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(54) Title: ECO-FRIENDLY ADDITIVES FOR HAY AND SILAGE INOCULANTS MINIMIZING GREENHOUSE GAS EMISSIONS

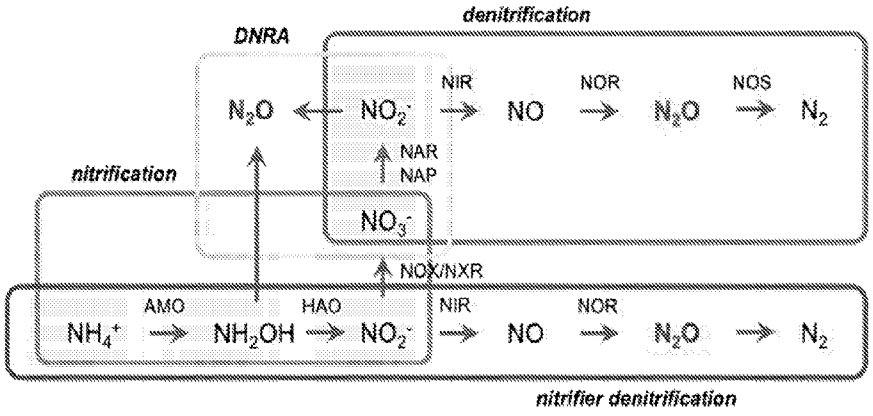

Fig. 1

(57) Abstract: The present invention relates to novel and eco-friendly additives for forage inoculants and to forage inoculants comprising the same. Notably, the forage inoculants, in addition to reducing non-CCh greenhouse gas emissions, are capable of fermenting forage while not reducing the nutritional value of the forage, thereby producing conserved forages with maintained nutritional values. Methods of reducing non-CCh greenhouse gas emissions produced during forage conservation are also described.

[Continued on next page]

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ECO-FRIENDLY ADDITIVES FOR HAY AND SILAGE INOCULANTS MINIMIZING GREENHOUSE GAS EMISSIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the priority benefit of U.S. Provisional Patent Application Serial No. 63/292,750, filed December 22, 2021, entitled ECO-FRIENDLY ADDITIVES FOR HAY AND SILAGE INOCULANTS MINIMIZING GREENHOUSE GAS EMISSIONS, incorporated by reference in its entirety herein.

BACKGROUND

Technical Field

[0002] The present disclosure relates to eco-friendly additives for forage inoculants and to forage inoculants comprising the same, which are notably capable of reducing non-CO_2 greenhouse gas emissions produced during the forage conservation process.

Description of Related Art

[0003] Sustainable food production is a way of producing a continuous supply of safe and nutritious foods while minimizing its environmental impacts. Forages are plants or parts of plants eaten by herbivores, and sustainable forage management is critical to livestock industry. Forages can be harvested and conserved during the peak growing season for use in time of scarcity. Historically, and throughout the world today, forage conservation is a key element for productive and efficient livestock farming. There are two major practices in forage conservation: hay and silage. Hay is dried below 20% moisture and densely packed in a rectangular or round shape, inducing anaerobic conditions in the central region. Silage is another form of conserved forage and the main principle is anaerobic environment with high moisture content (50~70%), which encourages fermentation. In short, conserved forages are nutrient-rich ecosystems that are maintained partly or completely under anaerobic conditions; hence, they can serve as ideal habitats for diverse microorganisms—especially those involved in the production of greenhouse gases. Particularly, forage conservation is the third-largest yet unaccounted nitrous oxide (N_2O) source in the agricultural sector. However, despite the high production volume and widespread

commercial use, little is known about these ecosystems and their greenhouse gas emission potentials.

[0004] As a result, there is a need for forage inoculants that are capable of reducing non-CO_2 greenhouse gas emissions produced during the forage conservation process and do not reduce the nutritional value of the forage.

SUMMARY OF THE INVENTION

[0005] The present disclosure is broadly concerned with an additive for a forage inoculant comprising at least two components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation. In preferred embodiments, the at least two components are selected from the group consisting of a component capable of inhibiting microbial denitrification activity or methanogenesis, a component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, an N_2O-reducing enrichment culture, and mixtures thereof.

[0006] In other embodiments, the present invention may be a forage inoculant, the forage inoculant comprising: starter bacteria capable of decreasing the pH in a quantity of forage to 5.5 or less; and an additive comprising one or more components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation. In preferred embodiments, when applied to forage, the forage inoculant does not reduce the nutritional value of the forage.

[0007] In yet another embodiment, the present invention may be a method of reducing non-CO_2 greenhouse gas emissions from forage conservation, the method comprising: applying to a quantity of forage: (1) a starter bacteria capable of decreasing pH in the quantity of forage to 5.5 or less, and (2) an additive comprising one or more components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation, wherein the additive has a concentration of about 0.05 to about 0.5 g per kg of forage.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] Figure (Fig.) 1 is a schematic drawing of the potential microbial processes in conserved forages responsible for N_2O emissions and the enzymes involved in each process.

[0009] Fig. 2 is a schematic drawing of the microbially driven CH_4 cycle and respective marker genes.

[0010] Fig. 3A is a graph illustrating N_2O production from corn, alfalfa, and sorghum silages.

[0011] Fig. 3B is a graph illustrating N_2O production from alfalfa silages using two different varieties (HVX MegaTron alfalfa from WINFIELD UNITED and HybriForce 3400 alfalfa from DAIRYLAND SEED) harvested in two different physiological stages (mid-bud and early flowering stages).

[0012] Fig. 3C is a graph illustrating CH_4 and N_2O production from forages under anerobic conditions.

[0013] Fig. 4A is a graph illustrating N_2O production from corn, alfalfa, and sorghum silages under different conditions.

[0014] Fig. 4B is a graph illustrating N_2O production from alfalfa silage amended with different concentrations of chlorate.

[0015] Fig. 4C is a graph illustrating total (cumulative) production volume of N2O and CH4 production simulated silage.

[0016] Fig. 5 is a schematic drawing of the genes associated with greenhouse gas cycles under aerobic conditions.

DETAILED DESCRIPTION

[0017] The present invention concerns novel additives for forage inoculants and forage inoculants comprising the same. Also described are methods of reducing non-CO_2 greenhouse gas emissions produced during the forage conservation process.

Additives

[0018] In one or more embodiments, the additives comprise, consist essentially of, or consist of one or more components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation. As used herein, the term "non-CO_2 greenhouse gas" refers to any greenhouse gas (i.e., a gas that contributes to the greenhouse effect by absorbing infrared radiation) recognized and/or classified as such by the U.S. Environmental Protection Agency. Examples of non-CO_2 greenhouse gases include, but are not limited to, methane (CH_4), nitrous oxide (N_2O), and fluorinated gases (e.g., hydrofluorocarbons (HFCs), perfluorocarbons (PFCs), and sulfur hexafluoride (SF_6)). Preferably, the additives and methods of using the additives according to

embodiments of the present invention are most concerned with reducing non-CO_2 greenhouse gases that are produced by a living organism.

[0019] In at least one embodiment, the one or more components capable of reducing non-CO_2 greenhouse gas emissions are capable of inhibiting microbial denitrification activity and/or methanogenesis. Examples of these components include, but are not limited to, analogs of nitrate reductase (e.g., chlorates and chlorites), phenylacetylene, and procyanidins. In preferred embodiments, the component capable of inhibiting microbial denitrification activity and/or methanogenesis is chlorate. In certain embodiments, the additive comprises about 1% to about 99% by weight, preferably about 20% to about 80% by weight, and more preferably about 40% to about 60% by weight of the component capable of inhibiting microbial denitrification activity and/or methanogenesis, wherein the % by weight is based upon the total weight of the additive taken as 100% by weight.

[0020] In at least one embodiment, the one or more components capable of reducing non-CO_2 greenhouse gas emissions includes a labile carbon source, which, if included, serves as an external carbon and/or energy source to alleviate low carbon-to-nitrogen ratios (i.e., one of the potential causes of N_2O accumulation during denitrification). In these embodiments, the component is preferably capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, such as denitrifiers, without N_2O accumulation. Examples of these components include, but are not limited to, labile carbon sources, carboxylic acids and their respective conjugate bases especially C_2-C_3 carboxylic acids including acetic acid (acetate) and lactic acid (lactate). In preferred embodiments, the component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms is acetate. In most embodiments, the additive comprises about 1% to about 99% by weight, preferably about 20% to about 80% by weight, and more preferably about 40% to about 60% by weight of the component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, wherein the % by weight is based upon the total weight of the additive taken as 100% by weight.

[0021] In at least one embodiment, the one or more components capable of reducing non-CO_2 greenhouse gas emissions includes an N_2O-reducing enrichment culture, preferably inoculated with fermented silage and wastewater sludge at a pH of 5 or less. In embodiments where the one or more components include an N_2O-reducing enrichment culture, the culture is *Gemmobacter*

serpentinus strain HB-1. In most embodiments, the additive comprises about 1% to about 20% by weight, preferably about 5% to about 15% by weight, and more preferably about 8% to about 10% by weight of the N_2O-reducing enrichment culture, wherein the % by weight is based upon the total weight of the additive taken as 100% by weight.

[0022] In some embodiments, the additive may comprise, consist essentially of, or consist of two or more components. In these embodiments, the first component is a component capable of inhibiting microbial denitrification activity and/or methanogenesis, preferably chlorate, and the second component is a component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, preferably acetate. In some embodiments, the additive may further comprise a third component, which preferably is an N_2O-reducing enrichment culture.

[0023] In at least one of the foregoing embodiments, the additive may further comprise one or more inactive ingredients.

Forage Inoculants

[0024] In one or more embodiments, the forage inoculants comprise, consist essentially of, or consist of the above-described additive(s), preferably the additive comprising chlorate or acetate, and starter bacteria capable of decreasing the pH in a quantity of forage to 5.5 or less. In preferred embodiments, the starter bacteria are members of the order Lactobacillales (i.e., lactic acid bacteria). Examples of starter bacteria include, but are not limited to, anaerobic lactic acid bacteria (e.g., *Lactobacillus buchneri*), *Lactobacillus plantarum*, *Enterococcus faecium*, and mixtures thereof. In most preferred embodiments, the starter bacteria are commercial inoculants containing lactic acid bacteria, such as Pioneer® 11H50, Pioneer® 1174, and Pioneer® 1129. In some embodiments, the forage inoculant comprises about 90% to about 99.99% by weight, preferably about 95% to about 99.9% by weight, and more preferably about 98% to about 99% of the additive and comprises about 0.01% to about 10% by weight, preferably about 0.1% to about 5%, and more preferably about 1% to about 2% by weight of the starter bacteria capable of decreasing the pH in a quantity of forage to 5.5 or less, wherein the % by weight is based upon the total weight of the forage inoculant taken as 100% by weight.

[0025] In at least one of the foregoing embodiments, the forage inoculant may further comprise one or more inactive ingredients.

