Dose–Response Effects of 3-Nitrooxypropanol Combined with Low- and High-Concentrate Feed Proportions in the Dairy Cow Ration on Fermentation Parameters in a Rumen Simulation Technique

Matthias Schilde 1,*D, Dirk von Soosten 1, Liane Hütber 1, Susanne Kersten 1, Ulrich Meyer 1, Annette Zeyner 2 and Sven Dänicke 1

1 Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, 38116 Braunschweig, Germany; Dirk.von_Soosten@fli.de (D.v.S.); Liane.Huether@fli.de (L.H.); Susanne.Kersten@fli.de (S.K.); Ulrich.Meyer@fli.de (U.M.); Sven.Daenicke@fli.de (S.D.)
2 Institute of Agricultural and Nutritional Sciences, Group Animal Nutrition, Martin Luther University Halle-Wittenberg, 06120 Halle (Saale), Germany; annette.zeyner@landw.uni-halle.de
* Correspondence: Matthias.Schilde@fli.de; Tel.: +49-531-58044-153

Abstract: Methane (CH\textsubscript{4}) from ruminal feed degradation is a major pollutant from ruminant livestock, which calls for mitigation strategies. The purpose of the present 4 × 2 factorial arrangement was to investigate the dose–response relationships between four doses of the CH\textsubscript{4} inhibitor 3-nitrooxypropanol (3-NOP) and potential synergistic effects with low (LC) or high (HC) concentrate feed proportions (CFP) on CH\textsubscript{4} reduction as both mitigation approaches differ in their mode of action (direct 3-NOP vs. indirect CFP effects). Diet substrates and 3-NOP were incubated in a rumen simulation technique to measure the concentration and production of volatile fatty acids (VFA), fermentation gases as well as substrate disappearance. Negative side effects on fermentation regarding total VFA and gas production as well as nutrient degradability were observed for neither CFP nor 3-NOP. CH\textsubscript{4} production was effectively reduced with an increasing dose of 3-nitrooxypropanol, which was, however, independent of concentrate feed proportion. Total gas production and fibre degradability were not affected by 3-nitrooxypropanol, indicating no negative side effects on fermentative capability. However, the hydrogen-liberating acetate production was reduced, whilst hydrogen gas was notably increased in a dose-dependent manner. The present in vitro study provides a deeper insight into a combined (3-nitrooxypropanol and high-concentrate feed proportions) methane abatement strategy under controlled conditions. The present combined approach reveals neither negative side effects nor additive effects between 3-nitrooxypropanol and varying concentrate feed proportions, which should be further investigated in future experiments in vivo.
Keywords: 3-nitrooxypropanol; concentrate feed proportion; RUSITEC; methane inhibitor; methane production

1. Introduction

Methane (CH\textsubscript{4}) is a climate-relevant greenhouse gas with a direct environmental impact insofar as its global warming potential exceeds 28 times that of carbon dioxide (CO\textsubscript{2}) on a 100-year time horizon [1]. In particular, enteric CH\textsubscript{4} from feed fermentation contributes to 46% of the total emissions from the dairy supply chain worldwide [2]. Accordingly, the development and implementation of CH\textsubscript{4} abatement strategies in ruminant livestock production systems can be expected to gain in importance [3].

The rumen simulation technique (RUSITEC) was introduced by Czerkawski and Breckenridge [4] as a semi-continuous-flow system to facilitate investigations on rumen fermentation processes, such as CH\textsubscript{4} production, and its manipulation under strictly controlled conditions. In parallel, dose–response relationships can be examined in the RUSITEC by incubating different dosage levels of CH\textsubscript{4} inhibitor substances on diet substrates in the juxtaposed reaction vessels.

Methane formation in ruminants, being catalysed by methyl Coenzyme M reductase (MCR) in hydrogenotrophic methanogenic Archaea, is the major pathway of removing metabolic hydrogen by reduction of CO\textsubscript{2} [5]. Apart from intraruminal volatile fatty acid (VFA) synthesis, CO\textsubscript{2} and hydrogen (H\textsubscript{2}) result from microbial degradation of fibre as well as non-fibre carbohydrates (NFC) supplied by the feed ration.

The synthetic substance 3-nitrooxypropanol (3-NOP) is a direct CH\textsubscript{4} inhibitor and structural analogue of methyl-coenzyme M (CoM). Thus, 3-NOP binds to the active site of the nickel enzyme methyl-coenzyme M reductase (MCR), causing its inactivation by oxidising the Ni(I) to Ni(II) in the cofactor F\textsubscript{430}. As a consequence, the MCR catalysed the reduction of CoM with coenzyme B to CH\textsubscript{4} and the heterodisulphide is intermitted during the last step of methanogenesis [5]. In contrast, increasing concentrate feed proportions (CFP) in the feed ration were previously proven as an indirect CH\textsubscript{4} abatement strategy [6], which can be related to diet-dependent effects on microbial community structures [7], reduced rumen pH values being detrimental to the growth of pH-sensitive methanogens and fibrolytic bacteria [8], and alterations in fermentation pathways. Thus, higher contents of NFC in high-concentrate diets are mainly degraded by propionate enhancers and, therefore, redirected to H\textsubscript{2}-consuming fermentation pathways, which results in substrate competition with methanogenesis [9].

Significant dose–response relationships of 3-NOP on CH\textsubscript{4} mitigation were observed in vitro [10] and in vivo [11–13]. Romero-Pérez et al. [10] tested 500, 1000, and 2000 mg of 3-NOP/kg of feed DM incubated with a high-forage diet substrate in a RUSITEC and observed quadratic effects of 3-NOP dose on CH\textsubscript{4} reduction (76.0%, 84.5%, and 85.6%). However, little information has been reported to reveal the dose–response relationships of 3-NOP in consideration of the potential additive effects with low and high CFP. Romero-Pérez et al. [14,15] supplemented 3-NOP in combination with the ionophore monensin to either high-forage [14] or high-grain [15] diets in a RUSITEC and reported additive effects of neither monensin nor high-grain diets on CH\textsubscript{4} reduction.

Regarding in vivo experiments, Vyas et al. [13] supplemented 50, 75, 100, 150, and 200 mg of 3-NOP/kg of feed DM to beef cattle provided high-forage and high-grain diets. The authors observed a significant dose response with regard to the higher dosage levels of 100, 150, and 200 mg of 3-NOP and a significant effect of the ration type. Thus, 3-NOP efficacy was greater in high-forage (26, 33, and 45% CH\textsubscript{4} reduction, resp.) when compared to high-forage (16, 21, and 23% CH\textsubscript{4} reduction, resp.) diets. However, CH\textsubscript{4} emissions at 50 and 75 mg 3-NOP dose/kg feed DM were not significantly different from the control. Correspondingly, in a meta-analysis of 3-NOP experiments including dairy and beef cattle, Dijkstra et al. [11] confirmed the dose-dependent 3-NOP effect on
Animals 2021, 11, 1784

CH$_4$ yield, which was modelled to $-2.48 \pm 0.0734\%$ CH$_4$ yield per 10 mg/kg DM increase in 3-NOP dose from its mean (123 mg 3-NOP/kg of feed DM). Melgar et al. [12] mixed 3-NOP into a forage-based total-mixed ration (TMR) for dairy cows and reported that CH$_4$ yield quadratically decreased by 24.3, 26.5, 22.5, 33.5, 35.9, and 31.8% for 40, 60, 80, 100, 150, and 200 mg of 3-NOP/kg feed DM, respectively, with no statistical difference among 40, 60, and 80 as well as between 100, 150, and 200 mg 3-NOP/kg DM.

Accordingly, in vitro studies investigating the dose–response relationships of 3-NOP in combination with low- and high-concentrate diets are scarce. Therefore, the present RUSITEC experiment aimed at investigating the dose–response relationships of 3-NOP and potential synergistic effects between 3-NOP dosage level and low- or high-concentrate diets on fermentation parameters. A novelty of the present approach encompasses the application of very low 3-NOP inclusion rates, which were experimentally chosen to enable comparisons to those recently supplemented to dairy [12,16] and beef [13] cattle (40–200 mg 3-NOP/kg feed DM) under practical conditions in vivo.

It was hypothesised that CH$_4$ production decreases with increasing 3-NOP dosage level and that supplementing high-concentrate feed proportions causes additive effects on CH$_4$ reduction in vitro.

2. Materials and Methods

The experiment was carried out at the experimental station and laboratory of the Friedrich-Loeffler Institut (FLI) in Braunschweig, Germany. Maintenance of the cannulated cows and collection of rumen fluid were in compliance with the German Animal Welfare Act and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (33.19-42502-04-15/1858).

