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(54) **Titre : UTILISATION DE MONONITRATE DE PROPANDIOL ET DE LIMONENE POUR REDUIRE LA FORMATION DE METHANE EMANANT ET POUR AMELIORER LA DIGESTIBILITE**

(54) **Title: USE OF PROPANDIOL MONONITRATE AND LIMONENE FOR REDUCING THE FORMATION OF METHANE EMANATING AND FOR IMPROVING THE DIGESTIBILITY**

(57) **Abrégé/Abstract:**
The present invention relates to the field of reduction of methane emission in ruminants. Particularly, it relates to the administration of limonene and propanediol mononitrate to a ruminant for reducing the production of methane emanating from the digestive activities of said ruminant.

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(54) **Title:** USE OF PROPANDIOL MONONITRATE AND LIMONENE FOR REDUCING THE FORMATION OF METHANE EMANATING AND FOR IMPROVING THE DIGESTIBILITY

(57) **Abstract:** The present invention relates to the field of reduction of methane emission in ruminants. Particularly, it relates to the administration of limonene and propanediol mononitrate to a ruminant for reducing the production of methane emanating from the digestive activities of said ruminant.

USE OF PROPANDIOL MONONITRATE AND LIMONENE FOR REDUCING THE FORMATION OF METHANE EMANATING AND FOR IMPROVING THE DIGESTIBILITY

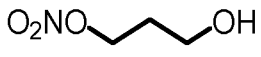
The present invention relates to the field of reduction of methane emission in ruminants. Particularly, it relates to the administration of limonene and propanediol mononitrate to a ruminant for reducing the production of methane emanating from the digestive activities of said ruminant.

The present invention further relates to animal feed compositions and feed additives comprising propanediol mononitrate and limonene as well as the use of said feed composition or feed additives for reducing the production of methane emanating from the digestive activities of ruminants.

The temperature of the air surrounding the earth is increasing, a process referred to as global warming. One of the main focuses to reduce this warming effect is to reduce the amount of greenhouse gases emitted into the atmosphere. Greenhouse gases are emitted from several different sources, both natural and artificial; however, the two sources with the most emphasis are the agricultural and fossil fuel industries. Within agriculture, ruminants and in particular cattle are the major contributors to the biogenic methane formation, and it has been estimated that the prevention of methane formation from ruminants would almost stabilize atmospheric methane concentrations.

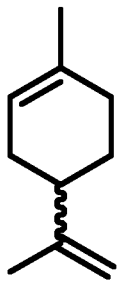
Methane emission from the ruminant livestock sector — a by-product from enteric fermentation of plant biomass in the ruminant digestive system — is produced by methanogenic archaea. Various attempts have been made in the last decade to mitigate methane production from ruminant animals. Although the approaches vary, the most popular method so far are feed additives which act in the rumen fluid by reducing respectively inhibiting the methane production by methanogenic archaea.

Propanediol mononitrate (also referred to herein as PDMN or 3-Nitrooxypropanol (3-NOP)) having the chemical structure of formula (I) has been reported to significantly reduce the methane production in ruminants (see e.g. WO2012/084629).



formula (I)

Limonene is a naturally occurring compound of formula (II)



formula (II)

e.g. found in citrus fruits.

Surprisingly, it has now been found, that the combined use of limonene and propanediol mononitrate leads to a synergistically reduced methane formation.

Thus, the combined use of a propanediol mononitrate and limonene has a great potential in the mitigation of climate change by significantly reducing the methane emissions emanating during the digestive activities of ruminants.

Therefore, in a first embodiment, the present invention provides the use of limonene and propanediol mononitrate for reducing the formation of methane emanating from the digestive activities of ruminants.

In a second embodiment, the invention further provides a method for reducing the production of methane emanating from the digestive activities of ruminants, said method comprising orally administering to the animal an effective amount of limonene and propanediol mononitrate.

In a third embodiment, the present invention relates to a (ruminant) feed composition or feed additive comprising limonene and propanediol mononitrate.

