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Biochemical constraints to the incorporation of dihydrogen into alternative electron acceptors in *in vitro* ruminal fermentation

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Abstract

Inhibitors of methane (CH_4) production are the most potent tool to decrease CH_4 emissions from domestic ruminants. However, inhibiting ruminal methanogenesis often leads to a buildup of ruminal dihydrogen (H_2), which may impair fermentation and is an energy loss when exhaled to the atmosphere. We hypothesized that carboxylic acid intermediates of ruminal fermentation could accept electrons to redirect excess H_2 toward the production of volatile fatty

acids (VFA), which are nutritionally useful to the host animal. In Experiment 1, it was hypothesized that fumaric, acrylic, or crotonic acids could incorporate accumulated H_2 into VFA production in methanogenesis-inhibited ruminal batch cultures. In Experiment 2, it was hypothesized that ruminal serial cultures could be adapted to the supplementation of crotonic acid and to elevated H_2 resulting from methanogenesis inhibition with 3-nitrooxypropanol (3-NOP) so as to improve the incorporation of H_2 into butyrate production. In Experiment 1, acrylic acid did not affect H_2 accumulation, and fumaric and crotonic acids had minimal effects on H_2 far below the stoichiometric expectations from their conversions to propionate and butyrate, respectively. However, most of the added fumaric, acrylic, and crotonic acids were recovered as their expected products of fermentation, propionate and butyrate, thus suggesting that reducing power for their reduction was largely provided by intracellular reduced cofactors rather than by external H_2. The decreases in CH_4 production caused by crotonic and acrylic acids, despite unchanged H_2 accumulation, further suggest that their reduction may have diverted intracellular reducing equivalents away from H_2 formation rather than stimulating extracellular H_2 uptake. In Experiment 2, supplementing 3-NOP in serial transfers 1 to 4 ameliorated H_2 accumulation in serial transfer 5 in ruminal serial cultures, suggesting adaptation over time of non-methanogenic hydrogenotrophs to utilize excess H_2. Supplementation of crotonic acid in serial transfers 1 to 4 greatly increased butyrate production but did not decrease H_2 accumulation. The available genomic and biochemical evidence suggests that crotonyl-CoA and acrylyl-CoA reduction in the rumen is primarily coupled to intracellular electron donors, potentially decreasing H_2 formation, rather than to extracellular H_2 oxidation.

The efficacy of supplemented fumarate to incorporate accumulated H_2 into propionate production would depend on the abundance and activity of hydrogenotrophic fumarate reducers in the ruminal microbiota. The results highlight that the capacity of electron acceptors to incorporate H_2 cannot be predicted solely from thermodynamics and depends also on whether the reduction is biochemically coupled to the oxidation of extracellular H_2.

Keywords: methanogenesis inhibition; dihydrogen; electron acceptors; hydrogen metabolism; rumen fermentation

Implications

Inhibiting methane production in the rumen often causes hydrogen to accumulate, which is a fermentation inefficiency. We found that supplemented fumaric, crotonic, and acrylic acids showed little or no capacity to incorporate accumulated hydrogen into propionate and butyrate production in *in vitro* ruminal cultures. Research on redirecting fermentation energy away from methane towards volatile fatty acid production should consider not only thermodynamic feasibility but whether the mixed microbiota can adapt to couple external hydrogen oxidation with sufficient affinity to the reduction of supplemented electron acceptors.

Introduction

The concentration of methane (CH_4) in the atmosphere has multiplied by nearly three-fold since the Industrial Revolution, and it is estimated to contribute for a 0.5 °C increase in the mean temperature of Earth (Reisinger, 2024). Because of CH_4 strong global warming potential and relatively short half-life in the atmosphere, decreasing the anthropogenic emissions of CH_4 can contribute to

decelerating global warming in the short-term (Reisinger, 2024; Shindell et al., 2024). About one third of anthropogenic emissions of CH_4 originate from the rumen of domestic ruminants (Szopa et al., 2021), and there is therefore ongoing research on the control of CH_4 production in the rumen (Beauchemin et al., 2022).

Various types of feed additives have been studied as strategies to decrease CH_4 formation in the rumen, including nitrates and sulfates (Van Zijderveld et al., 2010), tannins (Aboagye and Beauchemin, 2019), algae (Abbott et al., 2020; Wasson et al., 2022), and chemical inhibitors of methanogenesis (Honan et al., 2021; Hristov et al., 2025). In ruminal fermentation, CH_4 formation helps maintain the partial pressure of H_2 in the rumen at a level that allows NADH to reoxidize so that fermentation can continue (Wolin et al., 1997; Janssen, 2010), and maximize microbial ATP generation (Wolin et al., 1997). When methanogenesis is suppressed, H_2 buildup may impair the reoxidation of NADH and reduce fermentation efficiency (Morgavi et al., 2010) by favoring NADH-oxidation pathways that do not generate extra ATP, such as ethanol formation from pyruvate (Wolin et al., 1997). Managing H_2 accumulation with the aim of incorporating H_2 into metabolites that can be used by the host animal is an important aspect to be considered when inhibiting CH_4 production in the rumen (Martinez-Fernandez et al., 2017; Huang et al., 2023).

The supplementation of carboxylic acids intermediates of ruminal fermentation, or their analogs or precursors, has been evaluated as a means to compete with methanogenesis for electrons and simultaneously enhance the production of volatile fatty acids (VFA) (Carro and Ungerfeld, 2015). Promoting these alternative electron-incorporating pathways may become particularly relevant

when methanogenesis is inhibited and H_2 accumulates. Fumaric, acrylic, and crotonic acids incorporate metabolic hydrogen in their conversion to propionate (fumaric and acrylic acids) and butyrate (crotonic acid; Figure 1) and have been studied as electron acceptors competing with methanogenesis in rumen fermentation (Newbold et al., 2005; Ungerfeld et al., 2006; Ungerfeld et al., 2007). The reduction of fumarate, acrylate, and crotonate (activated to crotonyl-CoA) with H_2 as electron donor was estimated to be thermodynamically feasible under ruminal conditions (Figure 1) (Ungerfeld and Kohn, 2006). However, the actual electron donor in reduction reactions may be pathway- and microorganism-dependent, and not all thermodynamically feasible reductions in ruminal fermentation necessarily incorporate extracellular H_2 (i.e., H_2 produced and exchanged between rumen microorganisms). Here, we examined whether when methanogenesis is inhibited with 3-nitrooxypropanol (3-NOP) in ruminal cultures, some but not necessarily all supplemented carboxylic acids can redirect accumulated H_2 towards the production of VFA.

The objectives of the present study were (1) to evaluate the effect of supplementing fumaric, acrylic, or crotonic acids on the incorporation of H_2 into propionate and butyrate formation in ruminal batch cultures when inhibiting methanogenesis with 3-NOP (Experiment 1); and (2) to determine whether the supplementation with crotonic acid, 3-NOP, or their combination in serial transfers of ruminal cultures can induce microbial adaptation to incorporate elevated H_2 into crotonic acid as an electron acceptor, thereby enhancing the long-term potential of supplemented crotonic acid to incorporate H_2 into butyrate formation (Experiment 2).

Materials and Methods

The experiments were conducted at Instituto de Investigaciones Agropecuarias (INIA), Centro Regional de Investigación Carrillanca (Vilcún, Chile). All animal procedures were approved by Comité de Bienestar Animal, Instituto de Investigaciones Agropecuarias (Approval No. 03/2024).

Experiment 1

Arrangement of treatments

Three carboxylic acids: fumaric (BioChemica, AppliChem, Darmstadt, Germany), acrylic (Sigma-Aldrich, Zwijndrecht, The Netherlands), or crotonic acid (Sigma-Aldrich, Steinheim, Germany), each at four concentrations (0, 2, 4, or 8 mM), were combined with the methanogenesis inhibitor 3-nitrooxypropanol (3-NOP; RR Scientific, Shanghai, China) at 0 or 10 μM. As the three carboxylic acids shared their control treatments, the experiment comprised 20 combinations of treatments.

Donor animals and ruminal contents sampling and processing

Ruminal contents were obtained from two ruminally-cannulated non-lactating, non-pregnant, Holstein cows. Animals were fed *ad libitum* ryegrass hay (90.7% DM, and 6.1% total ash, 8.6% CP, 66.4% NDF, 39.5% ADF, and 1.9% EE, DM basis) and 1 kg/d of commercial concentrate (88.0%DM, and 3.3% total ash, 18.3% CP, 29.2% NDF, 8.6% ADF, and 3.4% EE, DM basis) in both experiments. Cows were adapted to their diets for two weeks prior to beginning the experiments.

Ruminal contents were collected from both cows before the morning feeding and strained through a 2-layer synthetic cloth (~0.5 mm mesh) to obtain fluid and solids fractions. Ruminal fluid and solids from each of the cows were

separately transported to the laboratory in insulated flasks. All laboratory procedures involving ruminal fluid and solids, inoculum, and inoculated culture medium, took place under O_2-free CO_2 to maintain anaerobic conditions. Approximately 200 mL of ruminal fluid and 200 mL ruminal solids from each cow were placed in a 500-mL Erlenmeyer flask and discontinuously (3 s bursts followed by 2 s pauses) blended with an eggbeater for 1 min to detach microbes adhered to plant particles. Blended ruminal contents were then strained through a 2-layer synthetic cloth (~0.5 mm mesh) to obtain the ruminal inoculum.

