Biology and Fertility of Soils (2022) 58:265–276
https://doi.org/10.1007/s00374-021-01573-1

ORIGINAL PAPER

Soil nitrification inhibition by urine of sheep consuming plantain (*Plantago lanceolata*)

M. E. Peterson[1] · P. M. Fraser[1] · D. Curtin[1] · J. W. van Klink[1] · N. I. Joyce[1]

Received: 13 October 2020 / Revised: 30 May 2021 / Accepted: 1 June 2021 / Published online: 17 June 2021

Abstract

Urine from sheep fed with different plantain cultivars (*Plantago lanceolata* L.) was characterised to determine if metabolites in the urine contributed to soil biological nitrification inhibition (BNI) activity. Chromatographic analyses, combined with mass spectrometry, revealed there were more than 800 mass features that were unique to, or present in much greater concentration, in urine from sheep fed plantain than in urine of ryegrass (*Lolium perenne* L.)-fed sheep. The plantain metabolites aucubin, verbascoside, and catalpol have been implicated in relation to BNI activity, but these compounds were not detected in the urine of sheep fed with plantain. However, based on the mass spectral fragmentation patterns, metabolic derivatives of these compounds were present. A series of incubation studies showed that short-term nitrification inhibition did occur when plantain urine was applied to soil. Inhibition depended on several factors, including length of plantain grazing period and plantain cultivar type. As the incubations progressed, the inhibitory effect declined, potentially because of degradation of the inhibitory compounds by soil microorganisms. Urine of sheep fed with the plantain cultivar 'Agritonic' had a particularly strong inhibitory effect, which persisted for more than 1 month at an incubation temperature of 20 °C. Furthermore, in a laboratory bioassay using a pure culture of ammonia-oxidising bacteria, the organic fraction of urine from sheep fed with 'Agritonic' plantain was shown to be a strong nitrification inhibitor. This work suggests that valuable environmental benefits can be obtained by including cultivars of plantain showing BNI activity in pastures.

Keywords Nitrification · Inhibition · *Plantago lanceolata* · Urine · Secondary metabolites

Introduction

Nitrate (NO_3^-) leaching from grazed pastures is an environmental concern due to the adverse effects of leached nitrogen (N) on water quality (Cameron et al. 2013). Grazing ruminants excrete 70–95% of their ingested herbage N, mostly in urine (Selbie et al. 2015) and urinary-N concentration is strongly correlated with the amount of N in the diet (Dijkstra et al. 2013). The N loading under a ruminant urine patch can be as high as 1000 kg N ha^{-1} (Haynes and Williams 1993), often exceeding the requirement of pasture plants by more than 400 kg N ha^{-1} $year^{-1}$ (Moir et al. 2011). Nitrate can accumulate in urine patches following the transformation of urea (the predominant form of N in urine) to NH_4^+, which is subsequently converted to NO_3^- by nitrifying microorganisms. Nitrate is highly mobile in most soils and leaching from urine patches can account for a significant proportion (average estimated at 20% but with high variability according to season) of total NO_3^- leaching losses from grazed pastures (Selbie et al. 2015).

Treating grazed pastures with synthetic nitrification inhibitors, such as dicyandiamide (DCD), to maintain urinary-N in the less mobile NH_4^+ form, is an effective means of reducing NO_3^- leaching (Monaghan and Barraclough 1992; Venterea et al. 2015). Nitrate leaching from animal urine patches can be reduced by up to 50% when DCD is applied at the recommended rate (Di and Cameron 2016). However, its use in New Zealand has been suspended following the detection of trace amounts of DCD in test samples of dairy products (Astley 2013).

A range of plants have been identified that exude compounds that inhibit nitrification. Tropical pasture grasses (e.g. *Brachiaria humidicola*), field crops (e.g. *Sorghum bicolor*), and weeds (e.g. *Raphanus raphanistrum*) have been shown to reduce soil nitrification through the exudation of

✉ M. E. Peterson
michelle.peterson@plantandfood.co.nz

[1] The New Zealand Institute for Plant and Food Research Limited, Christchurch, New Zealand

biological nitrification inhibitors (BNIs) (O'Sullivan et al. 2017b; Subbarao et al. 2009, 2013). The BNIs isolated from root exudates and plant tissues (root and shoot) have a diverse range of chemical structures including phenolic acids (Rice and Pancholy 1974), fatty acids (Subbarao et al. 2008), phenylpropanoids (Zakir et al. 2008), flavonoids (Subbarao et al. 2013), quinones (Tesfamariam et al. 2014), diterpenoids (Subbarao et al. 2009), monoterpenes (Ward et al. 1997), and isothiocyanates (Brown and Morra 2009).

The genus *Plantago* (Plantaginaceae) is known to contain a suite of plant secondary metabolites (PSMs) that belong to several of the chemical classes noted above (Goncalves and Romano 2016). In *Plantago* species, the iridoid (monoterpenoid) glucosides aucubin and catalpol, along with the phenylpropanoid glycoside verbascoside (syn: acteoside), have been identified as biologically active. These compounds have antimicrobial (Rombouts and Links 1956; Rumball et al. 1997), antifungal (Oyourou et al. 2013; Shirley et al. 2015), anti-inflammatory (Park 2013), and anti-oxidative (Yan-Chun and Rong-Liang 1991) properties.

Aucubin and verbascoside occur in ribwort plantain (*Plantago lanceolata*) at relatively high concentrations (up to 3 and 9%, respectively), depending on cultivar, season, and other factors (Adler et al. 1995; Al-Mamun et al. 2008; Navarrete et al. 2016; Tamura and Nishibe 2002) and there is evidence to suggest that these compounds may act as BNIs in soil. Rauber et al. (2008) observed a decline in N mineralisation in field plots when potatoes were under-sown with *P. lanceolata*, while soil mesocosms dominated by *P. lanceolata* had a significantly lower NO_3^- concentration, mineralisation, and nitrification rates than mesocosms dominated by *Anthoxanthum odoratum* or *Lotus corniculatus* (Massaccesi et al. 2015). Additionally, a soil incubation study has demonstrated the suppression of N mineralisation and nitrification following incorporation of plantain leaf material into soil (Dietz et al. 2013).

A domesticated and cultivated line of *P. lanceolata* ('Ceres Tonic', marketed as Tonic) was commercialised in New Zealand over 20 years ago (Stewart 1996) and has become a common forage plant for livestock, particularly sheep, beef cattle, and deer. Its success as a pasture species is due to excellent seasonal production, forage quality, palatability to livestock, and performance in a wide range of environments (Judson et al. 2018). The incorporation of Tonic plantain into the pasture sward has been observed to reduce N-leaching from urine patches (Carlton et al. 2019) with the suggestion that this reduction was partly due to the release of BNIs from the roots of the plant. Additionally, it has been shown that the inclusion of Tonic plantain in the diet of grazing dairy cows resulted in a reduction in total urinary-N output, with average urine N loading rates in the urine patch reduced from 700 to less than 500 kg N ha^{-1} (Box et al. 2017). An in vitro study suggested that urinary-N output may be reduced due to the influence of the plantain PSMs aucubin and verbascoside on rumen ammonia production (Navarrete et al. 2016).

Analyses of both cattle and goat urine have detected PSM-derived urinary metabolites with excretion profiles that are dependent on the species of plant ingested (Keir et al. 2001; Lane et al. 2006). Therefore, when plantain PSMs such as aucubin, catalpol, and verbascoside are consumed and digested by a ruminant, they and/or their derivatives may accumulate in ruminant urine. Given the BNI potential of these compounds, it is possible that an inhibitory effect within urine patches may be observed, reducing the rate of nitrification and ultimately the leaching of NO_3^-. However, the fate and transformations of these PSMs during ruminant digestion are not well understood, and the forms and concentrations of breakdown products excreted in ruminant urine are as yet unknown.

Reducing the environmental impact of agricultural livestock production in New Zealand is a key focus in the use of Tonic plantain in pasture systems (Minnée et al. 2019). In this study, sheep were used as the model ruminant to determine if metabolites excreted in the urine after the consumption of plantain can inhibit soil nitrification. We examined multiple plantain cultivars for their urine-excreted BNI potential, with the hypothesis that the degree of nitrification inhibition conferred by urinary metabolites, derived from the consumption of plantain by ruminants, is cultivar-dependent.

Methods and materials

Source of urine

The urine samples used in this study were obtained from sheep participating in trials carried out in the spring and autumn to compare effects of plantain-based versus ryegrass-based diets on animal performance. As there was only a single plot of each cultivar, significances are based on between-animal variation within the cultivars. The trials were conducted at the Agricom Kimihia Research Centre, in Canterbury, New Zealand.