2.1. Experimental Design and Diets

The experiment was conducted using the RUSITEC according to the general incubation procedure described by Czerkawski and Breckenridge [4]. The study was arranged as a $2 \times 4$ factorial design with low- (LC) and high- (HC) concentrate feed proportion (CFP) in the incubated diet and the methane inhibitor 3-nitrooxypropanol (3-NOP; DSM Nutritional Products AG, Kaiseraugst, Switzerland) supplied at four doses of 0 (placebo, PLA), 73 (LOW), 160 (MED), and 1200 (HIGH) mg of the active 3-NOP substance/kg of feed DM. Both the placebo and the 3-NOP supplement contained propylene glycol and SiO$_2$ acting as carriers for 10% of the active 3-NOP substance (1,3-propanediol mononitrate) in product DM, which was included in 3-NOP treatments only. On a DM basis, the experimental diet substrates were formulated according to the forage:concentrate ratio of 70:30 (LC) and 40:60 (HC). The forage proportion of the LC and HC diet was composed of 70% maize silage and 30% grass silage. The 3-NOP supplement was mixed into the ground concentrate feed and homogenised for 10 min (M4.REI; Gebr. Lödige Maschinenbau GmbH, Paderborn, Germany).

2.2. Experimental Procedure

In total, six incubation trials were conducted using a four-vessel RUSITEC apparatus. Each CFP $\times$ 3-NOP combination was tested in triplicate. Each incubation run consisted of an adaptation period lasting eight days, followed by a four-day sampling period.

The diet components were pre-dried at 60 °C for 48 h and ground to pass a 10 mm (forages) and a 3 mm (concentrates) screen (SM 1, Retsch, Haan, Germany). The diet (12.0 g fresh matter (FM) with 90.3% DM content) was weighed into nylon bags (50 ± 15 µm pore size; 10 × 20 cm; ANKOM Technol., Fairport, NY, USA). The ingredients and chemical composition of the incubated feedstuffs and diets are presented in Table 1.

Three rumen-fistulated cows were kept as donor animals for the inoculum of rumen liquid and solid digesta on a diet consisting of 40% concentrates, 30% maize silage, and 30% grass silage (DM basis) for ad libitum intake. Inocula were collected from three cows via the fistula one hour before the morning feeding. Rumen fluid was collected by introducing
a probe [17], which was attached to the flexible tube of a hand suction pump (SELEKT Rumen-Fluid Collector, Nimrod Veterinary Products Ltd., Gloucestershire, UK), into the ventral rumen. Solid rumen digesta were manually taken from the ventral, caudal, and cranial side of the rumen. The fluid was strained (cheesecloth of 250 µm mesh opening) into nitrogen-flushed and pre-warmed insulated bottles. Both the solid and liquid rumen contents were placed into a water bath (39 °C), transported to the laboratory immediately, and pooled together.

Table 1. Ingredients and chemical composition of the experimental diets.

| Item                                      | Experimental Diet † |
|-------------------------------------------|---------------------|
| Ingredients (g/kg of diet DM §)            | LC                  | HC                  |
| Maize silage                              | 495                 | 286                 |
| Grass silage                              | 212                 | 122                 |
| Rapeseed meal                             | 44.4                | 90.1                |
| Soybean meal                              | 37.2                | 74.7                |
| Wheat                                     | 97                  | 195.5               |
| Dried sugar beet pulp                     | 85                  | 172                 |
| Soybean oil                               | 4.5                 | 9.2                 |
| Calcium carbonate                         | 7                   | 14.2                |
| Urea                                      | 2.9                 | 6.1                 |
| Vitamin/Mineral premix ‡                  | 15                  | 30.2                |
| Chemical analysis of the ration DM (g/kg)| 908                 | 897                 |
| Nutrient (g/kg of DM)                     |                     |                     |
| Organic matter                            | 923                 | 938                 |
| Crude protein                             | 131                 | 171                 |
| Ether extract                             | 33                  | 33                  |
| aNDFom ¶                                 | 382                 | 308                 |
| ADFom #                                  | 217                 | 178                 |
| Starch                                    | 257                 | 284                 |

† Experimental diets with low- (LC) and high- (HC) concentrate feed proportion supplied at four doses of 0, 0.073, 0.16, and 1.2 mg of the active 3-nitrooxypropanol substance/g feed DM. § DM, dry matter. ‡ Ingredients according to the manufacturer’s specifications: minerals (g/kg of premix): Ca, 140; Na, 120; P, 70; Mg, 40; Zn, 6; Mn, 5.4; Cu, 1; I, 0.1; Se, 0.04; Co, 0.025; vitamins (IU/kg of premix): A, 1,000,000; D3, 100,000; E, 2235. ¶ aNDFom; α-amylase treated neutral detergent fiber expressed without residual ash; # ADFom; acid detergent fibre expressed without residual ash.

The incubation was initiated by inoculating the pre-warmed reaction vessels (volume of 900 mL), each with 550 mL of rumen fluid, 100 mL of warm artificial saliva [18], and with one nylon bag of 80 g wet weight of solid rumen digesta and one nylon bag containing the diet substrate. Subsequently, the bags were inserted into the perforated feed container of each vessel and the fermenters were immersed in the water bath (39 °C) of the RUSITEC apparatus. The food containers were moved up and down (vertical strokes of 65 mm) and agitated at 8 cycles/min. The feed bags were incubated for 48 h in the food container, whereas the initial bag containing the solid rumen inoculum was replaced after 24 h by a feed bag. After 48 h of incubation, the feed bags were removed from the vessel, gently washed with 40 mL of artificial saliva for 1 min in polyethylene bags, squeezed by hand, and replaced by a new one. The washed-out fluids were returned into the vessel. Both the vessels and effluent bottles were flushed with nitrogen every day after feed bag exchange. The McDougall buffer solution [18] was prepared daily and similarly across treatments and continuously infused into each vessel to achieve a dilution rate of 650 mL/24 h (3%/h) using a peristaltic pump. Buffer composition and infusion rate were not changed between treatments to investigate inherent effects of the diet composition in combination with 3-NOP.
2.3. Sampling and Analyses

Feed samples of the pre-incubated and fermented diet were analysed according to the standard methods of the Association of German Agricultural Analytic and Research Institutes [19] for DM (3.1), crude ash (CA; 8.1), crude protein (CP; Dumas method, 4.1.2), ether extract (EE) pre-treated with hydrochloric acid (5.1.1), starch (7.2.1), acid detergent fibre (ADFom; 6.5.2), and α-amylase treated neutral detergent fibre (aNDFom; 6.5.1), both expressed without residual ash.

During the four-day sampling period, all the samples were taken contemporaneously for one daily feed bag exchange. Each feed bag collected after 48 h incubation was dried at 60 °C for 72 h, weighed, and ground to pass a 1-mm mesh sieve (SM 1; Retsch, Haan, Germany). The feed residues of each treatment and incubation run were pooled over the sampling period and analysed for DM, CA, and aNDFom.

The pH and redox potential (Eh) in the fermenter fluid were measured using glass electrodes (SenTix 41 (pH) and SenTix PtR (Eh); pH 7110; WTW, Weilheim, Germany) which were calibrated every day.

The effluent was collected in 1-litre volumetric flasks placed on ice and effluent volume was noted daily. VFA were analysed from daily collected effluent samples (80 mL) according to Geissler et al. [20] using a gas chromatograph (Clarus 680; PerkinElmer LAS GmbH, Rodgau, Germany) equipped with a flame ionisation detector. Ammonia-N concentration (NH₃-N) was measured using steam distillation (DIN38406-E5-2, [21]).

Fermentation gases were collected over the whole sampling period in 10-litre gas bags (Plastigas; Linde GmbH, Pullach, Germany). After termination of the run, 10 mL of fermentation gases were withdrawn via the septum of the gas bag using a gas-tight syringe. The gas samples were injected on a chromatography column (Porapak QS; 80/100 mesh, 3 m × 3 mm, Agilent Technologies, Inc., Santa Clara, CA, USA) of a gas chromatograph equipped with a thermal conductivity detector (GC-14B; Shimadzu, Kyoto, Japan) and argon as carrier gas. Gas samples were determined for percentage of CH₄, CO₂, and H₂. The gas volume in the gas bags was measured using a drum-type gas meter (TG05; Ritter Apparatebau GmbH & Co. KG, Bochum, Germany) and added to the gas volume of the gas space in the effluent bottle.

2.4. Calculations and Statistical Analyses

Total gas volume was corrected for temperature (0 °C) and pressure (101.325 kPa) conditions. The daily production of VFA and NH₃-N resulted from multiplication of the measured concentrations by the effluent and gas volume, respectively. Apparent disappearance of organic matter (OMAD) and the degradability of DM (DMD) and aNDFom (NDFD) after 48 h of incubation were calculated by subtracting the pre- and post-incubated nutrient contents and substrate masses.

Statistical data analysis was carried out using PROC MIXED (version 9.4; SAS Institute Inc., Cary, NC, USA) and the mixed model was fitted by a restricted maximum likelihood (REML) method according to Littell et al. [22]. The 3-NOP dose level (DOSE), concentrate proportion in the diet substrate (CFP), incubation run, and their interaction were set as fixed effects and fermentation vessel was implemented as a random effect. Satterthwaite approximation was used for calculating the degrees of freedom. The variance components were estimated using the REML method and the variance-covariance structure was selected based on the lowest Akaike Information Criterion. Customised post-fitting hypothesis tests among LS means were conducted using the LSMESTIMATE statement in PROC MIXED with SCHEFFE-adjusted multiple comparisons.