Incubations

Ruminal inoculum (13 mL) was added to 100-mL serum bottles containing 19 mL of the Mould et al., 2005 medium, as modified by Raju, 2016 (Supplementary Table S1), and 400 ± 0.24 mg DM of a 75: 25 (DM basis) forage: concentrate substrate (Supplementary Table S2). Bottles received 8 mL of a solution of each organic acid solution (acrylic, crotonic, or fumaric) to achieve final concentrations of 0 mM (control with distilled water), 2, 4, or 8 mM. Bottles in which methanogenesis was inhibited received 50 µL of a 3-NOP solution to achieve a final concentration of 10 µM 3-NOP. Control bottles received 50 µL of distilled water. All bottles were sealed with butyl rubber stoppers and aluminum seals under a continuous flow of O_2-free CO_2 and incubated at 39 °C with linear oscillation (60 rpm) for 48 h. Samples (10 mL) of both ruminal inocula were taken and kept at - 20° C for subsequent determination of VFA initial concentration.

Sampling and measurements

At 48 h of incubation, headspace gas pressure was measured with a pressure transducer (Sper Scientific 840,065, Scottsdale, AZ, USA). Gas samples (25-mL; ambient pressure) were collected with a glass syringe and delivered overpressurized into previously evacuated 5.9-mL exetainers (Labco Limited, Lampeter, Ceredigion, UK).

Bottles were then opened and pH was measured (Orion Star A214, Thermo Scientific, Chelmsford, MA, USA), and a 1-mL fluid aliquot was sampled and delivered into 2-mL microcentrifuge tubes containing 0.2 mL of 20% (v/v) phosphoric acid and 0.2 mL of 35 mM 2-ethylbutyric acid as an internal standard (Sigma-Aldrich, St. Louis, MO, USA) for subsequent analysis of volatile fatty acids (VFA), and acrylate, crotonate, formate, lactate, and succinate. A second 1-mL aliquot was sampled into 2-mL microcentrifuge tubes containing 0.2 mL of 1% sulfuric acid to determine ammonium (NH_4^+) concentration. All samples were stored at -20°C until analyzed. The remaining contents of each bottle were then emptied into pre-weighted 50-mL centrifuge tubes and centrifuged at 10,956 × *g* and 4 °C for 15 min to isolate the undigested solid residues. The supernatant was discarded and the pellet dried for 48 h at 60 °C and weighed. Dried pellets were then incinerated in pre-weighted crucibles in a muffle furnace at 550 °C for 4 h. Crucibles containing ashes were cooled to room temperature and weighed.

Analytical procedures

Methane, H_2, and O_2 concentration in gas samples was analyzed in a Clarus 580 PerkinElmer GC equipped with a 60/80 Carboxen 1000 packed column (Supelco, Bellefonte, PA, USA) and a thermal conductivity detector. Chromatography conditions have been described (Ungerfeld et al., 2024). The

carrier gas was N_2 at 30 mL/min with an isothermal oven temperature of 180 °C. The CH_4 and H_2 concentrations measured were then adjusted to account for residual air remaining in the exetainers upon delivery of the gas samples; the residual air in the exetainers was determined from the O_2 concentration measured in the GC divided by 0.2095 (i.e., the molar proportion of oxygen in the atmosphere).

Samples for analysis of VFA, acrylate, crotonate, and succinate were thawed, vortexed, and centrifuged at 16,000 x g for 10 min. The supernatant was filtered through 0.45-µm pore cellulose filters into 2-mL GC vials, and 1-µL samples were injected into a Clarus 580 PerkinElmer GC equipped with an Elite-FFAP (PerkinElmer, Shelton, CT, USA) capillary column and a flame ionization detector. Helium at 1.5 mL/min was the carrier gas. Initial temperature was 90 °C with a 12-°C/min ramp until reaching 150 °C, which was held for 5 min. Temperature was then increased at 12 °C/min to 210 °C and held for 2 min (Ungerfeld et al., 2024). The concentration of formate, lactate, and fumarate was determined in a LaChrom Elite HPLC-DAD with a sample injection volume of 10 µL. Separation was performed with a Purospher® STAR RP-C18 (5 µm) LiChroCART® 250-4 column with isocratic elution gradient with 0.1% phosphoric acid/acetonitrile [95/5% (v/v)], at a flow rate of 1 mL/min. Oven temperature was 40 °C and the detector operated at 210 nm. Ammonium concentration was determined by colorimetry according to Kaplan (1989).

Calculations

In Experiment 1, apparent dry and organic matter disappearances (DMD and OMD, respectively) were calculated by subtracting the mass of the dried and

calcined residues, in each case, from the DM or OM mass of the incubated substrate, respectively, and expressing the differences as percentages.

Headspace gas pressure inside the bottles was determined as the sum of measured gauge pressure and atmospheric pressure (1 atm = 101,325 Pa). The number of moles in the headspace of each bottle was calculated using the ideal gas law, based on a headspace volume of 60.05 mL. Methane production and H_2 accumulation were estimated by multiplying their respective proportions in total gas by total gas volume.

Total and individual volatile fatty acids (VFA) production were calculated by subtracting the initial number of moles present in the inoculum from the final number of moles in each bottle, each calculated from concentration and volumes. Disappearance of fumaric, acrylic, and crotonic acids was determined by subtracting any remaining amounts from the amounts added, calculated from their concentrations. Both for Experiments 1 and 2, changes in production of VFA and the accumulation of H_2 are related to the actual disappearance of the added carboxylic acid to establish how close those were the actual relationships observed to the theoretical stoichiometries expected from known pathways of reduction.

Statistical analysis

The experiment was repeated in 3 consecutive weeks, resulting in six biological replicates (n = 6; 2 inoculum donors × 3 weeks; N = 120 incubation bottles). The results of Experiment 1 were analyzed using a mixed-effects model including the fixed effects of 3-NOP (0 or 10 μM), the linear and quadratic effects of the disappearance of each carboxylic acid (fumaric, acrylic, and crotonic acids), and

as their linear interactions with 3-NOP. Donor cow and week of incubation were included as random effects:

$$Y_{ijkl} = \beta_0 + \beta_1 \text{3-NOP}_i + \beta_2 Fm_j + \beta_3 Fm_j^2 + \beta_4 Ac_j + \beta_5 Ac_j^2 + \beta_6 Cr_j + \beta_7 Cr_j^2 + \beta_8(\text{3-NOP}_i \times Fm_j) + \beta_9(\text{3-NOP}_i \times Ac_j) + \beta_{10}(\text{3-NOP}_i \times Cr_j) + u_k + w_l + \varepsilon_{ijkl}$$

Where Fm, Ac, and Cr represent the disappearance (μM) of fumaric, acrylic, and crotonic acids, respectively; $u_k$is the random effect of donor cow, $w_l$is the random effect of incubation week, and $\varepsilon_{ijkl}$is the residual error. Statistical significance was declared at $P < 0.05$. Quadratic terms and interactions that were not significant ($P \geq 0.05$) were also removed, and the reduced models were refitted. Outliers were identified as observations that fell out of the central 0.999 *t*-distribution of studentized residuals. Observations identified as outliers were examined and eliminated if it was verified that they resulted from typos or likely technical problems. All analyses were performed using JMP® 19.1.2, 2026.

Experiment 2

Arrangement of treatments, incubations, and analytical procedures

In Experiment 2 we investigated whether the longer-term supplementation of crotonic acid and/or exposure to elevated H_2 caused by 3-NOP supplementation could increase the recovery of crotonic acid as butyrate and/or the incorporation of H_2 into butyrate production. Experiment 2 was conducted as a serial ruminal culture under a 2 × 2 × 2 × 2 factorial arrangement of treatments. The four

factors were two concentrations (0 or 8 mM) of crotonic acid in serial transfers 1 to 4 and in serial transfer 5, and two concentrations of the methanogenesis inhibitor 3-NOP (0 or 10 μM) in serial transfers 1 to 4 and in serial transfer 5.

Incubations and analytical procedures

Ruminal contents were collected and processed as described for Experiment 1. The culture medium by Mould et al., 2005, as modified by Raju, 2016, was dispensed (32 mL) into 100-mL serum bottles containing 400 ± 0.26 mg DM of the same substrate used in Experiment 1.

Each bottle received 8 mL of ruminal inoculum from one of the two donor cows. Crotonic acid and 3-NOP were delivered as 8-mL or 50 μL solutions, respectively, or the equivalent volumes of distilled water for control treatments. The resulting 8 bottles (two crotonic acid and two 3-NOP concentrations, with inocula from two cows) were capped and sealed under O_2-free CO_2 and incubated as in Experiment 1. At the end of the 48-h incubation, gas pressure and composition was measured as in Experiment 1.

Transfer of ruminal inocula from donor bottles to bottles with fresh medium and substrate then followed (Figure 2). First, donor bottles were vortexed at maximum speed for 2 min to detach cells adhered to solid particles and then left untouched for 1 min. Bottles were then opened under O_2-free CO_2, gently swirled twice, and an 8-mL aliquot was taken and delivered into a 100-mL bottle containing 32 mL of fresh medium and 400 mg ± 0.25 mg DM of the same substrate incubated before. Crotonic acid, 3-NOP, or distilled water were again delivered, repeating the same treatment of the donor bottle. The pH of donor

bottles was measured after transferring their inocula to bottles containing fresh medium (Supplementary Figure 1).

