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**Original article**

# Use of some novel alternative electron sinks to inhibit ruminal methanogenesis

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**Abstract** — Several compounds were evaluated in vitro as alternative electron sinks to ruminal methanogenesis. They were incubated with ruminal fluid, buffer mixture, and finely ground alfalfa hay for 24 h, at 0, 6, 12, and 18 mM initial concentrations. The propionate enhancer oxaloacetic acid, the butyrate enhancer β-hydroxybutyrate, and the butyrate unsaturated analog 3-butenoic acid were ineffective in decreasing methanogenesis. Nevertheless, β-hydroxybutyrate increased apparent fermentation of the alfalfa hay substrate from 58.0 to 63.4%, and 3-butenoic acid seemed to increase it from 62.0 to 73.7%. Almost all of added oxaloacetic acid disappeared during the incubation, while only between 30.3 and 53.4% of ß-hydroxybutyrate disappeared. The butyrate enhancers acetoacetate and crotonic acid, and the butyrate unsaturated analog 2-butynoic acid, decreased methanogenesis by a maximum of 18,9 and 9%, respectively. Crotonic acid at 18 mM initial concentration seemed to increase the substrate apparent fermentation from 57.0 to 68.2%. Between 78.6 and 100% of acetoacetate disappeared during the incubation. The propionate unsaturated analog propynoic acid, and the unsaturated ester ethyl 2-butynoate, decreased methanogenesis by a maximum of 76 and 79%, respectively. Less than 5% of propynoic acid disappeared. The substrate apparent fermentation was decreased by propynoic acid from 62.0 to 57.4%, and seemed to have been decreased by ethyl 2-butynoate from 62.0 to 29.3%. More accurate measurements of the disappearance of some of the compounds studied are needed to better understand how they are metabolized and how they affect fermentation.

**rumen / methane / inhibition / in vitro**

## 1. INTRODUCTION

Methane emission is an energy loss for ruminants, and also causes global warming [1]. It would be beneficial both for the efficiency of production and the environment to divert reducing equivalents from ruminal methanogenesis into alternative electron sinks with a nutritional value for the host animal [2], e.g., by enhancing propionate formation [3].

Intermediates of the fermentation pathways that lead to propionate ("propionate

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enhancers") have been studied as alternative electron sinks to ruminal methanogenesis. Compounds that accept one pair of electrons in their conversion into propionate include malate [3–5], fumarate [3, 4], lactate, and acrylate [4]. Oxaloacetate, however, accepts two pairs of electrons, and, theoretically, should be more effective in competing with methanogenesis as an alternative electron sink. To our knowledge, oxaloacetate has not been examined for this purpose.

Likewise, intermediates in the conversion of pyruvate into butyrate ("butyrate enhancers") also accept reducing equivalents [6]. Butyrate enhancers have not been studied as alternative electron sinks to ruminal methanogenesis. Also, unsaturated analogs of propionate and butyrate with double and triple bonds could be reduced to these VFA, redirecting reducing equivalents away from $CH_4$ formation. These compounds, which are not normal intermediates of ruminal fermentation (except for acrylate and crotonate), have not been studied as alternative electron sinks to ruminal methanogenesis. We hypothesized that the addition of oxaloacetate, butyrate enhancers, and unsaturated organic acids and esters, would decrease ruminal methanogenesis in vitro. The objective of this study was to assess the effects of these compounds on in vitro fermentation by mixed ruminal microbial cultures.

## 2. MATERIALS AND METHODS

### 2.1. Additives and concentrations

The intermediate of the propionate pathway, oxaloacetic acid [free acid, Sigma O 4126], three intermediates of the butyrate pathway [6], acetoacetate [Li salt, Sigma A 8509], β-hydroxybutyrate [Na salt, Sigma H 6501], and crotonic acid [free acid, Sigma C 4630], the unsaturated propionate analog propynoic acid [free acid, Acros 13150-0100], the unsaturated butyrate analogues 3-butenoic acid [free acid, Acros 15883-0250], and 2-butynoic acid [free acid, Acros 30806-0010] and the unsaturated ester ethyl 2-butynoate [Aldrich 4341-76-8] were examined as alternative electron sinks to ruminal methanogenesis in vitro. Each of the additives, except for ethyl 2-butynoate, was added to Wheaton bottles as 1 mL aqueous solutions, so as to achieve 6, 12 and 18 mM initial concentrations, respectively (without considering the substrate volume, which was very small in comparison to the volume of the fermentation medium; see Sect. 2.2). The hydrophobic ester, ethyl 2-butynoate, was added directly as a liquid (35.7, 71.3, and 107.0 μL, to achieve 6, 12 and 18 mM initial concentrations, respectively) together with 1 mL of deionized water. Controls received 1 mL of deionized water. The initial concentrations, which could be considered as relatively high, were chosen based on the additives hypothesized mode of action: the effectiveness of an additive for withdrawing electrons from methanogenesis should keep some stoichiometrical relation with the amount of additive reduced. As this was the first time these compounds were studied, a wide range of initial concentrations was chosen. Similar ranges of initial concentrations when other organic acids were studied as alternative electron sinks to ruminal methanogenesis have been used before [3–5].