[0026] The quantity of forage may be any plant material eaten by grazing livestock. In preferred embodiments, the quantity of forage is composed of highly digestible cell contents (e.g., protein, fat, soluble carbohydrates) and less digestible cell walls (e.g., cellulose, hemicellulose, lignin), which can be divided into two types: legumes and grasses. Legumes include, but are not limited to, Alfalfa (*Medicago sativa*), and grasses include, but are not limited to, corn (*Zea mays*) and sorghum (*Sorghum bicolor*). If the forage is ensiled, the silo/quantity of forage has a bulk density of about 400 kg/m^3 to about 1000 kg/m^3, preferably about 700 kg/m^3.

[0027] The forage preservation/conservation process may be performed using any methods known in the art. In most embodiments, however, by lowering the pH of the quantity of forage to 5.5 or less, the forage inoculant ferments the quantity of forage, thereby forming conserved forage, which may be in the form of silage, haylage, or baleage. Particularly, in embodiments where in the starter bacteria are members of the order Lactobacillales, during the fermentation process, indigenous or exogenous lactic acid bacteria convert water-soluble carbohydrates to organic acids, mainly lactic acid, which act as a natural preservative to inhibit undesirable microorganisms. It will be appreciated that, when the forage inoculant is applied to the quantity of forage, the forage inoculant does not reduce the nutritional value of the forage.

Method of Reducing Non-CO_2 Greenhouse Gas Emissions

[0028] In one or more embodiments, the method of reducing non-CO_2 greenhouse gas emissions comprises providing forage, preferably a quantity of forage, and applying the above-described forage inoculant. In these embodiments, the forage inoculant is applied to the quantity of forage at a loading of from about 0.001% by weight to about 0.2% by weight, preferably about 0.01% by weight to about 0.1% by weight, wherein the % by weight is based upon the total weight of the quantity of forage taken as 100% by wet weight. In preferred embodiments, the method of reducing non-CO_2 greenhouse gas emissions comprises providing a quantity of forage and applying the above-described starter bacteria to the quantity of forage before the above-described additive is applied. In these embodiments, the starter bacteria are applied to the quantity of forage at a loading of from about 0.000001% by weight to about 0.00005% by weight, preferably about 0.000001% by weight to about 0.00001% by weight, more preferably about 0.000001% by weight to about 0.000005% by weight, wherein the % by weight is based upon the total weight of the quantity of forage taken as 100% by wet weight. In these embodiments, the additive is applied to

the quantity of forage at a loading of from about 0.015% by weight to about 0.15% by weight, preferably about 0.015% by weight to about 0.06% by weight, more preferably about 0.015% by weight to about 0.0275% by weight, wherein the % by weight is based upon the total weight of the quantity of forage taken as 100% by wet weight. The additive is applied about 1 minute to about 1 hour, preferably about 10 minutes to about 50 minutes, and more preferably about 20 minutes to about 40 minutes after the starter bacteria is applied to the quantity of forage.

[0029] Regardless of when the additive is applied, the concentration of the additive is about 0.015 to about 0.03 g per kg of forage, preferably about 0.015 g per kg of forage (wet weight based). In embodiments where the additive comprises two components, the first component is preferably chlorate at a concentration of about 0.05 g per kg of forage, and the second component is preferably acetate at a concentration of about 0.1 g per kg of forage. In most preferred embodiments, the method reducing of non-CO_2 greenhouse gas emissions further comprises incubating the forage after the forage inoculant and/or the starter bacteria and additive are applied.

[0030] Additional advantages of the various embodiments described herein will be apparent to those skilled in the art upon review of the disclosure herein and the working examples below. It will be appreciated that the various embodiments described herein are not necessarily mutually exclusive unless otherwise indicated herein. For example, a feature described or depicted in one embodiment may also be included in other embodiments, but is not necessarily included. Thus, the present disclosure encompasses a variety of combinations and/or integrations of the specific embodiments described herein.

[0031] As used herein, the phrase "and/or," when used in a list of two or more items, means that any one of the listed items can be employed by itself or any combination of two or more of the listed items can be employed. For example, if a composition is described as containing or excluding components A, B, and/or C, the composition can contain or exclude A alone; B alone; C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and C in combination.

[0032] The present description also uses numerical ranges to quantify certain parameters relating to various embodiments of the disclosure. It should be understood that when numerical ranges are provided, such ranges are to be construed as providing literal support for claim limitations that only recite the lower value of the range as well as claim limitations that only recite the upper value

of the range. For example, a disclosed numerical range of about 10 to about 100 provides literal support for a claim reciting "greater than about 10" (with no upper bounds) and a claim reciting "less than about 100" (with no lower bounds).

EXAMPLES

[0033] The following examples set forth methods in accordance with the disclosure. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

Background

[0034] A preliminary study showed that a significant amount of N_2O, (38 mg CO_2 eq. per g-forage) and methane (CH_4, 0.2 mg CO_2 eq. per g-forage) were produced from laboratory incubations of alfalfa simulating silage process. Based on the annual silage production volume reported by USDA, the estimated N_2O emission potential was 0.3 million metric tons CO_2 equivalent per year, which places forage conservation as the third most important source of N_2O emissions in the agricultural sector. It should be noted that agriculture represents largest source of N2O emissions comprising 79%. After 3-month of incubation, DNA was extracted and analyzed by polymerase chain reaction. All five genes involved in denitrification (narG, napA, nirK, norB, and nosZ) were detected, suggesting that denitrifying bacteria were responsible for N_2O emission and indicating that the conserved forages do harbor microbial communities involved in the production and reduction of GHGs.

[0035] This research promotes sustainable agriculture, which will not only address many environmental and social concerns, but also offer innovative and economically viable opportunities for growers, laborers, consumers, policymakers and many others in the entire food system. The forage silage inoculant market is estimated to be valued at $808M in 2019 and is projected to rapidly grow to $1.2B by 2025. Further, the findings of the proposed study may have tremendous economic consequences for international carbon credit trading, which involves assigning a monetary value to a reduction or offset of GHG emissions. The world bank reported that the carbon price should be set $100 per metric ton CO_2 eq by 2030. The estimated N_2O

emission potential from forage conservation process – "the crisis" can be converted into "an opportunity" to earn extra revenues if efficiently controlled (0.3 million metric tons CO_2 eq per year × \$100 per metric ton CO_2 eq = \$30M per year). Based on the annual silage production volume in KS, the potential extra revenue is estimated to be \$0.3M per year. Overall, this project will accelerate the transformation of livestock industry towards a healthy and sustainable global food system.

[0036] Justification. In most parts of the world, forage conservation is a key element for productive and efficient ruminant livestock farms. Forages provide the primary feed source for cattle, horses, and small ruminants. Forages are conserved to feed livestock during periods of shortage caused by limited pasture growth or inadequate pasture conditions. Historically, throughout the world, forage conservation is a crucial element for productive and efficient livestock farming. Forages are composed of highly digestible cell contents (protein, fat, soluble carbohydrates) and less digestible cell walls (cellulose, hemicellulose, lignin). The relative proportions of each component vary from crop to crop. Alfalfa (*Medicago sativa*), corn (*Zea mays*), and sorghum (*Sorghum bicolor*) are three major crops used for conservation purpose in the US.

[0037] Forage conservation methods can be primarily classified into hay and silage. Hay is dried below 20% moisture (12~20%, w/w), baled in rectangular or round shape for ease of handling, transport, and storage, and stored under aerobic conditions. Silage is prepared with higher moisture content (40~70%, w/w) and stored under strictly anaerobic conditions. Silage can be further subcategorized into silage, haylage, and baleage based on the moisture content. Silage is a high moisture forage containing 60-70% moisture. Haylage and baleage contain lower-moisture content (40-55%). Haylage is chopped using a silage chopper and packed in a bunk, silo, or bag. Haylage is called baleage when baled (before chopping) and wrapped with at least six layers of 1 mm plastic. Typically, alfalfa is conserved as both baled hay and silage, and corn and sorghum are only conserved as silage under anaerobic conditions. Forage preservation, especially silage, replies on fermentation. Indigenous or exogenous lactic acid bacteria (LAB) convert water-soluble carbohydrates to organic acids, mainly lactic acid, which act as a natural preservative to inhibit undesirable microorganisms. The optimal pH of silage is approximately 5.5 or below. The silage inoculant market is estimated to be valued at \$808 million in 2019 and projected to rapidly grow to \$1.2 billion by 2025.

[0038] Despite high production volume and widespread use, the forage conservation process has been scarcely studied as a possible source of GHGs. However, many researchers did detect CH_4 and N_2O. N_2O production was observed from corn silages stored in an open bunker silo, and the ambient N_2O concentration in the silo was orders of magnitude greater than for rural background levels. CH_4 and ammonia (NH_3) were detected in a dairy barn suggesting that the true culprit of the repeated fire incidents may be not the "elevated temperature" caused by microbial activity, but biogenic CH_4. A few other studies reported CH_4 and N_2O production from silage, but a comprehensive quantitative assessment has not been performed.

[0039] **Forage Conservations.** GHGs are the main contributor to the global warming and climate change due to their absorption of infrared radiation from the earth's surface. Although carbon dioxide (CO_2) constitutes the majority of GHG emissions, other non-CO_2 gases such as methane (CH_4) and nitrous oxide (N_2O) also account for 25% of global GHGs emissions. N_2O is also a major source of ozone-depleting nitric oxide and nitrogen dioxide in the stratosphere and is currently the single most important ozone-depleting substance. Among others, agricultural activities are the largest anthropogenic source of N_2O comprising 79%. The emission will increase as it is closely linked to the most basic human need – food, unless substantial efforts are made to reduce the environmental footprint of agriculture.

[0040] Forages are plants or parts of plants eaten by grazing livestock, and they provide the main feed source for cattle, horses, and small ruminants. Forages can be conserved to feed livestock during periods of shortage caused by limited pasture growth or inadequate pasture conditions. Historically throughout the world, forage conservation is a key element for productive and efficient livestock farming. Typically, forages are conserved in the form of hay, usually below 20% moisture, and silage with high moisture content. Hay is packaged (or baled) with twine or plastic net in rectangular or round bales for ease of handling, transport, and storage. Rectangular bales are as small as 36 cm high, 46 cm wide, and 102 cm long, but they are usually stored as stacks in a covered storage facility. Typical round bales are larger, 1.2–1.8 m in diameter and 1.2–1.7 m in length. For both shapes, anaerobic regions are inevitably created in the core area. Silage is forage preserved by anaerobic storage, usually under conditions that encourage fermentation (1) to provide lactic acid as natural preservative to inhibit undesirable microorganisms by lowering pH, and (2) to improve nutritional value. Silages contain high moisture content (50~70%, w/w) and

have several variations in preservation type including silage, haylage, and baleage primarily depending on their moisture levels. In short, conserved forages are nutrient-rich ecosystems that are maintained partly or completely under anaerobic conditions. Therefore, it was hypothesized that conserved forages can serve as ideal habitats for diverse microorganisms not only for fermenters but also those involved in the production of GHGs, e.g., methanogens and denitrifiers, that are known to produce CH_4 and N_2O, respectively.