This process was repeated three times, i.e., for four serial incubations. After the fourth incubation, each bottle donated 8 mL of its content to each of four bottles containing fresh medium and 400 ± 0.17 mg of substrate, belonging to one of the same four treatments consisting of combinations of crotonic acid (0 or 8 mM) and 3-NOP (0 or 10 μM). Because the 32-mL total volume donated by each bottle (32 mL = 8 mL/bottle × 4 bottles of incubation 5) represented most of the donating bottle contents, the 8 mL bottle contents were delivered staggered to avoid biases: first, as a 4-mL aliquot to all four receiving bottles, followed by a 2-mL aliquot to all four receiving bottles, and finally a 1-mL aliquot delivered twice to each bottle. Bottles were then capped, sealed, and incubated as before.

At the end of the fourth and fifth incubations, gas production and composition was measured and the fluid was sampled for subsequent analysis of VFA and NH_4^+ concentration, as described for Experiment 1, and incubation residues obtained through centrifugation as per Experiment 1 were lyophilized.

Calculations

Production of VFA in serial incubation 5 was calculated by subtracting VFA present in the fluid from serial incubation 4 used to inoculate bottles of serial incubation 5. The rest of the calculations were conducted as in Experiment 1, except that DMD was calculated from lyophilized, rather than from oven-dried residues, and OMD was not calculated as residues were not incinerated.

Statistical analysis

The experiment was conducted thrice in different weeks. Responses were modelled as a function of the fixed effects of 3-NOP and crotonic acid in incubations 1 to 4, the fixed effects of 3-NOP and crotonic acid in incubation 5, six 2-way interactions, four 3-way interactions, and one 4-way interaction:

$$Y_{ijkl} = \beta_0 + Cr_{1to4i} + 3{-}NOP_{1to4i} + Cr_{5j} + 3 - NOP_{5j} + 2{-}, 3{-}, and\ 4 - way\ interactions + u_k + w_l + \varepsilon_{ijkl}$$

where $Cr_{1to4}$and 3-NOP_{1to4}represent crotonic acid and 3-NOP addition in incubations 1 to 4, respectively, and $Cr_5$and 3-NOP_5represent their addition in incubation 5.

All non-significant ($P \geq 0.050$) interactions were removed and the reduced model refitted. When interactions were present ($P < 0.050$), all pairwise comparisons among treatment combinations of the interacting factors were conducted using Tukey-adjusted contrasts. The rest of the statistical procedures were conducted as in Experiment 1.

Results

Experiment 1

Three observations were identified as outliers. Further examination revealed that two of them were likely consequences of errors of adding 3-NOP to control treatments, while the third one was a suspected gas leakage. All three outliers were excluded from the analysis across all response variables. A fourth observation was identified as an outlier for lactate concentration and was excluded from that analysis.

Accumulation of H_2 peaked at 2 mM fumaric acid (quadratic response; P = 0.019; Figure 3), while acrylic and crotonic acids had no effect ($P \geq 0.10$). Methane production decreased in response to acrylic ($P < 0.001$) and crotonic ($P = 0.014$) acids, whereas fumaric acid had no effect ($P = 0.93$; Figure 4). The addition of 3-NOP and acrylic acid decreased total gas production ($P \leq 0.043$; Supplementary Figure 1), whereas fumaric and crotonic acids increased total gas ($P \leq 0.001$). The addition of 3-NOP decreased total VFA production (P = 0.006; Supplementary Figure 2), whereas acrylic, crotonic, and fumaric acids increased total VFA production ($P \leq 0.040$). The addition of 3-NOP decreased acetate ($P < 0.001$; Figure 5) and increased propionate production ($P < 0.001$; Figure 6). Fumaric, crotonic, and acrylic acids had no effect on acetate ($P \geq$ 0.47). Acrylic and fumaric acids increased propionate ($P < 0.001$), whereas crotonic acid tended to decrease it ($P = 0.081$). Crotonic acid increased ($P <$ 0.001) and acrylic acid decreased ($P = 0.009$) butyrate production, with both effects being greater with 3-NOP (interactions crotonic/acrylic acid × 3-NOP $P \leq$ 0.010; Figure 7), with no effect of fumaric acid ($P = 0.80$). Effects on other VFA are shown in Supplementary Figures 3, 4, and 5.

3-Nitrooxypropanol increased formate concentration ($P < 0.001$; Supplementary Figure 6). Formate peaked at 2 mM fumaric acid (quadratic response $P <$ 0.001). Succinate concentration was decreased by 3-NOP ($P < 0.001$; Supplementary Figure 7) and by acrylic acid in the presence of 3-NOP (interaction 3-NOP × acrylic acid $P = 0.005$). Fumaric and acrylic acids increased lactate concentration ($P \leq 0.03$; Supplementary Figure 8) and crotonic acid decreased lactate (quadratic response; $P = 0.001$). Fumaric, crotonic, and acrylic acids decreased final pH ($P < 0.001$; Supplementary Figure

9), NH4+ concentration ($P \leq 0.003$; Supplementary Figure 10), and OMD ($P \leq 0.001$; Supplementary Figure 11).

Experiment 2

Results corresponding to incubation 5 are presented. Two observations were identified as outliers for DMD; because problems at the lyophilization and residue weighting steps were suspected, those observations were eliminated from that analysis.

There was a two-way interaction between 3-NOP supplementation in incubations 1 to 4 and 3-NOP supplementation in incubation 5 on H_2 accumulation ($P \leq 0.001$; Figure 8) by which those cultures supplemented 3-NOP in incubation 5 that had received 3-NOP in incubations 1 to 4 accumulated 37% less H_2 than those that had also received 3-NOP in incubation 5 but had not been previously supplemented 3-NOP ($P < 0.050$). There was a three-way interaction ($P = 0.028$) by which crotonic acid supplemented in incubation 5 decreased CH_4 production by 34% when 3-NOP was not supplemented at any incubation (Figure 9). Furthermore, 3-NOP supplemented in incubations 1 to 4 exerted a residual effect in incubation 5, as supplementing 3-NOP in incubation 5 did not exert any further suppression of methanogenesis when 3-NOP had been supplemented in incubations 1 to 4 (interaction 3-NOP in incubations 1 to 4 × 3-NOP in incubation 5 $P < 0.001$; Figure 9). Supplementing 3-NOP in incubations 1 to 4 and/or incubation 5 decreased total gas production ($P < 0.050$; Supplementary Figure 12).

Slightly less crotonic acid supplemented in incubation 5 disappeared in cultures not supplemented crotonic acid in incubations 1 to 4 compared to cultures that

had received crotonic acid ($P < 0.050$; Supplementary Figure 13). Supplementing 3-NOP in incubations 1 to 4 ($P = 0.024$) and crotonic acid in incubation 5 ($P = 0.041$) decreased, and supplementing crotonic acid in incubation 5 increased ($P < 0.001$), total VFA production (Supplementary Figure 14). Supplementing 3-NOP in incubations 1 to 4 ($P = 0.003$) and crotonic acid in incubation 5 ($P = 0.001$) both decreased acetate production (Supplementary Figure 15). Supplementing crotonic acid in incubations 1 to 4 and 3-NOP in incubation 5 both decreased propionate production ($P < 0.001$; Supplementary Figure 16). Supplementing crotonic acid in incubations 1 to 4 increased butyrate production ($P < 0.050$; Figure 10), without any further increase if crotonic acid was also supplemented in incubation 5. Effects on other VFA are shown in Supplementary Figures 17 to 19.

Crotonic acid supplementation in incubations 1 to 4 decreased pH ($P \leq 0.001$; Supplementary Figure 20). There was an interaction between 3-NOP supplementation in incubations 1 to 4 and in incubation 5 ($P = 0.030$) on NH_4^+ concentration, with no single Tukey-s adjusted pairwise contrast driving the effect ($P \geq 0.050$; Supplementary Figure 21). Supplementing crotonic acid in incubations 1 to 4 and/or in incubation 5 decreased DMD ($P < 0.050$; interaction crotonic acid in incubations 1 to 4 × crotonic acid in incubation 5 $P < 0.001$; Supplementary Figure 22).

