

UNIVERSITÀ CATTOLICA DEL SACRO CUORE

Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ph.D. in Agro-Food System

Cycle XXXVII

S.S.D. AGRI-09/B



UNIVERSITÀ
CATTOLICA
del Sacro Cuore

Measuring to reduce enteric methane: in vitro, in silico and in vivo approaches

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Candidate: Erica Fiorbelli

Matriculation n: 5116132

Academic Year 2023/2024



UNIONE EUROPEA
Fondo Sociale Europeo



Ministero dell'Università
e della Ricerca



REACT EU

La borsa di dottorato è stata cofinanziata con risorse del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione” e Azione IV.5 “Dottorati su tematiche Green”



UNIVERSITÀ
CATTOLICA
del Sacro Cuore

Measuring to reduce enteric methane: in vitro, in silico and in vivo approaches

Coordinator:

Ch.mo Prof. Paolo Ajmone Marsan

Tutor:

Prof. Antonio Gallo

Candidate: Erica Fiorbelli

Matriculation n: 5116132

Academic Year 2023/2024



UNIONE EUROPEA
Fondo Sociale Europeo



Ministero dell'Università
e della Ricerca



REACT EU

La borsa di dottorato è stata cofinanziata con risorse del Programma Operativo Nazionale Ricerca e Innovazione 2014-2020, risorse FSE REACT-EU Azione IV.4 “Dottorati e contratti di ricerca su tematiche dell’innovazione” e Azione IV.5 “Dottorati su tematiche Green”

Summary

<i>Abstract</i>	10
<i>List of scientific paper/manuscripts included in the PhD thesis</i>	11
Paper/Manuscript I	11
<i>List of other scientific contributions from the PhD program</i>	12
<i>List of Abbreviation</i>	13
1 General introduction	14
CLIMATE EMERGENCY AND THE 1.5°C TARGET	14
THE ROLE OF LIVESTOCK AND ENTERIC METHANE	15
Challenging on integrative and scalable approaches	16
Rumen microbial fermentation and biological basis of methane production	18
The rumen and its role on biochemical mechanisms of fermentation and methanogenesis	18
Rumen alternative hydrogen sinks	19
Fermentation and microbial adaptation	20
Experimental approaches for methane mitigation research	21
Overview of experimental approaches	21
In vitro models for rumen fermentation	21
In vivo validation and limitations	22
In silico dynamic modeling of rumen fermentation	22
Contribution of this thesis and research questions	22
<i>Reference</i>	23
2 Paper/Manuscript I	26
ABSTRACT	27

INTRODUCTION	29
MATERIALS AND METHODS	32
Animal and experimental design	32
Diets and feed ingredients	33
DMI and feeding behavior	34
Enteric gas emissions	35
Body weight, body condition, and behavior	35
Milk yield and sampling	35
Blood sampling and analysis	36
Rumen fluid sampling and analysis	37
Feed digestibility	38
Statistical Analysis	39
RESULTS	42
Diets	42
Gas production, yield, and intensity	42
Dry matter intake and feeding behavior	43
Milk production	43
Metabolic profile	44
Rumen fermentation profile and apparent nutrient digestibility	45
DISCUSSION	45
Enteric gas emissions	46
Dry matter intake and feed digestibility	47

Milk production and quality	49
Blood biomarkers and body condition	51
<i>CONCLUSIONS</i>	52
<i>NOTES</i>	53
<i>REFERENCES</i>	53
<i>SUPPLEMENTARY MATERIALS</i>	76
3 Paper/Manuscript II	80
<i>ABSTRACT</i>	81
<i>INTRODUCTION</i>	82
<i>MATERIALS AND METHODS</i>	85
Animal management and experimental design	85
Dry matter intake, Feeding behavior and meal-bout definition	86
Preprocessing of collected data	87
Statistical analysis	87
<i>RESULTS</i>	89
Feeding behavior and meal criterion	89
Meal pattern metrics	90
Repeatability of feeding behavior	91
<i>DISCUSSION</i>	91
Visit and meal pattern	91
Repeatability of feeding behavior	93

Diurnal pattern of feeding events	94
CONCLUSIONS	95
NOTES	96
REFERENCES	96
4 Paper/Manuscript III	109
ABSTRACT	110
Main body	111
Notes	119
References	119
5 Paper/Manuscript IV	127
Abstract	128
Introduction	129
Materials and methods	131
In vitro gas production method	132
Gas composition and ruminal volatile fatty acids concentration	134
Rumen model parameters estimation	134
Farm index determination	137
Statistical analysis	138
Results	139
Dietary ingredients and nutritional composition	139
Total gas production, composition and VFAs profile	140

Dynamic prediction of rumen fermentation	141
Rumen model parameters and correlations among farms' indexes	142
<i>Discussion</i>	143
Evaluation of fermentability of total mixed ration	143
Rumen fermentation model performance and limitations	144
Implications for Sustainable Nutritional Strategies	145
<i>Conclusions</i>	147
<i>References</i>	148
6 Paper/Manuscript V	164
ABSTRACT	165
INTRODUCTION	166
MATERIALS AND METHODS	168
Sample collection and preparation	168
In vitro experimental design	169
Rumen fluid collection	169
Inoculum procedure	170
Analysis of gas chromatography	171
Ruminal volatile fatty acids determination	172
In vitro screening of total phenolics and total flavonoids in macroalgae	172
Statistical analysis	172
RESULTS	174

Total Gas production and composition	174
Volatile Fatty Acid Profiles	174
Screening of total phenolics and total flavonoids	175
DISCUSSION	175
Dynamic Total Gas Production and CH ₄ Composition	175
VFA profiles	177
Total phenolics and total flavonoids profile	178
CONCLUSIONS	180
NOTES	180
REFERENCES	181
7 General discussion	194
An integrative framework for methane mitigation	194
Limitation of the PhD study	195
8 Conclusion and Perspective	197
9 Future perspectives	198
Refining feed additive strategies	198
Expanding the model framework	198
Omic screening	198
From experimental to practical applications	198

ABSTRACT

Enteric methane from ruminants is a potent greenhouse gas and a major contributor to agricultural emissions. This PhD research explored integrative strategies to measure and mitigate enteric methane through a multi-approach framework combining in vivo, in vitro, and in silico methodologies.

First, feeding behavior and enteric emissions were evaluated in dairy cows supplemented with 3-nitrooxypropanol (3-NOP), demonstrating significant methane reduction without impairing milk yield or animal health. Second, rumen inocula from 3-NOP-treated and control cows were used to assess fermentation kinetics in vitro, highlighting temporal shifts in gas production, volatile fatty acids profiles, and hydrogen dynamics, particularly during the initial incubation phases. Third, a dynamic mechanistic model of rumen fermentation was applied to 39 total mixed rations representative of Italian dairy systems, identifying biological parameters, such as hydrogen utilization rate and sugar degradation kinetics, linked to nutritional strategies and farm-level energy efficiency. Fourth, a comprehensive screening of Italian Mediterranean macroalgae revealed several species with promising methane mitigating potential and high levels of phenolic and flavonoid compounds, suggesting eco-compatible alternatives to tropical species like *Asparagopsis taxiformis*.

Together, these studies offer a coherent picture of the methane mitigation potential of nutritional strategies, the value of mechanistic modeling for predictive purposes, and the relevance of local biomasses. The integrative methodological approach enhances our understanding of microbial adaptations, hydrogen fluxes, and modeling accuracy, providing decision-support tools for more sustainable ruminant systems in the context of climate change.

LIST OF SCIENTIFIC PAPER/MANUSCRIPTS INCLUDED IN THE PHD THESIS

Paper/Manuscript I : Effects of 3-nitrooxypropanol on enteric methane emissions and milk production characteristics in dairy cows fed a high corn silage diet in different environmental conditions.

Paper/Manuscript II : Feeding behavior and meal patterns of mid-lactating dairy cows under methanogenesis inhibition with 3-nitrooxypropanol at different environmental conditions

Paper/Manuscript III : Kinetics of in vitro gas and methane production in presence of 3-nitrooxypropanol in rumen fluid of lactating dairy cows.

Paper/Manuscript IV : Evaluating in vitro nutritional strategies clusters for mitigating methane emissions in intensive dairy farms by a mechanistic model of in vitro rumen fermentation.

Paper/Manuscript V : A in vitro rumen screening of Italian mediterranean macroalgae on potential reduction of methane emission and its phenolic and flavonoid profile

LIST OF OTHER SCIENTIFIC CONTRIBUTIONS FROM THE PHD PROGRAM

Article in a journal

Fiorbelli, E., Lapris, M., Errico, M., Della Badia, A., Riahi, I., Rocchetti, G., & Gallo, A. (2024). Mycotoxin Challenge in Dairy Cows: Assessment of the Efficacy of an Anti-Mycotoxin Agent by Adopting an In Vitro Rumen Simulation Method. *Toxins*, 16(11), 490. <https://doi.org/10.3390/toxins16110490>

Abstract in proceedings

E. Fiorbelli, A.S. Atzori, L. Tedeschi, M. Santucci, A. Gallo. 55. Studying the predictive ability of a dynamic mechanistic model to forecast the oscillatory feeding behaviour of lactating dairy cows, *Animal - science proceedings*, Volume 13, Issue 4, 2022, Pages 575-576, ISSN 2772-283X, <https://doi.org/10.1016/j.anscip.2022.07.446>

E. Fiorbelli, A.S. Atzori, L. Tedeschi, A. Catellani, A. Gallo. 0407. A dynamic mechanistic model to forecast the oscillatory feeding behaviour of lactating dairy cows. Book of abstract of the 25th congress. *Italian Journal Animal Science* vol.22:s1, 2023 Pages, 112.

E. Fiorbelli, A.S. Atzori, A.T. Bushra, L. Tedeschi, and A. Gallo. A dynamic mechanistic model to forecast the oscillatory feeding behaviour of lactating dairy cows. Book of Abstracts of the 75th Annual Meeting of the European Federation of Animal Science 2023 Lyone, France. doi: 10.1016/j.anscip.2025.07.288

E. Fiorbelli, A.S. Atzori, R. Muñoz-Tamayo, A. Gallo. Use of a mechanistic model simulating in vitro rumen fermentation to evaluate the effect of nutritional strategies on rumen production and farm indices in intensive dairy farms, *Animal - Science proceedings*, Volume 16, Issue 3, 2025, Pages 458-459, ISSN 2772-283X, <https://doi.org/10.1016/j.anscip.2025.07.288>

LIST OF ABBREVIATION

ADF : acid detergent fiber	LS: alfalfa silage
ADL : acid detergent lignin	IEFC : income energy feed cost
AFOLU : Agriculture, Forestry, and Other Land Use	IOFC : income over feed cost
AOPP : advanced oxidation protein products	IPCC : Intergovernmental panel on climate change
BW : body weight	MCR : methyl coenzyme M reductase
CCC : Concordance correlation coefficient	MEE : metabolizable energy use efficiency
CH ₄ : methane	MF : milk fat
CL : cluster	MP : milk protein
CO ₂ : carbon dioxide	Mx : macroalgae x
CO ₂ -eq : carbon dioxide equivalent	MY : milk yield
COP : Conference of the Parties	NEE : net energy use efficiency
CP : crude protein	NDF : neutral detergent fiber
CTR : control group	NDFD : neutral detergent fiber digestibility
DEE : digestible energy use efficiency	NEFA : nonesterified fatty acids
DM : dry matter	NH ₃ : ammonia
DMI : dry matter intake	N ₂ O : nitrous oxide
dNDF : digestible NDF	NRC : National research council
DNDF 24h : NDF degradability evaluated after 24 h of rumen incubation	ODEs : ordinary differential equations
dOM : digestible organic matter	OM : organic matter
ECM : energy corrected milk	PCV : packed cell volume
EE : ether extract	P1 : phase 1
ECM : energy-corrected milk	P2 : phase 2
FA : fatty acid	PMR : partial mixed ration
FAO : Food and agriculture organization of the United Nations	ProteinCompoundFeed : compound feeds, used in more than 3.5 kg/cow per day, with a CP concentration higher than 30% as fed
FCM : fat corrected milk	ProteinRestrictedFeed : the compound feeds, used in less than 3.5 kg/cow per day, with a CP concentration between 21% and 30% as fed
FE : feed efficiency	REI : residual energy intake
FPCM : fat and protein corrected milk	RMSPE: root mean square prediction error
FRAP : ferric reducing antioxidant power	ROM : reactive oxygen metabolites
GAE : gallic acid equivalent	STA : starch
GC : gas chromatography	TFC : total flavonoid content
GE : gross energy	TMR : total mixed ration
GEI : gross energy intake	TPC : total phenolic content
GGT : γ -glutamyl transferase	TRT : treatment group
GHG : greenhouse gas	TRTProteinFeed : steam-flaked, expeller or extruded soybean
GLM : general linear model	uNDF : undigested NDF
GOL : <i>Asparagopsis taxiformis</i>	UNEP : Unit Nation environment program
GOT = glutamate oxaloacetate transferase	VFA : volatile fatty acids
GWP : global warming potential	YellowStarchFeed : corn meal
H ₂ : hydrogen	3-NOP : 3-nitrooxypropanol
H ₂ O : water	
HMC : hight moisture ear corn	
HVProteinFeed : solvent-extracted soybean meal and de-hulled soybean meal	

1 General introduction

CLIMATE EMERGENCY AND THE 1.5°C TARGET

Climate change is unequivocally recognized as one of the most critical points to ecosystems, economies, and human health. According to the Intergovernmental Panel on Climate Change (IPCC, 2018), human activities have already caused approximately 1.0°C of global warming above pre-industrial levels. If current trends continue, a global increase of 1.5°C is expected between 2030 and 2050. This threshold is of critical importance, in the sense that it represents the limit beyond which it is no longer possible to predict climate-related risks. These risks include, but are not limited to, extreme weather events, biodiversity loss and irreversible impacts on food systems.

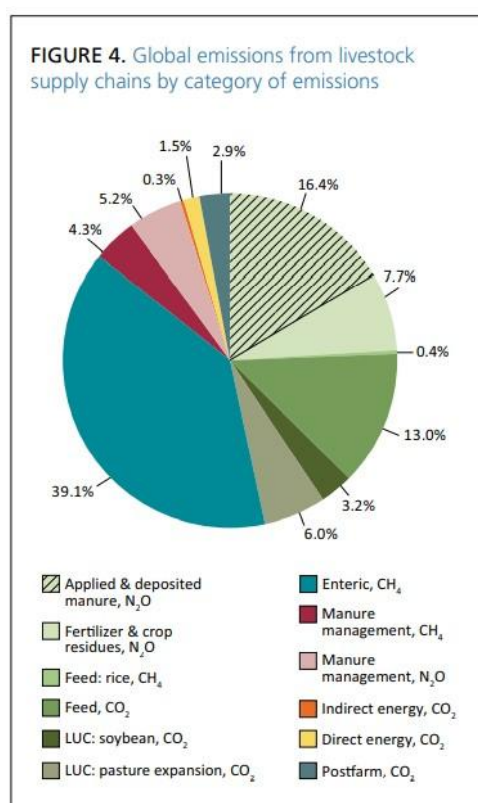
Methane (**CH₄**) is a potent short-lived climate pollutant with a global warming potential (**GWP**) 84 times greater than carbon dioxide (**CO₂**) over a 20-year time horizon (Myhre et al., 2013a). Despite its shorter atmospheric lifetime (~12 years), its high radiative forcing capacity makes it a crucial target for near-term climate mitigation. In fact, IPCC, 2018 and United Nations Environment Programme (UNEP), 2021 have highlighted that substantial reductions in CH₄ emissions are required to meet the 1.5°C scenario, recommending at least a 35% reduction in global CH₄ emissions by 2050 relative to 2010 levels.

In recent years, policy instruments have increasingly addressed CH₄ mitigation as part of broader climate goals. The Paris Agreement (2015) requires nations to set targets for greenhouse gases (**GHG**) reductions in line with limiting global warming to below 2°C, with aspirations for 1.5°C. Although methane is not directly regulated, its inclusion in national GHG inventories under the Paris Agreement makes agricultural enteric CH₄ a relevant mitigation target.

The Global Methane Pledge, launched at COP26 in 2021 by the United States and European Union, with over 150 signatory countries, has committed to reduce CH₄ emissions by 30% by 2030 compared to 2020 level. This initiative emphasizes key sectors including oil and gas, agriculture, and waste. At COP28 in 2023 in Dubai, new financial mechanisms and incentives were introduced to support actions such as the management of livestock manure and the reduction of enteric fermentation in ruminants.

