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(71) Applicant: **PGG WRIGHTSON SEEDS LIMITED** [NZ/NZ]; c/o - James & Wells, Level 12, KPMG Centre, 85 Alexandra Street, Private Bag 3140, Waikato Mail Centre, 3204 Hamilton (NZ).

(72) Inventors: **CURTIN, Denis**; c/o - James & Wells, Level 12, KPMG Centre, 85 Alexandra Street, Private Bag 3140, Waikato Mail Centre, 3204 Hamilton (NZ). **JUDSON, Howard Glenn**; c/o - James & Wells, Level 12, KPMG Centre, 85 Alexandra Street, Private Bag 3140, Waikato Mail Centre, 3204 Hamilton (NZ). **PETERSON, Michelle Emma**; c/o - James & Wells, Level 12, KPMG Centre, 85 Alexandra Street, Private Bag 3140, Waikato Mail Centre, 3204 Hamilton (NZ). **FRASER, Patricia Margaret**; c/o - James & Wells, Level 12, KPMG Centre, 85 Alexandra Street, Private Bag 3140, Waikato Mail Centre, 3204 Hamilton (NZ).

(74) Agent: **SCOTT, Andrew** et al.; James & Wells, Private Bag 3140, 3240 Hamilton (NZ).

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(54) Title: NITRATE FORMATION INHIBITION

(57) Abstract: The invention relates to methods, uses and products for modulating the conversion of nitrogenous waste to nitrate. Typically the modulation involves decreasing the rate of conversion of nitrogenous waste to nitrate. In particular the invention relates to methods, uses and plant material for reducing the rate at which nitrogenous compounds excreted in the urine of an animal are lost from practical utility. Mechanisms of loss may involve nitrification and possibly denitrification to produce compounds that can be leached and/or released to the atmosphere as gaseous compounds. This invention has particular application to domesticated animals, particularly sheep and cows, although this should not be seen as limiting.

NITRATE FORMATION INHIBITION

FIELD OF THE INVENTION

The invention relates to methods, uses and products for modulating the conversion of nitrogenous waste to nitrate. Typically the modulation involves decreasing the rate of conversion of nitrogenous waste to nitrate.

In particular the invention relates to methods, uses and plant material for reducing the rate at which nitrogenous compounds excreted in the urine of an animal are lost from practical utility. Mechanisms of loss may involve nitrification and possibly denitrification to produce compounds that can be leached and/or released to the atmosphere as gaseous compounds. This invention has particular application to domesticated animals, particularly sheep and cows, although this should not be seen as limiting.

BACKGROUND TO THE INVENTION

An inevitable consequence of systems that farm animals is the production of faeces and urine both of which contain nitrogen (N).

Farmed animals excrete approximately 75% of the nitrogen that they ingest in their urine (50%) and faeces (25%). The nitrogen contained in excreted urine is generally in the form of urea, which is readily broken down to provide ammonia/ammonium by, for example, urease present in the soil. Nitrification of the ammonium initially produces nitrite, which undergoes further nitrification to produce nitrate. High nitrate can be problematic because it is not retained by soil particles and readily leaches into groundwater before it can be utilised for plant growth. In some cases, such as under anaerobic conditions, the nitrate can denitrify to produce nitrogen gas, nitrous oxide, nitric oxide and ammonia, which in gaseous forms can be released to the air.

The risk of leaching nitrogenous products (particularly nitrate) into groundwater is especially acute in porous soils, such as gravels and after heavy rainfall events or in continually wet soils where water moves through the profile taking nitrogen below the root zone, where it could otherwise be utilised for plant growth.

Nitrogen is an essential macronutrient for pastures and any loss of nitrogen from the farm system must be accounted for primarily through the application of fertiliser or legume-Rhizobia fixation.

Where the groundwater serves as a source of drinking water, excessive nitrate levels have been linked with the infant disease commonly known as "blue baby syndrome" (methemoglobinemia) – particularly in bottle-fed babies younger than 4 months of age.

There are a number of methods that have been adopted to decrease the amount of nitrogen which enters the groundwater in farming regions, including the use of specific plant species which readily uptake nitrogen (as ammonium and/or nitrate) from the soil. One problem with relying on this technique is that as animal stocking levels increase, the plants cannot utilise enough of the excreted nitrogen to prevent leaching into the groundwater.

Another method employs the use of pasture species that, themselves, contain reduced levels of nitrogen. The urine produced by animals that consume these pasture species has a reduced level of nitrogen. At present, however, very few viable low-nitrogen plant species are available.

Another method to decrease groundwater pollution is to use stand-off pads which are areas that the animals are herded into to further digest the consumed herbage. These pads may comprise a hard (such as concrete) or soft (such as woodchip) surface and the animal excrement is contained and may be collected and further directed into storage ponds. A significant problem associated with this widely-used approach is the cost of building the infrastructure needed to collect, store and distribute the excrement.

A still further method to decrease nitrate leaching which has received recent interest is the use of a chemical nitrification inhibitor which is typically sprayed onto the pasture prior to animals being allowed to graze the area. Both dicyandiamide (DCD) and 3,4-dimethlpyrazole phosphate (DMPP) slow the conversion of ammonium to nitrite by *Nitrosomonas spp.* and therefore limit the amount of nitrate being produced. Despite no widely recognised standard for an acceptable level of DCD in milk, the widespread use of DCD has been curtailed by the discovery of low levels in milk products produced by cows grazed in areas in which DCD has been sprayed. Furthermore, with a growing trend towards organic production, the use of chemical nitrification inhibitors in the organic farming sector is becoming less popular.

There is a need for a farm management practice which leads to a reduction in nutrient leaching, particularly nitrate leaching, which addresses one or more of the foregoing problems or at least provides the public with a useful choice.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a method of modulating the conversion of nitrogenous waste to nitrite and/or nitrate, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

Typically the step of administering plant material derived from a plant of the genus *Plantago* to an animal will involve the animal consuming plant material derived from a plant of the genus *Plantago*.

In some embodiments the component or metabolite of the component from the plant of the genus *Plantago* from the urine will be applied as *whole* urine excreted by the animal. In other embodiments the urine excreted by the animal will undergo a subsequent processing step such as concentration, dilution and/or fractionation before being applied to the nitrogenous waste. Concentration may refer to the removal of at least a portion of the water in the urine excreted by the animal. Dilution may refer to the addition of at least a portion of water to the urine excreted by the animal. Fractionation may refer to the separation of fractions from the urine excreted by the animal. Such fractions may be separated based on any number of physical and/or chemical properties of the components of the urine, such as:

- Polarity (chromatography);
- Size (GPC/size exclusion chromatography); and/or
- Boiling point (distillation).

For example, a metabolomic profile of sheep urine (Figure 1) identified more than 2000 compounds (dark grey plus light grey) and differential analysis of the results found 75 compounds (light grey) that are unique to, or are present in much higher concentrations, in the plantain sheep urine than in the ryegrass sheep urine. Accordingly in some embodiments the subsequent processing step may include fractionating at least one of the 75 compounds that are unique to, or are present in much higher concentrations, in the plantain sheep urine than in the ryegrass sheep urine. In some embodiments the subsequent processing step may include fractionating at least two of the 75 compounds that are unique

to, or are present in much higher concentrations, in the plantain sheep urine than in the ryegrass sheep urine.

The nitrogenous waste may be nitrogenous animal waste. The nitrogenous waste may, or may not, include waste from the animal to which plant material derived from a plant of the genus *Plantago* has been administered.

In some embodiments the nitrogenous waste includes the urine from the animal. For example, the entire nitrogenous waste may be the urine from the animal. By way of further example, the urine from the animal may be only a part (such as a minor part) of the nitrogenous waste.

In some embodiments the nitrogenous waste will not include the urine from the animal. In such embodiments, the method of the invention may include the additional step of collecting the urine including a component or metabolite of a component from the plant of the genus *Plantago*.

In a second aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to modulate the conversion of nitrogenous waste to nitrate.

