Inhibited Methanogenesis in the Rumen of Cattle: Microbial Metabolism in Response to Supplemental 3-Nitrooxypropanol and Nitrate

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3-Nitrooxypropanol (3-NOP) supplementation to cattle diets mitigates enteric CH4 emissions and may also be economically beneficial at farm level. However, the wider rumen metabolic response to methanogenic inhibition by 3-NOP and the NO2– intermediary metabolite requires further exploration. Furthermore, NO3– supplementation potently decreases CH4 emissions from cattle. The reduction of NO3– utilizes H2 and yields NO2–, the latter of which may also inhibit rumen methanogens, although a different mode of action than for 3-NOP and its NO2– derivative was hypothesized. Our objective was to explore potential responses of the fermentative and methanogenic metabolism in the rumen to 3-NOP, NO3– and their metabolic derivatives using a dynamic mechanistic modeling approach. An extant mechanistic rumen fermentation model with state variables for carbohydrate substrates, bacteria and protozoa, gaseous and dissolved fermentation end products and methanogens was extended with a state variable of either 3-NOP or NO3–. Both new models were further extended with a NO2– state variable, with NO2– exerting methanogenic inhibition, although the modes of action of 3-NOP-derived and NO3–-derived NO2– are different. Feed composition and intake rate (twice daily feeding regime), and supplement inclusion were used as model inputs. Model parameters were estimated to experimental data collected from the literature. The extended 3-NOP and NO3– models both predicted a marked peak in H2 emission shortly after feeding, the magnitude of which increased with higher doses of supplement inclusion. The H2 emission rate appeared positively related to decreased acetate proportions and increased propionate and butyrate proportions. A decreased CH4 emission rate was associated with 3-NOP and NO3– supplementation. Omission of the NO2– state variable from the 3-NOP model did not change the overall dynamics of H2 and CH4 emission and other metabolites. However, omitting the NO2– state variable from the NO3– model did substantially change the dynamics of H2 and CH4 emissions indicated by a decrease in both H2 and CH4 emission after feeding. Simulations do not...
point to a strong relationship between methanogenic inhibition and the rate of NO$_3$ and NO$_2^-$ formation upon 3-NOP supplementation, whereas the metabolic response to NO$_3^-$ supplementation may largely depend on methanogenic inhibition by NO$_2^-$.

**Keywords:** 3-NOP, nitrite, cattle, feed supplement, bacteria, archaea, methane

### 1. INTRODUCTION

Animal agriculture emits about 7.1 gigatonnes of CO$_2$ equivalents of greenhouse gases per year, which represents approximately 14.5% of total global anthropogenic greenhouse gas emissions in 2005 (Gerber et al., 2013). Dairy and beef cattle emitted 4.6 gigatonnes CO$_2$ equivalents, of which CH$_4$ from enteric fermentation contributed about 45%. To decrease the latter enteric source of greenhouse gas emission, various dietary supplements with a potential inhibiting effect on ruminal methanogenesis have been tested. 3-nitrooxypropanol (3-NOP) is one of the most effective dietary supplements that was tested for cattle (e.g., Hristov et al., 2015), and may also be economically beneficial (Alvarez-Hess et al., 2019). The mode of action of 3-NOP was elucidated to be the inhibition of methyl co-enzyme-M reductase (MCR), with clear indications that NO$_2^-$ can be metabolized from 3-NOP and inhibit methanogenesis by blocking MCR activity as well (Duin et al., 2016). However, the wider effects of 3-NOP and NO$_3^-$ on methanogenic archaea in the rumen and the implications for the dynamics of ruminal metabolites require a more thorough exploration.

Nitrate is another dietary supplement (commonly in the form of a calcium salt, sometimes a sodium or potassium salt) that has been observed to decrease enteric CH$_4$ from cattle substantially and persistently (Van Zijderveld et al., 2011), although there seem no on-farm economical benefits (Alvarez-Hess et al., 2019). Nitrate is primarily reduced to NH$_3$ by ruminal bacteria, which may result in the utilization of four equivalents of H$_2$ per equivalent of NO$_3^-$. This reduction reaction causes less H$_2$ available for CH$_4$ production by the methanogens. However, NO$_3^-$ supplementation to dairy cattle diets was reported to increase H$_2$ emissions (Oljihoek et al., 2016). The latter increase was explained by NO$_3^-$ being reduced to NO$_2^-$, with NO$_2^-$ inhibiting the methanogenic metabolism (Latham et al., 2016). Therefore, the presence of NO$_2^-$ as an intermediate in the reduction of NO$_3^-$ to NH$_3$ may contribute to the CH$_4$ suppressing effect of NO$_3^-$ supplementation to cattle diets as well.

Various ruminal bacteria possess and express genes that result in the employment of periplasmic NO$_3^-$ and NO$_2^-$ reductases (Kern and Simon, 2009; Yang et al., 2016). The methanogens that reside in the rumen, however, were not observed to transcribe genes that encode for NO$_3$ and NO$_2$ reductases (Greening et al., 2019). Lack of these reductases may suggest that the conversion of 3-NOP into NO$_3^-$ inside methanogenic cells proceeds spontaneously or is catalyzed by different enzymes, which aligns with the formation of NO$_3^-$ and NO$_2^-$ upon the inactivation of the MCR enzyme (Duin et al., 2016). Although 3-NOP is transported across the methanogenic cell membrane, no evidence for NO$_2^-$ transportation across the methanogenic cell membrane is known to the authors. If NO$_3^-$ is transported across the methanogenic cell membrane, the NO$_3^-$ derived from NO$_3^-$ may even inhibit CH$_4$ production completely by blocking MCR at the commonly used dietary inclusion rates of NO$_3^-$, which is not commonly observed. On a molar basis, the relatively low inclusion rates of 3-NOP compared to NO$_3^-$ will likely result in lower NO$_2^-$ production. Therefore, the mechanisms by which NO$_2^-$ derived from NO$_3^-$ and 3-NOP act on archaea appear different, with 3-NOP derived NO$_2^-$ exerting its methanogenic inhibition inside the cell and NO$_3^-$ derived NO$_2^-$ potentially exerting methanogenic inhibition outside the cell.

Besides metabolic conversions and their enzyme kinetic implications, several studies suggested the inhibiting effect of 3-NOP and NO$_3^-$ on ruminal methanogenesis to be partly thermodynamically controlled (Van Zijderveld et al., 2011; Dijkstra et al., 2018). Both 3-NOP and NO$_3^-$ were found to increase H$_2$ emission, suggesting thermodynamic inhibition of NADH oxidation in fermentative microbes in the rumen (Van Lingen et al., 2016). This thermodynamic inhibition results in a shift from acetate to more propionate production, which decreases the yield of H$_2$ and next the yield of CH$_4$. The objective of this study is to explore putative mechanisms of methanogenic inhibition by 3-NOP and NO$_3^-$ and their implications for the dynamics of microbial fermentation in the bovine rumen using dynamic mechanistic modeling approaches. For this objective, an existing dynamic mechanistic model of microbial substrate degradation that incorporated various metabolic pathways (Van Lingen et al., 2019) is extended with putative kinetic downregulation mechanisms of methanogenesis by 3-NOP, NO$_3^-$ and their derivatives. These newly developed modeling approaches also enable the evaluation of the thermodynamic control of H$_2$ partial pressure ($p_{H_2}$) on volatile fatty acid (VFA) fermentation pathways via the NAD$^+$ to NADH ratio in fermentative microbes upon the supplementation of feed with 3-NOP and NO$_3^-$.

