

# *In vitro* Manipulation of Rumen Fermentation Efficiency by Fumaric acid – Bentonite Coupled Addition as an Alternative to Antibiotics

M. A. Abdl-Rahman

Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Egypt

Faculty of Veterinary Medicine, Cairo University, Giza-12211, Egypt

Tel: 202-2864-5669 E-mail: mahmod.abdelhafez@gmail.com

## Abstract

Short term *in vitro* incubations were used to evaluate the effect of fumaric acid - bentonite coupled addition on rumen fermentation efficiency. Ruminal contents from five steers were used for preparation of inoculums of mixed rumen microorganisms that were used in generation of three treatment systems, negative control, fumaric acid treated, and fumaric acid – bentonite coupled treated. The fermentation pattern revealed that, this coupled addition was associated with an additional increase in propionic acid production and fermentation efficiency and was related to an additional decrease in methanogenesis and VFAs utilization index. Furthermore, it increased total VFAs concentrations and decreased pH value, ammonia nitrogen (NH<sub>3</sub>-N) concentrations and butyrate proportions. Meanwhile, it did not alter the proportions of long chain VFAs or cellulase activity. Conclusively, this coupled addition would improve the impact of fumaric acid on rumen fermentation pattern and can be appropriate alternative for antibiotic feed additives in improving ruminants feed efficiency.

**Keywords:** Bentonite addition, Coupled addition, Fermentation efficiency, Fumaric acid, Rumen fermentation

## 1. Introduction

During the last decades, considerable amounts of antibiotics were used in ruminant production for optimization of rumen fermentation pattern. However, increasing worries with antibiotic residues in meat and milk led consumers all over the world to oppose the usage of antibiotics in animal feeds. At the same time this risk fuelled the search for nonantibiotic alternatives, which might have similar effects on animal performance.

According to Diebold and Eidelsburger (2006), fumaric acid is one of the most hopeful in this regard because of its potential to reduce methanogenesis by sinking hydrogen during its conversion to propionate (Newbold & Rode 2006). Increased hydrogen utilization by fumarate reducing bacteria could also stimulate cellulolytic bacteria and enhance cellulose digestion (Wallace *et al.* 2005). However, the inconsistent effects of fumaric acid on animal performance (Newbold & Rode 2006), have limited its adoption. One of the major constraints to induction of fumaric acid effects, is that, the affinity of fumarate reducing bacteria to hydrogen is lower than the affinity of methanogens, as a result, the maximum potential of fumarate to divert H<sub>2</sub> away from CH<sub>4</sub> is limited presumably because methanogens utilize H<sub>2</sub> more rapidly than fumarate-utilizing bacteria. Asanuma *et al.* (1999) suggested that fumarate-utilizing bacteria have a disadvantage in utilization of H<sub>2</sub> compared with methanogens when the partial pressure of H<sub>2</sub> is low. In this regard ciliate protozoa facilitate methanogenesis by consuming oxygen and establishing a high redox potential (Newbold *et al.* 1995). defaunating agents were found to strongly inhibit methanogenesis and direct hydrogen for propionates production (Santra *et al.* 1996).

Sodium bentonite is an expanded lattice clay of the montmorillonite group of minerals (Bates & Jackson 1980) with high ion exchange capacity that binds a wide range of cations (Fenn & Leng 1989). According to Wallace & Newbold (1991), bentonite interferes with the efficiency of protozoal ciliary motion and thereby reduces the activity of ciliate protozoa. Because of the huge surface area of bentonite and the electrical charges on its surface, it slows the capture rate of microbes by protozoa allowing higher bacterial and fungal populations to remain within the ruminal fluid (Heijnen *et al.* 1991; Wallace & Newbold 1991).

The present experiment was therefore conducted to investigate the effect of coupling bentonite to fumaric acid addition as a tool for potentiating fumaric acid effects on rumen fermentation pattern.

## 2. Materials and methods

This investigation was conducted in Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

### 2.1 Collection of rumen contents

Ruminal contents used to prepare the treatment systems were collected from the rumen of five slaughtered steers. Collected ruminal fluids were strained through four layers of cheesecloth into a separating flask previously gassed with oxygen-free CO<sub>2</sub> and brought immediately to the laboratory. Strained rumen liquors were mixed with the buffer solution of Goering and Van Soest (1970) in the proportion 1:2 (v/v), flushed with oxygen-free CO<sub>2</sub> and used as inoculums of mixed rumen microorganisms. Part of each buffered rumen fluid sample (blank) was not used as inoculum, immediately mixed with 0.3 mL H<sub>2</sub>SO<sub>4</sub> 10N and used for determination of total VFAs concentrations before incubation.