It is well understood, that limonene and propanediol mononitrate may be administered admixed together (i.e. pre-mixed) or separately to the ruminant, while in the latter case it is well understood that the administration of propanediol mononitrate and limonene occurs within a certain time window, i.e. within at most 6 h, preferably within 3 h, more preferably within 1h,

such as within 0.5 h. Most preferably, if administered separately, both ingredients are concomitantly administered to the ruminant e.g. by concomitant addition thereof into the animal's feed (diet) or into the feed rack.

Preferably, in all embodiments of the present invention limonene and propanediol mononitrate are administered together in form of a feed composition or feed additive comprising limonene and propanediol mononitrate.

It is well understood, that in all embodiments of the present invention limonene and propanediol mononitrate are administered to the ruminant in an effective amount, i.e. in an amount which results in a methane reduction, preferably of at least 10 %, more preferably of at least 20 %, most preferably of at least 30 %, when compared to the control, i.e. to ruminants not supplemented with limonene and propanediol mononitrate.

Thus, the present invention also relates to the (combined) use of limonene and propanediol mononitrate, wherein the methane production in ruminants is reduced by at least 10 % when compared to a control (i.e. in the absence of limonene and propanediol mononitrate). Preferably, the methane reduction is at least 15 %, more preferably at least 20 %, even more preferably at least 25 %, most preferably at least 30 %.

Propanediol mononitrate [CAS No: 100502-66-7] is a known compound which can e.g. be manufactured as outlined in WO2004043898 or WO2012084629 and which is available under the trademark Bovaer® at DSM Nutritional Products Ltd.

Limonene (CAS No: 138-86-3, unspecific) occurs as D-Limonene (5989-27-5) also known as (R)-(+)-Limonen and L-Limonene (5989-54-8) also known as (S)-(-)-Limonene as well as mixtures thereof. According to the present invention, limonene can be used in the form of the respective single enantiomer or as a mixture thereof. Preferably in all embodiments of the present invention limonene is used in the form of the D-isomer or as an enantiomeric mixture comprising the D-isomer, i.e. as (±) limonene. (R)-(+)-limonene as well as the isomeric mixture is e.g. commercially available from Sigma-Aldrich in ≥97% (HPLC).

The term 'an effective amount' as used herein refers to an amount necessary to obtain a reduction of the methane emissions resulting from the digestive activities of a ruminant. It is

well understood, that said reduction may be achieved by one single (daily) dose or by repeated (daily) doses. Furthermore, it is well understood by a person skilled in the art, that the effective amount of limonene and propanediol mononitrate in the uses, methods and compositions according to the invention may vary depending upon known factors, such as the characteristics of the particular composition and its mode and route of administration, the limonene content of the respective plant product, the age, health and weight of the ruminant, the frequency of treatment, all of which can be determined by the expert in the field with normal trials or with the usual considerations regarding the intake regime and/ or the formulation.

Preferably, in all embodiments of the present invention, the effective amount of propanediol mononitrate to be administered to the ruminants is selected in the range from 0.05 to 5 g PDMN/ animal/ day, more preferably in the range from 0.1 to 4 g PDMN/ animal/ day, most preferably in the range from 0.25 to 3 g PDMN/ animal/ day. Further suitable effective amounts are selected in the range from 0.5 to 3 g PDMN/ animal/ day or from 1 to 3 g PDMN/ animal/ day.

With regard to the feed, preferably, in all embodiments of the present invention, the effective amount of propanediol mononitrate in the feed is selected in the range from 10 mg to 300 mg PDMN/ kg DM/ day, more preferably in the range from 25 to 150 g PDMN/ kg DM/ day, most preferably in the range from 50 to 100 g PDMN/ kg DM/ day.

Preferably, in all embodiments of the present invention, the effective amount of limonene to be administered to the ruminants is selected in the range from 0.05 to 100 g limonene/ animal/ day, more preferably in the range from 0.1 to 50 g limonene/ animal/ day, most preferably in the range from 0.5 to 25 g limonene/ animal/ day, from 1 to 25 g limonene/ animal/ day, from 5 to 25 g limonene/ animal/ day, 10 to 25 g limonene/ animal/ day or from 15 to 25 g limonene/ animal/ day.