### 2. MODEL DESCRIPTION

An extant dynamic mechanistic rumen fermentation model with state variables for ruminal carbohydrate substrates, bacteria and protozoa, gaseous and dissolved fermentation end products and methanogens (Van Lingen et al., 2019) was extended with a representation of either the 3-NOP or NO$_3^-$ metabolism. The extant model represents the hydrolysis of carbohydrate polymers (viz., degradable fiber, degradable starch and sugars) into hexose, the thermodynamic control of $p_{H_2}$ on volatile fatty acid (VFA) fermentation pathways via the NAD$^+$ to NADH ratio in fermentative microbes,
and hydrogenotrophic methanogenesis in the bovine rumen. Four different extensions of the original model were made. These model extensions comprised a representation of 3-NOP and NO₂⁻ and with and without NO₃⁻, which is derived from both 3-NOP and NO₂⁻, respectively. The four extended models are diagrammatically represented in Figures 1, 2, while a schematic overview of physiological characteristics incorporated per model is provided in Table 1A. Mathematical notation of influxes and outfluxes of model state variables is \( P_{i,j,m} \) and \( U_{i,j,m,n} \), respectively, where the subscript represents the uptake or production of \( i \) by \( j \)-to-\( m \) transaction (generating \( n \)). To illustrate this, \( P_{3\text{NOP},\text{In},3\text{NOP}} \) represents the increase in 3-NOP as a result of the inflow of 3-NOP. Concentrations of \( i \) are computed as:

\[
C_i = \frac{Q_i}{V_{Fl}}
\]

for \( i = \{H_2, 3\text{-NOP}, NO_2^-, NO_3^-\} \) and \( V_{Fl} \) being the rumen fluid volume. State variables are expressed in [g] or [mol], with the corresponding fluxes and concentrations expressed in [mol·h⁻¹] or [g·h⁻¹], and [mol·L⁻¹] or [g·L⁻¹], respectively. Abbreviations and general notation are available in Table 2. Parameters specific for the new models are provided in Table 3.
2.1. Mathematical Representation of Model Extentions

2.1.1. 3-NOP Simple Model

3-nitrooxypropanol state variable, $Q_{3\text{NOP}}$ [mol]. The $Q_{3\text{NOP}}$ state variable receives input from 3-NOP contents in the feed that was supplemented:

$$P_{3\text{NOP};\text{In},3\text{NOP}} = D_{\text{DM}}(t) \cdot c_{3\text{NOP}}$$  \hspace{1cm} (2)

with $D_{\text{DM}}(t)$ the dry matter intake rate in time [kg·h$^{-1}$] and $c_{3\text{NOP}}$ the 3-NOP content of the feed [mol·kg$^{-1}$]. 3-NOP can easily diffuse through membranes (Duin et al., 2016) and was assumed to be absorbed across the rumen wall:

$$U_{3\text{NOP};\text{Ab},3\text{NOP}} = k_{3\text{NOP};\text{Ab}} \cdot Q_{3\text{NOP}}$$  \hspace{1cm} (3)

with $k_{3\text{NOP};\text{Ab}}$ the fractional absorption rate of 3-NOP (value and units in Table 3). Finally 3-NOP was assumed to flow out to the lower tract with the fluid fraction, which was represented as:

$$U_{3\text{NOP};\text{Ex},3\text{NOP}} = k_{\text{FL,Ex}} \cdot Q_{3\text{NOP}}$$  \hspace{1cm} (4)

with $k_{\text{FL,Ex}}$ the fractional outflow rate of the fluid fraction [h$^{-1}$] as in Van Lingen et al. (2019). The differential equation of the $Q_{3\text{NOP}}$ state variable is given by:

$$\frac{dQ_{3\text{NOP}}}{dt} = P_{3\text{NOP};\text{In},3\text{NOP}} - U_{3\text{NOP};\text{Ab},3\text{NOP}} - U_{3\text{NOP};\text{Ex},3\text{NOP}}$$  \hspace{1cm} (5)

Hydrogen state variable, $Q_{\text{H}_2}$ [mol]. As described by Van Lingen et al. (2019), inputs to the $Q_{\text{H}_2}$ state variable are
TABLE 1 | Overview of (A) physiological characteristics regarding methanogenic inhibition and H₂ sinks incorporated in 3-NOP, 3-NOP+nitrite, nitrate and nitrate+nitrite models, along with (B) the physiological response of various output variables to dietary inclusion of 3-NOP or NO₃⁻.

| (A) Physiological characteristic | 3-NOP | 3-NOP+nitrite | Nitrate | Nitrate+nitrite |
|----------------------------------|-------|---------------|---------|----------------|
| Methanogenic inhibition by 3-NOP (MCR⁴) | ✓ | ✓ | | |
| 3-NOP to NO₃⁻ + NO₂⁻ (MCR⁵) | ✓ | ✓ | | |
| Methanogenic inhibition by NO₂⁻ (MCR) | | | | ✓ |
| NO₃⁻ to NH₃ (H₂ sink) | | | ✓ | |
| NO₃⁻ to NO₂⁻ (H₂ sink) | | | | ✓ |
| NO₂⁻ to NH₃ (H₂ sink) | | ✓ | | |
| Methanogenic inhibition by NO₂⁻ (hypothesized⁶) | | | ✓ | |

| (B) Response to supplemented 3-NOP or NO₃⁻ | 3-NOP | 3-NOP+nitrite | Nitrate | Nitrate+nitrite |
|-------------------------------------------|-------|---------------|---------|----------------|
| H₂ emission rate and $p_{\text{H}_2}$ | ↑ | ↑ | ↓ | ↑ |
| CH₄ emission rate | ↓ | ↓ | ↓ | ↓ |
| Inhibition on NADH oxidation | ↑ | ↑ | ↓ | ↑ |
| Acetate proportion | ↓ | ↓ | - | - |
| Propionate proportion | ↑ | ↑ | - | ↑ |
| Butyrate proportion | ↑ | ↑ | - | - |

Check-marks indicate if a physiological characteristic was incorporated. Upward and downward arrows indicate up-regulation and down-regulation of the metabolism, respectively; a dash indicates a response was negligibly small.

⁴Reflects inhibition of archaeal methyl co-enzyme-M reductase. ⁵Mode of action of methanogenic inhibition by NO₂⁻ derived NO₃⁻ remains to be fully determined. See also sections 2.1.4 and 4.2.

TABLE 2 | Abbreviations used in mathematical expressions in the model.

| Symbol | Entity |
|--------|--------|
| Ab     | Absorption |
| Ac     | Acetate |
| AP     | Acetate + propionate |
| Bu     | Butyrate |
| DM     | Dry matter |
| Em     | Emission (from the rumen) |
| Ex     | Exit to lower tract |
| Fₙ     | Degradable fiber |
| Fl     | Fluid |
| He     | Hexose |
| In     | Intake |
| La     | Lactate |
| Me     | Methanogens |
| Mi     | Fermentative microbes |
| Pa     | Propionate |
| Pr     | Propionate |
| Pₙ     | Soluble protein |
| Sa     | Degradable starch |
| So     | Solid |
| Sₙ     | Soluble starch |
| Wₙ     | Water soluble carbohydrates |
| Cₙ     | CₙNO₂⁻,NO₃⁻ |
| JₙNO₂⁻/H₂,Me | JₙNO₂⁻/H₂,Me |
| JₙMCR.H₂,Me | JₙMCR.H₂,Me |

TABLE 3 | Preliminary parameter values used in the 3-NOP, 3-NOP+NO₃⁻, NO₃⁻, and NO₃⁻+NO₂⁻ models.

| Variable | Units | 3-NOP | 3-NOP+NO₃⁻ | NO₃⁻ | NO₃⁻+NO₂⁻ |
|----------|-------|-------|------------|------|-----------|
| $k_{\text{NO}_2^-/\text{H}_2}$ | h⁻¹ | - | - | 0.30 | 0.30 |
| $k_{\text{NO}_2^-/\text{Me}}$ | h⁻¹ | 0.30 | 0.30 | - | - |
| $k_{\text{NO}_2^-/\text{Me}}$ | mol⁻¹g⁻¹h⁻¹ | 6.99 | | | |
| $k_{\text{NO}_2^-/\text{NO}_3^-}$ | mol⁻¹g⁻¹h⁻¹ | 1.5 | | | |
| $k_{\text{NO}_2^-/\text{NO}_3^-}$ | mol⁻¹g⁻¹h⁻¹ | 0.113 | | | |
| $k_{\text{NO}_2^-/\text{NO}_3^-}$ | g⁻¹h⁻¹ | 1.55 | | | |
| $k_{\text{NO}_2^-/\text{NO}_3^-}$ | g⁻¹h⁻¹ | 0.44 | | | |
| $J_{\text{NO}_2^-/\text{H}_2,\text{Me}}$ | M | | | | 1.17e⁻3 |
| $J_{\text{MCR.H}_2,\text{Me}}$ | M | 1.93e⁻5 | 2.10e⁻5 |

