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The effects of dietary supplementation with 3-nitrooxypropanol on enteric methane emissions, rumen fermentation, and production performance in ruminants: a meta-analysis

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Abstract

The aim of this study was to investigate the effects of 3-nitrooxypropanol (NOP) on gas production, rumen fermentation, and animal performances depending on animal type using a meta-analysis approach. A database consisted of data from 14 studies, 18 experiments and 55 treatments. The supplementation of NOP linearly decreased methane (CH_4) emissions [g/kg dry matter intake (DMI)] regardless of animal type and length of experimental period (beef, $p < 0.0001$, $R^2 = 0.797$; dairy, $p = 0.0003$, $R^2 = 0.916$; and long term, $p < 0.0001$, $R^2 = 0.910$). The total volatile fatty acids (VFA) concentration and the proportion of acetate, based on beef cattle database, were significantly decreased with increasing NOP supplementation ($p = 0.0015$, $R^2 = 0.804$ and $p = 0.0003$, $R^2 = 0.918$), whereas other individual VFAs was increased. Based on the dairy database, increasing levels of NOP supplementation linearly decreased proportion of acetate ($p = 0.0284$, $R^2 = 0.769$) and increased that of valerate ($p = 0.0340$, $R^2 = 0.522$), regardless of significant change on other individual VFAs. In animal performances, the DMI, from beef cattle database, tended to decrease when the levels of NOP supplementation increased ($p = 0.0574$, $R^2 = 0.170$), whereas there was no significant change on DMI from dairy cattle database. The NOP supplementation tended to decrease milk yield ($p = 0.0606$, $R^2 = 0.381$) and increase milk fat and milk protein ($p = 0.0861$, $R^2 = 0.321$, $p = 0.0838$, $R^2 = 0.322$). NOP is a viable candidate as a feed additive because of its CH_4 mitigation effects, regardless of animal type and experiment period, without adverse effects on animal performances.

Keywords: Animal performance, Feed additive, Methane mitigation, 3-Nitrooxypropanol, Rumen fermentation

to this article was reported.

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Conceptualization: Kim HB, Lee HG, Seo JK.
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INTRODUCTION

Reducing methane (CH_4) emissions in rumen is a critical challenge to ruminant nutritionists. This is because CH_4 is a substantial anthropogenic greenhouse gas, possessing a global warming potential 28–34 times greater than carbon dioxide (CO_2) [1], and makes up 2%–12% of the loss of dietary gross energy (GE) intake to the ruminants [2]. Thus, there have been numerous global efforts to mitigate ruminal CH_4 emissions, using various feed additives such as tannin [3,4], dietary fats containing polyunsaturated fatty acids [5], plant essential oils [6,7], and phytochemicals [8,9].

3-Nitrooxypropanol (NOP) is a chemical compound, designed by Duval and Kindermann [10], which reduces CH_4 emissions produced by the rumen from microbial fermentation. The NOP is a structural analogue of methyl coenzyme-M, which inhibits the activity of methyl coenzyme-M reductase related to the final step of methanogenesis [11]. Until now, total 14 *in vivo* studies using NOP supplementation were performed on various domestic ruminants, including sheep [12], beef cattle [13–19], and dairy cattle [20–25]. According to the results of previous studies using NOP *in vivo*, the CH_4 emissions and proportion of acetate (% total volatile fatty acids, VFA) clearly decreased, whilst the proportion of propionate (% total VFA) significantly increased, but any adverse effects were not detected.

In a recent meta-analysis, Jayanegara et al. [26] observed that increasing NOP supplementation linearly decreased CH_4 emissions regardless of type of CH_4 unit, when a meta-analysis was investigated on 10 *in vivo* studies [12,16–24]. Dijkstra et al. [27] revealed that NOP supplementation has stronger CH_4 mitigation effects in dairy cattle than in beef cattle, and those effects were decreased in increasing dietary fiber content, when a meta-analysis was conducted using 9 *in vivo* studies [16–24]. With our knowledge, there is no meta-analysis study investigating the effects of supplementation of NOP on CH_4 reduction in a long term experiment, and the changes of rumen fermentation by NOP supplementation in a related with ruminant types was not analyzed as well.

In the present meta-analysis, therefore, we hypothesized that NOP supplementation might be affected differently on rumen fermentation characteristics depending upon animal type adding recent *in vivo* studies which was not included in previous meta-analysis studies [13–15,25]. The aim of this study was to investigate the effects of NOP on enteric gas production, rumen fermentation, and animal performances depending on animal type using a meta-analysis approach.