Discussion

In Experiment 1, we supplemented carboxylic acids as electron acceptors to methanogenesis-inhibited ruminal incubations aiming to incorporate accumulated H_2 into the production of propionate (from supplemented fumaric and acrylic acids) or butyrate (from supplemented crotonic acid). Tatsuoka et

al., 2008 and Ebrahimi et al., 2011 successfully decreased H_2 accumulation in methanogenesis-inhibited ruminal batch cultures by supplementing fumaric acid. Supplementation of fumarate´s precursor malate also decreased H_2 accumulation in methanogenesis inhibited ruminal cultures (Mohammed et al., 2004; Tatsuoka et al., 2008). However, in other studies, the addition of fumaric acid did not decrease H_2 accumulation in methanogenesis-inhibited ruminal batch cultures (Thorsteinsson et al., 2023), or H_2 emissions (except for the post-feeding H_2 peak) or H_2 dissolved in ruminal fluid in dairy cows (Maigaard et al., 2024) and sheep (Chowdhury et al., 2026) supplemented inhibitors of CH_4 production. Recently, encapsulated and non-encapsulated fumaric acid did not affect H_2 accumulation in ruminal batch cultures (Menni et al., 2026), although in that study, fumaric acid in both forms was supplemented separately from the methanogenesis inhibitors 3-NOP, bromoform, and iodoform, so that their interaction could not be evaluated. In our study, the disappearance of 320 μmol of fumaric acid at an initial concentration of 8 mM resulted in a decrease in H_2 accumulation of only 7.4 μmol in the methanogenesis-inhibited treatment. Considering that 1 mol of reducing equivalents reduces 1 mol of fumarate to 1 mol of succinate, which is then converted to propionate (Russell, 2002), and that 60% of added fumaric acid was recovered as propionate, the conversion of fumarate disappeared to propionate utilizing H_2 as an electron donor would have theoretically implied a decrease in accumulated H_2 of 192 μmol, a response much greater than the observed decrease of 7.4 μmol of accumulated H_2. The 60% fumarate recovery as propionate in our study was not affected by 3-NOP supplementation and, at the same range of fumarate concentration, is numerically similar to fumarate recovery in propionate in the meta-analysis by

Ungerfeld et al., 2007 of *in vitro* studies with functional methanogenesis. More recently, fumarate addition to ruminal batch cultures was shown to increase propionate production regardless of whether methanogenesis was inhibited by 3-NOP, although H_2 accumulation was not reported (Liu et al., 2022). Smaller than theoretical decreases in CH_4 as a response to fumaric acid addition has been explained based on supplemented fumarate partially being metabolized to acetate, releasing reducing equivalents rather than incorporating them (Ungerfeld et al., 2007). However, in Experiment 1 there were no increases in acetate production when fumaric acid was supplemented. The conversion of fumarate to butyrate via pyruvate and acetyl-CoA would also release reducing equivalents (Figure 1), but the same as acetate, fumarate supplementation did not affect butyrate production in Experiment 1. Hence, diversion of supplemented fumaric acid to pathways releasing reducing equivalents does not appear to explain the small effect of fumaric acid on H_2 in this experiment.

Some ruminal bacteria can utilize formate and H_2 at similar rates as electron donors for fumarate reduction (Asanuma et al., 1999). In Experiment 1, the disappearance of 320 μmol of fumaric acid at 8 mM initial concentration resulted in a decrease of approximately 40 μmol of accumulated formate in the methanogenesis-inhibited treatment with respect to 0 mM fumaric acid (calculations not shown). Thus, the decrease in accumulated formate represents only approximately 12% of the reductants required to recover the added fumarate as propionate in Experiment 1. Therefore, as fumarate conversion to propionate did not seem to draw electrons from the H_2 or formate pools to a large extent, it appears that electrons largely originated from intracellular metabolism. Asanuma et al., 1999 reported that, except for

458 *Fibrobacter succinogenes*, *Veillonella parvula*, *Selenomonas ruminantium*, *S.*
459 *lactilytica*, and *Wolinella succinogenes*, most of the ruminal bacteria they
460 examined that produced propionate or succinate did not grow or grew poorly on
461 fumarate as only substrate with added H_2 and/or formate as electron donors.
462 Zhang et al., 2023 showed that two species of the predominant ruminal genus
463 *Prevotella* balanced their intracellular redox cofactors by transferring electrons
464 from reduced ferredoxin to NAD^+ through Rnf ferredoxin-NAD+ oxidoreductase.
465 This mechanism allowed *Prevotella* to transfer reducing equivalents from
466 reduced ferredoxin generated in pyruvate oxidative decarboxylation to generate
467 NADH used as electron donor in fumarate reduction to succinate (Zhang et al.,
468 2023). It appears therefore likely that in Experiment 1 fumaric acid was mainly
469 reduced by organisms that utilize intracellular reducing power rather than H_2 of
470 formate.

471 In Experiment 1, crotonic acid did not affect H_2 accumulation. Similarly, in a
472 previous study, added crotonic acid slightly decreased CH_4 production in
473 ruminal batch cultures but it did not alleviate the buildup of H_2 or other reduced
474 end products that accumulated when methanogenesis was inhibited (Ungerfeld
475 et al., 2006). In that study, the increase in butyrate accounted for only 29 to 40%
476 of added crotonic acid (Ungerfeld et al., 2006). In Experiment 1, an average of
477 69% of added crotonic acid was on average recovered as butyrate, although
478 this was as high as 79% when methanogenesis was inhibited by 3-NOP. Lack of
479 effect of crotonic acid on H_2 accumulation suggests that, the same as with
480 fumaric acid, reducing equivalents incorporated into crotonate reduction to
481 butyrate were mostly supplied by intracellular metabolites rather than by the
482 accumulated H_2 pool. Inhibiting methanogenesis may have increased the NADH

to NAD^+ ratio (Hino and Russell, 1985) thus stimulating the bifurcative reduction of crotonate to butyrate (Hackmann and Firkins, 2015). Interestingly, the supplementation of crotonic acid decreased slightly propionate production, suggesting competition for electrons between both reductive pathways.

Unlike fumaric acid, which had some modest effects on H_2, and the same as crotonic acid, acrylic acid did not decrease the accumulation of H_2 or formate. However, in our study, acrylic acid was more effective than fumaric acid at stimulating propionate production, with approximately 83% of the disappeared acrylic acid being recovered as propionate disregarding of methanogenesis inhibition. In semicontinuous cultures, approximately 33% of supplemented acrylate was recovered as propionate, and the same as in our study, H_2 release was not affected (Newbold et al., 2005). Manlapig et al., 2025 observed that, contrary to expectations, the addition of 20 mM acrylic acid to ruminal batch cultures decreased propionate production and led to lactate accumulation; their results may be related to growth inhibition of *Megasphaera elsdenii*, which metabolizes lactate to propionate via acrylate, at acrylate concentrations greater than 10 mM (Prabhu et al., 2012). Similarly to our results, the supplementation of acrylic acid did not modify H_2 emissions in dairy cows supplemented inhibitors of methanogenesis (Maigaard et al., 2024). The same as added fumaric and crotonic acids, we speculate that acrylic acid reduction may have largely occurred via reduced intracellular metabolites acting as electron donors. As the addition of acrylic acid non-linearly decreased butyrate production, the incorporation of reducing equivalents into acrylate reduction to propionate seemed to occur partly at the expense of reducing equivalents otherwise incorporated into butyrate production.

Current biochemical understanding does not suggest that acrylyl-CoA reduction relies on H_2 as an electron donor, which would agree with the lack of effects observed of added acrylic acid on H_2. The acrylyl-CoA reductase of non-ruminal *Anaerotignum* (*Clostridium*) *propionicum* bifurcatively utilizes NADH as an electron donor to reduce acrylyl-CoA and oxidized ferredoxin to propionyl-CoA and reduced ferredoxin (Herrmann et al., 2008). Under high concentrations of lactate or when growing on acrylate, ruminal bacterium *Megasphaera elsdenii* disposes of electrons intracellularly through the reduction of acrylyl-CoA to propionyl-CoA as a means of balancing intracellular electron disposal (Prabhu et al., 2012). We are unaware of genomic evidence of ruminal or non-ruminal non-randomizing pathway propionate producers encoding unequivocally unidirectional H_2-incorporating hydrogenases.

In Experiment 1, supplementation of ruminal batch cultures with carboxylic acids was not effective at incorporating accumulated H_2 resulting from methanogenesis inhibition. However, most of the added carboxylic acids were indeed recovered as their hypothesized final products, suggesting that all three carboxylic acids were reduced utilizing intracellular electron donors as reductants in preference to H_2 and formate. Speculating that in Experiment 1 the incorporation of H_2 into the reduction of the added carboxylic acids may have been limited by insufficient adaptation to the carboxylic acids supplementation, in Experiment 2 we evaluated the effect of supplementing crotonic acid repeatedly in ruminal serial cultures. Crotonic acid was chosen for Experiment 2 because it had the highest recovery as its final product of the three carboxylic acids evaluated in Experiment 1. Following this rationale, we hypothesized that serial ruminal cultures could be adapted for enhanced H_2

uptake into crotonate reduction by repeatedly exposing them to elevated H_2 generated through inhibiting methanogenesis and to supplemental crotonic acid. In some studies, decreases over time in expelled H_2 in dairy cows supplemented 3-NOP have been observed (Hristov et al., 2015; Pitta et al., 2022), suggesting that amplification of non-methanogenic hydrogenotrophic pathways can potentially occur.

In Experiment 2, the interaction between short- (in incubation 5 only) and long-term (in incubations 1 to 4) supplementation of 3-NOP on H_2 accumulation suggests that the methanogenesis-inhibited serial cultures increased over time their capacity to utilize H_2. Supplementation of ruminants with 3-NOP stimulated bacterial groups such as *Lachnospiraceae* NK3 A20 with flexibility to dispose of electrons as H_2 or incorporate them into succinate and butyrate formation depending on H_2 pressure (Choi et al., 2025a). Choi et al., 2025b found that when methanogenesis was inhibited by 3-NOP there were short- and long-term reorganizations of the ruminal microbiota of beef cattle to cope with excess H_2. Shifts were observed in bacteria associated with flows of metabolic hydrogen, including decreases in the relative abundance of H_2-producing taxa such as *Clostridia* UCG-014, and increases in taxa involved in the production of propionate, such as *Succiniclasticum*, *Prevotella*, and *Prevotellaceae* UCG-001. Those changes, whilst not directly demonstrating increases in the incorporation of H_2 into propionate or butyrate production, are consistent with a general reorganization of reducing power disposal. In Experiment 2, repeated dosing of crotonic acid did not decrease H_2 accumulation despite butyrate production increasing more than 3-fold. Therefore, whilst repeated dosing of crotonic acid appeared to induce a strong adaptation to reduce crotonic acid to butyrate,

there was no evidence of enhanced incorporation of H_2 into crotonate reduction, which suggests that organisms reducing crotonate with H_2 were either absent or in low numbers, or inactive.