H₂ influxes associated with acetate and butyrate production ($P_{\text{H}_2/\text{He.Ac}}$ and $P_{\text{H}_2/\text{He.Bu}}$), whereas outputs that are copied to the present model are emission, outflow with rumen fluid and absorption of H₂ ($U_{\text{H}_2/\text{H}_2,\text{Em}}, U_{\text{H}_2/\text{H}_2,\text{Ex}},$ and $U_{\text{H}_2/\text{H}_2,\text{Ab}}$, respectively). In the present model, the outflow that represents H₂ utilization for 3-NOP inhibited methanogenic growth is given by:

$$U_{\text{H}_2/\text{H}_2,\text{CH}_4} = \frac{v_{\text{H}_2/\text{CH}_4} \cdot \mathcal{Q}_{\text{Me}}}{M_{\text{H}_2/\text{CH}_4} + \frac{C_{\text{NOP}}}{f_{\text{MCR.H}_2/\text{CH}_4}}}$$ (6)

where $v_{\text{H}_2/\text{CH}_4}$ denotes the maximum utilization rate of H₂ by archaea [mol·g⁻¹h⁻¹; from Van Lingen et al. (2019)], $Q_{\text{Me}}$ the methanogen state variable, $M_{\text{H}_2/\text{CH}_4}$ the saturation constant for H₂ utilization for methanogenesis [M; from Van Lingen et al. (2019)], $C_{\text{NOP}}$ the dissolved H₂ concentration, $C_{\text{NOP}}$ the 3-NOP concentration and $f_{\text{MCR.H}_2/\text{CH}_4}$ the inhibition constant of 3-NOP associated with hydrogenotrophic methanogenesis (Table 3). The differential equation is given by:

$$\frac{dQ_{\text{H}_2}}{dt} = P_{\text{H}_2/\text{He.Ac}} - P_{\text{H}_2/\text{He.Bu}} - U_{\text{H}_2/\text{H}_2,\text{Ex}} - U_{\text{H}_2/\text{H}_2,\text{Em}} - U_{\text{H}_2/\text{H}_2,\text{Ab}} - U_{\text{H}_2/\text{H}_2,\text{CH}_4}$$ (7)
2.1.2. 3-Nitrooxypropanol+Nitrite Model

According to Duin et al. (2016), 3-NOP is broken down to NO$_3^-$ and NO$_2^-$ along with the formation of 1,3-propanediol. These conversions may take place in the archael cytosol that contribute to the presence NO$_2^-$ that also inhibits MCR. For evaluating the implications of these metabolic steps, an extended 3-NOP model was developed that also comprised a $Q_{NO_2^-}$ state variable.

3-nitrooxypropanol state variable, $Q_{3NOP}$ [mol]. In addition to the inputs and outputs described for the simple 3-NOP model, the conversion of 3-NOP into NO$_3^-$ and NO$_2^-$ is described as output from the $Q_{3NOP}$ state variable in the present model by:

$$U_{3NOP;3NOP,NO_3^-} = k_{3NOP,NO_3^-} \cdot Q_{Me} \cdot Q_{3NOP} \quad (8)$$

and

$$U_{3NOP;3NOP,NO_2^-} = k_{3NOP,NO_2^-} \cdot Q_{Me} \cdot Q_{3NOP} \quad (9)$$

with $k_{3NOP,NO_3^-}$ and $k_{3NOP,NO_2^-}$ the fractional rate constants for the conversion of 3-NOP reduction to NO$_3^-$ and NO$_2^-$ (Table 3) and the reduction flow rate is assumed to be also dependent on the methanogenic biomass. It was assumed that NO$_3^-$ and NO$_2^-$ is not transported across the methanogenic cell membrane and no other outputs were represented. This resulted in the differential equation of the $Q_{3NOP}$ state variable in the 3-NOP extended model given by:

$$\frac{dQ_{3NOP}}{dt} = P_{3NOP;In,3NOP} - U_{3NOP;3NOP,NO_3^-} - U_{3NOP;3NOP,NO_2^-} - U_{3NOP;3NOP,Ab} - U_{3NOP;3NOP,Ex} \quad (10)$$

Nitrite state variable, $Q_{NO_2^-}$ [mol]. Input to the $Q_{NO_2^-}$ state variable was NO$_2^-$ production from 3-NOP reduction:

$$P_{NO_2^-;3NOP,NO_2^-} = U_{3NOP;3NOP,NO_2^-} \quad (11)$$

and outflow from the rumen to the lower tract is with the methanogens as in Van Lingen et al. (2019):

$$U_{NO_2^-;NO_2^-;Ex} = 0.4 \cdot (k_{Ex,Ex} + k_{SO,Ex}) \cdot Q_{NO_2^-} \quad (12)$$

with $k_{SO,Ex}$ the fractional outflow rate of the solid material as in Van Lingen et al. (2019). The differential equation is given by:

$$\frac{dQ_{NO_2^-}}{dt} = P_{NO_2^-;3NOP,NO_2^-} - U_{NO_2^-;NO_2^-;Ex} \quad (13)$$

Hydrogen state variable, $Q_{H_2}$ [mol]. Compared with the 3-NOP simple model, the outflow that represents H$_2$ utilization for methanogenesis in the 3-NOP+nitrite model also accounts for inhibition of methanogenic growth by NO$_2^-$, which is given by:

$$U_{H_2;H_2;CH_4} = \frac{\gamma_{H_2;CH_4} \cdot Q_{Me}}{1 + \frac{h_{MCR;H_2;CH_4}}{M_{H_2;CH_4}} + \frac{h_{3NOP;NO_2^-}}{h_{MCR;H_2;CH_4}}} \quad (14)$$

where $j_{MCR;H_2;CH_4}$ denotes the inhibition constant with respect to the aggregated concentrations of 3-NOP and NO$_2^-$. The differential equation for the 3-NOP+NO$_2^-$ extended model is given by:

$$\frac{dQ_{H_2}}{dt} = P_{H_2;He;Ac} - P_{H_2;He;Ba} - U_{H_2;H_2;Ex} - U_{H_2;H_2;Em} - U_{H_2;H_2;Ab} - U_{H_2;H_2;CH_4} \quad (15)$$

2.1.3. Nitrate Simple Model

The key mechanism for the decrease in CH$_4$ production after supplementing NO$_3^-$ is generally considered the utilization of H$_2$ (Yang et al., 2016). The model was extended with only a NO$_3^-$ state variable for evaluating the significance of this mechanism.

Nitrate state variable, $Q_{NO_3^-}$ [mol]. The $Q_{NO_3^-}$ state variable receives input from NO$_3^-$ contents in the feed that was supplemented:

$$P_{NO_3^-;In,NO_3^-} = D_{DM(t)} \cdot \epsilon_{NO_3^-} \quad (16)$$

with $\epsilon_{NO_3^-}$ the NO$_3^-$ content of the feed [mol·kg$^{-1}$]. Output comprised the reduction of NO$_3^-$ to NH$_3$ in the periplasm of fermentative microbes (Kern and Simon, 2009):

$$U_{NO_3^-;NO_3^-;NH_3} = k_{NO_3^-;NH_3} \cdot Q_{Mi} \cdot Q_{NO_3^-} \cdot Q_{H_2} \quad (17)$$

with $k_{NO_3^-;NH_3}$ the rate constant for NO$_3^-$ reduction to NH$_3$ (Table 3). The absorption of NO$_3^-$ across the rumen wall was represented as:

$$U_{NO_3^-;NO_3^-;Ex} = k_{Ex} \cdot Q_{NO_3^-} \quad (18)$$

The differential equation is given by:

$$\frac{dQ_{NO_3^-}}{dt} = P_{NO_3^-;In,NO_3^-} - U_{NO_3^-;NO_3^-;Ab} - U_{NO_3^-;NO_3^-;Ex} \quad (19)$$

with $k_{NO_3^-;Ab}$ the fractional absorption rate for NO$_3^-$ absorption (Table 3); NO$_3^-$ was assumed to flow out with the fluid fraction from the rumen to the lower tract:

$$U_{NO_3^-;NO_3^-;Ex} = k_{Ex} \cdot Q_{NO_3^-} \quad (19)$$

Hydrogen state variable, $Q_{H_2}$ [mol]. Influences and outfluxes that were taken from Van Lingen et al. (2019) were the same as for the 3-NOP model. In the NO$_3^-$ model, output represented H$_2$ utilization for NO$_3^-$ reduction to NH$_3$ while applying a 4:1 stoichiometric ratio:

$$U_{H_2;NO_3^-;NH_3} = 4 \cdot U_{NO_3^-;NO_3^-;NH_3} \quad (21)$$

The flux that represented H$_2$ utilization for methanogenic growth was copied from the Van Lingen et al. (2019) model:

$$U_{H_2;H_2;CH_4} = \frac{\gamma_{H_2;CH_4} \cdot Q_{Me}}{1 + \frac{h_{MCR;H_2;CH_4}}{M_{H_2;CH_4}}} \quad (22)$$
The differential equation is given by:

\[
\frac{dQ_{H_2}}{dt} = P_{H_2:He,Ac} + P_{H_2:He,Bu} - U_{H_2:H_2,Me} - U_{H_2:NO_3^-,NH_3} - U_{H_2:H_2,Ab} - U_{H_2:H_2,Em} - U_{H_2:H_2,Ex}
\]  

(23)

2.1.4. Nitrate+Nitrite Model

For evaluating the significance of the NO\textsubscript{2} intermediary metabolite on the metabolism, an extended NO\textsubscript{3} model was developed for which a Q\textsubscript{NO\textsubscript{3}} state variable was also included.