To fit the nested polynomial regression model and convert 3-NOP doses to equally spaced dosage levels, the linear LOGDOSE = LOG2(DOSE) and quadratic LOGDOSE_2 = LOGDOSE^2 regression parameters were created for 3-NOP doses within each CFP in the DATA step. As fixed regressive components were considered the effects of CFP, increasing 3-NOP dose (LOGDOSE) within the treatment (LC or HC) “LOGDOSE(CFP)”
(linear regression term) and, for calculating the quadratic regression term, additionally its square “LOGDOSE_2 (CFP)”. RUN × DOSE was set in the RANDOM statement to define the whole-plot error. The HTYPE option was set = 1 to enter and test the model terms (linear, quadratic) in sequential order.

The CONTRAST statement was used to test whether regression coefficients (linear (L), quadratic (Q)) were equal between both treatments of CFP ($H_0$: $\beta_{L,HC} = \beta_{L,LC}$ and $H_0$: $\beta_{Q,HC} = \beta_{Q,LC}$). The t-values from the regression model were used to test linear and quadratic effects of 3-NOP: $H_0$: $\beta_{L,3-NOP}$ or $\beta_{Q,3-NOP} = 0$, which is equivalent to the orthogonal polynomial contrasts.

PROCEDURE MIXED METHOD = REML;
CLASS CFP RUN DOSE;
MODEL Y = CFP LOGDOSE(CFP) LOGDOSE_2(CFP)/NOINT DDFM = KENWARD ROGER SOLUTION HTYPE = 1;
RANDOM RUN RUN × DOSE;
CONTRAST ‘LINEAR: coefficients equal’ LOGDOSE(CFP) 1 −1;
CONTRAST ‘QUADRATIC: coefficients equal’ LOGDOSE_2 (CFP) 1 −1;
RUN.
Effects were declared statistically significant at $p$-values $\leq 0.05$ and a trend was postulated at $p$-values between $>0.05$ and $0.10$. Results are presented as least square means (LS means) with the standard error of means (SEM). Pearson correlation coefficients were calculated with $N = 24$ observations.

3. Results

3.1. Diet Composition and Substrate Degradability

Ingredients and chemical composition of the incubated diets are presented in Table 1. The DMD (dose; $p = 0.041$) and OMAD (dose; $p = 0.052$) varied by 3-NOP dosage level (Table 2). In LC diets, DMD and OMAD increased from PLA to LOW by 11% and decreased by 7% in HIGH when compared to PLA. The DMD and OMAD were comparable between PLA and MED. In HC, DMD and OMAD were highest in diets with LOW and HIGH 3-NOP dosage levels but lower in PLA and MED. Percentage of DMD (%) was positively related to percentage proportion of CH$_4$ (Vol.-%) ($r = 0.471; p = 0.020$) and CO$_2$ (Vol.-%) ($r = 0.487; p = 0.016$) but negatively to H$_2$ (Vol.-%) ($r = -0.368; p = 0.077$) in total fermentation gas.

Table 2. Effects of 3-nitrooxypropanol (3-NOP) dosage levels (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (LC) or high- (HC) concentrate feed proportion in the diet (CFP) on dry matter degradation (DMD), apparent organic matter degradation (OMAD), and neutral-detergent fibre degradation (NDFD) (g/kg DM).

| Item   | Treatments | SEM | p-Values |
|--------|------------|-----|----------|
|        | CFP        | PLA | LOW | MED | HIGH | CFP | Dose | CFP × Dose | L | Q | $\beta_L > F$ | $\beta_Q > F$ |
| DMD    | LC         | 640 | 718 | 638 | 596  | 26  | 0.640 | 0.041 | 0.335 | 0.464 | 0.229 | 0.364 | 0.216 |
|        | HC         | 655 | 687 | 627 | 658  | 26  | 0.654 | 0.052 | 0.254 | 0.395 | 0.170 | 0.275 | 0.136 |
| OMAD   | LC         | 643 | 723 | 642 | 601  | 26  | 0.654 | 0.052 | 0.254 | 0.395 | 0.170 | 0.275 | 0.136 |
|        | HC         | 658 | 687 | 628 | 669  | 49  | 0.091 | 0.246 | 0.145 | 0.346 | 0.149 | 0.349 | 0.162 |
| NDFD   | LC         | 358 | 505 | 362 | 293  | 49  | 0.091 | 0.246 | 0.145 | 0.346 | 0.149 | 0.349 | 0.162 |
|        | HC         | 310 | 319 | 288 | 350  | 49  | 0.091 | 0.246 | 0.145 | 0.346 | 0.149 | 0.349 | 0.162 |

† Values presented as LS means. § SEM, standard error of the means. + Effects of CFP, 3-NOP dose, and interactions between them; L, Q, $p$-values for linear and quadratic effects of 3-NOP; $\beta_L > F$, $\beta_Q > F$, probability under $H_0$ that an F-distributed random variable exceeds observed $F$, for the difference in the linear and quadratic regression coefficients between LC and HC. Significant values ($p \leq 0.05$ are highlighted in bold).

Degradability of NDF tended to be higher in LC diets (CFP; $p = 0.091$; Table 2) irrespective of 3-NOP dose. The NDFD correlated negatively with pH in fermenter fluid ($r = 0.665; p < 0.001$) and NH$_3$-N (mg/g of DMD) ($r = -0.632; p = 0.001$) but positively to Eh ($r = 0.495; p = 0.014$) and acetic acid concentration (mmol/L) ($r = 0.734; p < 0.001$).
3.2. Gas Production and Gas Composition

Total GP (mL/d and mL/g of DMD) and CO$_2$ (%) and mL/g of DMD) were affected by neither 3-NOP dose nor CFP (Table 3) and both were negatively correlated to pH in the fermenter fluid (GP: $r = -0.436; p = 0.033$; CO$_2$: $r = -0.460; p = 0.024$). A trend was observed for a quadratic effect of 3-NOP dose on CO$_2$ (Vol.-%) regarding LC diets (Q: $p = 0.066$; Table 3) and the difference in quadratic regression coefficients between LC and HC diets ($f_Q > F: p = 0.086$).

Table 3. Effects of 3-nitrooxypropanol (3-NOP) dosage levels (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (LC) or high- (HC) concentrate feed proportion in the diet (CFP) on fermentation gas production and composition.

| Item                  | Treatments $^*$ | SEM $^b$ | p-Values $^c$ |
|-----------------------|----------------|----------|---------------|
|                       | CFP PLA LOW MED HIGH | CFP Dose | CFP × Dose L Q $f_L > F$ $f_Q > F$ |
| Total GP (mL/d)       |                |          |               |
| LC                    | 1902           | 1904     | 1664          | 1818 | 18 | 0.506 | 0.600 | 0.978 | 0.411 | 0.479 | 0.823 | 0.732 |
| HC                    | 1844           | 1797     | 1648          | 1654 | 0.543 | 0.705 |
| Gas production (mL/g of DMD) $^d$ |                |          |               |
| Total GP              |                |          |               |
| LC                    | 1091           | 977    | 938           | 1152 | 110 | 0.494 | 0.711 | 0.698 | 0.181 | 0.121 | 0.423 | 0.272 |
| HC                    | 1042           | 970    | 982           | 944  | 0.703 | 0.825 |
| CH$_4$                |                |          |               |
| LC                    | 15.8           | 12.2   | 5.2           | 0.5  | 1.2  | 0.419 | <0.001 | 0.241 | 0.001 | 0.145 | 0.028 | 0.045 |
| HC                    | 14.5           | 12.1   | 8.7           | 1.1  | 0.133 | 0.407 |
| CO$_2$                |                |          |               |
| LC                    | 100.2          | 94.2   | 92.7          | 98.4 | 13.8 | 0.538 | 0.962 | 0.981 | 0.642 | 0.637 | 0.835 | 0.745 |
| HC                    | 93.4           | 94.1   | 86.6          | 86.8 | 0.812 | 0.907 |
| H$_2$                 |                |          |               |
| LC                    | 0.44           | 2.11   | 6.03          | 11.89| 1.92 | 0.579 | 0.001 | 0.623 | 0.290 | 0.766 | 0.595 | 0.859 |
| HC                    | 1.42           | 3.21   | 3.80          | 8.96 | 0.749 | 0.584 |
| Gas composition (Vol.-%) |                |          |               |
| CH$_4$                |                |          |               |
| LC                    | 5.8            | 5.1    | 2.2           | 0.2  | 0.3  | 0.138 | <0.001 | 0.094 | 0.002 | 0.566 | 0.082 | 0.124 |
| HC                    | 5.5            | 5.0    | 3.6           | 0.5  | 0.241 | 0.119 |
| CO$_2$                |                |          |               |
| LC                    | 36.6           | 38.5   | 39.6          | 33.5 | 1.9  | 0.567 | 0.430 | 0.302 | 0.146 | 0.066 | 0.173 | 0.086 |
| HC                    | 35.6           | 37.8   | 35.0          | 36.6 | 0.979 | 0.959 |
| H$_2$                 |                |          |               |
| LC                    | 0.2            | 0.9    | 2.6           | 3.9  | 0.5  | 0.755 | <0.001 | 0.458 | 0.046 | 0.579 | 0.233 | 0.307 |
| HC                    | 0.5            | 1.2    | 1.5           | 3.7  | 0.699 | 0.368 |
| CH$_4$/CO$_2$         |                |          |               |
| LC                    | 0.157          | 0.131  | 0.055         | 0.004| 0.008 | 0.024 | <0.001 | 0.026 | <0.001 | 0.220 | 0.035 | 0.048 |
| HC                    | 0.155          | 0.133  | 0.102         | 0.014| 0.177 | 0.082 |
| CO$_2$/CH$_4$         |                |          |               |
| LC                    | 6.4            | 7.8    | 18.9          | 915.6| 193.2| 0.185 | 0.036 | 0.179 | 0.398 | 0.066 | 0.592 | 0.238 |
| HC                    | 6.5            | 7.6    | 10.0          | 163.6| 0.857 | 0.741 |
| CH$_4$/H$_2$          |                |          |               |
| LC                    | 39.45          | 5.84   | 0.86          | 0.04 | 2.65 | 0.002 | <0.001 | 0.001 | <0.001 | 0.001 | 0.001 | 0.002 |
| HC                    | 10.53          | 4.32   | 3.00          | 0.22 | 0.183 | 0.422 |