The inventors have now identified that when animals are fed plant material derived from a plant of the genus *Plantago*, the urine excreted by the animal can be used to modulate the conversion of nitrogenous waste to nitrite and/or nitrate. The modulation may result in an increase or a decrease in the rate of conversion of nitrogenous waste to nitrite and/or nitrate compared with the effect provided by urine excreted by an animal fed plant material that is not derived from a plant of the genus *Plantago*, such as ryegrass has been administered.

Without wishing to be bound by theory, it is believed that the chemical components in the urine, namely a component or metabolite of a component from the plant of the genus *Plantago*, inhibit the bacterial conversion of urea/ammonia/ammonium to nitrite and/or nitrate.

In a third aspect the invention provides a method of reducing the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In a fourth aspect the invention provides a method of reducing nitrogenous gases derived from nitrogenous waste, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In a fifth aspect the invention provides a method of reducing nitrite and/or nitrate levels in surface water and/or groundwater in the vicinity of nitrogenous waste, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to the animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*;

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In a sixth aspect, the invention provides a method of reducing nitrogen volatilisation from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system; and

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In a seventh aspect the invention provides a method of reducing greenhouse gas emissions from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system; and

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In an eighth aspect the invention provides a method of reducing nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system;

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

In a ninth aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water.

In a tenth aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogenous gases derived from nitrogenous waste.

In an eleventh aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate levels in surface water and/or groundwater in the vicinity of nitrogenous waste.

In a twelfth aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogen volatilisation from a pastoral system including nitrogenous waste.

In a thirteenth aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce greenhouse gas emissions from a pastoral system including nitrogenous waste.

In a fourteenth aspect the invention provides the use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste.

In a fifteenth aspect the invention provides urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered, wherein the urine has been concentrated, diluted, and/or fractionated following excretion by the animal.

In a sixteenth aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to modulate the conversion of nitrogenous waste to nitrite and/or nitrate.

In a seventeenth aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water.

In an eighteenth aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogenous

gases derived from nitrogenous waste.

In a nineteenth aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate levels in surface water and/or groundwater in the vicinity of nitrogenous waste.

In a twentieth aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogen volatilisation from a pastoral system including nitrogenous waste.

In a twenty-first aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce greenhouse gas emissions from a pastoral system including nitrogenous waste.

In a twenty-second aspect the invention provides the use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste.

BRIEF DESCRIPTION OF DRAWING

Further aspects of the present invention will become apparent from the following description which is given by way of example only and with reference to the accompanying drawings in which:

Figure 1 Figure 1 depicts a scatter plot showing the differential analysis of the mass spectroscopy results for sheep urine. Ryegrass urine data depicted at values ≤ -2 Log_2 Fold Change on x-axis, Tonic data depicted at values ≥ 2 Log_2 Fold Change on x-axis. Comparing these data points led to the conclusion that 75 compounds (shown in light grey) on the Tonic plot were compounds that were either unique to, or are in much higher concentrations in, the Tonic urine compared to the ryegrass urine.

Figure 2 Figure 2 depicts a line graph of changes in ammonium-N concentration in soil during the incubation period (DCD represents dicyandiamide).

Figure 3 Figure 3 depicts a line graph of the cumulative amount of nitrate-nitrogen produced from either urine or urea, in the absence and presence of DCD (dicyandiamide) or from control soil over the 42 d incubation.

Figure 4 Figure 4 depicts a line graph of the concentration of ammonium-N in the soil during the 42 d incubation in the presence or absence of DCD (dicayandiamide).

Figure 5 Figure 5 depicts a line graph of the production of nitrate-N in the soil over the incubation period of 42 d following the application of urine or urea (773 mg N/kg soil) plus or minus DCD (dicyandiamide), or from the control soil alone. The bars are LSD's.

Figure 6 Figure 6 depicts a line graph of changes in soil pH as observed following application of Tonic urine, WDA urine or ryegrass urine in the absence and presence of DCD (dicyandiamide), or from control soil over the 42 d incubation.

DETAILED DESCRIPTION OF THE INVENTION

As used herein "animal" includes reference to domesticated farm animals, including mammals. Examples of such mammals are cattle, horses, sheep, goats and pigs. Preferably the present invention is for use in modulating the conversion of nitrogenous waste to nitrite and/or nitrate from ruminant animals (such as cattle, goats, sheep, yaks, deer) – particularly cattle and sheep. Still more preferably the present invention is for use in modulating the conversion of nitrogenous waste to nitrite and/or nitrate from dairy cattle and sheep. Dairy cows are most preferred as they form the largest part of the dairy herd and are believed to contribute the most to nitrite and/or nitrate leaching.

Plants of the genus *Plantago* are not commonly used as forage crops, and are often thought of as weeds. Because of this, in many countries plants of the genus *Plantago* are eradicated from pasture systems. A preferred species of the genus *Plantago*, commonly referred to as plantain, that may be used in the present invention is *Plantago lanceolata*. Preferably a plantain having a Mediterranean genetic origin is used. Preferably the plantain used is characterised as providing winter growth, although some winter dormant cultivars (WDA+) are effective. A particularly preferred example of a plantain for use in the present invention is *Plantago lanceolata* cv. Ceres Tonic, or breeding lines largely derived from that breeding pool eg. Agritonic.

Aspects of the present invention use plant material derived from a plant of the genus *Plantago*. As used herein the expression "plant material" includes reference to:

(a) plant growing *in situ* at the time of grazing. For example, the feed may be at least part of a forage crop that is eaten by grazing livestock as pasture or as crop residue (such as the residue left after

a crop is harvested);

(b) propagating material of the plant of the genus *Plantago*;

(c) harvested material of the plant of the genus *Plantago*; and

(d) products obtained from harvested material of the plant of the genus *Plantago*.

Such products obtained from harvested material include material that has been conditioned into, for example, balage or conserved feed including, but not limited to, silage, balage, hay or haylage.

As used herein the expression "administering plant material derived from a plant of the genus *Plantago* to the animal" typically refers to any method of administering plant material derived from a plant of the genus *Plantago* to the animal orally, typically ingesting the plant material derived from a plant of the genus *Plantago* to the animal so that is enters the rumen in those cases where the animal is a ruminant. In its simplest form the plant material derived from a plant of the genus *Plantago* may be administered as at least part of the animal feed.

It is believed that any dosage of the plant material derived from a plant of the genus *Plantago* should produce a meaningful modulation of the conversion of nitrogenous waste to nitrite and/or nitrate. Such dosages are conveniently provided as dry matter equivalent per liveweight per day. By way of example, the dosage may be 0.1-1 kg dry matter per day for a 40 kg sheep which equates to 0.0025-0.025 kg/kg liveweight per day. By way of further example, for a 250 kg yearling the dosage may be 1-10 kg dry matter per day (such as 5 kg dry matter per day) which equates to about 0.004-0.04 kg/kg liveweight per day (such as 0.02 kg/kg/day). Such dosages may be suitable for any of the animals contemplated by the present invention, including sheep and cattle.

As used herein the term "decreasing", "decrease" (as the context requires) with respect to a parameter refers to the modulation of the level of that parameter compared to the level of that parameter in the absence of the modulation. For example with respect to decreasing the rate of conversion of nitrogenous waste to nitrite and/or nitrate, "decreasing", "decrease" (as the context requires) refers to a modulation of the rate of conversion with respect to that rate that would otherwise normally be found. For example, the decrease in the rate of conversion of nitrogenous waste to nitrite and/or nitrate by administering a component or metabolite of a component from the plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from a plant of the genus

Plantago has been administered, may be determined with respect to:

a) the rate of conversion of nitrogenous waste to nitrite and/or nitrate during a period prior to, or subsequent to, the period of time during which the component or metabolite of a component from the plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from a plant of the genus *Plantago* has been administered is applied to the nitrogenous waste; and/or
b) a control sample of nitrogenous waste to which the component or metabolite of a component from the plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from a plant of the genus *Plantago* has been administered has not been applied.