MATERIALS AND METHODS

Development of database

All studies used in the meta-analysis were collected from the Google Scholar database using NOP, CH_4, and ruminants as keywords. In total, variables from 14 studies, 18 experiments and 55 treatments were integrated into the database, as described in Table 1. The investigated factors were gas production (CH_4, H_2, and CO_2), rumen fermentation parameters [pH, total VFA production, acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, acetate to propionate ratio (A:P ratio), ammonia nitrogen (NH_3-N), bacteria, protozoa, and methanogen], and production performances [dry matter intake (DMI), dry matter digestibility (DMD), organic matter digestibility (OMD), neutral detergent fiber digestibility (NDFD), milk yield (MY), milk fat (MF), milk protein (MP), milk lactose (ML), and fat-corrected milk (FCM)]. Since all variables were not available across all experiments in the database, therefore, the number of observations used for regression analyses varied between independent and response variables. Units for NOP supplementation were expressed as NOP mg/kg of DMI. There were several differences on experimental animal, administration method of NOP, forage ratio in the diet, neutral detergent fiber (NDF) composition, and measure-

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Table 1. Summary of the studies used for the meta-analysis

Study no.	References	Animals	NOP level (mg/kg DMI)	Methods of administration	Forage ratio (in TMR, %)	NDF (%DM)	CH_4 measurement
1	Martínez-Fernández et al. [9]	Sheep	0 and 111.2	Direct administration via cannula	54.4	41.5	RCS
2	Romero-Perez et al. [15]	Beef	0, 47.4, 143.6, and 304.9	Top dressed	60.0	37.6	RCS
3	Romero-Perez et al. [16]	Beef	0 and 280.1	Mixed with diet	60.0	38.6	RCS
4	Vyas et al. [13]	Beef	0, 100, and 200	Mixed with diet	8 and 70.0	19.2 and 36.4	RCS
5	Vyas et al. [14]	Beef	0, 50, 75, 100, 150, and 200	Mixed with diet	8.0 and 65.0	27.0 and 41.7	RCS
6	Vyas et al. [12]	Beef	0, 125 and 200	Mixed with diet	8.0 and 65.0	18.4 and 40.5	RCS
7	Martinez-Fernandez et al. [11]	Beef	0 and 337.8	Mixed with roughage	100.0	66.1	RCS
8	Kim et al. [10]	Beef	0 and 100	Mixed with diet and direct administration via cannula	9.8 and 64.4	14.6 and 28.3	GFS
9	Haisan et al. [17]	Dairy	0 and 129.5	Mixed with diet	37.9	26.5	SF_6
10	Reynolds et al. [21]	Dairy	0, 26.6 and 135.1	Direct administration via cannula	51.2	39.8	RCS
11	Hristov et al. [19]	Dairy	0, 40.0, 60.0, and 80.0	Mixed with diet	60.7	27.6	GFS and SF_6
12	Lopes et al. [20]	Dairy	0 and 60.0	Mixed with diet	55.5	30.9	GFS
13	Haisan et al. [18]	Dairy	0, 68.3 and 132.3	Mixed with diet	60.0	33.8	SF_6
14	Van Wesemael et al. [22]	Dairy	0, 71.7 and 75.1	Mixed with roughage and mixed in pellet	65.8	34.6	GFS

NOP, 3-nitrooxypropanol; DMI, dry matter intake; TMR, total mixed ration; NDF, neutral detergent fiber; CH_4, methane; RCS, respiratory chamber system; SF_6, sulfur hexafluoride tracer; GFS, GreenFeed System; VFA, volatile fatty acids; MP, microbial population; MC, milk component.

ments methods of CH_4 emissions among all used studies [12–25] (Table 1). In short, the administration methods of NOP were direct administration via cannula, top dressed, and mixed with diet. forage ratio in dairy cattle showed narrow range (37.9% to 65.8%), although forage ratio in beef cattle were wide range (8% to 100%). Measurements of CH_4 emissions were conducted using a respiratory chamber system equipped with infrared CH_4 detectors, GreenFeed System (C-Lock Inc., Rapid City, SD, USA), and the sulfur hexafluoride (SF_6) tracer gas method.