[0041] Microbial processes controlling N_2O. N_2O is the third-largest contributor of GHG emissions to the atmosphere, after CO_2 and CH_4. N_2O is also a major source of ozone-depleting nitric oxide (NO) and nitrogen dioxide (NO_2) in the stratosphere, N_2O emission currently is the single most important ozone- depleting emission. N_2O is produced by both natural and anthropogenic sources, but the emissions from human activities have increased 20% since the pre-industrial era, and its concentration has increased 15% since 1750. Approximately 40% of N_2O emissions come from human activities, and of those, the majority are from agricultural practices. In the US, about 75% of all anthropogenic N_2O emissions are attributed to agriculture.

[0042] Although the mechanism of N_2O production is not completely understood, microbial production of N_2O is generally considered to be achieved through three different processes: (1) a by-product of nitrification by ammonia-oxidizing bacteria, (2) denitrification, and (3) nitrifier denitrification, as well as possibly by (4) anaerobic ammonia oxidation and (5) nitrification by ammonia-oxidizing archaea (see Fig. 1). Among them, denitrification is a sequence of reductive reactions and is thought to occur mostly in anaerobic environments. For this reason, the potential for N_2O production from denitrification process is highest in anaerobic ecosystems.

[0043] Under anaerobic conditions, microbial denitrification is known to be the primary source of N_2O emissions. Denitrification is one of the key processes in nitrogen cycle, where nitrate (NO_3^-) or nitrite (NO_2^-) are reduced to molecular nitrogen (N_2) via the gaseous intermediates, NO and N_2O. Each reduction step is catalyzed by one or more specific reductase enzymes encoded by *narG*, *napA*, *nirK*, *nirS*, *norB*, and *nosZ* genes. The accumulation of denitrification intermediates and the subsequent emission of N_2O to the atmosphere are highly undesirable. Although the mechanism of this phenomenon still needs to be clarified, several factors have been reported to result in N_2O accumulation during denitrification, including low pH, low C/N ratios, the presence of elevated levels of free nitrous acid or H_2S, and low oxygen concentrations. On the consumer

side, the only known sink for N_2O in the biosphere was its enzymatic reduction to N_2 by N_2O reductase, and this reaction has been attributed exclusively to denitrifying microorganisms. However, recent reports have demonstrated that diverse microbial taxa possessing divergent nos clusters with genes that are related yet evolutionarily distinct from the typical nos genes of denitirifers. Their environmental role in controlling N_2O emissions remains undefined, and these non-denitrifying N_2O-reducers may play an important role under conditions that are not favorable (e.g., low pH) for denitrifiers.

[0044] It should also be noted that different N-compounds are known to inhibit methanogenesis, which catalyze the terminal step of organic matter degradation to CO_2 and CH_4. Any change in denitrification activity may induce higher methanogenic activity.

[0045] **Non-denitrifying N_2O reducers.** On the consumer side, there is only one known sink for N_2O in the biosphere – the microbial reduction of N_2O to N_2, catalyzed by the N_2O reductase (N_2OR). This enzyme has typically been found among bacteria and archaea performing complete denitrification (microbes possessing "clade I" *nosZ*, a gene encoding N2OR). However, recent reports have demonstrated that diverse microbial taxa possessing divergent nos clusters with genes that are related yet evolutionarily distinct from the typical nos genes of denitirifers. Their environmental role in controlling N_2O emissions remains undefined, and these non-denitrifying N_2O-reducers may play an essential role under conditions that are not favorable for denitrifiers, such as, low pH.

[0046] **Microbial processes controlling CH4.** CH_4 is the second most important anthropogenic GHG after CO_2, contributing about 30% to the total net anthropogenic radiative forcing. The atmospheric concentration of CH_4 has been increasing from pre-industrial values of about 700 ppbv to currently about 1843 ppbv. Approximately 70% of the global CH_4 budget, which is on the order of 500–600 Tg CH_4 per year, is mainly the result of environmental microbial processes, such as methanogenesis in anaerobic environments and microbial CH_4 oxidation (methanotrophy) under anoxic and oxic conditions (see Fig. 2). Methanogenesis is anaerobic respiration that generates CH_4 as the final product of metabolism, and microbes capable of producing CH_4 are comprised exclusively of archaea, although many of them are closely associated with bacteria, e.g., fermentative bacteria. Methanogens can be subdivided into two primary functional groups: hydrogenotrophic methanogens (which use H_2 and CO_2 to produce CH_4) and acetoclastic

methanogens (which use acetate to produce CH_4). All known methanogens express an isozyme of methyl-coenzyme M reductase (MRT); the *mcrA* gene encoding the α-subunit of this enzyme is commonly used as a "functional gene" marker for the detection, enumeration, and classification of methanogens (see Fig. 2).

[0047] On the consumer side, CH_4 can be oxidized by methanotrophs, a group of bacteria that utilize CH_4 as its sole carbon and energy source. Although acidophilic bacteria belonging to the phylum Verrucomicrobia was recently isolated, traditionally, methanotrophs are classified into two classes gammaproteobacterial (or Type I) and alphaproteobacterial (or Type II) based on several characteristics such as cell morphology, membrane arrangement, carbon assimilation pathway, and predominant phospholipid fatty acids (PLFA). Despite the diversity of methanotrophs and the wide range of environments in which they are found, the general pathway by which these cells oxidize CH_4 to CO_2 is remarkably similar with methanol, formaldehyde, and formate as intermediates. The first step of the reaction, CH_4 oxidation, is catalyzed by methane monooxygenase (MMO). The MMO can be found in two different forms, particulate (pMMO) and soluble (sMMO) forms. The α subunit of pMMO, encoded by the *pmoA* gene, is present in all known methanotrophs except for the genus *Methylocella and Methyloferula spp.*, and is commonly used as a genetic marker for methanotrophs (see Fig. 2). Methanotrophs represent a major biological sink for CH_4 and are thus Earth's natural protection against this potent greenhouse gas.

[0048] **Fire and explosion risks of CH4 emissions.** Repeated fire incidents have been reported from conserved forages, and "elevated temperature" caused by microbial activity has been generally considered as a culprit. The temperatures as high as 75°C has been reported before bacterial activity ceases. It should be, however, noted that CH_4 is colorless, odorless, yet flammable gas with a lower explosive limit (LEL) of 5%. If ignited, CH_4 can pose a fire or explosion risk to people, infrastructure, or vegetation located nearby. The CH_4 flux measurement may decipher the causes of unexplained fire incidents at forage storage sites.

[0049] **Factors affecting GHG emissions from conserved forages.** GHGs production and consumption in conserved forages are controlled by microbial activity, and there are a variety of environmental factors affecting microbial growth including moisture content and nutrient availability.

[0050] In forage conservation practices, moisture content is governed by forage conservation method and nutrient availability varies from species to species. In the preliminary study, it was demonstrated that different cultivars and maturity stages do not affect the GHG production, and different crops and moisture content (i.e., conservation method) are used as independent variables.

Preliminary Studies

[0051] In preliminary studies, the greenhouse gas (GHG) emissions were measured from simulated silage and baled hay. The effects of different treatments were evaluated on GHG emissions, and microbial communities were examined using quantitative polymerase chain reaction (qPCR) and functional gene microarrays.

[0052] **GHG production from simulated silage.** Laboratory experiments were performed to estimate the GHG emission potentials from silage conservation (i.e. anaerobic conservation). In a first part of the study, N_2O production from forage silos was studied. The forages used were corn, alfalfa, and sorghum. The mini silos consisted of 1L glass jars connected to a 1-L Tedlar bag. The forage was chopped into 2-3 cm pieces with a laboratory chopper and ensiled in laboratory mini silos at a bulk density of 700 kg/m^3 (700 grams per silo). The experiments were performed in an incubator at 30°C in the dark, and the produced gas volume and composition were measured periodically for 30 days.

[0053] Three replicates for each crop were performed. Within a week of incubation, 4.1, 59.5, and 10.8 ml of N_2O were produced per bottle (see Fig. 3A). Two different alfalfa varieties including HVX MegaTron (WinField United LLC, Arden Hills, MN, USA) and HybriForce 3400 (Dairyland Seed Co.) were planted and harvested at two different maturity stages (mid-bud and early-flowering). There was no significant difference in N_2O production volume between the different varieties harvested at the same growth stages. However, the samples harvested at the later growth stages (i.e., early flowering) exhibited much lower N_2O production (see Fig. 3B).

[0054] In a second part of the study, N_2O and CH_4 production from forage silos were studied. The forage used was second cut alfalfa (HVX MegaTron) harvested at the early flower stage. The mini silos consisted of 1L glass jars connected to a 1-L Tedlar bag. The forage was chopped into

2-3 cm pieces with a laboratory chopper and ensiled in laboratory mini silos at a bulk density of 700 kg/m^3 (700 grams per silo). The experiments were performed in an incubator at 30°C in the dark, and the produced gas volume and composition were measured periodically for 35 days. 49±1 ml N_2O and 0.019±0.001 ml CH_4 were produced within two weeks of incubation, corresponding to 113 mg and 1.4 μg CO_2 eq. per g-forage based on global warming potential of 300 and 25, respectively.

[0055] In a third part of the study, N_2O and CH_4 production from forage samples were studied. In 160 ml serum bottles, 20 g of forage samples were added, and the moisture contents were adjusted to 40% and 70% (w/w) by adding sterilized deionized distilled water. Each bottle was closed with black rubber stopper and capped with aluminum cap. All the bottles were incubated at 30 °C in the dark, and the headspace samples were monitored for CH_4 and N_2O for 3 months. Within 2 months of incubation, up to 2.2 (±0.3) μmol of CH_4 and 13.0 (±1.2) μmol of N_2O were produced, which corresponds to 122.2 and 8,581.2 μg CO_2 eq per $g_{\text{-forage}}$, respectively (see Fig. 3C). (In Fig. 3C, the error bars represent standard errors of triplicate experiments and are not visible when smaller than the symbols.) The production rates of both gases were slower with lower moisture content (40%) but the total concentrations at the end were similar regardless of the moisture content.

[0056] After the incubation (in parts 1 and 3 of the study), DNA was extracted using the method optimized for forage samples in the lab. PCR amplification was performed using primers targeting genes associated with denitrification (*narG*, *napA*, *nirK*, *nirS*, *norB*, and *nosZ*), and they were all positive.

[0057] **Effects of different chemical treatments on GHG production**. The treatments compared in this research consisted of (1) no treatment, (2) commercial silage inoculant (Pioneer® inoculant), (3) inoculant + chlorate (0.1%, w/w), and (4) inoculant + acetate (0.1%, w/w). Three replicates for each treatment were performed.

