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(54) Title: USE OF ENCAPSULATED NITRATES AND SULFATES TO REDUCE METHANE EMISSION DERIVED FROM RUMINAL FERMENTATION

(57) Abstract: Nutritional additives and supplements in a granular shape for ruminants containing nitrates and sulfates encapsulated with vegetable fats in order to allow a slow release in the rumen being used to reduce methane emission.

USE OF ENCAPSULATED NITRATES AND SULFATES TO REDUCE METHANE EMISSION DERIVED FROM RUMINAL FERMENTATION

Field of the invention

The present invention is related to the field of livestock production, specifically to the field of animal nutrition, more specifically to the use of nutritional supplements and additives for ruminants, exactly to the use of nitrates and sulfates encapsulated with hydrogenated fats, used to reduce ruminal methane emission, in order to allow a slow-release of the active compounds in the rumen, maximizing their complete metabolism and reducing the risks of animal intoxication.

Background of invention

Greenhouse gases (GHG), mainly carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), partially absorb the infra-red radiation emitted by Earth's surface, which hampers its dissipation to the space. This process, however, is essential for the maintenance of life in Earth because hinders excessive heat loss and keeps the planet warmed.

Notwithstanding, an increase in the GHG concentration magnifies this natural phenomenon, thereby resulting in the raise of global mean temperature, a process called global warming.

Taking into account that industrialization process and world's population show a tendency to increase in the next years, the agricultural sector has been pressured to become more efficient in terms of GHG emissions.

Due to its shorter half-life (10 years) when compared with carbon dioxide (150 years) and nitrous oxide (150 years), methane mitigation plays a key role in the achievement of positive short-term climate effects derived from GHG mitigation.

In Brazil, methane generated by enteric fermentation represents 12% of total CO_2-eq (carbon dioxide equivalent) emitted by human activities, approximately. From this amount, 90% is represented by rumen fermentation.

Considering only the agricultural sector, enteric fermentation corresponds to 53% of Brazilian agricultural CO_2-eq emissions. In global terms, methane produced by ruminants represents around 22% of total methane produced by human activities.

Methane is naturally produced during microbial fermentation in the rumen, being the rumen the first stomach of a ruminant – an anaerobic fermentation chamber where cohabit different kinds of microbes inside, such as bacteria, protozoa, fungi, bacteriophages etc. Methane generation is essential for the maintenance of microbial processes, although methane production is always referred as an energy loss for the animal, ranging from 5 to 12% of gross energy intake.

Methane is produced by methanogenic *Archaea*, a population that consumes CO_2 and H_2 as substrates for energy production and eliminates methane as an end-product. In the rumen, methane production is necessary to keep a low hydrogen pressure, which is necessary for the processes of microbial fermentation responsible for feed degradation, basically cellulose, hemicellulose, starch, sugars, protein, peptides, aminoacids etc.

Ruminal interspecies hydrogen transfer is defined as the process when *Archaea* consume hydrogen disposed by the metabolic activities of other rumen microorganisms. When hydrogen is not eliminated from the rumen as methane, it occurs an increase in the hydrogen pressure that results in overall inhibition of microbial fermentation.

For instance, dairy cows produce about 500 L/day of CH_4, which corresponds to 357 g/day, approximately. Brazilian researches determined that dairy cows kept on pasture produce around 378 to 403 g/day of methane.

Basically, there is two ways of methane mitigation:

a) to stimulate metabolic pathways that are able to compete with methanogenesis, being examples the utilization of acetogenic microorganisms, organic acids (malate, fumarate etc), and hydrogen acceptors

(hydrogen peroxide, nitrates, sulfates etc);

b) to reduce ruminal hydrogen production, being examples the use of ionophores (*e.g.* monensin sodium), essential oils, and plant secondary compounds.

Besides the mentioned techniques, other potential strategies to reduce ruminal methane production are defaunation (elimination or reduction of protozoa), inoculation of live yeasts, control of *Archaea* population by immunization or vaccination, and nutritional strategies such as supplemental fats and an increase of concentrate feeds (*e.g.* grains) in the diet.

So far, all techniques to mitigate methane present limitations. Some of them show only transitory effects that disappear over time (*e.g.* essential oils, tannins, monensin, vaccines etc), while others show variable results (e.g. essential oils, tannins, saponins, vaccines etc). Moreover, some substances may be toxic to animals (e.g. some chemicals used to eliminate protozoa, chloroform, and high doses of unprotected and readily available nitrates), may not be viable due to elevated costs (e.g. organic acids), or having their use prohibited (e.g. ionophores such as monenin sodium, salinomycin, and lasalocid sodium in Europe). Finally, some techniques are too incipient, being examples the vaccination, immunization, and inclusion of acetogenic microorganisms.

Nitrate salts (NO_3^-) have a higher affinity to H_2 when compared with CO_2, allowing nitrate-reducing microorganisms to compete with methanogenic *Archaea* for substrate. The reduction of nitrate to nitrite (Equation 1) and its further reduction to ammonia (Equation 2) generate more energy than the reduction of CO_2 to methane (Equation 3). This greater energy production provides a competitive advantage towards nitrate-utilizing microbes in comparison with methanogenic *Archaea*.

$NO_3^- + 2\ H^+ \rightarrow H_2O + NO_2^-$ (Eq. 1; ΔG_0 = -130 kJ/mol of hydrogen)

$NO_2^- + 4\ H_2 \rightarrow NH_4^+ + 2\ H_2O$ (Eq. 2; ΔG_0 = -124 kJ/mol of hydrogen)

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$ (Eq. 3; $\Delta G_0 = -16.9$ kJ/mol of hydrogen)

According to Equations 1 and 2, each mol of nitrate reduced to ammonium avoids the production of 1 mol of methane. In addition, similarly to urea, ammonia originated from nitrate metabolism serves as a source of N for microbial protein synthesis. Consequently, there is a potential of using nitrate as a non-protein nitrogen (NPN) and, at the same time, anti-methanogenic agent. As a result, urea or true protein sources (soybean meal, cottonseed meal, etc) normally used as a NPN source in diet formulation for ruminants can be replaced by nitrate, combining the nutritional and anti-methanogenic potential to the diet.

Researches have showed that methane produced by rumen fermentation was reduced by 46.6% when using unprotected (uncoated) source of nitrate.

Nitrates when fed without prior adaptation – sudden inclusion – are toxic to animals including ruminants, causing a disease denominated methemoglobinemia. This disease is well-recognized in the field, being observed, as example, when animals ingest drinking water with high nitrate concentrations or when fed forages, mainly from temperate climates, that accumulated high levels of nitrate.

Once ingested, nitrate is metabolized by ruminal microorganisms to its intermediate compound, the nitrite (Equation 1). By a second reaction, nitrite is reduced sequentially to ammonium (Equation 2). The first reducing-reaction which leads to nitrite formation occurs in a rate much faster than the reaction that consumes nitrite. As a consequence, there is a ruminal nitrite accumulation, with nitrite being the toxic compound for the animal. Nitrite is readily absorbed by the wall of digestive tract and passes to blood circulation, converting the ferrous form of hemoglobin (Fe^{2+}) to the ferric form (Fe^{3+}). The ferric form is unable to transport oxygen to the tissues, resulting in death caused by anoxia – privation of O_2. In general, symptoms

are a rapid pulse rate and an increased respiration rate, followed by muscular tremors and general weakness. Membranes of eyes, mouth, and nose become a darker color due to oxygen deficit, with blood showing a brownish or "chocolate" pigmentation. Death occurs in extreme situations. In a chronic situation, the disease results in loss of performance (lower milk production, body weight gain, and wool production).

It is well established that gradual adaptation of ruminants to nitrate allows multiplication and increase in the activity of nitrate-reducing microorganisms, mainly *Selenomonas ruminantium* subsp. *lactilytica*, *Veillonella parvula*, *Wolinella succinogenes,* and *Megasphaera elsdenii*, thereby reducing the risks of nitrite accumulation. However, the adaptation of animals to nitrate brings some practical and operational problems to the ruminant production system. Dietary changes stress the animals, lowering the productive potential of animals during this period. Moreover, adaptation phases are potentially dangerous due to mistakes and errors caused by handlers during ration preparation and offering of feed to the animals.

Similarly to nitrate, the reduction of sulfate (SO_4^{2-}) to sulphydric acid (H_2S) are also an alternative route to sink hydrogen and to minimize the ruminal production of methane (Equation 4). In the rumen, similarly to the methanogenic *Archaea*, sulfate-reducing bacteria utilize hydrogen for their growth. As a result, stimulating the growth of sulfate-utilizing microorganisms is a strategy to reduce methane, thus enhancing an alternative pathway of hydrogen consumption.

The energy production derived from sulfate reduction (ΔG_0 = -152 kJ) is higher than the energy resulted from methane production (ΔG_0 = -131 kJ), allowing this alternative metabolic pathway to compete with methanogenesis.

$$SO_4^{2-} + 4\ H_2 + 2\ H^+ \rightarrow H_2S + 4\ H_2O \qquad \text{(Equation 4)}$$

The use of a sulfur source is especially important to minimize the risks of

intoxication by nitrate. Sulfur is reduced to H_2S, which acts as a hydrogen donator for the reduction of nitrite to ammonium. As a consequence, less accumulation of nitrites means a lower risk of intoxication. It is widely known by the scientific community that sulfur compounds are able to reduce the risks of nitrate intoxication.

It is realized, therefore, a gap in the art related to animal nutrition, of products that reduce methane emission without being harmful to animals, *e.g.* risks of intoxication, or being convenient to apply and use, not demanding high investments or, in addition, complex processes.

Based on this, and thinking on an uninterrupted development of products, it is proposed an innovation, at present claiming the privileges of its protection by its novelty and inventive activity, as exposed as follow. It is proposed, therefore, an encapsulated nutritional additive, in a granular form, thereby allowing the slow-release of nitrate and sulfates, and variations on its composition.

Such granules, or their variations, are manufactured with nitrates and sulfates, which are responsible by the mitigation of methane, and additives, or also similar compositions, coated/encapsulated with vegetable fats that are responsible for the reduction of releasing rate and solubilization of this salts in the rumen environment, with the purpose of avoiding animal intoxication and promoting the complete metabolism of nitrate and sulfates in the rumen.

In a similar way, alternatively to coating with vegetable fats, it is possible to use any other material compatible with the animal nutrition that shows equal or similar properties from those presented in fats in terms of promoting a controlled release of the substance. It is distinguished here natural materials, degradable in the rumen or not, such as cellulose and carboxycellulose-based emulsions (added, as example, with calcium carbonate, saccharose, vegetable oils, and xanthan gum), coatings containing

starch and other polysaccharides mixed with polyvinyl alcohols, as well as coatings based on lignin/lignosulfonates or chitosan biopolymers. Alternatively, coating may also be composed of synthetic polymers, degradable in the rumen or not, such as carboxyvinyl; polyacrylic acid (acrylic resins, polyethylenes etc); alginates; polyhydroxyalkanoates; polyhydroxyoctanoates; polyhydroxybutyrates (Biopols); polycaprolactones; polylactic acids; solutions of biuret with urethane and tungue oil; mixtures of isocyanates with alkydic resins, castor oil and peroxides; mixtures of stearamides with paraffin, magnesium stearate; other resins (polyurethanes, polyolefins, polyesthers, polyepoxides, silicones, polyvinylidene chloride etc, as well as mixtures thereof); alkyl and cycloalkyl amines; paraffins and waxes derived from petroleum.