### 2.2 Preparation of treatment systems and in vitro fermentation

Thirty milliliters of buffered rumen fluids were anaerobically transferred to 120-mL bottles containing 200 mg of feed sample (basal diet of steers, composition and chemical analysis is shown in table, 1) previously ground with a pestle and mortar to provide an even distribution of particle size. The following treatment systems were then prepared for each sample in duplicate tubes per treatment: negative control (no additives), fumaric acid treated (0.5 mg/mL), and fumaric acid – sodium bentonite coupled treated (0.5 and 0.3 mg/mL of each). The bottles were sealed (under continuous flushing of CO<sub>2</sub>) with rubber stoppers and aluminium caps and were placed in a shaking water bath at 39°C. for 24 hours.

### 2.3 Sampling and analysis

After termination of incubation, the bottles were uncapped and pH values were immediately measured using a digital pH meter. For determination of total VFAs concentrations and individual VFAs proportions 1 mL of 25% meta-phosphoric acid was added to 5 mL of fermentation fluids, centrifuged (7,000 x g for 10 min) and supernatants were stored at -20°C until analyzed. For NH<sub>3</sub>-N determination, a 2-mL sample of fermented fluid was acidified with 2 mL of 0.2 N HCl and frozen. Samples were centrifuged at 5000 x g for 20 min, and the supernatants were analyzed by spectrophotometry for NH<sub>3</sub>-N according to Chaney and Marbach (1962). Total VFAs concentrations were measured by steam distillation according to Eadie *et al.* (1967). The percentage concentrations of VFAs were analyzed using High Performance Liquid Chromatography (HPLC; Model Water 600; UV detector, Millipore Corp.) according to the method of Mathew *et al.* (1997). After the results of the percentage concentrations of VFAs had been received, the following fermentation parameters were calculated:

2.3.1 The amounts of VFA produced were obtained by subtracting the amounts present initially in the incubation medium (blanks) from those determined at the end of the incubation period.

2.3.2 The concentrations of acetic, propionic, butyric and valeric acids in their total concentration.

2.3.3 Acetic / propionic acid ratio

2.3.4 Fermentative CH<sub>4</sub> production in the buffered rumen fluid were estimated by the equations of Wolin (1960), which has been validated recently by Blummel *et al.* (1993), as following:

$$\text{Fermentative CO}_2 = A/2 + P/4 + 1.5B$$

Where A, P and B are moles of acetate, propionate, and butyrate respectively.

$$\text{Fermentative CH}_4 = (A+2B) - \text{CO}_2$$

Where A and B are moles of acetate and butyrate respectively and CO<sub>2</sub> is moles of CO<sub>2</sub> calculated from previous equation.

2.3.5 Percent of methane output per total VFAs production

2.3.6 The fermentation efficiency (FE): This was calculated on the basis of the equation worked out by Orskov (1975) and modified by Baran and Zitnan (2002):

$$\text{FE} = (0.622a + 1.092p + 1.56b) 100 / (a + p + 2b)$$

where: a, p and b express the concentrations (μmol) of acetic, propionic and butyric acids respectively in the total concentration of VFAs produced. The final result of this equation is expressed in percentage and shows an amount of energy stored in VFAs as a percentage participation of the initial energy.

2.3.7 The VFAs utilization index: This was expressed by non-glucogenic VFAs/glucogenic VFAs ratio (NGGR) according to Orskov (1975):

$$\text{NGGR} = (A + 2B + V) / (P+V)$$

where A, P, B and V express the concentrations (μmol) of acetic, propionic, butyric and valeric acids respectively. Valeric acid is classified as both glucogenic and non-glucogenic VFA because, its oxidation creates

1 mole of acetic acid and 1 mole of the propionic acid. Too high NGGR indicates high loss of energy in the form of gases.

#### 2.4 Measurement of cellulase activity

Supernatant from each fluid sample was separated by centrifugation at 3,000 x g for 20 minutes. Half mL of the supernatant (crude enzyme solution) was mixed with 0.5 mL of 1% carboxymethyl cellulose solution in 0.05 M sodium citrate buffer. The reaction proceeded for one hour at 55°C without shaking, and then stopped by boiling for 5 min. Boiled samples were centrifuged at 7,000 x g for 5 min, and reducing sugars produced in the supernatants were measured colorimetrically according to Miller *et al.* (1960). One unit of enzyme activity was defined as the amount of enzyme that produced 1 mmol of glucose equivalent of reducing sugar per minute.

#### 2.5 Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA) test according to Snedecor and Cochran (1980). Treatment means were compared by the least significance difference (LSD) at 5% level of probability.