With regard to the feed, in all embodiments of the present invention the effective amount of limonene in the feed is selected in the range from 5 mg to 5 g limonene/ kg DM/ day, more preferably in the range from 5 mg to 2.5 g limonene/ kg DM/ day, most preferably in the range from 5 mg to 2 g limonene/ kg DM/ day, such as in the range from 10 mg to 1.5 g limonene/ kg DM/ day, 10 mg to 1.25 g limonene/ kg DM/ day or 10 mg to 1 g limonene/ kg DM/ day.

In all embodiments of the present invention, it is furthermore advantageous if the molar ratio of limonene to propanediol mononitrate (i.e. mole limonene/ mole PDMN) is comprised between 100 and 1 (e.g. from 100 μM limonene/1 μM PDMN to 1 μM limonene/ 1 μM PDMN), preferably between 75 to 1, more preferably between 60 to 5 such as between 50 to 5, 40 to 5, 30 to 5 or 25 to 5. Further suitable ranges encompass 50:1 to 5:1, 40:1 to 7.5:1, 30:1 to 10:1, 25:1 to 10:1 as well as 25:1 to 15:1.

In all embodiments of the present invention, propanediol mononitrate is preferably administered in the form of a powderous formulation thereof.

Preferably, said powderous formulation is a powderous formulation comprising PDMN and a carrier material. Suitable carrier includes any carrier well known in the food and feed industry such as silicone dioxide (silica) without being limited thereto.

Powderous formulations comprising PDMN and a carrier material are usually prepared by PDMN being sprayed onto or admixed with the carrier material by standard methods in the art, e.g. by diluting PDMN in an organic solvent suitable for the preparation of food or feed products such as e.g. dichloromethane, spraying or admixing said solution with/ onto the carrier followed by evaporation of the organic solvent.

Alternatively, PDMN can be diluted in a suitable edible oil before being sprayed onto or admixed with the carrier material. In the latter cases the respective edible oil is generally not removed. The powderous formulation may in addition contain usual additives used in the preparation of powderous formulations for feed application.

The amount of PDMN in the powderous formulation according to the present invention is preferably selected in the range of 1 to 20 wt.-%, preferably in the range of 2 to 15 wt.-%, most preferably in the range of 4 to 12 wt.-%, based on the total weight of the composition.

Particularly suitable powderous formulation to be used in all embodiments of the present invention consists essentially of PDMN, propylene glycol and silica as e.g. outlined in WO2018149756 and WO2018149755 and are commercially available as Bovaer® 10 from DSM Nutritional Products Ltd.

In another preferred embodiment of the invention the limonene of the present invention is added to the feed (diet) of the animal in the form of a single feed additive formulation (also referred to herein as limonene additive formulation).

The term feed additive formulation as used herein refers to a (powderous) formulation, which can either be added directly into an animal feed (diet) or (preferably) be used to prepare a feed additive.

Preferably, said limonene additive formulation comprises limonene as defined herein, at least one carrier and optionally an antioxidant.

Preferably the (total) amount of limonene in the limonene additive formulations according to the present invention is selected in the range from 0.1 to 10 wt.-%, preferably 0.25 to 5 wt.-%, preferably 0.3 to 2.5%. Further suitable ranges include 0.3 to 1 % but also ranges from 10 to 50%, from 15 to 50% and from 20 to 30%.

In one preferred embodiment, PDMN and limonene are supplemented in the absence of six or more phenolic compounds, such as in particular in the absence of six or more phenolic compounds selected from the group consisting of 3-hydroxyphenol (CAS No.: 108-46-3), 2-methoxyphenol (CAS No.: 90-05-1), 3-methylphenol (CAS No.: 108-39-4), 2-(1-methylethyl)-5-methyl-phenol (CAS No.: 89-83-8), 4-allyl-2-methoxyphenol (CAS No.: 97-53-0), 4-hydroxy-3-methoxybenzaldehyd (CAS No.: 121-33-5), 5-isopropyl-2-methylphenol (CAS No.: 499-75-2), 2 hydroxybenzoates (salicylates) and tannic acid (CAS No.: 1401-55-4).

