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# Effects of increasing levels of stearidonic acid on methane production in a rumen *in vitro* system

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ABSTRACT

Enteric methane constitutes an energy loss for the animal and contributes to global warming and climate change. Stearidonic acid (SDA; C18:4*n*-3), a highly polyunsaturated *n*-3 fatty acid, could have potential to reduce methane production. Effects of increasing levels of SDA on methane production were evaluated in short-term batch incubations (24 h) of a mixed diet with buffered rumen fluid. Stearidonic acid was supplemented at 0 (SDA0; control), 1 (SDA1), 5 (SDA5), 20 (SDA20), and 50 (SDA50) mg/L incubation media. Increasing levels of SDA supplementation had no effect on total gas (mL) and methane production (mmol/g TMR dry matter, DM), or total concentration of volatile fatty acids (VFA; mmol/L). Stearidonic acid induced a shift to increased propionate production at the expense of acetate and butyrate, with the largest effect at the highest inclusion level. The apparent biohydrogenation of stearidonic acid was extensive, with less than 2% being detected after 24 h of incubation, and only at the highest level of addition. Increasing levels of SDA promoted an accumulation of C18:2 and C18:1 isomers, particularly vaccenic acid (*trans*-11 C18:11), with no effect on the end-product (stearic acid; C18:0). Effects on fermentation pattern and SDA biohydrogenation were not associated with a reduction in methanogenesis, suggesting that either higher levels of non-esterified SDA or supplementation in an alternative form might be needed to achieve methane mitigation.



## 1. Introduction

Methane is considered to be the greatest potential agricultural contributor to the global warming and climate change (Johnson et al., 2002), enteric fermentation from livestock being responsible for 25% of total atmospheric methane emissions (IPCC, 2007). Additionally, ruminal methanogenesis may represent an energy loss of 2–12% of the ingested gross energy, depending on the type of diet (Johnson and Johnson, 1995). Therefore, a growing interest has emerged in decreasing ruminal methane production.

Methanogenic archaea in the rumen convert carbon dioxide into methane through reduction of the hydrogen originating from oxidation reactions, thereby avoiding the negative impact of hydrogen accumulation. Ideally, methane production

*Abbreviations:* CP, crude protein; DM, dry matter; EE, ether extract; aNDFom, neutral detergent fiber assayed with a heat stable amylase and expressed exclusive of residual ash; OBCFA, odd- and branched-chain fatty acids; SDA, stearidonic acid; TMR, total mixed ration; VFA, volatile fatty acids.

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should be decreased without impairing fermentation and energy generation in the rumen. Strategies to reduce methane production vary from approaches that directly target the numbers or activity of methanogenic archaea to others that decrease hydrogen production, some having multiple modes of action (Moss et al., 2000).

Oil-rich supplements can be used in dairy diets to increase dietary energy density and decrease rumen methane emissions. They operate through a number of mechanisms, including reductions in organic matter fermentation, rumen ciliate protozoa numbers, methanogen activity and the use of hydrogen for biohydrogenation (Johnson and Johnson, 1995; Beauchemin et al., 2009). However, the amount of hydrogen used in the biohydrogenation process is small (1%) compared to the amount of hydrogen used to reduce carbon dioxide to produce methane (48%; Czerkawski, 1986). Oils rich in medium-chain fatty acids reduce methane production (Machmüller, 2006), with lauric (C12:0) and myristic (C14:0) acids being the most effective (Dohme et al., 2001; Soliva et al., 2003). Dietary inclusion of lipids containing high levels of polyunsaturated fatty acids in the diet of ruminants has also been promising. Indeed, some studies have shown a decrease in methane production by the dietary inclusion of linseed oil (Martin et al., 2008), sunflower oil (McGinn et al., 2004) and mixtures of sunflower oil and fish oil (Woodward et al., 2006).