### 3. Results

Data presented in table (2) reveals that, fumaric acid-bentonite coupled addition decreased pH values, NH<sub>3</sub>-N concentrations and butyrate proportions and increased total VFAs concentrations of the fermentation fluid relative to both control and fumaric acid addition. In contrast, addition of fumaric acid alone did not alter any one of the previously mentioned parameters. Furthermore, the overall means of VFAs molar proportions reveals that, both fumaric acid addition and fumaric acid-bentonite coupled addition were associated with increased propionates at the expense of acetates and therefore, a lowered A/P ratio was recorded for both treatments. however, the increment effect induced by coupled addition outdid that induced by fumaric acid addition (33.95% vs. 12.94%). Nevertheless, the molar proportions of the major long chain VFAs (valeric – isovaleric - isobutyric) were not affected by either fumaric acid addition or fumaric acid-bentonite coupled addition. Furthermore, the means of different treatment systems denote that, both fumaric acid addition and fumaric acid-bentonite coupled addition were associated with decreased CH<sub>4</sub> production. Nevertheless, when CH<sub>4</sub> production was calculated as a percent of total VFAs, it appeared that the decremenal effect of coupled addition had exceeded that of fumaric acid addition.

Table (3) identifies that, cellulase activity within the fermentation fluid did not alter by either fumaric acid addition or fumaric acid-bentonite coupled addition relative to control. Nevertheless, the overall means of the calculated fermentation efficiencies reveals that, both fumaric acid addition and fumaric acid-bentonite coupled addition were associated with higher values relative to control, however, the increment effect induced by coupled addition surpassed that induced by fumaric acid addition (3.72% vs. 1.6%). In contrast, VFAs utilization index expressed by (NGGR) in both fumaric acid addition and fumaric acid-bentonite coupled addition was lower than control. However, the lowering effect of coupled addition had exceeded that of fumaric acid addition (34.65% vs. 15.2%).

### 4. Discussion

*In vitro* studies have the advantage not only of being less expensive and less time-consuming, but they also allow maintaining experimental conditions more precisely than do *in vivo* trials. However, they reflect the pattern but not the extent of rumen fermentation as shown by Demeyer (1991).

Benefits of adding antibiotics to ruminants feed include a shift in the acetate-to-propionate ratio toward more propionate and an associated decrease in methanogenesis (Russell and Houlihan, 2003) that reflects positively on the efficiency of nutrient use by ruminants. Fumarate is an intermediate in one of the pathways of propionate formation (Russell & Wallace, 1997) and has been extensively studied as an alternative electron sink to ruminal methanogenesis (Castillo *et al.*, 2004). One mol of fumarate converted to propionate would stoichiometrically decrease CH<sub>4</sub> production by 5.6 liter (Newbold *et al.*, 2005). however the efficacy of fumarate to play this role is limited by the inability of fumarate-reducers to compete for H<sub>2</sub> with methanogens. this study is an attempt to potentiate the efficacy of fumarate reduction into propionates and improving fermentation efficiency by coupling bentonite to fumaric acid addition.

From the foregoing results it has been observed that, coupling bentonite to fumaric acid addition was associated with an additional increase in propionic acid production that was accompanied by additional decrease in CH<sub>4</sub> production relative to addition of fumaric acid singly. The influence of bentonite on rumen fermentation pattern was examined in several studies. Galyean and Chabot (1981) did not find any significant changes in the VFAs profile of bull rumen under the influence of a mineral mixture containing sodium bentonite. The rumen parameters were examined in sheep supplemented with bentonite, and no significant differences were found in