In a spring animal performance trial, 40 fifteen-month-old Romney cross ewes (45 kg live weight average) that had previously been grazing on ryegrass/clover pasture were transferred onto plots (10 sheep per 1.5-ha plot) with different plantain cultivars (*P. lanceolata* cv. Tonic, 'Agritonic', or WDA^+) or perennial ryegrass (*L. perenne* 'Samson'). Tonic is an upright, winter-active plantain and 'Agritonic' was bred from a Tonic population and selected for improved agronomic performance. WDA^+ is a breeding line from a gene pool that is winter-dormant with high aucubin concentrations relative to Tonic and 'Agritonic'. Concentrations of aucubin, catalpol, and verbascoside for these cultivars are

shown in Table 1. Aucubin concentrations ranged from 7 to 19 mg g^{-1}, depending on cultivar and season, whereas catalpol was low (≤ 0.5 mg g^{-1}) except in WDA^+, which contained up to 6 mg g^{-1}. Concentrations of verbascoside (~ 20–50 mg g^{-1}) were greater than those of aucubin or catalpol, with WDA^+ exhibiting the highest concentrations. The diet on offer in the plantain treatments comprised at least 90% plantain, with the balance being weed grasses. Urine was collected manually in a sheep handler at 48 h and 2 weeks after commencement of grazing.

In an autumn animal performance trial, 20 five-month-old Romney ewes (35 kg live weight average) that had previously been grazing on ryegrass/clover pasture were transferred onto plots (10 sheep per 1.5-ha plot) of either 'Agritonic' plantain (> 90% plantain) or 'Samson' ryegrass. Urine was collected manually in a sheep handler 2 weeks after commencement of grazing.

Analysis of urine

Immediately following collection, urine samples were analysed for total N after persulfate oxidation (Cabrera and Beare 1993) with concentrations of NH_4^+-N and NO_3^--N in each sample determined concurrently with total N on a Lachat Instruments QuikChem 8500 Series 2 Flow Injection Analysis System (Loveland, CO, USA). A subsample of each urine was set aside for mass spectrometry–based untargeted metabolomics analysis. Ultra-high-performance LC–MS analysis was performed on a Thermo Scientific™ (San Jose, CA, USA) Q Exactive™ Plus Orbitrap (HR/AM) LC–MS/MS coupled with a Vanquish™ UHPLC system (Binary Pump H, Split Sampler HT, Dual Oven). The samples were diluted 1:1 with cold acetonitrile within a Single Step® vial 0.22-µm PVDF (Thompson™ Part No. 65531–200) filter and a 2-µL aliquot of each sample (plus standards of aucubin, catalpol, and verbascoside) was separated by reverse phase chromatography (Accucore™ Vanquish™ C18 1.5 µm, 100 mm × 2.1 mm) maintained at 40 °C, flow rate of 400 µL min^{-1}; mobile phase of 0.1% formic acid in type 1 water (A) and 0.1% formic acid in acetonitrile (B), gradient 0–1 min/0% B, linear increase to 7 min/50% B, linear increase to 8 min/98% B, isocratic to 11 min/98% B, equilibration 11–12 min/0% B, isocratic to end 17 min/0% B. The eluent was scanned from 1 to 11 min by API-MS with electrospray ionisation in negative and positive ion modes. Data were acquired for precursor masses from *m/z* 110–1200 amu at 70 K resolution with data-dependent ms/ms product ions generated by normalised collision energy (NCE: 30) at 17.5 K resolution. Mass features identified include both the charged molecular ions of the parent molecule and any quasimolecular ions including adducts that might be formed during ionisation, e.g. formic acid adducts. Data were processed with the aid of Xcalibur® 4.0 and Compound Discoverer 2.1 (Thermo Electron Corporation).

Soil incubations with added urine

Soil collection and characterisation

A Templeton silt loam (Udic Haplustept (USDA)) from a site under low-input, long-term un-grazed Italian ryegrass (*Lolium multiflorum* 'Andy') was used in a series of incubation experiments with applied urine. The soil was sampled from the Plant & Food Research farm in Lincoln, Canterbury, New Zealand (43°38′17.8″ S 172°28′27.4″ E). Multiple soil cores were taken to a depth of 5 cm, mixed thoroughly into one composite sample, passed through a 4-mm sieve, and dried in a drying cupboard for 7 days at 20 ± 2 °C. Soil properties are listed in Table 2.

The potential of this soil to nitrify added NH_4^+-N was determined by the method of Belser and Mays (1980). Briefly, a soil slurry containing 140 mg N kg^{-1} (added as $(NH_4)_2SO_4$) was shaken for 24 h and subsamples obtained at intervals over this time period were analysed for NO_3^--N. From a linear regression of the NO_3^--N concentration over time, the potential nitrification rate was determined.

Incubation details

The urines collected from individual sheep were combined to provide sufficient volume for all treatments and the composite urines analysed for total N using the method above.

Table 1 Concentrations of aucubin, catalpol, and verbascoside in the three cultivars of plantain used in the grazing trials (adapted from Box and Judson (2018))

Season	Aucubin (mg g^{-1} DM)				Catalpol (mg g^{-1} DM)				Verbascoside (mg g^{-1} DM)			
	Tonic	'Agritonic'	WDA^+	$LSD_{5\%}$	Tonic	'Agritonic'	WDA^+	$LSD_{5\%}$	Tonic	'Agritonic'	WDA^+	$LSD_{5\%}$
Autumn (Mar–May)	11.8	11.6	19.1	7.4	< 0.1	0.2	1.8	1.2	23.5	22.4	32.8	17.0
Spring (Sep–Nov)	7.4	7.8	11.3	3.9	< 0.1	0.5	6.3	3.2	28.4	35.6	50.6	14.8

DM dry matter, $LSD_{5\%}$ least significant difference

Table 2 Properties of Templeton silt loam soil used in laboratory incubations

Soil property	Value	Method
pH	5.6	1:2 soil to water ratio
Total C	29 g kg^{-1}	} LECO C/N analyser
Total N	3 g kg^{-1}	
Ammonium	0.6 mg N kg^{-1}	} 2 M KCl extraction (Keeney and Nelson 1982)
Nitrate	1.1 mg N kg^{-1}	
Potential nitrification rate	9.0 mg N kg^{-1} day^{-1}	Soil slurry (Belser and Mays 1980)
Olsen P	21 mg L^{-1}	0.5 M $NaHCO_3$ extraction (Olsen et al. 1954)
Cation exchange capacity	16 $cmol_c$ kg^{-1}	1 M NH_4CH_3OO extraction (Chapman 1965)
Clay content	26%	Ultrasonic dispersion (Gee and Bauder 1986)

To ensure that urinary N inputs were standardised across treatments, the concentration of N was adjusted to that of the lowest N (composite) urine by addition of deionized water.

Samples of soil (20 g air-dry) were weighed into plastic containers and 6.3 mL of urine was added dropwise using an electronic pipette in titrate mode; this volume of urine brought the soil to field capacity (defined as the soil water content at − 10 kPa; actual water content of 32%). Urea (> 99.5%, UNIVAR®, Ajax Finechem) was applied in solution at the same N rate as the urines as a positive control, while a negative control received distilled water with no added N.

The microcosms were covered with Parafilm®, which was pierced to allow for rapid gas diffusion in and out of the microcosms. Microcosms were placed in 5.5-L boxes which were positioned in a split-plot arrangement in an incubator set at 20 ± 2 °C. During the incubation, the soils were maintained at − 10 kPa by gravimetric addition of water, if required. Experimental replicates of each treatment were destructively sampled to determine the net nitrification rate at 1, 7, 14, 21, 28, and 35 days. At each time point, NO_3^--N and NH_4^+-N were determined by 1-h extraction of 5 g soil with 25 mL of 2 M KCl and subsequent analysis of the filtered extract for NO_3^--N and NH_4^+-N on a Lachat Instruments QuikChem 8500 Series 2 Flow Injection Analysis System (Loveland, CO, USA). A further 5 g of soil was used to measure water-extractable C (WEC; 1:6 soil-to-water ratio, shaking time 30 min at 20 °C). The organic C content of the WEC extract was determined using a TOC-V_{CSH} analyser (Shimadzu Corporation, Kyoto, Japan). The phenolic content of the water extracts was determined using Folin-Ciocalteu's reagent with gallic acid as a standard (Ohno and First 1998).