Among the fats used for encapsulation, it is mentioned here soybean oil, castor oil, palm oil, cashew nut shell oil or cashew nut shell liquid, cotton-seed oil, linseed oil, peanut oil, babassu oil, sunflower oil, coconut oil, canola oil, wheat oil, rice oil, corn oil, cocoa oil, safflower oil, and waxes (from vegetable or animal sources), being examples carnauba wax, corn wax, castor wax, and bee wax. Here, it is not excluded the isolated use of just one fat source, as well as the use of a combination of two or more than two fat sources, aiming at bringing advantages such as the supply of functional fatty acids, in terms of melting point, plasticity, waxy properties, as well as shock and abrasion resistance.

Analysis of related art

The protection WO010921 contemplates the reduction of gastro-intestinal methanogenesis in ruminants, with the utilization of agents able to compete with methanogenesis by hydrogen atoms during the normal fermentation of ingested feeds. The products are offered comprehending high amounts of a combination of one compound based on nitrate and one compound based on sulfate and, alternatively, probiotic microorganisms for the reduction of

nitrite, as well as methods to reduce gastro-intestinal methanogenesis in ruminants by using such compositions. Such method does not consider the protection, coating, and encapsulation of nitrates and sulfates for a slow ruminal release, moving away from the proposed object characteristics.

The invention US 6231895 describes the offering of nutritional supplements for ruminants with a level of non-protein nitrogen (NPN) which results in a controlled and safe release of ammonia under conditions of ruminal incubation. In another form, this invention provides a nutritional supplement for ruminants with controlled release of non-protein nitrogen which comprehends urea particles encapsulated with a coating made with a rumen-degradable polymer. This invention moves away from the object proposed here because does not deal with supplements based on nitrates and sulfates.

The document WO03068256 deals with methods and compositions for an improvement of ruminal fermentation efficiency, enhancing the efficiency of dietary starch utilization, avoiding a deleterious increase in ruminal concentration of lactic acid/or a drop on ruminal pH, as well as promoting the benefit growth of ruminal microorganisms. Methods and compositions of the present invention can also include supplementation with yeasts, buffer agents, ionophores, or other agents to stimulate growth and productivity; however it does not cite any coating based on fats, thus moving away from the characteristics of the object proposed here.

The patent PI0608919 demonstrates a structural element suitable to use in the manufacturing of a releasing device for the administration of a intra-ruminal active agent composed of a compact material in a ruminant animal, which comprehends a mixture of iron, graphite and, optionally, powdered copper, with graphite being present in the mixture in an amount from 2% to 7% in weight, the copper in an amount from 0% to 5% in weight, and iron in an amount between 88% to 98% in weight, in relation to the total weight

of iron, copper and graphite. A variety of structural elements can be combined in order to achieve a structural unity of a releasing device. The patent describes a device for a slow ruminal release of a composition, and does not cite in its composition the use of nitrate or either the process of encapsulation, thus moving away from the characteristics of the innovation proposed here.

The protection PI0305047 consider a ration for ruminant animals composed mainly of starchy material from babassu nuts, which receives in its composition a mixture of urea, sulfur, babassu starch, babassu meal, in a proportion of 30% to 60%, 1.5% to 3.0%, 20% to 30%, and 20% to 30%, respectively. The process of compound preparation is comprehended by the stage of babassu nut selection, shelling of nuts, cleaning of starchy material, starch material grinding, product formulation, and thermal treatment. In this compound, NPN is protected by babassu starch, coated in a gelatinous form, which hampers solubilization in water. It also provides a slow ammonia release in the rumen, increasing, therefore, the utilization of NPN by rumen microorganisms during microbial protein synthesis. The compound is indeed a product that respects the N:S ratio of 10:1 and, besides providing protein to the ruminant, also provides energy which comes from starch. Using this product, intoxication risks are low and, in small quantities, it is possible to feed calves in creep-feeding system. The document is related to a composition based on starch and non-nitrate substances, moving away from the characteristics of the invention proposed here.

The document PI9201217 presents a slow-release capsule, adapted to be introduced in the rumen of an animal by its esophagus, kept inside the rumen for a long period for continuous liberation of the biological active composition held in the capsule. The capsule in a long and tubular-shape body, a tube and a terminal lid attached to its extremity to keep the biological active composition inside, and the other extremity being the dispenser.

The extremity of the dispenser shows an open in order to release the composition in the rumen. This invention deals with a capsule for a slow and gradual release of a biological active composition, not citing any nitrates, thus not colliding with the requirements proposed in the invention presented here.

The patent CA2725380 describes a method which includes a dispenser for ruminant feeding, plus one or more nutritional supplements, in which dispenser is attached a gas analyzer that stays close to the place where the animal introduces its head. The method determines if a specific ruminant accessed the feedbunk (dispenser), by reading the identification of a RFID ear-tag, and also release a nutritional supplement in order to reduce methane. The method includes a gas analyzer to determine the levels of carbon dioxide and methane, also including a data processor that modifies the type and amount of feed offered in the next feeding, in order to control de production of methane and achieve the animal performance desired. This protection is related to a feeding equipment, moving away from the characteristics of the invention proposed here.

The document WO2010071222 reports an inhibitor of ruminal methane emission in ruminants. Precisely, it is an inhibitor of methane emission by ruminant characterized by hydrogen peroxide as the active compound. The innovation is about mitigation of methane production with peroxides, moving away from the characteristics of the invention proposed here.

The patent WO2006040537 is about the inhibition of methane production in ruminants and/or improvement of meat and/or milk production and quality. In particular, this invention makes reference to the use of encapsulated organic acids, especially fumaric acid. It is also contemplated a composition comprehending ruminant feeding, by using encapsulated fatty acids, especially fumaric acid, for utilization in the reduction of methane production by ruminants. Such uses and compositions may also, alternatively, re-

sult in a weight gain increase and/or milk production. This protection describes encapsulated organic acids without mention of nitrates, moving away from the characteristics of the invention proposed here.

The patent JP2003088301 demonstrates a composition that inhibits the generation of methane without making the ruminal environment worse, by offering at least one selected strain of *Lactobacillus*, obtained from sheep milk derived products naturally fermented, yeasts and oligossacharides to a ruminant by oral administration. The inhibitory effect on methane may be improved with nitrate addition, and lactobacillus and yeasts comprises at least one type of microorganism, belonging to *Trichosporon, Candida, Leuconostoc, Lactococcus* and, in particular, oligossacharides, preferentially, galactoligossacharides. Such invention deals with milk-derived products to inhibit methane production, without mention of encapsulated nitrates, moving away from the characteristics of the invention proposed here.

The protection GB1445560 demonstrates a composed feed, supplemental block, liquid feed supplement, slow-release pellets, ensiled forage, hay or grain containing isobutyraldehyde with a mixture of adipic, glutaric and succinic acid, acetic acid, formol, sulfuric acid or trioxane in order to inhibit the production of methane in the rumen. The use this pelletted diet may contain barley, wheat, peanut, molasse, salt, limestone, bicalcium phosphate. The patent describes only an animal diet, moving away from the characteristics of the innovation proposed here.

Detailed description of the invention

Taking into account the gaps presented in the art, it is proposed, as an innovation, an encapsulated nutritional additive, in a granular form, composed of nitrates and sulfates, as well as its compositions.

Such granules, or their variations, are manufactured with nitrates and sulfates, which are responsible by mitigation of methane production, combined with additives or even similar compositions, recovered/encapsulated

with hydrogenated vegetable fats, being them responsible by the slow and gradual release/solubilization of nitrates and sulfates in the ruminal environment, with the purpose of avoiding animal intoxication and promoting the complete metabolism of nitrate and sulfates in the ruminal environment.

In a similar way, alternatively to coating with vegetable fats, it is possible to use any other material compatible with the animal nutrition that shows equal or similar properties from those presented in fats in terms of resulting in a controlled release of the substance. It is distinguished here natural materials, degradable in the rumen or not, such as cellulose and carboxycellulose-based emulsions (added, as example, calcium carbonate, saccharose, vegetable oils, and xanthan gum), coatings containing starch and other polysaccharides mixed with polyvinyl alcohols, as well as coatings based on lignin/lignosulphonates or chitosan biopolymers.

Alternatively, coating may also be composed of synthetic polymers, degradable in the rumen or not, such as carboxyvinyl; polyacrylic acid (acrylic resins, polyethylenes, etc); alginates; polyhydroxyalkanoates; polyhydroxyoctanoates; polyhydroxybutyrates (Biopols); polycaprolactones; polylactic acids; solutions of biuret with urethane and tungue oil; mixtures of isocyanates with alkydic resins, castor oil and peroxides; mixtures of stearamides with paraffin, magnesium stearate; other resins (polyurethanes, polyolefins, polyesthers, polyepoxides, silicones, polyvinylidene chloride etc, as well as mixtures thereof); alkyl and cycloalkyl amines; paraffins and waxes derived from petroleum. Besides the antimethanogenic property promoted by nitrates and sulfates, the encapsulation drastically reduces the risks of nitrate intoxication, protecting animal welfare and health, thus minimizing risks of loss by intoxication. The scenario of intoxication when using non-encapsulated nitrates is very likely in the practice.

Additionally, it is highlighted that the encapsulation process is able to re-

lease the active compounds nitrate and sulfate in a time interval matching the rumen fluid retention time (approximately 6 to 24 h), thus allowing the complete solubilization of these salts in the rumen.

In practice, there are several situations in which encapsulation brings advantages: management errors caused by animal handlers or people involved in animal feeding are very frequent. High amounts of nitrate may be ingested by animals due to lack of attention. The poor preparation of rations, mistakes during ingredient weighting and an inadequate mixture of them are common situations in the field, which may result in high levels of nitrate ingestion by the animals. As a consequence, encapsulation of nitrates and sulfates protects the animals when high amounts of nitrate are ingested by non-adapted animals. In summary, encapsulation ensures animal safety in case of a nitrate overdose.

An additional advantage of coated nitrates and sulfates is the "feedbunk safety" or "feedbunk protection", an usual term used in the livestock sector. If it rains, and offering uncoated nitrate in uncovered feedbunks, there would be a rapid solubilization of nitrate, since this salt is highly soluble in water. This water containing high nitrate concentrations increases the risk of intoxication, because once ingested may result in animal poisoning and death. Therefore, the coating process drastically delays the solubilization of nitrates and sulfates, resulting in animal safety in the situation described above.

The coating process also eliminates the necessity of gradual and progressive adaptation of animals to nitrate, which in practical conditions lasts around four weeks in order to achieve the doses required for adequate methane mitigation. The adaptation phase to nitrate also results in management problems, increasing the time expended during ration preparation and animal feeding, also making the process more complex which, in turn, increases the chance of operational errors. As a consequence, the encapsula-

tion brings a clear advantage, simplifying the animal feeding and allowing the direct offering of nitrates and sulfates in the recommended doses without risks to the animals.

The slow and gradual rumen release of nitrates and sulfates promoted by coating also ensures their complete metabolization in the ruminal environment. This avoids the absorption of nitrate and its intermediate compound – nitrite – by the rumen wall, therefore reducing their concentration in blood circulation.

Consequently, encapsulation allows complete reduction of nitrate to ammonia, which enhances the efficacy of methane mitigation. It is highlighted that nitrate and/or nitrite, if absorbed by rumen wall, will not drain hydrogen, thus reducing the efficiency of methane mitigation.

Moreover, encapsulation reduces or eliminates the circulation of nitrate and/or nitrites in the blood, avoiding their excretion in urine or milk. In high amounts, nitrate is a surface water and groundwater polluter. Although naturally found in milk, high concentrations of nitrate may be potentially dangerous, especially if ingested by neonates and children, also causing the disease called methemoglobinemia.