Such a comparison of modulation can be made in a number of different ways, including:

a) the amount of nitrite and/or nitrate accumulated over a period of time (such as 7, 14, 17, 28, 35 or 42 days); and/or
b) the amount of ammonia/ammonium lost over a period of time (such as 7, 14, 17, 28, 35 or 42 days).

The extent of the modulation may be measured as the percentage change in the rate of conversion of nitrogenous waste to nitrite and/or nitrate. The percentage change may be at least 1%, however will typically be greater than 5%, such as greater than 10%.

It will be understood that nitrate is a source of nitrogen for ground cover, including pasture useful for animal feed. In some instances, low nitrate levels can inhibit the proliferation of ground cover growth and so it could be expected that decreasing the rate of conversion of nitrogenous waste to nitrate could be deleterious to plant growth. Nitrogenous waste excreted in the urine of an animal typically falls to the ground in an area known as the urine patch, where the nitrogen is concentrated, and provided as a high loading, well above the nitrogen requirements of the ground cover located in the urine patch. As such, this surplus nitrogen cannot be taken up by the ground cover in the urine patch, and instead is lost through nitrification and/or denitrification with leaching and/or gaseous release to the environment. A primary source of loss of the nitrogen is through nitrification and subsequent leaching below the root zone of the ground cover. As such, it is believed that by decreasing the rate of conversion of nitrogenous waste to nitrate, thereby providing a form of controlled release of the nitrogen in the nitrogenous waste, that there will be less nitrogen lost to the environment and more nitrogen made available to the ground cover. In turn it is believed that this will typically lead to a reduction in the

amount of supplemental nitrogen (eg fertiliser) that needs to be applied to the ground cover, which is particularly desirable.

It is believed that it is particularly advantageous to control the release of nitrogen from the nitrogenous waste to a plant recovering from grazing during the first 20 days of regrowth.

It has been found that the modulation of the conversion of nitrogenous waste to nitrite and/or nitrate is affected by the duration of the step of administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*. It has been found that the modulation is most significant when the plant material is administered to the animal for a period of at least 24 hours, such as at least 5 days, more preferably at least 10 days, such as at least 2 weeks.

Advantageously, the present invention provides the user with a method of readily providing a feeding animal with nutrition whilst simultaneously reducing the deleterious environmental impact of the animal. At its simplest, the method of the invention is able to be performed by grazing an animal on a pasture which includes plant material derived from a plant of the genus *Plantago*, such as grazing an animal on pasture which includes live stands of a plant of the genus *Plantago*. The urine excreted by the animal includes the component or metabolite of a component from the plant of the genus *Plantago* and will therefore deliver *in situ* both the nitrogenous waste and the agent useful in modulating the rate of conversion of the nitrogenous waste to nitrite and/or nitrate.

In some embodiments the animal feed is derived from a pasture system which includes at least one plant of the genus *Plantago*, and optionally also includes at least one plant selected from one or more of the following: ryegrass (*Lolium* spp.), clover (*Trifolium* spp.), cocksfoot (*Dactylis glomerata*), chicory (*Cichorium intybus*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), birdsfoot trefoil (*Lotus corniculatus*), lucerne (*Medicago sativa*), and prairie grass (*Bromus willdenowii*).

In some embodiments the animal feed is derived from a pasture system which includes at least one plant of the genus *Plantago*, and optionally also includes at least one plant selected from between only one and four of the following: ryegrass (*Lolium* spp.), clover (*Trifolium* spp.), cocksfoot (*Dactylis glomerata*), chicory (*Cichorium intybus*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), birdsfoot trefoil (*Lotus corniculatus*), lucerne (*Medicago sativa*), and prairie grass (*Bromus willdenowii*).

In some embodiments the animal feed is derived from a pasture system which includes at least one plant of the genus *Plantago*, and optionally also includes at least one plant selected from between only one and three of the following: ryegrass (*Lolium* spp.), clover (*Trifolium* spp.), cocksfoot (*Dactylis glomerata*), chicory (*Cichorium intybus*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), birdsfoot trefoil (*Lotus corniculatus*), lucerne (*Medicago sativa*), and prairie grass (*Bromus willdenowii*).

In some embodiments the animal feed is derived from a pasture system which includes at least one plant of the genus *Plantago*, and optionally also includes at least one plant selected from only one or two of the following: ryegrass (*Lolium* spp.), clover (*Trifolium* spp.), cocksfoot (*Dactylis glomerata*), chicory (*Cichorium intybus*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), birdsfoot trefoil (*Lotus corniculatus*), lucerne (*Medicago sativa*), and prairie grass (*Bromus willdenowii*).

In some embodiments the animal feed is derived from a pasture system which includes at least one plant of the genus *Plantago*, and optionally also includes at least one plant selected from only one of the following: ryegrass (*Lolium* spp.), clover (*Trifolium* spp.), cocksfoot (*Dactylis glomerata*), chicory (*Cichorium intybus*), tall fescue (*Festuca arundinacea*), Kentucky bluegrass (*Poa pratensis*), birdsfoot trefoil (*Lotus corniculatus*), lucerne (*Medicago sativa*), and prairie grass (*Bromus willdenowii*).

Advantageously, plants of the genus *Plantago* allow for similar productivity to more diverse pasture systems, such that plants of the genus *Plantago* can be used as the predominant or even sole animal food source (H.G. Judson, R. McAnulty and R. Sedcole *Proceedings of the New Zealand Grassland Association* 71: 201-205 (2009)).

The invention uses urine including a component or metabolite of a component from the plant of the genus *Plantago*. A metabolomic profile of sheep urine (Figure 1) identified more than 2000 compounds and differential analysis of the results found 75 compounds (shown in light grey) that are unique to, or are present in much higher concentrations, in the plantain sheep urine than in the ryegrass sheep urine. This result is highly significant ($P<0.005$). In some studies, urine from cattle was shown to contain the three allelochemicals aucubin, catalpol and verbascoside. These three allelochemicals were not detected in the plantain sheep urine.

As used herein the expression "a component from the plant of the genus *Plantago*" refers to a chemical component that is administered to the animal and is excreted intact by the animal in the urine. By way

of example, aucubin is an iridoid glycoside that is found in plantain and can be excreted intact in the urine of cattle.

As used herein the expression "metabolite of a component from the plant of the genus *Plantago*" refers to an intermediate metabolic product or end metabolic product of a chemical component that is administered to the animal, that is excreted by the animal in the urine.

The present invention is particularly well suited to the use of urine that is excreted by domesticated farm animals, including mammals. Examples of such mammals are cattle, horses, sheep, goats and pigs. Preferably the present invention uses urine that is excreted by a ruminant animal (such as cattle, goats, sheep, yaks, deer) – particularly cattle and sheep. Still more preferably the present invention uses urine that is excreted by dairy cattle and/or sheep. Dairy cows are most preferred as they form the largest part of the dairy herd and are believed to contribute the most to nitrate leaching.

As used herein, the expression "in the vicinity of an animal", particularly with respect to reducing nitrite and/or nitrate levels in surface water and/or groundwater, refers to the sphere of influence of the animal on nitrate levels in that environment. For example, it will be understood that the impact of the animal on nitrite and/or nitrate levels in surface water and/or groundwater will most noticeably be felt downstream (surface water) or down-gradient (groundwater) and that effect will typically decay as a function of distance from the animal. Nonetheless the effect may be observed some kilometres from the animal, however typically the sphere of influence will extend the vicinity of the animal to less than 5 km, such as less than 1 km, such as less than 100 m from the animal. Most noticeably the effect of the invention herein will be observed within 5 m of where the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

Nonetheless, it will also be appreciated that where standoff pads are used to collect urine from animals, and where the urine is subsequently dispersed as liquid fertiliser, the sphere of influence of the animal may extend to those fertilised areas where the animal may not have passed for a significant period of time, if at all. In those cases, the reduced nitrite and/or nitrate levels achieved in that fertilised land could still justifiably be said to have benefited from the invention and accordingly would fall within the expression "in the vicinity of an animal", since those fertilised areas are typically on the same broader farm/pastoral system that the animal has inhabited.