An identical set of microcosms, prepared as described above, were used for determination of CO_2 production. Each microcosm was placed in a 500-mL air-tight jar (fitted with a rubber septum to facilitate gas sampling) and incubated at 20 °C. Production of CO_2 was assessed by periodically sampling the headspace of microcosm-containing jars using a gas-tight Hamilton syringe, fitted with a non-coring needle. At each sampling, the headspace was mixed by pumping the syringe three times before a 20-mL sample was withdrawn and the CO_2 concentration determined by infrared gas analysis (LI-COR, Lincoln, NE, USA). After sampling, the jars were opened and flushed with fresh air to return CO_2 concentrations to ambient before returning them to the incubator.

Nitrification inhibition bioassay

Nitrification inhibition by the urine organic fraction (UOF) was assessed by a bioassay in which the Griess reaction was used to detect NO_2^- produced by the nitrifying bacterium *Nitrosospira multiformis* (ATCC® 25198™) as it metabolises NH_4^+ (O'Sullivan et al. 2017a). To remove inorganic salts (including urea), 30 mL of urine was washed through a 20-mL Phenomenex StrataX C18 SPE column (Torrance, CA, USA) with three column volumes of type 1 water (Purite Select Fusion 40, Suez Water, Thame, UK). The adsorbed organic fraction was eluted with 30 mL of 90:10 v/v methanol:water, after which the methanol was driven off by Speedvac-assisted evaporation (Labconco, Kansas City, KS, USA) and the remaining fraction retained for use in the bioassay.

A cell culture of *N. multiformis* was revived and maintained in *Nitrosolobus* medium (ATCC medium 929, pH 7.8). Cultures were maintained in a shaking (70 rpm) incubator at 28 °C and sub-cultured weekly by aseptically transferring 1 mL of the culture into 100 mL of fresh, sterile medium. Prior to use in the bioassay, 500-mL suspensions of the cell culture were centrifuged at 8470 × *g* for 10 min and washed (3 ×) in N-free medium to ensure removal of residual NH_4^+ and NO_2^-. After washing, the pelleted cells were re-suspended in 6 mL of fresh *Nitrosolobus* medium and 1-mL aliquots were stored at 4 °C until required. These aliquots were centrifuged at 16,000 × *g* for 5 min and re-suspended in 6.5 mL of fresh medium immediately prior to their addition to the bioassay.

In a 96-well plate, 50 µL of the cell culture was assayed with an aliquot of the UOF, with water added to bring the

total well volume to 335 µL. Triplicate wells per UOF were included to enable the time course of NO_2^- production to be determined. Blank wells contained an aliquot of the UOF without cells. An inhibition control of allylthiourea (ATU), a synthetic nitrification inhibitor, was included at a concentration of 10 µM (determined, in our laboratory, as the minimum inhibitory concentration for our strain of *N. multiformis*). Assays were performed in triplicate and the production of NO_2^- was followed spectrophotometrically over a 100-min period at 28 °C in a temperature-controlled CLARIOstar microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). At intervals of 20 min, 15 µL of the Griess colour reagent (sulfanilamide 16.2 g L^{-1}, N-1-naphthylethylenediamine dihydrochloride 0.8 g L^{-1}, concentrated phosphoric acid 41 mL L^{-1}) was added to a set of wells and the colour change reaction allowed to proceed for 5 min before the absorbance was read at 548 nm. Absorbance values were converted to NO_2^- concentrations using the standard curve (0 to 2.5 mg L^{-1} NO_2^--N) and nitrification rates were calculated from a linear regression of NO_2^- formation over time. The BNI capacity of UOFs was calculated from the decrease in nitrification rate relative to the uninhibited controls.

Antibacterial/bacteriostatic effects of UOF were assessed using a bacterial growth assay. An aliquot of the UOF was combined with tryptic soy broth in a covered 48-well plate and the growth of an *Acidovorax* isolate, a facultative anaerobe (Garrity et al. 2005) isolated from soil at Plant & Food Research (Lincoln, New Zealand), was monitored by following the increase in optical density at 600 nm (OD_{600}) at 28 °C in the microplate reader described above. The UOF was tested (in triplicate) over a range of concentrations. A cell-only treatment was used as a positive control, while chloramphenicol antibiotic treatment was included as a negative control.

Statistical analysis

A differential analysis of the UOF constituent profiles was performed using Compound Discoverer™ 2.1 (Thermo Electron Corporation).

Statistical tests on the soil incubation data were performed using Genstat 64-bit Release 19.1 (VSN International Ltd, Hemel Hempstead, UK). Differences between treatment means were tested by one-way analysis of variance (ANOVA) and, if treatment effects were significant at $p < 0.05$, least significant difference ($LSD_{5\%}$) was used to separate means.

Results and discussion

Nitrogen concentrations in sheep urine

The total N concentration in plantain-fed sheep urine was generally less than half of that in urine of sheep fed with ryegrass (Table 3). This is consistent with results obtained in other studies (Box et al. 2017). The reduction in total N has been attributed to a diuretic effect (O'Connell et al. 2016) or to changes in ruminal fermentation (Navarrete et al. 2016), both thought to be caused by compounds in plantain leaves or compounds derived from a precursor contained in the leaves. Additionally, a number of studies have shown that N concentration was lower in urine of dairy cows grazing on "diverse" pastures that included a plantain component than in urine of cows grazing on grass-clover pasture (Bryant et al. 2017; Edwards et al. 2015; Minnée et al. 2017; Totty et al. 2013; Woodward et al. 2012).

The variability in urine-N concentration between individual sheep was large, with total N concentrations across the three experiments ranging from 1.3 to 17.4 g N L^{-1} in ryegrass-fed sheep and from 0.9 to 6.1 g N L^{-1} in sheep fed with plantain. This variability is consistent with other studies, with Hoogendoorn et al. (2010) observing a range of 1.4 to 17.8 g N L^{-1} in urine of sheep grazing a ryegrass/cocksfoot/white clover pasture while Bristow et al. (1992) reported a range of 3.0 to 13.7 g N L^{-1} for sheep on a ryegrass/white clover diet.

Nitrate–N was not detected in any of the urine samples. Ammonium-N comprised between 0.6 and 13% of urine-N with concentrations of NH_4^+-N usually greater in plantain-fed sheep urine than in urine of ryegrass-fed sheep (Table 4).

Organic constituent profiles in urine of sheep fed with plantain or ryegrass

Analysis of the metabolite profile of the whole urine samples (collected in the autumn animal performance trial) separated by reverse phase LC–MS identified almost 3000 mass features. Of these mass features, 94 were present in greater concentrations than creatinine, a biomarker commonly used to normalise other urinary metabolites. This normalisation was to account for differences in the time of urine collection, urine concentration, urine flow rate (Warrack et al. 2009), and in the type and availability of forage consumed by the

Table 3 Total N concentrations of composite sheep urines used in the laboratory soil incubation experiments

Trial	Grazing time†	Total N concentration (g N L^{-1})			
		Plantain			Ryegrass
		Tonic	WDA+	'Agritonic'	Samson
Spring	48 h	1.6	2.8	1.5‡	6.4
	2 weeks	2.4	3.1	3.1‡	9.9
Autumn	2 weeks	-	-	2.6	5.0

†Length of grazing prior to urine collection

‡Urine from this treatment was not included in the corresponding soil incubation experiment

Table 4 Ammonium-N concentration in the composite sheep urines used in laboratory incubation experiments

Trial	Diet fed	Grazing prior to urine collection	NH_4^+-N concentration (mg N L^{-1})	NH_4^+-N as a proportion of total urine N (%)
Spring	Tonic	2 weeks	92	4.0
	WDA^+		19	0.6
	'Agritonic'†		223	7.2
	Ryegrass		87	0.9
Autumn	'Agritonic'	2 weeks	334	13.1
	Ryegrass		30	0.6

†Urine from this treatment was not included in the corresponding soil incubation experiment

animal (David et al. 2015; Ma et al. 2014). Differential analysis ($p \leq 0.005$, $\log_2$ fold ≥ 2) showed there were more than 800 metabolites that were either unique to, or present in greater concentrations, in the plantain urine than in the ryegrass urine (designated in light grey in Fig. 1). Based on predicted formulae, N-containing mass features, defined as "non-urea nitrogenous components" (NUNCs), were almost twice as numerous in plantain urine as in the ryegrass urine (524 and 283, respectively).

Interrogation of the mass spectral data of the major metabolites detected in the urine suggested that many of these were sulfated, methylated, and glycine conjugates. A number of the metabolites were identified based on known ovine metabolites (http://lmdb.ca/metabolites). Hippuric acid, phenylacetylglycine, *p*-cresol sulfate, *N*-cinnamoyl glycine, and related ovine metabolites exhibited similar profiles regardless of whether the urine came from animals fed ryegrass or plantain diets.