Another additional advantage promoted by nitrate and sulfate coating is the slow release of NPN in the rumen. The gradual liberation of nitrogen allows the synchronization of carbohydrate degradation and microbial protein synthesis, permitting an adequate and complete amination of NPN. Concomitantly, the use of nitrates as a nitrogen source replacing more traditional sources (*e.g.* urea) shows as an advantage the maximization of microbial protein synthesis, since energy for microbial growth derived from nitrate reduction is greater than from methanogenesis. The maximization of microbial protein synthesis is crucial for animal performance improvement, because microbial protein is the most important and the best protein source for ruminant nutrition. In addition to nitrogen, the composition containing

coated nitrates and sulfates also provides sulfur, calcium, and magnesium to the animal.

The product is composed of nitrates, preferentially between 40% and 97%, more preferentially between 60% and 85%; oils and fats for coating, preferentially between 1% and 40%, more preferentially between 3% and 20%; sulfates, preferentially up to 50%, more preferentially between 5% and 40%; and other additives, preferentially up to 20%, more preferentially between 0.1 and 10%.

Preferentially, it is used calcium nitrate and magnesium sulfate. Alternatively, it is admitted the replacement of these salts by similar salts or by a combination of different nitrate and sulfate salts.

Nitrates used, as well as sulfates, must be sufficiently soluble in the rumen fluid, being accepted by animals and, consequently, physiologically suitable. Salts cannot carry heavy metals or other minerals in potentially toxic amounts, also attending the requirements of regulatory agencies for products used in animal feeding. Generally speaking, nitrates and sulfates are provided as inorganic salts.

The calcium nitrate is, preferentially, the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2.NH_4NO_3.10H_2O$], however it is not excluded the utilization of other salts, such as calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$], calcium nitrate anhydrous [$Ca(NO_3)_2$], magnesium nitrate [$Mg(NO_3)_2.6H_2O$], sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), cal-urea nitrate [$Ca(NO_3)_2.4CO(NH_2)_2$], the double salt of ammonium sulfate and nitrate [$(NH_4)_2SO_4.3(NH_4NO_3)$ or $(NH_4)_2SO_4.2(NH_4NO_3)$], as well as possible variations in the salts cited above due to number or absence of crystallization water. It has already been demonstrated that uncoated/unprotected calcium nitrate, potassium nitrate, sodium nitrate, and ammonium nitrate reduced methane emission in ruminants.

Similarly, it is not excluded here the utilization of mixtures of nitrates, aiming the addition of new properties or even to improve the mitigating effects of final product.

The magnesium sulfate is, preferentially, the monohydrate or anhydrous ($MgSO_4.1H_2O$ ou $MgSO_4$), however it is not excluded the use of magnesium sulfate heptahydrate [$MgSO_4.7H_2O$], sodium sulfate [Na_2SO_4 anhydrous, $Na_2SO_4.7H_2O$ and $Na_2SO_4.10H_2O$), ammonium sulfate [$(NH_4)_2SO_4$], potassium sulfate (K_2SO_4), calcium sulfate ($CaSO_4$ or $2CaSO_4.1H_20$), zinc sulfate ($ZnSO_4$ anhydrous or $ZnSO_4.7H_2O$), ferrous sulfate ($FeSO_4.1H_2O$, $FeSO_4.4H_2O$, $FeSO_4.5H_2O$ or $FeSO_4.7H_2O$), manganese sulfate ($MnSO_4$ anhydrous or $MnSO_4.4H_2O$), copper sulfate ($CuSO_4$ anhydrous or $CuSO_4.5H_2O$), as well as not mentioned variations in the salts cited above due to number or absence of crystallization water. It has already been demonstrated the effects of sodium sulfate and copper sulfate, as well as magnesium sulfate, in the reduction of ruminal accumulation of nitrite and in the minimization of intoxication risks.

Similarly, it is not excluded here the utilization of mixtures of sulfates or their potential replacers, aiming the inclusion of other properties or even to improve the mitigating effects of final product.

Similarly, in substitution of sulfate it is also not excluded here the use of elemental sulfur, as well as sulfides (as examples $Na_2S.9H_2O$, CaS, ZnS, K_2S) and sulphites (as examples Na_2SO_3, K_2SO_3, $CaSO_3$, $MgSO_3$).

It has already been demonstrated the properties of sulfides and sulphites in the reduction of ruminal accumulation of nitrite and in the minimization of intoxication risks, both *in vitro* and *in vivo*. Finally, here it is also considered the use of persulfates (SO_2^{-5}), thiosulfates ($S_2O_2^{-3}$) e hyposulphites (SO_2^{-2}). L-cysteine (anhydrous, monohydrate and chloridrates) can also be included, being one of the sulfur containing aminoacids that has well-known properties in the reduction of ruminal nitrite accumulation and, con-

sequently, in the minimization of nitrate and/or nitrite intoxication in ruminants. Here, it is not excluded the use of metals containing properties that inhibit nitrate reductase, as being demonstrated for sodium tungstate (Na_2WO_4).

In relation to additives that may preferentially be included in the formulation are cited those able to aggregate properties to the final product, such as aromatizers and flavours, natural or synthetics, but not harmful to animals (as examples monosodium glutamate, saccharine, sucrose, dextrose, glucose, guava essences, vanilla etc), antioxidants (such as vitamin C, beta-carotene, BHT – butylated hydroxytoluene, BHA – butylated hydroxyanisole), acidifiers (citric acid, acetic acid, tartaric acid, fumaric acid, malic acid), emulsifiers/stabilizing agents (such as lecithin, xathans, gums, polisorbates, propylene glycol, monostearates, mono-di-glycerides etc) and taste enhancers.

It is essentially important to consider the inclusion of anti-wetting and anti-caking agents which, by finality, are able to maintain the fluidity of granules during storage, such as calcium carbonate, starch, microcrystalline cellulose, tricalcium phosphate, silica/silicates, talcum powder, kaolin, calcium stearate etc.

Concurrently, other nutritional additives can also be included aiming at bringing novel properties to the final composition, such as macrominerals, trace minerals, vitamins (for instance A, B_1, B_2, B_3, B_5 B_6, B_7, B_9, B_{12}, C, D, E e K), essential oils (carvacrol, eugenol, thymol, cynamaldehyde, capsaicin, limonene etc), organic acids (lactate, malate, fumarate, aspartate etc), fatty acids (such as CLA – conjugated linoleic acid; myristic acid; anacardic acid; medium-chain fatty acids – capric acid, caprilic acid, caproic acid, lauric acid; as well as omega-6 and omega-3 fatty acids such as alpha-linolenic acid – ALA; eicosapentaenoic acid – EPA; docosahexaenoic acid – DHA; etc), aminoacids (mainly sulfur-containing

aminoacids as cysteine and methionine, but also considering histidine, threonine, leucine, isoleucine, tryptophan, phenylalanine, valine, glycine etc), enzymes (cellulases, hemicellulases, amylases, pectinases, xylases, β-glucanases, phytases, other glucanases etc), buffers and alkalizers (sodium bicarbonate, sodium sesquicarbonate, calcium carbonate, magnesium oxide etc), yeasts (*Trichosporon* sp., *Candida* sp., *Leuconostoc* sp., *Lactococcus* sp., *Candida kefyr, Saccharomyces cerevisiae* etc), fungi (such as *Aspergillus oryzeae* and *Aspergillus niger)*, probiotics and other live microorganisms (*Lactobacillus* sp. and mainly those that possess nitrate/nitrite reduction activity, such as *Selenomonas ruminantium*, *Veillonella parvula, Wollinela succinogenes, Megasphaera elsdenii*, *Propionibacterium acidipropionici*, *Escherichia coli* W3110; and intestinal bacteria, coryneform bacteria, *Bacillus subtilis*, *Methylophilus* sp., and *Actinomyces* sp).

It can also be included galactoligosaccharides and/or nisin, substances known by their properties in the reduction of nitrite accumulation and risks of nitrate poisoning. Finally, other additives potentially usable are antibiotics normally utilized in ruminant nutrition (ionophores – sodium monensin, salinomycin, lasalocid, narasin – other antibiotics such as virginiamycin, avilamycin, bacitracin, flavomycin, tylosin), natural substances with antimicrobial properties (propolis, beta-acids, alfa-acids, other hop-derived acids, cardanol, cardol, tannins, saponins), anthelmintic, and anticcocidials/coccidiostats.

The granules are coated preferentially with vegetable fats, which are responsible for the slow and gradual release/solubilization of nitrates and sulfates in the ruminal environment, in the sense of avoiding animal intoxication and maximizing their complete metabolism in the rumen.

The coating is, by itself, hydrophobic and allows the slow and gradual solubilization of nitrates/sulfates salts. The coating of granules permits the

synchronization of nitrates/sulfates release and reduction reactions, in the way of avoiding rumen accumulation of nitrate/nitrite, thus reducing the risks of animal poisoning. The gradual nitrate release permits the reduction of nitrite to ammonium occurring in a similar rate of reduction of nitrate to nitrite, thus avoiding the ruminal accumulation of nitrite. As an additional advantage, encapsulation with fats is biodegradable. Lipids are digested in the small intestine, also serving as a supplemental fat, therefore, providing additional energy.

When coated, granules of final product have 1.5 mm to 12 mm of diameter. The liberation rate of nitrates/sulfates varies between 1% to 30% per hour, more preferentially between 5% to 25% per hour. Considering the density of the final product, it varies between 0.85 g/cm^3 to 1.15 g/cm^3, more preferentially between 0.90 g/cm^3 to 1.10 g/cm^3.

The product is destined to all ruminant animals, either domestic or wild species. For instance, here are included cattle, sheep, goat, buffalos, cervids, camelids, giraffids, antelopes, bisons, and yaks. However, by convenience and importance, the technology here described is destined mainly to domestic species such as cattle, sheep, goat, and bubalines.

It is necessary a functional rumen in these animals, being excluded the utilization in pre-ruminant animals, being examples new-born calves and lambs. Additionally, the product is destined to feedlot animals as well as animals on pasture receiving supplementation.

The period of feeding is indetermined, being offered continuously since the moment that the animal possess a functional rumen until the moment of slaughtering. The product has a long-term effect on methane mitigation, without loss of efficiency due to prolonged utilization.

The product is offered in feed (by spontaneous animal ingestion), being a total mixed ration (TMR; mixture of all ingredients required by the animal, such as roughages/forages, concentrates/cereal grains, mineral supple-

ments, vitamin supplements, and additives), protein supplement, energy supplement, protein/energy supplement, or mineral supplement. Such supplements are generally fed to ruminants kept on pasture, being either a high-intake or low-intake supplement, preferentially a high-intake supplement. High and low intake supplements are terms generally used by professionals to designate mixtures of feeds ingested in high (2 g to 4 g per kg of body weight) and low (up to 1 g per kg of body weight) amounts, respectively.

Mixed in ration or supplement, granules of nitrates and sulfates composition can also be fed on top, which means that granules can be dispersed on the top of ration placed in feedbunk. It is also considered the isolated offering of the product, as long as the animal shows spontaneous preference.

The product can be mixed in the ration or supplement at the moment of animal feeding. Similarly, the product can be mixed in rations and supplements produced by feed companies and feed mills, being in that way stored for long periods of time. Due to its good abrasion resistance, in the moment of mixing, such process can be performed both manually and/or using mixing wagons.

The coating promotes protection against the high hygroscopicity naturally showed by nitrate salts. Exposed to air and heat, non-encapsulated nitrate absorbs air humidity and liquefies rapidly. Consequently, the encapsulation allows the pre-mixture of the product with rations or supplements, allowing a prolonged storage without quality loss of the final product.

In addition, the encapsulated product containing nitrates and sulfates permits a more homogenous mixing. Nitrate is generally found in a granular form, while sulfate is a powder salt. This granulometric and density variation results in problems related to the adequate homogenization and particle segregation during transport and storage. The encapsulated product containing nitrates and sulfates presented as a single granule has the advantage

of minimizing these problems.

