Nitrification inhibition by urine from cattle consuming *Plantago lanceolata*

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Abstract
Plantain (*Plantago lanceolata* L.) has the potential to indirectly reduce nitrate leaching from urine patches via compounds excreted in the urine of animals grazing the forb acting as biological nitrification inhibitors. Proof-of-concept research was previously undertaken using sheep urine, but it is important to examine whether this effect also occurs with cattle urine since cattle pose a greater N-leaching risk due to their higher urinary-N load. Housed dairy heifers (n=4) were assigned *ad libitum* dietary treatments of perennial ryegrass/ white clover or plantain for 14 days. On day 14, urine was collected through a sterile Foley catheter into a sealed container. Cattle then switched dietary treatment and urine was collected after a further 14 days. Urine samples were applied to soil microcosms and the net nitrification rate during a 35-day incubation determined. Similar urine-N concentrations were applied initially but a slower rate of soil nitrification was observed in the microcosms treated with urine from plantain-fed cows compared with those treated with urine from ryegrass/white clover-fed cows. The urine samples collected after the crossover showed a wider treatment difference in total N concentration, but also demonstrated a reduction in soil nitrification rate under the plantain urine. These results show similar trends to those previously reported for sheep urine.

Keywords: plantain, nitrate leaching, incubation, ammonium-N, crossover

Introduction
Reducing nitrate leaching from the livestock sector has become a strong research focus in New Zealand since the implementation of environmental regulations (Ministry for the Environment 2011). The nitrogen (N) deposited in urine patches of livestock-grazed perennial ryegrass pasture is a major contributor to on-farm N pollution, due to these swards delivering N in excess of animal requirements (Pacheco & Waghorn 2008). Grazing ruminants can return up to 95% of ingested N to the soil/pasture system (Selbie et al. 2015) and N loadings under cattle urine patches can be as high as 1000 kg N/ha (Haynes & Williams 1993). Therefore, the requirement of pasture plants for N (<600 kg/ha/y; Moir et al. (2007)) is often exceeded.

The inclusion of plantain (*Plantago lanceolata* L.) in grazed pasture systems has been identified as a potential tool for mitigation of nitrogen losses to the environment. Plantain contains a number of bioactive plant secondary metabolites that may be implicated in the reduction of nitrate leaching from urine patches via a number of mechanisms (Gardiner et al. 2016; Box et al. 2017; Carlton et al. 2018; Judson et al. 2018a; Mangwe et al. 2019). One of these mechanisms is a reduction in the rate at which nitrate accumulates in the soil following urine deposition. This reduction has been attributed to the presence of biological nitrification inhibition (BNI) compounds excreted in urine of plantain-fed animals that cause a delay in the conversion of ammonium to nitrate by soil dwelling nitrifying microorganisms. Judson et al. (2018a) showed that there was ~40% reduction in nitrate produced over at least 28 days following the application of urine from plantain-fed sheep to soil microcosms. A reduction in the rate of soil nitrification can potentially reduce nitrate leaching by slowing down the release of nitrogen into plant-available form and thereby increasing the time that plants have available to take up available nitrogen before it may be subject to leaching.

To date, research on this mechanism has focussed on sheep urine (Fraser et al. 2018; Judson et al. 2018a, b; Peterson et al. 2018) but cattle urine poses a greater N-leaching risk due to its higher urinary-N load. It is presently unclear whether BNI compounds are present in the urine of cattle grazing plantain, so the aim of this experiment was to assess if BNI occurs when urine from cattle fed plantain is deposited on soil.

Materials and Methods
A study was conducted indoors at the Johnson Memorial Laboratory, Lincoln University, Lincoln, New Zealand during Nov 2018. Four Jersey × Friesian heifers (body weight: 303±2.9 kg; age 448±4.2 days; and breeding worth 144±18.9 NZ$), were randomly assigned to one of two dietary treatments of either perennial ryegrass (*Lolium perenne* L.)–white clover (*Trifolium repens* L.) pasture (*cv* One^{50}) (RW) or plantain (*Plantago lanceolata* L.) (*cv* Agritonic) (PL), each cut fresh daily. Forage was cut from swards yielding approximately

2500 kg DM/ha, both containing minimal clover. Seed head was present in the plantain forage but was largely immature and thus palatable. Treatments and measurements of the heifers in this study were approved by the Lincoln University Animal Ethics Committee # AEC 2018-42.