The plantain PSMs aucubin, catalpol, and verbascoside were not identified in the urine samples. However, based on the mass spectral fragmentation patterns, metabolic derivatives of these compounds were present (Ghisalberti 1998; Hattori et al. 1990; Qi et al. 2013; Visioli et al. 2003).

Urine-induced nitrification inhibition in soil microcosms

The soil used in the incubation studies was a slightly acidic silt loam with an organic carbon content of 29 g kg^{-1} and nitrification potential of 9 mg kg^{-1} day^{-1} (Table 2). This soil type is representative of many sheep-grazed pastoral soils in the South Island of New Zealand.

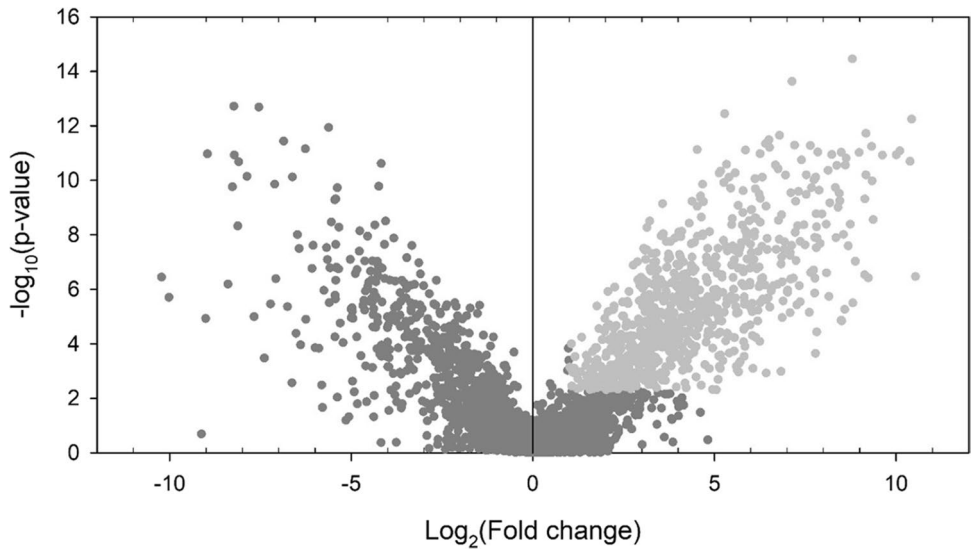

Fig. 1 Volcano plot of the mass feature profiles (separated by reverse phase chromatography) of whole ryegrass urine and whole plantain urine collected in the autumn animal performance trial. The x-axis specifies the fold-change in concentration and the y-axis specifies the negative logarithm (base 10) of the t-test p-values. Compound related mass features unique to, or present in much greater concentrations, in the plantain urine than in the ryegrass urine are indicated on the positive side of x-axis. Compound mass features designated in light grey are those that are either unique to, or present in greater concentrations, in the plantain urine than in the ryegrass urine ($p \leq 0.005$, $\log_2$ fold ≥ 2)

In the first incubation, urine collected from sheep grazing (in the spring) on plantain cultivars (Tonic or WDA$^+$) and Samson ryegrass for 48 h was applied to the soil at a rate of 511 µg N g^{-1} soil. The NO_3^--N production in the plantain cultivar (Tonic or WDA$^+$) urine treatments was similar to that from the ryegrass urine treatment (Fig. 2A). Over the first 21 days, significantly less NO_3^--N accumulated in the urine-treated soils than in the soil receiving urea (229, 236, and 223 µg NO_3^--N^{-1} g soil for Tonic, WDA$^+$, and ryegrass urine, respectively, versus 279 µg NO_3^--N g^{-1} soil for urea; $LSD_{5\%} = 12$). The synthetic inhibitor DCD (at the recommended rate, equivalent to 30 kg ha^{-1}) was effective in suppressing nitrification in urea-treated soil and less NO_3^--N was produced in the urea + DCD treatment compared to the control receiving no N (31 and 52 µg NO_3^--N g^{-1} soil over the 35 days, respectively; $LSD_{5\%} = 10$).

Tonic plantain has been shown to induce diuresis in sheep after only a 24-h grazing period (O'Connell et al. 2016). While diuresis was evident by way of a reduction in total N concentration (Table 3), the inhibitory effect on nitrification was not obvious. This finding is echoed in the work of Podolyan et al. (2019) and suggests that grazing plantain for 48 h is not adequate to confer a sufficient concentration of the plantain PSMs responsible for nitrification inhibition to the urine.

In the second incubation (Fig. 2B), the urines collected after 2 weeks of grazing (in spring) were applied at a slightly higher N application rate (773 µg N g^{-1} soil). Significantly less NO_3^--N was produced in the Tonic treatment than in the control or urea treatments at intervals up to 17 days. The cumulative amount of NO_3^--N produced in 17 days in the Tonic treatment was 146 µg NO_3^--N g^{-1} cf. 241 µg NO_3^--N g^{-1} ($LSD_{5\%} = 15$) when ryegrass urine was applied. There was no significant difference in NO_3^--N production between the WDA$^+$ and ryegrass treatments at any point during the incubation. Nitrate–N production in the urine-treated microcosms converged at approximately 25 days, although NO_3^--N produced at this point remained less than in the urea treatment (~ 320 µg versus ~ 390 µg NO_3^--N g^{-1} soil). There was no significant difference in the treatments at 28 days, and after 35 days, NO_3^--N production in the Tonic microcosms was significantly greater than in the other treatments. The results from these two incubations suggest that compounds in the urine of sheep grazing on plantain have nitrification inhibition capability when applied to soil but the degree of inhibition depends on the length of the grazing period prior to urination and on the cultivar.

In a third incubation, urine collected from sheep grazing 'Agritonic' plantain or 'Samson' ryegrass for 2 weeks (in autumn) was applied at a rate of 802 µg g^{-1} soil). In this incubation, the urine of sheep grazing 'Agritonic' plantain had a particularly strong inhibitory effect on the production of NO_3^--N (Fig. 3A). After 14 days of incubation, NO_3^--N produced in microcosms treated with urine of 'Agritonic'-fed sheep (44 µg g^{-1}) was significantly less than that produced in the negative control receiving no N (61 µg g^{-1}), and was ~ 5.5 times less than that produced in microcosms treated with urine of ryegrass-fed sheep. Throughout the incubation, there was no discernible difference in NO_3^--N production between the ryegrass urine and urea treatments. Until day 28, NO_3^--N production in the plantain urine microcosms was significantly less than in the ryegrass and urea treatments, but the difference diminished with time and was no longer statistically significant by day 35. These results indicate that the inhibitory effect of the plantain urine declined with time, presumably due to degradation and/or inactivation of the active compounds.

Measurements of NH_4^+-N during the incubation showed that hydrolysis of urea in both the plantain and ryegrass urine treatments was rapid, with near-complete conversion to NH_4^+-N within 1 day (Fig. 3B). In contrast, less than half the urea-N was in the NH_4^+-N form after 1 day. The rapid hydrolysis of urine-urea may have been caused by contamination of the urine by faecal ureases, or because of intrinsic

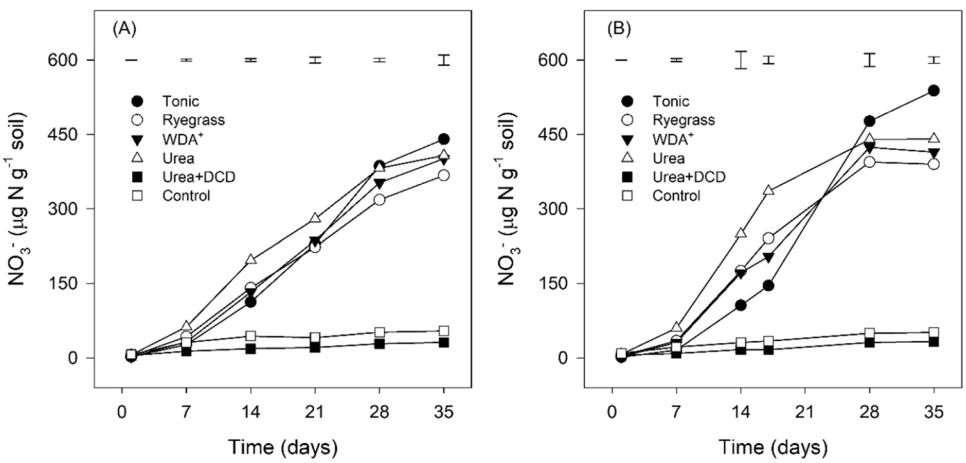

Fig. 2 Concentrations of nitrate–N (NO_3^--N) in soil microcosms during a 35-day incubation following the application of sheep urine collected after (**A**) 48 h or (**B**) 2 weeks of grazing on plantain cultivars (Tonic or WDA$^+$) compared with NO_3^--N concentrations in microcosms treated with ryegrass urine, urea or urea + DCD. The error bars represent LSDs ($p < 0.05$) at each incubation time

