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Effects of the methane-inhibitors Nitrophenol, 5-Nitrobenzimidazol and two new synthetic nitrocompounds on *in vitro* ruminal fermentation

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ABSTRACT

The objective of this study was to examine the effects of four nitrocompounds (Nitrophenol, 5-Nitrobenzimidazol and two synthetic nitrocompounds ABLE 244 and ABLE 245) on methane production and fermentation characteristics using *in vitro* rumen batch culture. 0, 2, 8 or 12 μM of each nitrocompound were incubated. The higher concentrations of Nitrophenol and 5-Nitrobenzimidazol produced 60% less CH_4 ($P < 0.05$) compared to controls, while two synthetic nitrocompounds ABLE 244 and ABLE 245 had no effect on CH_4 production. Quantification of fermentation end-products indicated that fermentation efficiencies were not compromised by the nitro-treatments.

1. Introduction

Methane is a greenhouse gas that contributes to global warming (Lassey, 2007). After carbon dioxide; methane is considered the most potent greenhouse gas (IPCC et al., 2001), due to the higher efficiency (20–30 times) of long-wave radiation absorption relative to CO_2 and involvement of CH_4 in chemical reactions that give ozone as the final product (Crutzen, 1995). Due to the increased concentration of CH_4 in the atmosphere in the post-industrial era, several investigations have been involved to identify sources and sinks of methane and to estimate their effects (Bodelier Paul and Laanbroek, 2004; Hilary et al., 2012; Guangming et al., 2013).

In the livestock sector, ruminants contribute significantly to global greenhouse gas emissions (Yáñez-Ruiz and Martín-García, 2016). In terms of the environment, ruminal methanogenesis accounts for about 12–14% of total greenhouse gas emissions (Zervas and Tsiplakou, 2012). But methane production results in a loss of raw energy (4–12%) for cattle fed on forage and fodder (Zhenming et al., 2012). In the rumen, CH_4 is produced by methanogens catalyzing the transfer of hydrogen and carbon dioxide into methane. In addition to methane production, the low hydrogen partial pressure by methanogenesis has a great influence on other products of the non-methanogenic and fermentative microbial community (Wolin et al., 1997). In many cases, the reduction of CH_4 production in the rumen may thus affect digestive function and microbial cell yields due to altered fermentation efficiencies associated with microbial hydrogen transfer reactions (Miller, 1995; Van Nevel and Demeyer, 1996; Anderson et al., 2008)

Several methods have been developed by ruminant microbiologists to reduce the energy losses associated with the production of ruminal CH_4 (Anderson et al., 2008), and many chemical inhibitors reduce methanogenesis (eg monensin and lasalocide) (Russell and Strobel, 1989), plant extracts (tannins for example) (Hariadi and Santoso, 2010) or new synthetic compounds (Patra et al., 2017). These strategies involve supplementing ruminants with anti-methanogenic compounds that directly inhibit methanogens or inhibit the biochemical reactions involved in methane production (Bozic et al., 2009). Among these methods; is the change in electron acceptors that consume more efficiently the reducing equivalents produced during fermentation to redirect the electron flux from the reduction of carbon dioxide to CH_4 (Anderson and Rasmussen, 1998; Sar et al., 2005). Several nitrocompounds have the ability to reduce ruminal methane *in vitro* up to 90% (Anderson et al., 2003), such as nitroethane, 2-nitroethanol, 2-nitro-1-propanol and 3- nitro-1-propionic inhibit the rumen. CH_4 production (Anderson and Rasmussen, 1998; Anderson et al., 2003, 2008; Bozic et al., 2009; Gutierrez-Banuelos et al., 2008). In addition, nitroethane and 2-nitro-1-propanol reduce CH_4-producing activity *in vivo* (Anderson et al., 2006; Gutierrez-Banuelos et al., 2008; Zhang and Yang, 2011), as well as ethyl-3. -nitrooxy propionate and 3-

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nitrooxypropanol have shown potential for successful use as anti-methanogenic additives in ruminants (Martínez-Fernández et al., 2014). Short-chain nitro compounds have demonstrated potently inhibiting methanogenesis and can serve as terminal electron acceptors (Zhenming et al., 2011).

The use of nitrates in reducing methane has been limited because of the risk of potential nitrite accumulation and its ability to cause methemoglobinemic cattle (Zhenming et al., 2012). Recent work, however, suggests that the risk of ruminal nitrite accumulation maybe alleviated by co-supplementation with nitrite-reducing bacteria or decreasing the rapidity of nitrate reduction in the rumen by feeding nitrate with a slow-release coating (Raphélis-Soissan et al., 2017). Therefore, nitrate may be a potential inhibitor to attenuate methane in cattle.

Literature survey revealed that benzimidazole derivatives have considerable interest as an antimicrobial (Ates-Alagoz, 2016) and anticancer agents (Yadav et al., 2016). However, *in vitro* antitumor screening of benzimidazoles toward cancer cell lines demonstrated that these compounds are the most potent analogs toward all tested cell lines (El-Gohary and Shaaban, 2017).

Smith et al. (1988) experiment's on male rats exposed for 2 weeks to up to 2 mg Nitrophenol have demonstrated that no histopathological alterations in the esophagus, stomach, small intestine, colon, and cecum. On the other hand, No studies were located regarding the carcinogenic effects in humans or animals following inhalation exposure to Nitrophenol or Nitrophenol derivatives.