THE ROLE OF LIVESTOCK AND ENTERIC METHANE

Globally, the area of Focus on Agriculture, Forestry, and Other Land Use (AFOLU) accounts for approximately 22% of net global GHG emissions (IPCC, 2023), with livestock systems contributing significantly through enteric fermentation and manure management. Ruminants, especially cattle, are the largest agricultural source of CH₄ (30%), where the agriculture sector is responsible for nearly 40% of total anthropogenic CH₄ emissions (UNEP and CCAC, 2021). Within



livestock emissions, enteric CH₄ alone contributes over 70% of total emissions from ruminants, making it a critical leverage point for mitigation strategies (Gerber et al., 2013)(Figure 1.).

Figure 1. Global emission from livestock supply chains by category of emissions (Figure of Gerber et al., 2013). From a biological perspective, enteric CH₄ is produced in the rumen by *archaea* through the hydrogenotrophic pathway, using hydrogen (H₂) and CO₂ as substrates (Figure 2). This process represents a loss of 2 to 12% of the gross energy intake (Johnson and Johnson, 1995),

thus reducing feed efficiency. Therefore, reducing enteric CH₄ not only helps to meet climate goals but can also improve animal productivity by optimizing energy use. Despite significant progress in reducing GHG from the energy and industry sectors, emissions from food systems alone are projected to surpass the 1.5°C-compatible threshold by 2050 without systemic changes (Clark et al., 2020). Therefore, CH₄ reduction in livestock systems is not only environmentally desirable but essential for sustainable development and food security.

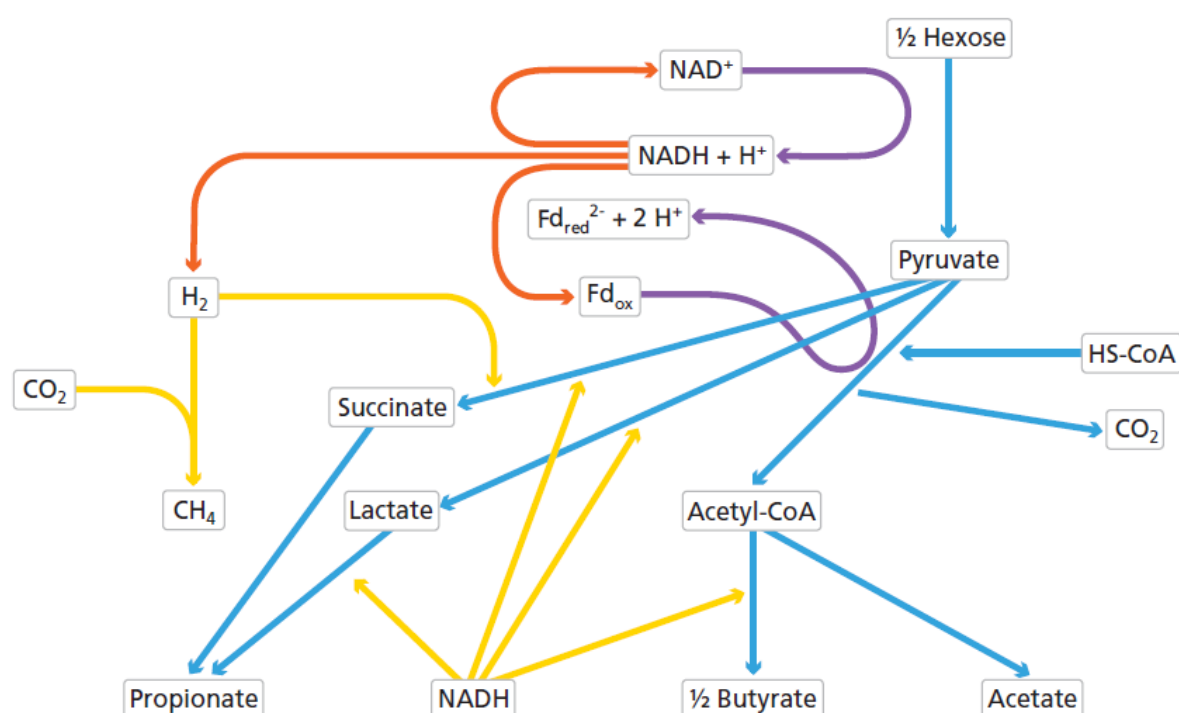


Figure 2. The main biochemical pathways in rumen fermentation (Figure of (FAO, 2023a))

Challenging on integrative and scalable approaches

Mitigating CH₄ emissions in livestock requires a systems-level perspective. According to FAO, (2023a), enteric fermentation is a major source of emissions in ruminants, contrasting with monogastric systems where emissions are primarily driven by feed production and manure. As such, ruminants represent a strategic target for CH₄ mitigation (Figure 3).

Gerber et al. (2013) emphasize that effective mitigation must span the entire production cycle, from feed source to digestion and waste management. Strategies must not only reduce CH₄ production but avoid increasing other GHG. Combining biological effectiveness with policy incentives and economic viability is therefore critical for broad implementation.

Within the livestock sector, cattle are by far the largest source of GHG emissions, accounting for approximately 65% of the total, equivalent to 4.6 gigatonnes CO₂-eq annually. This includes both dairy and beef production systems. Specifically, beef production is responsible for around 2.9 gigatonnes, or 41% of sector emissions, while milk production contributes about 1.4 gigatonnes, or 20%. The primary emission pathway in both systems is enteric fermentation, which generates approximately 1.1 gigatonnes CO₂-eq annually that represents 46% of emissions from dairy and 43% from beef supply chains (Gerber et al., 2013).

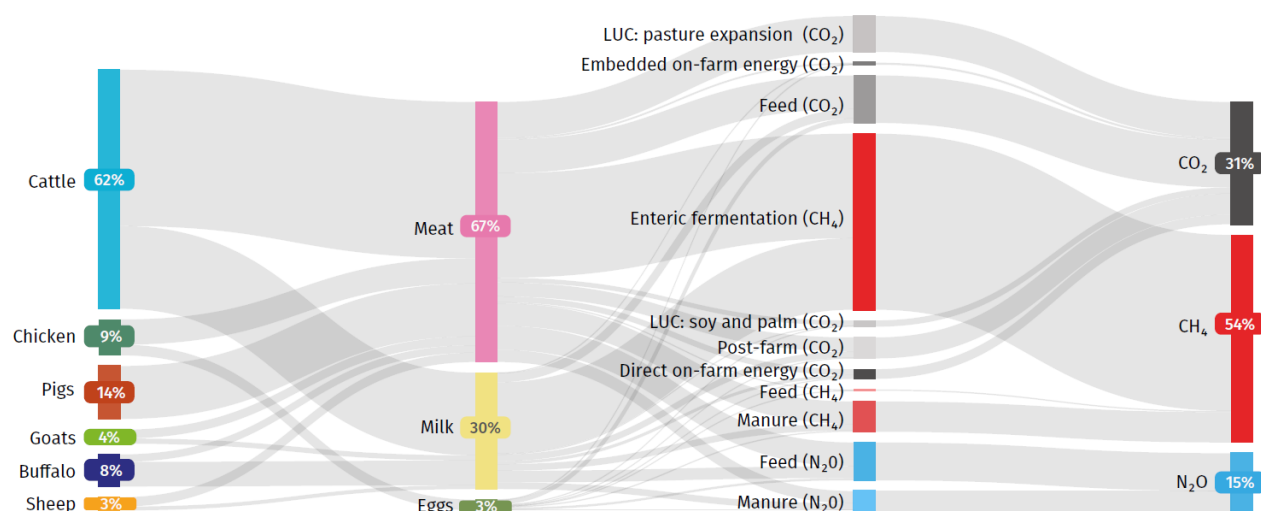


Figure 3. Sankey diagram of emission sources in 2015 by species, products, sources of emissions and gasses based on GLEAM 3 (Figure of FAO, 2023b)

Several CH₄ mitigation strategies are scientifically validated, their uptake on farms remains limited. Hristov et al., 2013 suggest that without economic benefits or policy support, farmers are

unlikely to implement these practices. This is especially true for feed-based interventions or additives, which may increase costs without immediate productivity gains.

A potentially valid approach could involve modifications to dietary composition and intake levels as a means to rapidly mitigate CH₄ emissions on farms. Moe and Tyrrell (1979) demonstrated that carbohydrate type affects CH₄ production more noticeably at high intake levels. Similarly, NRC, (2001) noted that high-fiber diets promote microbes that generate methane, while high-starch diets tend to favor microbes producing propionate, thereby reducing H₂ availability for methanogenesis.

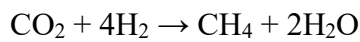
RUMEN MICROBIAL FERMENTATION AND BIOLOGICAL BASIS OF METHANE PRODUCTION

The rumen and its role on biochemical mechanisms of fermentation and methanogenesis

The rumen is the largest compartment of the ruminant stomach, accounting for approximately 80% of the total digestive volume in adult cattle. The environment was kept under anaerobic condition indispensable for diverse and microbial community composed of bacteria, protozoa, fungi, archaea, and viruses (Puniya et al., 2015). These microorganisms play a central role in the degradation of fibrous plant materials converting indigestible carbohydrates into energy-rich.

The digestion of feed in the rumen begins with microbial hydrolysis, during which complex carbohydrates are broken down into simple sugars. These monomers are subsequently fermented to VFAs, CO₂, H₂, ammonia (NH₃), and microbial biomass (Figure 2). The VFAs, in large percentage as acetate, propionate, and butyrate, are absorbed through the rumen wall and serve as the major energy source for the host animal, covering 60-80% of maintenance energy requirements (Puniya et al., 2015).

The CH₄ is produced by methanogenic archaea as a byproduct of anaerobic fermentation. These microorganisms utilize H₂ and CO₂ via the hydrogenotrophic pathway, following the reaction:



Methanogenesis acts as a hydrogen sink, maintaining low partial pressure of H₂ in the rumen to sustain microbial activity. However, this pathway represents an energetic loss for the host, as CH₄ carries away 2–12% of gross energy intake (Johnson and Johnson, 1995). Efforts to reduce enteric methane thus aim to redirect H₂ towards more energetically favorable pathways.

Rumen alternative hydrogen sinks

In the rumen environment, H₂ produced during fermentation can follow multiple metabolic routes, and its concentration could affect the production of VFA and the microbiota. The majority of H₂ within the rumen is metabolized into CH₄; a smaller portion (20–30%) contributes to propionate production, along with minor VFA like valerate. These two VFAs uniquely necessitate the net incorporation of metabolic H₂ during their respective production pathways, while the remainder is incorporated into microbial biomass (Morgavi et al., 2023).

While methanogenesis is the dominant H₂ sink, alternative pathways compete for H₂, potentially reducing CH₄ formation. These include propionate formation, nitrate and sulfate reduction, acetogenesis, and microbial biomass synthesis. Among them, the formation of propionate is the most energetically beneficial for the host and does not result in GHG emissions. Diets that favor propionogenic bacteria may enhance this pathway, diverting H₂ from methanogens (Ungerfeld, 2020).

The composition and nutrient concentration of the diet influence the rumen microbial ecology and fermentation outcomes. A more comprehensive impact of inhibition of rumen methanogenesis

on rumen and post-absorptive metabolism could facilitate the creation of dietary and management strategies. These strategies could leverage alterations in metabolite flows to enhance production or decrease the expenses associated with basic diets. Based on bibliography, high-fiber diets promote the growth of cellulolytic bacteria and methanogens, resulting in higher methane output. In contrast, high-starch diets stimulate amylolytic and propionogenic bacteria, increasing propionate production and reducing CH₄ emissions (NRC, 2001). Moreover, the physical form of the feed, feeding frequency, and rate of passage also influence microbial activity and H₂ balance in the rumen.

Fermentation and microbial adaptation

Rumen fermentation is a dynamic and adaptive process. Microbial communities respond to environmental pressures, including dietary changes and exposure to feed additives by reorganizing their metabolic pathways. In particular, inhibitors of methanogenesis, such as 3-nitrooxypropanol (3-NOP), can induce both immediate and delayed shifts in microbial composition and H₂ utilization routes.

Several in vitro and in vivo studies have reported that while 3-NOP effectively suppresses CH₄ production in the short term, microbial adaptation may emerge over time. Pitta et al. (2022b) documented that certain methanogen taxa, such as *Methanosphaera spp.*, exhibit delayed sensitivity to 3-NOP compared to *Methanobrevibacter spp.*, suggesting differential resilience among archaeal groups. Concurrently, shifts in fermentation end-products, including increased butyrate and isoacid formation, have been associated with alternative H₂ sinks becoming more active under methanogenesis inhibition. Despite short-term success in suppressing CH₄ production, longer studies of methanogenesis inhibition have shown persistent H₂ emissions due to microbial adaptation and the development of alternative hydrogenotrophic populations (Ungerfeld, 2020). Similar to CH₄, H₂ is primarily eliminated from the rumen headspace through eructation; however, the rate and potential

quantity of H₂ absorbed from the rumen and other parts of the ruminant gastrointestinal tract remain unclear (Morgavi et al., 2023).

Additionally, recent work by Hristov and Solomon (2025) estimated that global H₂ emissions from ruminants could increase by up to 6% under scenarios of high (75%) CH₄ reduction. Although this represents a minor offset (<1%) of the CO₂-equivalent savings, it introduces a new layer of complexity, since H₂ is now recognized as an indirect GHG due to its influence on atmospheric CH₄ lifespan. These findings underscore the importance of monitoring H₂ as a gas eructation alongside CH₄ in mitigation studies. The integration of dynamic fermentation models with microbial and chemical profiling can help anticipate long-term shifts and guide sustainable CH₄ reduction strategies.

Experimental approaches for methane mitigation research

Overview of experimental approaches

A comprehensive understanding of CH₄ methane mitigation requires the integration of complementary experimental approaches. These include in vitro batch fermentations that simulate rumen conditions under controlled environments, in vivo studies that evaluate the physiological and productive response of animals, and in silico modeling that formalizes biological mechanisms into mathematical frameworks. Each of these methodologies, largely reviewed by Storm et al. (2012); Zhao et al. (2020); Tedeschi et al. (2022a) has unique strengths and limitations, and their combined application enhances our capacity to evaluate mitigation strategies across multiple scales.

In vitro models for rumen fermentation

In vitro systems, particularly static batch cultures, are widely used to assess the fermentation kinetics and gas production from feed substrates or additives. These methodologies provide a less costly, reproducible, and ethically favorable program for high-through screening of CH₄ mitigation strategies. However, they are limited by the absence of host physiological factors such as absorption,

motility, and endocrine feedback (Tedeschi et al., 2022a). Despite this, dynamic sampling in in vitro setups allows detailed kinetic assessments of gas production, pH changes, VFA profiles, and H₂ dynamics, which are essential for characterizing the mechanistic impact of dietary interventions.

In vivo validation and limitations

In vivo experiments provide a holistic view of the animal's response to dietary interventions, encompassing not only CH₄ emissions but also feed intake, digestibility, milk yield, and overall animal health. These trials are essential for confirming the efficacy of additives under real-world conditions. Nevertheless, in vivo studies are time-consuming, expensive, and ethically constrained. Variability in animal responses, feed intake behavior, and environmental conditions can also complicate data interpretation, highlighting the need for robust experimental design and replication.

In silico dynamic modeling of rumen fermentation

Mechanistic models offer a powerful tool to simulate the rumen ecosystem under various dietary or microbial scenarios. Dynamic in silico models, often based on ordinary differential equations (ODEs), allow the prediction of fermentation end-products, H₂ dynamics, and the effect of additives (3-NOP, *Asparagopsis taxiformis* etc.) on methanogenesis (Muñoz-Tamayo et al., 2021; van Lingen et al., 2021). These models integrate mass balance, acid-base reactions, and microbial competition for substrates. Their strength lies in hypothesis testing, sensitivity analysis, and their use for extrapolating experimental results to larger temporal or population scales.

Contribution of this thesis and research questions

This thesis applies an integrative approach across in vitro, in vivo, and in silico methodologies to explore the biological basis and mitigation potential of enteric methane in dairy systems. It addresses critical knowledge gaps by (i) characterizing fermentation dynamics using individual

rumen inocula from 3-NOP treated animals, (ii) modeling Italian total mixed ration (TMRs) clustered into nutritional strategies using a dynamic mechanistic model of in vitro rumen fermentation, and (iii) screening the anti-methanogenic effects of Italian Mediterranean seaweeds in batch cultures.

These approaches are designed to identify sustainable feeding strategies, quantify microbial responses, and enhance our predictive understanding of methane mitigation under diverse dietary conditions.