Example 1

In order to prove the effects of this innovation, it was conducted an *in vitro* trial to measure the release of encapsulated and non-encapsulated nitrates, aiming at demonstrating the efficacy of two encapsulation methods with fats when comparing with non-encapsulated nitrate. The material used was calcium ammonium nitrate decahydrate.

In this trial, three treatments were used as follow:

i. Control: non-encapsulated calcium nitrate;
ii. Prototype 1: encapsulated calcium nitrate;
iii. Prototype 2: encapsulated calcium nitrate.

Three replicates were used per treatment. In each 1-L flask, 500 mL of distilled water were added with 2.482 g of calcium ammonium nitrate decahydrate. Prototypes were included in an amount corresponding to 2.482 g of pure calcium ammonium nitrate.

The incubation was performed in a circulation-forced incubator at 39 °C and 100 rpm. Samples were collected following treatment additions at 0 min, 5 min, 10 min, 15 min and 30 min; 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, and 48 h. In each sampling time, 5 mL were collected.

The water-solubilized nitrate was analyzed according to the colorimetric method with phenol disulphonic acid following by alcalinization with sodium hydroxide.

The trial results are showed in Figure 1 (Annex 1), being demonstrated that encapsulated nitrate sources presented a slower solubilization when compared with the non-encapsulated source. This supports that encapsulation with fats is effective and provides a slow and gradual nitrate release in aqueous medium. Therefore, coating of nitrate granules brings as an advantage the reduction of animal intoxication risks.

Example 2

The objective of this experiment was to evaluate the effects of two types of encapsulated (slow-release) nitrate on animal growth, methane production, rumen and blood constituents, digestibility, N balance, microbial N production, and carcass and meat characteristics.

This experiment was carried out at the Laboratory of Animal Nutrition, Centre for Nuclear Energy in Agriculture, University of São Paulo, Piracicaba, SP, Brazil. All animal use procedures followed guidelines recommended by the Internal Commission for Environmental Ethics and Experimentation with Animals of the same institution.

Material and Methods

The experiment consisted of 85-d period, with 21 days for dietary adaptation (from April 27th 2011 to May 17th 2011) and 64 days (from May 18th 2011 to July 20th 2011) for growth evaluation. After growth evaluation period, a digestibility trial was performed during 5 days, which occurred concomitantly with the last methane measurement.

Experimental design and treatments. Eighteen Santa Inês ram lambs (27.06 kg of initial BW) were assigned in randomized complete block design with 6 blocks and 3 treatments. Blocks defined by body weight (BW) and age at the beginning of the experiment. Animals were allocated in three dietary treatments as follow: **Control** – 1.5% urea in the dietary dry matter (DM) as a source of non-protein N (NPN); **NO_{3enc}** – 4.51% of encapsulated nitrate in the dietary DM as a replacement of urea; **NO_3+$CNSL_{enc}$** – 4.51% of encapsulated nitrate + cashew nut shell liquid in the dietary DM as a replacement of urea.

Housing and feeding. Lambs were housed in individual indoor pens with concrete floor, feed bunks, and water cups. At the onset of the experiment, animals were dewormed, vaccinated, and received a supplemental injection of vitamins A, D, and E.

Animals were fed *ad libitum* a 60:40 concentrate:forage diet (total mixed ration) formulated to meet NRC (2007) recommendations. The composition and chemical analyses of experimental diets are shown in Table 1. Animals were fed twice daily (morning and afternoon feeding) and had free access to fresh water.

Table 1: Ingredients and chemical composition of experimental diets (%, DM basis).

Ingredients	*Control*	*NO_{3enc}*	*NO_3+$CNSL_{enc}$*
Chopped coastcross hay	40.00	40.00	40.00
Ground corn	50.90	46.80	46.80
Soybean meal	5.00	5.00	5.00
Mineral premix	1.50	1.50	1.50
Urea	1.50	-	-
Limestone	1.10	-	-
Encapsulated nitrate	-	4.51	-
Encapsulated nitrate + CNSL	-	-	4.51
Magnesium sulfate	-	2.19	2.19

Where:

– Calcium ammonium nitrate decahydrate (5Ca(NO3)2.NH4NO3.10H2O) – 83.33% DM; 116.63% CP in DM basis; 75.77% NO3- (ion) in DM basis.

– Magnesium sulfate heptahydrate (MgSO4.7H2O) – 48.78% DM; 20% Mg in DM basis; 26.67% S in DM basis; 80% SO42- in DM basis.

– Encapsulated products: 86.17% of DM; 93.63% CP in DM basis; 17.84% Ca in DM basis; 61.15% NO3- (ion) in DM basis. Encapsulated product with CNSL contained 2.96% CNSL in DM basis.

– Urea – 281.25% CP in DM basis.

– CNSL – cashew nut shell liquid.

Amounts of feed offered to animals were calculated according to previous dry matter intake (DMI), and adjustments were made when needed so that refused feed did not exceed 10% of daily intake. Orts were recorded every day to determine daily DMI and not offered again to animals. Animals were weighed after a 16-h fast every two weeks.

Data collection and analysis.

Methane production was evaluated using six open-circuit respiration chambers (Abdalla et al., 2011). The eighteen animals (6 blocks) were divided in three groups of six animals each (2 blocks) and each group was placed in chambers for two consecutive days. Methane measurements were repeated three times (initial, middle, and end of experimental period) in order to evaluate persistency of effects on methane emission.

Digestibility was performed during 5 days at the end of growth period concurrently with the last methane measurement. Animals were placed in metabolism crates designed to allow the separation and collection of feces and urine. Crates were equipped with feeders and water cups and were kept in a shaded open-sided barn.

At the end of digestibility period, all animals were slaughtered. Carcass characteristics evaluated were hot carcass weight (HCW) and hot carcass yield (obtained at the time of slaughter), chilled carcass weight, chilled carcass yield, shrink after chilling, subcutaneous fat thickness over the 12^{th} rib, and rib-eye area (obtained after chilling for 24 h at 2°C). After weighing and immediately before data collection, chilled carcasses were separated into 2 symmetrical sections and ribbed between the 12^{th} and 13^{th} ribs to expose the Longissimus muscle (LM). The 12^{th}-rib fat thickness was measured using an outside caliper graduated in millimeters. The exposed rib-eye area was traced on acetate paper, and the area was determined by using a planimeter graduated in square centimeters. The presence of nitrate and ni-

trite in the lamb meat (*Longissimus dorsi*) was determined by the "Centro de Tecnologia de Carne" at "Instituto de Tecnologia de Alimentos" (ITAL), Campinas, São Paulo, Brazil (Brasil, 2005a,b).

Methane concentration was determined using a gas chromatograph (GC Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. no. 19809; Resteck, Bellefonte, PA, USA). Temperatures of column, injector, and flame ionization detector were 60, 200, and 240°C, respectively. Helium at 10 ml/min was the carrier gas. Methane concentration was determined by external calibration using an analytical curve prepared with pure CH_4 (White Martins PRAXAIR Gases Industriais Inc., Osasco, SP, Brazil; 995 mL/L purity).

Ruminal fluid was collected every two weeks at 3-h after morning feeding. Collection was performed using oral probes and aliquots stored at -20°C without preservatives. Short-chain fatty acids (SCFA) were determined according to manufacter's conditions (Hewlett Packard, 1998) with some modifications by using a gas chromatograph (GC HP 7890A, Automatic Injetor HP 7683B, Agilent Technologies, Palo Alto, CA, USA) equipped with a capillary column HP-FFAP (19091F-112; 0.320 mm OD, 0.50 μm ID, 25 m length , J&W Agilent Technologies Inc., Palo Alto, CA, EUA). A 1 μL aliquot were injected using a 20:1 split ratio with 31.35 mL/min of H_2 flux (9.20 psi). Injector and FID temperatures were kept at 260°C. Oven heating slope was: 80°C (1 min), 120°C (20°C/min; 3 min), 205°C (10°C/min; 2 min), with 16.5 min of total analytical time. Hydrogen at 1.35 mL/min was used as carrier gas. Detector hydrogen, synthetic air, and nitrogen fluxes (make up) were kept at 40, 400, and 40 mL/min, respectively.

Blood samples were collected every two weeks at 6-h after morning feeding into 4-mL BD vacutainer tubes (K_2-EDTA, BD, Franklin Lakes, NJ, USA). Blood was analyzed for methaemoglobin (MetHb) within 30 min

after blood collection according to Sato et al. (2005).

Results and discussion

Table 2 shows DMI, growth, and methane production data. Final BW, DMI, average daily gain (ADG), and feed efficiency were not affected by encapsulated types of nitrate. No differences of growth performance were also observed by Li et al. (*in press*), (van Zijderveld et al., 2010), and Huyen et al., (2010).

Table 2: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on performance and methane production of feedlot Santa Inês growing lambs.

Item	*Control*	NO_{3enc}	$NO_3 + CNSL_{enc}$	*SEM*	*P – value*		
					Treat.	*Time*	*Treat. x Time*
Initial BW, kg	26.75	27.25	27.17	-	-	-	-
Final BW, kg	37.80	36.83	37.03	0.882	0.80	< 0.01	0.17
Dry matter intake							
g/d	1112	1029	1037	44.7	0.39	< 0.01	0.05
g/kg BW	34.91	32.76	33.21	0.978	0.30	< 0.01	0.04
g/kg $BW^{0.75}$	82.75	77.39	78.24	2.52	0.31	0.02	0.04
ADG, g	173	156	153	18.3	0.71	0.15	0.06
Feed efficiency	0.159	0.150	0.147	0.0144	0.84	0.97	0.21
Methane production							
L/d	27.50a	18.27b	20.54ab	2.363	0.05	0.24	0.05
L/kg BW	0.92	0.61	0.70	0.082	0.06	< 0.01	0.09
L/kg $BW^{0.75}$	2.04a	1.37b	1.53ab	0.176	0.05	< 0.01	0.10
L/kg DMI	28.57a	19.14b	19.53b	2.178	0.02	0.03	0.72

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

– Treat. = treatment

– BW = body weight

– BW0.75 = metabolic weight

– DMI = dry matter intake

Methane production (expressed as L/d, L/kg $BW^{0.75}$, and L/kg DMI) was reduced when urea was replaced by encapsulated nitrate or encapsulated nitrate + CNSL. The addition of CNSL did not show any benefit related to methane production when added to encapsulated nitrate. In average, methane emission for NO_{3enc} and NO_3+$CNSL_{enc}$ was reduced by 32.3% (expressed as L/kg DMI) when compared with Control. Similar results were obtained by other, with reduction of 45% (van Zijderveld et al., 2010), 23% (Nolan et al., 2010), 35% (Li et al., in press), 27% (Hulshof et al., *in press*). Table 3 shows ruminal constituents data. Total SCFA and acetate concentrations increased for nitrate-fed treatments when compared with Control. NO_3+$CNSL_{enc}$ showed higher concentrations of total SCFA and acetate when compared with NO_{3enc}. NO_3+$CNSL_{enc}$ showed greater propionate and butyrate concentrations than Control, with NO_{3enc} showing intermediary results.

These results are in agreement with the greater energy available for microbial growth provided by nitrate reduction in the rumen, which could support a greater microbial activity. To our knowledge, this hypothesis has never been proved *in vivo*, but already demonstrated in *in vitro* conditions (Guo et al., 2009). Different results were obtained by others, which probably is in reason of a divergence in rumen collection time after feeding. Li et al. (*in press*) and (van Zijderveld et al., 2010) observed no variation in SCFA concentrations, but rumen fluid collection was performed before

feeding and approximately 24-h after last feeding, respectively. In general, *in vitro* studies have showed some consistency of effects, with an acetate increase and butyrate decrease when nitrate is used as NPN source (Guo et al., 2009; Zhou et al., *in press*).