EXAMPLES

EXAMPLE 1 - Sheep Study

Experimental

Soil sampling and characterisation

A Templeton silt loam (Udic Haplustept) under a low-input ryegrass pasture was used in the incubation experiments. It was sampled at PFR, Lincoln, in Canterbury (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 air-dried.

Total soil N and C were determined by Dumas combustion (LECO TruMac, Leco Corporation, St. Joseph, MI, USA). Mineral N was extracted with 2 M KCl (1:5 w/v soil:extractant ratio; 60 min shaking); the extracted NO_3-N and NH_4-N were determined using a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, CO).

The potential of this soil to nitrify added ammonium-N was determined by using the method of Verchot *et al.* (2001). Briefly, soil slurries, containing 140 mg N kg-1 of added NH_4-N, were shaken for 24 h. Subsamples were obtained at various intervals over this time period and analysed for NO_3-N. From a linear regression of the NO_3-N concentration versus time, the nitrification rate was determined.

Urine characterization

The concentration of N in the urines was determined by a Lachat QuikChem 8500 Series 2 Flow Injection Analysis System (Lachat Instruments, Loveland, Colorado, USA) fitted with an inline UV-catalysed persulphate oxidation unit. Each urine was subsampled for Liquid Chromatography Mass Spectrometry (LCMS) analysis; samples were diluted 1:1 with cold acetonitrile. The LCMS system consisted of 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, DAD HL, Dual Oven).

A 2 µL aliquot of each prepared urine extract (and reference materials) was separated with a mobile phase consisting of 0.1% formic acid in acetonitrile (A) and 5 mM ammonium acetate in water (B) by normal phase chromatography (Hypersil Gold HILIC 1.9 µm, 100mm x 2.1 mm, P/N:26502-102130) maintained at 55°C with a flow rate of 400 µl/min. A gradient was applied: as 0–1 min/5%B, 7–10

min/80%B, 11–14min/5%B. The eluent was scanned from 1–11 min by API-MS (Orbitrap) with electrospray ionisation (ESI) in the negative and positive mode. Data were acquired for precursor masses from m/z 110–1200 amu at 70 K resolution with data dependent ms/ms for product ions generated by normalised collision energy or 30 at 17.5 K resolution. Data were processed with the aid of Xcalibur® 4.0 and Compound Discoverer 2.0 SV1 (Thermo Electron Corporation).

Nitrification of urine N

Microcosms were prepared by weighing 20 g of air-dried soil (wet weight equivalent) into 70 mL plastic vials. A 6.3 mL aliquot of water containing urine was then added to bring the soil to field capacity (i.e. water content at -10 kPa determined using a tension table). Based on the results of the total N analysis, each urine was diluted with water so that equal amounts of N were added to the microcosms, regardless of the N concentration in the original urine. Urea, added at the same N concentration as in the urines, was included as a control treatment. The N application rate was 511 mg/kg in experiment 1 and 773 mg/kg in experiment 2. DCD was used as a positive control (maximum potential nitrification inhibition); it was added at a rate equivalent to 30 kg/ha (recommended field rate). Table 1 shows the range of treatments included in the incubation experiments.

Table 1: treatments included in the incubation experiments

	Nitrogen (N) added (mg/kg soil)	
Treatment	Experiment 1 (24 hour urine)	Experiment 2 (2 week urine)
Soil only	0	0
Urea	511	773
Urea + DCD	511	773
Tonic urine	511	773
Tonic urine + DCD	511	773
WDA urine	511	773
WDA urine + DCD	511	773

Ryegrass urine	511	773
Ryegrass urine + DCD	511	773

After treatment application, the microcosms were covered with parafilm which was pierced to allow gas diffusion in and out of the pottles. The microcosms were then placed in trays, and those trays placed on shelves according to a split plot design, in an incubator set at 20°C. Three replicates of each treatment were destructively sampled to determine the net nitrification rate at 1, 7, 14, 21, 28, 35 and 42 d. A 5 g soil sample was weighed into a 50 mL centrifuge tube and 25 mL of 2 M KCl added. The centrifuge tubes were tumbled for 1 h, centrifuged at 4000 rpm for 5 min and the extract filtered through a pre-leached filter paper (Advantec 5C, <5 µM retention).

The extracts were analysed for NO_3-N and NH_4-N, as previously described. A further 5 g from each replicate was sampled into a 50 mL centrifuge tube for the determination of cold water extractable C. Thirty millilitres of deionized water was added, the tubes tumbled for 30 mins and then centrifuged (3500 rpm for 20 min) after which the supernatant was filtered through a pre-leached filter paper (Advantec 5C, <5 µM retention) for collection. Hot water extractable C was then extracted by re-suspending the remaining soil in 30 mL of water and incubated at 80°C for 16 h (Ghani *et al.* 2003). The hot water extract was recovered by centrifugation and filtration as for the cold water extract. The organic C content of the cold and hot water extracts were determined by combustion catalytic oxidation using a TOC-VCSH analyser (Shimadzu Corporation, Kyoto, Japan). A 1 g sample from each replicate was set aside and frozen at -80°C pending methanol:water extraction and LCMS analysis for plantain biochemicals. The remaining soil from each replicate was used to determine the soil pH using a glass pH electrode at 1:2 field moist sample to water ratio (Hendershot *et al.* 2008).

Results

Soil characterisation

A low-input soil was chosen for this study so that background mineral N (nitrate- and ammonium-N) would not confound the soil assay. Mineral N was very low at 1.6 mg kg soil^{-1}. Total soil C & N were 3.0%

and 0.24% respectively. The nitrification rate assay showed that this soil was capable of nitrifying 9 mg N kg soil^{-1} day^{-1}.

Urine characterisation

Total N in the urines derived from plantain fed sheep was lower than in the urine derived from ryegrass fed sheep at both sampling points. Higher amounts of urine total N were observed in all three treatments when sheep had been grazing the feedstuffs for 2 weeks as compared to 24 h (Table 2). At both sampling times, total N concentration was less in urine derived from grazing the cultivar Tonic (Tonic urine) than in from urine derived from grazing the line WDA (WDA urine).

Table 2: Nitrogen in the sheep urine collected either 24 hours or 2 weeks after sheep had been introduced to plantain and ryegrass pastures.

Sampling	Forage	Total persulfate N (mg/L)	NH_4-N (mg/L)	Total N (mg/L)
48 hours	Ryegrass	6355	51.5	6407
	WDA	2750	25.0	2774
	Tonic	1598	24.1	1622
2 weeks	Ryegrass	9892	87.1	9979
	WDA	3061	19.0	3080
	Tonic	2363	92.3	2455

In the present study, there was no detection of the three allelochemicals aucubin, catalpol or verbascoside in the sheep urine.

A metabolomic profile of the sheep urine, however, identified more than 2000 compounds and differential analysis of the results found 75 compounds that are unique to, or are present in much higher concentrations, in the plantain sheep urine than in the ryegrass sheep urine. This result is highly significant (P<0.005).

Nitrification of urine-N

In the absence of DCD, a large part of the N added in urine or urea was converted to nitrate-N (nitrified) during the 42 d incubation at 20°C. In Experiment 1, where the N addition rate was 511 mg N/kg soil, we recovered between 431 and 481 mg/kg of nitrate-N on day 42. Corresponding values for Experiment 2, where N was applied at a rate of 773 mg/kg were 383 to 568 mg N/kg. As would be expected, ammonium-N decreased over time (Figure 2), in step with the increases in nitrate-N (Figure 3). By the first sampling point, 1 d after treatment application, most of the N added in the urines (commonly 80–90% urea-N) had already been converted to ammonium-N, confirming that this enzymatic process is very rapid in urine-affected soil. However, where pure urea was added, production of ammonium-N appeared slower, and a large increase in ammonium-N was observed between days 1 and 7 in both experiments.