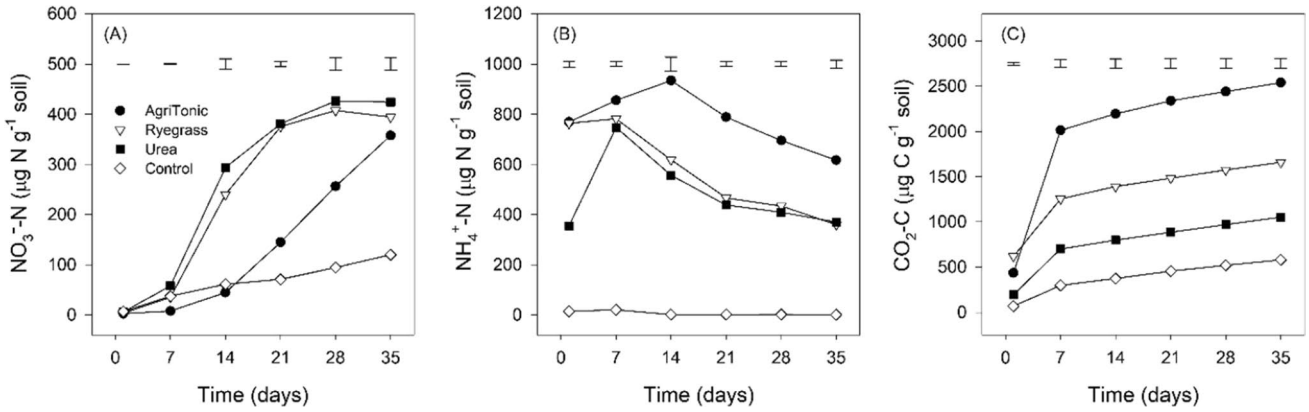

Fig. 3 NO_3^--N (**A**) and NH_4^+-N (**B**) concentrations and cumulative CO_2-C production (**C**) during a 35-day incubation for soil treated with urea or urine of sheep grazing on either 'Agritonic' plantain or ryegrass (N addition rate 802 µg N g^{-1}). The error bars represent LSDs ($p < 0.05$) at each incubation time

ureases produced by the urinary microbiome (Bao et al. 2017; Whiteside et al. 2015). However, in the case of the urea treatment, hydrolysis was reliant on ureases produced by the soil microbiota. In both the urea and ryegrass urine treatments, NH_4^+-N gradually decreased between days 7 and 35, with little difference between the two treatments for the remainder of the incubation.

In the plantain urine treatment, there was a significant increase (166 µg g^{-1} soil) in NH_4^+-N between days 1 and 14, which was not accounted for by urea-hydrolysed N. The probable source of this NH_4^+-N was organic matter mineralisation, brought on by the large increase in dissolved organic matter observed following application of plantain urine (discussed below). Additionally, the mass spectroscopy data suggested that 'Agritonic' urine was rich in nitrogenous compounds and these compounds may have contributed to the greater mineralisation of N in microcosms treated with 'Agritonic' urine. Between days 14 and 35, NH_4^+-N in the soil decreased, but concentrations remained greater than in the urea and ryegrass urine treatments (by ~ 250 µg N g^{-1} at day 35).

In the first 7 days of incubation, there was a pulse of respiration (CO_2 production), particularly where plantain urine was applied (plantain urine > ryegrass urine > urea > control) (Fig. 3C). In the urea treatment, a substantial part (~ 57%) of the pulse can be attributed to CO_2-C released by hydrolysis of urea (estimated at 344 µg C g^{-1}), with the remainder being due to mineralisation of soil organic matter solubilised as a result of the urea-induced pH increase (from pH 5.6 to 6.9, data not shown). Between 7 and 35 days, there was only a gradual increase in the production of CO_2 that was similar in all treatments, including the control. The additional quantity of CO_2-C generated in the plantain urine treatment relative to the urea treatment (i.e., CO_2-C released by mineralisation of UOF and/or soil organic matter) in the first 14 days amounted to ~ 1400 µg C g^{-1}. This quantum of C mineralisation is compatible with the additional N mineralised (~ 160 µg g^{-1}) in the first 14 days after application of plantain urine, assuming a ratio of ~ 10:1 between mineralised C and mineralised N. Although addition of ryegrass urine also increased CO_2-C production, there was no evidence of an associated increase in N mineralisation (total mineral N did not differ significantly from that in the urea treatment between days 7 and 35).

The respiration pulse following plantain urine addition was associated with a large increase in water-extractable organic C (WEC) in the soil (Fig. 4A). Within 1 day of incubation, WEC had increased from ~ 100 µg g^{-1} in the control to 1570 µg g^{-1} in soil treated with plantain urine, before declining to ~ 300 µg g^{-1} on day 7. This rapid decline in WEC was presumably due to its decomposition by soil microorganisms. The WEC was probably the source of a large part of the CO_2-C produced from the plantain urine treatment during the early part of the incubation. An increase in WEC was also observed following addition of ryegrass urine and urea, but it was a much smaller amount than in the plantain urine treatment. The WEC in the plantain urine treatment initially (day 1) had a large proportion (ca. 20%) of phenolic-C (Fig. 4B), the source of which was presumably phenolic compounds in the plantain urine. Phenolic compounds are known to be inhibitory to nitrification (Castaldi et al. 2009; Kholdebarin and Oertli 1994; Lodhi and Killingbeck 1980; Rice and Pancholy 1974) but a causal link between specific urinary phenolic metabolites and nitrification inhibition remains to be established.

A secondary finding in this incubation study was that while urea solution may closely mimic urine from a ryegrass system, it is not necessarily a good proxy for urine from animals fed other forages. Indeed, "artificial" urine has previously been shown to have different N dynamics compared

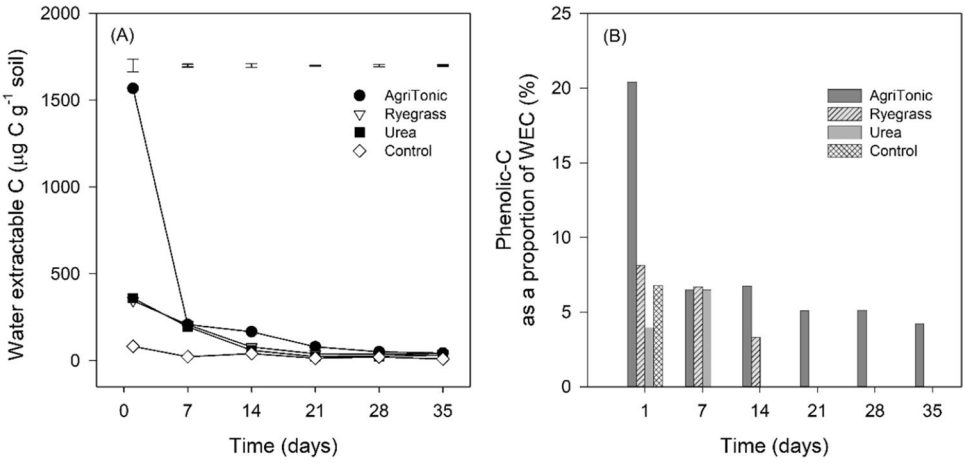

Fig. 4 Water-extractable C (**A**) and the proportion of phenolic-C (**B**) over 35 days of incubation for soil treated with urea or urine of sheep grazing on either 'Agritonic' plantain or ryegrass. The N addition rate was 802 µg N g^{-1}. The error bars represent LSD ($p < 0.05$) at each incubation time

to "natural" urine (Kool et al. 2006), and these results further support why natural urine has previously been recommended for experimental use (de Klein et al. 2003).