$^a$ Values presented as LS means. $^b$ SEM, standard error of the means. $^c$ Effects of CFP, 3-NOP dose, and interactions between them; L, Q, p-values for linear and quadratic effects of 3-NOP; $f_L > F$, $f_Q > F$, probability under $H_0$ that an F-distributed random variable exceeds observed $F$, for the difference in the linear and quadratic regression coefficients between LC and HC. $^d$ GP, gas production. $^*$ DMD, dry matter degradation. Significant values ($p \leq 0.05$ are highlighted in bold).

The greatest CH$_4$ proportion (5.8%) was recorded for the control (PLA), followed in descending order by LOW, MED, and HIGH 3-NOP treatment down to 0.2% as in the case referring to CH$_4$ production (from 15.8 to 0.5 mL/g of DMD) in LC diets and 5.5% (PLA) to 0.5% (HIGH) and 14.5 (PLA) to 1.1 mL/g of DMD (HIGH) in HC diets, respectively (Table 3; dose: $p < 0.001$). Increasing 3-NOP dosage levels reduced CH$_4$ (%) and mL/g of DMD) in a linear manner in LC diets only (L: $p < 0.01$; Table 3) and, with regard to CH$_4$ (Vol.-%), 3-NOP efficacy tended to be less pronounced in HC substrates (CFP × dose: $p = 0.094$; Table 3). The linear ($f_L > F: p = 0.028$) and quadratic ($f_Q > F: p = 0.045$) components of the regression were significantly different between LC and HC, indicating a variation in 3-NOP mitigation efficiency depending on the provided CFP, whereas the CFP main effect was not significant. In LC diets, 3-NOP supplementation reduced CH$_4$ (Vol.-%) by 12% (LOW), 61% (MED), and 97% (HIGH) relative to CH$_4$ (Vol.-%) analysed in the fermentation gas of the PLA treatment. Comparatively, CH$_4$ (Vol.-%) was mitigated to a lower extent in HC treatments, namely by 10% (LOW), 35% (MED), and 90% (HIGH) in relation to PLA. Methane proportion (Vol.-%) and production (mL/g of DMD) significantly differed among 3-NOP doses, except in the PLA versus LOW treatments (CH$_4$ (Vol.-%): LC: $p = 0.147$; HC:...
Figure 1. Effect of 3-nitrooxypropanol dose (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (□, dashed line) or high- (■, solid line) concentrate proportion in the incubated diet on (A) methane (CH₄) to carbon dioxide (CO₂) ratio and (B) hydrogen (H₂) proportion (Vol.-%) in fermentation gases; curve fitting according to (non)significant L and Q effects (Table 3).

Figure 1B illustrates a 27-fold increase in H₂ (% and mL/g of DMD) in LC and, to a lesser extent, a 6.2-fold increase in HC diets with increasing 3-NOP dose relative to PLA (Table 3; dose: p = 0.001). However, H₂ (% and mL/g of DMD) was not different between PLA and LOW. Both linear and quadratic effects of 3-NOP dose on H₂ (Vol.-%) remained not significant concerning the HC diet. In contrast, the linear regression coefficient was significant for the LC diet (L: p = 0.046), indicating that the slope of the curve increased more steadily when compared to HC (Figure 1B). In HC diets, H₂ (% and mL/g of DMD) was not significantly higher for 3-NOP dose MED in comparison to PLA, whereas contrast analysis revealed a significant variation between PLA and MED for LC diets (p < 0.05). Furthermore, H₂ (Vol.-%) significantly correlated to CH₄ (Vol.-%) (r = −0.872), acetate (mol %) (r = −0.535), propionate (mol %) (r = −0.379; p = 0.068), and iso-butyraye (mol %) (r = −0.570) in a negative manner, whereas positive relationships (p < 0.05) were found...
for iso-valerate (mol %) \((r = 0.601)\) and the production of butyrate (mmol/g of DMD) \((r = 0.606)\).

3.3. Fermentation Parameters and End-Products

The pH was significantly lower in fermenter fluids of LC diets (CFP: \(p = 0.019\); Table 4), which is due to the sharp drop in pH at the MED 3-NOP dose (Figure 2). The \(Eh\) was affected by 3-NOP dose (dose: \(p = 0.01\); Table 4) insofar as the \(Eh\) of 3-NOP dose LOW was significantly higher when compared to that of the PLA treatment \((p = 0.008)\). However, the \(Eh\) values of MED \((p = 0.548)\) and HIGH \((p = 0.120)\) were not significantly changed when compared to PLA, but MED differed from 3-NOP doses LOW \((p = 0.002)\) and HIGH \((p = 0.039)\) (Figure 2).

The effluent volume (mL/d) tended to be interactively affected by CFP and dose \((p = 0.065)\) and appeared to be significantly reduced at 3-NOP dose MED and HIGH in relation to LOW in HC diets (Table 4).

Irrespective of 3-NOP dose, NH3-N concentration (mg/L) was 41% lower in LC compared with HC diets (CFP: \(p = 0.009\); Table 4). In addition, NH3-N concentration increased when supplementing 3-NOP dose LOW but decreased at higher 3-NOP doses of MED and HIGH independently of the CFP in the incubated ration (dose: \(p = 0.006\)).

The NH3-N production ranged from 12.4 to 14.9 mg/g of DMD within LC and between 17.7 and 20.9 mg/g of DMD within HC treatment (Table 4). Moreover, NH3-N production significantly differed by 5.4 mg/g of DMD between LC and HC (CFP: \(p < 0.001\)) and tended to be influenced by the CFP \(\times\) dose interaction \((p = 0.081)\). In LC treatments, NH3-N production varied in a quadratic manner with increasing 3-NOP dose \((Q: p = 0.049)\) (Figure 3A). The curves of NH3-N production were shaped in an inverse manner between the incubated LC (convex) and HC (concave) substrates, and the quadratic regression coefficients differed significantly \((\beta_Q > F: p = 0.020)\).
Table 4. Effects of 3-nitrooxypropanol (3-NOP) dosage levels (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (LC) or high- (HC) concentrate feed proportion in the diet (CFP) on fermentation characteristics and production of volatile fatty acids (VFA).