The DCD treatment was effective in inhibiting nitrification and nitrate-N did not increase significantly during the incubation.

Where urine that was collected after sheep were exposed to the different feedstuffs for 24 h was applied to the soil, similar amounts of nitrate were produced at a relatively similar rate over time (Figure 3), although there was a small but perceptible difference at 14 days between the nitrate concentration provided when ryegrass urine is applied versus Tonic urine or WDA urine. The control soil (with no urine or fertiliser additions) mineralised about 57 µg/g of nitrate-N during the 42 d experiment.

Where urine from sheep fed on plantain for 2 weeks was used (Experiment 2), production of nitrate was slower for urine from animals fed a diet of Tonic plantain. This treatment showed significantly ($P<0.05$) lower nitrate levels during the first 17 days of the experiment (Figure 4). This may suggest that the Tonic urine contains a compound (or compounds) that may delay nitrification.

Within one day of applying Tonic urine to soil, we observed much elevated levels of dissolved organic matter (extractable using cold water) compared with the other urine treatments. Soil organic matter can be solubilised as a result of the pH increase that accompanies the conversion of urea-N to ammonium-N (Figure 6). However, as the pH increase where Tonic urine was applied was similar to that observed for the other urines, it is likely that the large increase in dissolved organic matter originated from organic compounds contained in the urine itself. The dissolved organic matter found in soil after Tonic urine

addition was readily biodegradable and, by day 7 of the incubation, its concentration had substantially decreased (from ~1200 to 330 mg C/kg).

Although data for urine from sheep fed on Tonic plantain for 2 weeks showed evidence of delayed nitrification, this result was less significant with urine taken after a brief exposure (24 h) to Tonic.

Again, in the absence of DCD, ammonium decreased over time (Figure 4) whilst nitrate increased (Figure 5).

Soil pH

Oxidation of ammonium to nitrate results in the production of H^+ ions and soil pH decreases. As expected, DCD completely inhibits nitrification and so the pH remained high (~2 pH units higher than soil with no additions) and changed very little throughout the incubation period. The pH of the soil to which Tonic urine had been applied (in the absence of DCD) was almost a pH unit higher at 17 days compared with soil treated with WDA urine, and more than a pH unit higher (at 17 days) than the ryegrass urine and urea treatments. This is consistent with our observation that the application of Tonic urine reduced the amount of nitrate produced.

The entire disclosures of all applications, patents and publications cited above and below, if any, are herein incorporated by reference.

All references, including any patents or patent applications cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like, are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense, that is to say, in the sense of "including, but not limited to".

The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features.

Where in the foregoing description reference has been made to integers or components having known equivalents thereof, those integers are herein incorporated as if individually set forth.

It should be noted that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications may be made without departing from the spirit and scope of the invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be included within the present invention.

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

CLAIMS:

1. A method of modulating the conversion of nitrogenous waste to nitrite and/or nitrate, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

2. A method of reducing the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

3. A method of reducing nitrogenous gases derived from nitrogenous waste, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

4. A method of reducing nitrite and/or nitrate levels in surface water and/or groundwater in the vicinity of nitrogenous waste, the method including the steps of:

a) administering plant material derived from a plant of the genus *Plantago* to the animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus

Plantago;

b) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

5. A method of reducing nitrogen volatilisation from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system; and

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

6. A method of reducing greenhouse gas emissions from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system; and

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

7. A method of reducing nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste, the method including the steps of:

a) growing a plant of the genus *Plantago* in the pastoral system;

b) administering plant material derived from the plant of the genus *Plantago* to an animal so that the animal excretes urine including a component or metabolite of a component from the plant of the genus *Plantago*; and

c) applying the component or metabolite of the component from the plant of the genus *Plantago* from the urine to the nitrogenous waste.

8. The method of any one of claims 1 to 7 wherein the step of administering plant material derived from a plant of the genus *Plantago* to an animal includes the animal consuming plant material derived from a plant of the genus *Plantago*.

9. The method of any one of claims 1 to 8 wherein the nitrogenous waste is nitrogenous animal waste.

10. The method of any one of claims 1 to 9 wherein the animal is sheep or cattle.

11. The method of any one of claims 1 to 10 wherein the animal is dairy cow.

12. The method of any one of claims 1 to 11 wherein the nitrogenous waste includes wastes from the animal to which plant material derived from a plant of the genus *Plantago* has been administered.

13. The method of any one of claims 1 to 12 wherein the nitrogenous waste includes the urine from the animal.

14. The method of any one of claims 1 to 13 wherein the entire nitrogenous waste includes urine from the animal.

15. The method of any one of claims 1 to 14 wherein the entire nitrogenous waste is urine from the animal.

16. The method of any one of claims 1 to 12 wherein the nitrogenous waste does not include the urine from the animal.

17. The method of any one of claims 1 to 16 wherein the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied as whole urine excreted by the animal.

18. The method of any one of claims 1 to 16 wherein the urine excreted by the animal undergoes a subsequent processing step selected from concentration, dilution and/or fractionation before being applied to the nitrogenous waste.

19. The method of any one of claims 1 to 18 including the additional step of collecting the urine including a component or metabolite of a component from the plant of the genus *Plantago*.

20. The method of any one of claims 1 to 19 wherein the plant of the genus *Plantago* is *Plantago lanceolata*.

21. The method of any one of claims 1 to 20 wherein the plant of the genus *Plantago* has a Mediterranean genetic origin.

22. The method of any one of claims 1 to 21 wherein the plant of the genus *Plantago* is characterised as providing winter growth.

23. The method of any one of claims 1 to 22 wherein the plant of the genus *Plantago* is characterised as being a winter dormant cultivar.

24. The method of any one of claims 1 to 23 wherein the plant of the genus *Plantago* is *Plantago lanceolata* cv. Ceres Tonic.

25. The method of any one of claims 1 to 24 wherein the plant of the genus *Plantago* is a breeding line largely derived from the breeding pool of *Plantago lanceolata* cv. Ceres Tonic.

26. The method of any one of claims 1 to 25 wherein the plant of the genus *Plantago* is *Plantago lanceolata* cv. Agritonic.

27. The method of any one of claims 1 to 26 wherein the plant material is plant growing *in situ* at the time of grazing.

28. The method of any one of claims 1 to 26 wherein the plant material is propagating material of the plant of the genus *Plantago*.

29. The method of any one of claims 1 to 26 wherein the plant material is harvested material of the plant of the genus *Plantago.*

30. The method of any one of claims 1 to 26 wherein the plant material is product obtained from harvested material of the plant of the genus *Plantago*.

31. The method of claim 30 wherein the plant material is in the form of silage, balage, hay or haylage.

32. The method of any one of claims 1 to 31 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of nitrite and/or nitrate accumulated over a period of time after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

33. The method of any one of claims 1 to 32 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of nitrite and/or nitrate accumulated over 7 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

34. The method of any one of claims 1 to 33 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of nitrite and/or nitrate accumulated over 14 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

35. The method of any one of claims 1 to 34 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of nitrite and/or nitrate accumulated over 20 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

36. The method of any one of claims 1 to 35 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of ammonia/ammonium lost after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

37. The method of any one of claims 1 to 36 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of ammonia/ammonium lost over 7 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

38. The method of any one of claims 1 to 37 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of ammonia/ammonium lost over 14 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

39. The method of any one of claims 1 to 38 wherein the conversion of nitrogenous waste to nitrite and/or nitrate provides a decrease in the amount of ammonia/ammonium lost over 20 days after the component or metabolite of the component from the plant of the genus *Plantago* from the urine is applied to the nitrogenous waste.

40. The method of any one of claims 32 to 39 wherein the decrease is at least 1%.

41. The method of any one of claims 32 to 40 wherein the decrease is greater than 5%.

42. The method of any one of claims 32 to 41 wherein the decrease is greater than 10%.

43. The method of any one of claims 1 to 42 wherein the plant material is administered to the animal for a period of at least 24 hours.

44. The method of any one of claims 1 to 43 wherein the plant material is administered to the animal for a period of at least 5 days.