Nitrification inhibition bioassay

With the incubation results demonstrating that the urine of plantain-fed sheep contained compounds capable of inhibiting nitrification in soil microcosms, the ability of the organic fraction of urine (UOF; derived from the urines sourced from the autumn animal performance trial) to inhibit nitrification was tested in vitro. Figure 5A shows that the addition of a small (1.4 µL) aliquot of 'Agritonic' plantain UOF substantially reduced the amount of NO_2^- produced by *N. multiformis* in a nitrification inhibition bioassay. The UOF from ryegrass urine produced no inhibition when added at doses up to 50 µL (maximum tested). The antibacterial/bacteriostatic effects of the UOF were assessed using a bacterial growth assay (Fig. 5B). The same aliquot of plantain UOF tested in the bioassay caused a slight delay in the growth of the *Acidovorax* isolate relative to the control. However, this delay was less than that caused by the synthetic inhibitor allylthiourea (ATU) and by the ryegrass UOF.

To further explore the inhibitory effect of 'Agritonic' plantain UOF, a preparation that had been freeze-dried and reconstituted to a known concentration was added in increasing quantities (up to 180 µg) to the bioassay (Fig. 6). The degree of nitrification inhibition increased asymptotically as the UOF rate increased, with 50% inhibition at the 25-µg addition rate and near-complete nitrification inhibition at 150-µg addition, indicating that compounds in plantain urine were strongly inhibitory.

These results suggest that the urine of 'Agritonic' plantain-fed sheep contained compounds that were not overtly bactericidal but were inhibitory toward ammonia monooxygenase (the enzyme that catalyses the conversion of NH_4^+ to NO_2^-); the amount of 'Agritonic' plantain UOF that caused a significant inhibition of nitrification relative to the control caused only a small and brief retardation of bacterial growth.

In this bioassay, a single bacterial species was challenged with the UOF, so caution is required when interpreting these results. Soils contain a consortium of nitrifying organisms

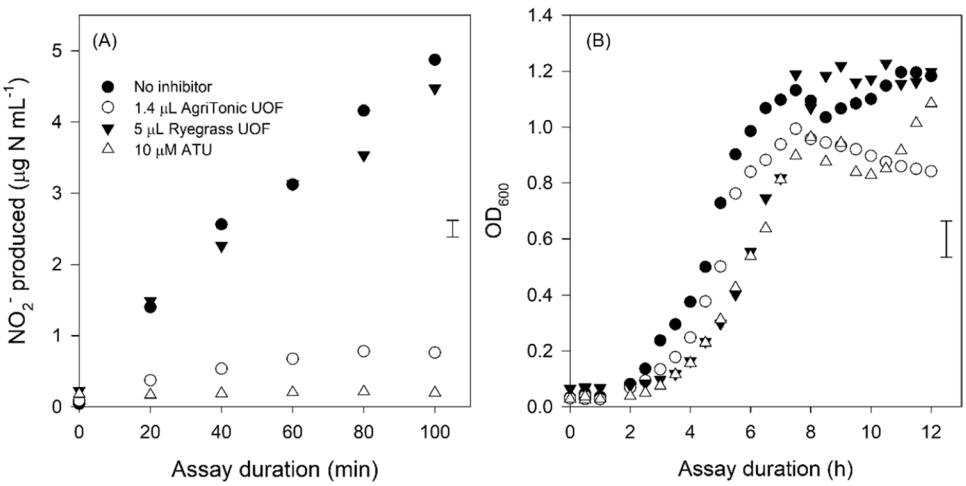

Fig. 5 The effect of plantain- and ryegrass-derived urine organic fraction (UOF) and allylthiourea (ATU) on (**A**) the production of nitrite (NO_2^-) by *Nitrosospira multiformis* and (**B**) the growth curve for *Acidovorax* isolate as monitored by an increase in optical density at 600 nm (OD_{600}). The error bars represent the $LSD_{5\%}$

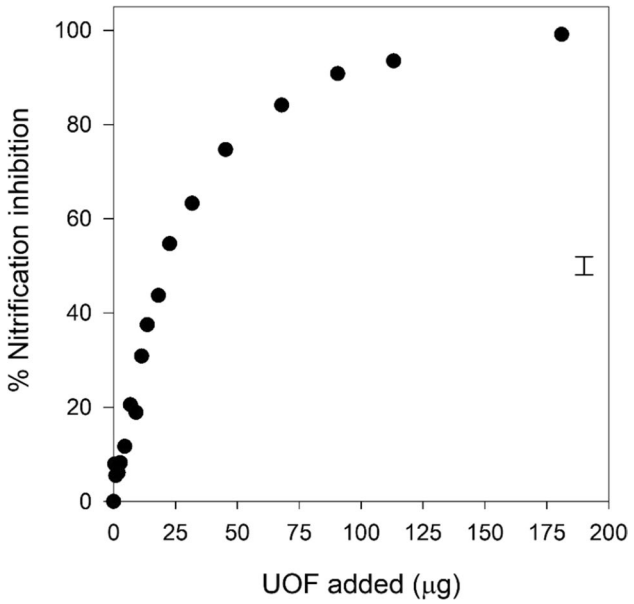

Fig. 6 Nitrification inhibition (% NO_2^--N production inhibited relative to uninhibited control) curve for *Nitrosospira multiformis* challenged with increasing concentrations of an 'Agritonic' plantain-derived urine organic fraction (UOF) in a bioassay volume of 335 µL. The error bars represent the $LSD_{5\%}$

that may differ in their sensitivity to the UOF. It has been shown that the response of nitrifying microorganisms (NH_4^+-oxidising archaea and bacteria) to synthetic nitrification inhibitors varies considerably (Lehtovirta-Morley et al. 2013; Shen et al. 2013). Therefore, it is essential to confirm that inhibition occurs under field conditions.

Conclusions

Soil incubation studies showed that net nitrification after the application of plantain urine was reduced compared to ryegrass urine, with the urine of sheep fed with the plantain cultivar 'Agritonic' producing the strongest reduction in NO_3^- produced. Mass spectroscopic analyses showed that plantain urine had many more N-containing compounds (excluding urea) than ryegrass urine; these included metabolic derivatives of the plantain bioactive compounds aucubin, verbascoside, and catalpol. In vitro assays suggested that the reduction in NO_3^- production by compounds excreted in plantain urine was due to the inhibition of nitrification, with ammonia monooxygenase being the target enzyme. In the future, targeted metabolomic analyses combined with bioassay-directed fractionation may be useful in identifying which organic metabolites have a role in suppressing nitrification and in providing targets for breeding programmes to develop cultivars that confer superior urinary-excreted BNI activity. However, the effect of these metabolites on other N transformations in the soil that reduce NO_3^- concentrations cannot be discounted and studies that consider gross N transformations should be considered. The activity of urinary BNI metabolites in soil may be affected by a multitude of factors and synergistic effects will likely influence the magnitude of their effect in mitigating NO_3^- leaching losses from agricultural systems.

Acknowledgements The authors thank Glenn Judson at Agricom for the collection of urine; Chris Dunlop, Kathryn Lehto, Rebekah Tregurtha, and Sandi Keenan at The New Zealand Institute for Plant & Food Research Limited for their technical assistance; Craig Anderson at The New Zealand Institute for Plant & Food Research Limited for internal review of the manuscript; and David Baird at VSN (NZ) Limited for his assistance with statistics.

Declarations

This work forms part of the Agricom-developed Greener Pastures Project with funding from Callaghan Innovation. The authors declare no competing interests.

References

Adler LS, Schmitt J, Bowers MD (1995) Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). Oecologia 101:75–85. https://doi.org/10.1007/BF00328903

Al-Mamun M, Abe D, Kofujita H, Tamura Y, Sano H (2008) Comparison of the bioactive components of the ecotypes and cultivars of plantain (*Plantago lanceolata* L.) herbs. Anim Sci J 79:83–88. https://doi.org/10.1111/j.1740-0929.2007.00501.x

Astley M (2013) DCD use suspended in New Zealand after residue discovered in dairy. William Reed Business Media Ltd. https://www.dairyreporter.com/Article/2013/01/24/DCD-use-suspended-in-New-Zealand-after-residue-discovered-in-dairy. Accessed 24 Jan 2013

Bao Y, Al KF, Chanyi RM, Whiteside S, Dewar M, Razvi H, Reid G, Burton JP (2017) Questions and challenges associated with studying the microbiome of the urinary tract. Ann Transl Med 5:1–8. https://doi.org/10.21037/atm.2016.12.14

Belser LW, Mays EL (1980) Specific-inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Appl Environ Microbiol 39:505–510. https://doi.org/10.1128/AEM.39.3.505-510.1980

Box LA, Edwards GR, Bryant RH (2017) Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. NZ J Agric Res 60:470–482. https://doi.org/10.1080/00288233.2017.1366924

Box LA, Judson HG (2018) The concentration of bioactive compounds in *Plantago lanceolata* is genotype specific. J NZ Grassl 80:113–118. https://doi.org/10.33584/jnzg.2018.80.334