In another preferred embodiment, PDMN and limonene are supplemented in the presence of one or more phenolic substances, more preferably however less than 6, i.e. in the presence of 1, 2, 3, 4 or 5 phenolic compounds. Preferably, the phenolic compounds are selected from the group consisting of 3-hydroxyphenol (CAS No.: 108-46-3), 2-methoxyphenol (CAS No.: 90-05-1), 3-methylphenol (CAS No.: 108-39-4), 2-(1-methylethyl)-5-methyl-phenol (CAS No.: 89-83-8), 4-allyl-2-methoxyphenol (CAS No.: 97-53-0), 4-hydroxy-3-methoxybenzaldehyd (CAS No.: 121-33-5), 5-isopropyl-2-methylphenol (CAS No.: 499-75-2), 2 hydroxybenzoates (salicylates) and tannic acid (CAS No.: 1401-55-4). Preferred salicylates are methyl, ethyl, 3-methyl-2-butanyl, isoamyl and benzyl salicylates. Most preferred in all embodiments of the present invention is the use of benzyl salicylate (CAS No.: 118-58-1), 3-methyl-2-butanyl and/

or isoamyl salicylate (CAS No.: 87-20-7), even more preferably benzyl salicylate and/ or isoamyl salicylate such as in particular a mixture of both.

In all embodiments of the present invention, it is further preferred if propanediol mononitrate and limonene are administered in the absence of (i.e. not concomitantly with) any antibiotic such a preferably monensin, one or more p-nitroaniline derivative such as p-nitroaniline and/ or one or more fatty acid derivative one fatty acid containing at least 5 carbon atoms such as lauric acid.

Limonene and propanediol mononitrate in all uses and methods according to the present invention are preferably administered concomitantly to the ruminant, either by prior admixing thereof or by separate addition to the diet of the animal.

In one preferred embodiment of the invention propanediol mononitrate and limonene are added to the feed as single (not premixed) ingredients.

In another preferred embodiment of the invention propanediol mononitrate and limonene are added to the feed as premixed ingredients.

Limonene and propanediol mononitrate are preferably administered via a (ruminant) feed composition or feed additive to the ruminant, e.g. by admixing the individual components with the ruminant's feed.

Thus, preferably, in all uses and methods according to the present invention propanediol mononitrate as well as limonene with all the definitions and preferences as given herein are administered to the ruminant incorporated into a (ruminant) feed composition or feed additive.

The term feed composition or feed additive as used herein means any preparation, mixture, or composition suitable for, or intended for oral intake by an animal. Exemplary feed for ruminants such as cows include forage (grass, legumes, silage), hay, grass, grain as well as soy without being limited thereto.
Said feed compositions or feed additives may be prepared by methods known per se in the art of feed formulation and processing.

Said feed compositions and feed additives are still novel. Thus, further aspects of the present invention are (ruminant) feed compositions and feed additives comprising limonene and propanediol mononitrate with all the definitions and preferences as given herein.

In one preferred embodiment, the feed compositions and feed additives are a mineral premix, a vitamin premix including vitamins and optionally minerals or a bolus.

Limonene and propanediol mononitrate may be used in combination with conventional ingredients present in an animal feed composition (diet) such as forages (raw, grass, hay, silages), co-products from industry (citrus pulp, soybean hulls, distillers dried or wet grains with solubles, minerals (calcium carbonates, electrolytes such as ammonium chloride, macro and trace minerals and in all forms, inorganic and organic), proteins such as soya bean meal, sunflower meal, meat and bone meal, fish meal, amino acids and others, energy ingredients such as grains, wheat, starch, barley, millet, sorghum, corn, animal and plants fat or oils, and vitamins without being limited thereto.

Particular examples of feed compositions of the invention are the following:

- An animal feed additive comprising (a) propanediol mononitrate and (b) limonene and (c) one or more of (c-1) fat-soluble vitamin(s), (c-2) water-soluble vitamin(s), (c-3) trace mineral(s) and (c-4) macro mineral(s);
- An animal feed composition comprising (a) propanediol mononitrate and (b) limonene and (c) one or more of (c-1) a crude protein content of 50 to 800 g/kg feed (50-80%), (c-2) fat from 5 – 100 g/kg feed (5-10%), (c-3) NDF from 150 – 700 g/kg feed (15-70%), (c-4) TDN from 300 – 800 (30 – 80%) and (c-5) starch from 150 – 700 g/kg feed (15 – 70%).