The objective of this study was to evaluate the effects of two newly synthesized nitrocompounds, 13- (4-nitrophenyl) -3,4-dihydro-2H-indazolo [1,2-b] phthalazine-1,6, 11 (2 H, 13 H) -trione (ABLE 244) and 16- (4-nitrophenyl) -1,16-dihydrophthalazino [2', 3′: 1,2] pyrazolo [4,3-a] carbazole-9,14 dione (ABLE 245), and two commercial nitrocompounds, Nitrophenol (NIP) and 5-Nitrobenzimidazol (5-NBZ) on the total *in vitro* production of volatile gas, methane and fatty acid in the rumen.

2. Materials and methods

2.1. Chemicals

Newly synthesized nitrocompounds (Fig. 1) were synthesized at the Crystallography Laboratory (University of Constantine, Algeria) as follows:

The 13-(4-nitrophenyl)-3,4-dihydro-2*H*-indazolo [1,2-b] phthalazine-1,6,11(2*H*, 13*H*)-trione (ABLE 244) was prepared according to the modified procedure (Sayyafi et al., 2008) *via* the multi component reaction of phthalhydrazide (1 mmol), 1,3-cyclohexadione (1.05 mmol) and 4-nitrobenzaldehyde (1.05 mmol) at reflux of acetic acid and in the presence of a catalytic amount of trifluoroacetic acid.

The 16-(4-nitrophenyl)-1,16-dihydrophthalazino [2′,3′:1,2] pyrazolo [4,3-a]carbazole-9,14 dione (ABLE 245)was obtained according to the previously described procedure (Lamera et al., 2017) from reaction of phthalhydrazide (1 mmol), 4-nitrobenzaldehyde (1 mmol), 1,3-cyclohexadione (1.05 mmol) and phenylhydrazine (1.7 mmol) using a sequential MCR/Fisher indolization strategy at reflux of acetic acid and in the presence of a catalytic amount of trifluoroacetic acid.

2.2. Experimental design and animal management

Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line to corresponding European Directive (2010/63/EU). The corresponding experimental protocol was approved by the Ethics and Animal Welfare Committee of the Estación Experimental del Zaidín (Spanish National Research Council).

Rumen content from three canulated goats was collected before morning feeding, through two layers of sterilized cheesecloth under a steady stream of oxygen-free CO_2 and maintained at 40 °C in a water bath. Then, in an interval of time lasting 30 min, the rumen fluid was used as inoculums for *in vitro* batch incubations as follow.

2.3. In vitro incubation

Tests for effects of inhibitors on ruminal methane production were accomplished by batch culture (Theodorou et al., 1987). The culture medium consisted of an artificial saliva (Menke and Steingass, 1988) that was bubbled with CO_2 until saturated before being used (Krishnamoorthy et al., 1991) and the clarified rumen fluid in a 3:1 ratio in crimp-top Wheathon bottles (capacity 120 ml). Each *in vitro* culture tube contained 30 ml medium and 10 ml fresh rumen fluid collected from three canulated goats before morning feeding; containing 0.3 g ground oats hay, and 0, 2, 8 and 12 μM of NIP, 5-NBZ, ABLE 244 and ABLE 254.

Culture tubes were immediately closed with rubber stoppers to contain the respective gas phase, and incubated upright without agitation at 39 °C during 24 h. Gas pressure in headspace was released and quantified using a Pressure Meter (Wide Range 840065) after 2, 4,6,12 and 24 h.

2.4. Gas and methane measurement

After 24 h incubation, a gas sample (about 5 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) to determine CH_4 produced from 12 to 24 h by gas chromatography using a HP Hewlett 5890 Packard Series II gas chromatograph (Waldbronn, Germany) equipped with a flame ionization detector (FID) and an HPINNOWAX cross linked polyethylene glycol column

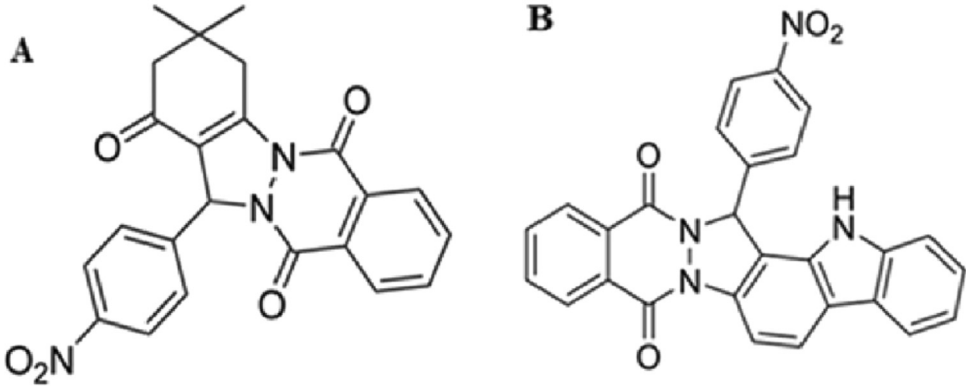

Fig. 1. Structure of ABLE 244 (A) (Sayyafi et al., 2008) and ABLE 245 (B) (Lamera et al., 2017).