Bristow AW, Whitehead DC, Cockburn JE (1992) Nitrogenous constituents in the urine of cattle, sheep and goats. J Sci Food Agric 59:387–394. https://doi.org/10.1002/jsfa.2740590316

Brown PD, Morra MJ (2009) Brassicaceae tissues as inhibitors of nitrification in soil. J Agric Food Chem 57:7706–7711. https://doi.org/10.1021/jf901516h

Bryant RH, Miller ME, Greenwood SL, Edwards GR (2017) Milk yield and nitrogen excretion of dairy cows grazing binary and multispecies pastures. Grass Forage Sci 72:806–817. https://doi.org/10.1111/gfs.12274

Cabrera ML, Beare MH (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Sci Soc Am J 57:1007–1012. https://doi.org/10.2136/sssaj1993.03615995005700040021x

Cameron KC, Di H, Moir J (2013) Nitrogen losses from the soil/plant system: a review. Ann Appl Biol 162:145–173. https://doi.org/10.1111/aab.12014

Carlton AJ, Cameron KC, Di HJ, Edwards GR, Clough TJ (2019) Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. N Z J Agric Res:1–23. https://doi.org/10.1080/00288233.2018.1461659

Castaldi S, Carfora A, Fiorentino A, Natale A, Messere A, Miglietta F, Cotrufo MF (2009) Inhibition of net nitrification activity in a Mediterranean woodland: possible role of chemicals produced by *Arbutus unedo*. Plant Soil 315:273–283. https://doi.org/10.1007/s11104-008-9750-x

Chapman HD (1965) Cation-exchange capacity. In: Norman AG (ed) Methods of soil analysis. ASA and SSSA, Madison, pp 891–901. https://doi.org/10.2134/agronmonogr9.2.c6

David DB, Poli CHEC, Savian JV, Amaral GA, Azevedo EB, Jochims F (2015) Urinary creatinine as a nutritional and urinary volume marker in sheep fed with tropical or temperate forages. Arq Bras Med Vet Zootec 67:1009–1015. https://doi.org/10.1590/1678-4162-7759

de Klein CAM, Barton L, Sherlock RR, Li Z, Littlejohn RP (2003) Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. Aust J Soil Res 41:381–399. https://doi.org/10.1071/SR02128

Di HJ, Cameron KC (2016) Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: a review. J Soils Sed 16:1401–1420. https://doi.org/10.1007/s11368-016-1403-8

Dietz M, Machill S, Hoffmann HC, Schmidtke K (2013) Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. Plant Soil 368:445–458. https://doi.org/10.1007/s11104-012-1524-9

Dijkstra J, Oenema O, van Groenigen JW, Spek JW, van Vuuren AM, Bannink A (2013) Diet effects on urine composition of cattle and N_2O emissions. Animal 7:292–302. https://doi.org/10.1017/s1751731113000578

Edwards G, Bryant R, Smith N, Hague H, Taylor S, Ferris A, Farrell L (2015) Milk production and urination behaviour of dairy cows grazing diverse and simple pastures. Proc NZ Soc Anim Prod 75:79–83

Garrity GM, Bell JA, Lilburn T (2005) Class II. Betaproteobacteria class. nov. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual® of systematic bacteriology: volume two The Proteobacteria Part C The Alpha-, Beta-, Delta-, and Epsilonproteobacteria. Springer US, Boston, pp 575–922. https://doi.org/10.1007/978-0-387-29298-4_2

Gee GW, Bauder JW (1986) Particle-size analysis. In: Klute A (ed) Methods of soil analysis. Part 1. Physical and mineralogical methods, 2 edn. ASA and SSSA, Madison, pp 383-411

Ghisalberti EL (1998) Biological and pharmacological activity of naturally occurring iridoids and secoiridoids. Phytomedicine 5:147–163. https://doi.org/10.1016/S0944-7113(98)80012-3

Goncalves S, Romano A (2016) The medicinal potential of plants from the genus *Plantago* (Plantaginaceae). Ind Crops Prod 83:213–226. https://doi.org/10.1016/j.indcrop.2015.12.038

Hattori M, Kawata Y, Inoue K, Shu Y, Che Q, Namba T, Kobashi K (1990) Transformation of aucubin to new pyridine monoterpene alkaloids, aucubinines A and B, by human intestinal bacteria. Phytother Res 4:66–70. https://doi.org/10.1002/ptr.2650040207

Haynes RJ, Williams PH (1993) Nutrient cycling and soil fertility in the grazed pasture ecosystem. Adv Agron 49:119–199. https://doi.org/10.1016/S0065-2113(08)60794-4

Hoogendoorn CJ, Betteridge K, Costall DA, Ledgard SF (2010) Nitrogen concentration in the urine of cattle, sheep and deer grazing a common ryegrass/cocksfoot/white clover pasture. NZ J Agric Res 53:235–243. https://doi.org/10.1080/00288233.2010.499899

Judson H, Fraser P, Peterson M, Kemp P, Edwards G (2018) Specific genotypes of plantain (*Plantago lanceolata*) vary in their impact on sheep urine volume and nitrification in the urine patch. J NZ Grassl 80:125–128. https://doi.org/10.33584/jnzg.2018.80

Keeney D, Nelson D (1982) Nitrogen-inorganic forms. In: Page A, Miller R, Keeney D (eds) Methods of soil analysis. Part 2. Chemical and microbiological properties. 2 Edn. ASA and SSSA, Madison, pp 643–698

Keir B, Mayes R, Ørskov E (2001) The potential of urinary metabolites of plant compounds as indicators of botanical composition of the diet of goats. Proc Br Soc Anim Sci 2001:146–146. https://doi.org/10.1017/S1752756200005287

Kholdebarin B, Oertli JJ (1994) Nitrification: interference by phenolic compounds. J Plant Nutr 17:1827–1837. https://doi.org/10.1080/01904169409364850

Kool DM, Hoffland E, Abrahamse S, van Groenigen JW (2006) What artificial urine composition is adequate for simulating soil N_2O fluxes and mineral N dynamics? Soil Biol Biochem 38:1757–1763. https://doi.org/10.1016/j.soilbio.2005.11.030

Lane GA, Fraser K, Cao M, Koulman A, Cosgrove GP, Death A (2006) Urinary biomarkers of forage feeding from ESI-MS/MS fingerprinting: exploratory studies. Proc NZ Soc Anim Prod 66:230–235

Lehtovirta-Morley LE, Verhamme DT, Nicol GW, Prosser JI (2013) Effect of nitrification inhibitors on the growth and activity of *Nitrosotalea devanaterra* in culture and soil. Soil Biol Biochem 62:129–133. https://doi.org/10.1016/j.soilbio.2013.01.020

Lodhi MAK, Killingbeck KT (1980) Allelopathic inhibition of nitrification and nitrifying bacteria in a Ponderosa pine (Pinus-Ponderosa Dougl) Community. Am J Bot 67:1423–1429. https://doi.org/10.2307/2442870

Ma T, Deng K-D, Tu Y, Jiang C-G, Zhang N-F, Li Y-L, Si B-W, Lou C, Diao Q-Y (2014) Effect of dietary concentrate:forage ratios and undegraded dietary protein on nitrogen balance and urinary excretion of purine derivatives in Dorper×thin-tailed Han crossbred lambs. Asian-Australas J Anim Sci 27:161–168. https://doi.org/10.5713/ajas.2013.13338

Massaccesi L, Bardgett RD, Agnelli A, Ostle N, Wilby A, Orwin KH (2015) Impact of plant species evenness, dominant species identity and spatial arrangement on the structure and functioning of soil microbial communities in a model grassland. Oecologia 177:747–759. https://doi.org/10.1007/s00442-014-3135-z

Minnée E, de Klein C, Dalley D (2019) Plantain helping farmers to achieve environmental targets. DairyNZ Technical Series. https://www.dairynz.co.nz/publications/technical-series/technical-series-april-2019/. Accessed April 2019

Minnée EMK, Waghorn GC, Lee JM, Clark CEF (2017) Including chicory or plantain in a perennial ryegrass/white clover-based diet of dairy cattle in late lactation: feed intake, milk production and rumen digestion. Anim Feed Sci Technol 227:52–61. https://doi.org/10.1016/j.anifeedsci.2017.03.008

Moir JL, Cameron KC, Di HJ, Fertsak U (2011) The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system. J Agric Sci 149:473–485. https://doi.org/10.1017/s0021859610001012