Statistical analysis

All statistical analyses were carried out using the PROC UNIVARIATE, PROC MIXED and PROC REG procedures of the SAS ver. 9.4 (SAS Institute Inc., Cary, NC, USA). Outliers in the dataset were screened using an absolute studentized residual value (> 2) before conducting the statistical analysis. The dataset was analyzed statistically using PROC MIXED of SAS (2008), according to St-Pierre [28]. The model was as follows:

$$Y_{ij} = B_0 + B_1X_{ij} + s_i + b_iX_{ij} + e_{ij}$$

Where Y_{ij} is the dependent variable, B_0 is the overall intercept across all experiments (fixed effect), B_1 is the slope of Y on X (fixed effect), X_{ij} is the value j of the continuous predictor variable X in experiment i (the concentration of dietary NOP supplementation), s_i is the random effect of experiment i, b_i is the random effect of the slope in experiment i, and e_{ij} is the unexplained residual error. The variable experiment was declared in the CLASS statement. The slops and intercepts by experiment were included as random effects, and an unstructured variance-covariance matrix (type = un) was performed at the random part of the model [28]. When random covariance between

slope and intercept was not significant, a variance-covariance matrix (type = vc) was performed [28]. Individual observed values of the dependent variables were corrected with corresponding residual errors and regressed on the *X* variable (the concentration of dietary NOP supplementation). The relationship between the dependent variables (CH_4 production, total VFA production, the proportion of each VFA, and DMI) and NOP supplementation was expressed in three types of linear regression under different animal databases (e.g., total, beef, and dairy). Along with the model statistics from the regression equations, the *p*-value of each intercept and slope, root mean square error (RMSE), and coefficient of determination (R^2) are also presented.

RESULTS

Description of the database

The description of all variables included in database listed in Table 2 and variables on beef and dairy cattle described in Table 3. The CH_4 emissions expressed in terms of g/kg DMI, were 17.29 ± 5.481 g/kg DMI (Table 2). The emission of H_2 and CO_2 and rumen fermentation parameters varied widely in different studies suggesting that relatively a wide range of data were included in the database. The mean value of CH_4 emission (g/kg DMI) on beef database and dairy database ranged from 3.10 to 28.20 and 7.18 to 23.50 g/kg DMI, respectively. The mean and standard deviation of

Table 2. Description of gas emission, rumen fermentation characteristics, and production performances in ruminant database

Parameter	Parameter estimates					
	n	Mean	SD	Median	MIN	MAX
Total data						
CH_4 (g/kg DMI)	55	17.29	5.481	17.80	3.10	28.20
H_2 (g/d)	26	1.95	3.091	0.89	0.00	12.43
CO_2 (g/d)	25	10,674.44	3,005.371	10,500.00	6,240.00	14,905.00
pH	22	6.43	0.210	6.43	6.13	6.96
Total VFA (mM)	30	108.26	20.857	103.45	74.50	160.50
Acetate (%)	30	58.74	7.291	58.70	44.10	74.40
Propionate (%)	30	22.66	5.976	21.25	14.30	42.60
Butyrate (%)	30	12.85	2.927	13.40	5.00	17.80
Iso-butyrate (%)	30	1.12	0.337	1.08	0.57	2.10
Valerate (%)	30	1.94	0.472	1.85	1.20	3.19
Iso-valerate (%)	30	1.92	0.642	1.97	0.66	3.18
A:P ratio	30	2.88	0.919	2.77	1.06	4.90
Ammonia (mg/dL)	28	12.75	12.368	7.90	2.72	51.00
Bacteria[1)]	15	8.18	9.223	7.13	0.00	34.50
Methanogen[2)]	15	4.24	3.919	2.78	0.01	15.46
Protozoa[3)]	13	2.90	1.552	2.57	1.35	5.56
DMI (kg/d)	55	12.85	6.967	10.30	0.84	28.00
DMD (%)	14	68.10	4.863	68.70	58.40	75.30
OMD (%)	14	70.26	4.291	70.45	62.00	77.40
NDFD (%)	14	49.29	9.970	50.50	30.70	64.40

[1)] 10^{10}/g of rumen digesta.

[2)] 10^{8}/g of rumen digesta.

[3)] 10^{5}/g of rumen digesta.

SD, standard deviation; MIN, minimum value in database; MAX, maximum value in database; CH_4, methane; DMI, dry matter intake; H_2, hydrogen; CO_2, carbon dioxide; VFA, volatile fatty acids; A:P ratio, acetate to propionate ratio; DMD, dry matter digestibility; OMD, organic matter digestibility; NDFD, neutral detergent fiber digestibility.

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total VFA concentration were higher on beef cattle studies than on dairy cattle studies (112.61 ± 25.142 and 99.22 ± 8.424 mM, respectively), although each VFA proportion of beef database was similar with those of dairy database (Table 3). There was a big difference on mean of DMI between beef database and dairy database (beef DMI, 9.09 ± 1.751; dairy DMI, 22.22 ± 3.732 kg/d).