Oxaloacetic acid, acetoacetate, β-hydroxybutyrate, and crotonic acid were examined together in two experimental runs, while propynoic acid, 3-butenoic acid, 2-butynoic acid, and ethyl 2-butynoate were examined in a third and a fourth experimental runs.

### 2.2. Ruminal fluid collection and incubation

The study was approved by the Michigan State University All-University Committee on Animal Use and Care (Animal Use Form 03/02-043-00). Ruminal fluid was withdrawn within two hours of the morning feeding from two mature Holstein cows fed alfalfa

hay. It was mixed together, and strained through two layers of cheesecloth. It was then blended for 15 s, and again strained through two layers of cheesecloth. One volume part of ruminal fluid was mixed with four volume parts of a bicarbonate and phosphate buffer [7], and 50 mL of the ruminal fluid and buffer mixture anaerobically delivered into 125-mL Wheaton bottles. All the bottles contained 300 mg of ground (0.2 mm screen mesh) alfalfa hay (11.4% CP in the DM) as substrate. Three samples of the ruminal fluid and buffer mixture were frozen for subsequent determination of VFA initial concentrations. Bottles were sealed under an $O_2$-free $CO_2$ atmosphere, and incubated in a shaking water bath at 39 °C for 24 h. At the end of the incubation, bottles were allowed to cool to room temperature, and total gas production volume was measured [3]. Fermentation was then stopped by adding 1 mL of a 10% phenol solution.

### 2.3. Analytical procedures

Methane and $CO_2$ were analyzed [3] using a Gow Mac series 750 flame ionization detector gas chromatograph (Gow Mac Instruments Co., Bridgewater, NJ) equipped with a 4' × 1/4" DC 200 stainless steel column (150 °C, carrier gas was $N_2$ at 820 kPa). A RGD2 Reduction Gas Detector (Trace Analytical, Menlo Park, CA) and the same type of column were used for $H_2$ analysis. The volume of gas produced was expressed as μmoles at 25 °C and 1 atm. A 5-mL aliquot was centrifuged (26 000 × *g*, 4 °C, 30 min), and the pH was measured in the supernatants (Digital Benchtop pH Meter, Cole-Parmer Instrument Company, Vernon Hills, IL). Volatile fatty acids, lactate, formate, ethanol, and the chemical additives were quantified by differential refractometry with a Waters 712 Wisp HPLC (Waters Associates Inc., Milford, MA) equipped with a BioRad HPX 87H column (BioRad Laboratories, Hercules, CA). Separation was done by ion moderated partition. Solvent was 0.005 M $H_2SO_4$ at 0.6 mL·min$^{-1}$. Column temperature was 65 °C. Sample injection volume was 15 μL. Ammonia was analyzed as reported before [8].

### 2.4. Calculations

Based on known biochemical pathways, some of the fermentation intermediates added were not expected to produce gases. Calculations based on VFA production stoichiometry [9] would have then overestimated apparently fermented OM (FOM). Therefore, FOM and substrate apparently fermented were calculated by mass balance from the net production of VFA, lactate, gases, and ammonia. As ethanol, formate, and succinate accumulated in some of the treatments, they were also included in the calculation:

FOM (%) = (gases + VFA + lactate + ethanol + formate + succinate + $NH_4^+$) × 100 / (substrate OM + additive OM),

with all fermentation products produced, substrate and additives expressed in grams.

Substrate apparently fermented (%) = (FOM (g) – additive disappeared (g)) × 100 / (substrate OM (g)).

Crotonic acid and 2-butynoic acid co-eluted off the HPLC column with isovalerate and isobutyrate, respectively. As the amounts of isovalerate and isobutyrate produced are relatively minor in comparison to the major VFA, acetate, propionate, and butyrate, isovalerate was not included in the calculations for estimating FOM in the crotonic acid treatments, and isobutyrate in the 2-butynoic acid treatments. Disappearance of crotonic acid and 2-butynoic acid are not reported. Similarly, disappearances are not reported for 3-butenoic acid and ethyl 2-butynoate, as these additives co-eluted off the HPLC column with propionate and butyrate, respectively. Organic matter and substrate fermentation are not reported for these additives.

Hydrogen balances was calculated [10], with net production of ammonia (one mole of ammonia produced releases one mole of reducing equivalent pairs [11]) also considered. Net production of ethanol, lactate, and formate were also considered, ethanol and lactate formations releasing and accepting one pair of reducing equivalents each [11], and formate incorporating one pair of reducing equivalents [12]:

H produced (μmol) = 2A + P + 4B + 3V + $NH_4^+$ + E + L,

H incorporated (μmol) = 2P + 2B + 4V + $4CH_4$ + $H_2$ + F + E + L,

H recovery (%) = H incorporated × 100 / H produced,

where A = acetate, P = propionate, B = butyrate, V = valerate, E = ethanol, L = lactate, and F = formate, all expressed as μmol. VFA and lactate were considered as nutritionally useful H sinks, while methane, dihydrogen, formate, and ethanol were considered as H sinks without a nutritional value. The H balance was not calculated for 3-butenoic acid and ethyl 3-butynoate, as these additives co-eluted off the HPLC column with propionate and butyrate.

### 2.5. Statistical analysis

Two replicates per compound and concentration were used in each of the two experimental runs. Orthogonal contrasts were performed to determine linear, quadratic, and cubic effects. The experimental run was modeled as a random block [13]. Significance was declared at $P < 0.05$.