REFERENCE

- FAO. 2023a. Methane emissions in livestock and rice systems – Sources, quantification, mitigation and metrics. Rome.
- FAO. 2023b. Pathways towards Lower Emissions. FAO ;
- Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., and Tempio, G. 2013. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO), Rome.
- Hristov, A.N., J. Oh, J.L. Firkins, J. Dijkstra, E. Kebreab, G. Waghorn, H.P.S. Makkar, A.T. Adesogan, W. Yang, C. Lee, P.J. Gerber, B. Henderson, and J.M. Tricarico. 2013. SPECIAL TOPICS — Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options¹. J. Anim. Sci. 91:5045–5069. doi:10.2527/jas.2013-6583.
- IPCC. 2018. Global Warming of 1.5°C: IPCC Special Report on Impacts of Global Warming of 1.5°C above Pre-industrial Levels in Context of Strengthening Response to Climate Change, Sustainable Development, and Efforts to Eradicate Poverty. Cambridge University Press.
- IPCC. 2023. Climate Change 2023 Synthesis Report. Intergovernmental Panel on Climate Change, Geneva, Switzerland.

- ISPRA (Italian Institute for Environmental and Protection and Research). 2024. Italian Greenhouse Gas Inventory 1990-2022. National Inventory Report 2024. ISPRA Reports. No. 398/24.
- Johnson, K.A., and D.E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73:2483–2492. doi:10.2527/1995.7382483x.
- van Lingen, H.J., J.G. Fadel, D.R. Yáñez-Ruiz, M. Kindermann, and E. Kebreab. 2021. Inhibited Methanogenesis in the Rumen of Cattle: Microbial Metabolism in Response to Supplemental 3-Nitrooxypropanol and Nitrate. *Front. Microbiol.* 12. doi:10.3389/fmicb.2021.705613.
- Moe, P.W., and H.F. Tyrrell. 1979. Methane Production in Dairy Cows. *J. Dairy Sci.* 62:1583–1586. doi:10.3168/jds.S0022-0302(79)83465-7.
- Morgavi, D.P., G. Cantalapiedra-Hijar, M. Eugène, C. Martin, P. Noziere, M. Popova, I. Ortigues-Marty, R. Muñoz-Tamayo, and E.M. Ungerfeld. 2023. Review: Reducing enteric methane emissions improves energy metabolism in livestock: is the tenet right?. *animal* 17:100830. doi:10.1016/j.animal.2023.100830.
- Muñoz-Tamayo, R., J.C. Chagas, M. Ramin, and S.J. Krizsan. 2021. Modelling the impact of the macroalgae *Asparagopsis taxiformis* on rumen microbial fermentation and methane production. *Peer Community J.* 1:e7. doi:10.24072/pcjournal.11.
- Myhre, G., Drew Shindell, François-Marie Bréon, William Collins, Jan Fuglestad, Jianping Huang, Dorothy Koch, Jean-François Lamarque, David Lee, Blanca Mendoza, Teruyuki Nakajima, Alan Robock, Graeme Stephens, Toshihiko Takemura, and Hua Zhang. 2013a. Anthropogenic and Natural Radiative Forcing. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.*
- NRC. 2001. *Nutrient Requirements of Dairy Cattle. Seventh Revised Edition.* National Academies Press, Washington, D.C.

- Pitta, D.W., N. Indugu, A. Melgar, A. Hristov, K. Challa, B. Vecchiarelli, M. Hennessy, K. Narayan, S. Duval, M. Kindermann, and N. Walker. 2022b. The effect of 3-nitrooxypropanol, a potent methane inhibitor, on ruminal microbial gene expression profiles in dairy cows. *Microbiome* 10:146. doi:10.1186/s40168-022-01341-9.
- Puniya, A.K., R. Singh, and D.N. Kamra. 2015. *Rumen Microbiology: From Evolution to Revolution*. 1st ed. Springer New Delhi.
- Tedeschi, L.O., A.L. Abdalla, C. Álvarez, S.W. Anuga, J. Arango, K.A. Beauchemin, P. Becquet, A. Berndt, R. Burns, C. De Camillis, J. Chará, J.M. Echazarreta, M. Hassouna, D. Kenny, M. Mathot, R.M. Mauricio, S.C. McClelland, M. Niu, A.A. Onyango, R. Parajuli, L.G.R. Pereira, A. Del Prado, M. Paz Tieri, A. Uwizeye, and E. Kebreab. 2022a. Quantification of methane emitted by ruminants: a review of methods. *J. Anim. Sci.* 100:skac197. doi:10.1093/jas/skac197.
- UNEP. 2021. *Annual Report*. United Nations Environment Programme.
- UNEP, and CCAC. 2021. *Global Methane Assessment: Benefits and Costs of Mitigating Methane Emissions*. United Nations Environment Programme, Nairobi.
- Ungerfeld, E.M. 2020. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Front. Microbiol.* 11:589. doi:10.3389/fmicb.2020.00589.
- Zhao, Y., X. Nan, L. Yang, S. Zheng, L. Jiang, and B. Xiong. 2020. A Review of Enteric Methane Emission Measurement Techniques in Ruminants. *Animals* 10:1004. doi:10.3390/ani10061004.

2 Paper/Manuscript I

Effects of 3-nitrooxypropanol on enteric methane emissions and milk production characteristics in dairy cows fed a high corn silage diet in different environmental conditions

M. Moschini¹, F. Frolidi¹, F. Piccioli-Cappelli¹, P. Bani¹, E. Fiorbelli¹, A. Gallo^{1,2}, I. Mueller³, L. F. M Tamassia⁴, R. Zihlmann⁴, P. Bulgarelli⁵, S. Agazzi⁶, E. Trevisi^{1,2*}, and L. Cattaneo¹

¹Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

²Romeo and Enrica Invernizzi Research Center for Sustainable Dairy Production of the Università Cattolica del Sacro Cuore (CREI), 29122 Piacenza, Italy

³dsm-firmenich, 3131 Getzersdorf, Austria

⁴dsm-firmenich Animal Nutrition & Health, 4303 Kaiseraugst, Switzerland

⁵Lactalis Italia, Italy Milk Collection, 43044 Collecchio (PR), Italy

⁶Lactalis Italia, Laboratorio Centrale Parmalat, 43038 Sala Baganza (PR), Italy

*Corresponding author: Erminio Trevisi, erminio.trevisi@unicatt.it

Article published : Journal Dairy Science

<https://doi.org/10.3168/jds.2025-26640>

ABSTRACT

This study examined the impact of 3-nitrooxypropanol (3-NOP) on enteric greenhouse gas (GHG) emissions, milk production, and the blood metabolic profile of lactating Holstein dairy cows across two experiments, one conducted during the summer and the other in fall. Thirty mid-lactation multiparous cows, with an average (mean $\pm$ SD) of 190 ± 72 days in milk and a milk yield of 36.8 ± 7.5 kg/d, were included in the summer experiment. In the fall experiment, cows averaged 158 ± 74 days in milk and a milk yield of 36.8 ± 4.6 kg/d. Experiments were structured as longitudinal studies with repeated measurements in a completely randomized design, with an initial 14-d covariate period, a 63-d treatment period, and a 14-d washout period. Cows were randomly assigned to 1 of 2 treatment groups: (i) the control group (CTR), receiving a corn silage-based diet supplemented with a placebo, (ii) the 3-NOP group, receiving the same diet supplemented with 60 mg/kg DM of 3-NOP. Cows were housed in a free-stall barn, with individual monitoring of feed intake and fed a TMR ad libitum delivered once a day. The enteric CH₄, CO₂, and H₂ emissions were measured using a GreenFeed unit. 3-NOP reduced enteric CH₄ production (46%), yield (46%), and intensity (44%), regardless of the season, but increased H₂ and CO₂ emissions. In the summer, 3-NOP cows reduced DMI (5.7%) and meal size and increased the feed (4.8%) and dairy (4%) efficiencies, but not in the fall. Adding 3-NOP increased the apparent OM, CP, and NDF digestibility, independently of the season. There was no effect of 3-NOP on milk yield, although supplementing 3-NOP increased the milk protein and casein and decreased milk urea contents. Saturated FA increased with 3-NOP, regardless of the phase of the study. In the summer season, 3-NOP supplementation increased de novo FA (7.3%) and decreased mixed FA (6.7%), whereas in the fall, SFA decreased (2.3%) and MUFA increased (6.8%) compared with CTR. Supplementing 3-NOP affected the molar proportions of rumen VFA. Acetate was

reduced, whereas butyrate, valerate, isovalerate, and caproate were increased by 3-NOP. Plasma concentrations of urea, NEFA, Zn, and AOOP decreased with 3-NOP, but blood cholesterol and myeloperoxidase increased. In conclusion, including 3-NOP in a high corn silage and high starch diet almost halved enteric CH₄ emissions without adversely affecting milk yield and composition.

Keywords: dairy cows, anti-methanogenic compound, GHG emission, milk composition, climate change

List of nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; AOPP = advanced oxidation protein products; CTR = control group; FA = fatty acid; FPCM = fat and protein corrected milk; FRAP = ferric reducing antioxidant power; GGT = γ -glutamyl transferase; GOT = glutamate oxaloacetate transferase; NEFA = nonesterified fatty acids; PCV= packed cell volume; P1 = phase 1; P2 = phase 2; ROM = reactive oxygen metabolites; uNDF = undigested NDF.

INTRODUCTION

The contribution of livestock to the total anthropogenic GHG emissions has been estimated to be 14.5% (Gerber et al., 2013), with a remarkable worldwide variability, and CH₄ is the second most important GHG contributor to climate change after CO₂. Recent data indicate that, in Italy, livestock accounts for 5.8% of the total GHG emissions (ISPRA, 2024). Agriculture is the major source of CH₄ emissions (45.6%), and livestock, with CH₄ derived from enteric fermentation and manure storage accounting for over 90% of the agriculture-derived CH₄ emissions (ISPRA, 2024). Therefore, reducing enteric CH₄ production represents a valuable opportunity to improve the environmental sustainability of the dairy sector and contribute to climate change mitigation. Indeed, as CH₄ has a short lifetime in the atmosphere but a short-term high global warming potential (about 80 on a 20-year time horizon) (Forster et al., 2021), mitigation of its emissions represents a strategic option in the short term (Myhre et al., 2013b). Among the possible strategies that can be adopted to reduce enteric CH₄ production in ruminants, the use of feed additives that inhibit rumen methanogenesis showed positive results (Arndt et al., 2022). Among those compounds, 3-nitrooxypropanol (**3-NOP**) can reduce CH₄ production by, on average, 32.7% in dairy cattle (Kebreab et al., 2023) by targeting methyl-coenzyme M reductase in the last step of CH₄ formation by the rumen *Archaea* (Duin et al., 2016). Research on dairy cows underlined reductions in CH₄ yield (g/kg DMI) between 15% to 60%, depending on the diet composition, method of supplementation, and dose of 3-NOP (Kebreab et al., 2023). Though no adverse effects on milk production and DMI are reported (Haisan et al., 2017; Melgar et al., 2020b), according to a recent meta-regression based on published and unpublished studies (Martins et al., 2025) 3-NOP would slightly decrease DMI and milk yield but improve milk fat and protein contents, so that yield of FPCM remains unchanged and feed efficiency improves.

The impact of 3-NOP on CH₄ emissions appears to be more pronounced when the diet is rich in starch and low in fiber (Dijkstra et al., 2018; van Gastelen et al., 2022) and fat (Kebreab et al., 2023). Increasing dietary starch tends to lower the rumen pH (Humer et al., 2018) and redirects the ruminal fermentations, enhancing the production of propionate at the expense of acetate, reducing the availability of hydrogen for methanogenesis and lowering the methyl-coenzyme M concentration, so disadvantaging the methanogenic population (Grainger and Beauchemin, 2011; van Gastelen et al., 2022). Furthermore, the roughage composition of the diet could affect the anti-methanogenic action of 3-NOP, with more favorable results obtained when diets, though similar for fiber (NDF) and NFC, are based on corn silage, instead of grass silage, as the main forage (van Gastelen et al., 2022). In Italy, Grana Padano PDO cheese production utilizes about 22% of the national milk production (CLAL, 2023), mostly coming from the Po Valley, where the diets for lactating cows are usually high in starch (usually ranging from 25 to 35% DM) and based mainly on corn silage (ranging from 28 to 38% of dietary DM) and where lactating cows are exposed to high temperatures and humidity during the summer season (Abeni et al., 2007; Bonaldo et al., 2023). Although the effects of 3-NOP appear to be highly specific, targeting the ruminal methanogenic microbial population without major effects on protozoa and bacteria (Gruninger et al., 2022), modifications of the ruminal microbiome are also reported (Lopes et al., 2016; Pitta et al., 2022), that might be influenced by dietary features, such as forage-to-concentrate ratio (Haisan et al., 2017; Choi et al., 2025). Despite these low impacts on rumen bacteria and protozoa, 3-NOP administration impacts ruminal fermentations patterns, frequently lowering total VFA and acetate molar proportion in favor of propionate, butyrate and other minor VFA (Melgar et al., 2020a; Kebreab et al., 2023; Orzuna-Orzuna et al., 2024; Martins et al., 2025) suggesting that the bacterial and protozoal populations react to the increased availability of H₂ favoring more reduced end products.

Environmental temperature and humidity, in particular when heat stress conditions are reached, affect dairy cows performances and health and alter rumen physiology, with a decrease in feed intake (Baumgard and Rhoads, 2013) and a frequently reported reduction of pH and increase of lactic acid concentrations (Zhao et al., 2019). Additionally, heat stress leads to changes in the rumen microbial population, largely driven by the reduced DMI, and a decrease in rumen total VFA concentrations coupled with a decrease of acetate and an increase of propionate and butyrate molar proportions is often reported (Nonaka et al., 2008; Yadav et al., 2013). Therefore, the availability of dissolved H₂ for methanogenic *Archaea* is altered. Furthermore, CH₄ emissions vary during prolonged heat stress as they would be temporarily reduced after heat stress onset to recover later (Souza et al., 2023).

Moreover, the metabolic, inflammatory, and immune responses to 3-NOP supplementation have been investigated. The studies available in the literature limited their analysis to some blood biomarkers of energy metabolism and indicated no or limited effects of 3-NOP on blood concentrations of glucose, non-esterified fatty acids (**NEFA**), BHB, urea, and insulin (Jayanegara et al., 2018; Melgar et al., 2020a; Kjeldsen et al., 2024). Therefore, in-depth investigations on the systemic effects of 3-NOP are points of interest for further investigations.

We hypothesized that 3-NOP would effectively reduce enteric CH₄ emissions in lactating cows fed a corn silage-based and high starch diet without impairing the cow's performance, milk quality, and the health. The objectives of this study were to comprehensively investigate the effects of 3-NOP supplemented in the summer and the fall seasons to a typical diet for Grana Padano PDO cheese on enteric GHG emissions, feed intake and digestibility, milk production and composition, rumen fermentations, and metabolic and inflammatory conditions.

MATERIALS AND METHODS

The cows involved were cared for according to the guidelines of the Committee for the Protection and Welfare of Experimental Animals of the Catholic University of the Sacred Heart. The study was authorized by the Italian Ministry of Health (number 355/2022-PR).

Animal and experimental design

Two experiments were conducted from May 23rd to August 21st 2022 (Phase 1: **P1**; average temperature, relative humidity, and THI of 25.7°C, 58.9%, and 72.8, respectively) and from September 20th to December 19th 2022 (Phase 2: **P2**; average temperature, relative humidity, and THI of 11.7°C, 86.2%, and 52.8, respectively).

Both phases were performed at CERZOO (Research Center for Livestock and Environment, s.r.l., Piacenza, Italy). Animals were housed in a freestall barn equipped with a Roughage Intake Control system – RIC (Hokofarm Group, The Netherlands) for individual monitoring of feed intake and eating behavior and with one GreenFeed unit (C-Lock Inc., Rapid City, SD) for continuous measurements of enteric CH₄, CO₂, and H₂ emissions.