Table 3: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on ruminal constituents of feedlot Santa Inês growing lambs.

Item	*Control*	*NO_{3enc}*	*NO_3 + $CNSL_{enc}$*	*SEM*	*P – value*		
					Treat.	*Time*	*Treat. x Time*
Total SCFA, mM	90.46a	99.47b	110.07c	2.091	< 0.01	0.18	0.62
Acetate, mM	48.88a	55.21b	64.04c	1.317	< 0.01	< 0.01	0.43
Propionate, mM	14.77a	15.97ab	17.87b	0.811	0.04	0.64	0.97
Butyrate, mM	9.32a	11.25ab	13.01b	0.600	< 0.01	0.89	0.22
Isobutyrate, mM	6.68	7.46	6.26	0.438	0.16	0.02	0.74
Valerate, mM	6.04	5.74	5.56	0.593	0.85	0.52	0.77
Isovalerate, mM	4.76	3.85	3.33	0.539	0.19	< 0.01	0.84
C_2:C_3	3.57	3.61	3.67	0.204	0.94	0.05	0.89
pH	6.76	6.78	6.74	0.063	0.92	< 0.01	0.36
NH_3, mg/100 mL	34.93a	26.37b	22.29c	0.281	< 0.01	0.10	0.39
Protozoa, x 10^5/mL	22.55a	19.90b	19.87b	0.408	< 0.01	< 0.01	0.29
Nitrate, µM	17506	12638	12490	3379	0.50	0.60	0.88
Nitrite, µM	4.02a	5.00b	4.76b	0.133	< 0.01	0.42	0.99

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

– Treat. = treatment

– Rumen samples were collected 3 h after morning feeding

Nitrate-fed animals had lower ammonia concentrations than Control. This result is explained because urea is rapidly hydrolyzed in the rumen, producing ammonia. However, in the rumen nitrate is reduced to nitrite and consecutively reduced to ammonia. Since rumen fluid was collected 3h after feeding, it is reasonable to observe lower ammonia concentration at this time in the rumen of nitrate-fed lambs. In accordance, nitrite concentration was greater for $Nitrate_{enc}$ and $Nitrate+CNSL_{enc}$ in comparison with Control. However, nitrate concentration did not differ among treatments, which is explained by the very fast reduction of nitrate to nitrite when the first reaches the ruminal environment. Despite this, it is important to mention that nitrite concentration in nitrate-fed animals were not very high in comparison with Control. This shows that encapsulated nitrate is effective in the slow release of nitrate in the rumen and, at the same time, an adapted rumen is able to metabolize nitrate effectively. Protozoa count was also reduced by nitrate inclusion, which is in agreement with lower ruminal ammonia concentration, as well as methane production.

Table 4 shows blood constituents data. Red blood cell concentration increased for NO_{3enc} and NO_3+CNSL_{enc}. This was probably an animal metabolism adaptation due to oxygen transport deficiency promoted by nitrate feeding. However, methaemoglobin was not affected by both types of encapsulated nitrates. This demonstrated that encapsulation was effective in delaying nitrate release in the rumen, and that an adapted rumen promotes a total reduction of nitrate to ammonia. This idea is supported by similar ADG and feed efficiency observed for NO_{3enc} and NO_3+CNSL_{enc} when compared with Control.

Table 4: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on blood constituents of feedlot Santa Inês growing lambs

Item	*Control*	*NO_{3enc}*	*NO_3 + $CNSL_{enc}$*	*SEM*	*P – value*		
					Treat.	*Time*	*Treat. x Time*
Packed cell vol., %	34.15	35.35	33.21	1.454	0.59	< 0.01	0.13
Red blood cells, x $10^6/\mu L$	10.36a	12.75b	12.01b	0.246	< 0.01	< 0.01	0.71
Hemoglobin, g/100 mL	11.81	12.29	11.65	0.416	0.54	< 0.01	0.05
Methaemoglobin, %	0.62	1.08	0.92	0.131	0.08	0.23	0.30
Nitrate, µM	30498	36426	36219	3689	0.46	< 0.01	0.73
Nitrite, µM	2.04a	2.40b	2.19ab	0.093	0.05	0.03	0.06
Total protein, g/100 mL	7.00	7.31	7.27	0.153	0.35	0.12	0.30
Albumin, g/100 mL	3.19	3.20	3.12	0.069	0.72	0.03	0.26
Urea, mg/100 mL	36.74	33.09	30.58	2.087	0.16	< 0.01	0.06

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

– Treat. = treatment

– Packed cell volume = hematocrit

– Blood samples were collected 6 h after morning feeding

Blood nitrate was not influenced by diets, but nitrite concentration increased when nitrate was fed. This occurred because nitrite is the predominantly form of N-oxide absorbed. It is important to notice that even with greater nitrite blood concentration, there was no increase in blood methaemoglobin. Total protein, albumin, and urea in plasma were not affected by treatments.

Tables 5 and 6 show digestibility and N-balance data. Any digestibility or

N-balance variable was influenced by urea replacement with nitrate. These results show that nitrate is able to promote similar growth rates than urea, which was in accordance with ADG and feed efficiency measured in the present experiment.

Table 5: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on dietary digestibility of feedlot Santa Inês growing lambs

Item	*Control*	*NO_{3enc}*	*$NO_3 + CNSL_{enc}$*	*SEM*	*P – value*
DM Intake, g	1068	999	1119	67.1	0.48
Digestibility, %					
DM	62.26	61.49	62.65	1.638	0.88
OM	64.06	63.33	63.99	1.535	0.93
CP	64.43	66.84	68.04	2.118	0.50
NDF	58.44	57.72	58.26	1.549	0.94
ADF	29.53	32.69	29.93	1.487	0.31
EE	74.23	66.55	67.22	2.771	0.15

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

– DM = Dry matter

– OM = Organic matter

– CP = Crude protein

– NDF = neutral detergent fiber

– ADF = acid detergent fiber

– EE = ether extract

Nitrate in urine was not affected by NO_{3enc} and NO_3+$CNSL_{enc}$, but there was an increase in nitrite concentration of nitrate-fed treatments. This result is in accordance with the greater blood nitrite observed when nitrate was fed. On the other hand, urinary urea was reduced when feeding nitrate as

NPN source. Consequently, N excretion in the form of urea was reduced, coupled by an increase of excretion in the form of nitrite. Despite this, efficiency of N-use did not differ among treatments.

Table 6: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on N balance of feedlot Santa Inês growing lambs

Item	*Control*	*NO_{3enc}*	*NO_3 + $CNSL_{enc}$*	*SEM*	*P – value*
N intake, g/day	24.67	21.96	24.41	1.428	0.37
Fecal N, g/day	8.45	7.31	7.60	0.458	0.24
Urinary N, g/day	9.82	7.66	7.51	1.751	0.60
N retention					
g/day	6.41	6.98	9.31	1.785	0.50
g/kg of N intake	260.22	310.19	386.58	70.673	0.47
g/kg of N absorbed	405.03	467.71	558.72	103.900	0.59
Urine					
Nitrate, µM	32367	58417	37650	6970	0.06
Nitrite, µM	1.57a	2.32b	2.14b	0.078	< 0.01
Urea, g/d	5.87a	3.31b	3.80b	0.498	0.01

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

Table 7 show microbial production data. Microbial N supply and efficiency of microbial production did not differ among treatments.

Table 7: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on purine derivatives and estimation of microbial N synthesis of feedlot Santa Inês growing lambs

Item	*Control*	*NO_{3enc}*	*NO_3 + $CNSL_{enc}$*	*SEM*	*P – value*
Purine derivatives, mmol/d					
Allantoin	10.80	13.18	12.79	1.643	0.57
Uric acid	3.35	3.09	2.84	0.242	0.37
Hypoxanthine + xanthine	1.06	1.02	0.85	0.070	0.13
Total	15.21	17.29	16.48	1.851	0.73
Creatinine, mmol/d	7.19	6.92	6.93	0.530	0.92
Daily absorbed microbial purine, mmol/kg $BW^{0.75}$	5.42	5.71	5.56	0.256	0.72
MN supply, g/d	3.94	4.15	4.04	0.186	0.73
DOMI, kg/d	0.655	0.599	0.673	0.0448	0.50
DOMR, kg/d	0.426	0.390	0.438	0.0291	0.50
Efficiency of MN production, g/kg DOMR	9.45	10.76	9.41	0.574	0.21
MN fermented OM, g/d	10.22	9.34	10.50	0.699	0.50

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– MN = microbial nitrogen

– DOMI = Digestible organic matter intake

– DOMR = Digestible organic matter fermented in the rumen

– SEM = standard error of the mean

Finally, all carcass characteristics, carcass components as well as meat characteristics were not affected by nitrate feeding (Tables 8 and 9). Particularly, sodium nitrate in lamb meat was below the detection limit for all treatments, whilst no residue of sodium nitrite was observed for any treatment. Thus, no accumulation of nitrate or nitrite occur in meat when encap-

sulated nitrate was fed to lambs.

Table 8: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on carcass and meat characteristics of feedlot Santa Inês growing lambs

Item	*Control*	*NO_{3enc}*	*NO_3 + $CNSL_{enc}$*	*SEM*	*P – value*
Carcass characteristics					
Slaughter weight, kg	37.09	37.83	37.77	1.425	0.93
Hot carcass weight, kg	17.69	17.61	18.13	0.492	0.81
Chilled carcass weight, kg	17.61	17.51	18.02	0.639	0.83
Hot carcass yield, %	47.70	46.39	48.08	0.567	0.15
Chilled carcass yield, %	47.49	46.16	47.80	0.573	0.17
Shrink after chilling, %	0.44	0.58	0.65	0.087	0.27
Rib eye area, cm^2	13.81	14.85	14.61	1.052	0.79
Carcass fatness, mm	2.70	2.37	2.17	0.205	0.23
Carcass components					
Half carcass weight, kg	8.97	8.93	9.12	0.348	0.92
Shoulder, kg	1.81	1.79	1.85	0.065	0.82
Leg, kg	2.79	2.77	2.84	0.121	0.91
Rib, kg	0.54	0.50	0.54	0.022	0.42
Carcass length, cm	76.02	73.80	74.00	0.937	0.26

Meat characteristics					
Lightness (L*)	39.01	38.78	38.24	0.906	0.83
Redness (a*)	16.62	16.79	16.15	0.349	0.41
Yellowness (b*)	4.42	4.88	4.71	0.545	0.85
pH at slaughter	7.26	7.26	7.16	0.215	0.51
pH after chilling	6.82	6.69	6.58	0.088	0.20
Sodium nitrate, mg/kg of fresh meat	< 6.155	< 6.155	< 6.155	-	-
Sodium nitrite, mg/kg of fresh meat	0	0	0	-	-

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– Rib eye area = LM area (Longissimus muscle area) = Eye muscle area

– Chilled (hot) carcass yield = cold (hot) carcass dressing

– Nitrate and nitrite in meat expressed and sodium nitrate and sodium nirite. Detection limit of analytical method for sodium nitrate is < 6.155 mg/kg of fresh meat.