Nitrate state variable, Q\textsubscript{NO\textsubscript{3}} [mol]. The \( U_{NO_3^-:NO_3^-:NH_3} \) of the Q\textsubscript{NO\textsubscript{3}} state variable in the simple model was broken up in two parts in the extended model. The first part resulted in output that comprised the reduction of NO\textsubscript{3} to NO\textsubscript{2} in the periplasm of fermentative microbes (Kern and Simon, 2009):

\[
U_{NO_3^-:NO_3^-:NO_2^-} = k_{NO_3^-:NO_3^-} \cdot Q_{Mi} \cdot Q_{NO_3^-} \cdot Q_{H_2}
\]  

(24)

with \( k_{NO_3^-:NO_3^-} \) the rate constant for NO\textsubscript{3} reduction to NO\textsubscript{2} by fermentative microbes (Table 3). Inflow, absorption across the rumen wall and outflow to the lower gastrointestinal tract were represented identical to the nitrate simple model, which resulted in a differential equation given by:

\[
\frac{dQ_{NO_3^-}}{dt} = P_{NO_3^-:In,NO_3^-} - U_{NO_3^-:NO_3^-:Ab} - U_{NO_3^-:NO_3^-:Ex}
\]  

(25)

Nitrite state variable, Q\textsubscript{NO\textsubscript{2}} [mol]. Input to the Q\textsubscript{NO\textsubscript{2}} state variable was NO\textsubscript{2} production from NO\textsubscript{3} reduction:

\[
P_{NO_2^-:NO_2^-:NO_2^-} = U_{NO_2^-:NO_2^-:NO_2^-},
\]  

(26)

whereas output from this state variable comprised absorption of NO\textsubscript{2} across the rumen wall:

\[
U_{NO_2^-:NO_2^-:Ab} = k_{NO_2^-:Ab} \cdot Q_{NO_2^-}
\]  

(27)

with \( k_{NO_2^-:Ab} \) the fractional absorption rate for NO\textsubscript{2}, which was also used for NO\textsubscript{3} absorption. The outflow of NO\textsubscript{2} was with the fluid fraction from the rumen to the lower tract:

\[
U_{NO_2^-:NO_2^-:Ex} = k_{FleX} \cdot Q_{NO_2^-}
\]  

(28)

and the reduction of NO\textsubscript{2} to NH\textsubscript{3}:

\[
U_{NO_2^-:NO_2^-:NH_3} = k_{NO_2^-:NH_3} \cdot Q_{Mi} \cdot Q_{NO_2^-} \cdot Q_{H_2}
\]  

(29)

where \( k_{NO_2^-:NH_3} \) denotes the rate constant for NO\textsubscript{2} reduction to NH\textsubscript{3} by fermentative microbes (Table 3). The differential equation is given by:

\[
\frac{dQ_{NO_2^-}}{dt} = P_{NO_2^-:In,NO_2^-} - U_{NO_2^-:NO_2^-:Ab} - U_{NO_2^-:NO_2^-:Ex} - U_{NO_2^-:NO_2^-:NH_3}
\]  

(30)

Hydrogen state variable, Q\textsubscript{H_2} [mol]. Influxes and outfluxes that were taken from Van Lingen et al. (2019) were the same as for the 3-NOP models and the reduced NO\textsubscript{3} model. In the full NO\textsubscript{3} model, output represented H\textsubscript{2} utilization for NO\textsubscript{3} reduction to NO\textsubscript{2} while applying a 1:1 stoichiometric ratio:

\[
U_{H_2:NO_3^-:NO_2^-} = U_{NO_3^-:NO_3^-:NO_2^-}
\]  

(31)

and H\textsubscript{2} utilization for NO\textsubscript{2} reduction to NH\textsubscript{3} while applying a 3:1 stoichiometric ratio:

\[
U_{H_2:NO_2^-:NH_3} = 3 \cdot U_{NO_2^-:NO_2^-:NH_3}
\]  

(32)

Rumen methanogens without cytochromes were suggested to be inhibited by NO\textsubscript{3} (Latham et al., 2016) at their electron-carrier system (Yang et al., 2016). Therefore, the flux that represented H\textsubscript{2} utilization for methanogenic growth that was incorporated accounted for inhibition by NO\textsubscript{3}:

\[
U_{H_2:H_2,CH_4} = \frac{V_{H_2,CH_4} \cdot Q_{Me}}{1 + \frac{M_{H_2,CH_4}}{C_{NO_3^-} \cdot k_{FleX} \cdot Q_{NO_2^-}}}
\]  

(33)

where \( C_{NO_3^-} \) denotes the H\textsubscript{2} concentration, \( J_{NO_3^-:H_2,CH_4} \) the inhibition constant for NO\textsubscript{3} of the H\textsubscript{2} uptake rate for methanogenesis (Table 3). The differential equation is given by:

\[
\frac{dQ_{H_2}}{dt} = P_{H_2:He,Ac} + P_{H_2:He,Bu} - U_{H_2:H_2,Me} - U_{H_2:NO_3^-:NO_2^-} - U_{H_2:NO_2^-:NH_3} - U_{H_2:H_2,Em} - U_{H_2:H_2,Ab} - U_{H_2:H_2,Ex}
\]  

(34)

2.2. Model Input and Parameter Values

Inputs to the model were intake rate (shown in Figures 1, 2) and nutrient composition of DM (Table 4). These inputs were taken from Van Zijldeveld et al. (2011), Veneman et al. (2015), and Olijhoek et al. (2016) for the NO\textsubscript{3} models, whereas the inputs were taken from Haisan et al. (2014), Hristov et al. (2015), Lopes et al. (2016), Haisan et al. (2017), and Van Wesenael et al. (2019) for the 3-NOP models. Every simulation was based on a dietary treatment with the inclusion rates of 3-NOP and NO\textsubscript{3} that was supplemented. If the feed intake rate in time was not reported, feed intake rates were scaled to Olijhoek et al. (2016) for ad libitum feeding and scaled to Van Lingen et al. (2017) for restricted feeding. This scaling was done based on the fraction of daily feed intake consumed per hour of a day. The dietary nutrient contents and \( k_{FleX} \) for the different studies were set per dietary treatment and taken in accordance with Bannink et al. (2010) and CVB (2018). Non-identified fractions that may include pectin and fructan were assigned to Fg, Sg, and Wra as in Van Lingen et al. (2019). An overview of all nutrient contents and degradation characteristics is given in Table 4. For evaluating the biological significance of 3-NOP and NO\textsubscript{3}
TABLE 4 | Degradable fiber (Fg), degradable starch (Sg), degradable protein (Pg) soluble sugars (Wr), acetate (Ac'), propionate (Pr'), butyrate (Bu'), and lactate (La') feed contents [g·kg⁻¹], and fractional hydrolysis rates [h⁻¹] of degradable fiber and degradable starch per experiment and/or treatment assigned (ExpTr) for 3-NOP and NO₃ model fitting data from Ōjioke et al. (2016, O), Ven Zijderveld et al. (2011, VZ), Veneman et al. (2015, VM), Haisan et al. (2014, Hn1), Haisan et al. (2017, Hn2), Lopes et al. (2016, Ls) and Van Wesemael et al. (2019, VW), and model evaluation data from Van Lingen et al. (2017, VL, average across all treatments and cows).