Crotonic acid stimulating butyrate production without stimulating H_2 incorporation is actually consistent with genomic and biochemical evidence. Main ruminal butyrate producers butyrivibrios encode the Bcd-Etf complex, which catalyzes the electron-bifurcative reduction of crotonyl-CoA and oxidized ferredoxin with NADH as electron donor, producing butyryl-CoA and reduced ferredoxin (Li et al., 2008; Hackmann and Firkins, 2015). Hence, butyryl-CoA can be produced in butyrivibrios from intracellular reducing equivalents without the input of an intercellular electron carrier such as H_2 or formate, which is consistent with the lack of effects of crotonic acid on H_2 accumulation with simultaneous apparent conversion of crotonate to butyrate occurring in both Experiments 1 and 2. Main ruminal butyrate producers such as *Butyrivibrio fibrisolvens* are considered to be H_2 producers (Stewart et al., 1997). The only ruminal butyrate producer encoding a putative uptake hydrogenase we are aware of is *Selenomonas ruminantium* subsp. *lactilytica* DSM 2872 (Hackmann and Zhang, 2023; Zhang et al., 2023); if this or other organisms have the capacity to couple H_2 oxidation to crotonyl-CoA reduction, they may not have been stimulated enough by repeated crotonic acid supplementation under the conditions of our experiment.

Therefore, current knowledge of rumen microbial metabolism suggests that H_2 incorporation into added crotonic or acrylic acids is improbable for most rumen organisms (Figure 11). As for fumaric acid, some but not most ruminal bacteria in pure culture grew by incorporating H_2 or formate into the reduction of

583 fumarate to succinate (Asanuma et al., 1999). Thus, whether adapting ruminal
584 cultures to utilize fumarate under elevated H_2 ---as attempted with crotonic acid
585 in Experiment 2- could enhance the capacity of the cultures to incorporate H_2
586 into propionate production would depend on the predominant organisms
587 responsible for any enhanced fumarate-reducing activity. Such activity can be
588 mediated by hydrogenotrophs or by organisms that reduce fumarate using
589 intracellular electron donors without taking up external H_2 or formate (Figure
590 11). Among fermentative prokaryotes of diverse origin producing acetate along
591 with succinate and/or propionate, most organisms encoded Rnf ferredoxin-
592 NAD^+ oxidoreductases, whilst a relatively minor proportion were found to
593 encode H_2-uptake hydrogenases (Zhang et al., 2023). In the sheep rumen,
594 however, group 1d [NiFe] hydrogenases, which are thought to transfer electrons
595 from H_2 to fumarate and nitrate reduction in *Selenomonas*, were among the
596 most abundant H_2-uptake hydrogenases (Greening et al., 2019). It is therefore
597 possible that, under some conditions, hydrogenotrophic fumarate-reducing
598 organisms may be prevalent or could be stimulated in the rumen. Whilst most
599 research in this area has focused on stimulating propionate or succinate
600 production, it therefore important to distinguish organisms capable of taking up
601 extracellular H_2 from those that produce succinate or propionate primarily as a
602 means to balance intracellular redox cofactors. Bioaugmentation with
603 hydrogenotrophic fumarate reducers may help redirect H_2 towards propionate
604 production in the methanogenesis-inhibited rumen, provided that the organisms
605 remain metabolically active and competitive. However, it should be considered
606 that, if fumarate reducers have lower apparent affinity for H_2 than methanogens,
607 as found by Asanuma et al., 1999, even cultures adapted to incorporate H_2 into

608 fumarate reduction may not be able to decrease H_2 to the low concentration
609 maintained by functional methanogenesis.

610 Crotonic and acrylic acids did not decrease H_2 accumulation but both
611 decreased CH_4 production by ratios close to the expected theoretical decrease
612 if 1 mol of reducing equivalent pairs were incorporated per mole of carboxylic
613 acid reduced i.e., -0.25 mol CH_4 per mole of carboxylic acid disappeared.
614 Therefore, their reduction may have diverted electrons away from H_2 formation,
615 thereby indirectly decreasing CH_4 production. We propose a model through
616 which, depending on their capacity for H_2 uptake, supplemented electron
617 acceptors may decrease H_2 accumulation or indirectly decrease CH_4 formation
618 through competing for electrons with H_2 production, ultimately not altering
619 accumulated H_2 (Figure 11).

620 Other electron acceptors have been evaluated for incorporating accumulated H_2
621 resulting from the inhibition of ruminal methanogenesis. Phenolic compound
622 phlorglucinol is reduced to acetate and has decreased H_2 accumulation in
623 methanogenesis-inhibited ruminal serial cultures (Huang et al., 2023; Romero et
624 al., 2023) and H_2 expelled in vivo (Martinez-Fernandez et al., 2017; Romero et
625 al., 2024). Although phlorglucinol reduction utilizes NAD(P)H as the direct
626 electron donor (Conradt et al., 2016), the decrease in H_2 accumulation implies
627 the presence of a metabolic network capable of coupling the oxidation of
628 extracellular H_2 to the reduction of $NAD(P)^+$ and ultimately phlorglucinol
629 reduction. Autotrophic reductive acetogenesis provides another example in
630 which H_2 reduces CO_2, an electron acceptor plentiful in the rumen, to acetate
631 (Müller, 2003; Karekar et al., 2022). Although reductive acetogens are
632 thermodynamically outcompeted by methanogens for H_2 in the rumen with

functional methanogenesis due to the lower H_2 threshold of methanogens (Ungerfeld, 2020), reductive acetogenesis could be successfully induced and H_2 accumulation decreased when methanogenesis was inhibited in ruminal batch (Nollet et al., 1997; Le Van et al., 1998; Li et al., 2025; Ni et al., 2025) and semicontinuous (Karekar and Ahring, 2023) cultures. Metagenomic evidence also suggests that inhibiting methanogenesis with 3-NOP in calves redirected electrons towards reductive acetogenesis (Ni et al., 2025).

In conclusion, the supplementation of ruminal fermentation intermediates fumaric, crotonic, or acrylic acids as electron acceptors along with 3-NOP was ineffective to incorporate accumulated H_2 into propionate or butyrate production in ruminal batch cultures. An attempt to adapt ruminal cultures to crotonic acid in serial ruminal cultures was unsuccessful to improve the incorporation of H_2 into butyrate production, although the microbiota seemed to partially adapt to utilize elevated H_2 and to independently adapt to reduce the added crotonic acid to butyrate. The present results demonstrate that thermodynamic feasibility alone is insufficient to predict whether a supplemented electron acceptor will be able to alleviate H_2 accumulation, and selection of electron acceptors to incorporate H_2 in the methanogenesis-inhibited ruminal fermentation should also consider the physiology and enzymology of the reductions by the mixed ruminal microbiota. The existence of intracellular metabolic networks coupling the uptake and oxidation of extracellular H_2 to the reductions of the supplemented electron acceptors appears to be pivotal for the success of strategies seeking H_2 incorporation into VFA production.

Ethics approval

The study followed Chilean law 20380 on animal protection and was approved by Comité Institucional de Bienestar Animal, Instituto de Investigaciones Agropecuarias (approval number 03/2024).

Data availability statement

The datasets supporting the conclusions of this article are available in the Open Science Framework repository, project c2bqu: https://osf.io/c2bqu/overview

Declaration of Generative IA and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence assisted technologies in the writing process.

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Declaration of interest

None.

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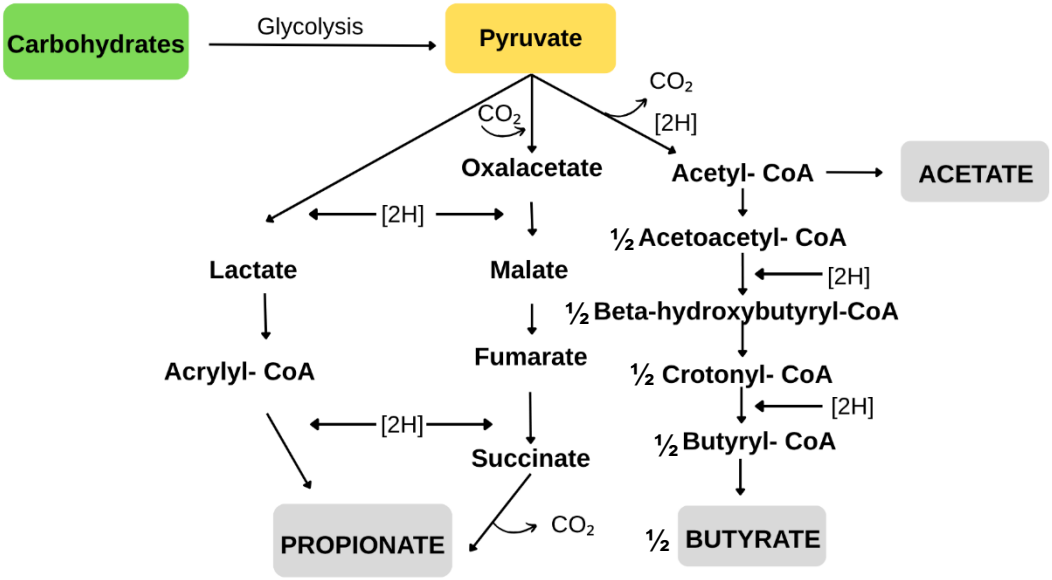

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908 Figure 1. Pathways of acetate, butyrate, and propionate formation from

909 carbohydrate fermentation in the rumen. [2H] = reducing equivalent pairs; CO_2

910 = carbon dioxide.