## 3. RESULTS

### 3.1. Oxaloacetic acid

Production of $CH_4$ linearly increased ($P < 0.001$) by 7, 8, and 13%, at 6, 12, and 18 mM initial concentration, respectively (Tab. I). The release of $CO_2$ was linearly increased ($P < 0.001$). $H_2$ accumulation was not affected.

Oxaloacetic acid was almost totally fermented. There was a linear increase in total VFA concentration ($P < 0.001$), and production of acetate ($P < 0.001$), propionate ($P < 0.05$), butyrate ($P < 0.01$), valerate ($P < 0.05$; data not shown), and isovalerate ($P < 0.05$; data not shown). Production of isobutyrate (data not shown), the final pH, and $NH_4^+$ concentration were not affected. Oxaloacetic acid linearly decreased ($P < 0.001$) the alfalfa substrate apparent fermentation from 58.0 to 35.8%. Oxaloacetic acid linearly increased ($P < 0.001$; data not shown) both H produced and incorporated, but decreased ($P < 0.001$; data not shown) H recovery from 81.8 to 66.5%. The percentage of nutritionally useful H incorporated was not affected by oxaloacetate (data not shown).

### 3.2. Acetoacetate

Addition of acetoacetate linearly decreased ($P < 0.05$) $CH_4$ production by 5, 18, and 10% at 6, 12, and 18 mM initial concentration, respectively (Tab. II). Release of $CO_2$ was not affected, while $H_2$ accumulation was linearly decreased ($P < 0.001$) by 32%.

Acetoacetate co-eluted off our HPLC column with formate. As formate concentration in the rumen is normally very small [14], reasonable disappearances could be calculated by assuming that there was not formate present. The percentage of acetoacetate disappeared decreased linearly ($P < 0.001$) with the initial concentration. Total VFA concentration, and production of acetate, butyrate, and isovalerate (data not shown) were linearly increased ($P < 0.001$) by the addition of acetoacetate. Propionate, isobutyrate (data not shown), and valerate (data not shown) production, the substrate apparent fermentation, and the final pH, were not affected. Ammonia concentration was lowest ($P < 0.05$) at 12 mM initial

**Table I.** Effects of the addition of oxaloacetic acid on in vitro ruminal fermentation.

| | Initial concentration, mM | | | | Linear effect | Quadratic effect | Cubic effect | SEM |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 18 | | | | |
| $CH_4$ (µmol)[1] | 421 | 452 | 454 | 476 | < 0.001 | 0.46 | 0.13 | 4.27 |
| $CO_2$ (µmol)[1] | 911 | 1091 | 1276 | 1327 | < 0.001 | 0.37 | 0.98 | 40.1 |
| $H_2$ (µmol)[1] | 0.41 | 0.46 | 0.44 | 0.61 | 0.12 | 0.36 | 0.47 | 0.16 |
| Additive disappearance (%) | – | 98.7 | 99.0 | 99.3 | 0.64 | 0.98 | – | 0.896 |
| Total VFA (mM) | 54.5 | 59.4 | 64.2 | 66.5 | < 0.001 | 0.13 | 0.49 | 0.783 |
| Acetate (µmol)[1] | 1111 | 1330 | 1547 | 1688 | < 0.001 | 0.14 | 0.51 | 23.8 |
| Propionate (µmol)[1] | 345 | 365 | 377 | 372 | < 0.05 | 0.09 | 0.81 | 6.67 |
| Butyrate (µmol)[1] | 136 | 151 | 165 | 172 | < 0.01 | 0.51 | 0.75 | 5.84 |
| Substrate apparently fermented (%) | 58.0 | 52.3 | 41.2 | 35.8 | < 0.001 | 0.96 | 0.44 | 3.09 |
| Final pH | 6.86 | 6.99 | 6.79 | 6.94 | 0.92 | 0.87 | 0.10 | 0.080 |
| $NH_4^+$ (mg·$L^{-1}$) | 262 | 249 | 234 | 252 | 0.32 | 0.13 | 0.41 | 9.06 |

[1] 24 h incubation.

**Table II.** Effects of the addition of acetoacetate on in vitro ruminal fermentation.

| | Initial concentration, mM | | | | Linear effect | Quadratic effect | Cubic effect | SEM |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 18 | | | | |
| $CH_4$ (µmol)[1] | 421 | 400 | 346 | 377 | < 0.05 | 0.13 | 0.12 | 15.2 |
| $CO_2$ (µmol)[1] | 911 | 987 | 936 | 998 | 0.56 | 0.92 | 0.51 | 78.6 |
| $H_2$ (µmol)[1] | 0.41 | 0.35 | 0.35 | 0.28 | < 0.001 | < 0.10 | 0.62 | 0.011 |
| Additive disappearance (%) | – | 100 | 93.2 | 78.6 | < 0.001 | < 0.10 | – | 1.79 |
| Total VFA (mM) | 54.5 | 63.1 | 70.9 | 75.8 | < 0.001 | 0.12 | 0.68 | 1.08 |
| Acetate (µmol)[1] | 1111 | 1542 | 1922 | 2110 | < 0.001 | < 0.05 | 0.51 | 45.7 |
| Propionate (µmol)[1] | 345 | 336 | 330 | 337 | 0.20 | 0.11 | 0.54 | 4.51 |
| Butyrate (µmol)[1] | 136 | 172 | 208 | 259 | < 0.001 | < 0.10 | 0.38 | 3.79 |
| Substrate apparently fermented (%) | 58.0 | 58.3 | 56.6 | 58.8 | 0.93 | 0.55 | 0.41 | 1.53 |
| Final pH | 6.86 | 6.88 | 6.84 | 6.91 | 0.69 | 0.56 | 0.43 | 0.046 |
| $NH_4^+$ (mg·$L^{-1}$) | 262 | 248 | 237 | 253 | 0.41 | < 0.05 | 0.42 | 6.80 |