Gas emissions

3-NOP supplementation linearly decreased CH_4 production (g/kg DMI) of total ruminant ($p < .0001$, $R^2 = 0.744$). The CH_4 emissions in both beef and dairy cattle significantly decreased with increasing NOP supplementation ($p < 0.0001$, $R^2 = 0.797$ and $p = 0.0003$, $R^2 = 0.916$, respectively), however, the slope value in the linear regression for dairy is smaller than that for beef. The significant linear decrease in CH_4, with increasing levels of NOP supplementation, was also observed in the long-term *in vivo* studies ($p < 0.0001$, $R^2 = 0.910$). The H_2 emissions increased with increasing

Table 3. Description of gas emission, rumen fermentation characteristics, and performances in beef and dairy cattle database

Parameter	Parameter estimates					
	n	Mean	SD	Median	MIN	MAX
Beef						
CH_4 (g/kg DMI)	36	17.52	6.031	17.85	3.10	28.20
pH	14	6.46	0.249	6.45	6.13	6.96
Total VFA (mM)	18	112.61	25.142	104.65	74.50	160.50
Acetate (%)	18	58.41	8.498	59.65	44.10	74.40
Propionate (%)	18	23.49	7.216	21.00	15.90	42.60
Butyrate (%)	18	12.28	3.475	12.95	5.00	17.80
Iso-butyrate (%)	18	1.11	0.180	1.07	0.88	1.52
Valerate (%)	18	1.92	0.552	1.84	1.20	3.19
Iso-valerate (%)	18	2.08	0.558	1.97	1.20	3.18
A:P ratio	18	2.85	0.998	2.89	1.06	4.70
DMI (kg/d)	36	9.09	1.751	9.13	6.05	12.10
Dairy						
CH_4 (g/kg DMI)	17	16.81	4.560	17.80	7.18	23.50
pH	8	6.37	0.106	6.38	6.20	6.50
Total VFA (mM)	10	99.22	8.424	99.95	85.80	109.00
Acetate (%)	10	57.70	4.195	57.94	52.10	65.70
Propionate (%)	10	22.53	2.144	22.39	19.30	26.40
Butyrate (%)	10	14.13	1.380	14.21	11.10	15.90
Iso-butyrate (%)	10	0.94	0.238	1.00	0.57	1.19
Valerate (%)	10	2.03	0.331	2.09	1.57	2.57
Iso-valerate (%)	10	1.63	0.768	1.93	0.66	2.58
A:P ratio	10	2.65	0.455	2.64	2.02	3.51
DMI (kg/d)	17	22.22	3.732	21.30	18.30	28.00
Milk yield (kg/d)	17	32.69	7.803	28.20	25.80	46.40
Milk fat (%)	17	3.93	0.332	4.02	3.31	4.35
Milk protein (%)	17	3.28	0.198	3.19	3.06	3.61
Milk lactose (%)	14	4.61	0.195	4.65	4.26	4.81
FCM (kg/d)	17	32.25	8.143	29.00	23.90	46.41

SD, standard deviation; MIN, minimum value in database; MAX, maximum value in database; CH_4, methane; DMI, dry matter intake; H_2, hydrogen; CO_2, carbon dioxide; VFA, volatile fatty acids; A:P ratio, acetate to propionate ratio; FCM, fat corrected milk.

Table 4. Equations for linear regression of gas parameters on 3-nitrooxypropanol levels (mg/kg of DMI)

Parameter	Parameter estimates							Model statistics	
	n	intercept	SE intercept	*p*-value	Slope	SE slope	*p*-value	RMSE	R^2
CH_4 (g/kg DMI, total)	54	20.636	1.0186	< 0.0001	–0.041	0.0047	< 0.0001	1.793	0.744
CH_4 (g/kg DMI, beef)	35	21.365	1.4766	< 0.0001	–0.037	0.0043	< 0.0001	1.678	0.797
CH_4 (g/kg DMI, dairy)	16	20.068	1.1647	< 0.0001	–0.073	0.0084	0.0003	1.010	0.916
CH_4 (g/kg DMI, long term)	19	21.379	2.1144	< 0.0001	–0.053	0.0055	< 0.0001	1.482	0.910
H_2 (g/d)	24	–0.105	0.2949	0.7304	0.024	0.0079	0.0234	2.391	0.361
CO_2 (g/d)	24	10,622.0	1,028.20	< 0.0001	–0.785	1.1842	0.5286	268.34	0.065

DMI, dry matter intake; CH_4, methane; H_2, hydrogen, CO_2, carbon dioxide; SE, standard error; RMSE, residual mean square error; R^2, coefficient of determination.

levels of NOP (p = 0.0234, R^2 = 0.361), the increasing of NOP did not affect CO_2 emission (p = 0.5286, R^2 = 0.065).