The so-called premixes are examples of animal feed additives of the invention. A premix designates a preferably uniform mixture of one or more micro-ingredients with diluents and/or carrier. Premixes are used to facilitate uniform dispersion of micro-ingredients in a larger mix.

Apart from the active ingredients of the invention (i.e. limonene and propanediol mononitrate), the premix of the invention preferably contains at least one fat-soluble vitamin, and/or at least one water soluble vitamin, and/or at least one trace mineral, and/or at least one macro mineral. In other words, the premix of the invention comprises the propanediol mononitrate

and limonene together with at least one additional component selected from the group consisting of fat-soluble vitamins, water-soluble vitamins, trace minerals, and macro minerals.

Macro minerals may be separately added to the feed. Therefore, in a particular embodiment, the premix comprises limonene and propanediol mononitrate with at least one additional component selected from the group consisting of fat-soluble vitamins, water-soluble vitamins, and trace-minerals.

The following are non-exclusive lists of examples of these components:

- Examples of fat-soluble vitamins are vitamin A, vitamin D3, vitamin E, and vitamin K, e.g. vitamin K3.
- Examples of water-soluble vitamins are vitamin B12, biotin and choline, vitamin B1, vitamin B2, vitamin B6, niacin, folic acid and panthothenate, e.g. Ca-D-panthothenate.
- Examples of trace minerals are manganese, zinc, iron, copper, iodine, selenium, manganese, and cobalt.
- Examples of macro minerals are calcium, phosphorus, potassium, magnesium and sodium.

As regards feed compositions for ruminants such as cows, as well as ingredients thereof, the ruminant diet is usually composed of an easily degradable fraction (named concentrate) and a fiber-rich less readily degradable fraction (named hay, forage, or roughage).

Hay is made of dried grass, legume or whole cereals. Grasses include among others temperate or tropical grasses, timothy, ryegrasses, fescues, brachiaria, panicum, tifton. Legumes include among others clover, lucerne or alfalfa, peas, beans and vetches. Whole cereals include among others barley, maize (corn), oat, wheat, sorghum. Other forage crops include sugarcane, sugarcane bagasse, citrus pulp, kales, rapes, and cabbages. Also root crops such as turnips, swedes, mangles, fodder beet, and sugar beet (including sugar beet pulp and beet molasses) are used to feed ruminants. Still further crops are tubers such as potatoes, cassava and sweet potato. Silage is an ensiled version of the fiber-rich fraction (e.g. from grasses, legumes or whole cereals) and grains (e.g. high moisture corns silage) whereby material with a high water content is treated with a controlled anaerobic fermentation process (naturally-fermented or additive treated) without being limited thereto.

Concentrate feed is largely made up of cereals grains (such as barley including brewers grain and distillers grain, maize, wheat, sorghum), but also often contain protein-rich feed ingredients such as soybean meal, rapeseed meal, palm kernel and sunflower meal without being limited thereto.

Ruminants (males, females and in all growth stages and adults) may also be fed total mixed rations (TMR), where all the dietary components, e.g. forage, silage, other feed ingredients and concentrate, are mixed before serving.

As mentioned above a premix is an example of a feed additive which may comprise limonene and propanediol mononitrate. It is understood that the compounds may be administered to the animal in different other forms. For example the compounds can also be included in a bolus that would be placed in the rumen and that would release a defined amount of the active compounds continuously in well-defined dosages over a specific period of time.

In a particular advantageous embodiment, the feed composition according to the present invention is a ruminant feed (often also referred to as ruminant diet) where all the dietary components, e.g. macro and micro ingredients, forage, silage and concentrate feed and additives including limonene and propanediol mononitrate are included. Such ruminant feed or diet is also often referred to as total mixed ration (TMR) or Partial mixed ration (PMR) or nutritional supplement for grazing animals.