[0058] In the fourth part of the study, three replicates for each treatment were added to forage silos prepared as described in paragraph [0052]. As shown in Fig. 4A, the addition of the silage inoculant did not affect N_2O production. The addition of 0.1% (w/w) chlorate significantly reduced N_2O production by up to 99%, confirming that denitrifiers were responsible for N_2O production in this ecosystem. Acetate amendment also diminished N_2O production by up to 25% suggesting low

C/N ratio could be one of the causes for N_2O accumulation. These results provide (1) insights into N_2O production mechanisms in the forage conservation process and (2) a basis to develop improved forage management practices minimizing environmental impact. Fig. 4B shows the effect of different concentrations of chlorate. Lower concentrations up to 0.01% (w/w) exhibited higher than 90% reduction of N_2O.

[0059] In the fifth part of the study, three replicates for each treatment were added to forage silos prepared as described in paragraph [0054]. In the control treatment (SI-), 49±1 ml N_2O and 0.019±0.001 ml CH_4 were produced within two weeks of incubation, corresponding to 113 mg and 1.4 μg CO_2 eq. per g-forage based on global warming potential of 300 and 25, respectively. As shown in Fig. 4C, the addition of the silage inoculant did not affect N_2O production but CH_4 production was significantly decreased, suggesting that low pH induced by the silage inoculant inhibited methanogenesis. The addition of 0.1% (w/w) chlorate significantly reduced N2O production by more than ten times, confirming that denitrifiers were responsible for N2O production in this ecosystem. Like in the fourth part of the study, the acetate amendment also significantly diminished N_2O production by 25% ($P<0.01$) suggesting low C/N ratio could be one of the causes for N_2O accumulation. Again, these results provide (1) insights into N2O production mechanisms in the forage conservation process and (2) a basis to develop improved forage management practices minimizing environmental impact.

[0060] **GHG production from round hay bales.** In the field experiments, two round alfalfa hay bales were monitored for surface GHG fluxes to evaluate the GHG emission potentials from hay. The surface fluxes were measured using a flow-through chamber method, which is the most common technique to measure surface GHG fluxes.

[0061] Briefly, a custom-built closed chamber with two gas ports was installed on the surface of round hay bales, and CH_4-free argon controlled by a flowmeter passed into the chamber at 5 mL/min. The chamber headspace was continuously mixed with a battery-powered fan installed inside. The outflow gas samples were collected, measured in the laboratory. The flux was calculated using the following equation, $F = C \cdot q/A$, where F is the emission flux (μg/m²×hour), C is the concentration (μmol/m^3), q is the air flow rate (m^3/hour), and A is surface area (m^2). CH_4 was not observed throughout the study, but N_2O was observed after two months of incubation. The concentrations were 0.26 and 0.13 μmol/L at 1 and 2 months of age, which

correspond to 0.66 and 0.33 μmol/day/m^2, respectively. During each field sampling event, forage core samples were collected for molecular biological analysis using a hay coring probe at two different depths representing oxic region (5-25 cm) and anoxic/anaerobic region (35-55 cm), respectively, at 0, 1, and 2 months. After sampling, the holes were sealed with spray polyurethane foam to prevent an oxygen intrusion.

[0062] Given that CH_4 was not detected from the surface flux samples, it was hypothesized that CH4 was microbially oxidized by methanotrophs in the outer layer where oxygen (O_2) is available. To verify the hypothesis, 20 g of forage samples obtained from the outer layer of each bale were incubated with 10 μL of CH4 in 160 ml serum bottles. CH_4 in each bottle was completely disappeared in two days, indicating that methanotrophs are present in the outer layer.

[0063] **Optimization of microbial nucleic acid extraction from forage samples.** Analysis of phyllosphere microbial communities can be challenging due to the interference with plant DNA, especially when applying shotgun sequencing. To minimize the release of plant DNA during the DNA extraction, the microbial DNA extraction method was optimized using a proteinase K-based DNA extraction kit by varying the incubation time and temperature with the enzyme.

[0064] The copy number of 16S rRNA gene per ng DNA was used as a dependent variable. Briefly, 5 g of forage sample was mixed with 35 mL of TE-buffer (pH 8) and incubated on a shaker at 120 rpm for 15 min at room temperature. The mixture was centrifuged at 14,100 g at 4°C for 20 min, and the resulting pellets were used to extract DNA using the Qiagen Blood and Tissue kit. After mixing with proteinase K, each sample was incubated at 40°C for 20 min.

[0065] **Microbial community analysis.** DNA was extracted using the Blood and Tissue kit (Qiagen, Hilden, Germany), and Geochip 5.0S, a functional gene microarray, was used to examine the relative abundance of functional genes. Compared to the initial time point, genes associated with N cycles, i.e., *amoA, hao, norB, napA*, and *nrfA*, significantly increased in the anoxic/anaerobic region in a month, which coincided with the N_2O emissions.

[0066] After two months of incubation, presumably due to the depletion of nutrients, the N-related gene abundances plateaued or decreased (*amoA, hao,* and *napA*) (data not shown). CH_4 oxidation functional gene (*pmoA*) abundance increased in the oxic region of forage at one month, suggesting CH_4 was oxidized by methanotrophs and evaded detection on the surface. In conclusion, conserved forages do harbor microorganisms associated with N_2O and CH_4 cycles, and further

research is warranted to comprehensively evaluate their role in controlling GHG emissions from the forage conservation process.

[0067] The forage materials contain organic compounds that spanned a wide range of recalcitrance from the labile starch to the recalcitrant lignin, allowing us to examine how active microbial communities utilized substrates with different recalcitrance. Within one month of incubation, the gene abundances associated with labile C degradation increased. After another month, the gene abundances associated with labile C degradation decreased and those associated with recalcitrant C degradation increased, presumably due to the depletion of labile organic C.

Isolation of Novel N_2O-respiring Bacterium

[0068] With the goal to isolate microorganisms that can be used to minimize N_2O emissions from conserved forages, a novel N_2O-respiring bacterium was isolated from the round hay bales and characterized. A cream-colored colony found on the plate after four days of incubation, designated strain HB-1^T, was isolated using anoxic mineral salt medium buffered with 5 mM phosphate at pH 7 amended with 5 mM acetate and 5 mL of N2O as a sole electron acceptor. Strain HB-1^T had a unique cellular fatty acid composition with a substantially higher abundance of palmitic acid (C16:0). The average nucleotide identity values suggest that strain HB-1^T represents a new species. Further isolation efforts targeted at N2O reducing bacteria at low pH are in progress.

Quantification and Mechanisms of Non-CO_2 Greenhouse Gas Emissions from Forage Conservation

[0069] As explained below, a temperature-controlled silage reactor is used to simulate the forage conservation process under anaerobic conditions. In the preliminary experiments, a significant volume of CO_2 was produced, which may disturb the microbial community unless properly released. A gas bag is connected to the headspace to collect the evolved gas and maintain atmospheric pressure in the reactor. The GHG emission potentials of conserved forages are assessed using three different crops; alfalfa, corn, and sorghum. These crops were selected because (1) alfalfa is legume and corn and sorghum are grass, and (2) they have different harvest windows, spaced at least two months apart. The moisture content is adjusted to 70%.

[0070] Chemical and biological treatments are evaluated for their impact on GHG emissions and

nutrient contents. For the chemical treatments, chlorate and acetate are used. Low C/N ratio is one of the potential causes for N_2O accumulation during denitrification, and acetate serves as an external carbon source. For the biological treatment, N_2O-reducing enrichment cultures inoculated with fermented silage and wastewater sludge at low pH are used as an inoculant to consume N_2O.

[0071] The microbial community composition and their functional potential are investigated using a combination of culture-independent and culture-dependent approaches. Microbial diversities are assessed through culture-independent community analysis based on high coverage, high-throughput sequencing. Direct quantification of gene abundances and their transcripts through quantitative PCR (qPCR) and reverse transcriptase quantitative PCR (RT-qPCR), respectively, are performed. Key here is the use of transcript:gene abundance ratios as a measure of physiological activity for different microbial communities. This ratio can serve a direct predictor of GHG flux. In addition to the primer sets targeting bacterial/archaeal 16S rRNA genes, a functional gene related to CH_4 production (*mcrA*) and N_2O cycle (*narG*, *napA*, *nirK*, *nirS*, *norB*, *nosZ*, and *nrfA*) are used.

[0072] **Quantification of non-CO2 GHG emissions from silages.** To quantitatively assess GHG emissions from silage, the three major silage crops, including alfalfa, corn, and sorghum, are ensiled at different moisture contents in a 1-L mini silo connected to a 1-L Tedlar bag at a bulk density of 700 kg/m^3 as described above. The experimental design and harvesting windows of each crop are summarized in Table 1.

[0073] **Table 1. Experimental design and harvesting window for each species.**

Experimental Design Species	Conservation Method	Moisture Content (%, w/w)	Harvesting Window in Kansas
Alfalfa	Hay/silage	20*/40/55/70	May-Aug
Corn	Silage	40/55/70	Sep-Nov
Sorghum	Silage	40/55/70	Sep-Nov

[0074] The gas bags are replaced with new ones to characterize the temporal variations in gas production volume and composition on days 1, 2, 3, 7, 14, and 21. Whenever the gas bags are replaced, the total gas production volumes is measured by the water displacement method. The forage samples are collected and stored at -80 °C for chemical and molecular biological analysis.

A subset of samples are submerged in RNAlater® buffer for RNA analysis. The detailed description of molecular analysis is presented below. The forage sample collection is carried out in the anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) containing a nitrogen/ hydrogen (97/3; v/v) atmosphere to prevent any oxygen intrusion. The sampling frequency was determined based on the preliminary study. The inoculants are provided by Pioneer Hi-Bred International, Inc. The alfalfa, corn, and sorghum samples are provided by Thurlow Farms Inc. Fresh harvest is wilted to targeted moisture content (45, 55, and 70%) and chopped into approximately 1.5-cm length particles.

[0075] Low concentration of CH_4 are measured using Agilent 7890 gas chromatograph equipped with a Flame Ionization Detector and a DB-624 capillary column (60 m × 0.32 mm × 1.8 μm), and high concentration of CH_4 and CO_2 are measured using Shimadzu GC 2010 Plus equipped with Thermal Conductivity Detector (TCD). For N2O measurement, the Agilent 7890 gas chromatograph system equipped with an electron capture detector and HP-PLOT/Q column (30 m × 0.53 mm × 40 μm) is used. For inorganic N (i.e., NH_4^+, NO_3^- and NO_2^-) and pH, "haylage juice" is prepared by mixing the forage sample and water (1:1, w/w). Dionex ICS 5000 ion chromatography is used for inorganic N measurement.