Ruminal parameters and animal performances

The linear regressions of ruminal fermentation parameters, with increasing levels of NOP supplementation, from the all *in vivo* studies are shown in Table 5.

Total VFA concentrations, based on the database from whole studies, appeared to have a signi-

Table 5. Equations for linear regression of ruminal fermentation parameters on 3-nitrooxypropanol levels (mg/kg of DMI) from ruminant database

Parameter	Parameter estimates							Model statistics	
	n	intercept	SE intercept	*p*-value	Slope	SE slope	*p*-value	RMSE	R^2
Ruminal fermentation parameters									
pH	21	6.368	0.0643	< 0.0001	0.0007	0.00018	0.0071	0.048	0.678
Total VFA (mM)	27	107.230	3.8455	< 0.0001	–0.0366	0.01090	0.0073	4.897	0.388
Acetate (%)	29	61.303	2.0731	< 0.0001	–0.0310	0.00303	< 0.0001	0.875	0.898
Propionate (%)	28	20.000	1.1388	< 0.0001	0.0128	0.00329	0.0031	1.250	0.448
Butyrate (%)	28	12.364	0.6298	< 0.0001	0.0108	0.00333	0.0087	0.899	0.404
Iso-butyrate (%)	28	1.004	0.0618	< 0.0001	0.0005	0.00019	0.0301	0.068	0.366
Valerate (%)	28	1.719	0.1083	< 0.0001	0.0018	0.00038	0.0007	0.117	0.581
Iso-valerate (%)	29	1.737	0.1595	< 0.0001	0.0021	0.00071	0.0135	0.217	0.381
A:P ratio	28	3.153	0.2602	< 0.0001	–0.0034	0.00037	< 0.0001	0.110	0.884
Ammonia (mg/dL)	26	10.606	1.9932	0.0005	–0.0112	0.00408	0.0228	1.658	0.001
Bacteria[1)]	14	7.535	2.8407	0.0453	0.0068	0.00748	0.4157	1.211	0.052
Methanogen[2)]	14	4.114	1.3232	0.0266	–0.0076	0.00288	0.0574	0.641	0.610
Protozoa[3)]	12	2.426	0.4245	0.0046	0.0003	0.00270	0.9243	0.550	0.457
Animal performances									
DMI (g/kg)	50	12.074	1.5541	< 0.0001	–0.0017	0.00072	0.0304	0.329	0.170
DMD (%)	13	68.022	2.6669	0.0001	0.0024	0.00559	0.6979	1.119	0.055
OMD (%)	14	69.818	2.9233	0.0002	0.0047	0.00899	0.6359	1.273	0.086
NDFD (%)	14	48.338	6.0701	0.0041	0.0111	0.01125	0.3952	1.944	0.123

[1)] 10^{10}/g of rumen digesta.

[2)] 10^{8}/g of rumen digesta.

[3)] 10^{5}/g of rumen digesta.

VFA, volatile fatty acids; A:P ratio, acetate to propionate ratio; DMI, dry matter intake; DMD, dry matter digestibility; OMD, organic matter digestibility; NDFD, neutral detergent fiber digestibility; SE, standard error; RMSE, residual mean square error; R^2, coefficient of determination.

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ficant linear reduction with increasing NOP supplementation (p = 0.0073, R^2 = 0.388; Table 5). The NOP supplementation linearly decreased the proportion of acetate (p < 0.0001, R^2 = 0.898), whereas the proportion of propionate was linearly increased with increasing levels of NOP supplementation (p = 0.0031, R^2 = 0.448). This led to a linear reduction of the A:P ratio (p < 0.0001, R^2 = 0.884; Table 5). There was linear increase on the proportion of butyrate, iso-butyrate, valerate, and iso-valerate with increasing levels of NOP supplementation (Table 5). The pH was slightly increased (p = 0.0071, R^2 = 0.678) with increasing levels of NOP supplementation (Table 5). In the microbial population, methanogen counts were tended to decrease with increasing levels of NOP supplementation (p = 0.0574, R^2 = 0.610), although there was no significant change on the counts of total bacteria and protozoa (p = 0.4157, R^2 = 0.052 and p = 0.9243, R^2 = 0.457, respectively). In animal performances, based on the database from total *in vivo* studies, DMI was slightly decreased when NOP supplementation was increased (p = 0.0304, R^2 = 0.170), although increase of NOP supplementation did not affect digestibility of DM, OM, and NDF (Table 5).