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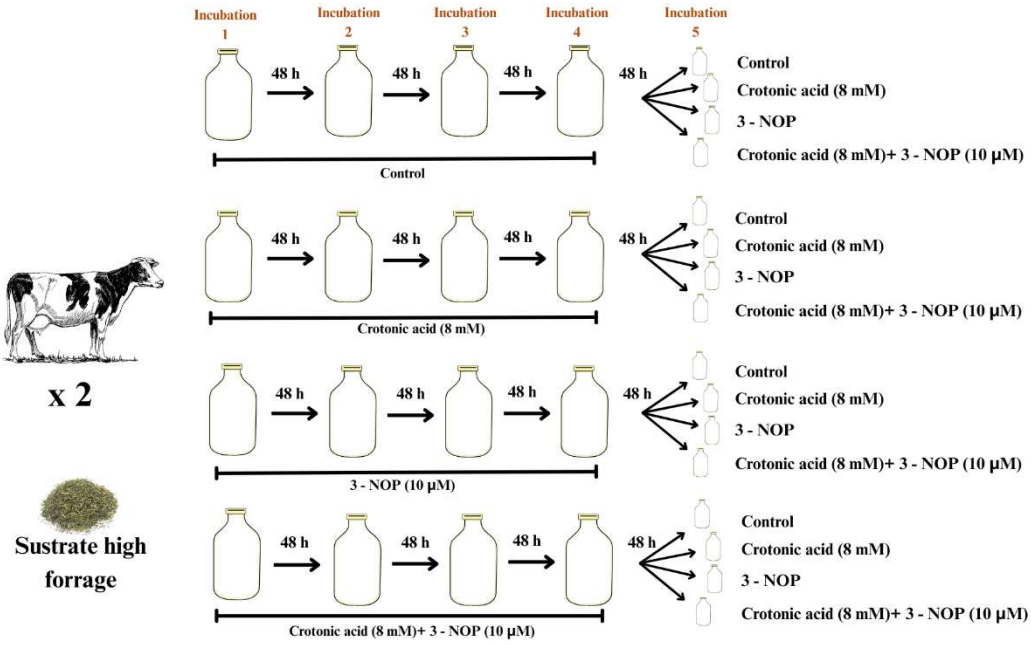

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922 Figure 2. Representative diagram of Experiment 2 showing mixed ruminal serial
923 culture incubations with a high forage substrate, each transferred through five
924 serial 48-h incubations.

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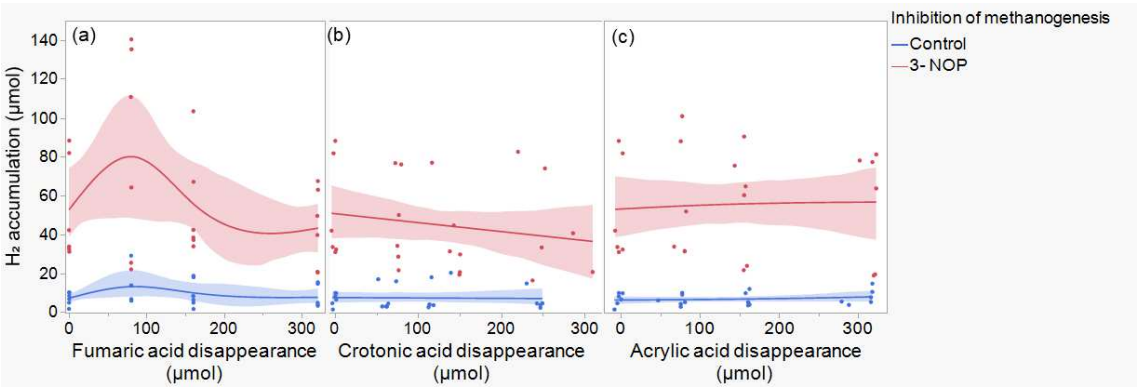
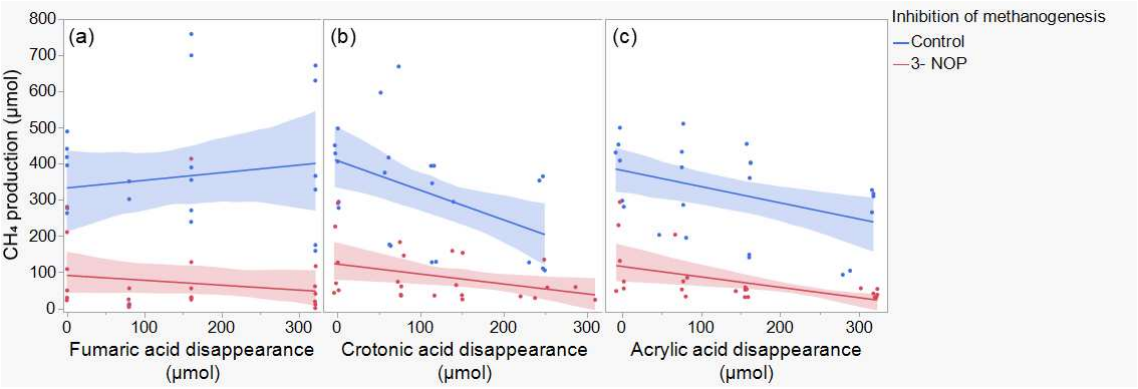


Figure 3. Effect of disappearance of (a) fumaric acid, (b) crotonic acid, and (c) acrylic acid (0, 2, 4, or 8 mM) with or without 3-nitrooxypropanol (3-NOP) on H_2 accumulation after 48 h ruminal incubations (N = 117): H_2 (µmol) = 33.5 (±9.86; P = 0.038) + [22.3 (±1.65; P < 0.001) × if 3-NOP] + [0.076 (±0.044; P = 0.092) × fumaric acid (µmol disappearance)] – [0.00048 (±0.00020; P = 0.019) × (fumaric acid (µmol disappearance) – 56.42)]² – [0.039 (±0.025; P = 0.13) × crotonic acid (µmol disappearance)] + [0.00010 (±0.020; P = 0.10) × acrylic acid (µmol disappearance)] + cow (random) + week (random) + error; all other terms, $P \geq$ 0.32. Curves and 95% confidence bands fit the observations displayed without accounting for the random effects of the donor cow and incubation run (Experiment 1).

948 

949 Figure 4. Effect of disappearance of (a) fumaric, (b) crotonic, and (c) acrylic
950 acids (0, 2, 4, or 8 mM) with or without 3-nitrooxypropanol (3-NOP) on CH_4
951 production at 48 h. (*N* = 117): CH_4 (µmol) = 219 (±63.1; *P* = 0.046) - [132.06
952 (±9.02; *P* < 0.001) × if 3-NOP] + [0.0091 (±0.10; *P* = 0.93) × fumaric acid (µmol
953 disappearance)] – [0.33 (±0.13; *P* = 0.014) × crotonic acid (µmol
954 disappearance)] – [0.34 (±0.10; *P* = 0.001) × acrylic acid (µmol disappearance)]
955 + cow (random) + week (random) + error; all other terms, *P* ≥ 0.15. Curves and
956 95% confidence bands fit the observations displayed without accounting for the
957 random effects of the donor cow and incubation run (Experiment 1).

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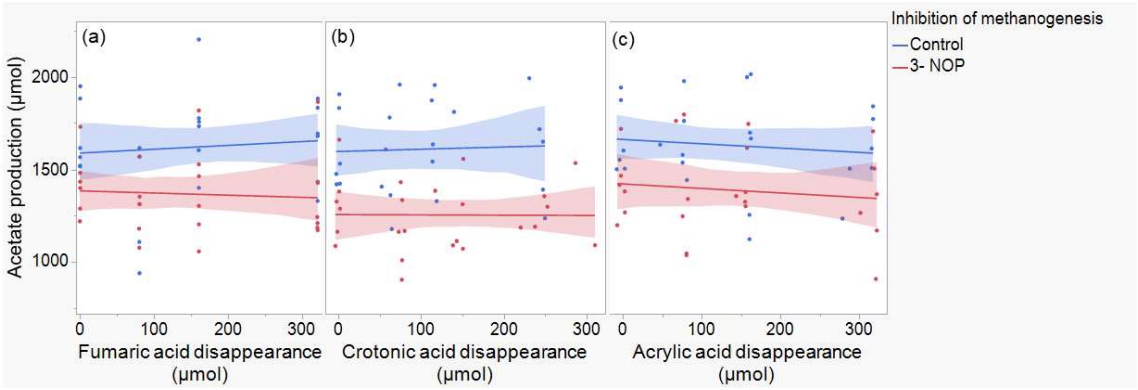


Figure 5. Effect of the disappearance of (a) fumaric, (b) crotonic, and (c) acrylic acids (0, 2, 4 or 8 mM) with or without 3-nitroxypropanol (3-NOP) on acetate production at 48 h ruminal incubations (N = 117): acetate production (µmol) = 1511 (±132; $P < 0.002$) – [142 (±14.6; $P < 0.001$) × if 3-NOP] – [0.033 (±0.17; $P = 0.84$) × fumaric acid (µmol disappearance)] + [0.15 (±0.21; $P = 0.47$) × crotonic acid (µmol disappearance)] – [0.041 (±0.17; $P = 0.81$) × acrylic acid (µmol disappearance)] + cow (random) + week (random) + error; all other terms, $P \geq 0.29$. Curves and 95% confidence bands fit the observations displayed without accounting for the random effects of the donor cow and incubation run (Experiment 1).