[1] 24 h incubation.

concentration of acetoacetate. H produced was linearly ($P < 0.001$; data not shown) increased by acetoacetate. As H incorporated was not affected, H recovery was decreased from 81.8 to 48.0% ($P < 0.001$; data not shown). The percentage of nutritionally useful H incorporated was linearly increased ($P < 0.001$) by acetoacetate from 38.4 to 45.8% (data not shown).

### 3.3. β-hydroxybutyrate

Addition of β-hydroxybutyrate did not affect $CH_4$ production or $H_2$ accumulation (Tab. III). The release of $CO_2$ was linearly increased ($P < 0.05$). Similar to acetoacetate, β-hydroxybutyrate co-eluted off our HPLC column with formate, and no formate present was assumed when calculating its disappearance. The percentage of β-hydroxybutyrate disappeared decreased linearly ($P < 0.001$) with its initial concentration. Total VFA concentration, and acetate and butyrate production were linearly increased ($P < 0.001$) by the addition of β-hydroxybutyrate. The substrate apparent fermentation was linearly increased ($P < 0.05$) from 58.0 to 63.4%. However, as not all the additive disappeared, FOM tended ($P < 0.10$; data not shown) to decrease from 58.0 to 55.0%. Propionate production, the final pH, and $NH_4^+$ concentration (Tab. III), isobutyrate, valerate, and isovalerate production (data not shown) were not affected. H produced and incorporated were linearly increased ($P < 0.001$; data not shown) by β-hydroxybutyrate, but H recovery was decreased ($P < 0.001$; data not shown) from 81.8 to 66.5%. The percentage of nutritionally useful H incorporated was linearly increased ($P < 0.001$; data not shown) by β-hydroxybutyrate from 38.4 to 43.6% (data not shown).

**Table III.** Effects of β-hydroxybutyrate on in vitro ruminal fermentation.

| | Initial concentration, mM | | | | Linear effect | Quadratic effect | Cubic effect | SEM |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 18 | | | | |
| $CH_4$ (μmol)[1] | 421 | 425 | 423 | 442 | 0.24 | 0.52 | 0.61 | 10.9 |
| $CO_2$ (μmol)[1] | 911 | 927 | 937 | 1041 | < 0.05 | 0.22 | 0.52 | 33.0 |
| $H_2$ (μmol)[1] | 0.41 | 0.39 | 0.50 | 0.43 | 0.28 | 0.36 | < 0.10 | 0.029 |
| Additive disappearance (%) | – | 53.4 | 38.8 | 30.3 | < 0.001 | 0.30 | – | 2.23 |
| Total VFA (mM) | 54.5 | 59.4 | 61.2 | 65.0 | < 0.001 | 0.59 | 0.31 | 1.01 |
| Acetate (μmol)[1] | 1111 | 1296 | 1379 | 1511 | < 0.001 | 0.47 | 0.35 | 34.1 |
| Propionate (μmol)[1] | 345 | 348 | 337 | 353 | 0.59 | 0.36 | 0.16 | 5.89 |
| Butyrate (μmol)[1] | 136 | 207 | 242 | 281 | < 0.001 | 0.17 | 0.44 | 10.6 |
| Substrate apparently fermented (%) | 58.0 | 58.4 | 58.4 | 63.4 | < 0.05 | 0.13 | 0.43 | 1.38 |
| Final pH | 6.86 | 6.84 | 6.85 | 6.93 | 0.27 | 0.24 | 0.81 | 0.038 |
| $NH_4^+$ ($mg \cdot L^{-1}$) | 262 | 249 | 254 | 248 | 0.51 | 0.79 | 0.58 | 11.7 |

[1] 24 h incubation.

### 3.4. Crotonic acid

Production of $CH_4$ was 4, 9, and 2% lower ($P < 0.05$; data not shown) than the control at 6,12, and 18 mM initial concentration, respectively. The release of $CO_2$ was linearly increased ($P < 0.05$) by 24%, and $H_2$ accumulation was not affected (data not shown).

Crotonic acid disappearance was not estimated because it co-eluted off the HPLC column with isovalerate. Total VFA concentration ($P < 0.001$), and production of acetate ($P < 0.001$), butyrate ($P < 0.001$), isobutyrate ($P < 0.05$), and valerate ($P < 0.001$) were increased by crotonic acid (data not shown). Propionate production and $NH_4^+$ concentration were not affected (data not shown). If control levels of isovalerate are assumed, crotonate increased ($P < 0.05$; cubic response; data not shown) the substrate apparent fermentation from 57.0 to 68.2%. Final pH was linearly decreased ($P < 0.01$; data not shown). H produced and incorporated were linearly increased ($P < 0.001$; data not shown) by crotonic acid, but H recovery was linearly decreased ($P < 0.001$; data not shown) from 81.8 to 56.7%. The percentage of H incorporated into nutritionally useful products was linearly increased ($P < 0.001$; data not shown) from 38.4 to 49.1%.