Herbage samples were collected from the field immediately after forage was cut, once during the measurement periods, to determine chemical composition. Feed samples were freeze-dried, ground through a 1.0-mm sieve and analysed for N content, neutral detergent fibre (NDF), acid detergent fibre (ADF), water-soluble carbohydrate and dry organic matter digestibility (DOMD) using calibrated near infra-red spectroscopy (Hill Laboratories). Metabolisable energy (ME) of feed was estimated based on the equation ME (MJ/kg) = DOMD% × 0.16 (AFRC 1993).

Heifers from each treatment were housed in pens (two heifers per pen) with sawdust bedding during the first 7 days and then in individual metabolic crates during the next 7 days (Measurement 1). Freshly cut herbage was offered to heifers over two meals at 10:00 and 16:00 hr. Herbage allocation was set to allow 400-500 g dry matter (DM) feed refusals in feeding bins after each meal, and each heifer allowed *ad libitum* access to water.

On day 11, a sterile Foley catheter (14 Fr, 55 cm, 30 cc; ClearView, Smiths Medical, USA) was placed into the urethra and, on day 14, urine samples (200 mL) were collected through the catheters into sealed containers and immediately prepared for total N analysis and subsequently application to soil. After urine collection, heifers were returned to pens, diets reversed to create a crossover design and the experiment repeated (Measurement 2).

Immediately following collection, urine samples from each animal were transferred to the laboratory and total N determined on a QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, CO, USA) fitted with an online UV-catalysed persulfate oxidation unit (Cabrera & Beare 1993).

A soil-incubation experiment (adapted from Curtin et al. 2017) was used to evaluate soil nitrification following deposition of urine from heifers fed different diets. For this purpose, a Templeton silt loam (Typic Immature Pallic) soil from under a low-input ungrazed Italian ryegrass (*Lolium multiflorum* cultivar 'Andy') pasture was collected at Plant & Food Research farm, at Lincoln, Canterbury. Multiple soil cores (0–5 cm depth) were mixed thoroughly to form a composite sample, before passing through a 4-mm sieve and air-drying. Next, a total of 72 soil microcosms were prepared from this composite sample as detailed below.

Urine samples from each treatment were bulked to provide sufficient volume of homogenous composition. The urines were standardised for N concentration by dilution of the urine with deionised water. In Measurement 1, the urine from the heifers fed RW was diluted to match that from the heifers fed PL; both urines were then further diluted (to 2260 µg/mL) to avoid inhibition by high ammonium concentrations in the microcosm system. The latter step was not required for the urine collected in Measurement 2 as the standardised N concentrations (3325 µg/mL) were already sufficiently low to avoid this complication.

Soil microcosms were prepared in 70 mL plastic containers containing 20 g of air-dried equivalent soil. The urines were added to the microcosms drop-wise (6.3 mL added using an electronic pipette in titrate mode) to bring the soil to field capacity (defined here as the soil moisture content at -10 kPa). Water (in the place of urine) was added to additional microcosms to provide a control treatment. The microcosms were covered with Parafilm® which was pierced to allow for gas diffusion and placed in 5.5 L boxes in a specially designed split-plot arrangement in an incubator set at 20°C. During the incubation, the soil was maintained at -10 kPa by the addition of water as required.

Four experimental replicates of each treatment (PL urine, RW urine and control) were destructively sampled to determine the net nitrification rate after 1, 7, 14, 21, 28 and 35 days. At each time point, 5 g of soil from each replicate was used to measure inorganic N (NH_4^+-N and NO_3^--N) by shaking the soil with 2M KCl (soil/solution ratio 1:5) for 1 h (Keeney & Nelson 1982). The soil suspensions were centrifuged at 4000 rpm for 5 minutes and the supernatant filtered through a pre-leached Munktell 393 (Munktell Inc., Raleigh, NC, USA) filter paper before analysis of the filtered extract for NH_4^+-N and NO_3^--N on a QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, CO, USA) .

Statistical tests 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 the least significant difference ($LSD_{5\%}$) was used to separate means if treatment effects were found to be significant ($P<0.05$).