Preferably, in all embodiments of the present invention, in said ruminant feed the amount of the propanediol mononitrate is selected in the range from 1 mg to about 25 g per kg dry matter feed, preferably from about 1 mg to about 10 g per kg dry matter feed, more preferably from about 10 mg to about 1 g per Kg dry matter feed, most preferably from 20 mg to 500 mg per Kg of dry matter feed, such as from about 20 mg to 250 mg per Kg of dry matter feed, or even mor preferably from 10 mg to 300 mg per dry matter kg feed such as in the range from 50 mg to 150 mg per kg dry matter feed or 60 mg to 100 mg per kg dry matter feed.

Preferably, in all embodiments of the present invention, in said ruminant feed the amount of limonene is selected in the range from 5 mg to about 5 g limonene per kg dry matter feed, preferably from about 5 mg to about 2.5 g of limonene per kg dry matter feed, more preferably 5 mg to about 2 g limonene per Kg dry matter feed, most preferably from 10 mg to 1.5 g

limonene per Kg of dry matter feed. Further particular suitable ranges are from 10 mg to 1.25 g limonene per Kg of dry matter feed or 10 mg to 1 g limonene per Kg of dry matter feed.

Daily dry matter intake for cattle is generally in the range of 1 to 3.5 % of dry matter per kg live weight. The amount of dry matter intake (DMI) for dairy cows is, for example, about 2-3 % dry matter per kg live weight, the amount of dry matter intake for beef cattle is generally between 1.0 - 3% of live weight depending on the feeding system as feedlot or grazing.

It is also well understood that in all uses and methods according to the present invention limonene and the propanediol mononitrate have to be supplemented timely together to excerpt the synergistic effect, can however been added separately to the diet of the animal, e.g. can be added separately into the respecting feeding rack.

Thus, the present invention relates to a method of supplementing limonene and propanediol mononitrate to a ruminant, said method encompassing the step of adding limonene and propanediol mononitrate with all the definitions and preferences as given herein concomitantly to a feed rack.

The amount of ruminant feed administered to a ruminant may vary dependent on the kind and age. Generally, the amount of dry matter fed to beef cattle or dairy cows is selected in the range of 1.5% to 3.5% of live weight, such as for an animal of 500 kg of live weigh it means to feed 7.5 to 17.5 kg dry matter/d.

In all embodiments of the present invention, it is to be understood by oral administration, a simple feeding, or manual administration of a bolus. It is also well understood, that propanediol mononitrate and the limonene can be pre-mixed before the administration or can be added separately to the animal feed compositions and feed additives.

Methane emission by ruminants can easily be measured in individual animals in metabolic chambers by methods known in the art (Grainger *et al.*, 2007 J. Dairy Science; 90: 2755-2766). Moreover, it can also be assessed at barn level by an emerging technology using laser beam (McGinn *et al.*, 2009, Journal of Environmental Quality; 38: 1796-1802) or Sulfur hexafluoride or just SF6 or GreenFeed system. Alternatively, methane produced by a dairy

ruminant can also be assessed by measurement of fatty acid profiles in milk according to WO 2009/156453.

The present invention also relates to the use of a feed composition or feed additive according to the present invention which feed composition or feed additive comprises one or more additional active substance which shows similar effects with regard to methane formation in the rumen and which is selected from the group consisting of diallyl disulfide, garlic oil, allyl isothiocyanate, deoxycholic acid, chenodeoxycholic acid and derivatives thereof.

Further components that could be given together with the limonene and the propanediol mononitrate are for example yeasts, oregano extracts, tannins and tannic acids and essential oils e.g., thymol, 3-methylphenol, vaniline, guajacol and eugenol.

It is at present contemplated that diallyl disulfide, garlic oil, allyl isothiocyanate deoxycholic acid, chenodeoxycholic acid and derivatives thereof are independently administered in dosage ranges of for example 0.01-500 mg active substance per kg feed (ppm). These compounds are either commercially available or can easily be prepared by a skilled person using processes and methods well-known in the prior art.

Ruminating mammals according to the present invention include cattle, goats, sheep, giraffes, American Bison, European bison, yaks, water buffalo, deer, camels, alpacas, llamas, wildebeest, antelope, pronghorn, and Nilgai.