### 3.5. Propynoic acid

Methane production was decreased ($P < 0.001$) by 65, 72, and 76%, at 6, 12, and 18 mM initial concentration, respectively (Tab. IV). The release of $CO_2$ was linearly decreased ($P < 0.05$). Propynoic acid caused ($P < 0.001$) a 42, 53, and 51-fold increase in $H_2$ accumulation, at 6, 12, and 18 mM initial concentration, respectively.

Less than 5% of propynoic acid disappeared. Disappearance was not affected by initial concentration (Tab. IV). Total VFA

**Table IV.** Effects of the addition of propynoic acid on in vitro ruminal fermentation.

| | Initial concentration, mM | | | | Linear effect | Quadratic effect | Cubic effect | SEM |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 18 | | | | |
| $CH_4$ (µmol)[1] | 447 | 155 | 123 | 108 | < 0.001 | < 0.001 | < 0.001 | 5.18 |
| $CO_2$ (µmol)[1] | 1010 | 874 | 906 | 794 | < 0.05 | 0.84 | 0.25 | 55.7 |
| $H_2$ (µmol)[1] | 0.93 | 38.6 | 49.5 | 47.5 | < 0.001 | < 0.001 | < 0.05 | 0.81 |
| Additive disappearance (%) | – | 3.38 | 3.42 | 4.48 | 0.39 | 0.54 | – | 0.849 |
| Total VFA (mM) | 56.8 | 52.5 | 51.5 | 49.3 | < 0.001 | 0.13 | 0.14 | 0.62 |
| Acetate (µmol)[1] | 1224 | 961 | 922 | 880 | < 0.001 | < 0.001 | < 0.01 | 13.5 |
| Propionate (µmol)[1] | 342 | 391 | 335 | 290 | < 0.001 | < 0.001 | < 0.01 | 7.23 |
| Butyrate (µmol)[1] | 144 | 152 | 155 | 135 | 0.35 | < 0.05 | 0.50 | 5.74 |
| Substrate apparently fermented (%) | 62.0 | 56.6 | 62.5 | 57.4 | 0.16 | 0.95 | < 0.01 | 1.15 |
| Final pH | 7.01 | 6.94 | 6.83 | 6.80 | < 0.001 | 0.63 | 0.46 | 0.033 |
| $NH_4^+$ ($mg \cdot L^{-1}$) | 239 | 180 | 183 | 168 | < 0.001 | < 0.01 | < 0.05 | 6.4 |

[1] 24 h incubation.

concentration, acetate production, the final pH, and $NH_4^+$ concentration, were all linearly decreased ($P < 0.001$). Propionate production was maximum at 6 mM initial concentration, and then decreased ($P < 0.001$). Butyrate production increased at 6 and 12 mM concentration of propynoic acid, and decreased at 18 mM ($P < 0.05$; quadratic response). Isobutyrate and isovalerate production were minimum at 6 mM initial concentration ($P < 0.05$; cubic response; data not shown). Valerate production was not affected (data not shown). The substrate apparent fermentation was decreased from 62.0 to 56.6 and 57.4% at 6 and 18 mM initial concentration, respectively, but not affected at 12 mM ($P < 0.01$; cubic response). Although FOM (%) was decreased ($P < 0.001$) by propynoic acid, $CH_4$ production per milligram of FOM was decreased by 61, 73, and 73%, at 6, 12, and 18 mM initial concentration, respectively (data not shown).

Propynoic acid caused the accumulation of some compounds unusually observed as end products of ruminal fermentation (data not shown). Formate was increased ($P < 0.001$) from 0.48 to a maximum of 5.59 mM, and ethanol from 0.11 to a maximum of 3.17 mM ($P < 0.001$), both at 12 mM propynoic acid. Also, succinate concentration was increased ($P < 0.001$) from 0.02 to 1.10 mM.

Propynoic acid decreased ($P < 0.001$; data not shown) H produced and incorporated. H recovery decreased ($P < 0.001$; data not shown) from 81.2 to 66.0%. The percentage of H incorporated into nutritionally useful fermentation end products was increased ($P < 0.001$; data not shown) from 37 to 50%.

### 3.6. 3-Butenoic acid

Methane production tended ($P < 0.10$) to decrease linearly by 5%, and $H_2$ accumulation was not affected (data not shown). There was a 25% linear increase ($P < 0.05$; data not shown) in $CO_2$ release with the addition of 3-butenoic acid. Acetate and butyrate production were linearly ($P < 0.001$; data not shown) increased. Valerate and isovalerate production were maximum ($P < 0.01$; data not shown) at 6 mM initial concentration of 3-butenoic acid. Isobutyrate tended ($P < 0.10$; data not shown) to increase linearly. The co-elution of 3-butenoic acid and propionate off the HPLC column prevented us from finding propionate production and 3-butenoic acid disappearance. If 100% disappearance of 3-butenoic acid is assumed, it would have increased ($P < 0.05$; cubic response; data not shown) the substrate apparent fermentation from 62.0 to 74.0, 68.3, and 73.7%, at 6, 12, and 18 mM initial concentration, respectively. Final pH tended ($P < 0.10$) to decrease linearly (data not shown). Ammonia concentration was not affected (data not shown).