The total VFA concentration and the proportion of acetate, based on beef cattle database, were significantly decreased with increasing NOP supplementation (p = 0.0015, R^2 = 0.804 and p = 0.0003, R^2 = 0.918; Table 6). The increase of NOP significantly increased the proportion of propionate, butyrate, iso-butyrate, and valerate when analyzed using beef cattle database (Table 6). The response of NOP supplementation on A:P ratio (Slop = –0.0036, p = 0.0002, and R^2 = 0.924) and DMI (Slop = –0.0016, p = 0.0574, and R^2 = 0.170) in beef was similar with those from total database.

When analyzed using dairy database, similarly for total and beef cattle database, the proportion of acetate (p = 0.0284, R^2 = 0.769) and A:P ratio (p = 0.0628, R^2 = 0.552) were decreased, whereas that of valerate (p = 0.0340, R^2 = 0.522) was linearly increased with increasing NOP supplementation (Table 7). However, there was no significant change on the proportion of propionate (p = 0.1591), butyrate (p = 0.3667), iso-butyrate (p = 0.3832), and iso-valerate (p = 0.2395). In the dairy production performances, the NOP supplementation had no significant linear relationship with DMI (p = 0.1760), FCM (p = 0.5718), and milk lactose percentage (p = 0.2263). The percentage of milk fat (p = 0.0861, R^2 = 0.321) and protein (p = 0.0838, R^2 = 0.322) tended to increase, although the milk yield (p = 0.0606, R^2 = 0.381) tended to decrease with increasing levels of NOP addition (Table 7).

Table 6. Equations for linear regression of ruminal fermentation parameters on 3-nitrooxypropanol levels (mg/kg of DMI) from beef database

Parameter	Parameter estimates							Model statistics	
	n	intercept	SE intercept	*p*-value	Slope	SE slope	*p*-value	RMSE	R^2
Total VFA (mM)	16	113.370	8.5296	< 0.0001	–0.0622	0.01131	0.0015	2.886	0.804
Acetate (%)	15	61.209	3.3136	< 0.0001	–0.0298	0.00336	0.0003	0.902	0.918
Propionate (%)	17	22.215	2.9321	0.0003	0.0112	0.00429	0.048	1.514	0.425
Butyrate (%)	17	11.298	1.2231	< 0.0001	0.0087	0.00332	0.0473	0.975	0.452
Iso-butyrate (%)	17	1.072	0.0571	< 0.0001	0.0005	0.00019	0.0396	0.078	0.426
Valerate (%)	17	1.775	0.2273	0.0002	0.0015	0.00042	0.0158	0.126	0.636
Iso-valerate (%)	18	1.843	0.1876	< 0.0001	0.0020	0.00092	0.0733	0.240	0.349
A:P ratio	17	3.187	0.3969	0.0002	–0.0036	0.00037	0.0002	0.107	0.924
DMI (g/kg)	36	9.103	0.5638	< 0.0001	–0.0016	0.00075	0.0574	0.331	0.170

VFA, volatile fatty acids; A:P ratio, acetate to propionate ratio; DMI, dry matter intake; SE, standard error; RMSE, residual mean square error; R^2, coefficient of determination.

Table 7. Equations for linear regression of ruminal fermentation parameters on 3-nitrooxypropanol levels (mg/kg of DMI) from dairy database

Parameter	Parameter estimates							Model statistics	
	N	intercept	SE intercept	*p*-value	Slope	SE slope	*p*-value	RMSE	R^2
Total VFA (mM)	10	99.762	4.0520	0.0001	–0.0240	0.02516	0.4102	3.404	0.051
Acetate (%)	10	59.653	2.0003	< 0.0001	–0.0339	0.00851	0.0284	1.070	0.769
Propionate (%)	10	21.831	0.9936	0.0002	0.0124	0.00662	0.1591	0.851	0.466
Butyrate (%)	9	13.855	0.2088	< 0.0001	0.0068	0.00588	0.3667	0.680	0.528
Iso-butyrate (%)	10	0.922	0.1383	0.0069	0.0002	0.00021	0.3832	0.051	0.000
Valerate (%)	10	1.952	0.1569	0.0011	0.0025	0.00068	0.0340	0.115	0.522
Iso-valerate (%)	10	1.456	0.3296	0.0215	0.0049	0.00335	0.2395	0.304	0.160
A:P ratio	10	2.811	0.2202	0.001	–0.0028	0.00098	0.0628	0.157	0.552
DMI (g/kg)	17	22.051	1.4831	< 0.0001	–0.0032	0.00202	0.1760	0.316	0.211
Milk yield (kg/d)	17	32.557	2.9927	0.0001	–0.0122	0.00507	0.0606	0.791	0.381
Milk fat (%)	17	3.845	0.1514	< 0.0001	0.0012	0.00058	0.0861	0.092	0.321
Milk protein (%)	17	3.275	0.0873	< 0.0001	0.0005	0.00023	0.0838	0.033	0.322
Milk lactose (%)	14	4.597	0.0897	< 0.0001	0.0001	0.00010	0.2263	0.015	0.214
FCM (kg/d)	14	31.771	3.7451	0.0011	0.0072	0.01169	0.5718	1.109	0.008

VFA, volatile fatty acids; A:P ratio, acetate to propionate ratio; DMI, dry matter intake; FCM, 4% fat corrected milk; SE, standard error; RMSE, residual mean square error; R^2, coefficient of determination.