| Item                      | Treatments $^+$ | SEM $^§$ | $p$-Values $^+$ | CFP | Dose | CFP $\times$ Dose | L  | Q  | $f_L > F$ | $f_Q > F$ |
|---------------------------|-----------------|----------|-----------------|-----|------|-------------------|----|----|-----------|-----------|
| **pH**                    |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | 6.39            | 6.29     | 6.44            | 6.42| 0.05 | 0.019             | 0.0552| 0.083| 0.912     | 0.830     | 0.880 | 0.495     |
| HC                        | 6.49            | 6.51     | 6.48            | 6.40| 0.007| 0.009             | 0.011 | 0.017| 0.623     | 0.645     | 0.100 | 0.178     |
| **Eh (mV)** $^\ddagger$   |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | −279            | −244     | −302            | −268| 0.006| 0.010             | 0.517 | 0.623| 0.621     | 0.743     |
| HC                        | −308            | −271     | −299            | −281| 0.004| 0.004             | 0.007 | 0.032| 0.442     | 0.328     | 0.433 | 0.361     |
| **Effluent (mL/d)**        |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | 656             | 588      | 650             | 655 | 0.055| 0.009             | 0.006 | 0.499| 0.344     | 0.611     | 0.187 | 0.125     |
| HC                        | 622             | 666      | 625             | 642 | 0.007| 0.007             | 0.008 | 0.505| 0.608     | 0.228     |
| **NH$_3$-N (mg/L)**       |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | 157             | 165      | 136             | 142 | 0.008| 0.008             | 0.007 | 0.083| 0.065     | 0.912     |
| HC                        | 214             | 235      | 207             | 191 | 0.004| 0.004             | 0.007 | 0.552| 0.621     | 0.743     |
| **NH$_3$-N (mg/g DMD)** $^\#$ |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | 14.9            | 12.4     | 12.5            | 14.7| 0.011| <0.001            | 0.081 | 0.065| 0.049     | 0.040     | 0.020     |
| HC                        | 18.3            | 20.9     | 19.1            | 17.7| 0.007| 0.007             | 0.008 | 0.260| 0.151     |           |
| **Total VFA (mmol/L)**    |                 |          |                 |     |      |                   |    |    |           |           |
| LC                        | 76.3            | 83.4     | 78.0            | 71.1| 0.010| 0.010             | 0.011 | 0.203| 0.466     | 0.287     | 0.547 | 0.229     |
| HC                        | 69.0            | 70.6     | 71.3            | 80.5| 0.004| 0.004             | 0.005 | 0.955| 0.587     |           |

- Values presented as LS means.
- SEM, standard error of the means.
- Effects of CFP, 3-NOP dose, and interactions between them; L, Q, $p$-values for linear and quadratic effects of 3-NOP; $f_L > F$, $f_Q > F$, probability under $H_0$ that an F-distributed random variable exceeds observed $F$, for the difference in the linear and quadratic regression coefficients between LC and HC.
- $Eh$, redox potential.
- DMD, dry matter degradation.
- $C_2/C_3$ ratio, acetate/propionate ratio. Significant values ($p \leq 0.05$ are highlighted in bold).
compared to the relatively constant fluctuations observed in the HC treatment. Accordingly, a quadratic effect of 3-NOP dose on propionate (mol %) was noted in LC diets (Q: \( p = 0.017 \)) (Figure 3C).

It was noted that CFP and 3-NOP dose affected the acetate/propionate ratio (\( C_2/C_3 \)) in an interactive manner (CFP × dose: \( p = 0.032 \)). The quadratic effect of 3-NOP dose on \( C_2/C_3 \) in LC diets (Q: \( p = 0.008 \)) corresponded to the continual decrease from 3.03 to 2.77 in \( C_2/C_3 \) with increasing 3-NOP dose and the peak of 3.80 at 3-NOP dose HIGH. In HC diets, \( C_2/C_3 \) fluctuated non-significantly between 3-NOP treatments.

Neither CFP nor 3-NOP dose affected butyric acid (mol % and mmol/g of DMD). Valeric acid (mol % and mmol/g of DMD) was only affected by 3-NOP dose (dose: \( p < 0.05 \)). The steady decrease in valerate (mol % and mmol/g of DMD) with increasing 3-NOP dose was interrupted by a notable peak at 3-NOP dose MED, being significantly different from LOW and HIGH dose (\( p < 0.05 \)), independently of the incubated diet type.

The production (mmol/g of DMD) of the branched-chain fatty acid (BCVFA) iso-valerate increased with increasing 3-NOP dose in a convex parabolic-shaped manner in both LC (Q: \( p = 0.048 \)) and, as a trend, in HC (Q: \( p = 0.069 \)) substrates (Figure 3D). However, quadratic regression coefficients were not different between LC and HC (Table 4), but iso-valerate was approximately 22% lower in HC when compared to LC diets (CFP: \( p < 0.05 \)).

Total VFA concentration (mmol/L) was not modified by treatments. In contrast, higher total VFA production (mmol/g of DMD) was observed with reduced dietary CFP (CFP: \( p = 0.012 \)) and increasing 3-NOP dose level (dose: \( p = 0.05 \)) (Table 4).

Molar acetate proportion (mol %) ranged from 49.8% to 52.3% in LC and 49.4% to 53.4% in the HC treatment. Acetate (mol %) was not influenced by CFP but decreased with 3-NOP dose increment (dose: \( p = 0.044 \)) and dropped to the greatest extent at 3-NOP dose MED (Figure 3B). In contrast, acetate production (mmol/g of DMD) was independent of 3-NOP dose but 9.4% higher in LC than in HC diets (CFP: \( p = 0.004 \); Table 4).

The molar percentage of propionate (mol %) and its production (mmol/g of DMD) were numerically increased in LC diets and affected by a CFP × dose interaction (\( p < 0.05 \)), which was related to the notable drop at 3-NOP dose HIGH in the LC treatment when compared to the relatively constant fluctuations observed in the HC treatment. Accordingly, a quadratic effect of 3-NOP dose on propionate (mol %) was noted in LC diets (Q: \( p = 0.017 \)) (Figure 3C).
It was noted that CFP and 3-NOP dose affected the acetate/propionate ratio ($C_2/C_3$) in an interactive manner (CFP × dose: $p = 0.032$). The quadratic effect of 3-NOP dose on $C_2/C_3$ in LC diets (Q: $p = 0.008$) corresponded to the continual decrease from 3.05 to 2.77 in $C_2/C_3$ with increasing 3-NOP dose and the peak of 3.80 at 3-NOP dose HIGH. In HC diets, $C_2/C_3$ fluctuated non-significantly between 3-NOP treatments.

Neither CFP nor 3-NOP dose affected butyric acid (mol % and mmol/g of DMD). Valeric acid (mol % and mmol/g of DMD) was only affected by 3-NOP dose (dose: $p < 0.05$). The steady decrease in valerate (mol % and mmol/g of DMD) with increasing 3-NOP dose was interrupted by a notable peak at 3-NOP dose MED, being significantly different from LOW and HIGH dose ($p < 0.05$), independently of the incubated diet type.

The production (mmol/g of DMD) of the branched-chain fatty acid (BCVFA) iso-valerate increased with increasing 3-NOP dose in a convex parabolic-shaped manner in both LC (Q: $p = 0.048$) and, as a trend, in HC (Q: $p = 0.069$) substrates (Figure 3D). However, quadratic regression coefficients were not different between LC and HC (Table 4), but iso-valerate was approximately 22% lower in HC when compared to LC diets (CFP: $p < 0.05$).

Iso-butyrate (mol %) decreased with increasing 3-NOP dose in a different manner depending on whether LC and HC diets were incubated (CFP × dose: $p = 0.014$). In HC diets, iso-butyrate (mol %) tended to decrease in a curvilinear-shaped manner (Q: $p = 0.052$). The 3-NOP dose levels LOW and MED did not significantly differ from PLA but noticeably declined from MED to HIGH 3-NOP dose level ($p < 0.001$). In LC treatments, iso-butyrate decreased more or less steadily, which led to numerically increased levels at 3-NOP dose HIGH when compared to HC treatment (Table 4).

4. Discussion

In the present in vitro experiment, it was hypothesised that CH$_4$ production would decrease with increasing inclusion levels of 3-NOP and concentrate feeds in the incubated diet in an interactive manner.

4.1. 3-NOP Dosage Level

The magnitude of CH$_4$ reduction was highly affected by 3-NOP inclusion level, but this occurred independently of concentrate proportion in the diet substrate. A wider range of CH$_4$ inhibition was covered by the presently applied 3-NOP doses and diet substrates (Table 3) when compared to previous in vitro studies. Comparatively, Romero-Pérez et al. [10,14] incubated a forage-based substrate with 200, 500, 1000, and 2000 mg of 3-NOP/kg of feed DM in RUSITEC apparatuses. In the course of a saturation curve, they observed a high 3-NOP efficacy of reduction of 71.5, 76.0, and 84.5% at 200, 500, and 1000 mg of 3-NOP/kg of feed DM but no further CH$_4$ reduction with 3-NOP dose increment from 1000 to 2000 mg of 3-NOP/kg of feed DM (84.5 and 85.6%, resp.). Interestingly, in the present study, higher CH$_4$ mitigation maxima of 97 and 90% in LC and HC diets, respectively, were observed for the highest 3-NOP inclusion rate of 1200 mg/kg of feed DM applied. These different dose–response relationships may result from inherent sources of variation in the RUSITEC experiments, such as the use of different apparatuses and experimental protocols between laboratories. In the present experiment, the CH$_4$ reduction (Vol.-%) increased in a linear (LC) and convex parabolic (HC) shaped manner (Figure 1A) but not as a saturation curve, as had been previously reported in vitro [10,14] and in vivo [12]. Hence, 3-NOP inhibited CH$_4$ production at even lower doses (73 mg 3-NOP/kg feed DM: 12 and 10%; 160 mg 3-NOP/kg feed DM: 61 and 35% CH$_4$ reduction in LC and HC diets, resp.) when compared to the 71.5% CH$_4$ reduction at the minimum 3-NOP dose of 200 mg/kg of feed DM reported previously [10,23]. The differences in CH$_4$ production between LOW 3-NOP dose and the PLA treatment were, however, not significant (Figure 1A). This may indicate a compensatory response by the archaeal community attempting to counterbalance the 3-NOP inhibiting effect, which was likely metabolically feasible only at the lowest 3-NOP dose. Accordingly, methanogens can reactivate MCR.
through internal repair systems. In fact, Duin et al. [5] concluded that CH₄ inhibition is reversible.

