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Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep

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ABSTRACT

The aim of this work was to investigate the effect of feeding ethyl-3-nitrooxy propionate (E3NP) and 3-nitrooxypropanol (3NP), 2 recently developed compounds with potential antimethanogenic activity, in vitro and in vivo in nonlactating sheep on ruminal methane production, fermentation pattern, the abundance of major microbial groups, and feed degradability. Three experiments were conducted, 1 in vitro and 2 in vivo. The in vitro batch culture trial (experiment 1) tested 2 doses of E3NP and 3NP (40 and 80 μL/L), which showed a substantial reduction of methane production (up to 95%) without affecting concentration of volatile fatty acids (VFA). The 2 in vivo trials were conducted over 16 d (experiment 2) and 30 d (experiment 3) to study their effects in sheep. In experiment 2, 6 adult nonpregnant sheep, with permanent rumen cannula and fed alfalfa hay and oats (60:40), were treated with E3NP at 2 doses (50 and 500 mg/animal per day). After 7, 14, and 15 d of treatment, methane emissions were recorded in respiration chambers and rumen fluid samples were collected for VFA analysis and quantification of bacterial, protozoal, and archaeal numbers by real-time PCR. Methane production decreased by 29% compared with the control with the higher dose of E3NP on d 14 to 15. A decrease in the acetate:propionate ratio was observed without detrimental effects on dry matter intake. In experiment 3, 9 adult nonpregnant sheep, with permanent rumen cannula and fed with alfalfa hay and oats (60:40), were treated with E3NP or 3NP at one dose (100 mg/animal per day) over 30 d. On d 14 and d 29 to 30, methane emissions were recorded in respiration chambers. Rumen fluid samples were collected on d 29 and 30 for VFA analysis and quantification of bacterial, protozoal, and archaeal numbers by real-time PCR. In addition, on d 22 and 23, samples of oats and alfalfa hay were incubated in the rumen of sheep to determine dry matter ruminal degradation over 24 and 48 h, respectively; no effect was observed (78.6, 78.3, and 78.8% of alfalfa and 74.2, 74.0, and 70.6% of oats in control, E3NP, and 3NP groups, respectively). A reduction in methane production was observed for both additives at d 14 and d 29 to 30. In both treatments, the acetate:propionate ratio was significantly decreased. Likewise, total concentrations of the analyzed microbial groups in the rumen showed no difference among treatments and doses for both experiments. Both tested compounds showed promise as methane inhibitors in the rumen, with no detrimental effects on fermentation or intake, which would need to be confirmed in lactating animals.

Key words: ethyl-3-nitrooxy propionate, 3-nitrooxypropanol, rumen, methane, sheep

INTRODUCTION

The inhibition of methane (CH_4) production by ruminants has long been an objective of ruminant nutritionists. Methane is produced within the rumen by methanogenic archaea. It is a byproduct of ruminal fermentation and constitutes a pathway for the disposal of metabolic hydrogen produced by microbial metabolism. The production of CH_4 represents an energy loss of between 2 and 12% of dietary gross energy (Johnson and Johnson, 1995) and contributes significantly to total anthropogenic greenhouse gas emissions (Hristov et al., 2013). It is obvious that if the energy lost as methane were to be conserved as fermentation products, improved energy retention would lead to increased productivity, in addition to decreasing production of this important greenhouse gas (Moss et al., 2000). Various approaches aimed at reducing methane emissions from enteric fermentation have been studied in many countries (McAllister and Newbold, 2008). The development of new feed additives (mainly based on

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plant extracts) to decrease methane production within the rumen has attracted research efforts over the last 20 yr. The results remain variable and contradictory (Benchaar and Greathead, 2011), thus restricting the uptake and use of these new compounds in the animal feeding market. The reasons behind such restriction may be related to several factors, including the lack of persistency of the effects when they are tested in vivo due to the adaptation of the microbial ecosystem, the variability in concentration of active compounds in plant extracts, the stability of the active compound within the rumen, and side effects that compromise overall ruminal fermentation (Hart et al., 2008).