[0076] **Quantification of non-CO_2 GHG emissions from hay.** In the preliminary experiment, surface N_2O emission was monitored but accurate estimation was challenging due to the irregular shape of the surface. To better assess GHG emissions from hay (Table 1), an open circuit hay bale respiration chamber is installed at the K-State Beef Stocker Unit. The Unit has a metal-sided hay barn for indoor storage. A round bale (4 ft width $\times$ 4 ft diameter) from second-cut alfalfa wrapped with plastic mesh wrap is placed inside of the respiration chamber. The chamber is built using bolted steel angles and flexible PVC sheets to form a gas-tight envelop. The inside dimensions of the chamber are 2 m (length) x 2 m (width) x 2 m (height). The chamber is continuously flushed with ambient air (20 ml·min^{-1}) to simulate the natural wind using Zero Air Generator HP2A-3500 (Parker-Balston, Haverhill, MA).

[0077] The chamber headspace is continuously mixed with a fan installed inside. The exhaust air escaping through the outlet on the opposite side is collected and measured in the laboratory. The flux is calculated using the following equation, F = (C·q), where F is the emission flux (μg·hour^{-1}), C is the concentration (μg·m^3), and q is the air flow rate (m^3·hour). The ambient

temperature and humidity of the headspace are monitored using a Govee H5072 Bluetooth thermometer (OH, USA).

[0078] Identification of mechanisms of non-CO_2 GHG emissions from silage. In the preliminary study, most N_2O was produced within the first week of silage incubation (Fig. 4), and the redox conditions of the silage were assumed to be microaerobic to anoxic. Under these conditions, both nitrification and denitrification can play roles in N_2O production.

[0079] Indeed, *amoA* (encoding ammonia monooxygenase subunit A) abundance also significantly increased in the anoxic region of the hay bales as well as denitrification gene abundances, suggesting that nitrifiers may play a role in N_2O emissions from silage. Several factors have been reported to result in N_2O accumulation during denitrification, including low pH, low carbon to nitrogen (C/N) ratios, consumption of internal storage compounds, the presence of elevated levels of free nitrous acid or H_2S, and low dissolved oxygen (DO) concentrations.

[0080] The types of carbon sources also affect N_2O accumulation. In this task, the cause of N_2O emission from silage process is investigated, focusing on pH, C/N ratio, and oxidation-reduction potential (ORP). A silage reactor is made using stainless steel cylinder (25 cm ø $\times$ 30 cm H). The reactor is made in-house at the Department of Electrical and Computer Engineering at K-State. Using a pH meter and ORP meter, pH and ORP, respectively, are recorded automatically on the computer through a data logger, and gas volume and composition are monitored manually using a water displacement gas collector. For the forage sampling, four sampling holes (1 cm ø) are drilled on the sidewall of the reactor and closed using a rubber stopper. The samples are collected using a sample corer. Each sampling hole is sacrificed at each sampling event, and the holes are closed using the same rubber stopper. The samples are stored at -80°C for molecular biological analysis, and a subset of samples is submerged in RNAlater® buffer for RNA analysis.

[0081] Characterization of the microbial community structure within conserved forages elucidates how they produce and reduce GHGs and eventually allow for estimates and control of GHG emissions. DNA are extracted from the samples collected (from the experiments performed as described above) for molecular analysis using the microbial nucleic acid extraction method optimized in the preliminary study as described above. To address the question of GHG fluxes in conserved forages are driven by changes in microbial communities, direct quantification of gene abundances and their transcripts through quantitative PCR (qPCR) and reverse transcriptase

quantitative PCR (RT-qPCR) is performed, respectively. The key here is the use of transcript:gene abundance ratios as a measure of physiological activity for different microbial communities. This ratio can serve a direct predictor of GHG flux. In addition to the primer sets targeting bacterial/archaeal 16S rRNA genes, different functional genes of CH4 cycle – *pmoA* (encoding particulate CH4 monooxygenase), *mmoX* (soluble CH4 monooxygenase), and *mcrA* (α subunit of the methyl coenzyme M reductase), and N2O cycle – *narG* (the membrane-bound nitrate reductase), *napA* (periplasmic nitrate reductase), *nirK* (copper-containing nitrite reductase), *nirS* (te cytochrome cd1 nitrite reductase), *norB* (nitric oxide reductase), *nosZ* (nitrous oxide reductase), *nrfA* (periplasmic nitrite reductase), and bacterial/archaeal *amoA* (ammonia monooxygenase) are used. For the samples obtained from the hay method, the genes associated with GHG cycles under aerobic conditions, i.e., bacterial/archaeal 16S rRNA, *pmoA*, *mmoX*, and bacterial/archaeal *amoA,* are examined in addition to *mcrA, nrfA,* and denitrification genes. All the primers except for *pmoA, mmoX,* and *amoA* are used for the silage samples. In addition to 16S rRNA, N cycle, and CH4 cycle genes, genes associated with essential C-degradation (*amyA, ara, pme* etc.) are monitored as shown in Fig. 5.

[0082] A high-throughput metagenomic sequencing technique is used for in-depth community profiling. Reads are screened for accuracy using the Illumina quality control filter, and sequences shorter than 300 nt are removed from analysis to prevent inaccurate identification. Then, quality control of raw Illumina reads is performed, and sequences with imprecise matches to primer sequences, poor quality sequence reads, and sequences with ambiguous bases ($\geq$1) are filtered out. Next, the Ribosomal Database Project II – FunGene (Functional Gene Pipeline and Repository) database is taken advantage of for accurate classification at the genus and family level for each gene. The database uses a reference sequence dataset and employs Hidden Markov models for alignment based on protein homology, allowing for statistical classification of query reads. Then, sequencing and analysis is performed as described above using both DNA and RNA (converted to cDNA) extracted from the samples. Analysis of DNA provides an estimation of the total microbial communities, while RNA analysis provides an estimate of that portion of the community physiologically active at the time of sampling. Both alpha (within-sample) and beta (among-samples) diversities are quantified to test hypotheses about variation in microbial communities along temporal and environmental gradients (crop type, moisture content). This is done using

both taxonomic and phylogenetic methods. Taxonomic alpha diversity is measured as an operational taxonomic unit (OTU) and taxonomic group richness and equitability (Shannon diversity) within individual communities. Taxonomic dissimilarity of different communities is measured as the Bray-Curtis distance among samples based on OTU and taxonomic group membership. Phylogenetic diversity is measured based on a phylogenetic tree linking representative sequences from all OTUs. Phylogenetic relationships are inferred among representative sequences using FastTree software, which results in an approximate maximum likelihood tree. The resulting phylogeny is used to compute Faith's PD, the sum of branch lengths within samples. A standardized version of Faith's PD is computed based on comparing the observed PD to the PD expected under a random null model of community assembly to account for variation in the number of sequences in different samples. Phylogenetic beta diversity is measured using the UniFrac dissimilarity metric as implemented in QIIME.

[0083] To examine the effects of different treatments on forage nutritional quality, forage samples are sent to Rock River Laboratory, Inc., Watertown, WI, and are analyzed with NIR spectrophotometry and wet chemistry for dry matter, crude protein, crude fiber, total tract neutral detergent fiber digestibility, carbohydrates, and fermentation products. The same sample is sent to Hi-Bred International, Inc for pH and fatty acids analysis.

[0084] To find the strength of the relationship between the gas production and the quantified microbial populations (gene abundances) and between these populations and the chemical parameters, correlation analyses (with Pearson's correlation coefficient and Spearman's rank correlation coefficient) is performed. All quantitative data are examined by analysis of variance (ANOVA) and t-test among the control and treatment groups.

Framework to Control of N_2O emissions from forage conservations

[0085] In the preliminary study, the amendment of chlorate and acetate (0.1%, w/w) significantly repressed N_2O production ($P<0.001$). Chlorate has been known to inhibit microbial denitrification activity by blocking nitrate reductase. The same concentration of chlorate was used as a ruminant supplement to reduce E. Coli O157 population, suggesting that chlorate is promising as a silage additive minimizing N_2O emissions. Also, a low C/N ratio is one of the potential causes of N_2O accumulation during denitrification. It is hypothesized that acetate served as an external

carbon source to alleviate the possible low C/N ratio stress. Denitrifiers are known to produce N_2O at low pH. On the consumer side, recent reports have demonstrated that diverse microbial taxa possessing divergent *nos* clusters with genes that are related yet evolutionarily distinct from the typical *nos* genes of denitrifiers. Their environmental role in controlling N_2O emissions remains undefined, and these non-denitrifying N_2O-reducers may play an important role under conditions that are not favorable for denitrifiers (e.g., low pH). In this objective, it is investigated how GHG production can be reduced by regulating microbial processes.

[0086] Investigation of the Effects of Nitrification and Denitrification Inhibitors. Denitrification may contribute significantly to N_2O emissions in the silage process, and the contribution of nitrification is less likely. However, nitrification should not be ruled out, because forages are ensiled under aerobic conditions and low concentrations of residual oxygen may have favored a marginal metabolism of microaerobic bacteria. Further, in the preliminary study, most N_2O was produced within the first week where microaerobic condition is maintained (Fig. 3, Yang et al., in preparation). The same mini silos connected to a Tedlar bag (see Section "Quantification of non-CO2 GHG emissions from silages" in Example 4) is used to evaluate the effect of different concentrations of chemical inhibitors that are known to inhibit nitrification and denitrification on GHG production. In this task, phenylacetylene and chlorate (as potassium salt) are used as inhibitors of nitrification and denitrification, respectively, and their effects on GHG emissions is examined. Phenylacetylene is added at a concentration of 0.01-1 mg per kg-forage (wet weight), and chlorate is added at a concentration of 0.01-1 g per kg-forage (wet weight). The gas volume and composition are periodically monitored, and the forage samples are collected for chemical and molecular analysis as described above. Chlorate and phenylacetylene concentrations are monitored using IC and GC-FID, respectively. In addition to the genes of interest listed in Example 4, cld gene (encoding chlorite dismutase) are monitored.

[0087] Investigation of the effects of carbon source amendment. Decrease in carbon sources increases N_2O emissions during denitrification as the various enzymes in that process compete for the electrons where nitrous oxide reductase is the weakest competitor, which ultimately leads to incomplete denitrification. A study using a pure culture of *Alcaligenes faecalis* verified that as soon as the availability of carbon compounds became exhausted and the culture entered starvation, the main product of denitrification was N_2O instead of N_2, and the conversion rate increased up to

64% of the total N-feed. In the preliminary study, the total amount of N_2O was reduced by 25% by the amendment of acetate (as a sodium salt, 1 g per kg-forage). In this task, acetate and lactate are evaluated as external carbon sources to reduce N2O accumulation during denitrification at a concentration of 0.1-10 g per kg-forage (wet weight). The gas volume and composition are periodically monitored, and the forage samples are collected for chemical and molecular analysis, as described above.