45. The method of any one of claims 1 to 44 wherein the plant material is administered to the animal for a period of at least 10 days.

46. The method of any one of claims 1 to 45 wherein the plant material is administered to the animal for a period of at least 2 weeks.

47. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to modulate the conversion of nitrogenous waste to nitrite and/or nitrate.

48. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water.

49. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogenous gases derived from nitrogenous waste.

50. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the

urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate levels in surface water and/or groundwater in the vicinity of nitrogenous waste.

51. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogen volatilisation from a pastoral system including nitrogenous waste.

52. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce greenhouse gas emissions from a pastoral system including nitrogenous waste.

53. Use of a component or metabolite of a component from a plant of the genus *Plantago* from the urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste.

54. Urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered, wherein the urine has been concentrated, diluted, and/or fractionated following excretion by the animal.

55. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to modulate the conversion of nitrogenous waste to nitrite and/or nitrate.

56. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce the amount of nitrite and/or nitrate derived from nitrogenous waste that is leached into groundwater and/or surface water.

57. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogenous gases derived from nitrogenous waste.

58. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate levels in surface water and/or

groundwater in the vicinity of nitrogenous waste.

59. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrogen volatilisation from a pastoral system including nitrogenous waste.

60. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce greenhouse gas emissions from a pastoral system including nitrogenous waste.

61. Use of urine excreted by an animal to which plant material derived from the plant of the genus *Plantago* has been administered to reduce nitrite and/or nitrate leaching from a pastoral system including nitrogenous waste.

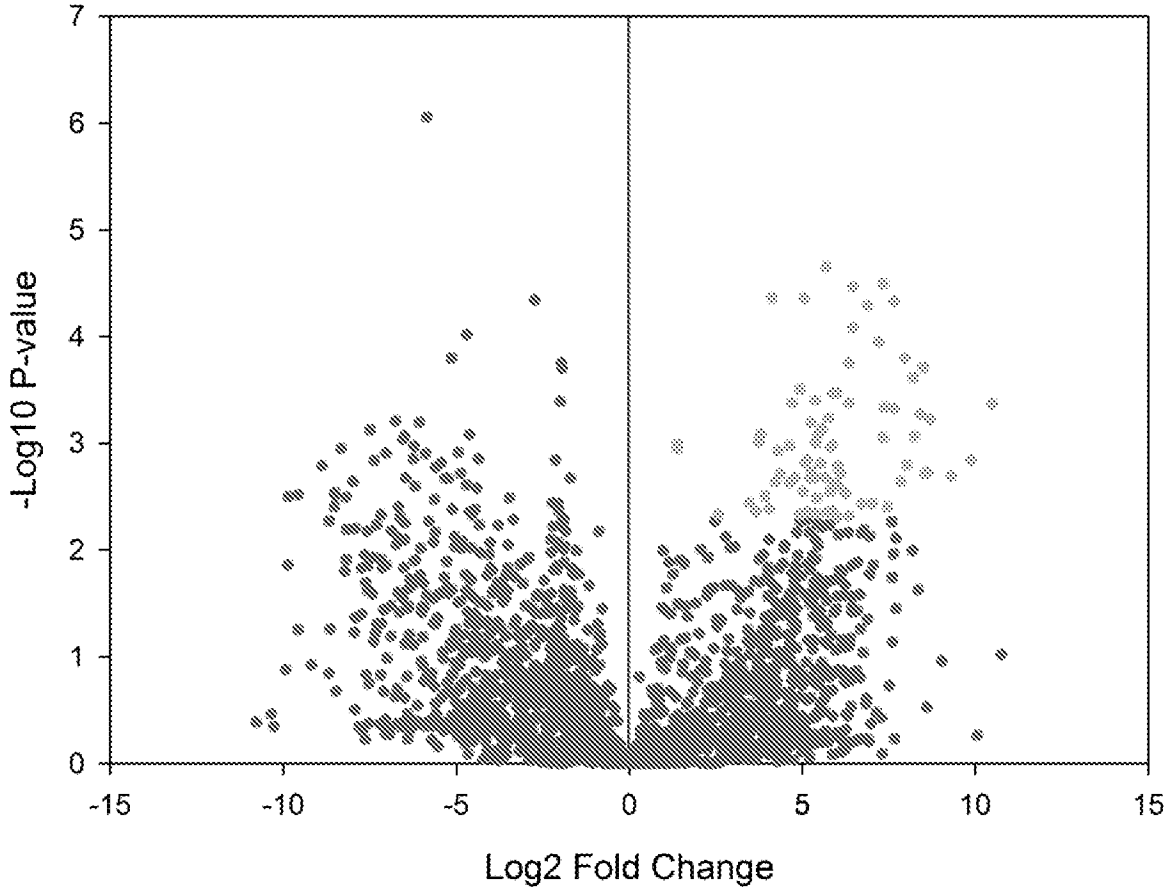

Figure 1

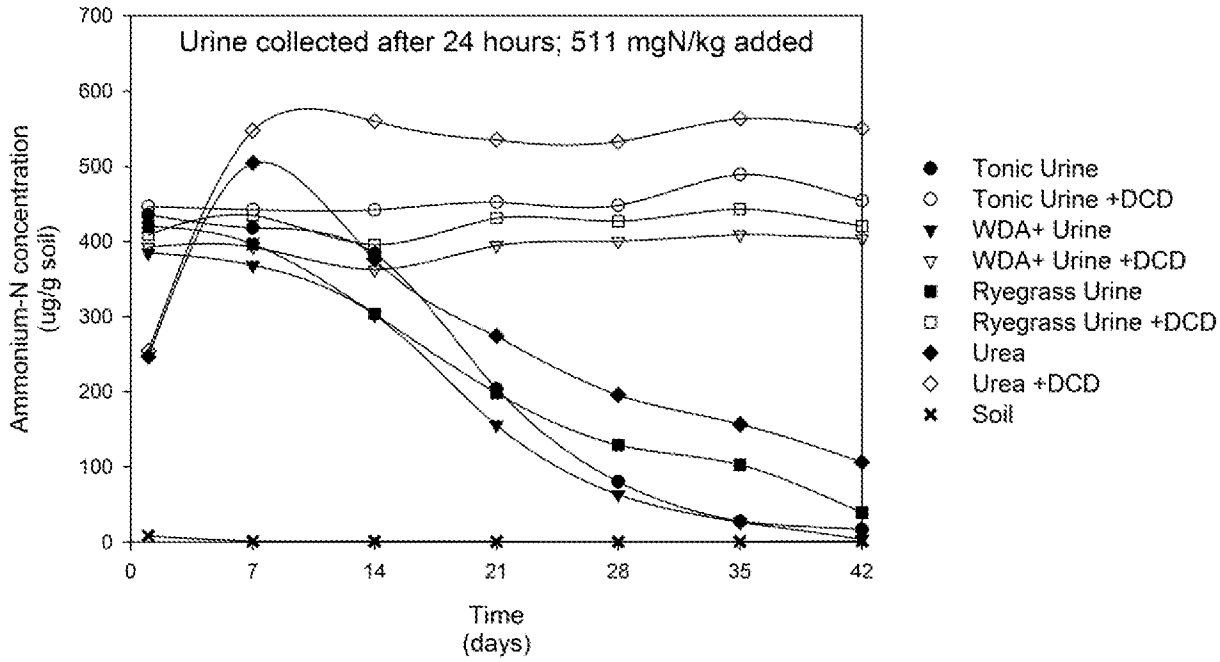
Urine collected after 24 hours; 511 mgN/kg added
Ammonium-N concentration (ug/g soil)
700
600
500
400
300
200
100
0
0
7
14
21
28
35
42
Time (days)
Tonic Urine
Tonic Urine +DCD
WDA+ Urine
WDA+ Urine +DCD
Ryegrass Urine
Ryegrass Urine +DCD
Urea
Urea +DCD
Soil