DISCUSSION

Methane mitigation

The present study conducted a meta-analysis using total 14 *in vivo* studies published from 2014 to 2019, and the meta-analysis showed that supplementation of NOP was effective to a significant linear decrease in CH_4 yield (g/kg DMI), regardless of animal type compared with those fed a diet without NOP. It is similar with a result from Jayanegara et al. [26] who reported NOP supplementation had an effect of CH_4 mitigation regardless of type of CH_4 unit (CH_4 g per BW, DMI, milk, DMI, and digested OM). Dijkstra et al. [27] revealed that NOP supplementation has stronger CH_4 mitigation effects in dairy cattle than in beef cattle, when a meta-analysis was analyzed using 9 *in vivo* studies from 2014 to 2018 [16–24]. In present study including the latest articles(reference addition), we also observed that the effects of CH_4 mitigation by increasing levels of NOP supplementation in dairy cattle were more critical than those in beef cattle, indicating that the appropriate level of NOP to reduce CH_4 emissions may vary depending upon the animal type.

The most important factor in investigating an effective CH_4 mitigation strategy in rumen is persistent efficacy. With our knowledge, total 5 *in vivo* studies were conducted to investigate the effects of NOP supplementation on sustained mitigation of CH_4 emission [15,16,19,22,25]. Romero-Perez et al. [19], conducted a long-term study where eight ruminally cannulated heifers were fed a TMR, with a 60% forage ratio, supplemented with NOP (2 g/d of NOP) for about 146 d. Methane emissions were reduced up to 59% for both the g/d and g/kg of DMI in the NOP supplemented groups. Hristov et al. [22], reported that dairy cows that were fed diets containing NOP (40, 60, and 80 mg/kg of feed DM) produced up to 30% less enteric CH_4 throughout 12 weeks. In addition, two studies conducted a long-term experiment (238 d) including a backgrounding phase (105 d) and finishing phase (105 d) using beef cattle as the experimental animal [15,16]. In the backgrounding phase, Vyas et al. [16] reported that a significant linear reduction of CH_4 (g/d) was observed with increasing levels of NOP supplementation. Whereas in the finishing phase, the significant CH_4 (g/d) reduction was only observed when a high dose of NOP supplementation

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was applied (84% decrease compared to control). Vyas et al. [15] observed that NOP could decrease the CH_4 production (g/kg DMI) by 42% with improving gain-to-feed ratio (G:F) by 5% when NOP was added by 200 mg/kg DM with backgrounding diet, and they also stated 37% reduction of CH_4 production (g/kg DMI) with increasing G:F by 3% by supplementation of 125 mg/kg DM of NOP in the finishing period. More recently, Van Wesemael et al. [25] reported NOP can reduce CH_4 emissions (g/kg DMI) about 20% regardless of type of NOP supplementation (NOP incorporated into a concentrate pellet vs. NOP mixed with basal roughage), when dairy cattle fed 1.6 g/d of NOP throughout 10 weeks. With consistent previous results, this meta-analysis revealed that there was the significant linear decrease in CH_4 production (g/kg DMI) by supplementation of NOP on long-term *in vivo* studies, indicating that NOP might be an effective feed additive to mitigate CH_4 emissions sustainably.

Ruminal parameters

In the present study, a meta-analysis, based on the database including all experiments, revealed that NOP supplementation linearly decreased total VFA concentration and proportion of acetate, on the other hand linearly increased proportion of other individual VFAs, which was similar with a previous meta-analysis study [26]. On the other hand, NOP supplementation had different an effect intensity on total VFA and individual VFA proportion depending on animal type, although CH_4 emissions (g/kg DMI) were decreased with increasing levels of NOP regardless of animal type.