| ExpTr | Fg  | Sg  | Wr  | Ac' | Pr' | Bu' | La' | k_NO₃,Ab | k_Sg,He | k_Fg,Ps |
|-------|-----|-----|-----|-----|-----|-----|-----|---------|--------|--------|
| O     | 329 | 207 | 49  | 10  | 2   | 2   | 20  | 0.036   | 0.100  | 0.044  |
| VZ    | 245 | 252 | 76  | 6   | 1   | 1   | 11  | 0.029   | 0.094  | 0.065  |
| VM    | 294 | 230 | 90  | 13  | 2   | 2   | 26  | 0.025   | 0.100  | 0.064  |
| Hn1   | 281 | 250 | 140 | 0   | 0   | 0   | 0   | 0.056   | 0.091  | 0.067  |
| Hv    | 198 | 244 | 157 | 3   | 0   | 0   | 5   | 0.043   | 0.087  | 0.070  |
| Hn2   | 322 | 230 | 118 | 0   | 0   | 0   | 0   | 0.049   | 0.085  | 0.071  |
| Ls    | 193 | 257 | 151 | 3   | 0   | 0   | 5   | 0.045   | 0.087  | 0.065  |
| VW    | 315 | 149 | 116 | 6   | 1   | 1   | 12  | 0.042   | 0.081  | 0.063  |
| VL    | 287 | 159 | 125 | 11  | 2   | 2   | 21  | 0.043   | 0.078  | 0.054  |

on the rumen microbial metabolism, the 3-NOP models were run for supplement inclusion rates of 0, 0.5, and 1.0 mmol-(kg DMI)⁻¹, whereas the NO₃ models were run for inclusion rates of 0, 0.16, and 0.32 mol-(kg DMI)⁻¹. Dry matter intake rate and composition input data were from Van Lingen et al. (2017) on which various parameters of the extant model were fitted previously.

The differential equations of all state variables were numerically integrated for a given set of initial conditions and parameter values. The equations were solved using the lsoda numerical integration method (Petzold, 1983), a robust implicit integrator for stiff and non-stiff systems. This numerical integrator changes step size automatically to minimize computation time while maintaining calculation accuracy. The DM intake profile caused dramatic changes in QH₂ during the first 0.5 h and 10⁻² h during the remaining hours of every consecutive 12 h period. Based on the absorption rate of NO₃ and NO₂ that was discussed to be slowly (Nolan et al., 2016), the k_NO₃,Ab parameter was assigned a value of 0.30 h⁻¹, which is slightly lower than used for NH₃ and VFA absorption in the Dijkstra et al. (1996) model. Given the lack of data on 3-NOP absorption, the same value was used for the k₃NOP,Ab parameter. Simulations based on the aforementioned collection of literature data were used for estimating the fMCR:H₂,Me and k_NO₃,NH₃ parameters of both 3-NOP models and the NO₃ model to average daily CH₄ emission output. The k_NO₃,NH₃ and fNO₃:H₂,Me parameters of the NO₃+NO₂ model were estimated to the diurnal H₂ and CH₄ emission rates that were extracted from the graphs presented in Van Zijderveld et al. (2011), Veneman et al. (2015) and Olijhoek et al. (2016). Including the k₃NOP,NO₂, k₃NOP,NO₃, and k_NO₃,NO₂ in the parameter estimation procedure resulted in limited identifiability and these three parameters were assigned values more arbitrarily, but such that NO₂ concentrations in the 3-NOP+nitrite and nitrate+nitrate models approached the order of magnitude of the 3-NOP and NO₃ concentrations, respectively.

To avoid numerical dispersion during the parameter estimation procedure and to correct for the model inaccuracy, the model was run using control treatment input (i.e., no supplementation of 3-NOP and NO₃) for every study, after which the observed CH₄ emission data for all dietary treatments for which a certain dose of 3-NOP and NO₃ was administered were multiplied by the ratio of the observed and predicted values. A 240 h run of the model was considered to have converged to quasi steady-state. Model output of the final 24 h vs. the experimental data were calculated to assess the model performance given the model parameter values. The parameters were optimized to minimize the sum of squared residuals values using the BFGS algorithm (Conn et al., 1991).

2.3. Global Sensitivity Analysis

The sensitivity of the CH₄ emission rate to the parameters directly related to the inhibition was evaluated using a global sensitivity analysis. For this evaluation, the fMCR:H₂,CH₄, fNO₃:H₂,CH₄, k_NO₃,NO₂, k_NO₃,NH₃, k₃NOP,NO₂, k₃NOP,NO₃, k_NO₃,Ab, and k₃NOP,Ab parameters of the 3-NOP+NO₃ and NO₃+NO₂ models drawn from 0.75 to 1.25 times their optimum value using Latin hypercube sampling and a sample size of 1,000. The sensitivity of CH₄ production was evaluated using the highest inclusion rates of 3-NOP and NO₃ and the Van Lingen et al. (2017) feed input. Correlation coefficients were calculated to quantify the sensitivity of the CH₄ emission rate to the parameter values at 0, 0.5, 1, 2, 4, 6, and 10 h from the last meal of a 240 h simulation. All analyses were performed using the base...
3. RESULTS

3.1. Models Solutions

Parameter estimates of the optimized parameters of the four different models are provided in Table 3. In response to the assumed feed intake rate and all other parameters that were adopted from Van Lingen et al. (2019), all reference simulations in Figures 3–6, i.e., zero inclusion of 3-NOP and NO$_2$$_3$, are identical to the simulations shown in this study by definition. The present 3-NOP model predicts a 3-NOP concentration up to about 0.055 mM at 1.5 h from in silico feeding for the highest inclusion rate (Figure 3). Predicted 3-NOP concentrations then steadily approached zero at 12 h at which the next portion of feed was delivered. The diurnal dynamics of the total VFA concentration appeared largely unaffected by the inclusion of 3-NOP, whereas $p_{H_2}$ clearly increased in response to 3-NOP inclusion, with a peak of 0.3 atm at about 1 h from feeding for the 1.0 mmol·kg$^{-1}$ inclusion rate. The emission rate of $H_2$ followed a similar dynamic pattern as $p_{H_2}$ (result not shown). In contrast to the increased peak in $p_{H_2}$, the $CH_4$ emission rate in response to 3-NOP decreased almost immediately after feeding and then increased to the reference emission rate, while $C_{3NOP}$ approached zero. Increased $p_{H_2}$ exerted increased thermodynamic inhibition of NADH oxidation, as indicated by the decreased minima of the thermodynamic potential factor ($F_T$; a dimensionless factor that corrects a predicted kinetic reaction rate for the thermodynamic control exerted; $F_T = 1$ indicates no thermodynamic inhibition; $F_T = 0$ indicates equilibrium between forward and reverse reaction or, in other words, complete inhibition of the chemical reaction) and the prolonged decrease of $r_{NAD}$. It should be noted that for both non-zero inclusion rates of 3-NOP, $r_{NAD}$ starts reconditioning toward basal level at about 3 and 5 h from feeding when $F_T$ is equal to zero ($F_T = 0$ indicates neither the forward nor the reverse reaction of NADH oxidation are thermodynamically feasible). The decrease in $r_{NAD}$ after feeding was prolonged by 3-NOP supplementation that also resulted in decreased acetate, increase propionate and increased butyrate proportions that were prolonged. Extending the 3-NOP model to the 3-NOP+nitrite
model had negligible effect on the dynamics of total VFA concentration (result not shown), whereas non-zero basal $C_{NO_2}$ and peaks of 0.2 and 0.5 µM at 3.25 and 2.75 h from feeding appeared for the two inclusion rates, respectively (Figure 4). Other dynamics predicted by the 3-NOP+nitrite model appeared similar to the 3-NOP model.