Figure 2

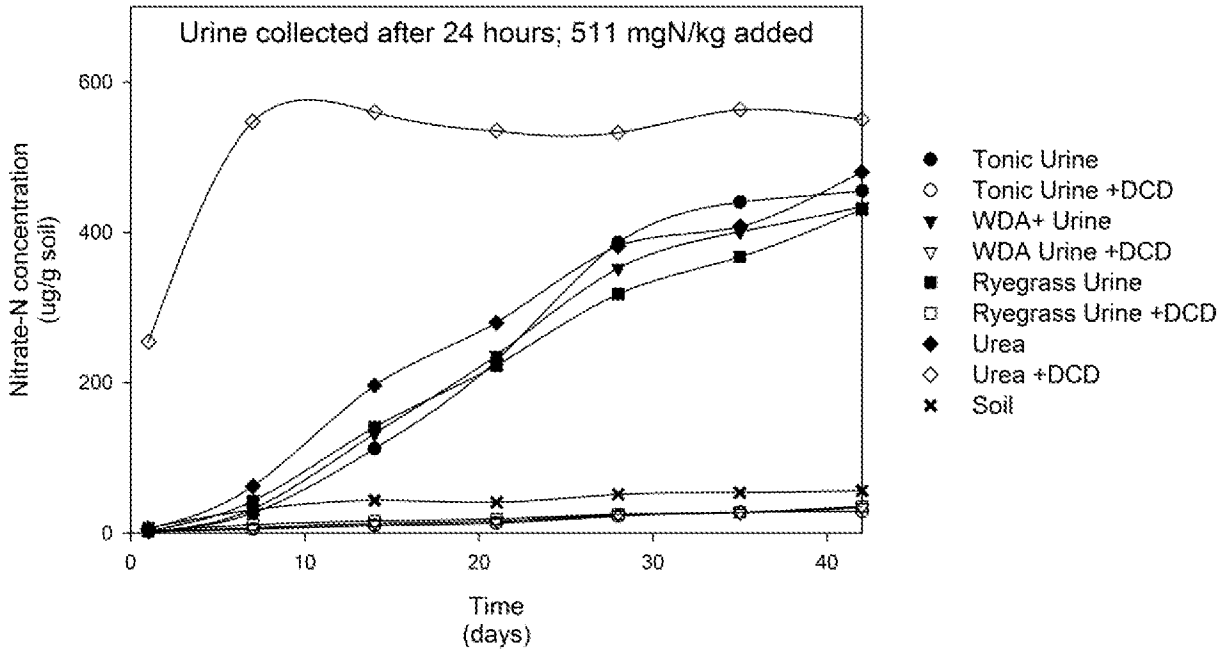
Urine collected after 24 hours; 511 mgN/kg added
Nitrate-N concentration (ug/g soil)
600
400
200
0
0
10
20
30
40
Time (days)
Tonic Urine
Tonic Urine +DCD
WDA+ Urine
WDA Urine +DCD
Ryegrass Urine
Ryegrass Urine +DCD
Urea
Urea +DCD
Soil

Figure 3

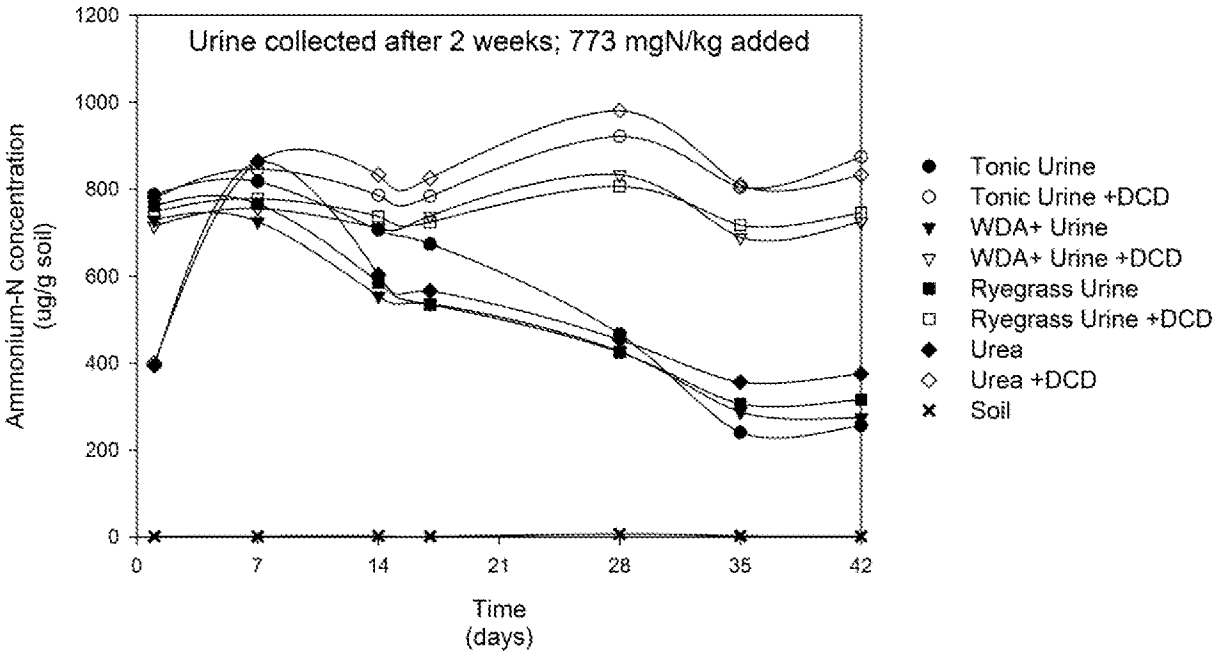
Urine collected after 2 weeks; 773 mgN/kg added
Ammonium-N concentration (ug/g soil)
1200
1000
800
600
400
200
0
0
7
14
21
28
35
42
Time (days)
Tonic Urine
Tonic Urine +DCD
WDA+ Urine
WDA+ Urine +DCD
Ryegrass Urine
Ryegrass Urine +DCD
Urea
Urea +DCD
Soil

Figure 4

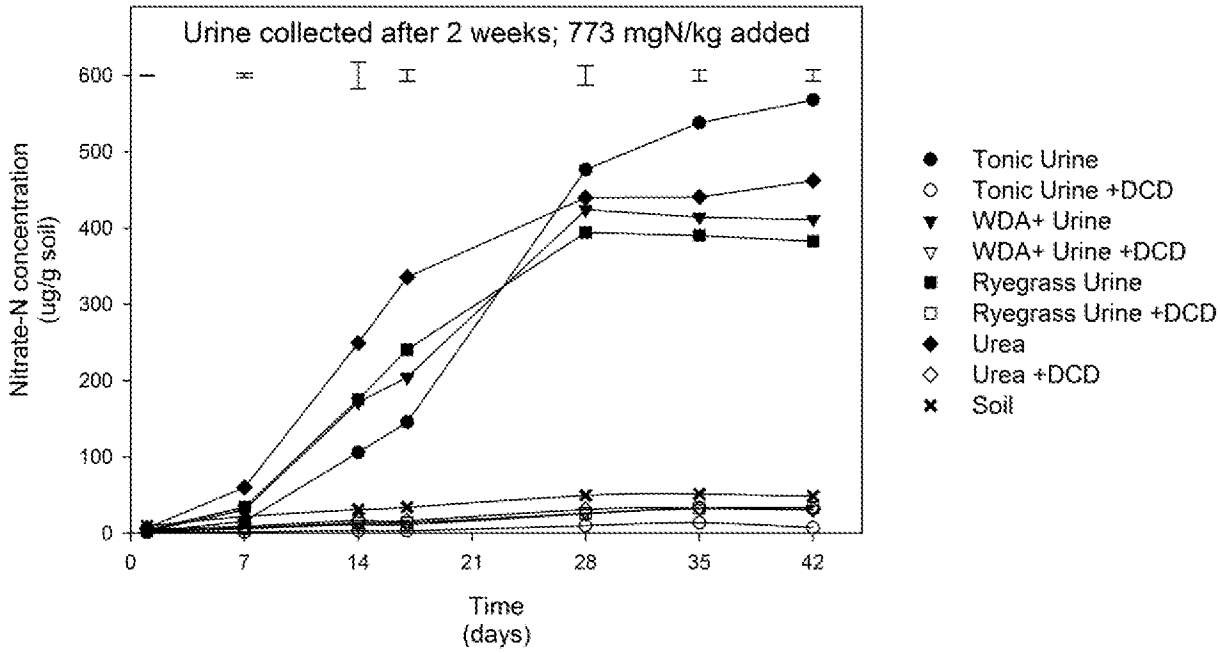
Urine collected after 2 weeks; 773 mgN/kg added
Nitrate-N concentration (ug/g soil)
600
500
400
300
200
100
0
0
7
14
21
28
35
42
Time (days)
Tonic Urine
Tonic Urine +DCD
WDA+ Urine
WDA+ Urine +DCD
Ryegrass Urine
Ryegrass Urine +DCD
Urea
Urea +DCD
Soil