In each phase, 30 multiparous Holstein lactating dairy cows in mid and late lactation with no clinically diagnosed diseases for at least 3 wk before enrollment were used. The DIM, milk yield, milk fat and protein contents of cows entering the experiments were (mean ± SD) 190 ± 72, 36.8 ± 7.5 kg/d, 3.73 ± 0.33%, and 3.47 ± 0.19%, respectively, for P1, and 158 ± 74, 36.8 ± 4.6 kg/d, 3.78 ± 0.65 %, and 3.28 ± 0.28 %, respectively, for P2. All cows were housed in the same pen, with free access to drinking water and were fed *ad libitum* (aiming at 5-10% refusals) a corn silage-based diet (Table 1), formulated to meet or exceed a mid-lactation cow's NE_L and MP requirements according to NRC (2001) and suitable for producing milk for Grana Padano cheese-making (G.U., 2022). The chemical composition of the forages used is reported

in Supplemental Table S1. Both phases were structured as longitudinal studies with repeated measurements in a completely randomized design: a 10-d adaptation period (no data were collected) followed by a 14-d covariate period with all animals fed the same diet, then a 63-d treatment period, and a final 14-d washout period in which all cows continued to receive the same diet. In both phases, at the end of the initial covariate period, cows were randomly assigned to one out of two groups: (i) the treated group (**3-NOP**; n = 15/phase), that received the basal diet supplemented with 600 mg/kg DM of Bovaer®10 (dsm-firmenich Animal Nutrition & Health, Kaiseraugst, Switzerland; containing 100 g/kg of 3-NOP on silicon dioxide + 1,2-propanediol) included in a premix, and (ii) the control group (**CTR**; n = 15/phase), that received the same basal diet supplemented with a placebo (i.e., silicon dioxide + 1,2-propanediol) premix. The 3-NOP and placebo premixes were prepared weekly by mixing ground corn grain (65%), soybean oil (5%), and either Bovaer®10 or a placebo, both provided by dsm-firmenich. Then, premixes were kept in sealed containers at 4°C before being incorporated into the diets. The delivered diets were formulated by mixing the basal diets with the premixes and were distributed using a self-moving mixer (Rotomix 5000, Bravo s.r.l., Savigliano, Italy). To avoid cross-contamination, the TMR for CTR was mixed and distributed before the 3-NOP TMR, and 3 other unsupplemented TMR for other animals (i.e. dry cows and heifers) were prepared before the next day's TMR for the CTR group.

Diets and feed ingredients

Samples of the TMR were collected daily during the covariate, treatment, and washout periods and stored frozen at -20 °C until composited. Additional samples of the concentrate offered in the GreenFeed were also sampled weekly. Samples collected during the treatment periods were composited on a 3-wk basis, whereas samples collected during the covariate and washout periods were composited over 2 wk. Then, collected samples were dried at 65°C until

constant weight in a forced-air oven for dry matter determination, then ground in a Wiley Mill (Thomas Scientific; Swedesboro, NJ) through a 1-mm sieve for wet chemistry analyses. Samples were analyzed for dry matter content (934.01), crude protein (990.03), crude fat (920.39), ash (942.05), starch (2014.10), ADF, and ADL (973.18) (AOAC International, 2016), NDF (Mertens, 2002), and water-soluble carbohydrates (Hall, 2014).

DMI and feeding behavior

Cows were fed once daily at 0900 h. The daily feed intake was determined as the amount recorded by the RIC system between 2 consecutive TMR deliveries. The DMI was calculated according to the DM contents of collected diet samples, then pooled by treatment and timepoint. Then, the amount of DMI from concentrate delivered at the GreenFeed was added to the DMI calculated from the RIC recording.

To investigate feeding behavior, the meal criterion (i.e., the minimum time interval between gate visits to be considered a new meal) was calculated by fitting a mixture of 3 normal distributions to the distributions of log₁₀-transformed intervals using the method of exact maximum likelihood (Tolkamp and Kyriazakis, 1999; Horvath and Miller-Cushon, 2019), using the *mixdist* package in R (Macdonald and Du, 2018). The 3 distributions were interpreted as those within meals, within meals including drinking, and between meals, respectively. The meal criterion was determined as the point at which the conditional probability corresponding to the second distribution intersected the third distribution curve. In this study, the meal criterion was set at 44.48 min. A visit to the bin consisted of a cow entering a gate feeder, followed by a negative weight change from eating. Eating time (min/d) was defined as the time that a cow had her head in the gate feeder. Meal frequency (number of meals/d) was computed using the previously defined meal criterion. Meal length (min/meal) and size (kg of DM/meal) were determined by dividing eating time and DMI, respectively, by meal frequency.

Enteric gas emissions

The GreenFeed unit was calibrated weekly and the CO₂ recovery was performed monthly, following the manufacturer's recommendations (<http://greenfeed.c-lockinc.com>). Cows were identified with a unique radio-frequency identification ear tag and were adapted (10-d) to using the GreenFeed before the beginning of the covariate period. Cows were provided with pelletized bait feed (ME = 2.78 Mcal/kg; CP = 184 g/kg DM; starch = 316 g/kg DM; NDF = 259 g/kg DM) as attractive when visiting GreenFeed. Each cow was allowed a maximum of 6 visits/d, with a 3-hour interval between visits and no more than eight feed drops (35.94 g $\pm$ 1.96 per drop) per visit. The daily drops of pellets were recorded individually, and the weight was added to the daily TMR intake upon calculating the daily individual feed intake. Overall, average intake was 0.75 $\pm$ 0.25 kg/d for CTR and 0.76 $\pm$ 0.25 kg/d for 3-NOP.

Body weight, body condition, and behavior

Cow's BW was recorded twice daily upon exiting the milking parlor using an AfiFarm 3.04E scale system (Afimilk Ltd., Kibbutz Afikim, Israel) and averaged by day. In each phase, the BCS was evaluated using a 1 to 4 scale (ADAS, 1986) by the same trained researcher at the beginning and the end of the covariate and treatment periods. The daily rumination time was measured using collar tags (SenseHub Dairy, Merck & Co., Inc., Rahway, NJ, USA) and daily lying time with pedometers (AfiAct II, Afimilk Ltd., Kibbutz Afikim, Israel).

Milk yield and sampling

Cows were milked twice daily in a side-by-side parlor at 0400 and 1600 h. Milk production and composition (fat, protein, lactose) were recorded at each milking by in-line measurement (Afilab, Afimilk, Israel). The daily milk yield was reported as the sum of milk

produced in two consecutive milkings (p.m., a.m.), and the milk composition from continuous reading during milking was calculated as the weighted average of the milk composition.

Milk samples were collected from continuous flow samples of the morning milking at the end of the covariate period and every 21 d of the treatment period. Milk was analyzed for milk fat, protein, lactose and urea contents (MilkoScan™ FT3, Foss Italia s.r.l., Padua, IT), SCC (Fossomatic, Foss Italia s.r.l., Padova, IT), and fatty acid (FA) composition (Parmalat Central Laboratory) (C4-C24; ISO 14156:2001/Amd 1:2007; ISO 15884:2002; ISO 15885:2002).

Fat (4%) and protein (3.3%) corrected milk (FPCM) and energy corrected milk (ECM) were calculated according to IDF (2015) and Sjaunja et al. (1990), respectively, as:

$$\text{FPCM} = \text{milk yield(kg)} \cdot [0.1226 \times \text{fat(\%)} + 0.0776 \cdot \text{protein(\%)} + 0.2534]$$

$$\text{ECM} = \frac{\text{milk yield(kg)} \cdot [0.383 \cdot \text{fat(\%)} + 0.242 \cdot \text{protein(\%)} + 0.7832]}{3.14}$$

Moreover, feed efficiency (FE) was calculated as:

$$\text{feed efficiency} = \frac{\text{daily milk yield (kg/cow/day)}}{\text{Total DMI (kg/cow/day)}}$$

Blood sampling and analysis

Blood samples were collected at the end of the covariate period and every 21-d of the treatment period. The samples were drawn in the morning before the TMR distribution from the jugular vein in Lithium-heparinized vacuum tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ). After collection, the tubes were immediately put in iced water until centrifugation, performed within 1 h. Plasma aliquots were obtained after centrifugation (3500 × g for 10 min at 4°C) and stored at –20°C before the analysis. The analyses were performed with a clinical

auto-analyzer (ILab Taurus, Werfen, Barcelona, Spain) for glucose, NEFA, BHB, triglycerides, urea, cholesterol, creatinine, Ca, P, Mg, Na, K, Cl, Zn, total protein, albumin, myeloperoxidase, glutamate oxaloacetate transferase (**GOT**), γ -glutamyl transferase (**GGT**), bilirubin, alkaline phosphatase, paraoxonase, reactive oxygen metabolites (**ROM**), and thiol groups using the commercial kits reported in Supplemental Table S2. Moreover, ceruloplasmin (Sunderman and Nomoto, 1970), haptoglobin (Skinner et al., 1991), myeloperoxidase (Bradley et al., 1982), paraoxonase (Ferré et al., 2002), advanced oxidation protein products (**AOPP**; Hanasand et al., 2012), and ferric reducing antioxidant power (**FRAP**; Benzie and Strain, 1999) were determined following previously proposed methods, adapted to the ILab Taurus. Globulin was calculated as the difference between total protein and albumin.

Rumen fluid sampling and analysis

Rumen fluid was collected at 0830 h, before the morning feeding, immediately following the blood sampling at the conclusion of the experimental period, utilizing oral stomach probes specially designed for cattle (Ruminator; profs-products.com) (Muizelaar et al., 2020). The first 0.5 L of fluid was discarded to reduce saliva contamination, and the probes were thoroughly rinsed with tap water between cows. After immediate pH measurement (pH meter model GLP 21, Crison Strumenti SpA, Modena, IT), an aliquot of the fluid was immediately cooled in iced water. Aliquots of 2.5 ml for VFA, ammonia, and lactate analyses were transferred into tubes with 0.5 ml of 25% metaphosphoric acid, centrifuged at 1000g for 3 min and the supernatant transferred to 1.5 ml Eppendorf Safe-Lock tubes and frozen at -20°C until laboratory analysis. Analysis of VFA was carried out by GC (7820A; Agilent Technologies, Santa Clara, CA) according to methods described in Minuti et al. (2014). Data about individual VFA were reported as molar proportions of the total VFA concentration. Ammonia and D- and L-lactate concentrations were determined with commercial kits

(EnzytecTM Liquid Ammonia, R-Biopharm AG, Darmstadt, Germany; D-/L-Lactate assay kit, Megazyme International Ltd., Wicklow, Ireland) using a clinical auto-analyzer (ILab Taurus, Werfen, Barcelona, Spain).

Feed digestibility

During each phase of the study, fecal samples were collected from each cow twice daily over 2 consecutive days at 2 different timepoints. Specifically, collections were conducted at 0900 and 1400 h on the first day and at 1100 and 1600 h on the second day. Samples were collected at 41-d and 42-d (first timepoint) and 62-d and 63-d (second timepoint) on treatment. The pH was measured, after which samples were pooled by cow and time point, and subsequently frozen at -20 °C. Upon analysis, samples were thawed and dried in a forced-air oven (60°C, 72 h) for DM determination, then ground to pass at a 1 mm screen sieve. The samples were subsequently analyzed with a spectrophotometer system (NIR Foss 5000, Hilleroed, Denmark), as previously reported by Gallo et al. (2022). The calibrations for chemical characterizations were produced by a commercial forage testing laboratory (CRPA Lab, Reggio Emilia, Italy) for CP, NDF, ADF, ADL, starch, and ash. About one-third of the samples were further analyzed using wet chemical analysis, as reported above for the feeds, to verify the precision and accuracy of the calibration.

The undigested NDF (**uNDF**) was used as an internal marker (Huhtanen et al. 1994; Gallo et al., 2019). Briefly, the in situ uNDF evaluation was performed in the rumen of 2 cannulated dry Holstein dairy cows (625 ± 10 kg BW and 38 ± 0.3 months old) fed at maintenance (NRC, 2001) with a TMR characterized by 120 g/kg CP and 505 g/kg aNDFom on a DM basis. Ground samples (about 300 mg) were inserted into acetone-prewashed polyester/polyethylene filter bags (Ankom F57; ANKOM Technology Corporation; 2 bags per sample/cow and sample size to net surface area ratio of about 10 mg/cm^2 , which were placed

in string net bags (10 by 15 cm and 15-mm pore size; 2 filter bags for each). The *in situ* ruminal incubations lasted for 240 h. At the end of each incubation, bags were rinsed in a household washing machine (Smeg GW 3000; Smeg S.p.A – Instruments Division, Guastalla, Italy), three washings of 2.5 minutes each, two with cold water and one with distilled water. Then, samples were oven-dried at 65°C, and the residual NDF was quantified. The OM, NDF, starch, CP, and uNDF contents of feces and TMR were used to obtain apparent digestibility coefficients. The uNDF was used as an internal marker to calculate digestion coefficients (Kotb and Luckey 1972) according to the following:

$$\text{digestibility X (\%)} = \frac{1 - (\text{uNDF}_{\text{diet}} \cdot [\text{X}]_{\text{feces}})}{\text{uNDF}_{\text{feces}} \cdot [\text{X}]_{\text{diet}}} \cdot 100$$

Where:

digestibility X = digestibility coefficient of nutrient X (i.e., NDF, starch, CP).

Statistical Analysis

The final data set included 57 dairy cows. Three cows were excluded from P2, 2 cows (1 in the CTR and 1 in the 3-NOP group) due to the lack of persistence in the lactation curve, which was attributed to erratic recording of the pregnancy date, and 1 cow (CTR) due to foot problems, unrelated to the experimental treatment. In DMI data, two days of data collection in P1 were excluded ahead of analysis because of erratic feed intake behaviors of cows due to extreme weather conditions in one case and detected RIC malfunction in the other. Two nonconsecutive days of milk data measurements were excluded in P2 because of erratic measurements during milking. Milk quality data from samples collected at the end of the covariate period and every 21-d of the treatment period were screened for the SCC content, and samples with SCC greater than 300/mL were not considered for statistical analysis.

Data from GreenFeed were validated by C-Lock Inc. (<http://greenfeed.c-lockinc.com>) before being subjected to statistical analysis: 6147 visits in P1 and 5874 visits in P2 were validated. Then, the number of visits to GreenFeed was calculated for each animal over each considered timepoint. To ensure data accuracy, we applied a minimum threshold of 20 visits per timepoint, as recommended by Manafiazar et al. (2016), to consider gas emission estimates valid. The observation windows were 14 days for the covariate period and 21 days for the treatment period. Across both phases, approximately 15% of the animal-period combinations were excluded due to an insufficient number of visits to the GreenFeed unit. These data points were therefore not used for estimating gas emissions or related calculated variables. Gas production (g/d), yield (g/kg DMI), and intensity (g/kg ECM) were calculated.

Data about feed intake, daily milk yield and quality, and gas emissions were averaged per cow over a 14-d for the covariate period and over 21-d for the treatment period. Then, all data were screened for possible outliers according to quantile distribution. The considered outlier threshold was 1.5 times the interquartile range past the tail quantiles of 0.25 and 0.75. Data were tested for normality with the Shapiro–Wilk test before statistical analysis. Variables not normally distributed were log-transformed. Each trial included a 14-d period of adaptation to the base diet.

A linear mixed model with repeated measures was used for data analysis (JMP®, Version 17.0.0, SAS Institute Inc., Cary, NC, 1989–2021), with treatment (Trt), timepoint (T), phase (P), and the first-order interactions (Trt×T, Trt×P, T×P) as fixed effects and the cow nested within treatment as a random effect. Five covariance structures were investigated to account for the relationship of dependence of repeated measurements over time on the same subject: unstructured, compound symmetry, auto-regressive-of-order-1, toeplitz, and antedependent. The equal variance assumption was verified, and from the resulting fit statistics,

the Bayesian Information Criterion (BIC) was used to select the model that best fits the data. Data were adjusted with the covariate period when the homogeneity of regression slopes was not violated (Hamilton, 1977).

The general statistical model was as follows:

$$y_{ijkh} = \mu + \gamma x_{ij} + a_i + \tau_k + (a\tau)_{ik} + b_h + (ab)_{ih} + (b\tau)_{hk} + \delta_c + e_{ijkh}$$

where:

y_{ijkh} = response at the h phase, at the k timepoint of the j cow in the i treatment group

μ = overall mean

γx_{ij} = fixed effect of the covariate measurement for the j cow in the i treatment group

a_i = fixed effect of the i treatment group

τ_k = fixed effect of the k timepoint

$a\tau_{ik}$ = interaction effect of the k timepoint with the i treatment group

b_h = effect of the h phase

ab_{ih} = interaction effect of the h phase with the i treatment group

$b\tau_{hk}$ = interaction effect of the k timepoint with the h phase

δ_c = random effect of cow

e_{ijkh} = random residual error assumed to be independent and $\sim N(0, \sigma_e^2)$

When the treatment had a significant interaction effect with the phase, the effect of treatment was analyzed within the phase. Data were presented as LSM. For non-normally distributed variables, results are shown as back-transformed LSM with corresponding 95% CI.

Statistical differences were declared at $P < 0.05$. Differences between treatments with $0.05 \leq P < 0.10$ were considered a tendency toward significance.