case of animals fed bulky feed, whereas when they were fed concentrate, an increase in the percentage participation of acetate at the expense of propionate was obtained (Murray *et al.*, 1990), thus the effect was opposite to the one obtained in this research. Moreover, addition of magnesium-mica to heifers did not cause any significant changes in ruminal VFAs content (Coffey *et al.*, 2000). Therefore, increased propionates under the influence of fumaric acid-bentonite coupled addition seems to be first of all, a result of fumaric acid itself. However, the additional increase in propionic acid production achieved by coupling bentonite to fumaric acid could be really attributed to the antagonistic actions that bentonite exerts on protozoal – methanogens and protozoal – amylolytic bacteria interrelationships. Bentonite interferes with protozoal activity (Wallace & Newbold, 1991) and some ruminal methanogens associate metabolically with protozoa for greater H<sub>2</sub> availability (Finlay *et al.*, 1994) a relation that may augment the capacity of methanogens to compete for H<sub>2</sub> with fumarate-reducers. In agreement, fumarate effects on methanogenesis were more pronounced in protozoa-depleted than in protozoa-enriched ruminal fluid (Asanuma *et al.*, 1999). In this regard coupling bentonite to fumaric acid could give fumarate-reducers an advantage in the competition for H<sub>2</sub> with methanogens. Additionally, protozoa ingest both starch grains and amylolytic bacteria associated with them (Jouany, 1997). These amylolytic bacteria are succinate producing and act synergistically with succinate decarboxylating *Selenomonas ruminantium* to give propionates leading to better energy use since propionate metabolism is more favorable than acetate and butyrate ones (Eugene *et al.*, 2004). In this regard and because of its small particle size, protozoa are probably deceived into gathering bentonite particles instead of starch and bacterial particles. Bentonite slows the capture rate of microbes by protozoa because of its huge surface area and the electrical charges on its surface (Heijnen *et al.*, 1991, Wallace & Newbold, 1991). This also could provide more substrates for amylolytic bacterial attack and probably this might be the cause of higher VFAs concentrations achieved by coupled addition. Moreover, the observed decrease in butyrates molar proportions associated with coupled addition is probably also related to decreased protozoal activities since it is well established that protozoa are important butyrate producers (Jouany, 1991).

In this research, neither fumaric acid addition nor fumaric acid-bentonite coupled addition altered the profile of the major long chain VFAs (valeric – isovaleric - isobutyric). This probably reveals a balanced microbial deaminative activity as deamination of branched chain amino acids represents the major source of long chain VFAs (Hino & Russell, 1985).

The tendency to lower NH<sub>3</sub>-N concentrations in the fermentation fluid observed by coupled addition could be attributed either to a greater ammonia utilization by rumen microbes or to the great ability of bentonite to adsorb ammonia when present at high concentrations (Saleh, 1994). Furthermore, inhibition of protozoa is often associated with reduced ruminal NH<sub>3</sub>-N concentration (Williams & Withers, 1993) presumably resulting from a reduction in protozoal proteolytic and deaminative activity.

The recorded pH values here were within the normal range required for optimum microbial activities (Russell and Wilson, 1996). One could expect that the concentrations of VFAs and NH<sub>3</sub> in the fermentation fluid have a decisive influence on the recorded pH, as they are the main sources of H<sup>+</sup> and OH<sup>-</sup> and hence, reduction of pH associated with coupled addition is corresponded well with VFAs and NH<sub>3</sub> concentrations.

Advantageously, cellulase activity within the fermentation fluid did not alter by coupled addition which points to efficient H<sub>2</sub> disposal without negative drawbacks on cellulolytic bacterial activity. Conversely, bentonite can improve fungal cellulolytic activity by protecting fungal rhizobium against predation by rumen protozoa as suggested by Heijnen *et al.* (1991).

The fermentation efficiencies calculated in this study were in the following order: fumaric acid-bentonite coupled addition > fumaric acid addition > control. The value for coupled addition amounted to 80.82 % and this was higher than the value calculated for fumaric acid addition by about 1.6% and higher than that calculated for control by about 2.9%. Calculation of fermentation efficiency is based on conversion of hexose energy to VFAs energy on the basis of equations worked out by Orskov (1975) and modified by Baran and Zitnan (2002). Metabolism of VFAs is less efficient than metabolism of glucose as more energy is used for formation of one mole of ATP (Livesey and Elia, 1995). It is clear that increased fermentation efficiency achieved by coupled addition is actually the end result of its decremental effect on methane production and its ability to increase propionates at the expense of acetates and butyrates. In contrast to fermentation efficiency, VFAs utilization index (NGGR) being optimum at values amounts to 3.5 and higher values indicate the worse use of VFA (Czerkawski, 1986). The lowest value of NGGR, which indicates the best utilization of VFA, was achieved by fumaric acid-bentonite coupled addition (2.15). The 34.6% improvement in VFAs utilization under the influence of coupled addition versus 15.2% for addition of fumaric acid singly indicates an increase in the contribution of glucogenic VFAs in their total amount.

## 5. Conclusion

The principal effects of coupling bentonite to fumaric acid addition on *in vitro* rumen fermentation include increased contribution of propionate in the total VFAs concentrations, decreased methane production, improved fermentation efficiency and the rise of glucogenic VFA content compared to non-glucogenic ones. These results suggest that coupling bentonite to fumaric acid addition could be a feasible strategy for intensifying the response of rumen microbial system for addition of fumaric acid to smooth the progress of its use as a feed additive instead of ionophores. Even so, additional *in vivo* studies are needed to settle on the probable adaptation of rumen microflora to this coupled addition and its effect on animal performance.