Monaghan RM, Barraclough D (1992) Some chemical and physical factors affecting the rate and dynamics of nitrification in urine-affected soil. Plant Soil 143:11–18. https://doi.org/10.1007/bf00009124

Navarrete S, Kemp PD, Pain SJ, Back P (2016) Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata* L.) and their effect on in vitro rumen fermentation. Anim Feed Sci

Technol 222:158–167. https://doi.org/10.1016/j.anifeedsci.2016.10.008
O'Connell CA, Judson HG, Barrell GK (2016) Sustained diuretic effect of plantain when ingested by sheep. Proc NZ Soc Anim Prod 76:14–17
O'Sullivan CA, Duncan EG, Whisson K, Treble K, Ward PR, Roper MM (2017a) A colourimetric microplate assay for simple, high throughput assessment of synthetic and biological nitrification inhibitors. Plant Soil 413:275–287. https://doi.org/10.1007/s11104-016-3100-1
O'Sullivan CA, Whisson K, Treble K, Roper MM, Micin SF, Ward PR (2017b) Biological nitrification inhibition by weeds: wild radish, brome grass, wild oats and annual ryegrass decrease nitrification rates in their rhizospheres. Crop Pasture Sci 68:798–804. https://doi.org/10.1071/CP17243
Ohno T, First PR (1998) Assessment of the Folin and Ciocalteu's method for determining soil phenolic carbon. J Environ Qual 27:776–782. https://doi.org/10.2134/jeq1998.00472425002700040008x
Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. US Dept Agric Circ 939:1–18
Oyourou JN, Combrinck S, Regnier T, Marston A (2013) Purification, stability and antifungal activity of verbascoside from *Lippia javanica* and *Lantana camara* leaf extracts. Ind Crops Prod 43:820–826. https://doi.org/10.1016/j.indcrop.2012.08.028
Park KS (2013) Aucubin, a naturally occurring iridoid glycoside inhibits TNF-α-induced inflammatory responses through suppression of NF-κB activation in 3T3-L1 adipocytes. Cytokine 62:407–412. https://doi.org/10.1016/j.cyto.2013.04.005
Podolyan A, Di HJ, Cameron KC (2019) Effect of plantain on nitrous oxide emissions and soil nitrification rate in pasture soil under a simulated urine patch in Canterbury, New Zealand. J Soils Sed. https://doi.org/10.1007/s11368-019-02505-1
Qi M, Xiong A, Li P, Yang Q, Yang L, Wang Z (2013) Identification of acteoside and its major metabolites in rat urine by ultra-performance liquid chromatography combined with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. J Chromatogr B 940:77–85. https://doi.org/10.1016/j.jchromb.2013.09.023
Rauber R, Liebenau S, Friedrichs E, Schmidtke K (2008) Agronomic effects of underseeding ribwort plantain (*Plantago lanceolata* L.) in organically grown potatoes. Pflanzenbauwissenschaften 12:32–40
Rice EL, Pancholy SK (1974) Inhibition of nitrification by climax ecosystems: 3. Inhibitors other than tannins. Am J Bot 61:1095–1103. https://doi.org/10.2307/2441927
Rombouts JE, Links J (1956) The chemical nature of the antibacterial substance present in *Aucuba japonica* thunbg. Experientia 12:78–80. https://doi.org/10.1007/bf02164691
Rumball W, Keogh RG, Lane GE, Miller JE, Claydon RB (1997) 'Grasslands Lancelot' plantain (*Plantago lanceolata* L.). NZ J Agric Res 40:373–377. https://doi.org/10.1080/00288233.1997.9513258
Selbie DR, Buckthought LE, Shepherd MA (2015) The challenge of the urine patch for managing nitrogen in grazed pasture systems. Adv Agron 129:229–292. https://doi.org/10.1016/bs.agron.2014.09.004
Shen T, Stieglmeier M, Dai J, Urich T, Schleper C (2013) Responses of the terrestrial ammonia-oxidizing archaeon Ca. *Nitrososphaera viennensis* and the ammonia-oxidizing bacterium *Nitrosospira multiformis* to nitrification inhibitors. FEMS Microbiol Lett 344:121–129. https://doi.org/10.1111/1574-6968.12164
Shirley KP, Windsor LJ, Eckert GJ, Gregory RL (2015) In vitro effects of Plantago major extract, aucubin, and baicalein on *Candida albicans* biofilm formation, metabolic activity, and cell surface hydrophobicity. J Prosthodont 26:508–515. https://doi.org/10.1111/jopr.12411
Stewart AV (1996) Plantain (*Plantago lanceolata*) - a potential pasture species. In: Proceedings of the New Zealand Grassland Association, pp 77–86
Subbarao GV, Nakahara K, Hurtado MP, Ono H, Moreta DE, Salcedo AF, Yoshihashi AT, Ishikawa T, Ishitani M, Ohnishi-Kameyama M, Yoshida M, Rondon M, Rao IM, Lascano CE, Berry WL, Ito O (2009) Evidence for biological nitrification inhibition in *Brachiaria* pastures. Proc Natl Acad Sci USA 106:17302. https://doi.org/10.1073/pnas.0903694106
Subbarao GV, Nakahara K, Ishikawa T, Ono H, Yoshida M, Yoshihashi T, Zhu Y, Zakir HAKM, Deshpande SP, Hash CT, Sahrawat KL (2013) Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366:243–259. https://doi.org/10.1007/s11104-012-1419-9
Subbarao GV, Nakahara K, Ishikawa T, Yoshihashi T, Ito O, Ono H, Ohnishi-Kameyama M, Yoshida M, Kawano N, Berry WL (2008) Free fatty acids from the pasture grass *Brachiaria humidicola* and one of their methyl esters as inhibitors of nitrification. Plant Soil 313:89–99. https://doi.org/10.1007/s11104-008-9682-5
Tamura Y, Nishibe S (2002) Changes in the concentrations of bioactive compounds in plantain leaves. J Agric Food Chem 50:2514–2518. https://doi.org/10.1021/jf011490x
Tesfamariam T, Yoshinaga H, Deshpande SP, Rao PS, Sahrawat KL, Ando Y, Nakahara K, Hash CT, Subbarao GV (2014) Biological nitrification inhibition in sorghum: the role of sorgoleone production. Plant Soil 379:325–335. https://doi.org/10.1007/s11104-014-2075-z
Totty VK, Greenwood SL, Bryant RH, Edwards GR (2013) Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. J Dairy Sci 96:141–149. https://doi.org/10.3168/jds.2012-5504
Venterea RT, Clough TJ, Coulter JA, Breuillin-Sessoms F (2015) Ammonium sorption and ammonia inhibition of nitrite-oxidizing bacteria explain contrasting soil N_2O production. Sci Rep 5.https://doi.org/10.1038/srep12153
Visioli F, Galli C, Grande S, Colonnelli K, Patelli C, Galli G, Caruso D (2003) Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. J Nutr 133:2612–2615. https://doi.org/10.1093/jn/133.8.2612
Ward BB, Courtney KJ, Langenheim JH (1997) Inhibition of *Nitrosomonas europaea* by monoterpenes from coastal redwood (*Sequoia sempervirens*) in whole-cell studies. J Chem Ecol 23:2583–2598. https://doi.org/10.1023/B:JOEC.0000006668.48855.b7
Warrack BM, Hnatyshyn S, Ott K-H, Reily MD, Sanders M, Zhang H, Drexler DM (2009) Normalization strategies for metabonomic analysis of urine samples. J Chromatogr B 877:547–552. https://doi.org/10.1016/j.jchromb.2009.01.007
Whiteside SA, Razvi H, Dave S, Reid G, Burton JP (2015) The microbiome of the urinary tract—a role beyond infection. Nat Rev Urol 12:81. https://doi.org/10.1038/nrurol.2014.361
Woodward SL, Waghorn GC, Bryant MA, Benton A (2012) Can diverse pasture mixtures reduce nitrogen losses? In: Jacobs J (ed) Proceedings of the 5th Australasian Dairy Science Symposium. Australasian Dairy Science Symposium, Melbourne, Australia, pp 463–464
Yan-Chun Z, Rong-Liang Z (1991) Phenolic compounds and an analog as superoxide anion scavengers and anti oxidants. Biochem Pharmacol 42:1177–1179. https://doi.org/10.1016/0006-2952(91)90251-Y
Zakir HAKM, Subbarao GV, Pearse SJ, Gopalakrishnan S, Ito O, Ishikawa T, Kawano N, Nakahara K, Yoshihashi T, Ono H, Yoshida M (2008) Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). New Phytol 180:442–451. https://doi.org/10.1111/j.1469-8137.2008.02576.x