RESULTS

Diets

The chemical composition of the basal diets used in the two phases is presented in Table 1. The base diet formulation was the same within phases, with only minimal differences between experimental groups within each phase. However, there were some adjustments between the 2 phases of the study to adapt to the different batches of forages used. Overall, for the experimental periods, the diets fed in P2 had a higher CP (158.5 vs. 148.7 g/kg DM) and starch (295.2 vs. 287.7 g/kg DM), but lower crude fat (28.5 vs. 37.5 g/kg DM) contents compared to the diets fed in the P1.

Gas production, yield, and intensity

No differences were observed among groups regarding the daily visits to GreenFeed, which averaged 2.7 visits (Table 2). The enteric CH₄ production, yield, and intensity were reduced ($P < 0.01$) in the 3-NOP group compared with CTR, regardless of the phase, by 46.3%, 46.0%, and 44.2%, respectively. During the washout period, CH₄ production was similar between groups (438.0 vs 426.9 ± 21.7 g/d in CTR and 3-NOP; Supplemental Figure S1). Supplementation with 3-NOP increased H₂ and CO₂ emissions. Significant interactions ($P < 0.01$) were observed between phase and treatment concerning H₂ production and yield and CO₂ yield. In contrast, there was no interaction for CO₂ production, with a tendency for higher emission (2.3%) in the 3-NOP-fed cows. The H₂ production and yield were increased ($P < 0.01$) by 3-NOP supplementation (Table 3), with a greater increment in P1 (7.71 g/d and 0.31 g/kg DMI, respectively) compared to P2 (5.59 g/d and 0.21 g/kg DMI, respectively). Also,

providing 3-NOP into the diet increased (8.7%, $P < 0.01$) the CO₂ yield in P1, whereas no effect was observed in P2.

Dry matter intake and feeding behavior

Overall, DMI tended to be reduced by 3-NOP ($P = 0.10$), with a significant interaction ($P < 0.01$) between treatment and phase regarding DMI (Table 4). The analysis of the DMI within the phase showed a reduction of the DMI (1.5 kg/d, $P < 0.01$) in the 3-NOP group compared with CTR in P1, whereas no difference between experimental groups was observed in P2, with an average DMI of 26.3 kg/head/d (Table 5). In both phases, the DMI was not affected by the timepoint, but there was a tendency for interaction between 3-NOP and the timepoint in P2.

Treatment did not affect the daily eating time, the number of visits to the bins, the number of meals, or the average meal length (Supplemental Table S3). A significant interaction ($P = 0.02$) between treatment and phase was observed for the meal size, with the 3-NOP group having a meal size 7.6% lower than the CTR group in P1 ($P < 0.01$), but no differences were observed in P2 (Table 5). The 3-NOP did not affect the rumination time, with cows ruminating, on average, for 528 min/d (Supplemental Table S3). No effects of 3-NOP were observed on BCS and BW, with average values throughout the study of 2.47 and 651 kg, respectively (Table 4).

Milk production

The analysis of milk production revealed no significant interaction between treatment and phase. The mean values were consistent across the groups, with averages of 33.8, 33.3, and 33.2 kg/d for raw milk, FPCM, and ECM yield, respectively (Table 4). The protein and casein contents were increased (3.5% and 4.2%, respectively; $P < 0.01$) when supplementing 3-NOP,

whereas no differences were observed in milk fat and lactose contents. Milk urea was lower in 3-NOP ($P < 0.01$), regardless of the phase of the study. A significant interaction ($P < 0.01$) between treatment and phase was observed for feed efficiency (Table 4). Feeding 3-NOP increased ($P < 0.01$) the feed (4.8%) efficiency in P1 (Table 5), but not in P2. Milk SCC was not affected by 3-NOP supplementation.

The FA profile of milk fat is reported in Table 6. There was a significant interaction between treatment and phase for *de novo* FA ($P < 0.01$), MUFA, and mixed FA ($P = 0.03$), and a tendency ($P = 0.07$) for saturated FA. The *de novo* FA were greater in 3-NOP cows in P1 (7.3%, $P < 0.01$) but tended to be lower in P2 compared with CTR, whereas mixed FA were reduced (6.7%, $P < 0.01$) in P1 but not in P2 (Table 7). The saturated FA decreased in 3-NOP compared with CTR, whereas no difference was observed in PUFA. The MUFA increased (6.8%, $P = 0.01$), and SFA decreased (2.3%, $P = 0.02$) in 3-NOP compared with CTR in P2 but not in P1. Interestingly, the odd SFA (i.e., C11, C13, C15, and C17) increased in 3-NOP cows compared with CTR ($P < 0.01$; Table 7), as well as the proportions of C17:1 ($P < 0.01$), C18:1n7 ($P = 0.03$), C20:3n3 ($P < 0.01$) and a tendency for higher C18:2 content ($P = 0.06$).

Metabolic profile

The LSM of plasma biomarkers is reported in Table 8. Most of the variables investigated changed with timepoint and phase of the study. However, the interaction Trt×P was never significant, enabling us to focus on the overall response to 3-NOP, regardless of the study phase. Hematocrit did not differ between groups ($P = 0.11$). Among markers of energy metabolism, glucose and BHB were not affected by 3-NOP supplementation, whereas NEFA and urea were overall lower in 3-NOP compared with CTR ($P \leq 0.03$). Similarly, cholesterol was lower in 3-NOP ($P = 0.01$). Creatinine concentration did not differ between groups. Among minerals, Ca tended to be reduced in 3-NOP compared with CTR ($P = 0.06$), Zn was lower (P

< 0.01), whereas Mg tended to be greater in 3-NOP ($P = 0.09$). Treatment did not affect Ca, P, Na, K, and Cl.

No differences were noted in plasma protein fractions (i.e., total protein, albumin, and globulin). Moreover, either positive (i.e., haptoglobin and ceruloplasmin) and negative acute-phase protein concentrations (i.e., bilirubin – as an indirect marker of liver enzymes for its glucuronidation - and paraoxonase) were not different between groups. In contrast, plasma myeloperoxidase was greater in 3-NOP cows compared with CTR ($P < 0.01$). Liver enzyme markers of its functionality (GOT and GGT) did not reveal any change, nor did alkaline phosphatase. The concentration of ROM was not different, and that of AOPP was lower in 3-NOP compared with CTR ($P = 0.04$), but the concentration of FRAP tended to be reduced ($P = 0.08$). Plasma thiol groups were not altered by treatment.

Rumen fermentation profile and apparent nutrient digestibility

Feeding 3-NOP did not affect rumen pH, total VFA, ammonia, D- and L-lactate concentrations (Table 9), but it significantly ($P < 0.01$) affected the VFA molar proportions. Feeding 3-NOP decreased the proportion of acetate ($P < 0.01$), the acetate to propionate ratio ($P < 0.01$), and the acetate plus butyrate to propionate ($P = 0.02$) and tended to increase the molar proportion of propionate ($P = 0.09$). Butyrate and valerate were increased by feeding 3-NOP in P2 more than in P1 (Supplemental Table S4), whereas isobutyrate and isovalerate were increased without any interaction with the phase.

Fecal DM content and pH were not affected by treatment. In 3-NOP compared with CTR higher ($P < 0.01$) apparent digestibilities were recorded for OM (1.0%), CP (2.2%), and NDF (3.7%), but starch digestibility remained unaltered (Table 10).

DISCUSSION

This study evaluated the effects of adding 3-NOP to a diet for dairy cows based on corn silage as the main forage in two phases, the first carried out in summer and the second in the fall of 2022.

Enteric gas emissions

Several works reported the anti-methanogenic effect of supplementing 3-NOP to dairy cows. and models to predict this effect from meta-analysis were first proposed by Dijkstra et al. (2018) and then updated by Kebreab et al. (2023). The anti-methanogenic effect was positively related to the dose of 3-NOP, and according to Kebreab et al. (2023), a dose of 70.5 mg/kg DM resulted in a reduction of 32.7%, 30.9%, and 32.6% for CH₄ production, yield, and intensity, respectively. In our conditions, supplementing 3-NOP at 60 mg/kg DM consistently lowered CH₄ production, yield, and intensity, with average reductions of 46.3%, 46.0%, and 44.2%, respectively, compared to CTR. These values are greater than those that can be calculated from the average characteristics of our diets based on the equations proposed by Kebreab et al. (2023) (i.e., 32.7, 29.7, and 30.8% reduction for CH₄ production, yield, and intensity, respectively). The diets fed in our study differ from those used in the studies evaluated in that meta-analysis, particularly for the level of starch content, limiting their applicability to our conditions. Nevertheless, explaining the more significant reduction observed remains challenging. Feeding 3-NOP increased the H₂ production four times on average in our study, slightly higher in the summer than in the fall, but this increase accounts for only 13.4% of the H₂ not emitted with CH₄. These results agreed with van Gastelen et al. (2022), whereas in other studies where the same dose of 3-NOP was used (60 mg/kg DMI), lower recoveries of hydrogen directly emitted as H₂ are reported (Hristov et al., 2015a; Melgar et al., 2020a; c; Van Gastelen et al., 2020). These differences are not related to the 3-NOP efficacy in reducing CH₄ production and could be partially explained by the different

techniques adopted to measure gas emissions, i.e., GreenFeed or respiratory chambers, as the sharp peaks of H₂ emissions after feeding the diet added with 3-NOP could not be adequately captured by GreenFeed (Van Gastelen et al., 2020). The higher availability of dissolved hydrogen, as methanogenesis is inhibited (Melgar et al., 2020a), tends to increase the involvement of other H₂ sinks like other VFA more reduced compared to acetate, as well as formate, methanol, and microbial biomass (Czerkawski, 1986), that were not monitored in this experiment.

Dry matter intake and feed digestibility

The average DMI was lower in the summer than in the fall due to the elevated temperatures (West et al., 2003). Supplementing 3-NOP tendentially decreases DMI in beef cattle but not dairy cows (Yu et al., 2021). A lower intake was reported by Almeida et al. (2021) in dairy cows supplemented with 3-NOP, but this effect disappeared when expressed on a BW basis. The different impact on beef vs. dairy cattle has been attributed to the higher dose used in beef trials, hypothesizing a reduction in diet palatability as 3-NOP concentration increases (Yu et al., 2021). However, Melgar et al. (2020a) found no effect of 3-NOP in this respect. Kim et al. (2020) attempted to explain the lower DMI induced by 3-NOP with the increase of propionate proportion in total VFA of rumen fluid, as previously suggested by Allen et al. (2000) for high starch diets in dairy cows. This explanation does not appear relevant to our findings, as we only noted a modest increase in the propionate molar proportion, which was consistent across both seasons. The last meta-regression carried out by Martins et al. (2025) predicts a dose-dependent depressing effect of 3-NOP on DMI, with the effects being more pronounced for diets richer in starch and lower in fiber, i.e. where 3-NOP is tendentially less effective in reducing enteric emissions, but no clear conclusions on the explanatory mechanisms were reported. The reduced DMI measured in summer did not affect rumen total

VFA concentration and pH, though these measures carried out before feeding could have not adequately captured the daily changes.

In our conditions, the OM, CP, and NDF apparent total-tract digestibility slightly but significantly increased with 3-NOP supplementation. The use of NIR to estimate nutrient digestibility might have reduced the variability among samples, contributing to the significance of the differences between groups. The effect of 3-NOP on total-tract digestibility is not clearly defined, but it ranges from null to positive. The results of the meta-analysis by Jayanegara et al. (2018) showed only a numeric increase in OM, NDF, and CP total-tract digestibility. More recently, Kim et al. (2020) found no significant effect of 3-NOP on OM and NDF digestibility due to the inconsistent results reported in the literature. Romero-Perez et al. (2014) in beef cattle and Hristov et al. (2015) in dairy cows reported quadratic but opposite effects of 3-NOP dosages on DM digestibility. Moreover, Haisan et al. (2017) reported that the apparent total tract digestibility of DM and NDF increased by 7.4% and 23.8%, respectively, in lactating cows receiving 2500 mg/d of 3-NOP. Additionally, they observed a reduction in acetate and an increase in propionate, butyrate, and certain iso-acids, which support our findings. Notably, the observed increase in ruminal iso-acid concentrations may align with the enhanced apparent CP digestibility reported for 3-NOP.

Incorporating 3-NOP into the diet of dairy cows resulted in reduced DMI during the summer, but not in the fall, while milk yield remained unaffected, thereby enhancing feed efficiency in the summer. Although most parameters related to feeding behavior were not influenced by seasonal variations, the administration of 3-NOP led to smaller meal sizes in the summer. Kjeldsen et al. (2024), in a study conducted from April to June, observed an 11% reduction in DMI in cows fed 3-NOP compared to the control group, attributing this effect to a probable decrease in rumen passage rate. We did not measure rumen passage rate, and the

literature lacks data on the impact of 3-NOP on this parameter. Nutrient digestibility, which could be affected by alterations in rumen retention times, was not influenced by seasonal changes. However, de novo milk FA, considered an indicator of optimal rumen functionality, as well as odd-chain FA from C11 to C17, originating from rumen microbes, were elevated in the 3-NOP group during the summer but not in the fall. It is speculated that a slightly altered feeding pattern in cows fed 3-NOP during the summer may have influenced the pattern of 3-NOP entering the rumen, with beneficial effects on the rumen environment. To elucidate the mechanisms underlying this hypothesized effect, further investigation into changes in the rumen microbiome is warranted.

Milk production and quality

In our conditions, supplementing 60 mg 3-NOP/kg DM did not impact milk yield and fat content, but, in agreement with van Gastelen et al. (2024), increased the protein content, that is a relevant parameter for long-ripened cheese making (Pretto et al., 2013) and might suggest an improved nitrogen use efficiency in those cows. The decreased ammonia in the rumen, previously reported (Reynolds et al., 2014a), may also be an indication of decreased proteolysis or increased ammonia uptake by the rumen bacteria (Lopes et al., 2016). Moreover, *Clostridium spp.*, which can have proteolytic and deaminative activities, was reported to be decreased by 3-NOP (Lopes et al., 2016). Recent meta-analysis (Kim et al., 2020) and meta-regression (Martins et al., 2025) reported that the supplementation with 3-NOP tends to decrease milk yield but to increase milk fat and protein content, resulting in a not significant reduction of ECM yield.

The observed increased proportions of milk short-chain fatty acids (C4 – C10) induced by 3-NOP agree with previous research (Hristov et al., 2015; Melgar et al., 2021; Lokuge et al., 2024), but took place only in the summer, when a more marked increase of rumen butyrate

was also observed, and not in the fall. Melgar et al. (2020a) hypothesized that inhibiting the methanogenic process with 3-NOP can make the role of butyrate more critical for the synthesis of short-chain FA. Interestingly, an analogous increase of short-chain FA has been reported when bromoform containing seaweed was used as an anti-methanogenic additive (Stefenoni et al., 2021; Alvarez-Hess et al., 2024), although in these research studies ruminal butyrate was not consistently increased. Feeding 3-NOP consistently increased odd chain FA C11:0, C13:0, C15:0 and C17:0. These FA are largely synthesized from rumen propionate and valerate (Fievez et al., 2012), that were increased by 3-NOP in the present study. The relationship between the CH₄ reduction obtained in different ways and the increase in milk short-chain and odd chain FA is not clearly defined in the literature (de Souza et al., 2010) and would warrant further investigation. In 3-NOP supplemented cows, we observed a consistent decrease in SFA, mainly led by changes in C16:0, and minor effects in MUFA and PUFA. In previous studies (Hristov et al., 2015; Melgar et al., 2021), 3-NOP caused an increase in SFA, whereas Lokuge et al. (2024) did not find any effects on them. When the availability of hydrogen is augmented, an increase in SFA could be expected. However, FA biohydrogenation represents only a minor hydrogen sink (Melgar et al., 2020b). De novo FA were increased by 3-NOP only in the summer, when reduced DMI and greater feed efficiency were recorded. These FA are proposed to be an indicator of rumen health (Woolpert et al., 2017) and might suggest that 3-NOP cows had better rumen efficiency during summer, counteracting the adverse effect on DMI.

Even though supplementing 3-NOP increased the protein content of milk, the lack of effect on the milk yield resulted in no effect on FPCM and ECM. However, the feed efficiency increased in the summer because of the lower DMI in 3-NOP-fed cows. Previous works reported no effect on feed efficiency in mid-to-late-lactation dairy cows (Hristov et al., 2015; van Wesemael et al., 2019), whereas feed efficiency was improved in early lactating cows

because of lower intake (Melgar et al., 2020a). The increased feed efficiency observed during the hot season, together with the greater proportion of de novo FA in milk, might suggest an improvement in the rumen metabolism, likely due to reduced heat production during the methanogenesis.