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Table 1. Composition and chemical analysis of the used basal diet

| Ingredients                | % as fed | Chemical analysis                | % of dry matter |
|----------------------------|----------|----------------------------------|-----------------|
| Barely grain               | 39.56    | Crude fibers                     | 31.00           |
| Berseem hay                | 40.00    | Crude proteins                   | 13.00           |
| Wheat straw                | 20.14    | Ether extract                    | 2.80            |
| Vitamin and mineral premix | 0.30     | Nitrogen free extract            | 32.50           |
|                            |          | total ash                        | 10.60           |
|                            |          | Digestible energy (kcal/kg diet) | 2200            |

Table 2. Effect of treatment systems on fermentation pattern by mixed rumen micro-organisms after 24 hours *in vitro* incubation

| Parameter                       | Control (no additives)    | Fumaric acid addition     | Fumaric acid-bentonite coupled addition | L.S.D. |
|---------------------------------|---------------------------|---------------------------|---|--------|
| P <sup>H</sup> value            | 6.58 <sup>a</sup> ±0.49   | 6.52 <sup>a</sup> ±0.05   | 6.19 <sup>b</sup> ±0.11                 | 0.148  |
| Total VFAs conc. (µmol)         | 898.2 <sup>a</sup> ±22.84 | 916.2 <sup>a</sup> ±24.82 | 1091.4 <sup>b</sup> ±16.36              | 66.698 |
| Acetic acid (mol/100 mol)       | 49.89 <sup>a</sup> ±0.25  | 46.99 <sup>b</sup> ±0.56  | 44.95 <sup>c</sup> ±0.21                | 1.16   |
| Propionic acid (mol/100 mol)    | 24.80 <sup>a</sup> ±0.34  | 28.01 <sup>b</sup> ±0.53  | 33.22 <sup>c</sup> ±0.34                | 1.277  |
| Butyric acid (mol/100 mol)      | 19.04 <sup>a</sup> ±0.43  | 18.49 <sup>a</sup> ±0.29  | 15.00 <sup>b</sup> ±0.32                | 1.093  |
| Acetic / propionic ratio        | 2.01 <sup>a</sup> ±0.03   | 1.67 <sup>b</sup> ±0.05   | 1.35 <sup>c</sup> ±0.02                 | 0.108  |
| Valeric (mol/100 mol)           | 3.20±0.198                | 3.02±0.199                | 2.86±0.150                              | NS     |
| Isovaleric (mol/100 mol)        | 1.08±0.09                 | 1.30±0.19                 | 1.25±0.20                               | NS     |
| Isobutyric (mol/100 mol)        | 1.97±0.28                 | 2.17±0.28                 | 2.28±0.20                               | NS     |
| CH <sub>4</sub> (µmol)          | 253.42 <sup>a</sup> ±6.61 | 234.78 <sup>b</sup> ±6.55 | 236.49 <sup>b</sup> ±5.52               | 12.264 |
| CH <sub>4</sub> /total VFAs (%) | 28.26 <sup>a</sup> ±0.12  | 25.63 <sup>b</sup> ±0.44  | 21.66 <sup>c</sup> ±0.23                | 1.012  |
| Ammonia N. conc.(mg/dl)         | 11.62 <sup>a</sup> ±0.49  | 11.55 <sup>a</sup> ±0.54  | 7.69 <sup>b</sup> ±0.51                 | 1.594  |

Data presented as means ± SE, N =5

Values having different letters in the same row are significantly different at  $P < 0.05$

Table 3. Effect of treatment systems on cellulase activity, fermentation efficiency and VFAs utilization index (NGGR) after 24 hours *in vitro* incubation

| parameter   | Control (no additives)    | Fumaric acid addition     | Fumaric acid-bentonite coupled addition | L.S.D. |
|---|---------------------------|---------------------------|---|--------|
| Cellulase activity (mmol of glucose equivalent / min) | 5.29±0.119                | 5.18±0.149                | 5.46±0.113                              | NS     |
| Fermentation efficiency (%)                           | 77.92 <sup>a</sup> ±0.072 | 79.17 <sup>b</sup> ±0.228 | 80.82 <sup>c</sup> ±0.307               | 0.692  |
| VFAs utilization index (NGGR)                         | 3.30 <sup>a</sup> ±0.08   | 2.79 <sup>b</sup> ±0.06   | 2.15 <sup>c</sup> ±0.04                 | 0.198  |

Data presented as means ± SE, N =5

Values having different letters in the same row are significantly different at  $P < 0.05$