The concentration of $NO_3^−$ predicted by the $NO_3^−$ model showed an increase from 0 to 1.75 and 5.75 mM in 0.5 h for $NO_3^−$ inclusion rates of 0.16 and 0.32 mol·kg$^{-1}$ DMI, respectively, and then steadily approached zero at 12 h at which the next portion of feed was delivered (Figure 5). Peak $pH_2$ was clearly decreased and delayed in response to $NO_3^−$ inclusion with a $pH_2$ value of $2.4 \times 10^{-3}$ atm at 3.1 h from feeding for the 0.32 mol·kg$^{-1}$ inclusion rate vs. $1 \times 10^{-2}$ atm at 0.5 h for zero $NO_3^−$ inclusion. A qualitatively similar decrease was simulated for the emission rate of $H_2$ (result not shown). In line with this decrease in $H_2$, the $CH_4$ emission rate was decreased compared to the reference simulation as well. Decreased $pH_2$ alleviated the thermodynamic inhibition of NADH oxidation, as indicated by $F_T$ approaching one throughout almost the entire 24 h simulation period for the highest $NO_3^−$ inclusion rate. The $r_{NAD}$ and the proportions of acetate, propionate and butyrate were negligibly affected by the inclusion of $NO_3^−$, as was the total VFA concentration. Extending the nitrate model to the nitrate+nitrite model negligibly affected the dynamics of total VFA concentration (result not shown), whereas the $C_{NO_3^−}$ diurnal pattern qualitatively followed the $C_{NO_3^−}$ diurnal pattern (Figure 6). In contrast to the nitrate model, the nitrate+nitrite model predicted an increase in $pH_2$, $r_{NAD}$ and the proportions of acetate, propionate and butyrate decreased, decreased, increased and increased, respectively. When zooming in on the highest inclusion rate of $NO_3^−$ using the nitrate+nitrite model, 2% passes out from the rumen after reduction to $NO_2^−$, 3% is absorbed after reduction to $NO_2^−$, 13% passes out from the rumen to the lower gastrointestinal tract, 32% is absorbed, and 51% undergoes complete reduction to $NH_3$. These percentages indicate that 51% + 0.25 × (3% + 2%) = 52% of the potential of

![FIGURE 4 | Solutions of the 3-NOP dynamic model with NO2 representation for 3-NOP concentration [mM], NO2 concentration [µM], rumen headspace pH2 [atm], CH4 emission rates [mol·h$^{-1}$], thermodynamic potential factor ($F_T$; [–]), NAD+ to NADH ratio ($r_{NAD}$), acetate proportion (Ac$^−$), propionate proportion (Pr$^−$), and butyrate proportion (Bu$^−$).]
NO$_3$ as a H$_2$ sink is utilized, where 0.25 relates to one of the four H$_2$ equivalents for complete reduction of NO$_3$ are consumed by fermentative microbes. Lastly, a qualitative overview of the output of the four different models in response to 3-NOP and NO$_3$ supplementation is provided in Table 1B.

### 3.2. Global Sensitivity Analysis

The $J_{\text{MCR;H$_2$Me}}$ inhibition parameter showed the strongest positive correlation with the CH$_4$ emission rate ($r = 0.6$ to 0.90) by the 3-NOP+nitrite model for the different time points for which the global sensitivity analysis was performed (Figure 7). The $k_{\text{3NOP,Ab}}$ parameter related to 3-NOP reduction also showed positive correlations, although the magnitude of the correlations was slightly stronger for the $J_{\text{MCR;H$_2$Me}}$ parameter. The $k_{\text{3NOP,NO$_3$}}$ absorption parameter was negligibly correlated to CH$_4$ emission rate at any of the time points. Correlations between the $k_{\text{3NOP,NO$_3$}}$ parameter and CH$_4$ emission rate were also very minor, $|r| \leq 0.10$, but were consistently negative. For the nitrate+nitrite model, the $J_{\text{NO$_3$;H$_2$Me}}$ inhibition parameter showed correlations of 0.61 to 0.97 from 0.5 to 6 h and correlations of approximately 0.5 at 0.0 and 10 h, whereas the $k_{\text{NO$_2$;NH$_3$}}$ parameter related to NO$_2$ reduction showed the correlations from roughly 0.22 to 0.76 at the various time points. The $k_{\text{NO$_3$;NO$_2$}}$ parameter related to NO$_3$ reduction showed very weakly negative correlations varying from $-$0.02 to $-$0.13. The $k_{\text{NO$_2$;Ab}}$ parameter related to absorption of NO$_3$ and NO$_2$ had the highest correlations of 0.78 and 0.57 at basal level, that is at 0 and 10 h, respectively, with the correlations at the other time points varying from 0.09 to 0.28.

### 4. DISCUSSION

The present paper presents models for simulating the dynamics of rumen metabolic physiology after supplementing two effective inhibitors of enteric CH$_4$ emissions from cattle, viz. 3-NOP and NO$_3$. It should be noted that 3-NOP is also economically profitable at farm level, whereas this could not be clearly indicated for NO$_3$ (Alvarez-Hess et al., 2019). Furthermore, NO$_3$ supplementation may increase the concentration of the NO$_2$ intermediate to levels that are poisonous to the
animal. To the authors’ knowledge, the present study is the first effort that describes the metabolism of methanogenic inhibition in the rumen using dynamic mechanistic modeling. Presenting 3-NOP and NO$_3^-$ models aids to distinguish the mode of action of decreased CH$_4$ caused by supplementation of 3-NOP and NO$_3^-$ to diets of cattle and other domestic ruminants, and explores further metabolic implications of H$_2$ accumulation and its impact on VFA dynamics. The latter metabolic changes were most clearly indicated by the two 3-NOP models. The 3-NOP to NO$_3^-$ conversion rate of the 3-NOP+nitrite model did not affect the inhibition potential of administered 3-NOP, whereas the 3-NOP to NO$_3^-$ conversion rate appeared to alleviate methanogenic inhibition. The different metabolic dynamics of the two NO$_3^-$ models point to the significance of the impact of NO$_2^-$ as an inhibitor of methanogenic archaea, in addition to the metabolic steps that reduce NO$_3^-$ to NH$_3$ and serve as H$_2$ sinks. The present modeling framework by which methanogenesis is inhibited by the concentration of an inhibitor (3-NOP models and nitrate+nitrite model) is possibly applicable to a wider variety of methanogenic inhibitors that are fed to various ruminant species.

4.1. Parameter Estimation Procedure

Data availability is an important determinant of model parameter identifiability (e.g., Brun et al., 2001). Data used for parameter estimation of the present models comprised average daily CH$_4$ emissions for both 3-NOP models and the nitrate model, whereas data describing diurnal dynamics of H$_2$ and CH$_4$ emission rates were used for the nitrate+nitrite model. It would be ideal, however, to obtain data that describes the diurnal dynamics of metabolites and also includes rumen 3-NOP, NO$_3^-$ and NO$_2^-$ concentrations. A dataset that comprises the concentrations of all these metabolites would increase the identifiability of the parameters, particularly of the nitrate+nitrite and 3-NOP+nitrite models for which the $k_{3\text{NO}_3,\text{NO}_2}$, $k_{3\text{NO}_3,\text{NO}_2}$, $k_{\text{NO}_3,\text{NO}_2}$ and $k_{\text{NO}_3,\text{Ab}}$ parameters were not estimated to data. Such data would likely also increase the accuracy of the simulated diurnal profiles of the various metabolites. Despite a relatively large variation of ruminal NO$_3^-$ and NO$_2^-$ concentrations across published studies.
Van Lingen et al. (2017); Veneman et al., 2015; Wang et al., 2018) this increase in dissolved concentration and 

The similar responses of the 

Wenner et al., 2015; NO

observed predictions align with changes in VFA proportions that were 

VFA proportions after feeding 3-NOP supplemented feed. These 

oxidation and next more pronounced minima and maxima in 

NAD

2

fermentation by representing a H 

2

and CH

4

emission by both H 

2

emission rate and parameter values obtained from global sensitivity analysis for the 3-NOP+nitrite and nitrate+nitrite model 

FIGURE 7 | Correlation between CH

4

emission rate and parameter values obtained from global sensitivity analysis for the 3-NOP+nitrite and nitrate+nitrite model when using inclusion rates of 1.0 mmol·kg

−1

for 3-NOP and 0.32 mol·kg

−1

for nitrate and the Van Lingen et al. (2017) feed input. Parameters values were drawn from the interval of 0.75 to 1.25 times their optimum value (see Table 3) using latin hypercube sampling and a sample size of 1,000. Correlation coefficients were calculated at 0, 0.5, 1, 2, 4, 6, and 10 h from the last meal of a 240 h simulation.