Blood biomarkers and body condition

The use of 3-NOP as a mitigating strategy for enteric CH₄ emissions has been widely studied (Kebreab et al., 2023). Nevertheless, its effects on the cow's physiology have been less frequently investigated and little information is available about the metabolic and innate immune response to 3-NOP supplementation in lactating dairy cows. Literature data only suggest a mild or negligible effects on blood concentrations of glucose, NEFA, BHB, and urea (Melgar et al., 2020a). In the present study, we monitored the animals using a comprehensive plasma immunometabolic profile to investigate the potential impact of 3-NOP beyond methane mitigation. This approach aims to provide a deeper understanding of its physiological effects and to shed light on the metabolic pathways and mechanisms involved, also in different environmental conditions.

Consistent with previous reports (Melgar et al., 2020a), we did not observe any effect of 3-NOP on glycemia and ketonemia. However, in our study, 3-NOP supplementation reduced plasma concentrations of urea and NEFA. The difference observed for the NEFA concentrations between CTR and 3-NOP groups, though limited as absolute value (~0.03 mmol/L), it is of some importance, considering mid-lactating cows were monitored. A similar decrease in blood NEFA was observed in primiparous dairy cows supplemented with 3-NOP (Yanibada et al., 2020) and during the postpartum period (Schilde et al., 2022). It can be hypothesized that the increased propionate flux from the rumen improved the availability of oxaloacetate in the mitochondria and, in turn, the liver NEFA oxidation (White, 2015), though

in our experiment we observed a minor and only numerical increase of propionate, only. The concurrent greater butyrate molar proportion in rumen fluid, paired with a decreased proportion of acetate, might have masked the expected decrease in plasma BHB as a marker of improved energy balance, as Schilde et al. (2022) suggested. Therefore, these results might help understand the action of 3-NOP on cow's physiology, but may warrant further investigation in early lactating dairy cows. Plasma urea, instead, was markedly reduced by 3-NOP. This outcome is consistent with the reduced milk urea, as previously reported by Martins et al. (2025), and the numerically lower ammonia recorded in the rumen fluid (Nousiainen et al., 2004; Huhtanen et al., 2015).

Nevertheless, the slightly reduced plasma cholesterol, that, despite not being a protein itself, can be interpreted as a negative acute phase protein being an indicator of liver-derived lipoproteins (Bertoni and Trevisi, 2013)), and Zn, paired with the greater myeloperoxidase concentration observed in 3-NOP, might suggest that some differences between groups in the inflammatory status. Nevertheless, the concentrations of these markers measured in the 3-NOP group, despite being significantly different than in CTR, were not of particular concern and were within physiological limits. Other markers of inflammation, as positive (i.e., haptoglobin, ceruloplasmin, and globulin) and negative (albumin, paraoxonase, and bilirubin) acute phase proteins, were unaltered, and AOPP, markers of oxidative stress, were reduced in 3-NOP. These outcomes suggest that other mechanisms than inflammation can be the reason for those differences and may warrant further investigation.

CONCLUSIONS

Results from this study confirm the marked anti-methanogenic effect of 3-NOP in lactating dairy cows. Supplementing 60 mg/kg DM of 3-NOP to a high-starch diet over a 9-wk treatment period decreased the enteric CH₄ production by 46.3%, the CH₄ yield by 46%, and

the CH₄ intensity by 44.2%. The H₂ production and yield increased, with the magnitude of the responses being season-dependent. Supplementing 3-NOP in summer decreased the DMI by 5.7% but did not affect milk yield, with little changes in milk composition and FA profile. Overall, including 3-NOP in a corn silage-based diet for mid-lactating cows represents a valuable tool to tackle enteric CH₄ emissions without adversely affecting the cow's performance and milk quality.

NOTES

This study was funded by dsm-firmenich (Kaiseraugst, Switzerland). Research activities in the Department of Animal Science, Food and Nutrition of the Università Cattolica del Sacro Cuore are supported by the Romeo and Enrica Invernizzi Foundation. I. Mueller, L. Tamassia, and R. Zihlmann are employees of dsm-firmenich. The authors have not stated any other conflicts of interest.

REFERENCES

- Abeni, F., L. Calamari, and L. Stefanini. 2007. Metabolic conditions of lactating Friesian cows during the hot season in the Po valley. 1. Blood indicators of heat stress. *Int J Biometeorol* 52:87–96. doi:10.1007/s00484-007-0098-3.
- ADAS (Agricultural Development and Advisory Service). 1986. Condition Scoring of Dairy Cows. Publ. 612, Agricultural Development and Advisory Service, Ministry of Agriculture, Fisheries and Food. Lion House, Alnwick, Northumberland, UK.
- Allen, M.S., 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *Journal of Dairy Science* 83, 1598–1624. [https://doi.org/10.3168/jds.S0022-0302\(00\)75030-2](https://doi.org/10.3168/jds.S0022-0302(00)75030-2).

- Almeida, A. K., R. S. Hegarty, and A. Cowie. 2021. Meta-analysis quantifying the potential of dietary additives and rumen modifiers for methane mitigation in ruminant production systems. *Anim. Nutr.* 7:1219–1230. <https://doi.org/10.1016/j.aninu.2021.09.005>.
- Alvarez-Hess, P.S., A.L. Thomson, S.R.O. Williams, A. Logan, C. Taylor, T. Singh, B.M. Roque, A.S. O Neachtain, R.D. Kinley, and J.L. Jacobs. 2024. The influence of feeding canola oil steeped *Asparagopsis armata* on resulting fatty acid profile and dairy processing properties of cow's milk. *Animal Feed Science and Technology* 310:115924. doi:10.1016/j.anifeedsci.2024.115924.
- AOAC International. 2016. *Official Methods of Analysis*. 20th ed. AOAC Int., Gaithersburg, MD.
- Arndt, C., A. N. Hristov, W. J. Price, S. C. McClelland, A. M. Pelaez, S. F. Cueva, J. Oh, J. Dijkstra, A. Bannink, A. R. Bayat, L. A. Crompton, M. A. Eugene, D. Enahoro, E. Kebreab, M. Kreuzer, M. McGee, C. Martin, C. J. Newbold, C. K. Reynolds, A. Schwarm, K. J. Shingfield, J. B. Veneman, D. R. Yanez-Ruiz, and Z. Yu. 2022. Full adoption of the most effective strategies to mitigate methane emissions by ruminants can help meet the 1.5 °C target by 2030 but not 2050. *Proc. Natl. Acad. Sci. USA* 119:e2111294119. <https://doi.org/10.1073/pnas.2111294119>.
- Baumgard, L.H., and R.P. Rhoads. 2013. Effects of heat stress on postabsorptive metabolism and energetics. *Annual Review of Animal Biosciences* 1:311–337. doi:10.1146/annurev-animal-031412-103644.
- Benzie, I.F.F., and J.J. Strain. 1999. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration.

- Bertoni, G., and E. Trevisi. 2013. Use of the Liver Activity Index and other metabolic variables in the assessment of metabolic health in dairy herds. *Veterinary Clinics of North America: Food Animal Practice* 29:413–431. doi:10.1016/j.cvfa.2013.04.004.
- Bonaldo, D., D. Bellafigliore, C. Ferrarin, R. Ferretti, A. Ricchi, L. Sangelantoni, and M.L. Vitelletti. 2023. The summer 2022 drought: a taste of future climate for the Po valley (Italy)?. *Reg Environ Change* 23:1. doi:10.1007/s10113-022-02004-z.
- Bradley, P.P., D.A. Priebat, R.D. Christensen, and G. Rothsein. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *Journal of Investigative Dermatology* 78:206–209. doi:10.1111/1523-1747.ep12506462.
- Choi, Y., M. Zhou, Y. Ban, M. Oba, S. Duval, and L.L. Guan. 2025. Differential alteration of rumen microbial composition in response to 3-nitrooxypropanol supplementation in dairy cattle fed high-grain and high-forage diets. *Journal of Dairy Science*. doi:10.3168/jds.2024-26206.
- CLAL.it. 2023. ITALY: Milk production and utilization 2023. https://www.clal.it/en/?section=bilancio_approv2&year=2023.
- Czerkawski, J. W. 1969. Methane production in ruminants and its significance. *W. Rev. Nut. Diet.*, 11: 240–280.
- Dijkstra, J., A. Bannink, J. France, E. Kebreab, and S. van Gastelen. 2018. Short communication: Anti-methanogenic effects of 3-nitrooxypropanol depend on supplementation dose, dietary fiber content, and cattle type. *J. Dairy Sci.* 101:9041–9047. <https://doi.org/10.3168/jds.2018-14456>.
- Duin, E. C., T. Wagner, S. Shima, D. Prakash, B. Cronin, D. R. Yáñez-Ruiz, S. Duval, R. Rumbeli, R. T. Stemmler, R. K. Thauer, and M. Kindermann. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small

- molecule 3-nitrooxypropanol. *Proc. Natl. Acad. Sci. USA* 113:6172–6177.
<https://doi.org/10.1073/pnas.1600298113>.
- Ferré, N., J. Camps, E. Prats, E. Vilella, A. Paul, L. Figuera, and J. Joven. 2002. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage.. *Clinical chemistry* 48:261–8.
- Fievez, V., E. Colman, J.M. Castro-Montoya, I. Stefanov, and B. Vlaeminck. 2012. Milk odd- and branched-chain fatty acids as biomarkers of rumen function—An update. *Animal Feed Science and Technology* 172:51–65. doi:10.1016/j.anifeedsci.2011.12.008.
- Forster, P., T. Storelvmo, K. Armour, W. Collins, J.L. Dufresne, D. Frame, D.J. Lunt, T. Mauritsen, M.D. Palmer, M. Watanabe, M. Wild, and H. Zhang. 2021. 2021: The Earth’s Energy Budget, Climate Feedbacks, and Climate Sensitivity. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Gallo A., Bruchi S., Masoero F. 2019. Technical note: Evaluation of a novel enzymatic method to predict in situ undigested neutral detergent fiber of forages and nonforage fibrous feeds. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2019-16279>.
- Gallo A., Valsecchi C., Masseroni M., Cannas A., Ghilardelli F., Masoero F. & Atzori A. S. (2022) An observational study to verify the influence of different nutritional corn silage-based strategies on efficient use of dietary nutrients, faecal fermentation profile, and profitability in a cohort of intensive dairy farms, *Italian Journal of Animal Science*, 21:1,228-243, doi:10.1080/1828051X.2022.2025932.
- Garcia, F., C. Muñoz, J. Martínez-Ferrer, N. L. Urrutia, E. D. Martínez, M. Saldivia, I. Immig, M. Kindermann, N. Walker, and E. M. Ungerfeld. 2022. 3-Nitrooxypropanol substantially decreased enteric methane emissions of dairy cows fed true protein- or urea-containing diets. *Heliyon* 8:e09738. <https://doi.org/10.1016/j.heliyon.2022.e09738>.

- Gerber, P. J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013. Tackling climate change through livestock—a global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. 115 p. Available at: <http://www.fao.org/docrep/018/i3437e/i3437e.pdf>.
- Grainger, C., and K. Beauchemin. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? *Anim. Feed Sci. Technol.* 166–167:308–320. <https://doi.org/10.1016/j.anifeedsci.2011.04.021>.
- Gruninger, R.J., X.M. Zhang, M.L. Smith, L. Kung, D. Vyas, S.M. McGinn, M. Kindermann, M. Wang, Z.L. Tan, and K.A. Beauchemin. 2022. Application of 3-nitrooxypropanol and canola oil to mitigate enteric methane emissions of beef cattle results in distinctly different effects on the rumen microbial community. *anim microbiome* 4:35. doi:10.1186/s42523-022-00179-8.
- G.U. - Gazzetta Ufficiale della Repubblica Italiana. 2022. n. 187: 84 – 92.
- Haisan, J., Y. Sun, L. Guan, K. A. Beauchemin, A. Iwaasa, S. Duval, M. Kindermann, D. R. Barreda, and M. Oba. 2017. The effects of feeding 3-nitrooxypropanol at two doses on milk production, rumen fermentation, plasma metabolites, nutrient digestibility, and methane emissions in lactating Holstein cows. *Anim. Prod. Sci.* 57:282–289. <https://doi.org/10.1071/an15219>.
- Hall, M.B. 2014. Selection of an empirical detection method for determination of water-soluble carbohydrates in feedstuffs for application in ruminant nutrition. *Anim. Feed Sci. Technol.* 198:28-37. 10.1016/j.anifeedsci.2014.08.009.
- Hamilton, B. L. (1977). An Empirical Investigation of the Effects of Heterogeneous Regression Slopes in Analysis of Covariance. *Educational and Psychological Measurement*, 37(3), 701-712. <https://doi.org/10.1177/001316447703700313>

- Hanasand, M., R. Omdal, K.B. Norheim, L.G. Gøransson, C. Brede, and G. Jonsson. 2012. Improved detection of advanced oxidation protein products in plasma. *Clinica Chimica Acta* 413:901–906. doi:10.1016/j.cca.2012.01.038.
- Horvath, K.C., and E.K. Miller-Cushon. 2019. Characterizing grooming behavior patterns and the influence of brush access on the behavior of group-housed dairy calves. *Journal of Dairy Science* 102:3421–3430. doi:10.3168/jds.2018-15460.
- Hristov, A. N., J. Oh, F. Giallongo, T. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moate, M. H. Deighton, S. R. O. Williams, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc. Natl. Acad. Sci. USA* 112:10663–10668. <https://doi.org/10.1073/pnas.1504124112>.
- Huhtanen, P., K. Kaustell, and S. Jaakkola. 1994. The use of internal markers to predict total digestibility and duodenal flow of nutrients in cattle given six different diets. *Animal Feed Science and Technology* 48:211–227. doi:10.1016/0377-8401(94)90173-2.
- Huhtanen, P., E.H. Cabezas-Garcia, S.J. Krizsan, and K.J. Shingfield. 2015a. Evaluation of between-cow variation in milk urea and rumen ammonia nitrogen concentrations and the association with nitrogen utilization and diet digestibility in lactating cows. *Journal of Dairy Science* 98:3182–3196. doi:10.3168/jds.2014-8215.
- Humer, E., R.M. Petri, J.R. Aschenbach, B.J. Bradford, G.B. Penner, M. Tafaj, K.-H. Südekum, and Q. Zebeli. 2018. Invited review: Practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. *Journal of Dairy Science* 101:872–888. doi:10.3168/jds.2017-13191.
- IDF, 2015. Bulletin of the International Dairy Federation N479/2015. In: A common carbon footprint approach for the dairy sector-the IDF guide to standard life cycle assessment methodology. Available at: <https://shop.fil-idf.org/collections/publications/>.

- ISPRA (Italian Institute for Environmental and Protection and Research). 2024. Italian Greenhouse Gas Inventory 1990-2022. National Inventory Report 2024. ISPRA Reports. No. 398/24.
- Jayanegara, A., K. A. Sarwono, M. Kondo, H. Matsui, M. Ridla, E. B. Laconi, and Nahrowi. 2018. Use of 3-nitrooxypropanol as feed additive for mitigating enteric methane emissions from ruminants: A meta-analysis. *Ital. J. Anim. Sci.* 17:650–656. <https://doi.org/10.1080/1828051X.2017.1404945>.
- Kebreab, E., A. Bannink, E. M. Pressman, N. Walker, A. Karagiannis, S. van Gastelen, and J. Dijkstra. 2023. A meta-analysis of effects of 3-nitrooxypropanol on methane production, yield, and intensity in dairy cattle. *J. Dairy Sci.* 106:927–936. <https://doi.org/10.3168/jds.2022-22211>.
- Kim, H., Le, H.G., Beek, Y.C., Lee, S., Seo, J., 2020. The effects of dietary supplementation with 3-nitrooxypropanol on enteric methane emissions, rumen fermentation, and production performance in ruminants: a metaanalysis. *Journal of Animal Science and Technology* 62, 31–42. <https://doi.org/10.5187/jast.2020.62.1.31>.
- Kjeldsen, M.H., Weisbjerg, M.R., Larsen, M., Højberg, O., Ohlsson, C., Walker, N., Hellwing, A.L.F., Lund, P., 2024. Gas exchange, rumen hydrogen sinks, and nutrient digestibility and metabolism in lactating dairy cows fed 3-nitrooxypropanol and cracked rapeseed. *J. Dairy Sci.* 107:2047–2065. <https://doi.org/10.3168/jds.2023-23743>.
- Kotb, A.R., Luckey, T.D. 1972. Markers in nutrition. *Nutr Abstr Rev.* 42:813–845.
- Lokuge, G.M.S., C. Kaysen, M. Maigaard, P. Lund, L. Wiking, and N.A. Poulsen. 2024. Effects of feeding whole-cracked rapeseeds, nitrate, and 3-nitrooxypropanol on composition and functional properties of the milk fat fraction from Danish Holstein cows. *Journal of Dairy Science* 107:5330–5342. doi:10.3168/jds.2024-23980.