Results

Chemical composition and ME of the ryegrass-white clover pasture and plantain fed to the cattle are presented in Table 1. Dry matter (DM) content of forage was greater for RW than PL. The content of N was also numerically greater for ryegrass than plantain (34 vs 28 g N/kg DM for RW and PL, respectively). Dry organic matter digestibility (DOMD) and subsequent metabolisable energy (ME) content were numerically

lower for PL than RW.

The total N concentration in the PL-fed heifer urine was slightly lower than the RW-fed heifer urine in Measurement 1, but was half the concentration of the RW-fed heifer urine in Measurement 2 (Table 2). Nitrate-N was not detected in any of the urine samples. Concentrations of NH_4^+-N were greater in the urine of PL-fed heifers (13% of total N) compared to the urine of RW-fed heifers (<1% of total N) in Measurement 1 but were similar in Measurement 2.

The soil incubation with the urines collected in Measurement 1 (Figure 1A) showed that, after 14 days of incubation, NO_3^--N produced in microcosms treated with urine of PL-fed heifers (59 µg/g) was less than that produced in the control (68 µg/g). The NO_3^--N was also ~3 times less than was produced in microcosms treated with urine of RW-fed heifers (171 µg/g). Throughout the incubation, NO_3^--N production in the PL-urine microcosms was significantly less than in the RW-urine treatment, with the largest difference between the two treatments observed at 21 days incubation (164 and 392 µg/g for PL and RW respectively, $LSD_{5\%} = 18$).

Measurements of NH_4^+-N suggest that hydrolysis of urea and/or organic N species in the RW urine treatment was rapid, with near-complete conversion to NH_4^+-N within 1 day (Figure 1B). In contrast, the conversion of urea/organic N species to NH_4^+-N was much slower in the PL urine treatment. Beyond day 7 of the incubation, NH_4^+-N consumption in the RW-urine microcosms was significantly greater than in the PL-urine treatment, with the largest difference between the two treatments observed at 28 days incubation (503 and 238 µg/g for PL and RW respectively, $LSD_{5\%} = 26$).

The soil incubation of the urines collected in

Table 1 Chemical composition (g/kg DM) and metabolisable energy (MJ/kg DM) of ryegrass-white clover and plantain

	Ryegrass-white clover (RW)	Plantain (PL)
Dry matter	190	148
Nitrogen	34	28
Water-soluble carbohydrate	79	123
Neutral detergent fibre	500	371
Acid detergent fibre	261	234
Dry organic matter digestibility	725	669
Metabolisable energy	11.6	10.7

Table 2 Concentrations (µg/mL) of N species in the bulked, undiluted heifer urine used in the two laboratory soil incubation experiments (Measurements 1 & 2).

	Measurement 1		Measurement 2	
	Ryegrass-white clover (RW)	Plantain (PL)	Ryegrass-white clover (RW)	Plantain (PL)
Total N	4910	4520	6120	3325
NH_4^+-N	44	657	46	33
NO_3^--N	0	0	0	0