Interestingly, 3-NOP dose LOW (73 mg of 3-NOP/kg of feed DM) seems to cause, in relative terms, a lower CH₄ reduction potential under in vitro conditions (10–12%; Table 3) when compared to supplementing comparable 3-NOP doses to dairy cows in vivo (23% with 68 mg of 3-NOP/kg DM [24]; 26.5% and 22.5% with 60 and 80 mg of 3-NOP/kg DM, resp. [12]). Conversely, a considerably high CH₄ reduction of more than 77.7% with 200 mg of 3-NOP/kg of feed DM can apparently only be achieved in vitro [15,23]. However, the maximum CH₄ reduction potential seems to be limited to 40% under in vivo conditions when 3-NOP is continuously supplied at an equal dose of 200 mg/kg of feed DM to dairy cows by mixing in the compound with the TMR [12]. Thus, Melgar et al. [12] quantified the maximum CH₄ mitigation effect to 40% at a 3-NOP dose of 100 mg/kg of feed DM without any statistical improvement in 3-NOP efficacy when supplementing higher doses of 150 and 200 mg of 3-NOP/kg of feed DM into the TMR of lactating cows. In conclusion, when compared to 3-NOP supplementation in vivo, the 3-NOP efficacy seems to be reduced at low but increased at high 3-NOP dose levels in vitro. This leads to the assumption that the dose–response relationships and 3-NOP effect mechanisms find expression in a different manner depending on whether 3-NOP is supplemented in vitro or in vivo and corresponding technical as well as rumen physiological factors affecting the mode of action of 3-NOP.

In the present experiment, the 3-NOP compound was mixed into the concentrate feed and therefore supplemented once per day as a ‘single dose’ with the feed bag into the fermenter but not as a continuous infusion. The 3-NOP compound is supposed to be water-soluble and rapidly metabolised in rumen liquid [25] and, therefore, recommended to be dosed at sufficient amounts synchronously to the MCR activity stimulating feed degradation [11]. In the present experiment, it is likely that the compound was rapidly disaggregated into 1,3-propanediol and nitrate [5] and further washed out of the vessel with the liquid outflow due to the high dilution rate of 3%/h, which could, conclusively, explain the general need for higher 3-NOP inclusion rates under conditions in vitro. In correspondence, Vyas et al. [26] observed that 3-NOP efficacy decreased 16 h after feeding when supplementing only 100 mg of 3-NOP/kg of feed DM to beef cattle, whereas a persistent CH₄ inhibition over 24 h was achieved at higher 3-NOP inclusion levels of 200 mg/kg DM. Thus, the highest 3-NOP dose applied in the present study could have prevented the complete washing out of the 3-NOP supplement from the fermenter, which could have resulted in sufficient amounts of the feed additive remaining in the fermenter fluid for targeting archaeal MCR over the whole 24 h incubation time horizon until the next feed bag exchange. This would become even more important during the course of the incubation with regard to inactivating the MCR activity arising time-delayed from slow fermentable fibre fractions in the LC diets. Thus, rates of fermentation of NDF are significantly lower as compared to that of rapidly fermentable NFC [27]. However, the CFP × dose interaction was not significant. Moreover, RUSITEC experiments are limited to investigating the short-term gas production kinetics of fast and slow fermentable fractions between feeding bag exchange. Therefore, 3-NOP effects on 24 h fermentation kinetics should be the focus in future experiments, e.g., using the Hohenheim Gas Test, according to Menke et al. [28].

Interestingly, Duin et al. [5] found that a 100-fold increase in 3-NOP concentration is required to suppress the growth of the methanogenic Archaea *Methanomicrobium mobile* and *Methanosarcina barkeri* when compared to the required 3-NOP amounts for inhibiting the growth of the predominant species in the bovine rumen, i.e., *Methanobrevibacter ruminantium* [29]. The reasons for the different degrees of sensitivity of methanogenic species towards 3-NOP remain to be elucidated, yet 3-NOP’s effects on individual methanogenic lineages were recently observed in vivo [30,31]. However, the possibility that not all of the methanogenic species were captured by the lower 3-NOP dose could have favoured those methanogenic Archaea being less sensitive to 3-NOP, causing a shift in the methanogenic
community structure to those occupying this ecological niche. As a consequence, the 3-NOP dose HIGH could have targeted a greater number and wider range of methanogenic archaeal species, causing a more comprehensive and effective blocking of those MCR amounts arising from immediate feed fermentation processes directly after feed bag exchange. This could have led to a more sustained suppressive effect on methanogenic activity until the next feed bag exchange and could further explain the high CH$_4$ reduction of more than 90%. In conclusion, the 3-NOP stability and its CH$_4$ inhibiting persistency in vitro should be investigated in future experiments by conducting continual 3-NOP infusion into the fermenter paralleled with frequent gas sampling from the gas bag for CH$_4$ analyses between feeding events.

4.2. Effects of the Diet Substrate and 3-NOP on Fermentation Parameters

In the present in vitro study, the nonsignificant combination effect of 3-NOP and CFP on CH$_4$ reduction contrasts findings from in vivo experiments [13,16]. This leads to the assumption that diet type per se does not contribute to synergistic effects but, rather, specific diet-induced rumen physiological factors and, more importantly, those being controlled in a RUSITEC. Thus, feeding HC diets may cause additive indirect effects on CH$_4$ inhibition that are related to the increased production of propionate from H$_2$-consuming fermentation pathways, passage rate (thereby limiting the time available for degradation of slowly fermentable carbohydrates), and reduced pH values (thereby inhibiting pH-sensitive methanogens) [7,32], affecting fermentation kinetics and microbial community structures [11,16,33]). As is typical for RUSITEC experiments, the fermentation conditions (e.g., particle retention time, flow rate of the (artificial) saliva, size of feed particles, motility, temperature, ratio of feed to liquid content, and liquid outflow rate) were standardised and strictly controlled in the present study. This could have equalised the abovementioned potential concentrate feed effects on fermentation characteristics and, therefore, explain the lack of synergistic effects between high CFP and 3-NOP on CH$_4$ inhibition.

In the performed trial, pH values remained within the physiological range of pH-sensitive rumen bacteria and methanogenic Archaea [29]. Therefore, inhibition of rumen microorganisms due to low pH values was excluded, particularly as a high buffering capacity and controlled infusion rate of the artificial saliva were pre-set in the apparatus. The wide ratio between the liquid and solid phase in the fermenter may have prevented significant acidification solely by the diet substrate. The infusion rate of the buffer was, however, not changed with regard to the lack of effects of a comparable HC diet on rumen pH values, as previously observed in vivo [16]. In the conducted experiment, pH values were marginally lower in LC when compared to HC diets (pH: CFP: $p = 0.019$), which was related to the increased total VFA production (mmol/g of DMD) observed in LC diets (CFP: $p = 0.012$; Table 4).