[0088] Isolation of N2O-reducing microorganisms at low pH. In general, the vast majority of microbial biodiversity cannot yet be maintained in laboratory culture. This is true on GHG-cycling microorganisms as well; only a small percentage of CH_4 cycling microbes are currently in culture collections, and non-denitrifier N_2O-reducing organisms were only recently discovered. Although multiple sources of N_2O exist, there is only one known sink for N2O in the biosphere – the microbial reduction of N_2O to N_2, catalyzed by the N_2O reductase. This enzyme has traditionally been assigned to bacteria and archaea performing complete denitrification until recent discoveries unveiled the unexpectedly broad diversity of *nosZ*. *nosZ* genes of the novel clade II are often found in organisms lacking the genes encoding the key denitrification enzymes, namely *nirK* or *nirS*, indicating that these organisms utilize N_2O reduction as a respiratory reaction independent from denitrification. Such non-denitrifying N_2O reducers potentially can function as N_2O sinks. In the preliminary study, a novel N_2O-respiring bacterium, designated as *Gemmobacter serpentinus* strain HB-1, was isolated from a round hay bale.

[0089] For an application toward N_2O mitigation, characterization of more N_2O-reducing isolates is required. Efforts are continued on enrichment and isolation of N_2O-reducing bacteria and characterize their roles in conserved forages particularly at pH 5.5 or below (optimal silage pH). Further, the application of N_2O-reducing bacteria as a silage additive is examined. A continuous microbial enrichment is used as a tool to search for such unknown N_2O-reducing metabolisms. To develop enrichment cultures, 10 ml of sludge from a local wastewater treatment plant and 10 g of fermented silage samples is used to inoculate two double-jacket glass bioreactors (BioFlo®/CelliGen® 115, New Brunswick/Eppendorf, Germany) with 1 L working volume of sterile phosphate-buffered mineral salt media using N_2O as sole electron acceptor and acetate as sole carbon/energy source. Initially, organisms are grown under batch mode until biomass concentration reaches 1.2 g L^{-1}, and then continuous mode of operation is initiated. In one reactor,

the initial pH is maintained at pH 7 and then gradually lowered to pH 5 by feeding fresh media adjusted to different pH. In another reactor, the pH is maintained at pH 5. The pH is monitored and maintained automatically by adding 1 M HCl and NaOH. The headspace N2O concentration ismonitored and maintained at a targeted level by feeding pure gas manually. Once enrichment cultures are obtained, purification is accomplished by repeated serial dilution with liquid media and streaking on solid media. At the same time, DNA is extracted and subjected to 16S rRNA gene amplicon sequencing. Both pure cultures and mixed enrichment cultures is used as an additive in simulated silage experiments (Task 1.1), and their effects on GHG emissions are examined.

Validation of Laboratory Results by Performing Field Scale Evaluations

[0090] In order to validate the laboratory results, a field study is carried out at the K-State Beef Stocker Unit. The unit has 1,120 acres of native warm season grass subdivided into 20 functional paddocks. The unit has a well-drained site for outdoor forage storage and a metal-sided hay barn for indoor storage. The optimized forage amendment selected from the laboratory experiments is applied to prepare three silage bags (2.5 m×2.5 m) along with additional three bags as controls. Four gas bags are connected, apart from each other. Similar to the laboratory experiments, the gas bags are periodically replaced with new ones, and the gas composition and total volumes are monitored. The gas/forage samples are collected once in two weeks and used for chemical, nutritional, and molecular biological analyses.

[0091] The proposed studies in Examples 4 and 5 should provide insights into the GHG emission mechanisms from the forage conservation process and the strategies to control the emissions. In this objective, the results from these Examples are further validated in the field scale experiments at the K-State Beef Stocker Unit and the Thurlow Farms.

[0092] Round bale silages (baleages) made with alfalfa, corn, and sorghum are obtained and monitored for GHG production at the K-State Beef Stocker Unit. Baleage is a relatively new technique for conserving forage as silage, wrapped with at least 6 layers of 1 mil plastic and maintained under gas-tight conditions. Moisture content of the forage is adjusted to 50%. Two control baleages receive silage inoculant (Pioneer® 11H50, 1174, and 1129, respectively), and the other sets of two baleages are amended with treatments optimized in addition to the inoculant. Two gas bags (2 L capacity, SKC Inc. Eighty Four, PA) are connected on the other side. The forage

samples are also collected for chemical and molecular analysis as described above. Similar to the laboratory experiments, the effect of different treatments on GHG emissions, silage nutritional quality, and microbial community are examined.

[0093] There are four bunker silos (20'W x 8'H x 80'L) for corn silage at the Thurlow Farms, and two of the silos are used for the study. The control silo receives silage inoculant (Pioneer® 1174), and the other silo is amended with treatments optimized in addition to the inoculant. The GHG emissions are monitored once a week by measuring the surface fluxes using a flow-through chamber method. The forage samples are collected for chemical and molecular analysis at each sampling event as described above. The effect of the treatment on GHG emissions, silage nutritional quality, and microbial community are examined.

CLAIMS:

1. An additive for a forage inoculant, the additive comprising at least two components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation.

2. The additive of claim 1, wherein the at least two components are selected from the group consisting of a component capable of inhibiting microbial denitrification activity or methanogenesis, a component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, an N_2O-reducing enrichment culture, and mixtures thereof.

3. The additive of claims 1-2, wherein the first component is a component capable of inhibiting microbial denitrification activity or methanogenesis and the second component is a component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms.

4. The additive of claims 2 or 3, wherein the component capable of inhibiting microbial denitrification activity or methanogenesis is chlorate or chlorite.

5. The additive of any of claims 2-4, wherein the component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms is acetate or lactate.

6. The additive of claim 2, wherein the N_2O-reducing enrichment culture is *Gemmobacter serpentinus* strain HB-1 inoculated with fermented silage and wastewater sludge at a pH of 5 or less.

7. A forage inoculant, the forage inoculant comprising:

starter bacteria capable of decreasing the pH in a quantity of forage to 5.5 or less; and

an additive comprising one or more components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation.

8. The forage inoculant of claim 7, wherein the starter bacteria are members of the order Lactobacillales.

9. The forage inoculant of claim 8, wherein the starter bacteria are selected from the group consisting of *Lactobacillus buchneri*, Pioneer® 11H50, Pioneer® 1174, and Pioneer® 1129, and mixtures thereof.

10. The forage inoculant of any of claims 7-9, when applied to the quantity of forage, the forage inoculant does not reduce the nutritional value of the quantity of forage.

11. The forage inoculant of any of claims 7-10, wherein the forage inoculant is capable of fermenting the quantity of forage, thereby forming conserved forage.

12. The forage inoculant of claims 10 or 11, wherein the forage is selected from the group consisting of legumes or grasses.

13. The forage inoculant of claims 11 or 12, wherein the conserved forage is in the form of silage, haylage, or baleage.

14. A method of reducing non-CO_2 greenhouse gas emissions from forage conservation, the method comprising:

applying to a quantity of forage:

(i) a starter bacteria capable of decreasing pH in the quantity of forage to 5.5 or less, and

(ii) an additive comprising one or more components capable of reducing non-CO_2 greenhouse gas emissions from forage conservation,

wherein the additive has a concentration of about 0.15 to about 0.3 g per kg of forage.

15. The method of claim 14, wherein the additive comprises one or more components that are selected from the group consisting of a component capable of inhibiting microbial denitrification

activity or methanogenesis, a component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms, an N_2O-reducing enrichment culture, and mixtures thereof.

16. The method of claim 14, wherein the starter bacteria are applied to the quantity of forage before the additive.

17. The method of claim 15, wherein the component capable of inhibiting microbial denitrification activity or methanogenesis is chlorate or chlorite.

18. The method of claim 15, wherein the component capable of facilitating the conversion of N_2O to N_2 by non-denitrifying N_2O-reducing organisms is acetate or lactate.

19. The method of claim 15, wherein the N_2O-reducing enrichment culture is *Gemmobacter serpentinus* strain HB-1 inoculated with fermented silage and wastewater sludge at a pH of 5 or less.

20. The method of claim 14, wherein the additive comprises two components, wherein the first component is chlorate and the second component is acetate.

21. The method of claim 20, wherein the first and second components each have a concentration of about 0.015 g per kg of forage.

22. The method of claim 14, wherein the starter bacteria are members of the order Lactobacillales.

23. The method of claim 22, wherein the starter bacteria are selected from the group consisting of *Lactobacillus buchneri*, Pioneer® 11H50, Pioneer® 1174, and Pioneer® 1129, and mixtures thereof.

24. The method of claim 14, wherein the method further comprises incubating the quantity of forage.

25. The method of claim 14, wherein the quantity of forage has a bulk density of about 400 kg/m^3 to about 1000 kg/m^3.

26. The method of claim 14, wherein the non-CO_2 greenhouse gas is N_2O, CH_4, or combinations thereof.

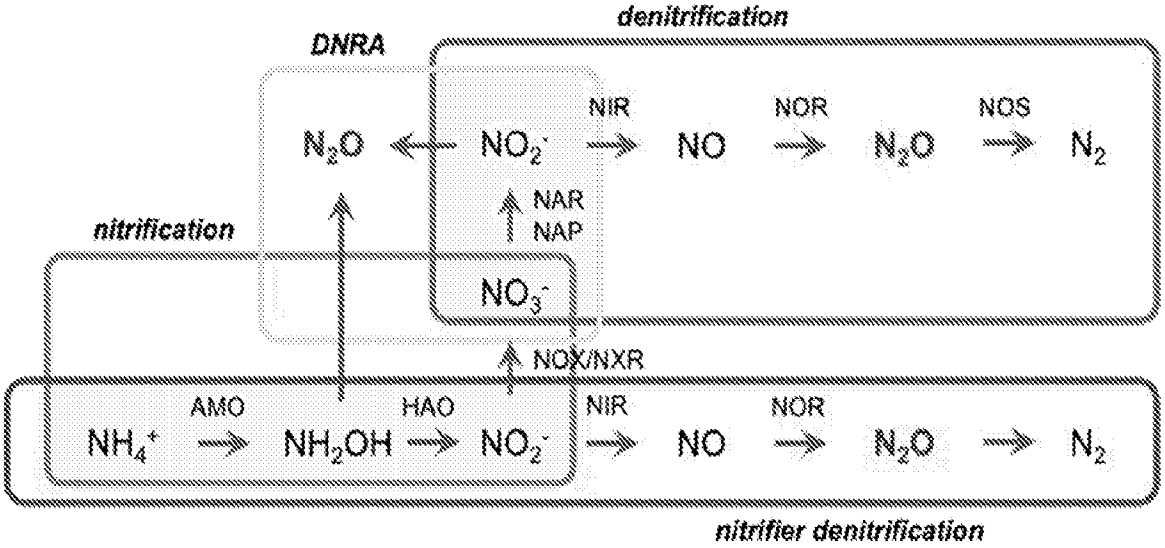

denitrification
DNRA
NIR
NOR
NOS
N_2O
NO_2^-
NO
N_2O
N_2
NAR
NAP
nitrification
NO_3^-
NOX/NXR
AMO
HAO
NIR
NOR
NH_4^+
NH_2OH
NO_2^-
NO
N_2O
N_2
nitrifier denitrification