(e.g., Veneman et al., 2015; Wang et al., 2018), NO

3

and NO

2

concentrations of the same study were within the same order of magnitude for both studies. The NO

3

and NO

2

concentrations simulated using the nitrate+nitrite model are in the same order of magnitude as well, suggesting that our k

NO

3

,NO

2

−

estimate has a fair degree of accuracy, given that the k

NO

3

,NH

3

was highly identifiable to the diurnal profiles of H 

2

and CH

4

emission. Although various parameters may not have the utmost accuracy, different estimates may not result in different conclusions being drawn regarding the mechanisms by which CH

4

production is inhibited and the sensitivity of the CH

4

emission rate to these parameters may not change and be more related to the overall developed model structures.

4.2. Inhibited Methanogenesis and Metabolism

The 3-NOP models predicted increased and decreased emission rates of H 

2

and CH

4

upon 3-NOP supplementation, respectively, which indicated the model behavior was in line with various responses observed in vivo (e.g., Van Gastelen et al., 2020). The present models that are extensions of the Van Lingen et al. (2019) model, which accounts for the thermodynamic control of rumen fermentation by representing a H 

2

pool and the inclusion of NAD

+ and NADH, predict thermodynamic inhibition of NADH oxidation and next more pronounced minima and maxima in VFA proportions after feeding 3-NOP supplemented feed. These predictions align with changes in VFA proportions that were observed in vivo (e.g., Haisan et al., 2014, 2017; Romero-Perez et al., 2015; Lopes et al., 2016). The similar responses of the 3-NOP and 3-NOP+nitrite models and the weakly negative correlation between the CH

4

emission rate and the k

3-NOP,NH

3

parameter obtained from the global sensitivity analysis indicate that the rate of NO

3

production from 3-NOP has a minor effect on the inhibition of methanogenesis.

Extension of the nitrate model with a NO

2

representation reversed the pattern of pH 

2

and the H 

2

emission rate in response to NO

3

supplementation. The increased H 

2

emission rate simulated using the nitrate+nitrite model reproduces the in vivo experiments used for model calibration (Van Zijderveld et al., 2011; Veneman et al., 2015; Olijhoek et al., 2016), and is also in line with increased dissolved H 

2

concentration observed in faunated and defaunated in vitro systems (Wenner et al., 2020). This increase in dissolved concentration and emission rate of H 

2

supports the role of NO

3

as an inhibitor of methanogenesis (Iwamoto et al., 2002), which makes H 

2

accumulate. The positive correlations observed from the global sensitivity analysis for the CH

4

emission rate with the J

NO

2

·H

2

,Me and k

NO

3

,NH

3

parameters point to the significance of the contribution of NO

3

to the inhibition in CH

4

emission observed upon NO

3

supplementation. The positive relationship between the k

NO

3

,NH

3

Parameter and the CH

4

emission rate suggests that the major mode of action of decreased CH

4

production after NO

3

supplementation is caused by NO

2

inhibition rather than H 

2

that is consumed by the reduction of NO

3

to NH

3
.

The very weakly negative correlations obtained for k

NO

3

,NO

2

−
could be associated with decreased CH

4

emission by both H 

2

sink reinforcement and NO

2
 accumulation resulting in inhibited methanogenesis, although the effect may be negligibly small based on the low
absolute correlations. If H\textsubscript{2} sink mechanisms were the key controller of the CH\textsubscript{4} emission rate, a negative relationship between the $k_{NO_2-NH_4}$ parameter and the CH\textsubscript{4} emission rate should have been obtained from the global sensitivity analysis, with increased reduction of NO\textsubscript{3} and NO\textsubscript{2} resulting in less CH\textsubscript{4}. However, possibly in line with the low absolute correlations, Welty et al. (2019) only observed a numerical increase in dissolved H\textsubscript{2} concentration upon NO\textsubscript{3} supplementation to a continuous culture and no increase in H\textsubscript{2} production. Therefore, the lack of H\textsubscript{2} accumulation in this specific study does not point to substantial methanogenic inhibition by NO\textsubscript{2} in continuous cultures. Moreover, another possible explanation for unaffected H\textsubscript{2} concentration or production aligning with the present modeling study might be that their experimental conditions favored a rapid reduction of NO\textsubscript{2} to NH\textsubscript{3} that alleviated the methanogenic inhibition by NO\textsubscript{2}.

In line with Duin et al. (2016), the present 3-NOP+nitrite model also represents NO\textsubscript{3} formation. Nitrate production from 3-NOP would alleviate the methanogenic inhibition as it does not block MCR, indicating that the proportion in which NO\textsubscript{3} and NO\textsubscript{2} are formed from 3-NOP may determine the persistence of the methanogenic inhibition of 3-NOP supplementation to cattle diets. However, the sensitivity analysis did not indicate the formation rates of NO\textsubscript{3} and NO\textsubscript{2} were substantially influential for the area of the parameters space that was explored. Lack of evidence for the presence of NO\textsubscript{3} and NO\textsubscript{2} reductases in rumen methanogens (Greening et al. 2019) may conceptually support the fact that NO\textsubscript{3} formation alleviates methanogenic inhibition, because NO\textsubscript{3} may then not be reduced to NO\textsubscript{2}. However, Duin et al. (2016) observed 0.7 mol of NO\textsubscript{3} and 0.2 mol of NO\textsubscript{2} per mol of MCR when titrating with 3-NOP, which then requires one or more alternative mechanisms for the production of NO\textsubscript{3} and NO\textsubscript{2}. 1,3-propanediol also being formed from 3-NOP may suggest the production of NO\textsubscript{2} that is subsequently converted into NO\textsubscript{3} and NO\textsubscript{2}. The latter conversion has been described as a disproportionation reaction, which results in equimolar production of NO\textsubscript{3} and NO\textsubscript{2} (e.g., Park and Lee, 1988; Holleman and Wiberg, 2007). The production of 0.7 and 0.2 mol of NO\textsubscript{3} and NO\textsubscript{2}, respectively, may suggest either alternative NO\textsubscript{3} production or NO\textsubscript{2} utilization. If MCR deactivation by 3-NOP results in the formation of NO\textsubscript{2} (Duin et al., 2016), MCR deactivation by NO\textsubscript{2} may then result in the formation of NO (disproportionation also described by Park and Lee, 1988), which could explain why more NO\textsubscript{3} than NO\textsubscript{2} was observed. Furthermore, nitrate esters, which include 3-NOP, may hydrolyze and yield NO\textsubscript{3} and an alkanediol (Baker and Easty, 1950, 1952). Although it is unknown if the latter hydrolysis reaction proceeds inside archaeal cells, it describes the production of NO\textsubscript{3} and 1,3-propanediol from 3-NOP.

Nitrate at the outside or inside of archaeal cells will have consequences for the inhibition of archaeal physiology and methanogenesis. Whether or not transportation of NO\textsubscript{2} across archaeal cell membranes takes place affects our understanding of methanogenic inhibition by NO\textsubscript{2} derived from 3-NOP. Cabello et al. (2004) described some archaea, which are not abundant in the rumen, that possess NO\textsubscript{3} transporters and NO\textsubscript{2} and NO\textsubscript{3} reductases. Therewith, these enzymes were not indicated in rumen methanogens. Furthermore, genes for nitrate and nitrite transporters were searched using the IGM/M online database (https://img.jgi.doe.gov/m/; Chen et al., 2019) using “Methanobrevibacter,” “nitrate,” “nitrite,” and “transporter” did not point to any enzyme that possibly facilitates transportation of NO\textsubscript{2} across the archaeal cell membrane, indicating that NO\textsubscript{2} transportation across archaeal cell membranes is unlikely to occur. Nitrite inside archaeal cells, which is formed from 3-NOP that is transported across the archaeal cell membrane, contributes to blocking MCR and enhances methanogenic inhibition (Duin et al., 2016), although this specific study did not investigate if MCR inhibition is the only way in which NO\textsubscript{2} inhibits CH\textsubscript{4} production. Besides MCR, membrane-associated enzyme complexes catalyze several metabolic steps of the methanogenic pathway in archaea without cytochromes (Thauer et al., 2008), which are the common methanogens in the rumen. Nitrite at the outside of archaeal cells may inhibit the membrane-associated enzyme complexes or disrupt the electron transport system of the membrane (Yang et al., 2016). In contrast to NO\textsubscript{3} supplementation, 3-NOP supplementation results in substoichiometric ruminal concentrations of NO\textsubscript{2}, which may indicate that the actual membrane-associated inhibition of methanogenesis is negligible based on the $V_{NO_2-H_2,Me}$ parameter for the NO\textsubscript{3} model that is about two orders of magnitude greater than the $J_{MCR,H_2,Me}$ parameter for the two 3-NOP models. Furthermore, the value of the latter parameter could be taken as an additional indication for absence of NO\textsubscript{2} transportation across archaeal cell membranes, because the methanogenic metabolism may be completely ceased by blocking of MCR if NO\textsubscript{2} concentrations predicted after NO\textsubscript{3} supplementation to cattle diets occur inside archaea. To the authors’ knowledge, ceased methanogenic metabolism has not been observed upon ruminal NO\textsubscript{3} supplementation, which may rule out that NO\textsubscript{2} is transported into archaeal cells.