For all embodiments of the present invention, domestic cattle, sheep and goat are the more preferred species. For the present purposes most preferred species are domestic cattle. The term includes all races of domestic cattle, and all production kinds of cattle, in particular dairy cows and beef cattle. It is well understood that the term dairy cows and beef cattle encompasses animals in all ages and physiological stage of life and production systems such as confined, semi-confined and grazing.

In further embodiments, the present invention relates to the use of limonene to synergistically enhance the methane reducing properties of propanediol mononitrate in ruminants. It is well understood that all the definitions and properties as defined herein also apply to said use.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples

In vitro test for methane production: A modified version of the "Hohenheim Forage value Test (HFT)" was used for testing the effect of specific compounds on the rumen functions mimicked by this in-vitro system.

Principle: Feed is gadded into a syringe with a composition of rumen liquor and an appropriate mixture of buffers. The solution is incubated at 39°C. After 8 hours the quantity (and composition) of gas phase produced is measured and put into a formula for conversion.

Reagents:

Mass element solution:

- 6.2 g potassium dihydrogen phosphate (KH_2PO_4)
- 0.6 g magnesium sulfate heptahydrate ($MgSO_4 * 7H_2O$)
- 9 ml concentrated phosphoric acid (1 mol/l)
- dissolved in distilled water to 1 l (pH about 1.6)

Buffer solution:

- 35.0 g sodium hydrogen carbonate ($NaHCO_3$)
- 4.0 g ammonium hydrogen carbonate ($(NH_4)HCO_3$)
- dissolved in distilled water to 1 l

Trace element solution:

- 13.2 g calcium chloride dihydrate ($CaCl_2 * 2H_2O$)
- 10.0 g manganese(II) chloride tetrahydrate ($MnCl_2 * 4H_2O$)
- 1.0 g cobalt(II) chloride hexahydrate ($CoCl_2 * 6H_2O$)
- 8.0 g iron(III) chloride ($FeCl_3 * 6H_2O$)
- dissolved in distilled water to 100 ml

Sodium salt solution:

- 100 mg sodium salt
- dissolved in distilled water to 100 ml

Reduction solution:

- first 3 ml sodium hydroxide (c = 1 mol/l), then 427.5 mg sodium sulfide hydrate ($Na_2S * H_2O$) are added to 71.25 ml H_2O
- solution must be prepared shortly before it is added to the medium solution

Procedure:

Sample weighing: The feed stuff (i.e. TMR (44 % concentrate, 6 % hay, 37 % maize silage and 13 % grass silage) is sieved to 1 mm and weighed exactly into 64 syringes. 4 of these syringes are the substrate controls, which display the gas production without the effect of the tested compounds. 4 other syringes are positive control (3-NOP, 10 µM). When needed, 4 syringes contain a carrier control (if the test compounds need a carrier). The remaining syringes contain the test substances, by groups of 4 syringes, in the amounts as indicated in table 1.

Preparation of the medium solution:

The components are mixed in a Woulff bottle in following order:

- 711 ml water
- 0.18 ml trace element solution
- 355.5 ml buffer solution
- 355.5 ml mass element solution

The completed solution is warmed up to 39 °C followed by the addition of 1.83 ml sodium salt solution and the addition of reduction solution at 36 °C. The rumen liquor is added, when the indicator turns colourless.

Extraction of the rumen liquor: 750 ml of rumen liquor are added to approximately 1,400 ml of medium solution under continued agitation and CO_2-gassing.

Filling the syringes, incubation and determining gas volumes and VFA values: The diluted rumen fluid (24 ml) is added to the glass syringe. The syringes are then incubated for 8 hours at 39 °C under gentle agitation. After 8 hours, the volume of gas produced is measured, and the percentage of methane in the gas phase is determined by gas chromatography.

Results

The food fermented was artificial TMR (44 % concentrate, 6 % hay, 37 % maize silage and 13 % grass silage). D-Limonene was obtained from Sigma-Aldrich (G8761) and used at a concentration as outlined in table 1. 3-Nitrooxy-propanol (PDMN) was used at the concentration as outlined in table 1. The respective in vitro data was linked to dosage regimes correlating to the same methane reduction in vivo.