- Lopes, J. C., L. F. de Matos, M. T. Harper, F. Giallongo, J. Oh, D. Gruen, S. Ono, M. Kindermann, S. Duval, and A. N. Hristov. 2016. Effect of 3-nitrooxypropanol on methane and hydrogen emissions, methane isotopic signature, and ruminal fermentation in dairy cows. *J. Dairy Sci.* 99:5335–5344. <https://doi.org/10.3168/jds.201510832>.
- Macdonald, P., and J. Du. 2018. *mixdist: Finite Mixture Distribution Models*. R package version 0.5–5.
- Manafiazar, G., S. Zimmerman, and J. Basarab. 2016. Repeatability and variability of short-term spot measurement of methane and carbon dioxide emissions from beef cattle using GreenFeed Emissions Monitoring System. *Can. J. Anim. Sci.* CJAS-2015-0190. doi:10.1139/CJAS-2015-0190.
- Martins L.F., Maigaard M., Johansen M., Lund P., Ma X., Niu M., Hristov A.N. 2025. Lactational performance effects of 3-nitrooxypropanol supplementation to dairy cows: A meta-regression. *J. Dairy Sci.*, 108: 1538-1553. <https://doi.org/10.3168/jds.2024-25653>.
- Melgar, A., M. T. Harper, J. Oh, F. Giallongo, M. E. Young, T. L. Ott, S. Duval, and A. N. Hristov. 2020a. Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. *J. Dairy Sci.* 103:410–432. <https://doi.org/10.3168/jds.2019-17085>.
- Melgar, A., K. Nedelkov, C. M. M. R. Martins, K. C. Welter, X. Chen, M. T. Harper, S. Duval, and A. N. Hristov. 2020b. Short communication: Short-term effect of 3-nitrooxypropanol on feed dry matter intake in lactating dairy cows. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2020-18331>.
- Melgar, A., C. F. A. Lage, K. Nedelkov, S. E. Räisänen, H. Stefenoni, M. E. Fetter, X. Chen, J. Oh, S. Duval, M. Kindermann, N. D. Walker, and A. N. Hristov. 2021. Enteric

- methane emission, milk production and composition of dairy cows fed 3-nitrooxypropanol. *J. Dairy Sci.* 104:357–366. <https://doi.org/10.3168/jds.2020-18908>.
- Mertens, D. R. (2002). Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. *Journal of AOAC International*, 85(6), 1217–1240. <https://doi.org/10.1093/jaoac/85.6.1217>.
- Minuti, A., S. Ahmed, E. Trevisi, F. Piccioli-Cappelli, G. Bertoni, N. Jahan, and P. Bani. 2014. Experimental acute rumen acidosis in sheep: Consequences on clinical, rumen, and gastrointestinal permeability conditions and blood chemistry. *Journal of Animal Science* 92:3966–3977. doi:10.2527/jas.2014-7594.
- Muizelaar, W., P. Bani, B. Kuhla, I. Tapio, D.R. Yáñez-Ruiz, and S. Van Gastelen. 2020. Rumen fluid sampling via oral stomach tubing method. S. Danesh Mesgaran, H. Ferguson, L. Munksgaard, M. Terré, E. Kennedy, D. Humphries, J. Dijkstra, B. Kuhla, R. Dewhurst, & R. Baumont (Eds.).
- Myhre, G., D. Shindell, F.M. Bréon, W. Collins, J. Fuglestedt, J. Huang, D. Koch, J.F. Lamarque, D. Lee, B. Mendoza, T. Nakajima, A. Robock, G. Stephens, T. Takemura, and H. Zhang. 2013. *Anthropogenic and Natural Radiative Forcing*. 1st ed. Cambridge University Press.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*. 7th rev. ed. Natl. Acad. Press, Washington, DC.
- Nonaka, I., N. Takusari, K. Tajima, T. Suzuki, K. Higuchi, and M. Kurihara. 2008. Effects of high environmental temperatures on physiological and nutritional status of prepubertal Holstein heifers. *Livestock Science* 113:14–23. doi:10.1016/j.livsci.2007.02.010.
- Nousiainen, J., K.J. Shingfield, and P. Huhtanen. 2004. Evaluation of Milk Urea Nitrogen as a Diagnostic of Protein Feeding. *Journal of Dairy Science* 87:386–398. doi:10.3168/jds.S0022-0302(04)73178-1.

- Orzuna-Orzuna, J.F., J.E. Godina-Rodríguez, J.R. Garay-Martínez, L.D. Granados-Rivera, J.A. Maldonado-Jáquez, and A. Lara-Bueno. 2024. A Meta-Analysis of 3-Nitrooxypropanol Dietary Supplementation on Growth Performance, Ruminal Fermentation, and Enteric Methane Emissions of Beef Cattle. *Fermentation* 10:273. doi:10.3390/fermentation10060273.
- Pitta, D.W., N. Indugu, A. Melgar, A. Hristov, K. Challa, B. Vecchiarelli, M. Hennessy, K. Narayan, S. Duval, M. Kindermann, and N. Walker. 2022. The effect of 3-nitrooxypropanol, a potent methane inhibitor, on ruminal microbial gene expression profiles in dairy cows. *Microbiome* 10. doi:10.1186/s40168-022-01341-9.
- Pretto D., De Marchi M., Penasa M., Cassandro M.. 2013. Effect of milk composition and coagulation traits on Grana Padano cheese yield under field conditions. *J Dairy Res.* Feb;80(1):1-5. doi: 10.1017/S0022029912000453. Epub 2012 Sep 24. Erratum in: *J Dairy Res.* 2013 Feb;80(1):6. PMID: 22998758.
- Reynolds, C.K., D.J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. *Journal of Dairy Science* 97:3777–3789. doi:10.3168/jds.2013-7397.
- Romero-Perez, A., E.K. Okine, S.M. McGinn, L.L. Guan, M. Oba, S.M. Duval, M. Kindermann, and K.A. Beauchemin. 2014. The potential of 3-nitrooxypropanol to lower enteric methane emissions from beef cattle¹. *Journal of Animal Science* 92:4682–4693. doi:10.2527/jas.2014-7573.
- Schilde, M., D. Von Soosten, J. Frahm, S. Kersten, U. Meyer, A. Zeyner, and S. Dänicke. 2022. Assessment of Metabolic Adaptations in Periparturient Dairy Cows Provided 3-Nitrooxypropanol and Varying Concentrate Proportions by Using the GreenFeed

- System for Indirect Calorimetry, Biochemical Blood Parameters and Ultrasonography of Adipose Tissues. *Dairy* 3:100–122. doi:10.3390/dairy3010009.
- Sjaunja, L.O., Baevre, L., Junkkarinen, L., Pedersen, J., Setälä, J. 1990. A Nordic proposal for an energy corrected milk (ECM) formula. 26th session of the International Committee for Recording the Productivity of Milk.
- Skinner, J., R. Brown, and L. Roberts. 1991. Bovine haptoglobin response in clinically defined field conditions. *Veterinary Record* 128:147–149. doi:10.1136/vr.128.7.147.
- Souza, V.C., L.E. Moraes, L.H. Baumgard, J.E.P. Santos, N.D. Mueller, R.P. Rhoads, and E. Kebreab. 2023. Modeling the effects of heat stress in animal performance and enteric methane emissions in lactating dairy cows. *Journal of Dairy Science* 106:4725–4737. doi:10.3168/jds.2022-22658.
- Stefenoni, H.A., S.E. Räisänen, S.F. Cueva, D.E. Wasson, C.F.A. Lage, A. Melgar, M.E. Fetter, P. Smith, M. Hennessy, B. Vecchiarelli, J. Bender, D. Pitta, C.L. Cantrell, C. Yarish, and A.N. Hristov. 2021. Effects of the macroalga *Asparagopsis taxiformis* and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. *Journal of Dairy Science* 104:4157–4173. doi:10.3168/jds.2020-19686.
- Sunderman, F.W., and S. Nomoto. 1970. Measurement of human serum ceruloplasmin by its p-phenylenediamine oxidase activity. *Clinical Chemistry* 16:903–910. doi:10.1093/clinchem/16.11.903.
- Tolkamp, B.J., and I. Kyriazakis. 1999. To split behaviour into bouts, log-transform the intervals. *Animal Behaviour* 57:807–817. doi:10.1006/anbe.1998.1022.
- van Gastelen, S., J. Dijkstra, G. Binnendijk, S. M. Duval, J. M. L. Heck, M. Kindermann, T. Zandstra, and A. Bannink. 2020. 3-nitrooxypropanol decreases methane emissions and increases hydrogen emissions of early lactation dairy cows, with associated changes in

- nutrient digestibility and energy metabolism. *J. Dairy Sci.* 103:8074–8093.
<https://doi.org/10.3168/jds.2019-17936>
- van Gastelen, S., J. Dijkstra, J. M. L. Heck, M. Kindermann, A. Klop, R. de Mol, D. Rijnders, N. Walker, and A. Bannink. 2022. Methane mitigation potential of 3-nitrooxypropanol in lactating cows is influenced by basal diet composition. *J. Dairy Sci.* 105:4064–4082.
<https://doi.org/10.3168/jds.2021-20782>.
- van Gastelen, S., Burgers, E. E. A., Dijkstra, J., de Mol, R., Muizelaar, W., Walker, N., & Bannink, A. (2024). Long-term effects of 3-nitrooxypropanol on methane emission and milk production characteristics in Holstein Friesian dairy cows. *Journal of Dairy Science*, 107, 5556–5573. <https://doi.org/10.3168/jds.2023-24198>.
- van Wesemael, D., L. Vandaele, B. Ampe, H. Cattrysse, S. Duval, M. Kindermann, V. Fievez, S. De Campeneere, and N. Peiren. 2019. Reducing enteric methane emissions from dairy cattle: Two ways to supplement 3-nitrooxypropanol. *J. Dairy Sci.* 102:1780–1787.
<https://doi.org/10.3168/jds.2018-14534>.
- West, J.W., B.G. Mullinix, and J.K. Bernard. 2003. Effects of Hot, Humid Weather on Milk Temperature, Dry Matter Intake, and Milk Yield of Lactating Dairy Cows. *Journal of Dairy Science* 86:232–242. doi:10.3168/jds.S0022-0302(03)73602-9.
- White, H. 2015. The Role of TCA Cycle Anaplerosis in Ketosis and Fatty Liver in Periparturient Dairy Cows. *Animals* 5:793–802. doi:10.3390/ani5030384.
- Woolpert, M.E., H.M. Dann, K.W. Cotanch, C. Melilli, L.E. Chase, R.J. Grant, and D.M. Barbano. 2017. Management practices, physically effective fiber, and ether extract are related to bulk tank milk de novo fatty acid concentration on Holstein dairy farms. *Journal of Dairy Science* 100:5097–5106. doi:10.3168/jds.2016-12046.
- Yadav, B., G. Singh, A.K. Verma, N. Dutta, and V. Sejian. 2013. Impact of heat stress on rumen functions. *Vet World* 6:992–996. doi:10.14202/vetworld.2013.992-996.

- Yanibada, B., U. Hohenester, M. Pétéra, C. Canlet, S. Durand, F. Jourdan, J. Boccard, C. Martin, M. Eugène, D.P. Morgavi, and H. Boudra. 2020. Inhibition of enteric methanogenesis in dairy cows induces changes in plasma metabolome highlighting metabolic shifts and potential markers of emission. *Scientific Reports* 10:15591. doi:10.1038/s41598-020-72145-w.
- Yu, G., Beauchemin, K. A., & Dong, R. (2021). A review of 3-nitrooxypropanol for enteric methane mitigation from ruminant livestock. *Animals*, 11(12), 1–16. <https://doi.org/10.3390/ani11123540>.
- Zhao, S., L. Min, N. Zheng, and J. Wang. 2019. Effect of Heat Stress on Bacterial Composition and Metabolism in the Rumen of Lactating Dairy Cows. *Animals* 9:925. doi:10.3390/ani9110925.

Table 1. Formulated TMR ingredients and analyzed chemical composition of diets fed in phase 1 (P1) and 2 (P2) to lactating Holstein cows supplemented with a placebo (CTR) or 3-NOP^{1,2}.

Item, g/kg DM	Diet			
	P1		P2	
Corn silage	369.0		392.4	
Alfalfa hay	238.5		213.6	
Barley straw	21.4		19.7	
Soybean meal	88.3		89.5	
Corn grain, fine ground	135.2		139.6	
Barley grain, ground	90.2		93.3	
Sunflower meal	36.5		35.8	
Mineral and vitamin mix ³	10.5		9.9	
Limestone	3.2		2.9	
Sodium bicarbonate	3.2		2.9	
Salt	0.4		0.4	
	CTR	3-NOP	CTR	3-NOP
DM, % of fresh matter	52.5 ± 0.47	52.2 ± 0.35	53.1 ± 1.17	52.7 ± 0.86
CP, g/kg DM	148.7 ± 1.53	148.7 ± 1.53	158.3 ± 3.06	158.7 ± 2.52
Starch, g/kg DM	286.0 ± 8.19	289.3 ± 4.73	294.7 ± 2.89	295.7 ± 14.57
Crude fat, g/kg DM	36.3 ± 1.53	38.7 ± 1.15	28.7 ± 2.08	28.3 ± 3.21
NDF, g/kg DM	319.0 ± 11.14	317.0 ± 5.29	322.0 ± 8.54	316.0 ± 8.66
ADF, g/kg DM	224.0 ± 15.1	213.3 ± 9.07	192.7 ± 6.11	197.0 ± 8.54
Ash, g/kg DM	69.3 ± 2.31	74.7 ± 2.08	72.0 ± 6.08	75.0 ± 4.58

¹Dose of 3-NOP: 60 mg/kg DM.

²Means ± SD; n = 3.

³Mineral and vitamin mix: vitamin A = 150,000 IU, vitamin D = 10,000 IU, vitamin E = 200 mg, vitamin K = 100 mg, vitamin H = 100 mg, vitamin B1 = 150 mg, vitamin B12 = 0.5 mg, vitamin PP = 500 mg, choline = 4,000 mg, Mn = 350 mg, Zn = 800 mg, Cu = 40 mg, I = 20 mg, Co = 1 mg, Se = 1 mg, DL-Methionine = 2 g.