Figure 5

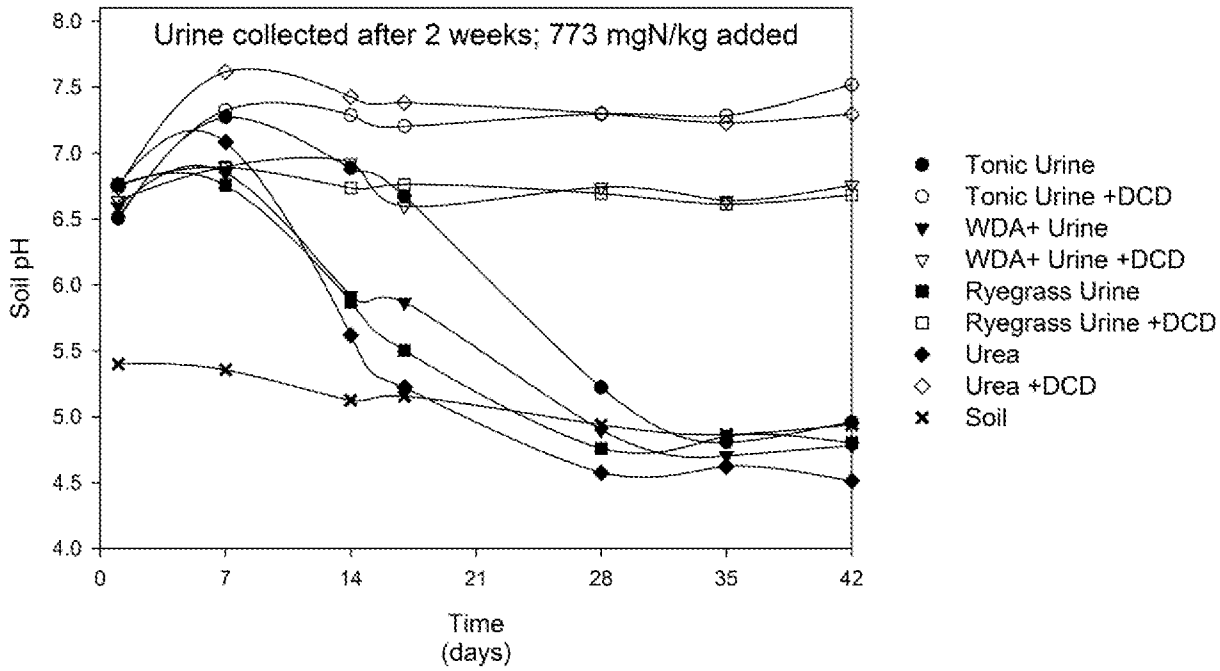
Urine collected after 2 weeks; 773 mgN/kg added
Soil pH
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
7
14
21
28
35
42
Time
(days)
Tonic Urine
Tonic Urine +DCD
WDA+ Urine
WDA+ Urine +DCD
Ryegrass Urine
Ryegrass Urine +DCD
Urea
Urea +DCD
Soil

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2018/050044

A. CLASSIFICATION OF SUBJECT MATTER

A23K 10/30 (2016.01) A23K 50/10 (2016.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPOQUE (PATENW): Applicant/Inventor names (PGG Wrightson Seeds Limited, D Curtin, H G Judson, M E Peterson, P M Fraser); IPC/CPC marks (A23K 10/30, 50/10); Keywords (plantago, plantain, lanceolata, nitrogen, nitrate, nitrite, cow, cattle, sheep, urine, urina+ and like terms). **NEW STN (CAPlus, DWPI, BIOSIS, CABA):** Applicant/Inventor names (as above); Keywords (plantago, plantain, lanceolata, nitrogen, nitrate, nitrite, cow, cattle, sheep, animal, urine, urina* and like terms). **ONLINE DATABASES (Espacenet, Google Scholar/Patents/Search):** Applicant/Inventor names (as above); Keywords (plantago, plantain, lanceolata, ceres tonic, agritonic, urine, nitrogen, nitrate, nitrite, ammonium, ammonia, greenhouse, cow, cattle, sheep, livestock and like terms and alternatives).

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 2018	04 June 2018

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
Email address: pct@ipaustralia.gov.au

Authorised officer

Austin Smith
AUSTRALIAN PATENT OFFICE
(ISO 9001 Quality Certified Service)
Telephone No. +61262832381

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/NZ2018/050044**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JUDSON, H. G. *et al.*, "Urinary nitrogen concentration from dairy heifers grazing kale supplemented with either plantain or perennial ryegrass baleage in winter", *Journal of New Zealand Grasslands* (2016) Vol 78, pages 99-102. Abstract, p99 col 2 par 1-3, p100 col 1 par 3, Table 1, p101 col 1 par 2 to col 2 par 2	1-15, 17-26 & 29-61
X	GARDINER, C. A. *et al.*, "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* (2016) Vol 59, No 3, pages 301-317. Abstract, p302 par 2, p303 par 1-2, p307 par 2, p310 par 2-3, p311 par 2 to p312 par 1	1-15, 17, 20-28, 32-53 & 55-61
X	CHENG, L. *et al.*, "Live weight gain, urinary nitrogen excretion and urinary behaviour of dairy heifers grazing pasture, chicory and plantain", *Journal of Agricultural Science* (2017) Vol 155, pages 669-678 [published online 19 January 2017]. Abstract, p670 col 1 par 3 to col 2 par 4, p672 col 1 par 1, p676 col 1 par 3 to col 2 par 3, Table 3	1-15, 17-28 & 32-61
X	DI, H. J. *et al.*, "The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions", *J. Soils Sediments* (2016) Vol 16, pages 2252-2262. Abstract, p2253 col 1 par 1, Experiments 2-3 (Sections 2.2.2-3 & 3.2-3), p2259 col 2 par 3-4	1-15, 17-28 & 32-61
X	NAVARRETE, S. *et al.*, "Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata* L.) and their effect on *in vitro* rumen fermentation", *Animal Feed Science and Technology* (2016) Vol 222, pages 158-167. Abstract, Sections 1, 2.1, 4.2 & 5	1-15, 17, 20-28, 32-53 & 55-61
P,X	WO 2017/065619 A1 (PGG WRIGHTSON SEEDS LIMITED) 20 April 2017 Abstract; pp 3-4, 7, 9, 16, 36; Experiment 1; Claims	1-15, 17-25, 27, 29-39 & 43-61
P,X	WO 2017/078544 A1 (PGG WRIGHTSON SEEDS LIMITED) 11 May 2017 Abstract; p15 par 2; Claims	1-15, 17-25, 27-39, 43-44 & 47-61

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ2018/050044**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2017/065619 A1	20 April 2017	WO 2017065619 A1	20 Apr 2017
		AU 2016339720 A1	10 May 2018
WO 2017/078544 A1	11 May 2017	WO 2017078544 A1	11 May 2017
		AU 2016348215 A1	10 May 2018

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.