– SEM = standard error of the mean

Table 9: Effect of encapsulated nitrate and encapsulated nitrate + cashew nut shell liquid on 12th rib composition of feedlot Santa Inês growing lambs

Item	*Control*	NO_{3enc}	$NO_3 + CNSL_{enc}$	*SEM*	*P – value*
Rib weight, g	104.74	99.88	102.24	9.50	0.94
Muscle weight, g	49.29	47.17	48.86	3.980	0.92
Fat weight, g	27.02	28.16	26.77	4.649	0.97
Bone weight, g	27.57	23.92	25.93	2.966	0.70

Muscle, %	46.96	48.02	48.89	2.685	0.89
Fat, %	25.50	28.15	25.07	2.649	0.67
Bones, %	26.79	23.14	25.42	1.594	0.32
Rib fatness, mm	1.28	1.67	1.68	0.329	0.65

Where:

– NO3enc = encapsulated nitrate

– NO3+CNSLenc = encapsulated nitrate + cashew nut shell liquid

– SEM = standard error of the mean

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Example 3

The objective of this experiment was to evaluate the effects of non-encapsulated and encapsulated (slow-release) types of nitrate and sulfate on acute intoxication (methemoglobinemia) of Nellore beef steers.

This experiment was carried out at the Experimental Feedlot Facility of the Department of Animal Production, College of Veterinary and Animal Science, Federal University of Goiás, Goiânia, Goiás, Brazil. All animal use procedures followed guidelines recommended by the Internal Ethics Commission of the same institution.

Material and Methods

Experimental design and treatments. Five castrated Nellore steers (5 years old and 450 kg of BW in average) fitted with rumen cannula were assigned in a 5 x 5 Latin square design. The experimental period lasted 70 days, divided in 5 periods of 14-d each.

Animals were allocated in five dietary treatments as follow: **Control** – without addition of nitrate or sulfate; **NE123 (non-encapsulated)** – inoculation of 123 g/d of nitrate (NO_3^-) + 16.5 g/d of sulfate (SO_4^{2-}), corresponding to 195 g/d of calcium ammonium nitrate decahydrate and 24 g/d of magnesium sulfate monohydrate. It was equivalent to 1.82% calcium ammonium nitrate and 0.23% magnesium sulfate in the dietary dry matter (DM); **NE246 (non-encapsulated)** – inoculation of 246 g/d of nitrate (NO_3^-) + 33 g/d of sulfate (SO_4^{2-}), corresponding to 390 g/d of calcium ammonium nitrate decahydrate and 48 g/d of magnesium sulfate monohydrate. It was equivalent to 3.64% calcium ammonium nitrate and 0.47% magnesium sulfate in the dietary dry matter (DM); **E123 (encapsulated)** – inoculation of 123 g/d of nitrate (NO_3^-) + 16.5 g/d of sulfate (SO_4^{2-}) as a single encapsulated product, corresponding to 266 g/d of final product. It was equivalent to 2.58% final encapsulated product in the dietary DM; **E246 (encapsulated)** – inoculation of 246 g/d of nitrate (NO_3^-) + 33 g/d of

sulfate (SO_4^{2-}) as a single encapsulated product, corresponding to 532 g/d of final product. It was equivalent to 5.16% final encapsulated product in the dietary DM.

Housing and feeding. Steers were kept in individual outdoors pens with covered feed bunks and automatic water cups. At the onset of the experiment, animals were dewormed, vaccinated, and also received a supplemental injection of vitamins A, D, and E.

Animals were fed *ad libitum* a 50:50 concentrate:forage diet (total mixed ration) formulated according to the approximate chemical composition of feedstuffs (Valadares Filho et al., 2010) in order to meet NRC (1996) recommendations. The composition and calculated chemical analyses of experimental diets are shown in Table 1. Animals were fed once daily at morning and had free access to fresh water.

Data collection and analysis. In each period, during 12 days animals were fed *ad libitum* the Control diet at 0800 am. At day 13, animals were inoculated through rumen cannula with non-encapsulated nitrate/sulfate or encapsulated nitrate/sulfate according to treatments.

Inoculation was performed at 0, 3, 6, 9, and 12 h after morning feeding as described in Tables 2 and 3. Inoculated doses according to hour after feeding were defined after estimating average total feed intake and feed intake pattern (intake rate per time interval) of animals prior to the experimental onset. Average feed intake was 16 kg/d (as-fed) and estimated feed intake rate was 31.3% from hour 0 to 3; 21% from hour 3 to 6; 21% from hour 6 to 9; 13.3% from hour 9 to 12; and 13.3% from hour 12 to 24.

Blood samples used for methemoglobin determination were collected from jugular vein at 0h, 3h, 6h, 9h, 12h, 18h, 24h, and 30 hours after morning feeding at d 13. Methemoglobin analysis was performed using a spectrophotometer according to Hegesh et al. (1970).

Blood samples for hemogram, biochemical analyses (liver enzymes, glu-

cose, urea, and bilirubin), and hemogasometry (acid-base balance) were collected from jugular vein at 0, 6, 12, 18, 24, and 30 h after morning feeding at d 13. Hemogram was performed by the microhematocrit method using vacutainer tubes with EDTA for blood collection. Blood samples for biochemical analysis were obtained using vacutainer tubes without additives.

Physical examination (heart rate, respiratory rate, and body temperature), as well as rumen pH were performed at 0, 3, 6, 9, 12, 18, 24, and 30 hours after morning feeding at d 13. Physical examination was performed according to Radostits et al. (2007). Animals were carefully monitored by two experienced veterinarians throughout inoculation period. Animals at risk, if presenting visual signs of intoxication, a well-defined brownish mucosa, and based on the immediately previous blood analysis were treated with an intravenous injection of 100 mL methylene blue at 4%.

Table 1: Ingredients and calculated chemical composition of experimental diets.

Item	*Treatments*				
	Control	*NE123*	*NE246*	*E123*	*E246*
Ingredients					
Sorghum silage	25.00	25.00	25.00	25.00	25.00
Sugarcane bagasse	25.00	25.00	25.00	25.00	25.00
Ground silage	30.18	32.98	35.77	32.33	34.47
Soybean meal	18.57	13.72	8.87	13.84	9.12
Mineral premix	1.25	1.25	1.25	1.25	1.25
Calcium nitrate	-	1.82	3.64	-	-
Magnesium sulfate	-	0.23	0.47	-	-
Encapsulated nitrate + sulfate	-	-	-	2.58	5.16

Calculated chemical composition					
DM, %	55.92	55.88	55.83	55.90	55.88
CP, %	14.00	14.00	14.00	14.00	14.00
NDF, %	43.27	42.93	42.59	42.85	42.43
TDN, %	66.28	64.77	63.27	64.30	62.32

Where:

–Nitrate source: calcium ammonium nitrate decahydrate (5Ca(NO3)2.NH4NO3.10H2O); 83.33% DM, 116.63% CP, 75.77% NO3- in DM basis.

– Sulfate source: magnesium sulfate monohydrate (MgSO4.1H2O); 86.96% DM, 80% SO42- in DM basis.

– Encapsulated product containing calcium ammonium nitrate decahydrate and magnesium sulfate monohydrate; 86.70% DM, 81.56% CP, 52.97% NO3- in DM basis, 7.296% SO42- in DM basis.

– NDF: neutral detergent fiber

– TDN: total digestible nutrients

Table 2: Inoculation protocol of nitrate and sulfate salts through rumen cannula according to hour after feeding (g of salts in as-fed basis).

Inoculation time, h	*Treatments*						
	Control	*NE123*		*NE246*		*E123*	*E246*
		Nitrate	*Sulfate*	*Nitrate*	*Sulfate*	*Nitrate + Sulfate*	*Nitrate + Sulfate*
0	-	61	8	122	16	84	168
3	-	41	5	82	10	56	112
6	-	41	5	82	10	56	112
9	-	26	3	52	6	35	70

12	-	26	3	52	6	35	70
Total	-	195	24	390	48	266	532

Where:

–Nitrate: calcium ammonium nitrate decahydrate (5Ca(NO3)2.NH4NO3.10H2O); 83.33% DM, 116.63% CP, 75.77% NO3- in DM basis.

– Sulfate: magnesium sulfate monohydrate (MgSO4.1H2O); 86.96% DM, 80% SO42 in DM basis.

– Encapsulated product containing calcium ammonium nitrate decahydrate and magnesium sulfate monohydrate; 86.70% DM, 81.56% CP, 52.97% NO3- in DM basis, 7.296% SO42- in DM basis.

Table 3: Inoculation protocol of nitrate (NO_3^-) and sulfate (SO_4^{2-}) ions through rumen cannula according to hour after feeding (in g of DM).

Inoculation time, h	*Treatments*						
	Control	*NE123*		*NE246*		*E123*	*E246*
		NO_3^-	SO_4^{2-}	NO_3^-	SO_4^{2-}	NO_3^- + SO_4^{2-}	NO_3^- + SO_4^{2-}
0	-	38.52	5.56	77.04	11.12	38.58 + 5.31	77.16 + 10.62
3	-	25.89	3.48	51.78	6.96	25.72 + 3.54	51.44 + 7.08
6	-	25.89	3.48	51.78	6.96	25.72 + 3.54	51.44 + 7.08
9	-	16.42	2.09	32.84	4.18	16.07 + 2.21	32.14 + 4.42
12	-	16.42	2.09	32.84	4.18	16.07 + 2.21	32.14 + 4.42

Total	-	123.14	16.70	246.28	33.40	122.16 + 16.81	244.32 + 33.62

Where:

– Nitrate: calcium ammonium nitrate decahydrate (5Ca(NO3)2.NH4NO3.10H2O); 83.33% DM, 116.63% CP, 75.77% NO3- in DM basis.

– Sulfate: magnesium sulfate monohydrate (MgSO4.1H2O); 86.96% DM, 80% SO42- in DM basis.

– Encapsulated product containing calcium ammonium nitrate decahydrate and magnesium sulfate monohydrate; 86.70% DM, 81.56% CP, 52.97% NO3- in DM basis, 7.296% SO42- in DM basis.

Results and Discussion

Hemogram and methemoglobin data are presented in Table 4. In a short-term (up to 30 h after inoculation onset), 246 g of non-encapsulated or encapsulated nitrate increased blood methemeglobin concentration. However, encapsulation was efficient in the reduction of methemoglobinemia risks, because methemoglobin concentration stayed in tolerable levels (up to 30%) whereas non-encapsulated nitrate peaked at up to 50%.

Table 4: Hemogram and methemoglobin concentration of Nellore steers inoculated with pure or encapsulated nitrate/sulfate trough rumen cannula

Item	*Treatments*					*SEM*	*P-value*		
	Control	*NE123*	*NE246*	*E123*	*E246*		*Treat.*	*Time*	*Treat*Time*
Hemoglobin, g/dL	10.28 a	10.34a	10.84b	10.25a	10.49 a	0.115	<0.01	<0.0001	0.84
Methaemoglobin, %	0.59a	1.74 a	23.16c	1.99 a	13.55 b	1.257	< 0.0001	< 0.0001	< 0.0001

Red blood cells, x 10^{12}/L	7.00a	7.12 a	7.47 b	7.06 a	7.12a	0.0 84	<0.0 1	<0.01	0.65
Packed cell volume, %	34.10 a	34.0 3a	35.8 5b	33.8 7a	34.49 ab	0.3 93	<0.0 1	<0.00 01	0.45
Mean corpuscular volume, x 10^{-15} L	48.85	48.3 4	48.4 1	48.2 3	48.99	0.2 08	0.03	0.03	0.95
Mean corpuscular hemoglobin, x 10^{-12} g/cell	14.69	14.6 5	14.5 3	14.5 0	14.72	0.1 01	0.43	<0.00 1	0.86
Mean corpuscular hemoglobin concentration, g/dL	30.27	30.5 0	30.4 0	30.2 5	30.43	0.2 39	0.93	0.02	0.86
Platelets, x 10^{9}/L	202a b	211a b	193b	216a	198a b	5.6	0.03	0.09	0.91

Where: Packed cell volume = Hematocrit

For both NE246 and E246, peak of methemoglobin occurred 18 h after inoculation onset or 6 h after last dose of nitrate inoculation. The concentration of methemoglobin over time was very similar between NE246 and E246, but higher levels were observed for NE246. This emphasizes that nitrate encapsulation was effective in the reduction of nitrate release in the rumen, thus reducing the acute risks of intoxication. (Figure 2 Annex II).