### 3.7. 2-Butynoic acid

Methane production was linearly decreased ($P < 0.01$) by 4, 6, and 9% at 6, 12, and 18 mM initial concentration, respectively (data not shown). The release of $CO_2$, and $H_2$ accumulation, were not affected (data not shown). Total VFA concentration was maximum ($P < 0.001$; data not shown) at 6 mM 2-butynoic acid. Acetate and propionate production were decreased ($P < 0.01$; data not shown) at 12 and 18 mM initial concentration. Butyrate, valerate, and isovalerate production were maximum ($P < 0.001$; data not shown) at 6 mM initial concentration. Apparently FOM was decreased ($P < 0.001$; data not shown) by 2-butynoic acid from 61.4 to 48.6%. The final pH and $NH_4^+$ concentration were both linearly decreased ($P < 0.01$; data not shown) by 2-butynoic acid. H produced and incorporated were highest ($P < 0.001$; data not shown) at 6 mM 2-butynoic acid, but H recovery was not affected (data not shown). The percentage of H incorporated into nutritionally useful end products was highest

($P < 0.001$; data not shown) at 6 mM 2-butynoic acid.

### 3.8. Ethyl 2-butynoate

Methane production was linearly decreased ($P < 0.001$) by 24, 64, and 79%, at 6, 12, and 18 mM initial concentration, respectively (Tab. V). Release of $CO_2$ was also linearly decreased ($P < 0.01$). Ethyl 2-butynoate caused ($P < 0.001$) a 12, 28, and 37-fold increase in $H_2$ accumulation, at 6, 12, and 18 mM initial concentration, respectively. Acetate production and $NH_4^+$ concentration were linearly decreased ($P < 0.001$). Propionate ($P < 0.001$), valerate ($P < 0.001$; data not shown), and isovalerate ($P < 0.001$; data not shown) production were maximum at 6 mM initial concentration, and dropped at 12 and 18 mM. Butyrate production was not determined because it co-eluted off the HPLC column with ethyl 2-butynoate. Isobutyrate production was linearly increased ($P < 0.001$; data not shown) 7, 13, and 18-fold, at 6, 12, and 18 mM initial concentration, respectively. Apparent fermentation of OM and the substrate were not estimated as the co-elution of ethyl 2-butynoate and butyrate off the HPLC column prevented us from finding butyrate production and ethyl 2-butynoate disappearance. If 100% disappearance is assumed, ethyl 2-butynoate would have linearly decreased ($P < 0.001$; data not shown) the substrate fermentation from 62.0 to 29.3%. The final pH was not affected.

Ethyl 2-butynoate caused the accumulation of some unusual end products of ruminal fermentation (data not shown). Formate concentration was increased ($P < 0.01$) from 0.48 to 6.11 mM at 18 mM ethyl 2-butynoate. Also, ethanol concentration was increased ($P < 0.001$) from 0.12 to 10.4 mM at 18 mM ethyl 2-butynoate.

**Table V.** Effects of the addition of ethyl 2-butynoate on in vitro ruminal fermentation.

| | Initial concentration, mM | | | | Linear effect | Quadratic effect | Cubic effect | SEM |
|---|---|---|---|---|---|---|---|---|
| | 0 | 6 | 12 | 18 | | | | |
| $CH_4$ (μmol)[1] | 447 | 340 | 160 | 93.9 | < 0.001 | 0.63 | 0.33 | 40.3 |
| $CO_2$ (μmol)[1] | 1010 | 909 | 704 | 748 | < 0.01 | 0.30 | 0.26 | 65.6 |
| $H_2$ (μmol)[1] | 0.93 | 11.1 | 25.8 | 34.4 | < 0.001 | 0.80 | 0.47 | 3.10 |
| Additive disappearance (%) | – | NA[2] | NA | NA | NA | NA | NA | NA |
| Total VFA (mM) | 56.2 | NA[2] | NA | NA | NA | NA | NA | NA |
| Acetate (μmol)[1] | 1224 | 1181 | 931 | 792 | < 0.001 | 0.32 | 0.16 | 45.5 |
| Propionate (μmol)[1] | 342 | 427 | 358 | 329 | < 0.05 | < 0.001 | < 0.01 | 9.89 |
| Butyrate (μmol)[1] | 144 | NA[2] | NA | NA | NA | NA | NA | NA |
| Substrate apparently fermented (%) | 62.0 | NA[2] | NA | NA | NA | NA | NA | NA |
| Final pH | 7.01 | 6.97 | 6.91 | 6.87 | 0.17 | 0.93 | 0.92 | 0.071 |
| $NH_4^+$ ($mg \cdot L^{-1}$) | 239 | 200 | 183 | 170 | < 0.001 | < 0.10 | 0.53 | 5.5 |

[1] 24 h incubation.
[2] NA = not available. As ethyl 2-butynoate co-eluted the HPLC with butyrate, their final concentrations, and total VFA, could not be determined, and fermentation could not be estimated.