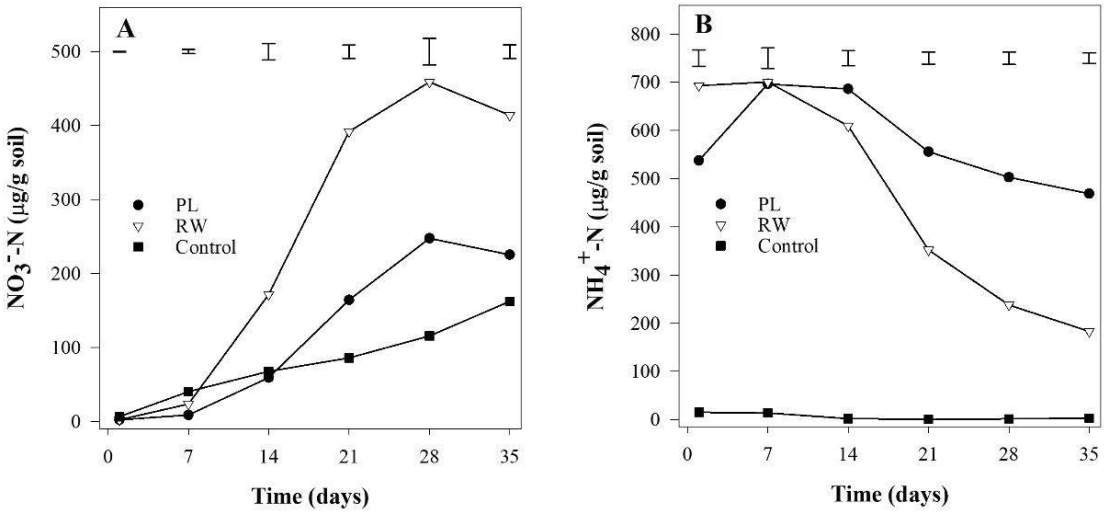

Figure 1 Concentrations of (A) soil nitrate-N and (B) soil ammonium-N during a 35-day incubation for soil treated with the urine collected in Measurement 1 from heifers fed either plantain (PL) or ryegrass-white clover (RW). Urines were added to these microcosms at a rate of 712 mg N/g soil. The control treatment received no N. The error bars represent LSDs (P<0.05) at each sampling time.

Measurement 2 (after treatment crossover and where urine was added at 1057 mg N/g soil, a slightly higher rate than in Measurement 1) showed a longer delay in the production of NO_3^--N compared to the first incubation experiment (Figure 2A). There was a small but statistically significant difference in the concentration of NO_3^--N produced in the microcosms after 14 days of incubation, but the BNI effect was not apparent until after 21 days of incubation. At this point, the concentration of NO_3^--N in the microcosms treated with urine of PL-fed heifers was similar to that produced in the control (91 and 101 µg/g, respectively) while the microcosms treated with urine of RW-fed heifers had produced 215 µg/g soil. The largest difference in NO_3^--N produced between the two treatments was observed after 28 days incubation. (206 and 403 µg/g for PL and RW respectively, $LSD_{5\%} = 40$). There was an associated delay in the consumption of NH_4^+-N in this incubation but in contrast to the NO_3^--N data, no significant difference in the urine treatments for NH_4^+-N during the timeframe of the incubation. With the higher concentration of N applied in Measurement 2 resulting in a notable delay in nitrate production, a longer incubation timeframe may have been beneficial, since a considerable amount of NH_4^+-N remained in urine treated soil (630–750at µg/g for PL and RW respectively) at the end of the incubation period.

Discussion

Pasture plants are unable to take up the large amounts of nitrogen deposited in cattle urine over a short time frame but are able to utilise N more effectively where it is made available to them slowly. Therefore, a slower rate of nitrification may be beneficial where high rates of nitrogen (such as found in cattle urine patches) are returned to the soil. In the current experiments, soil nitrification occurred at a significantly slower rate in soil over the first month following application of urine from cattle that had been fed plantain than where they had been fed ryegrass/white clover. This result is similar to findings previously reported for sheep urine (Fraser et al. 2018; Judson et al. 2018a, b; Peterson et al. 2018) where the effects were attributed to inhibition of the nitrification reaction by unidentified compounds present in the urine that had likely originated in plantain. The current results suggest that there is a similar mode of action for the urine from cattle fed plantain.

Several concomitant mechanisms have been suggested to account for the reduction in nitrate leaching from the urine patch (Judson 2018b). One mechanism, a lower urine patch N loading, has been partially attributed to the presence of a diuretic substance (O'Connell et al. 2016). In the current experiment, despite similar N intake, the urinary N concentrations in Measurement 1 were comparable between treatments suggesting that significant diuresis had not occurred. There was no effect of volume or N concentration when urine was deposited onto soil but the urine did exhibit a strong BNI effect. This result suggests that the diuretic and BNI effects are caused by independent mechanisms. Further, in Measurement 2, total N for plantain urine was half that of ryegrass urine proposing a strong diuretic effect had occurred in this instance. Although urine produced from the heifers