Methanogenesis is a main part of removing metabolic hydrogen in the rumen, and accumulated H_2 resulting from methanogenesis inhibition may be incorporated into propionate producing pathway and reductive acetogenesis [29]. Accumulated H_2 were also involved in the reduction of rumen fermentation through the inhibition of the re-oxidation of cofactors [30], therefore, it is consistent with present study based on beef database showing the reduction of total VFA concentration when NOP supplementation was increased (Table 6). Inconsistent with beef cattle, based on dairy cattle, it was revealed that increasing NOP supplementation only had linear relationship on proportion of acetate and valerate. Lopes et al. [23], who studied the effect of a dietary NOP addition on rumen microbial diversity, observed a decrease of *Ruminococcus* spp. known as acetate producing fibrolytic bacteria ($p < 0.01$), an increase of *Selenomonadales* including propionate producing bacteria ($p < 0.05$), and an increase of *Butyrivibrio* spp. known as butyrate producing bacteria. This indicated that changes of microbial compositions by NOP supplementation, might affect the concentration of each VFA, although NOP is not a material that directly manipulate the growth of rumen microbes. Generally, starch amount in feed ration could affect especially proportion of propionate in VFAs. When we investigated starch content in feed ration, starch (%DM) was 21.3 ± 4.98 (data not shown, dairy cattle database) [20,22,24,25], 39.3 ± 13.11 (data not shown, beef cattle database) [15,16,18,19]. Considering the lower starch content in the dairy cattle, increased metabolic hydrogen generated in methane reduction may be diverted to different hydrogen sink than the propionate producing pathway. Bleicher and Winter [31] revealed that formate was increased by *Methanobacterium formicicum*, when methanogenesis was inhibited by bromoethanesulphonic acid, suggesting that increased H_2 was utilized to produce formate. Ungerfeld [29] also stated alone both hydrogen sink (propionate and reductive acetogenesis) could not explain all incorporation of hydrogen generated by inhibition of methane production, thus, Ungerfeld [32] reported considering other hydrogen sinks, such as other fermentation products (formate, valerate, caproate, ethanol, and lactate) and microbial protein or fatty acid synthesis is important in studying about inhibition of methanogenesis. Several studies revealed that NOP supplementation might increase proportion of caproate when high dose of NOP supplemented [21,24], although other studies showed no sig-

nificant change [15,20] or significant decrease [14]. Reynolds et al. [24] reported NOP significantly increased ethanol production when was supplemented to 2,500 mg/d, and Kim et al. [13] observed significant increase of lactic acid when NOP was ruminally infused in high grain diet. A few studies showed increase of several fermentation products as hydrogen sink, more evidence will be needed to understand mechanism of metabolic hydrogen produced from CH_4 reduction by NOP supplementation in rumen.

Animal performances

Animal performances in response to NOP supplementation were presented in Table 4. The DMI, from beef cattle database, was tended to decrease when the levels of NOP supplementation increased, whereas there was no significant change on DMI from dairy cattle database. This is consistent with all previous studies using dairy cattle, which reported that the use of NOP did not change the DMI significantly [20–24]. Allen [33] stated that DMI might be decreased by increased starch digestion in reticulo-rumen and absorbed propionate might affects satiety and ingestion patterns. It is speculated that the higher starch content in beef cattle than dairy cattle might affect not only rumen fermentation, but also DMI, although the results should be interpreted with caution because various conditions can affect DMI, such as chemical composition (NDF and starch), particle size, silage fermentation products [33].

In the present study, the MY tended to decrease with increasing NOP supplementation, although all previous studies from dairy cattle, consistently observed no significant difference between the control and NOP groups [20–25]. These differences might be caused by numerical decreases in MY from most studies [20–22,24,25]. In the present study, results of the meta-analysis showed a tendency of increasing MF and MP, affecting milk price importantly, suggesting the use of NOP did not negatively affect the milk proportion.

Romero-Perez et al. [18], reported that NOP supplementation had quadratic effects on the DMD (p = 0.05) and OMD (p = 0.06). Hristov et al. [22], reported quadratic effects on the DMD (p = 0.006) and OMD (p = 0.06) with increasing levels of NOP, except for the NDFD. Haisan et al. [21] observed an increase in the DMD and OMD with NOP supplementation. Reynolds et al. [24] observed a tendency of DMD (p = 0.08) and OMD (p = 0.06) to decrease, when comparing doses between the control group and 2,500 mg/d of NOP. Thus, inconsistent results of nutrient digestibility would have affected the results of the present meta-analysis. Many studies postulated that CH_4 mitigation might affect the increase of available dietary GE. However, in this study, available dietary GE from reduced CH_4 emissions might not be totally utilized for animal production.

In conclusion, NOP is a viable candidate as a feed additive because of its strong CH_4 mitigation effects, regardless of animal type and experiment period, without adverse effects on animal performances. The magnitude of NOP supplementation effect was varied in relation to animal types. Thus, further research will be needed to identify the relationship between NOP supplementation and dietary content (starch, non-fiber carbohydrate, and NDF).

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