## 4. DISCUSSION

### 4.1. Oxaloacetate and butyrate enhancers

Acetate, followed by $CO_2$, seemed to be the major C sink of the metabolism of added oxaloacetic acid. Therefore, most of added oxaloacetic acid was not fermented to propionate, as it was hypothesized, but perhaps decarboxylated to pyruvate, and subsequently decarboxylated again to acetate, releasing one pair of reducing equivalents. The increase observed in the release of $CO_2$ suggests that oxaloacetic acid in fact underwent decarboxylation. The slight increase in $CH_4$ production might have been a consequence of the release of reducing equivalents in the oxidative decarboxylation of pyruvate into acetate. Oxaloacetate, is, however, an intermediate of a ruminal fermentation pathway leading to propionate [12]. It was expected to be metabolized to propionate, rather than to acetate. It is possible that most of externally added oxaloacetic acid was taken up by microbial species whose main fermentation end product is acetate, rather than propionate.

Acetate, rather than butyrate, as we had hypothesized, also seem to have been the major C sink of added acetoacetate and β-hydroxybutyrate. More of the latter, however, seemed to be converted to butyrate. Acetoacetate could have been broken down into two moles of acetate, which agrees with the fact that $CO_2$ release did not increase. The existence of a preferred pathway towards acetate could have allowed the greater disappearance observed for acetoacetate as compared to β-hydroxybutyrate, as β-hydroxybutyrate would need to be oxidized to acetoacetate in order to be converted to acetate. Similar to oxaloacetic acid, it is possible that microbial species different from the butyrate producers that normally metabolize these compounds took the externally added additives, and metabolized them to acetate.

More of the added crotonic acid was fermented to butyrate, as compared to the other additives, but acetate still was an important C sink. Similarly, the sewage anaerobic bacterium *Syntrophomonas wolfei* catabolized crotonate to acetate and smaller proportions of butyrate and caproate [15]. Thus, the added organic acids did not seem to have been metabolized only by the pathways of which they are intermediates in ruminal fermentation.

Oxaloacetic acid and β-hydroxybutyrate did not inhibit $CH_4$ production. Acetoacetate caused a small decrease in $CH_4$ production, without inhibiting fermentation or causing the accumulation of end products of fermentation without a nutritional value. The small decrease in $CH_4$ production caused by crotonic acid could be partly due to the decrease in pH that it caused [16], as it was added as a free acid. Furthermore, the fact that the pH was not measured at the $CO_2$ partial pressure present in the Wheaton bottles before opening them, probably resulted in some overestimation of the final pH, due to loss of dissolved $CO_2$ [17]. β-Hydroxybutyrate at 18 mM initial concentration stimulated the substrate apparent fermentation. Crotonic acid seemed to have the same effect. Stimulation of fermentation of a high roughage substrate has been reported for pyruvate, lactate, fumarate, malate, 2-oxoglutarate and tartrate [4]. Due to its low disappearance, β-hydroxybutyrate did not affect OM apparent fermentation. Butyrate absorbed through the rumen and omasal walls is converted into β-hydroxybutyrate, and used as an energy source [18]. Then, externally added β-hydroxybutyrate not disappearing in ruminal fermentation would be usable for the ruminant, if it could be absorbed as such.

All of the additives decreased H recovery. The inhibition of methanogenesis may have stimulated some H sinks that were not measured, like sulfate and nitrate reductions [19], and fatty acids biohydrogenation and synthesis [20]. Acetoacetate, β-hydroxybutyrate, and crotonic acid, increased the percentage

of H incorporated into nutritionally useful sinks. In case of β-hydroxybutyrate, which did not inhibit $CH_4$ production, this can be understood if the fermentation of the additive itself did not produce additional $CH_4$, or produced less $CH_4$ than the substrate fermentation.

### 4.2. Unsaturated organic acids and esters

A shift of the VFA profile from acetate to propionate when methanogenesis is inhibited has been previously reported [10, 21, 22]. However, the acetate to propionate ratio decreased from 3.56 to 2.44 at 6 mM propynoic acid, and then increased to 2.74 and 3.05 at 12 and 18 mM, respectively ($P < 0.001$; quadratic response; data not shown). Ethyl 2-butynoate linearly decreased ($P < 0.001$) the acetate to propionate ratio from 3.56 at 0 mM to 2.75, 2.56, and 2.38, at 6, 12, and 18 mM initial concentration, respectively (data not shown).

Some unusual end products of fermentation accumulated when methanogenesis was inhibited by propynoic acid or ethyl 2-butynoate. Hydrogen accumulation has been observed with other methanogenesis inhibitors, like 2-bromoethanesulfonate [23] and 9,10-anthraquinone [22]. Under normal conditions, methanogenesis keeps a low partial pressure of $H_2$ in the rumen [24]. The inhibition of methanogenesis also resulted in the accumulation of formate, because the increase in $H_2$ partial pressure displaces the equilibrium from $HCO_3^-$ and $H_2$ towards formate formation [25]. It is also possible that the inhibition of methanogenesis stimulated the disposal of reducing equivalents from pyruvate oxidative decarboxylation to acetyl-CoA into formate, a reaction catalyzed by formate lyases [26] instead of pyruvate oxidoreductases [27]. Reducing equivalents spared from methanogenesis would also have been used to reduce acetyl-CoA to ethanol, as happens in pure cultures of *Ruminococcus albus* and *Neocallimastix frontalis* in the absence of methanogens [24]. The accumulation of $H_2$, formate, and ethanol, indicates that the electrons not captured by methanogenesis could not be efficiently disposed into other alternative pathways like propionate formation, or fatty acids synthesis and biohydrogenation [20].