The development of synthetic compounds with activity specific to metabolic pathways essential to ruminal archaea has shown promising results in recent studies. This may overcome the restrictions associated with the use of plant-derived compounds (Liu et al., 2011; Soliva et al., 2011). Methyl-Coenzyme M (**CoM**) reductase catalyzes the last step of reduction of CO_2 to CH_4 by hydrogenotrophic methanogenic archaea (Attwood and McSweeney, 2008). Preliminary observations using an in silico screening approach (Halgren et al., 2004) identified some nitrooxy carboxylic acids with potential to dock into the active site of methyl-CoM reductase.

Therefore, the present study was designed to investigate the effects of 2 compounds—ethyl-3-nitrooxy propionate (**E3NP**) and 3-nitrooxypropanol (**3NP**)—on antimethanogenic activity in vitro and in vivo, ruminal fermentation, and microbial abundances.

MATERIALS AND METHODS

One in vitro and 2 in vivo experiments were conducted. In experiment 1, the antimethanogenic activity of E3NP and 3NP was assessed in batch cultures over 24 h. In experiment 2, the effects of 2 doses of E3NP on ruminal fermentation, methane production, and microbial abundance were studied in sheep over 16 d, and in experiment 3, single doses of E3NP or 3NP were tested in sheep over 30 d.

Animals, Diet, and Compound

Fifteen adult, dry Segureña sheep (44.3 ± 4.7 kg of BW) fitted with permanent rumen cannulas were used in experiments 1, 2, and 3. Throughout the trials, they had free access to water and were fed, twice a day (0900 and 1600 h), a diet that consisted of alfalfa hay chopped at 15 to 20 cm and grain oats in a proportion of 60:40 at approximately 1.1 times the energy requirements for maintenance (Aguilera et al., 1986) and a mineral-vitamin supplement (Table 1). The same diet was used as the substrate for the in vitro experiment. Animals were cared for by trained personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree No. 1201/2005; Boletin del Estado, 2005) and the European Convention for the Protection of Vertebrates used for Experimental and Other Scientific Purposes (European Commission, 2007). The experimental procedures involved in this study were approved by the Animal Welfare Committee at the Institute of Animal Nutrition (Consejo Superior de Investigaciones Científicas, Madrid, Spain). The temperature, humidity, and air turnover in chambers were carefully monitored with respect to animal welfare conditions. The CO_2 concentration was also continuously monitored to ensure good air quality and air flow in the chambers. Animals did not show any stress-related behavior while housed in chambers.

Table 1. Chemical composition of alfalfa hay and oats (g/kg of DM unless otherwise noted)

Item	Alfalfa hay	Oats
DM, g/kg of fresh matter	907	912
OM	875	975
CP[1]	193	103
NDF	517	263
ADF	334	67.4
ADL	103	13.2
Ether extract	9.4	19.9
Gross energy, MJ/kg of DM	18.5	21.1

[1]CP = N × 6.25.

The compounds to be tested were E3NP (99.7% purity) and 3NP (99.5% purity), both classified as nitrooxy alkanoic derivates (Figure 1). Both compounds were provided by DSM Nutritional Products (Saint-Louis Cedex, France; Duval and Kindermann, 2012). In experiment 1, the compounds were directly pipetted into the bottles before inoculation. In experiments 2 and 3, the compounds were provided twice a day through the ruminal cannulas at the time the animals were fed. The corresponding amount of each additive was pipetted onto 10 g of ground oats and wrapped in cellulose paper immediately before being placed in the rumen via the cannula.

Experimental Design and Sampling

Experiment 1. Three 24-h incubation runs were carried out with 2 bottles per treatment (including blanks). Treatments were as follows: control (no additive treatment), E3NP at 40 and 80 μL/L, 3NP at 40 and 80 μL/L, and bromochloromethane (**BCM**) at 160 and 320 μL/L, as a positive antimethanogenic control (Goel et al., 2009). The corresponding molar doses were 124 and 247 μ*M* (BCM), 33 and 66 μ*M* (3NP), and 25 and 50 μ*M* (E3NP). The substrate incubated was the