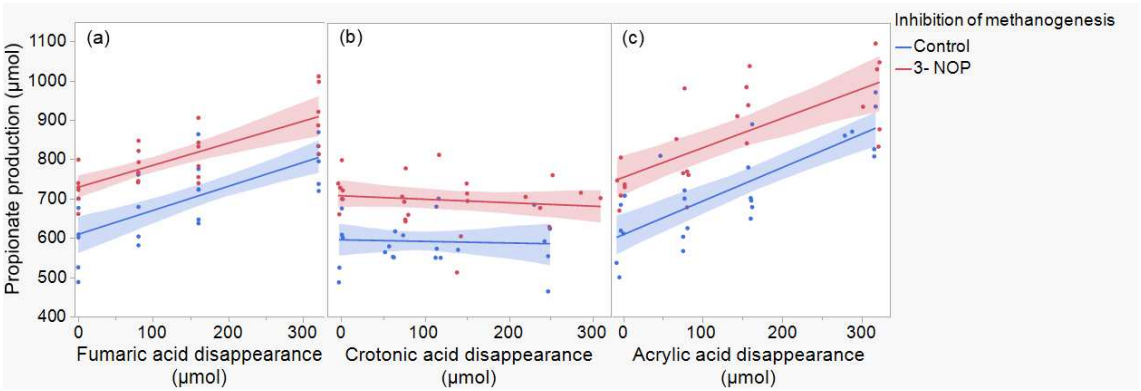
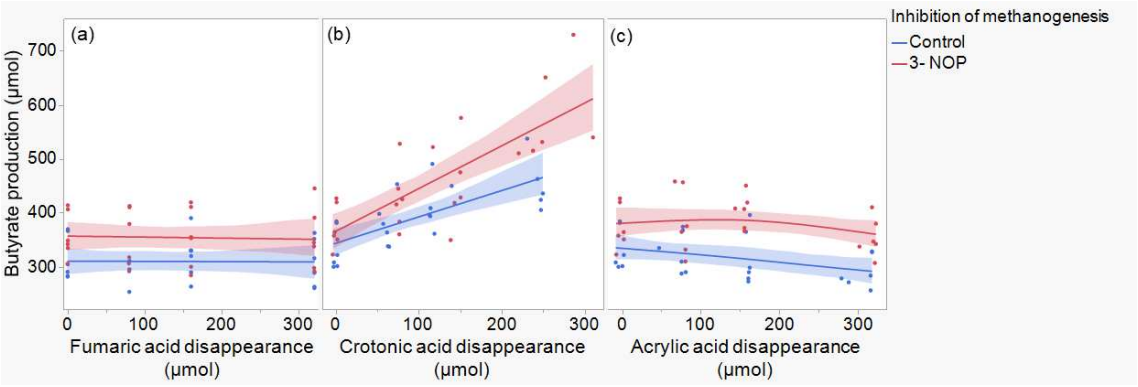


Figure 6. Effect of the disappearance of (a) fumaric, (b) crotonic, and (c) acrylic acids (0, 2, 4 or 8 mM) with or without 3-nitroxypropanol (3-NOP) on propionate production at 48 h ruminal incubations (N = 117): propionate production (µmol) = 670 (±35.3; $P < 0.001$) + [58.4 (±5.63; $P < 0.001$) × if 3-NOP] + [0.60 (±0.064; $P < 0.001$) × fumaric acid (µmol disappearance)] – [0.14 (±0.083; $P = 0.081$) × crotonic acid (µmol disappearance)] + [0.83 (±0.065; $P < 0.001$) × acrylic acid (µmol disappearance)] + cow (random) + week (random) + error; all other terms, $P \geq 0.32$. Curves and 95% confidence bands fit the observations displayed without accounting for the random effects of the donor cow and incubation run (Experiment 1).



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1003 Figure 7. Effect of the disappearance of (a) fumaric, (b) crotonic, and (c) acrylic
1004 acids (0, 2, 4 or 8 mM) with or without 3-nitroxypropanol (3-NOP) on butyrate
1005 production at 48 h ruminal incubations (N = 117): butyrate production (µmol) =
1006 340 (±28.3; P = 0.006) + [28.19 (±2.89; P < 0.001) × if 3-NOP] – [0.0088
1007 (±0.035; P = 0.80) × fumaric acid (µmol disappearance)] + [0.69 (±0.045; P <
1008 0.001) × crotonic acid (µmol disappearance)] + [0.10 (±0.038; P = 0.010) ×
1009 (crotonic acid (µmol disappearance) – 43.16)] × if 3-NOP + [0.11 (±0.083; P =
1010 0.20) × acrylic acid (µmol disappearance)] – [0.001 (±0.0003; P = 0.009) ×
1011 (acrylic acid (µmol disappearance) – 53.81)]^2 + [0.079 (±0.029; P = 0.009) ×
1012 (acrylic acid (µmol disappearance) – 53.81)] × if 3-NOP + cow (random) + week
1013 (random) + error; all other terms, $P \geq 0.41$. Curves and 95% confidence bands
1014 fit the observations displayed without accounting for the random effects of the
1015 donor cow and incubation run (Experiment 1).

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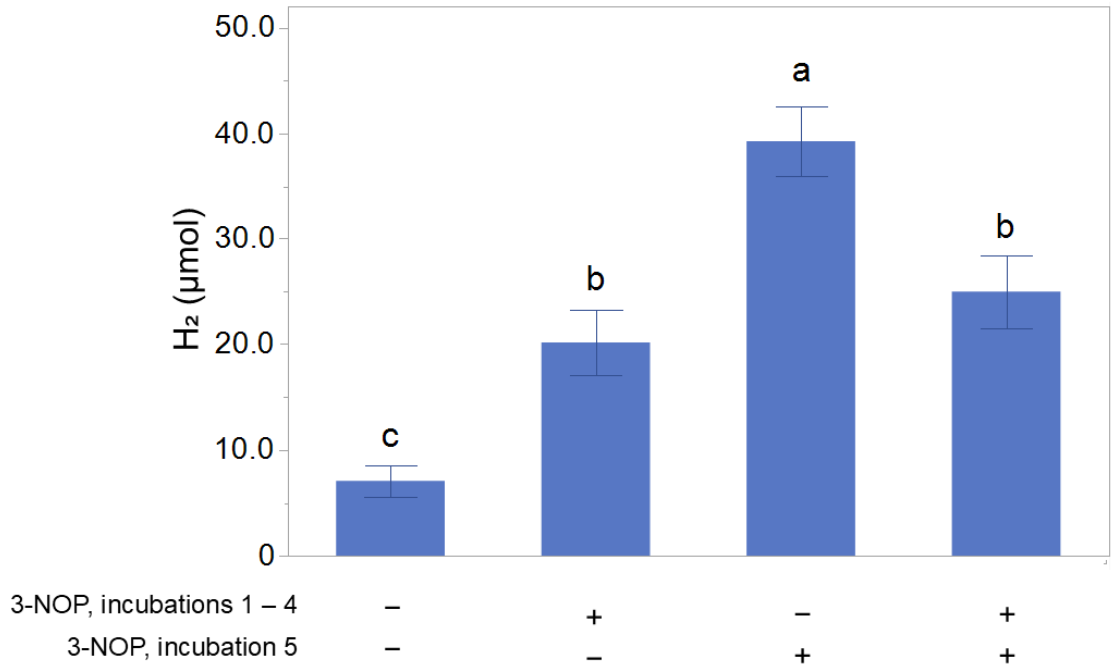


Figure 8. Effect of 3-NOP at 10 µM and crotonic acid at 8 mM on dihydrogen (H_2) accumulation in incubation 5 of 5-transfer serial ruminal cultures. Under a 2 × 2 × 2 × 2 factorial arrangement of treatments, cultures received 3-NOP in incubations 1 to 4 and/or incubation 5, and crotonic acid in incubations 1 to 4 and/or incubation 5 (N = 96). Only significant (P < 0.050) main effects and interactions are shown. 3-NOP in incubations 1 to 4, P < 0.001; Crotonic acid in incubations 1 to 4, P = 0.31; 3-NOP in incubation 5, P = 0.69; Crotonic acid in incubation 5, P = 0.14; 3-NOP in incubation 1 to 4 × 3-NOP in incubation 5, P < 0.001; all other interactions, P ≥ 0.094. Bars represent least square means ± SEM. Different letters indicate Tukey-s adjusted significant (P < 0.050) differences between treatment means. Cow donor of inoculum and week of incubation were included as random effects (Experiment 2).