Succinate is a fermentation intermediate that normally does not accumulate in the rumen or in mixed ruminal cultures, as it is converted to propionate by succinate utilizers [24]. It is interesting that the greatest accumulation of succinate occurred at 12 and 18 mM initial concentration of propynoic acid (0.85 and 1.10 mM, respectively), while propionate production was maximum at 6 mM. It is possible that succinate utilizers could have been overwhelmed by the amount of succinate formed at 12 and 18 mM propynoic acid. Added succinate at 34 mM initial concentration was metabolized to both acetate and propionate, although disappearance was not reported [28]. There might then be opportunities to increase the amounts of propionate formed by adding succinate utilizers to the fermentations, or by stimulating the ones already present. Alternatively, the fact that propionate production decreased, rather than remained constant, at 12 and 18 mM propynoic acid, suggests a direct inhibition by propynoic acid on succinate utilizers. In support of this, it was found that added succinate at 29.7 mM was completely consumed by a mixed ruminal culture, and that more than 90% of it was decarboxylated to propionate [29]. The initial concentration of succinate of 29.7 mM was much greater that the accumulation we observed, yet succinate utilization was not overwhelmed in that study.

Despite the formation of unusual fermentation end products, propynoic acid increased the percentage of H incorporated into products nutritionally useful for the host animal. However, due to the decrease

in the H produced and to the formation of non-useful H sinks, H spared from methanogenesis by propynoic acid did not cause an increase in the absolute amount of H incorporated into useful sinks. Between two thirds and four fifths of the electrons released by fermentation were accounted by measured sinks. Propynoic acid was little metabolized, so it could have not acted as an electron sink itself. A H balance was not calculated for ethyl 2-butynoate, because its co-elution with butyrate off the HPLC column prevented us from finding butyrate production.

It was hypothesized that propynoic acid and ethyl 2-butynoate would inhibit $CH_4$ production by being alternative electron sinks. However, it is doubtful that methanogenesis was inhibited based on electron withdrawal from the medium since: (1) accumulation of unusual, reduced end products like $H_2$, formate, and ethanol, was observed, and (2) almost all of the propynoic acid remained after 24 h incubation. Therefore, it was not hydrogenated to propionate or acrylate.

Propynoic acid decreased apparent OM fermentation partly because the additive itself was not fermented. Apparent fermentation of the alfalfa substrate was decreased at 6 and 18 mM, but unaffected at 12 mM initial concentration. However, a higher proportion of the fermented products were nutritionally non-usable at 12 mM, compared to 6 mM initial concentration. As most of the inhibition of methanogenesis was already achieved at 6 mM initial concentration, the utilization of lower initial concentrations could be a way of minimizing the negative effects of propynoic acid on fermentation. This would decrease the proportion of the OM that is not fermentable.

The inhibition of methanogenesis caused by 3-butenoic acid was small, but it might have stimulated the substrate fermentation. Acetate, followed by butyrate, seemed to be the most important C sink of this additive.

2-Butynoic acid also caused small decreases in $CH_4$ production. Fermentation was inhibited at 12 and 18 mM initial concentration, but not at 6 mM. At 6 mM initial concentration, most 2-butynoic acid seemed to have been metabolized into butyrate, valerate, and isovalerate. Disappearance of 2-butynoic acid could not be measured as it co-eluted off the HPLC column with isobutyrate; however, as changes in total VFA production were relatively small at 12 and 18 mM compared to the control, it is possible that most of 2-butynoic acid was not metabolized at those initial concentrations.

### 4.3. Implications

Propynoic acid and ethyl 2-butynoate decreased ruminal methanogenesis in vitro. Propynoic acid had some adverse effects on the substrate apparent fermentation, and ethyl 2-butynoate also seemed to be inhibitory for fermentation, although its disappearance could not be measured. Both propynoic acid and ethyl 2-butynoate caused the formation of products without nutritional value. It is possible that organic acids that seemed to benefit fermentation, like β-hydroxybutyrate, crotonic acid, or 3-butenoic acid, could be fed to ruminants together with propynoic acid or ethyl 2-butynoate to relieve the negative effects on fermentation caused by the inhibitors of methanogenesis. Propynoic acid oral $LD_{50}$ to rodents is 100 $mg \cdot kg^{-1}$ [30], although its toxicity to ruminants at the doses inhibitory to ruminal methanogenesis would need to be evaluated. We are not aware of toxicity trials with ethyl 2-butynoate or 3-butenoic acid. Crotonic acid $LD_{50}$ to rodents is between 1 and 4.8 $g \cdot kg^{-1}$ [30]. It might be less toxic to ruminants as it is a naturally occurring intermediate in ruminal fermentation [6]. Likewise, acetoacetate and ß-hydroxibutyrate may be mildly toxic to ruminants because of the same reason. Accurate measurements of the disappearances of some of the compounds studied are needed in order

to understand what happened to, and as a consequence of, the addition of these chemicals. Their toxicity to ruminants, as well as the potential hazards for humans and the environment, would also need to be assessed.

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