Fig. 1

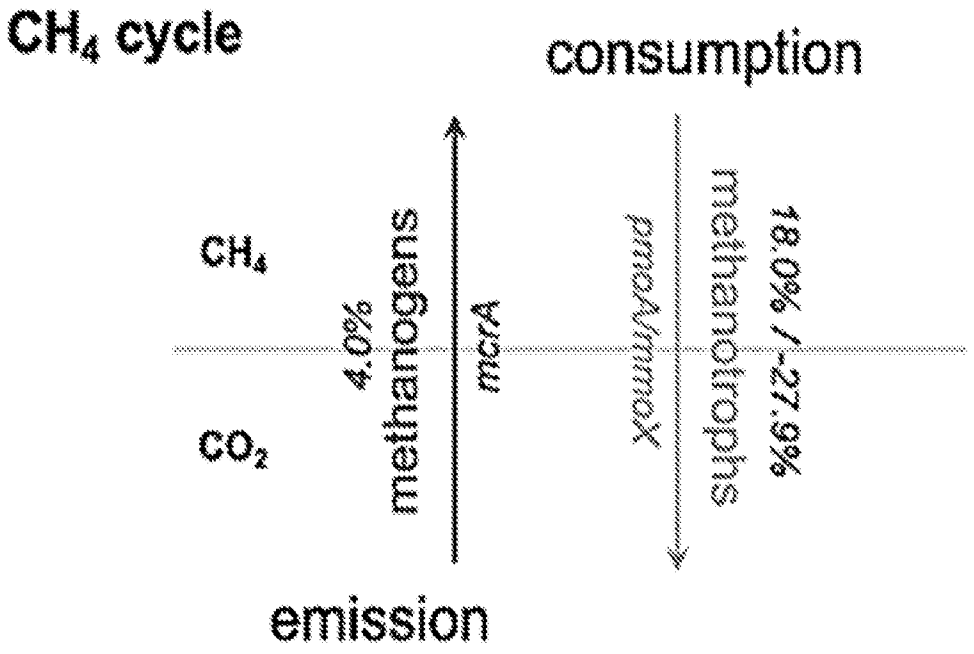

CH_4 cycle
consumption
CH_4
CO_2
4.0%
methanogens
mcrA
anammox
methanotrophs
18.0% / -27.9%
emission

Fig. 2

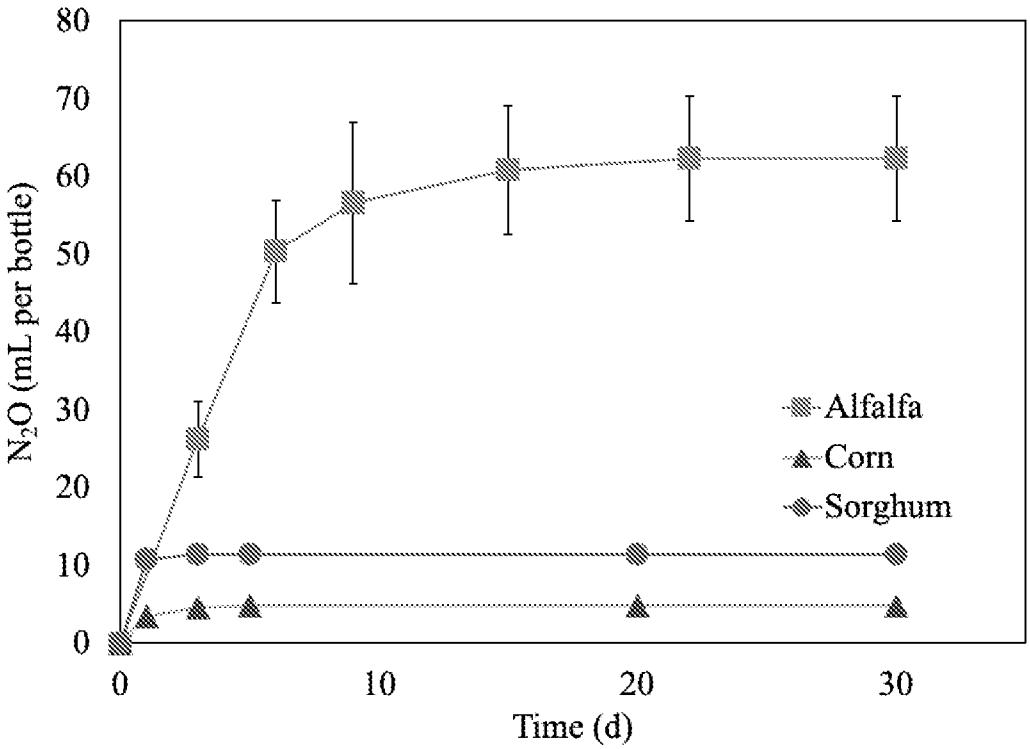

80
70
60
50
40
30
20
10
0
N_2O (mL per bottle)
0
10
20
30
Time (d)
Alfalfa
Corn
Sorghum

Fig. 3A

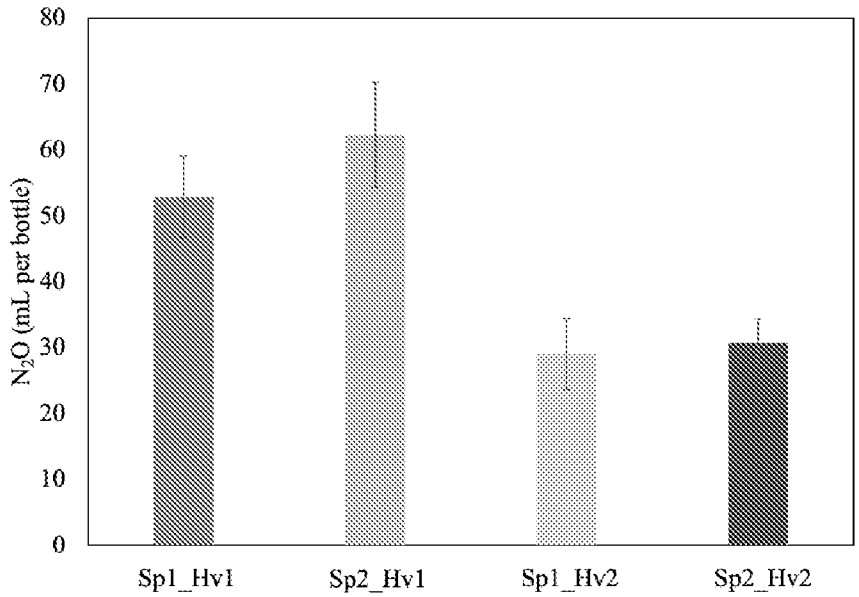

80
70
60
50
40
30
20
10
0
N_2O (mL per bottle)
Sp1_Hv1
Sp2_Hv1
Sp1_Hv2
Sp2_Hv2

Fig. 3B

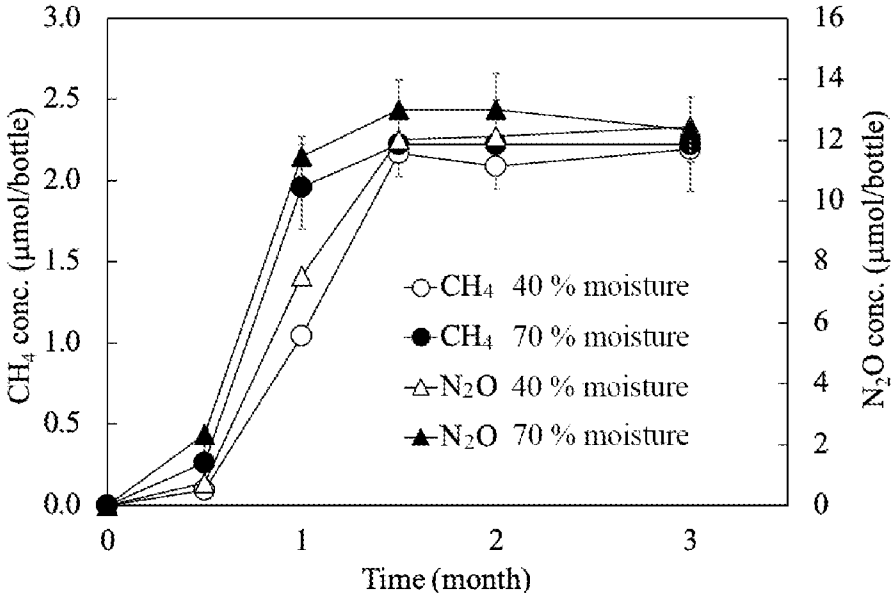

3.0
2.5
2.0
1.5
1.0
0.5
0.0
CH4 conc. (µmol/bottle)
16
14
12
10
8
6
4
2
0
N2O conc. (µmol/bottle)
0
1
2
3
Time (month)
CH4 40 % moisture
CH4 70 % moisture
N2O 40 % moisture
N2O 70 % moisture

Fig. 3C

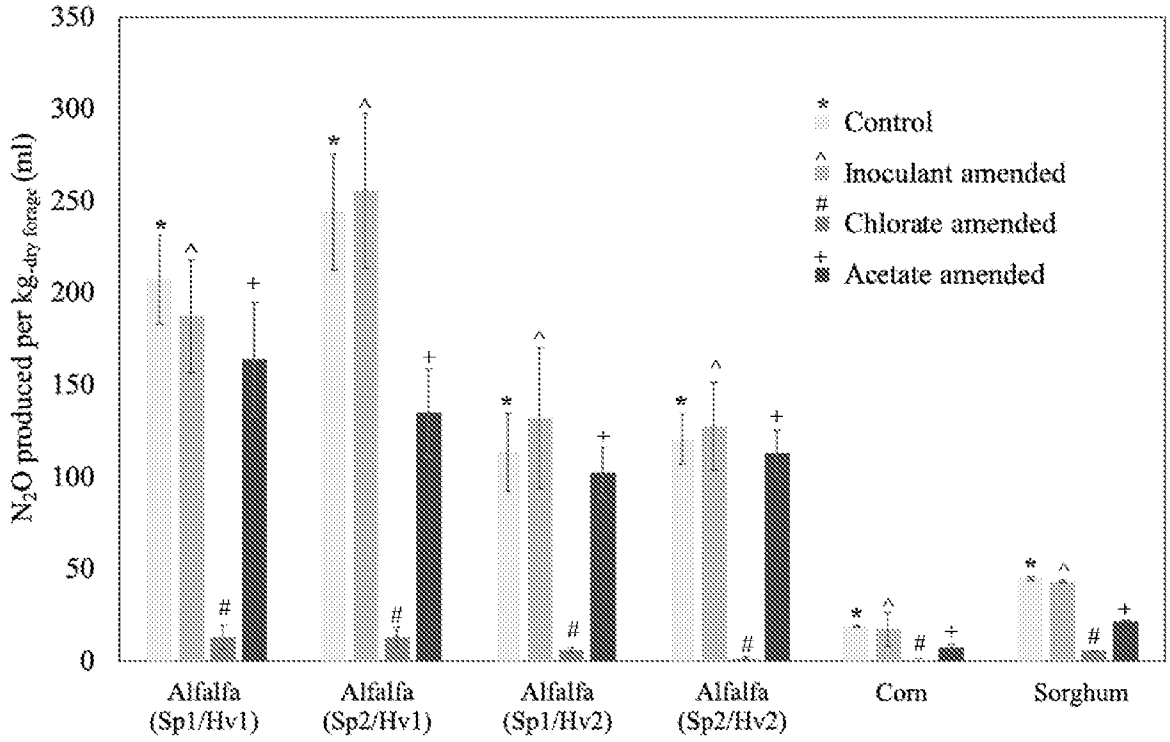

350
300
250
200
150
100
50
0
N_2O produced per $kg_{dry\ forage}$ (ml)
Control
Inoculant amended
Chlorate amended
Acetate amended
Alfalfa (Sp1/Hv1)
Alfalfa (Sp2/Hv1)
Alfalfa (Sp1/Hv2)
Alfalfa (Sp2/Hv2)
Corn
Sorghum