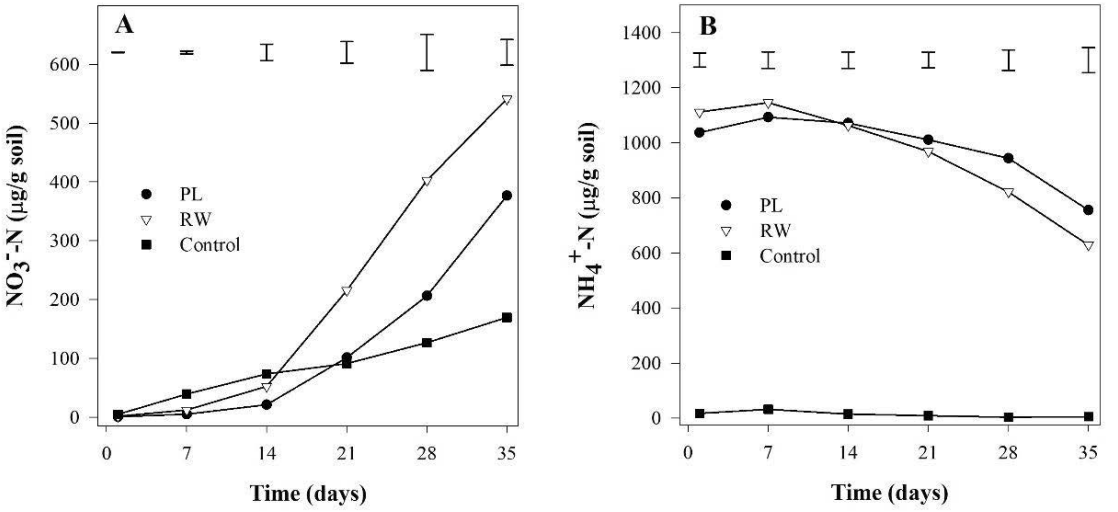

Figure 2 Concentrations of (A) soil nitrate-N and (B) and soil ammonium-N concentrations during a 35-day incubation for soil treated with the urine collected in Measurement 2 from heifers fed either plantain (PL) or ryegrass-white clover (RW). Urines were added at a rate of 1057 mg N/g soil. The control treatment received no N. The error bars represent LSDs ($P<0.05$) at each incubation time.

fed plantain had considerably lower N concentration in Measurement 2, the nitrification inhibition effect when plantain urine was added to soil was still apparent. This finding implies BNI activity may not be sensitive to large changes in urine volume or N concentration which is positive from a practical perspective.

The results of this laboratory study suggest that the excretion of BNI compounds in the urine via the inclusion of plantain in the diet of grazing animals, is just one factor in a potentially multiplicative system effect that would serve to mitigate excess N loss from the urine patch. The compound/s responsible for the BNI observed in these microcosms are yet to be identified and their mode of inhibition on the nitrification reaction also remains unknown. Additionally, further work is required to establish if factors such as soil type and management history affect the efficacy of these BNI compounds to inhibit the conversion of ammonium to nitrate.

Studies of leaching and gaseous losses are a potential next step in evaluating the contribution of urine-excreted BNI compounds to reducing urine patch N losses in a pasture system. To date there have been a few studies published that focus on the plant effect of incorporating plantain in the sward with a reduction in N leaching observed when urine from ryegrass-fed dairy cows was applied to swards containing plantain (Carlton et al. 2018; Welten et al. 2019) and when urine from dairy cows fed diets containing up to 42% plantain was applied to plots with the corresponding percentage of plantain in the sward (Woods et al. 2018). Additionally, it has been suggested that the plant effect, in tandem with a reduction in urinary N exertion, are the drivers behind plantain's efficacy in the mitigation of nitrous oxide (N_2O) (Simon et al. 2019). The latter study found that the proportion of plantain in the diet of dairy cows and thus the concentration of secondary metabolite concentrations in urine, did not result in any difference in N_2O emissions when urine from animals on a diet of 0% or 45% plantain were applied to a standard ryegrass/white clover pasture. Despite this, the results presented here and in Judson et al. (2018a) suggest further quantification and qualification of urine-excreted BNI compounds is warranted, in conjunction with assessment of the various mechanisms working in unison, and the contribution of each mechanism to the overall mitigation of N loss from the urine patch.

Conclusions

When urine from two dairy heifers fed plantain was collected and deposited onto soil, the ensuing rate of soil nitrification over the following month was considerably slower compared with where urine from ryegrass/white clover-fed heifers was deposited. The result from this small study suggests that BNI compounds do occur in plantain-fed cattle and is consistent with our previous research conducted with sheep urine. It appears that BNI compounds in the urine of animals grazing plantain act to delay the transformation of ammonium ions to nitrate ions. Therefore, under field conditions, it is expected that plants growing in the urine patch of animals that have grazed plantain would have more time to slowly utilise the urine nitrogen. This situation would result in more efficient utilisation of the urine nitrogen, leaving less nitrate present in the soil and thereby less nitrate available for leaching.

