3[a]	296.3[a]	344.6[a]	21.8[a]	36.6	46.1[a]	10.6[b]	12.4[a]	13.3[a]	9.4[b]	12.6[b]	14.0[a]	1.6[a]
	ACT-100	213.5[a]	306.6[a]	354.5[a]	21.3[a]	34.7	40.6[b]	10.0[d]	11.3[b]	11.4[b]	9.3[b]	11.4[c]	14.6[a]	1.7[a]
aucubin treatments[c]	AUC-50	200.2[a]	292.5[a]	340.1[a]	23.0[a]	38.4	47.9[a]	11.5[a]	13.1[a]	14.0[a]	9.7[b]	12.4[b]	13.9[a]	1.7[a]
	AUC-75	198.0[b]	294.9[a]	350.2[a]	22.2[a]	37.7	47.3[a]	11.2[a]	12.8[a]	13.4[a]	10.2[b]	12.7[b]	13.5[b]	1.7[a]
	AUC-100	187.3[d]	286.8[b]	360.5[a]	20.4[b]	36.4	50.4[a]	10.9[a]	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[a]	310.4[a]	372.0[a]	21.8[a]	37.7	48.8[a]	10.3[c]	12.1[a]	13.0[a]	10.1[b]	13.3[a]	14.0[a]	1.7[a]
	ACT75+ AUC50[e]	205.9[a]	294.2[a]	336.7[a]	21.6[a]	36.6	45.1[a]	10.5[c]	12.5[a]	13.5[a]	9.2[c]	12.6[b]	14.5[a]	1.7[a]
catalpol	CAT-50[f]	200.0[a]	298.94[a]	353.7[a]	21.8[a]	38.0	48.7[a]	10.9[a]	12.7[a]	13.6[a]	10.23[b]	13.2[a]	13.8[a]	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	<0.0001	<0.0001	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 corresponding 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 corresponding 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 GP, PCH_4: potential methane production, GP: gas production, CH_4: methane production, h: hours, $T^{1/2}$: 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.

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Table 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 PSM in Experiment 3 over Time

treatments		absolute concentration		molar proportion (mol per 100 mol of total SCFA)							ratios	
		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[a]	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[a]	3.72[b]
acteoside treatments[f]	ACT-50	68.8[a]	4.9[a]	67.0[a]	10.2[d]	19.7[c]	1.62	0.19[b]	0.70[a]	0.70[a]	3.41[c]	3.63[d]
	ACT-75	69.6[a]	4.6[a]	66.6[c]	10.2[d]	20.0[b]	1.64	0.23[a]	0.69[a]	0.67[b]	3.35[d]	3.56[e]
	ACT-100	68.8[a]	4.1[a]	66.3[d]	10.2[d]	20.4[a]	1.62	0.26[a]	0.64[c]	0.63[d]	3.26[g]	3.48[f]
aucubin treatments[g]	AUC-50	69.2[a]	4.2[a]	67.3[a]	10.5[c]	19.1[d]	1.65	0.09[c]	0.67[a]	0.71[a]	3.53[a]	3.75[a]
	AUC-75	68.5[a]	3.6[b]	66.9[b]	11.0[b]	18.9[e]	1.73	0.09[c]	0.67[a]	0.70[a]	3.54[a]	3.78[a]
	AUC-100	65.6[b]	3.0[c]	66.5[c]	11.4[a]	18.8[e]	1.76	0.11[c]	0.67[a]	0.70[a]	3.54[a]	3.79[a]
combined treatment	ACT50+ AUC75[h]	70.5[a]	3.6[b]	65.9[e]	11.0[b]	19.8[c]	1.76	0.24[a]	0.66[b]	0.65[c]	3.33[e]	3.57[e]
	ACT75+ AUC50[i]	72.3[a]	3.0[c]	65.8[e]	10.7[c]	20.2[a]	1.73	0.27[a]	0.62[d]	0.67[b]	3.26[f]	3.49[f]
catalpol	CAT-50[j]	70.4[a]	4.2[a]	67.0[a]	10.5[c]	19.3[d]	1.71	0.08[c]	0.69[a]	0.73[a]	3.47[b]	3.68[c]
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	<0.0001	0.003	<0.0001	0.042	<0.0001	0.048	0.021	<0.0001	<0.0001
ACT-50 *vs* AUC-75		0.842	0.029	0.586	0.005	<0.0001	0.019	<0.0001	0.113	0.833	<0.0001	<0.0001
ACT75 + AUC50 *vs* (ACT-75 + AUC-50)/2		0.015	0.004	<0.0001	<0.0001	<0.0001	0.04	<0.0001	<0.0001	0.03	<0.0001	<0.0001
ACT-75 *vs* AUC-50		0.792	0.478	<0.0001	0.177	<0.0001	0.744	<0.0001	0.285	0.002	<0.0001	<0.0001

[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 corresponding 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 corresponding 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.