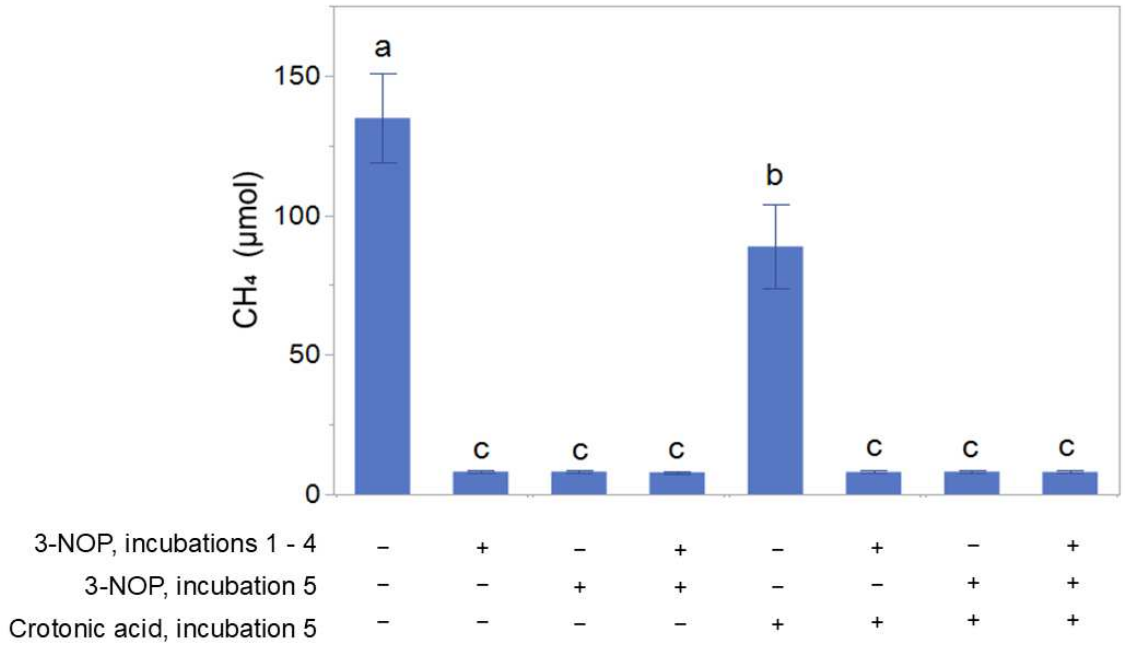


Figure 9. Effect of 3-NOP at 10 µM and crotonic acid at 8 mM on methane (CH_4) production in incubation 5 of 5-transfer serial ruminal cultures. Under a 2 × 2 × 2 × 2 factorial arrangement of treatments, cultures received 3-NOP in incubations 1 to 4 and/or incubation 5 and crotonic acid in incubations 1 to 4 and/or incubation 5 (N = 96). Only significant (P < 0.050) main effects and interactions are shown. 3-NOP in incubations 1 to 4, P < 0.001; Crotonic acid in incubations 1 to 4, P = 0.16; 3-NOP in incubation 5, P < 0.001; Crotonic acid in incubation 5, P = 0.029; 3-NOP in incubations 1 to 4 × Crotonic acid in incubation 5, P = 0.029; 3-NOP in incubations 1 to 4 × 3-NOP in incubation 5, P < 0.001; 3-NOP in incubation 5 × Crotonic acid in incubation 5, P = 0.028; 3-NOP in incubation 1 to 4 × 3-NOP in incubation 5 × Crotonic acid in incubation 5, P = 0.028; all other interactions, P ≥ 0.14. Bars represent least square means ± SEM. Different letters indicate Tukey-s adjusted significant (P < 0.050) differences between treatment means. Cow donor of inoculum and week of incubation were included as random effects (Experiment 2).

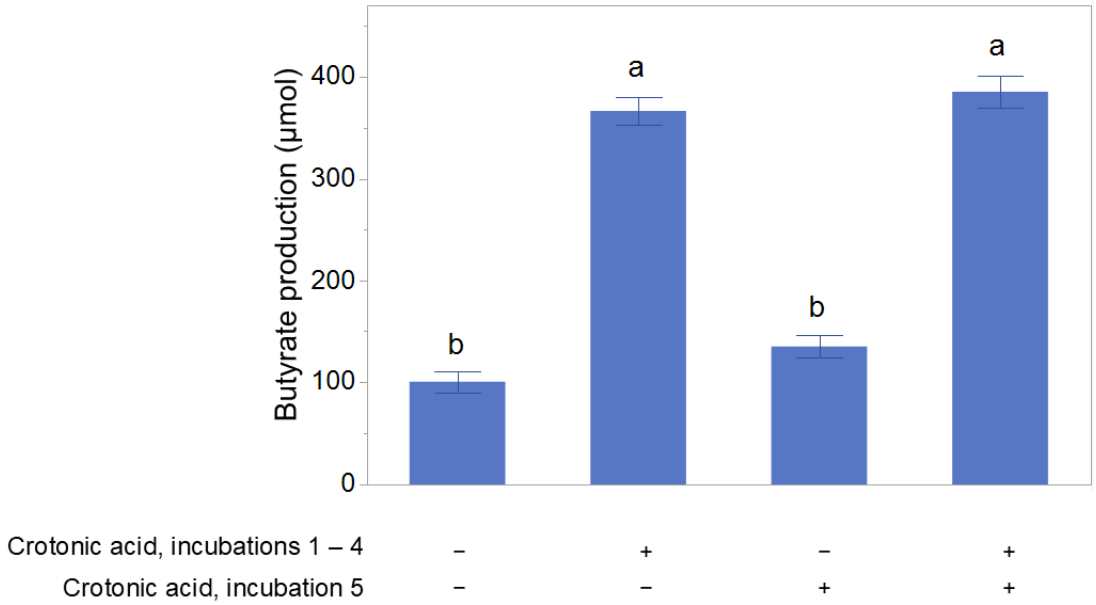
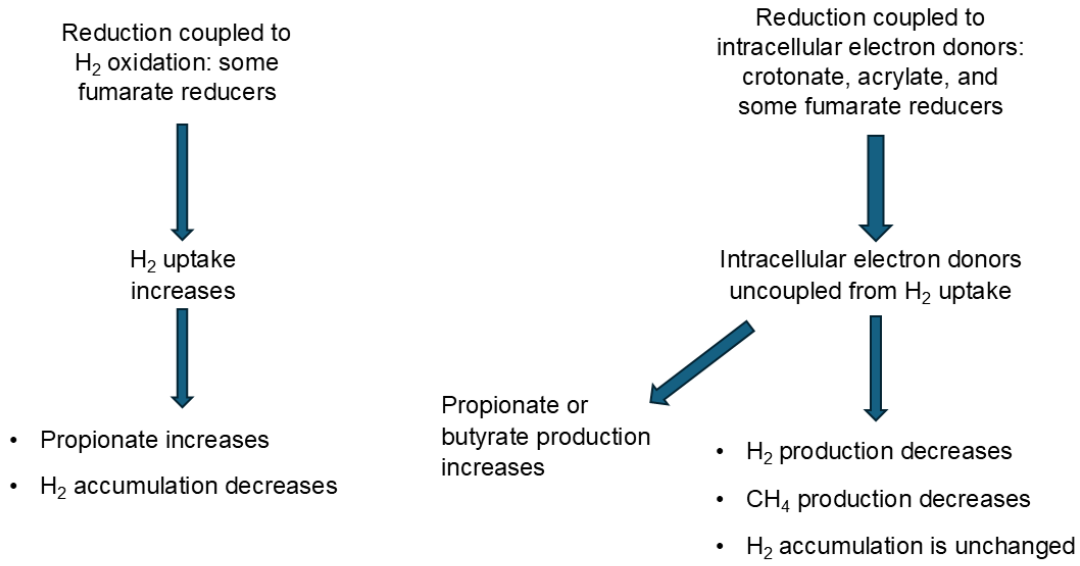


Figure 10. Effect of 3-NOP at 10 µM and crotonic acid at 8 mM on butyrate production in incubation 5 of 5-transfer serial ruminal cultures. Under a 2 × 2 × 2 × 2 factorial arrangement of treatments, cultures received 3-NOP in incubations 1 to 4 and/or incubation 5, and crotonic acid in incubations 1 to 4 and/or incubation 5 (N = 95). Only significant (P < 0.050) main effects and interactions are shown. 3-NOP in incubations 1 to 4, P = 0.54; Crotonic acid in incubations 1 to 4, P < 0.001; 3-NOP in incubation 5, P = 0.49; Crotonic acid in incubation 5, P = 0.037; all interactions, P ≥ 0.47. Bars represent least square means ± SEM. Different letters indicate significant (P < 0.050) effects of crotonic acid supplementation in incubations 1 to 4 within each treatment of crotonic acid supplementation in incubation 5. Cow donor of inoculum and week of incubation were included as random effects (Experiment 2).



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1069 Figure 11. Proposed model illustrating how the source of reducing equivalents
1070 used in the reduction of supplemented electron acceptors may differentially
1071 affect dihydrogen (H_2) accumulation and methane (CH_4) formation in rumen
1072 fermentation.

Supplementary Files

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