End-products of microbial fermentation, i.e., VFA, act as electron acceptors, which maintain the strongly reductive rumen milieu and can, therefore, directly be linked to microbial activity [34]. Correspondingly, the $Eh$ in the fluid, reflecting the redox homeostasis and electron transfer, is hypothesised to be a control of enzymatic processes in rumen microorganisms [34,35]. However, $Eh$ positively correlated to total VFA ($r = 0.563$; $N = 24$; $p = 0.004$) in the present investigation, which contrasts findings of an inverse relationship [36]. From a literature review, Huang et al. [36] indicated that $Eh$ increased with CFP ($r = 0.497$; $p = 0.015$) and negatively correlated to pH, whereby the latter was also observed in the study at hand ($Eh$ vs. pH: $r = -0.57$; $N = 24$; $p = 0.004$). Hydrogen produced from microbial fermentation preserves reducing conditions in the rumen. In the present experiment, though, negative correlations between $Eh$ and H$_2$ concentrations in fermentation gases of 3-NOP treatments were not observed and $Eh$ fluctuated inconsistently over the 3-NOP dose levels. However, interpretation should be made with caution as $Eh$ was measured during feed bag exchange so that oxygen entrance, notably affecting oxidation-reduction conditions in the fermenter fluid, was unavoidable.
The present RUSITEC experiment confirmed the previous studies of Guayder et al. [23] and Romero-Peréz et al. [10] reporting H₂ accumulation in fermentation gases and decreased molar acetate proportions with increasing 3-NOP dose and CH₄ mitigation (H₂ (Vol.-%): dose: p < 0.001 (Table 3); acetate (mol %): dose: p = 0.044 (Table 4)). As an H₂-liberating fermentation process, acetate production could have been downregulated to prevent a further increase in H₂ partial pressure in the fermenter fluid, which would be detrimental to the growth of cellulolytic bacteria. However, negative side effects of reduced NDF degradability (Table 2) or total gas production (Table 3) were not consistently observed in the present experiment. Starch fermentation in the HC diets and the 3-NOP-induced H₂ increase (Figure 1B) were both assumed to promote a shift in fermentation balance from acetate to alternative H₂ sinks of valerate and propionate, as previously observed under conditions in vivo [16,37]. However, contrary to these expectations, the propionate proportion (mol %) was lower in HC when compared to LC diet, whereas the opposite held true concerning valerate proportions (Table 4). However, valerate formation can be traced back not only to NFC fermentation but also to the deamination of proline. In addition, a consistent increase in propionate and valerate proportions due to the 3-NOP supply was, interestingly, observed in neither the present study nor in previous in vitro experiments [10,23], except for valerate, which was previously found to be increased with 3-NOP inclusion in vitro [23]. These observations could be explained by the unphysiological longer retention time of small-sized feed particles in a RUSITEC (fixed time of 48 h) when compared to rumen conditions in vivo. For instance, Prigge et al. [38] reported that the retention time of 3 mm particles amounts to 20 h in vivo. Martinez et al. [39] incubated a 30:70 alfalfa hay:concentrate diet in RUSITEC fermenters and observed that reducing the retention time of concentrates from 48 to 24 h and increasing the dilution rate from 3.78 to 5.42%/h increased the production and molar proportions of propionate. Furthermore, retention time depends on dry matter intake, stratification of the rumen content, and the size and density of feed particles [40]. These influencing factors were, however, standardised in the reaction vessels, which could have negatively affected the adaptation of propionate enhancers to the environmental conditions in the RUSITEC [41]. The question about a possible redirection of H₂ spared from methanogenesis to further alternative H₂ utilising pathways other than propionate synthesis becomes even more interesting as it can be assumed that H₂ accumulation does not exclusively occur in fermentation gas but also in the liquid phase. Thus, in the performed experiment, the calculative amounts of the H₂ excess from CH₄ inhibition (assuming that 4 moles of metabolic hydrogen are spared from the inhibition of 1 mole of CH₄) were not completely recovered in alternative H₂ removals (H₂ emission via fermentation gas, H₂ incorporation into propionate and valerate), which can be deduced from the decreasing CH₄/H₂ ratio with increasing 3-NOP dose (Table 3).

In conclusion, a rechannelling of the spared H₂ to further alternative metabolic routes not analysed in the present experiment may have occurred. Regarding this, Guyader et al. [23] observed increased concentrations of atypical H₂ sinks of ethanol, formate, caproate, and heptanoate in the fermenter fluid at a 200 mg inclusion rate of 3-NOP/kg of feed DM.

The convex parabolic curve of the NH₃-N production in LC diets was inversely shaped to that of the HC diets (Figure 3A). These interrelations might be of multifactorial origin, such as reduced proteolysis or an increased microbial NH₃-N uptake in LC fermenter fluid [42], but also with regard to the lower CP content in LC diets (Table 1). Molar proportions of iso-butyrate decreased with increasing 3-NOP dose, which could indicate a decreased deamination of amino acids (AA). In contrast, as reported previously [10], iso-valerate production increased in a quadratic manner with 3-NOP dose, which contradicts the hypothesis of a decreased AA deamination as BCVFA results from both deamination and decarboxylation of valine and leucine, respectively [43]. There are possibly different metabolic processes of these BCVFA under CH₄ inhibition and H₂ accumulation which need to be clarified in future (Figure 3D). Microbial uptake and release of BCVFA thus are the main determinants of BCVFA concentrations and microbial protein synthesis is regarded as a H₂ sink apart from methanogenesis [43,44].
5. Conclusions

The hypothesis of synergistic effects between 3-NOP and increased CFP on CH\textsubscript{4} inhibition was rejected for the applied in vitro conditions. The present RUSITEC experiment evidenced that 3-NOP effectively inhibited methanogenesis in a dose-dependent manner irrespective of CFP in the incubated diet. Negative side effects on nutrient degradability and, correspondingly, total VFA and gas production were not consistently observed for 3-NOP or CFP. However, 3-NOP dose increment was paralleled by H\textsubscript{2} gas accumulation, whereas alternative H\textsubscript{2} sinks of propionate and valerate remained unaffected. Increasing 3-NOP dosage decreased H\textsubscript{2}-liberating acetate formation, whereas butyrate proportion remained unchanged. Conclusions from in vitro experiments cannot be fully transferred to the rumen environment in vivo. This study and others suggest that extrapolating findings from dose-dependent dynamics of the 3-NOP efficacy under conditions in vitro should be treated with caution for planning 3-NOP application in vivo. The present research should be broadened by focusing on potential changes in microbial community structures when 3-NOP is supplemented to different dietary concentrate:forage ratios.

Author Contributions: Conceptualisation, M.S.; Formal analysis, M.S.; Funding acquisition, D.v.S. and U.M.; Investigation, M.S. and L.H.; Methodology, M.S.; Project administration, M.S. and D.v.S.; Resources, S.K. and U.M.; Supervision, D.v.S., A.Z. and S.D.; Validation, M.S.; Visualisation, M.S.; Writing—original draft, M.S.; Writing—review and editing, M.S., D.v.S., L.H., S.K., U.M., A.Z. and S.D. All authors have read and agreed to the published version of the manuscript.

Funding: The project is supported by funds from the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme (grant number 281B101416).

Institutional Review Board Statement: The experiment was conducted according to the guidelines of the German Animal Welfare Act and approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany) (approval number: 33.19-42502-04-15/1858).

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to legal issues.

Acknowledgments: The authors thank the staff of the Institute of Animal Nutrition, Friedrich-Loeffler Institut (FLI) and the co-workers of its experimental station for taking care of the experimental animals and supporting the sample collection and analysis. The authors also thank DSM Nutritional Products AG (Kaiseraugst, Switzerland) for their support in providing the 3-NOP compound and 3-NOP analyses in the feedstuffs.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. IPCC, Intergovernmental Panel on Climate Change. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2014; p. 1535.

2. Gerber, P.J.; Steinfeld, H.; Henderson, B.; Mottet, A.; Opio, C.; Dijkman, J.; Falcucci, A.; Tempio, G. Tackling Climate Change through Livestock: A Global Assessment of Emissions and Mitigation Opportunities; Food and Agriculture Organization of the United Nations (FAO): Rome, Italy, 2013; ISBN 9789251079201.  
3. Hristov, A.N.; Oh, J.; Lee, C.; Meinen, R.; Montes, F.; Ott, T.; Firkins, J.; Rotz, A.; Dell, C.; Adesogan, A.; et al. Mitigation of greenhouse gas emissions in livestock production: A review of technical options for non-CO2 emissions. In FAO Animal Production and Health; Gerber, P.J., Henderson, B., Makkar, H.P.S., Eds.; FAO: Rome, Italy, 2013; Volume 177.

4. Czerkawski, J.W.; Breckenridge, G. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr. 1977, 38, 371–384. [CrossRef] [PubMed]

5. Duin, E.C.; Wagner, T.; Shima, S.; Prakash, D.; Cronin, B.; Yanez-Ruiz, D.R.; Duval, S.; Rumbeli, R.; Stemmler, R.T.; Thauer, R.K.; et al. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proc. Natl. Acad. Sci. USA 2016, 113, 6172–6177. [CrossRef] [PubMed]

6. Beauchemin, K.A.; Kreuzer, M.; O’Mara, F.; McAllister, T.A. Nutritional management for enteric methane abatement: A review. Aust. J. Exp. Agric. 2008, 48, 21–27. [CrossRef]
Animals 2021, 11, 1784

7. Hook, S.E.; Steele, M.A.; Northwood, K.S.; Wright, A.D.; McBride, B.W. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. Microb. Ecol. 2011, 62, 94–105. [CrossRef]

8. Lana, R.P.; Russell, J.B.; Van Amburgh, M.E. The role of pH in regulating ruminal methane and ammonia production. J. Anim. Sci. 1998, 76, 2190–2196. [CrossRef]

9. McAllister, T.; Newbold, C.J. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 2008, 48, 7–13. [CrossRef]

10. Romero-Pérez, A.; Okine, E.K.; Guan, L.L.; Duval, S.M.; Kindermann, M.; Beauchemin, K.A. Effects of 3-nitrooxypropanol on methane production during rumen fermentation simulation technique (Rusitec). Anim. Feed Sci. Technol. 2015, 209, 98–109. [CrossRef]

11. Dijkstra, J.; Bannink, A.; France, J.; Kebreab, E.; van Gastelen, S. Short communication: Antimethanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fiber content, and cattle type. J. Dairy Sci. 2018, 101, 9041–9047. [CrossRef]