Stearidonic acid (SDA; C18:4*n*-3) is a highly polyunsaturated *n*-3 fatty acid found in seed plants of Aracauriaceae, Boraginaceae, Caryophillaceae, Cannabinaceae, Primulaceae, Saxifragaceae and Loasaceae families (Guil-Guerrero, 2007), and of genetically modified soybeans (Ursin, 2003). Interest in SDA has arise from evidence of beneficial health properties of eicosapentaenoic acid (C20:5*n*-3), and docosahexaenoic acid (C22:6*n*-3; Calder, 2006). Although these fatty acids can be synthesized in mammal tissues by α-linolenic acid (C18:3*n*-3) desaturation and elongation (Brenna et al., 2009), its efficiency is very low, Δ6-desaturase being the limiting step. Stearidonic acid is the reaction product of Δ6-desaturase, thereby more readily converted to C20:5*n*-3 than C18:3*n*-3 (Yamazaki et al., 1992). However, polyunsaturated fatty acids are known to be toxic to ruminal microorganisms (Prins et al., 1972). We hypothesized that being an octadecatetraenoic acid, SDA might exert a greater inhibitory effects on microbial population than linoleic acid (C18:2*n*-6) and C18:3*n*-3, as the inhibitory effects of C18 unsaturated fatty acids were found to be related to the number and geometrical configuration of double bonds (Demeyer and Henderickx, 1967). Additionally, the recent observation that SDA is extensively biohydrogenated *in vitro* (Maia et al., 2012), thus providing an alternative acceptor of hydrogen, suggests that SDA inclusion may be useful to reduce methane production. The objective of this study was to evaluate increasing supplementation levels of SDA on methane production *in vitro*. Stearidonic acid inclusion levels were chosen to prevent eventual toxic effects from this octadecatetraenoic acid on the rumen micro-organisms and to include practical dairy cow lipid supplementation strategies.

## 2. Materials and methods

### 2.1. Rumen inoculum and diet

Rumen contents were obtained from 3 adult dairy cows immediately after slaughter (Matadouro Central de Entre Douro e Minho, Vila Nova de Famalicão, Portugal), and placed in a pre-heated thermos container at 39 °C. At the laboratory, the ruminal digesta was mixed, strained through 4 layers of linen-cloth, and maintained at 39 °C under $O_2$-free $CO_2$. The length of time between collection of rumen contents and incubation was less than 60 min. A commercial total mixed ration (TMR) for lactating dairy cows, containing 491 g maize silage, 85 g wheat straw and 424 g concentrate mixture (per kg dry matter, DM, basis; Table 1), was dried for 48 h at 65 °C, milled through 1 mm sieve, and used as substrate in the *in vitro* incubations. The TMR contained (DM basis) 179 g/kg of CP, 383 g/kg of aNDFom and 252 g/kg of starch, C16:0, *cis*-9 C18:1, and C18:2*n*-6 were the major fatty acids present (Table 2).

### 2.2. In vitro incubations

Effects of increasing levels of SDA on methane production were evaluated in short-term batch incubations (24 h) with the TMR. Stearidonic acid was supplemented at 0 (SDA0; control), 1 (SDA1), 5 (SDA5), 20 (SDA20), and 50 (SDA50) mg/L incubation media. Briefly, a working solution of 10 mg SDA/mL ethanol was prepared from a commercial SDA solution (100 mg/mL ethanol; Sigma–Aldrich Inc., St. Louis, MO, USA) and stored at 4 °C. Increasing levels of SDA working solution were added to, approximately, 400 mg ground TMR, corresponding to 0, 0.125, 0.625, 2.5, and 6.25 g SDA/kg TMR (DM basis). Ethanol was also added to equal its final volume in all tubes, removed under $N_2$ flow at 37 °C, and tubes kept at −20 °C until incubation. One part of strained ruminal fluid was diluted anaerobically into two parts of the medium described by Menke and Steingass (1988), and mixed at 39 °C under $O_2$-free $CO_2$. Fifty milliliters of buffered ruminal fluid were dispensed anaerobically into 125 mL serum bottles (Sigma–Aldrich Inc., St. Louis, MO, USA) containing the experimental treatments, sealed with butyl rubber stoppers (Sigma–Aldrich Inc., St. Louis, MO, USA), and incubated, at 39 °C in triplicate. Reactions were stopped after 24 h by cooling in an ice-slurry.

### 2.3. Incubation media sampling and measurements

Bottles were warmed up to a temperature of 25 °C and head-space gas volume was measured with a pressure transducer (Bailey & Mackey Ltd., Birmingham, UK) as described by Theodorou et al. (1994). The composition of the head-space gas was determined by gas chromatography, using a GC-4000A (East & West Analytical Instruments, Inc, Beijing, China) equipped