The results are presented in the following Table 1. Clear synergistic effects were obtained for the methane reduction when propanediol mononitrate was combined with D-limonene, which also translates into additional performance benefit for the animal.

Table 1: Effect on Methane production resulting from the average of four to eight experiments with either D-limonene (DL), propanediol mononitrate (PDMN), or combination of both.

#	PDMN [µM]	DL [µM]	Total methane change [% vs. control]		Synergy# [%]
			expected*	found	
1 (Control)	—	—	—	—	—
2 (Positive control)	10	—	—	-98	—
3 (Ref)	5	—	—	-27	—
5 (Ref)	—	100	—	-1	—
9 (Inv)	5	100	-27	-44	+ 16

*expected = sum of individual contribution of limonene and PDMN

#synergy = found/expected *100%

Table 2: Correlation of in vitro data to respective dosage/ feeding regime

PDMN [µM]	DL [µM]	Dosage§ [g/animal/d]	Dosage° [g/kg feed]
10	—	2	0.1
5	—	1	0.05
—	100	23	1.14

§based on correlation of in vitro/ in vivo data

°based on the respective average feed intake per animal/day

Claims

1. Use of limonene and propanediol mononitrate (PDMN) for reducing the formation of methane emanating from the digestive activities of ruminants.

2. The use according to claim 1, wherein the limonene is D-limonene.

3. The use according to claim 1 and/ or 2, wherein propanediol mononitrate is administered to the ruminant in an amount selected in the range from .05 to 5 g PDMN/ animal/ day, preferably 0.1 to 4 g PDMN/ animal/ day, most preferably from 0.25 to 3 g PDMN/ animal/ day.

4. The use according to anyone or more of the preceding claims, wherein limonene is administered to the ruminant in an amount selected in the range from 0.05 to 100 g limonene/ animal/ day, preferably from 0.1 to 50 g limonene/ animal/ day, most preferably from 0.2 to 25 g limonene/ animal/ day.

5. The use according to anyone or more of the preceding claims, wherein the molar ratio of limonene to propanediol mononitrate is comprised between 100 and 1, preferably between 75 to 1, more preferably between 60 to 5, most preferably between 25 to 5.

6. The use according to anyone or more of the preceding claims, wherein the ruminant animal is selected from the group of domestic cattle, most preferably from beef cattle or dairy cows.

7. A method for reducing the production of methane emanating from the digestive activities of ruminants, said method comprising orally administering to the ruminant an effective amount of limonene and propanediol mononitrate, wherein the effective amount of propanediol mononitrate is selected in the range from 0.05 to 5 g PDMN/ animal/ day and the effective amount of limonene is selected in the range from 0.05 to 100 g limonene/ animal/ day.

8. The method according to claim 7, wherein the effective amount of propanediol mononitrate is selected in the range from 0.1 to 4 g PDMN/ animal/ day, most preferably from 0.25 to 3 g PDMN/ animal/ day.

9. The method according to claim 7 and/ or 8, wherein the effective amount of limonene is selected in the range from 0.1 to 50 g limonene/ animal/ day, most preferably from 0.2 to 25 g limonene/ animal/ day.

10. A feed composition or feed additive comprising limonene and propanediol mononitrate, wherein the feed composition is a ruminant feed comprising limonene in an amount selected in the range from 5 mg to about 5 g limonene per kg dry matter feed and propanediol mononitrate in an amount selected in the range from 20 mg to 250 mg propanediol mononitrate per kg dry matter feed.

11. The ruminant feed according to claim 10, wherein the molar ratio of limonene to propanediol mononitrate is comprised between 100 and 1, preferably between 75 to 1, more preferably between 60 to 5, most preferably between 25 to 5.

12. The ruminant feed according to claim 10 and/ or 11, wherein the limonene is D-limonene.

13. The feed composition according to claim 10, wherein the feed composition or feed additive is a mineral premix, a vitamin premix or a bolus.

14. Use of limonene, preferably D-limonene to synergistically enhance the methane reducing properties of propanediol mononitrate in ruminants.

15. Use according to claim 14, wherein the molar ratio of limonene to propanediol mononitrate is comprised between 100 and 1, preferably between 75 to 1, more preferably between 60 to 5, most preferably between 25 to 5.

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