Fig. 4A

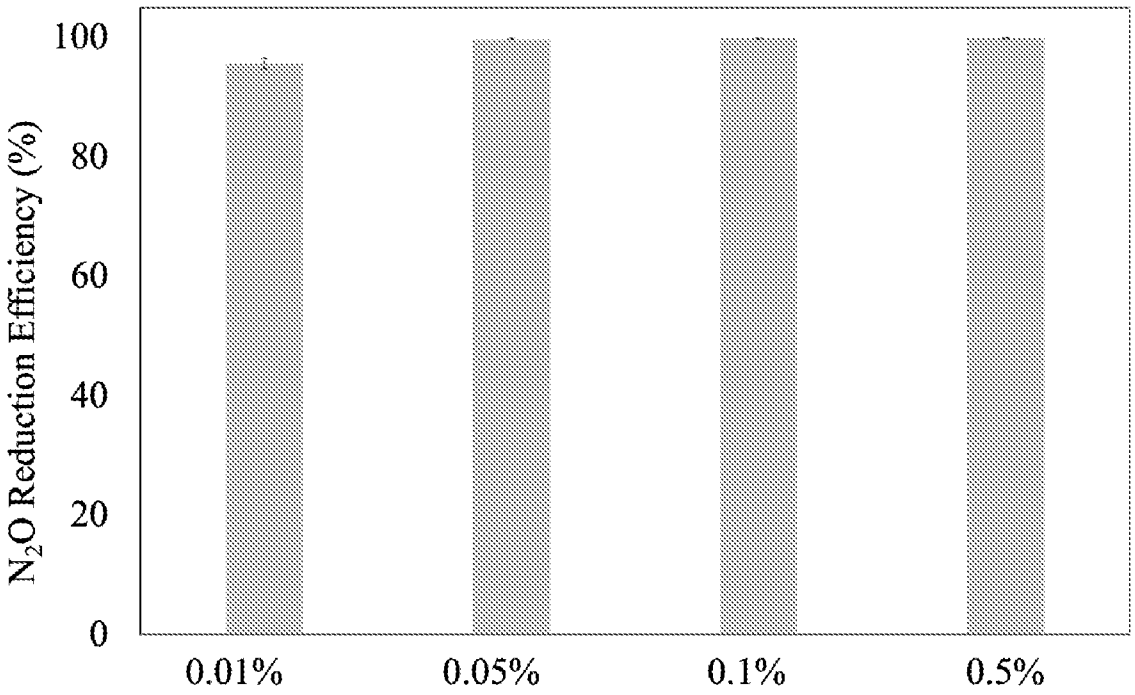
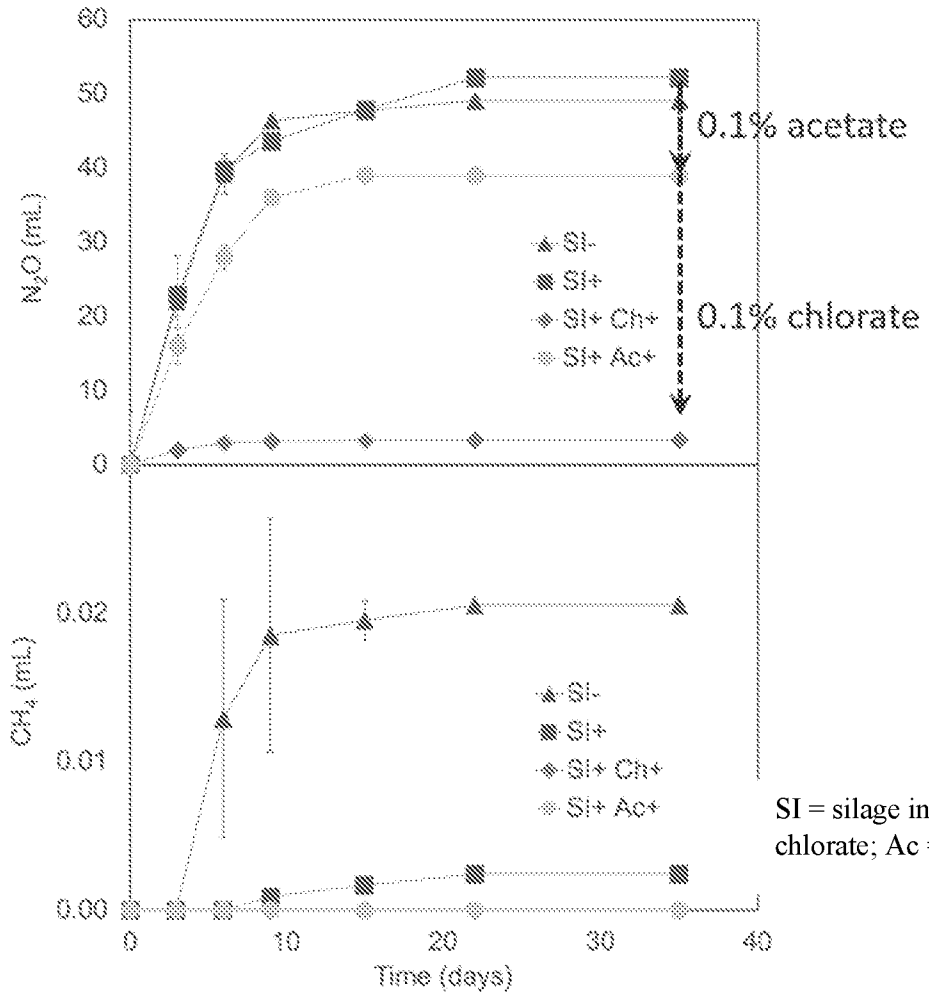

100
80
60
40
20
0
N_2O Reduction Efficiency (%)
0.01%
0.05%
0.1%
0.5%

Fig. 4B



SI = silage inoculant; Ch = chlorate; Ac = acetate.

Fig. 4C

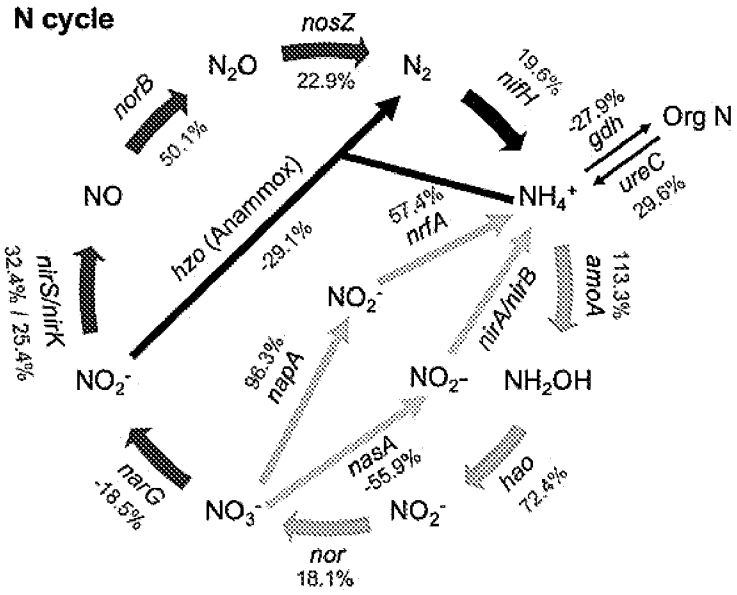

N cycle
nosZ
N2O
22.9%
N2
19.6%
nifH
norB
50.1%
-27.9%
gdh
Org N
ureC
29.6%
NO
hzo (Anammox)
-29.1%
57.4%
nrfA
NH4+
nirS/nirK
32.4% / 25.4%
NO2-
NO2-
86.3%
napA
nirA/nirB
amoA
113.3%
NO2-
NH2OH
narG
-18.5%
NO3-
nasA
-55.9%
hao
72.4%
NO2-
nor
18.1%

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/082198

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - INV. - A23K 30/18; A23K 10/10; A23K 10/30 (2023.01)
ADD.

CPC - INV. - A23K 30/18; A23K 10/10; A23K 10/30 (2023.02)

ADD.

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)
See Search History document

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

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	CN 108740366 A (JIESHOU LIMIN GRASS CO LTD) 06 November 2018 (06.11.2018) see machine translation	1-3 --- 7-10, 14, 15, 17, 18, 20, 22-26
Y	US 2017/0208836 A1 (PIONEER HI-BRED INTERNATIONAL INC) 27 July 2017 (27.07.2017) entire document	7-10, 14, 15, 17, 18, 20, 22-26
Y	EP 0 578 479 A2 (SWEENEY) 12 January 1994 (12.01.1994) entire document	17, 20
A	LIM et al., Gemmobacter serpentinus sp. nov., isolated from conserved forages, International Journal of Systematic and Evolutionary Microbiology, Vol. 70, 2020 [retrieved on 07 March 2023]. Retrieved from the Internet: <URL: https://www.microbiologyresearch.org/content/journal/ijsem/10.1099/ijsem.0.004276>. Pgs. 4224–4232	1-3, 6-10, 14-26
A	MAHMOOD, Role of microbial diversity in controlling greenhouse gas emissions from conserved forages, A thesis submitted to the Department of Civil Engineering at Kansas State University, 28 July 2021 [retrieved on 07 March 2023]. Retrieved from the Internet: <URL: https://krex.k-state.edu/handle/2097/41576?show=full>. Pgs. v-viii, 1-28	1-3, 6-10, 14-26
P, A	WO 2022/137149 A1 (FONTERRA CO-OPERATIVE GROUP LIMITED et al) 30 June 2022 (30.06.2022) entire document	1-3, 6-10, 14-26

☐ Further documents are listed in the continuation of Box C. ☐ 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
"D" document cited by the applicant in the international application
"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
11 March 2023	MAY 17 2023
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Taina Matos Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/082198

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4, 5, 11-13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.