In two of five sub-periods, animals inoculated with 246 g/d of pure nitrate had to be treated with antidote (100 mL per 450 kg BW of methylene blue at 4%) in reason of clear visual signs of intoxication, one animal at 9 h and the other at 18 h after inoculation onset. It is very important to take in con-

sideration that data of these two treated animals were excluded from statistical analysis (hour 24 and 30 for the first animal and hour 12, 18, 24, and 30 for the second animal). For this reason, methemoglobin concentration would be even greater for NE246 if animals had not been treated with antidote. However, this decision could be very dangerous to the animals, being not allowed by the Internal Ethics Committee. In contrast, during the five sub-periods none of the animals receiving 246 g of encapsulated nitrate had to be treated.

Methemoglobin occurs in ruminants due to high nitrite absorption through the rumen wall in a short period of time. Nitrite accumulates in the rumen because unadapted ruminal microbes are not able to totally reduce nitrate to ammonia. In the blood, nitrite converts the ferrous (Fe^{2+}) iron of hemoglobin into ferric iron (Fe^{3+}). When this occurs, hemoglobin (now named methemoglobin) is unable to transported oxygen to tissues (Cockburn et al., 2010). This is responsible to the general anoxia symptoms of nitrite intoxication, which in severe cases may be lethal.

Animals receiving both encapsulated and non-encapsulated nitrate/sulfate at 123/16 g did not present any increase in methemoglobin concentration when compared with Control. These results demonstrate that, up to this level of nitrate inclusion, ruminal nitrate reduction to ammonia and/or blood methemoglobin-reductase (convertion of blood methemoglobin back to hemoglobin) are able to avoid intoxication problems. However, it is important to mention that in this experiment nitrate inoculation simulated only a one-day nitrate ingestion, being not possible to speculate about accumulative effects caused by a subsequent nitrate inoculation in the following day.

Hemoglobin concentration was greatest for NE246. It has been reported that animals with elevated MetaHg concentration had increased Hb concentration, which is a physiological response to compensate for the decreased blood capacity to transport oxygen (Winter and Hokanson, 1964). A greater

number of red blood cells for NE246 is also in agreement with this observation.

Glucose, liver enzymes, and bilirubin levels are presented in Table 5. Glucose concentration was greatest for NE246, as well as AST. The AST is an enzyme that indicates acute inflammation in liver, heart, and kidneys, thus also indicating the intoxication symptoms caused by inoculation of pure nitrate/sulfate.

GGT, creatinine, alkaline phosphatase, creatinine kinase, and bilirubin were not affected by treatments.

Table 5: Blood glucose, liver enzymes, and bilirubin concentration of Nellore steers inoculated with pure or encapsulated nitrate/sulfate trough rumen cannula

Item	*Treatments*					*SEM*	*P-value*		
	Control	*NE123*	*NE246*	*E123*	*E246*		*Treat.*	*Time*	*Treat*Time*
Glucose, mg/dL	62.68 a	65.90 a	84.43 b	65.92 a	69.07 a	2.759	< 0.001	0.03	0.02
GGT, IU/L	21.51	20.19	20.28	21.80	20.93	0.666	0.32	0.16	0.95
Creatinine, mg/dL	1.70	1.75	1.73	1.70	1.79	0.037	0.38	< 0.0001	0.99
AST, IU/L	66.79 ab	62.72 a	73.54 c	65.83 ab	71.19 bc	1.853	<0.001	<0.0001	0.96
Alkaline phosphatase, IU/L	84.32	95.20	93.54	95.88	92.35	3.782	0.20	<0.001	0.92

Creatinine kinase, IU/L	141.95	139.84	165.06	123.32	152.69	13.245	0.29	0.10	0.63
Urea, mg/dL	52.59 a	48.68 a	52.51 a	47.76 b	50.72 a	1.244	0.02	< 0.001	0.36
Total bilirubin, mg/dL	0.47	0.45	0.49	0.48	0.45	0.027	0.85	0.05	0.59
Direct bilirubin, mg/dL	0.11	0.11	0.11	0.09	0.08	0.010	0.15	0.18	0.30
Indirect bilirubin, mg/dL	0.365	0.345	0.382	0.385	0.375	0.0276	0.85	0.28	0.71

Where:

– GGT: Gamma Glutamyl Transferase

– AST: Aspartate transaminase

Heart rate, respiratory rate, and blood temperature were not influenced by nitrate inoculation (Table 6). Rumen pH increased for all nitrate treatments, which is in reason of calcium nitrate buffer capacity.

Table 6: Heart and respiratory rates, body temperature, and rumen pH of Nellore steers inoculated with pure or encapsulated nitrate/sulfate trough rumen cannula

Item	*Treatments*					*SEM*	*P-value*		
	Control	*NE123*	*NE246*	*E123*	*E246*		*Treat.*	*Time*	*Treat*Time*
Heart rate, per min	47.35	45.35	47.30	47.00	46.78	0.656	0.18	<0.0001	0.81
Respiratory rate, per min	27.23	26.55	28.01	27.53	26.38	0.446	0.07	0.01	0.12

Body temperature, °C	38.63	38.55	38.56	38.57	38.35	0.123	0.58	< 0.001	0.62
Ruminal pH	6.91a	7.10b	7.10b	7.06 b	7.14 b	0.0326	< 0.0001	<0.0001	< 0.01

Table 7: Hemogasometry analysis of Nellore steers inoculated with pure or encapsulated nitrate/sulfate trough rumen cannula

Item	*Treatments*					*SEM*	*P-value*		
	Control	*NE123*	*NE246*	*E123*	*E246*		*Treat.*	*Time*	*Treat*Time*
Plasma pH	7.49	7.50	7.46	7.47	7.49	0.0127	0.18	< 0.01	0.58
pO_2, mmHg	97.34	104.91	84.93	83.05	92.60	6.383	0.10	0.25	0.35
pCO_2, mmHg	25.43	26.27	28.66	30.07	26.30	1.353	0.08	0.90	0.61
Plasma bicarbonate, mmol/L	18.36a	19.35a	20.09 a	20.93 b	19.08a	0.610	0.03	0.35	0.51
Total CO_2 in arterial plasma, mmol/L	19.14a	20.15a	21.37 a	21.92 b	19.88a	0.653	0.02	0.42	0.42
Base excess, mmol/L	-3.48a	-2.46a	-2.31a	-1.62b	-2.60a	0.421	0.04	<0.01	0.64
Anion gap, mmol/L	15.26a	12.17a b	6.80b	14.93 a	9.53ab	1.719	<0.01	0.07	0.92

Calculated blood oxygen saturation, %	95.10	95.31	92.83	93.21	93.80	1.493	0.70	0.36	0.70
Packed cell volume, %	28.21	27.49	29.26	28.29	28.87	0.657	0.40	<0.01	0.88
Na^+, mmol/L	136.51	135.40	135.42	136.96	135.24	0.462	0.02	< 0.01	0.97
K^+, mmol/L	3.25	3.31	3.05	3.36	3.09	0.090	0.07	0.11	0.68
iCa^{2+}, mmol/L	0.44	0.45	0.44	0.54	0.45	0.038	0.26	0.35	0.81
Cl^-, mmol/L	106.97 bc	105.20 bc	112.65a	104.54c	110.11 ab	1.324	<0.0001	0.04	0.58

Where:

– pO2: partial pressure of O2.

– pCO2: partial pressure of CO2.

– iCa: ionized calcium.

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This innovation is not limited to the representations here mentioned or illustrated, must being comprehended in its broad scope. Many modifications and other representations of this innovation will come up in the mind of those skilled in the technique in which this innovation belongs, having the benefit of teaching presented in the previous descriptions and sketches attached. Besides that, it must be understood that this innovation is not limited to the specific form revealed, and modifications and other forms are comprehended as included inside the scope of the attached claims. Although specific terms were used here, they are employed only as a generic and descriptive form and not with a purpose of limitation.

CLAIMS

1. Composition based on nitrate and sulfates, utilized in ruminant nutrition for reduction of methane emission, **characterized by** presenting the following preferential composition:
   i. 40% to 97% in weight of calcium nitrate, preferentially the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)_2.NH_4NO_3.10H_2O$], more preferentially from 60% to 85% in weight;
   ii. Up to 50% in weight of magnesium sulfate, preferentially the monohydrate or anhydrous ($MgSO_4.1H_2O$ or $MgSO_4$), preferentially from 3% to 20% in weight;
   iii. 1% to 40% in weight of coating, preferentially hydrogenated vegetable fats, preferentially from 3% to 20% in weight and
   iv. up to 20% of additives in weight, preferentially from 0.1 to 10% in weight, presented as covered granules, preferentially with vegetable fats, among them, soybean oil, castor oil, palm oil, babassu oil, cashew nut shell liquid or oil and, alternatively, coconut oil, linseed oil and canola oil.
2. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting, alternatively, the utilization of other nitrates or the mixture of themselves, such as calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$], calcium nitrate anhydrous [$Ca(NO_3)_2$], magnesium nitrate [$Mg(NO_3)_2.6H_2O$], sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3), cal-urea nitrate [$Ca(NO_3)_2.4CO(NH_2)_2$], the double salt of ammonium sulfate and nitrate [$(NH_4)_2SO_4.3(NH_4NO_3)$ or $(NH_4)_2SO_4.2(NH_4NO_3)$], as well as possible variations in the salts cited above due to the number or absence of crystallization water and other compatible nitrates.
3. Composition based on nitrates and sulfates, according to claim 1,

characterized by presenting, alternatively, the utilization of other sulfates or mixtures thereof, such as magnesium sulfate heptahydrate [$MgSO_4.7H_2O$], sodium sulfate [Na_2SO_4 anhydrous, $Na_2SO_4.7H_2O$ and $Na_2SO_4.10H_2O$), ammonium sulfate [$(NH_4)_2SO_4$], potassium sulfate (K_2SO_4), calcium sulfate ($CaSO_4$ or $2CaSO_4.1H_20$), zinc sulfate ($ZnSO_4$ anhydrous or $ZnSO_4.7H_2O$), ferrous sulfate ($FeSO_4.1H_2O$, $FeSO_4.4H_2O$, $FeSO_4.5H_2O$ or $FeSO_4.7H_2O$), manganese sulfate ($MnSO_4$ anhydrous or $MnSO_4.4H_2O$), copper sulfate ($CuSO_4$ anhydrous $CuSO_4.5H_2O$), other compatible sulfates and also cysteine, sulfides, sulphites, elemental sulfur, and sodium tungstate.

4. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting, alternatively, coating with at least one fat, originating from a group consisted of soybean oil, castor oil, palm oil, cashew nut shell liquid or oil, cottonseed oil, linseed oil, peanut oil, babassu oil, sunflower oil, coconut oil, canola oil, wheat oil, rice oil, corn oil, cocoa oil, safflower oil, and vegetable and animal waxes, such as carnauba wax, corn wax, castor wax, and bee wax.

5. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting, alternatively, coating with any other material compatible with the animal nutrition that shows equal or similar properties from those presented in fats in terms of promoting a controlled release of the substance, such as natural materials, degradable in the rumen or not, such as cellulose and carboxycellulose-based emulsions added with calcium carbonate, saccharose, vegetable oils, and xanthan gum; coatings containing starch and other polysaccharides mixed with polyvinyl alcohols; as well as coatings based on lignin/lignosulphonates or chitosan biopolymers.

6. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting, alternatively, coating with synthetic

polymers, degradable in the rumen or not, such as carboxyvinyl; polyacrylic acid (acrylic resins, polyethylenes, etc); alginates; polyhydroxyalkanoates; polyhydroxyoctanoates; polyhydroxybutyrates (Biopols); polycaprolactones; polylactic acids; solutions of biuret with urethane and tungue oil; mixtures of isocyanates with alkydic resins, castor oil and peroxides; mixtures of stearamides with paraffin, magnesium stearate; other resins (polyurethanes, polyolefins, polyesthers, polyepoxides, silicones, polyvinylidene chloride etc, as well as mixtures thereof); alkyl and cycloalkyl amines; paraffins and waxes derived from petroleum.

7. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting, alternatively, aromatizers, flavours, and taste enhancers, being them natural or synthetic (monosodium glutamate, saccharine, sucrose, dextrose, glucose, guava essences, vanilla etc); antioxidants such as vitamin C, beta-carotene, BHT (butylated hydroxytoluene), BHA (butylated hydroxyanisole), acidifiers such as citric acid, acetic acid, tartaric acid, fumaric acid, malic acid; emulsifiers/stabilizing agents such as lecithin, xathans, gums, polisorbates, propylene glycol and monostearates; anti-wetting and anti-caking agents, such as calcium carbonate, starch, microcrystalline cellulose, tricalcium phosphate, silica/silicates, talcum powder, kaolin, calcium stearate; other nutritional additives, such as macrominerals, trace minerals, and vitamins, for instance A, B_1, B_2, B_3, B_5 B_6, B_7, B_9, B_{12}, C, D, E e K); essential oils, such as carvacrol, eugenol, thymol, cynamaldehyde, capsaicin, limonene; organic acids, such as lactate, malate, fumarate, aspartate; fatty acids, such as CLA – conjugated linoleic acid, myristic acid, anacardic acid, medium-chain fatty acids (capric acid, caprilic acid, caproic acid, lauric acid), as well as omega-6 and omega-3 fatty acids, such as alpha-linolenic acid – ALA;

eicosapentaenoic acid – EPA; docosahexaenoic acid – DHA); aminoacids, mainly sulfur-containing aminoacids as cysteine and methionine, but also considering histidine, threonine, leucine, isoleucine, tryptophan, phenylalanine, valine, glycine; enzymes, such as cellulases, hemicellulases, amylases, pectinases, xylases, β-glucanases, phytases and other glucanases; buffers and alkalizers, such as sodium bicarbonate, sodium sesquicarbonate, calcium carbonate, magnesium oxide; yeasts, such as *Trichosporon* sp., *Candida* sp., *Leuconostoc* sp., *Lactococcus* sp., *Candida kefyr, Saccharomyces cerevisiae* etc); fungi, such as *Aspergillus oryzeae* and *Aspergillus niger*; probiotics and other live microorganisms, such as *Lactobacillus* sp. and mainly those that possess nitrate/nitrite reduction activity, such as *Selenomonas ruminantium*, *Veillonella parvula, Wollinela succinogenes* , *Megasphaera elsdenii*, *Propionibacterium acidipropionici*, *Escherichia coli* W3110; and intestinal bacteria, coryneform bacteria, *Bacillus subtilis*, *Methylophilus* sp., and *Actinomyces* sp); galactooligosaccharides and/or nisin; ionophoric antibiotics, such as sodium monensin, salinomycin, lasalocid, narasin; other antibiotics, such as virginiamycin, avilamycin, bacitracin, flavomycin, tylosin; natural substances with antimicrobial properties, such as propolis, beta-acids, alfa-acids, other hop-derived acids, cardanol, cardol, tannins, saponins; anthelmintic agents, and anticcocidials/coccidiostats.

8. Composition based on nitrates and sulfates, according to claim 1, **characterized by** presenting a shape approximately spherical with 1.5 mm to 12 mm of diameter, more preferentially varying from 3 to 7 mm and density varying from 0.85 g/cm^3 to 1.15 g/cm^3, more preferentially between 0.90 g/cm^3 to 1.10 g/cm^3.

9. Composition based on nitrates and sulfates, according to claim 1,

characterized by presenting a liberation rate of nitrates/sulfates varying from 1% to 30% per hour, more preferentially between 5% to 25% per hour.

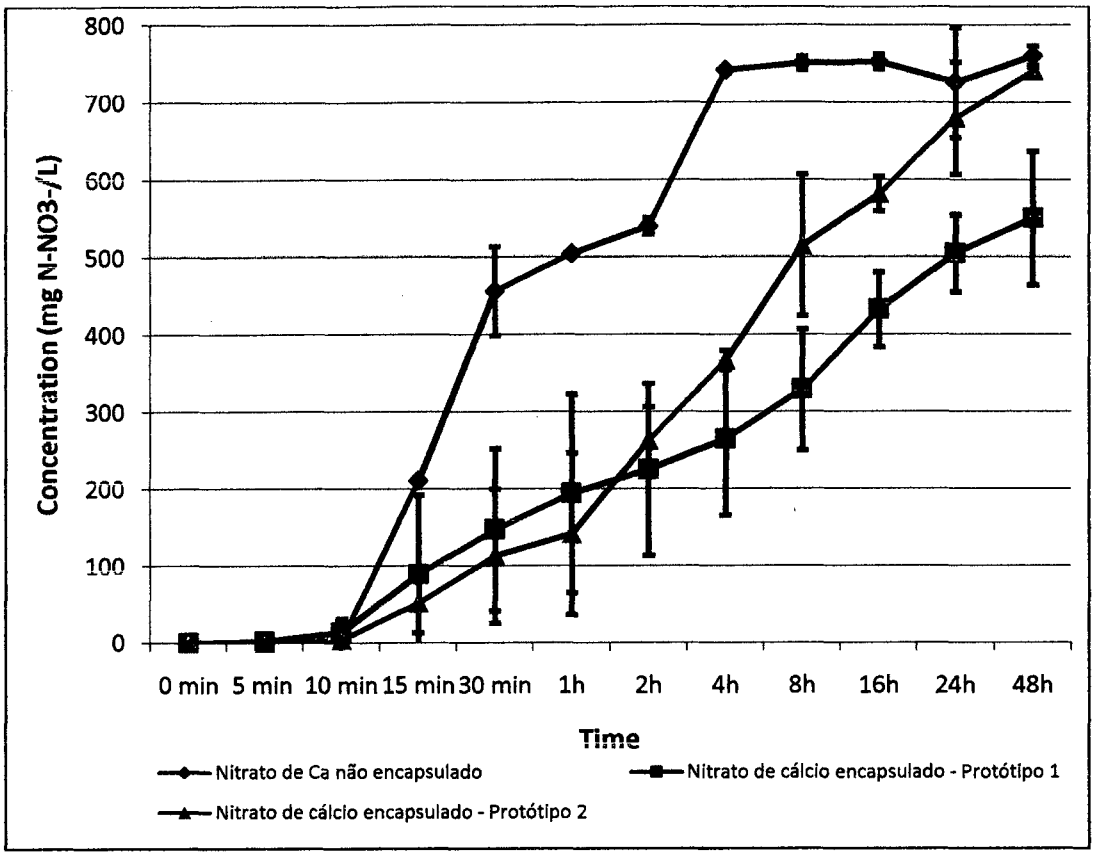

Figure 1: Nitrate release curves of non-encapsulated and encapsulated calcium nitrate decahydrate.

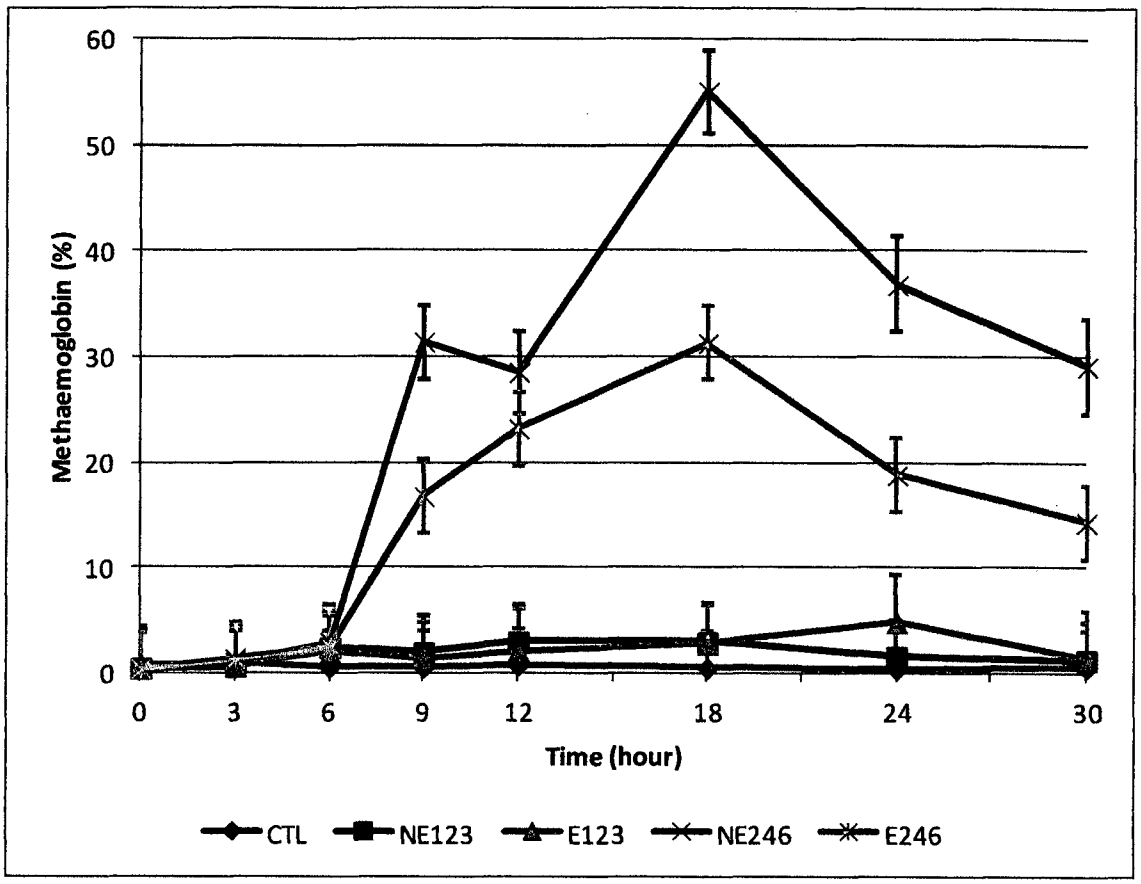

Figure 2: Methemoglobin concentration in beef steers inoculated with encapsulated or non-encapsulated nitrate/sulfate according to hour after inoculation onset.

INTERNATIONAL SEARCH REPORT

International application No
PCT/BR2012/000157

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23K1/175 A23K1/18
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)
A23K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 1 475 492 A (KRASE HERBERT J ET AL) 27 November 1923 (1923-11-27) page 1, line 50 - line 105	1-7,9
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X	WO 2011/010921 A2 (PROVIMI HOLDING B V [NL]; PERDOK HINDRIK BENE [BE]; VAN ZIJDERVELD SAN) 27 January 2011 (2011-01-27) the whole document	1-9
X	US 2004/234650 A1 (MISHRA SURENDRA K [US]) 25 November 2004 (2004-11-25) the whole document	1-9

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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"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
16 July 2012	24/07/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rooney, Kevin

INTERNATIONAL SEARCH REPORT

International application No
PCT/BR2012/000157

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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