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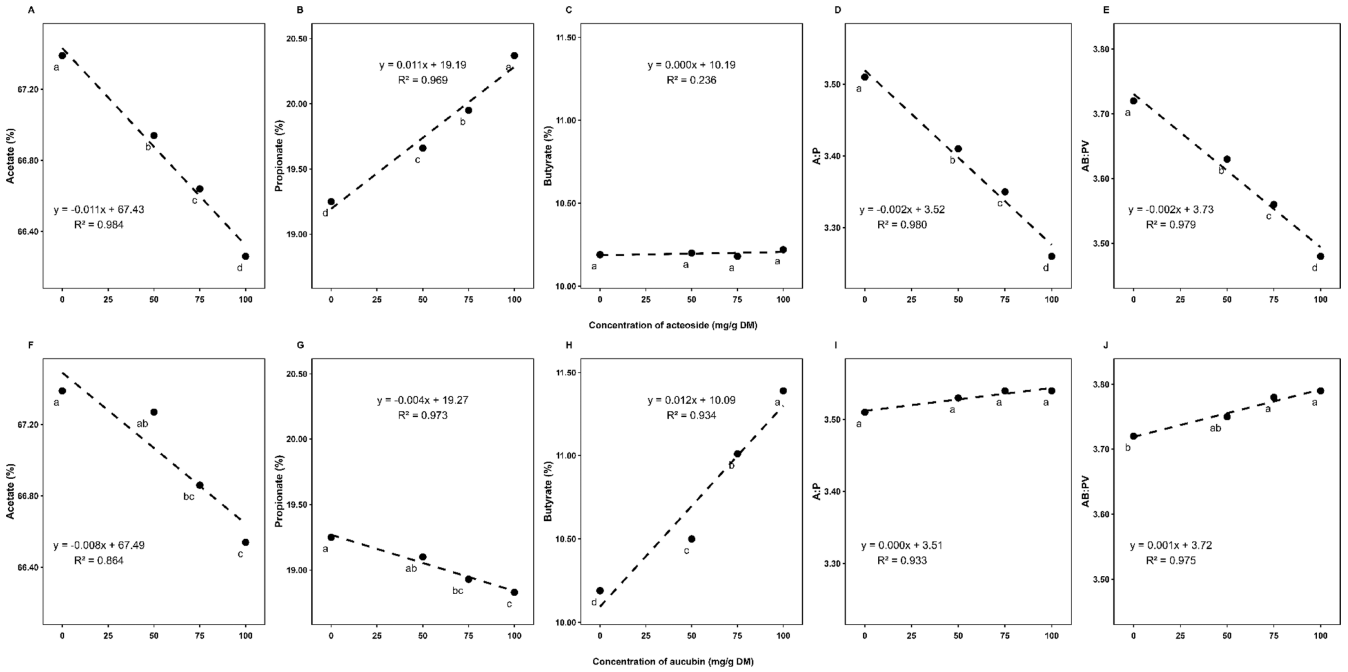

Figure 2. Effect of acteoside on molar proportion of short-chain fatty acids (SCFA) [mol per 100 mol of total SCFA, (A) to (C)], effect of aucubin on molar proportion of SCFA [mol per 100 mol of total SCFA, (F) to (H)], A/P; acetate to propionate ratio, AB/PV; acetate + butyrate to propionate + valerate 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).

comparison and to explore the potential of plantain in reducing CH_4 emissions.

Despite a 5% reduction in % CH_4 was observed in the PL2 at 12 h compared to other PL substrates which had similar OMD. 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.

A reduction in net NH_3 in both PL-mixed pasture and with PSM added treatments was observed. 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,[45] 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 a similar CP content when naturally occurring PSM concentrations were tested (Figure 1). The results from E3 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.

The management of CH_4 emissions and N losses from the 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. Acteoside and aucubin, particularly at high concentrations and in combination, significantly reduced CH_4 production, highlighting the potential for PSM in PL to be a mitigation option, but further research is needed to confirm our results *in vivo*. Aucubin extended the lag phase of CH_4 production, likely due to its antimicrobial properties, while acteoside lowered CH_4 emissions by increasing propionate production, an alternative hydrogen sink. Additionally, the role of aucubin and acteoside in decreasing NH_3 production *in vitro* was also confirmed in the present study. 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. Moreover, this study suggests incorporating PL-mixed pasture with higher acteoside and aucubin concentrations may reduce CH_4 emissions and potentially lower nitrogen losses to the environment by decreasing rumen NH_3 production.

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■ ABBREVIATIONS

% CH_4, methane proportion; AP, acetate to propionate ratio; ABPV, acetate + butyrate to propionate + valerate ratio; ADF, acid detergent fiber; CH_4, methane; CP, crude protein; DM, dry matter; DMF, dimethylformamide; E1, experiment 1; E2, experiment 2; E3, experiment 3; GP, gas production; N, nitrogen; N_2O, nitrous oxide; NDF, neutral detergent fiber; NH_3, ammonia; OMD, organic matter digestibility; PCH_4, potential methane production; PGP, potential gas production; PL, plantain; PSM, Plant secondary metabolites; $R^{1/2}$ GP, rate of GP at half-time; RC, red clover; RG, ryegrass; SCFA, short-chain fatty acids; $T^{1/2}$ GP, time at which the incubation reached 1/2 of PGP; WC, white clover

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