12. Melgar, A.; Welter, K.C.; Nedelkov, K.; Martins, C.M.M.R.; Harper, M.T.; Oh, J.; Räsänen, S.E.; Chen, X.; Cueva, S.F.; Duval, S.; et al. Dose-response effect of 3-nitrooxypropanol on enteric methane emissions in dairy cows. J. Dairy Sci. 2020, 103, 6145–6156. [CrossRef]

13. Vyas, D.; McGinn, S.M.; Duval, S.M.; Kindermann, M.K.; Beauchemin, K.A. Optimal dose of 3-nitrooxypropanol for decreasing enteric methane emissions from beef cattle fed high-forage and high-grain diets. Anim. Prod. Sci. 2016, 58, 1049–1055. [CrossRef]

14. Romero-Pérez, A.; Okine, E.K.; Guan, L.L.; Duval, S.M.; Kindermann, M.; Beauchemin, K.A. Effects of 3-nitrooxypropanol and monensin on methane production using a forage-based diet in Rusitec fermenters. Anim. Feed Sci. Technol. 2016, 220, 67–72. [CrossRef]

15. Schilde, M.; von Soosten, D.; Hüther, L.; Meyer, U.; Zeyner, A.; Dänicke, S. Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows. Arch. Anim. Nutr. 2021, 75, 79–104. [CrossRef] [PubMed]

16. Geishauser, T. An instrument for collection and transfer of ruminal fluid and for administration of water soluble drugs in adult cattle. Bov. Pract. 1993, 27, 38–42. [CrossRef]

17. McDougall, E.I. Studies on ruminant saliva. 1. The composition and output of sheep’s saliva. Biochim. J. 1948, 43, 99–109. [CrossRef]

18. VDLUFA. Bd. III Die chemische Untersuchung von Futtermitteln. In Handbuch der Landwirtschaftlichen Versuchs-Und Untersuchungsmethodik (VDLUFA-Methenbuch); VDLUFA-Verlag: Darmstadt, Germany, 2006.

19. Disser, J.; Hoffmann, M.; Hoikel, B. Ein Beitrag zur gaschromatographischen Bestimmung flüchtiger Fettsäuren. J. Anim. Sci. 1979, 40, 2441–2447. [CrossRef]

20. Geissler, C.; Hoffmann, M.; Hiokel, B.; Kretschmer, F.-J. Ein Beitrag zur gaschromatographischen Bestimmung flüchtiger Fettsäuren. J. Anim. Sci. 1979, 40, 2441–2447. [CrossRef] [PubMed]

21. Anonymous. DIN 38406–E5-2. Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. J. Agric. Sci. 1948, 79, 154–164. [CrossRef]

22. Littell, R.C.; Henry, P.R.; Ammerman, C.B. Statistical analysis of repeated measures data using SAS procedures. J. Anim. Sci. 1998, 76, 1216–1231. [CrossRef]

23. Goyder, J.; Ungerfeld, E.M.; Beauchemin, K.A. Redirection of Metabolic Hydrogen by Inhibiting Methanogenesis in the Rumen Simulation Technique (RUSITEC). Front. Microbiol. 2017, 8, 393. [CrossRef]

24. Haisan, J.; Sun, Y.; Guan, L.; Beauchemin, K.A.; Iwaasa, A.; Duval, S.; Kindermann, M.; Barreda, D.R.; Oba, M. The effects of feeding 3-nitrooxypropanol at two doses on milk production, rumen fermentation, plasma metabolites, nutrient digestibility, and methane emissions in lactating Holstein cows. Anim. Prod. Sci. 2017, 57, 282–289. [CrossRef]

25. Reynolds, C.K.; Humphries, D.J.; Kirton, P.; Kindermann, M.; Duval, S.; Steinberg, W. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. J. Dairy Sci. 2014, 97, 3777–3789. [CrossRef]

26. Vyas, D.; McGinn, S.M.; Duval, S.M.; Kindermann, M.; Beauchemin, K.A. Effects of sustained reduction of enteric methane emissions with dietary supplementation of 3-nitrooxypropanol on growth performance of growing and finishing beef cattle. J. Anim. Sci. 2016, 94, 2024–2034. [CrossRef]

27. Keim, J.P.; Alvarado-Gilis, C.; Arias, R.A.; Gandarillas, M.; Cabanilla, J. Evaluation of sources of variation in in vitro fermentation kinetics of feedstuffs in a gas production system. Anim. Sci. J. 2017, 88, 1547–1555. [CrossRef]

28. Menke, K.H.; Raab, L.; Salewski, A.; Steingass, H.; Fritz, D.; Schneider, W. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when they are incubated with rumen liquor in vitro. J. Agric. Sci. 1979, 93, 217–222. [CrossRef]

29. Whitford, M.F.; Teather, R.M.; Forster, R.J. Phylogenetic analysis of methanogens from the bovine rumen. BMC Microbiol. 2001, 1, 1–5. [CrossRef]

30. Pitta, D.W.; Melgar, A.; Hristov, A.N.; Indugo, N.; Narayan, K.S.; Pappalardo, C.; Hennessy, M.L.; Vecchiarelli, B.; Kaplan-Shabtai, V.; Kindermann, M.; et al. Temporal changes in total and metabolically active ruminal methanogens in dairy cows supplemented with 3-nitrooxypropanol. J. Dairy Sci. 2021. [CrossRef]

31. Zhang, X.M.; Gruninger, R.J.; Alemu, A.W.; Wang, M.; Tan, Z.L.; Kindermann, M.; Beauchemin, K.A. 3-Nitrooxypropanol supplementation had little effect on fiber degradation and microbial colonization of forage particles when evaluated using the in situ ruminal incubation technique. J. Dairy Sci. 2020, 103, 8986–8997. [CrossRef]
32. Van Kessel, J.A.S.; Russell, J.B. The effect of pH on ruminal methanogenesis. *FEMS Microbiol. Ecol.* **1996**, *20*, 205–210. [CrossRef]
33. Aguerre, M.J.; Wattiaux, M.A.; Powell, J.M.; Broderick, G.A.; Arnldt, C. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. *J. Dairy Sci.* **2011**, *94*, 3081–3093. [CrossRef] [PubMed]
34. Dijkstra, J.; van Gastelen, S.; Dieho, K.; Nichols, K.; Bannink, A. Review: Rumen sensors: Data and interpretation for key rumen metabolic processes. *Animal* **2020**, *14*, 176–186. [CrossRef] [PubMed]
35. Barry, T.N.; Thompson, A.; Armstrong, D.G. Rumen fermentation studies on two contrasting diets: 1. Some characteristics of the in vivo fermentation, with special reference to the composition of the gas phase, oxidation/reduction state and volatile fatty acid proportions. *J. Agric. Sci.* **1977**, *89*, 183–195. [CrossRef]
36. Huang, Y.; Marden, J.P.; Julien, C.; Bayourthe, C. Redox potential: An intrinsic parameter of the rumen environment. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 393–402. [CrossRef]
37. Jayanegara, A.; Sarwono, K.A.; Kondo, M.; Matsui, H.; Ridla, M.; Laconi, E.B.; Nahrowi. Use of 3-nitrooxypropanol as feed additive for mitigating enteric methane emissions from ruminants: A meta-analysis. *Ital. J. Anim. Sci.* **2018**, *17*, 650–656. [CrossRef] [PubMed]
38. Frigge, E.C.; Stuthers, B.A.; Jacquemet, N.A. Influence of forage diets on ruminal particle size, passage of digesta, feed intake and digestibility by steers. *J. Anim. Sci.* **1990**, *68*, 4352–4360. [CrossRef]
39. Martinez, M.E.; Ranilla, M.J.; Ramos, S.; Tejido, M.L.; Carro, M.D. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in Rusitec fermenters. *J. Dairy Sci.* **2009**, *92*, 3930–3938. [CrossRef] [PubMed]
40. Clauss, M.; Lechner, I.; Barboza, P.; Collins, W.; Tervoort, T.A.; Südekum, K.-H.; Codron, D.; Hummel, J. The effect of size and density on the mean retention time of particles in the reticulorumen of cattle (Bos primigenius f. taurus), muskoxen (Ovibos moschatus) and moose (Alces alces). *Br. J. Nutr.* **2011**, *105*, 634–644. [CrossRef]
41. Ungerfeld, E.M. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. *Front. Microbiol.* **2015**, *6*, 1–17. [CrossRef] [PubMed]
42. Carro, M.D.; Lebzien, P.; Rohr, K. Influence of yeast culture on the in vitro fermentation (Rusitec) of diets containing variable portions of concentrates. *Anim. Feed Sci. Technol.* **1992**, *37*, 209–220. [CrossRef]
43. Mansfield, H.R.; Endres, M.I.; Stern, M.D. Comparison of microbial fermentation in the rumen of dairy cows and dual flow continuous culture. *Anim. Feed Sci. Technol.* **1995**, *55*, 47–66. [CrossRef]
44. Asanuma, N.; Iwamoto, M.; Hino, T. Effect of the Addition of Fumarate on Methane Production by Ruminal Microorganisms In Vitro. *J. Dairy Sci.* **1999**, *82*, 780–787. [CrossRef]