### 4.3. Hydrogen as a Controller of Fermentation

Inhibited methanogenesis resulted in increased $p_{H_2}$ and H\textsubscript{2} emissions from the rumen, as simulated by both 3-NOP models using different inclusion rates as well as implementing methanogenic inhibition by NO\textsubscript{2} when transitioning from the nitrate to the nitrate+nitrite model. Increased $p_{H_2}$ exerted inhibition of NADH oxidation, which resulted in decreased proportions of acetate and increased proportions of propionate and butyrate (Van Lingen et al., 2016, 2019). These respective shifts in VFA proportions in response to NO\textsubscript{2} supplementation seem less consistent in the literature. Observations were that acetate proportion was unaffected or increased, propionate proportion was unaffected, increased or decreased, and butyrate proportion was unaffected or increased across various studies (e.g., Guyader et al., 2015; Troy et al., 2015; Veneman et al., 2015; Olijhoek et al., 2016; van Lingen et al., 2019).
et al., 2016; Wang et al., 2018). This somewhat diverse picture in response to NO\textsubscript{3} may be related to the methanogenic inhibition that is likely employed, which is adverse to the H\textsubscript{2} sink mechanism in relation to thermodynamic inhibition of NADH oxidation and associated VFA proportions. Ruminal conditions that control the favorability of NO\textsubscript{3} reduction may determine the occurrence of the H\textsubscript{2} sink mechanism and the methanogenic inhibition by NO\textsubscript{3} mechanism. A mixed culture in vitro experiment by Anderson et al. (2016) indicated a decreased acetate to propionate ratio and an increased headspace pH\textsubscript{H} in response to increased NO\textsubscript{3} supplementation, whereas these changes were impaired when the mixed culture was also inoculated with Denitrovibacterium detoxificans, despite a more pronounced decrease of headspace CH\textsubscript{4} partial pressure. This inoculation may have stimulated the reduction of NO\textsubscript{3} and alleviated methanogenic inhibition and H\textsubscript{2} accumulation, and next affected the production of the different VFA. Therefore, these observations will likely be reproduced by a nitrate model such as the present nitrate+nitrite model in which both the H\textsubscript{2} sink mechanism and the nitrite inhibition of methanogenesis mechanism are implemented.

Thermodynamic inhibition of NADH oxidation was greatest for the highest pH\textsubscript{H} that was simulated and changed VFA proportions the most, perhaps more than observed in vivo. Electron-bifurcating hydrogenases that are able of reoxidizing NADH oxidation (e.g., Buckel and Thauer, 2018), were found to be the primary mediators of H\textsubscript{2} production by a metatranscriptomics analysis, but this analysis did not indicate that these hydrogenases were expressed differently in high and low CH\textsubscript{4} emitting sheep (Greening et al., 2019). No differences between hydrogenase enzyme expressions in these two groups of sheep may not suggest that VFA proportions in ovine rumens were changed (Van Lingen et al., 2016) and also that the present modeling framework of rumen fermentation metabolism that did predict changes in VFA proportions is too simple. However, Greening et al. (2019) did not relate actual H\textsubscript{2} emissions to enzyme expressions, nor were their samples collected from animals that were fed diets known to induce inhibition of methanogenic archaea, which point to the need for future studies that explore these relationships. Nonetheless, the latter recent study did report evidence for differences in enzyme expression associated with various alternative H\textsubscript{2} utilizing pathways in high and low CH\textsubscript{4} emitting sheep. Besides decreased expression of methanogenic enzymes, they reported increased expression of enzymes that mediate fumarate reduction. Fumarate reduction produces succinate, which is a precursor of propionate. Therefore, increased fumarate reduction upon elevated pH\textsubscript{H} is expected to stimulate propionate production in the rumen, which qualitatively supports the present model predictions of increased propionate proportions upon feeding dietary substrate that induces methanogenic inhibition. Furthermore, a decrease in H\textsubscript{2} recovered as the sum of propionate, butyrate, H\textsubscript{2} and CH\textsubscript{4} was observed when inhibiting methanogenesis in both batch and continuous culture (Ungerfeld, 2015), although the specific energetic benefits of methanogenic inhibition depended on the type and concentration of the inhibitor and on the in vitro system.

A more exhaustive metabolic framework of ruminal H\textsubscript{2} dynamics may comprise more than the key mechanism by which hydrogenases produce H\textsubscript{2} and mediate NADH oxidation. Ungerfeld (2015) speculated that H\textsubscript{2} was incorporated in formate and microbial biomass, and perhaps taken away via reductive acetogenesis in continuous cultures. For the latter H\textsubscript{2} utilizing pathway, the pH\textsubscript{H} threshold may be as high as 2.5×10\textsuperscript{-3} atm (Poehlein et al., 2012). Administration of methanogenic inhibitors to the rumen increases the number of hours per day that this threshold is exceeded and may, therefore, stimulate reductive acetogenesis. Upon supplementing bromochloromethane as an methanogenic inhibitor to goats, a metagenomic analysis indicated that, apart from increased Prevotella and Selenomonas species that are able to produce propionate using the randomizing pathway, reductive acetogenic populations were also affected significantly suggesting that they provide minor contributions to the redirection of H\textsubscript{2} (Denman et al., 2015). In the previously cited metatranscriptomics analysis for sheep rumens (Greening et al., 2019), reductive acetogenesis was indicated and enzyme expression was negatively correlated to CH\textsubscript{4} yield. Therefore, the incorporation of the reductive acetogenic pathway in the present models may shed further light on the metabolic dynamics in the rumen upon supplementation of inhibitors. However, further studies are required to discover other so far unidentified H\textsubscript{2} sinks for a better understanding of the metabolic pathways involved in H\textsubscript{2} production and utilization (Guyader et al., 2017).

### 4.4. Summary of Main Findings

In conclusion, both 3-NOP models and the nitrate+nitrite model predicted that the H\textsubscript{2} emission rate and pH\textsubscript{H} increased with the inclusion rate of 3-NOP and NO\textsubscript{3}, whereas a decreased CH\textsubscript{4} emission rate was simulated for these supplements. Omission of the NO\textsubscript{3} state variable from the 3-NOP model did not qualitatively change the overall dynamics of H\textsubscript{2} and CH\textsubscript{4} emission and other metabolites. However, omitting the NO\textsubscript{3} state variable from the NO\textsubscript{3} model substantially changed the dynamics of H\textsubscript{2} and CH\textsubscript{4} emissions indicated by a decrease in the emission rates of these two gases after feeding. Increased pH\textsubscript{H} induced by methanogenic inhibition, after 3-NOP supplementation particularly, resulted in decreased proportions of acetate and increased proportions of propionate and butyrate, although the incorporation of alternative H\textsubscript{2} consuming pathways may contribute to less pronounced responses in VFA proportions being predicted. The findings of this modeling study provide deeper insights into the metabolic physiology of ruminal bacteria, protozoa and archaea in response to two effective inhibitors of enteric CH\textsubscript{4} production. These insights will contribute to a better use of antimethanogenic additives and therefore help reducing enteric CH\textsubscript{4} production and the total ecological footprint of ruminant livestock production in the future.

### DATA AVAILABILITY STATEMENT

R code and data files that support the model simulations of this study can be found online at the GitHub
repository through: https://github.com/linge006/Modeling-inhibited-methanogenesis.

**AUTHOR CONTRIBUTIONS**

HL designed the research, performed all simulations of this study, and wrote the paper. HL, DY-R, and MK did the conceptualization. JF, EK, and MK supervised the work. EK and MK were responsible for the project administration. All authors reviewed drafts of the manuscript and approved the final version.

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Conflict of Interest: MK is affiliated with DSM Nutritional products, which is the funder of the present study and patented 3-NOP.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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