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REFERENCES

AFRC. 1993. *Energy and protein requirements of ruminants: an advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients.* Commonwealth Agricultural Bureaux, Farnham Royal, UK.

Box LA, Edwards GR, Bryant RH. 2017. Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research 60*: 470-482.

Cabrera ML, Beare MH. 1993. Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. *Soil Science Society of America Journal 57*: 1007-1012.

Carlton AJ, Cameron KC, Di HJ, Edwards GR, Clough TJ. 2018. Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. *New Zealand Journal of Agricultural Research*: 1-23.

Curtin D, Beare MH, Lehto K, Tregurtha C, Qiu W, Tregurtha R, Peterson M. 2017. Rapid assays to predict nitrogen mineralization capacity of agricultural soils. *Soil Science Society of America Journal 81*: 979-991.

Fraser P, Peterson M, Curtin D, Judson H. 2018. Soil nitrification inhibition by urine of sheep fed on plantain (*Plantago lanceolata*) is cultivar-dependent. Presented at: *Diverse Soils, Productive Landscapes, New Zealand Society of Soil Science Conference*. Napier, New Zealand.

Gardiner CA, Clough TJ, Cameron KC, Di HJ, Edwards GR, de Klein CAM. 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N_2O emissions: a review. *New Zealand Journal of Agricultural Research 59*: 301-317.

Haynes RJ, Williams PH. 1993. Nutrient cycling and soil fertility in the grazed pasture ecosystem. In: Sparks DL Ed. *Advances in Agronomy 49*: 119-199. Academic Press.

Judson H, Fraser P, Peterson M, Kemp P, Edwards G. 2018a. Specific genotypes of plantain (*Plantago lanceolata*) vary in their impact on sheep urine volume and nitrification in the urine patch. *Proceedings of the New Zealand Grasslands Association 80*: 125-128.

Judson H, Fraser P, Peterson M, Kemp P, Edwards G. 2018b. Reducing nitrate leaching from the urine patch – a plant based approach. Presented at: *Australasian Dairy Symposium*. Palmerston North, New Zealand.

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*. 2nd ed, pp. 643-698. American Society of Agronomy, Madison, WI, USA.

Mangwe MC, Bryant RH, Beck MR, Beale N, Bunt C, Gregorini P. 2019. Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. *Animal Feed Science and Technology 252*: 11-22.

Ministry for the Environment. 2011. *National Policy Statement for Freshwater Management 2011*. Retrieved 8 March 2019 from www.mfe.govt.nz.

Moir J, Cameron K, Di H. 2007. Effects of the nitrification inhibitor dicyandiamide on soil mineral N, pasture yield, nutrient uptake and pasture quality in a grazed pasture system. *Soil Use and Management 23*: 111–120.

O'Connell CA, Judson HG, Barrell GK. 2016. Sustained diuretic effect of plantain when ingested by sheep. *Proceedings of the New Zealand Society of Animal Production 76*: 14-17.

Pacheco D, Waghorn GC. 2008. Dietary nitrogen-definitions, digestion, excretion and consequences of excess for grazing ruminants. *Proceedings of the New Zealand Grassland Association 70*: 107-116.

Peterson M, Anderson C, Fraser P, Curtin D, Joyce N. 2018. Soil microbial community structure and function is influenced by plant secondary metabolites excreted in sheep urine. Presented at: *Diverse Soils, Productive Landscapes, New Zealand Society of Soil Science Conference*. Napier, New Zealand.

Selbie DR, Buckthought LE, Shepherd MA. 2015. The challenge of the urine patch for managing nitrogen in grazed pasture systems. In: Sparks DL Ed. *Advances in Agronomy, Vol 129*, pp. 229-292.

Simon PL, de Klein CAM, Worth W, Rutherford AJ, Dieckow J. 2019. The efficacy of *Plantago lanceolata* for mitigating nitrous oxide emissions from cattle urine patches. *Science of the Total Environment 691*: 430-441.

Welten BG, Ledgard SF, Judge AA, Sprosen MS, McGowan AW, Dexter MM. 2019. Efficacy of different temperate pasture species to reduce nitrogen leaching from cattle urine applied in different seasons: A soil lysimeter study. *Soil Use and Management* https://doi.org/10.1111/sum.12512.

Woods RR, Cameron KC, Edwards GR, Di HJ, Clough Tim J. 2018. Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. *Grass and Forage Science 73*: 878-887.