Table 2. Visits to the GreenFeed, GHG productions (g/d), yields (g/kg DMI), and intensities (g/kg ECM) in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item	Treatment (Trt)		SEM	P-value ²				
	CTR	3-NOP		Trt	T	Trt×T	P	Trt×P
Visits, n/d	2.74	2.62	0.134	0.48	0.14	0.94	0.11	0.65
CH ₄								
Production, g/d	427	230	9.3	<0.01	<0.01	0.06	0.37	0.57
Yield, ³ g/kg DM	16.3 (15.4-17.3)	8.8 (8.3-9.4)	-	<0.01	<0.01	<0.01	0.29	0.79
Intensity, ⁴ g/kg ECM	12.9	7.2	0.49	<0.01	<0.01	0.64	0.48	0.46
Intensity, ⁴ g/kg Milk	12.8	7.1	0.51	<0.01	<0.01	0.88	0.24	0.40
H ₂								
Production, g/d	2.10	8.79	0.220	<0.01	0.01	0.01	0.02	<0.01
Yield ⁴ , g/kg DM	0.08	0.35	0.011	<0.01	0.04	0.05	<0.01	<0.01
CO ₂								
Production, kg/d	14.11	14.44	0.143	0.07	<0.01	<0.01	0.05	0.29
Yield, kg/kg DM	0.55	0.57	0.008	0.02	<0.01	0.92	<0.01	0.01

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

⁴Means without adjustment for the covariate measurement (adjustment period)

Table 3. Gas productions and yields in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹, in phase 1 (P1, summer) or 2 (P2, fall) of the study

Item, unit	Treatment (Trt)		SEM	P-value ²		
	CTR	3-NOP		Trt	T	Trt×T
P1						
H ₂ production, g/d	2.16	9.87	0.288	<0.01	0.04	0.04
H ₂ yield, g/kg DM	0.09	0.40	0.011	<0.01	0.05	0.04
CO ₂ yield, g/kg DM	542	589	10.9	<0.01	0.07	0.48
P2						
H ₂ production, g/d	2.01	7.60	0.343	<0.01	0.04	0.31
H ₂ yield, g/kg DM	0.08	0.29	0.013	<0.01	0.15	0.61
CO ₂ yield, g/kg DM	554	554	10.6	0.99	<0.01	0.54

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 4. BCS, BW, DMI, milk yield and composition in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item	Treatment (Trt)		SEM	P-value ²				
	CTR	3- NOP		Trt	T	Trt×T	P	Trt×P
DMI, kg/d	26.2	25.6	0.29	0.10	0.96	0.37	<0.01	<0.01
BCS ³	2.46 (2.41- 2.50)	2.48 (2.44- 2.53)	-	0.44	<0.01	0.92	0.10	0.88
BW, ³ kg	651 (645- 656)	650 (645- 655)	-	0.96	<0.01	0.41	0.24	0.21
Milk yield, kg/d	33.9	33.5	0.43	0.50	<0.01	0.94	0.05	0.20
FPCM, kg/d	33.4	33.1	0.41	0.51	<0.01	0.90	<0.01	0.11
ECM, kg/d	33.3	33.0	0.41	0.51	<0.01	0.90	<0.01	0.11
Feed efficiency, kg milk/kg DMI	1.29	1.29	0.015	0.77	<0.01	0.05	0.89	<0.01
Milk fat, ⁴ %	3.88	3.89	0.138	0.97	<0.01	0.96	0.06	0.47
True protein, %	3.40	3.52	0.031	<0.01	<0.01	0.34	0.02	0.07
Lactose, %	4.65	4.59	0.029	0.09	0.01	0.07	0.14	0.95
Casein, %	2.64	2.75	0.027	<0.01	<0.01	0.36	0.03	0.12
Urea, ⁴ mg/dL	24.3	22.1	0.61	<0.01	<0.01	0.11	<0.01	0.37
SCC, ³ n/mL	28 (23- 34)	23 (18- 28)	-	0.17	<0.01	0.74	0.96	0.39

¹Dose of 3-NOP: 60 mg/kg DM; Means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

⁴Means without adjustment for the covariate measurement (adjustment period)

Table 5. DMI, meal size, and feed efficiency in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹, in phase 1 (P1, summer) or 2 (P2, fall) of the study

Item, unit	Treatment (Trt)		SEM	P-value ²		
	CTR	3-NOP		Trt	T	Trt×T
P1						
DMI, kg/d	26.2	24.7	0.50	<0.01	0.18	0.25
Meal size, ³ kg DM/meal	4.47 (4.27-4.47)	4.13 (3.95-4.27)	-	0.01	<0.01	0.37
Feed efficiency, kg milk/kg DMI	1.25	1.31	0.025	0.03	<0.01	0.74
P2						
DMI, kg/d	26.1	26.5	0.43	0.36	0.56	0.06
Meal size, ³ kg DM/meal	4.81 (4.62-5.01)	4.89 (4.71-5.08)	-	0.57	0.28	0.01
Feed efficiency, kg milk/kg DMI	1.32	1.28	0.020	0.12	0.02	0.03

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 6. Fatty acid profile of milk fat in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item, %	Treatment (Trt)			P-value ²				
	CTR	3-NOP	SEM	Trt	T	Trt×T	P	Trt×P
C4:0	3.13	3.28	0.045	0.02	<0.01	0.01	<0.01	<0.01
C6:0	2.10	2.21	0.031	0.01	0.02	0.24	<0.01	<0.01
C8:0	1.28	1.37	0.022	0.01	0.12	0.39	0.16	<0.01
C10:0	3.24	3.37	0.062	0.13	0.38	0.74	0.37	<0.01
C10:1	0.36	0.38	0.008	0.04	0.37	0.04	0.59	<0.01
C11:0 ³	0.12	0.17	0.011	<0.01	0.77	0.02	0.11	0.57
C12:0	4.13	4.11	0.075	0.85	0.56	0.72	0.02	<0.01
C13:0	0.16	0.21	0.011	<0.01	0.51	0.02	<0.01	0.38
C14:0	12.41	11.85	0.117	<0.01	0.08	0.29	0.79	0.32
C14:1	1.32	1.32	0.026	0.91	0.17	<0.01	0.90	0.12
C15:0	1.32	1.60	0.059	<0.01	0.33	0.01	0.02	0.83
C16:0	37.28	35.80	0.337	<0.01	<0.01	0.37	0.02	0.24
C16:1	1.84	1.93	0.039	0.11	0.29	0.02	0.59	0.01
C17:0	0.48	0.53	0.011	<0.01	0.16	0.80	0.17	0.79
C17:1	0.23	0.26	0.006	<0.01	0.08	0.22	0.38	0.18
C18:0	6.94	6.66	0.183	0.29	0.11	<0.01	0.31	0.37
C18:1n-7	2.11	2.24	0.041	0.03	<0.01	0.07	0.74	0.53
C18:1n-9 cis+trans ⁴	15.42	15.82	-	0.18	<0.01	0.40	0.21	0.02
	(15.03-15.83)	(15.43-16.21)						
C18:2	0.41	0.44	0.011	0.07	<0.01	0.11	0.87	0.13
C18:2n-6 trans ³	0.45	0.45	0.014	0.96	0.20	0.39	<0.01	0.56
C18:2n-6 cis ³	2.18	2.16	0.057	0.83	<0.01	0.18	0.02	0.95
C18:3n-3 ³	0.30	0.28	0.008	0.13	0.97	0.01	<0.01	0.38
C20:0	0.084	0.082	0.002	0.48	<0.01	<0.01	0.60	0.17
C20:1	0.118	0.114	0.002	0.11	<0.01	0.44	0.62	0.02
C20:3n-6 ^{3,4}	0.088	0.086	-	0.45	<0.01	0.21	<0.01	0.47
	(0.084-0.093)	(0.082-0.091)						
C20:3n-3	0.116	0.131	0.002	<0.01	<0.01	0.66	0.79	0.81
SFA	72.44	71.40	0.333	0.04	<0.01	0.78	0.33	0.09
MUFA	21.46	22.24	0.288	0.07	<0.01	0.83	0.50	0.04
PUFA ³	3.55	3.52	0.090	0.78	<0.01	0.30	<0.01	0.95
De novo	28.81	29.18	0.268	0.35	0.06	0.65	0.82	<0.01
Mixed	39.11	37.75	0.351	0.01	<0.01	0.30	0.02	0.19
Preformed	28.06	28.59	0.371	0.33	<0.01	0.08	0.34	<0.01

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP; SFA: Σ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0); MUFA: Σ (C10:1, C14:1, C17:1, C18:1n-9 cis+trans, C18:1n7, C20:1); PUFA: Σ (C18:2n-6 trans, C18:2n-6 cis, C18:3n-3, C20:3n-6, C20:3n-3); De Novo: Σ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0); Mixed: Σ (C16:0, C16:1, C17:0); Preformed: Σ (C18:0, C18:1n-7, C18:1n-9 cis+trans, C18:2, C18:2n-6 trans, C18:2n-6 cis, C18:3n-3, C20:0, C20:1, C20:3n-6, C20:3n-3).

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Means without adjustment for the covariate measurement (adjustment period)

⁴Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 7. Fatty acid profile of milk fat in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹, in phase 1 (P1, summer) or 2 (P2, fall) of the study

Item, %	Treatment (Trt)		SEM	P-value		
	CTR	3-NOP		Trt	T	Trt×T
P1						
C4:0	3.03	3.49	0.067	<0.01	0.01	<0.01
C6:0	2.03	2.34	0.037	<0.01	0.16	0.22
C8:0	1.22	1.45	0.028	<0.01	0.16	0.30
C10:0	2.98	3.54	0.078	<0.01	0.06	0.62
C10:1	0.33	0.39	0.007	<0.01	0.89	0.43
C12:0	3.83	4.20	0.106	0.03	0.09	0.75
C16:1	1.84	1.79	0.058	0.52	0.75	0.10
C18:1n-9	15.46	15.50	-	0.94	<0.01	0.61
cis+trans ²	(14.74-16.21)	(14.81-16.23)				
C20:1	0.126	0.115	0.003	0.01	<0.01	0.08
MUFA	21.63	22.02	0.422	0.55	<0.01	0.73
De novo	27.81	29.84	0.336	<0.01	<0.01	0.78
Preformed	28.46	28.53	0.412	0.90	<0.01	<0.01
P2						
C4:0	3.24	3.07	0.056	0.06	<0.01	0.76
C6:0	2.19	2.06	0.050	0.08	0.07	0.81
C8:0	1.37	1.27	0.037	0.08	0.21	0.83
C10:0	3.51	3.18	0.099	0.03	0.32	0.97
C10:1	0.38	0.37	0.015	0.62	0.10	0.07
C12:0	4.47	4.04	0.117	0.02	0.04	0.73
C16:1	1.84	2.07	0.046	<0.01	0.03	0.17
C18:1n-9	15.34	16.26	-	0.04	0.03	0.37
cis+trans ²	(14.75-15.96)	(15.66-16.88)				
C20:1	0.108	0.112	0.002	0.20	0.10	0.37
MUFA	21.12	22.55	0.373	0.01	0.15	0.47
De novo	29.84	28.57	0.446	0.06	0.01	0.73
Preformed	27.63	28.60	0.634	0.28	<0.01	0.13

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP; MUFA: Σ (C10:1, C14:1, C17:1, C18:1n-9 cis+trans, C18:1n7, C20:1); De Novo: Σ (C4:0, C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0); Preformed: Σ (C18:0, C18:1n-7, C18:1n-9 cis+trans, C18:2, C18:2n-6 trans, C18:2n-6 cis, C18:3n-3, C20:0, C20:1, C20:3n-6, C20:3n-3).

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 8. Plasma biomarkers in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item, unit	Treatment (Trt)		SEM	P-values ²				
	CTR	3-NOP		Trt	T	Trt×T	P	Trt×P
PCV, L/L	0.299	0.292	0.003	0.11	0.25	0.05	0.39	0.29
Glucose, mmol/L	4.29	4.35	0.03	0.19	0.10	0.10	0.54	0.10
NEFA, mmol/L	0.219	0.190	0.009	0.03	0.11	0.81	<0.01	0.79
BHB, mmol/L	0.396	0.410	0.013	0.47	0.33	0.85	0.10	0.73
Urea, mmol/L	4.17	3.78	0.10	0.01	<0.01	0.11	0.47	0.94
Cholesterol, mmol/L	5.85	5.55	0.08	0.01	0.10	0.16	0.97	0.45
Creatinine, µmol/L	84.49	84.63	0.59	0.87	0.52	0.70	0.37	0.48
Ca, mmol/L	2.53	2.48	0.02	0.07	<0.01	0.99	0.07	0.17
P, mmol/L	1.79	1.74	0.05	0.47	<0.01	0.04	0.80	0.11
Mg, mmol/L	1.035	1.062	0.011	0.09	<0.01	0.92	0.85	0.05
Zn, µmol/L	11.85	10.42	0.34	<0.01	0.45	0.42	0.89	0.14
Na, mmol/L	145.20	145.38	0.34	0.72	<0.01	0.15	0.34	0.60
K, mmol/L	4.16	4.21	0.04	0.36	0.54	0.26	0.01	0.10
Cl, mmol/L	103.66	104.41	0.32	0.11	<0.01	0.03	<0.01	0.87
Total protein, g/L	81.77	81.66	0.46	0.87	0.02	0.53	0.14	0.08
Albumin, g/L	37.45	37.57	0.19	0.67	0.80	0.48	<0.01	0.91
Globulin, g/L	44.27	44.14	0.48	0.85	0.01	0.31	0.98	0.10
Haptoglobin, ³ g/L	0.011	0.009	-	0.28	<0.01	0.90	<0.01	0.46
	(0.009-0.013)	(0.008-0.011)						
Ceruloplasmin, µmol/L	2.29	2.37	0.05	0.26	0.23	0.97	<0.01	0.53
Bilirubin, µmol/L	0.855	0.849	0.035	0.91	<0.01	0.96	<0.01	0.44
Paraoxonase, U/mL	98.82	98.76	1.17	0.97	0.36	0.90	0.06	0.37
Myeloperoxidase, U/L	364.51	392.10	6.22	<0.01	<0.01	0.42	<0.01	0.87
GOT, U/L	122.74	125.21	3.34	0.61	0.99	0.02	0.08	0.74
GGT, U/L	36.56	37.72	1.07	0.45	0.04	<0.01	0.16	0.63
Alkaline phosphatase, U/L	51.40	52.67	1.19	0.45	<0.01	0.80	0.50	0.63
Thiol groups, µmol/L	319.6	317.57	4.63	0.75	<0.01	0.23	0.20	0.14
ROM, mg H ₂ O ₂ /100 mL	12.30	12.59	0.28	0.47	<0.01	0.08	0.08	0.33
FRAP, µmol/L	147.41	142.21	2.10	0.09	0.34	0.16	0.99	0.60
AOPP, µmol/L	24.75	23.17	0.54	0.04	<0.01	0.21	<0.01	0.52

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP.

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 9. Rumen fluid pH, ammonia, lactate, and volatile fatty acid molar proportions in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item, unit	Treatment (Trt)		SEM	P-value ²		
	CTR	3-NOP		Trt	P	Trt x P
pH	6.80	6.86	0.036	0.23	0.62	0.21
Ammonia, mmol/L	5.06	4.56	0.353	0.31	<0.01	0.70
L-lactate, mmol/L	0.49	0.50	0.034	0.79	0.65	0.23
D-lactate, mmol/L	0.49	0.50	0.035	0.94	0.77	0.30
Total VFA, mmol/L	85.81	84.06	2.785	0.65	<0.01	0.52
Individual VFA, mol %						
Acetate	64.17	59.25	0.696	<0.01	<0.01	0.16
Propionate	21.70	23.14	0.592	0.09	<0.01	0.57
Butyrate	9.68	11.80	0.237	<0.01	0.06	<0.01
Isobutyrate	0.97	1.03	0.032	0.16	<0.01	0.86
Valerate ³	1.14	1.56	-	<0.01	<0.01	0.03
	(1.04-1.24)	(1.43-1.71)				
Isovalerate	1.45	1.87	0.083	<0.01	<0.01	0.96
Acetate:Propionate	3.08	2.62	0.106	<0.01	<0.01	0.54
(Acetate+Butyrate):Propionate	3.55	3.15	0.120	0.02	<0.01	0.27

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

Table 10. Fecal DM, pH, and apparent digestibility of OM, CP, starch, and NDF in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item	Treatment (Trt)		SEM	P-value ²				
	CTR	3-NOP		Trt	T	Trt×T	P	Trt×P
DM, ² %	14.14 (13.78-14.52)	14.15 (13.77-14.51)	-	0.98	0.70	0.62	0.51	0.47
pH	6.85	6.89	0.030	0.35	<0.01	0.01	<0.01	0.20
Apparent digestibility								
OM, ³ %	80.26 (79.93-80.58)	81.05 (80.73-81.37)	-	<0.01	<0.01	<0.01	<0.01	0.94
CP, ³ %	79.93 (79.53-80.34)	81.70 (81.29-82.11)	-	<0.01	<0.01	<0.01	0.40	0.57
Starch, %	98.60	98.47	0.060	0.08	<0.01	0.27	<0.01	0.55
NDF, ³ %	60.35 (59.86-60.86)	62.56 (62.06-63.07)	-	<0.01	<0.01	<0.01	0.01	0.16

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

SUPPLEMENTARY MATERIALS

Effects of 3-nitrooxypropanol on enteric methane emissions and milk production characteristics in dairy cows fed a high corn silage diet in different environmental conditions. Moschini et al.

Supplemental Table S1. Average chemical composition of the forages fed in phase 1 (P1) and 2 (P2) to lactating Holstein cows

	Corn silage		Alfalfa hay		Barley straw	
	P1	P2	P1	P2	P1	P2
DM, g/kg of fresh matter	376	306	887	878	891	903
CP, g/kg DM	83	76	173	186	33	34
Starch, g/kg DM	331	316	23	25	-	-
Crude fat, g/kg DM	31	27	27	29	19	16
NDF, g/kg DM	385	402	421	427	773	775
ADF, g/kg DM	248	261	278	263	543	539
Ash, g/kg DM	42	59	89	92	82	80

Supplemental Table S2. List of commercial kits used for the plasma analyses

Analyte ¹	CV		Catalog number	Method
	Inter-assay	Intra-assay		
Glucose	1.05	0.67	18250840 ²	
NEFA	2.14	0.85	434-91795 ³ , 436-91995 ³	
BHB	1.71	0.96	RB1007 ⁴	
Triglycerides	2.95	0.49	18255640 ²	
Urea	1.97	0.68	18255440 ²	
Cholesterol	1.96	0.34	18250540 ²	
Creatinine	1.51	0.93	18255540 ²	
Ca	1.59	1.15	18164340 ²	
P	0.82	0.26	18251240 ²	
Mg	1.51	0.52	18164540 ²	
Na	0.88	0.37		
K	1.01	0.36	0018253400 ² ,	
Cl	0.97	0.25	0018253500 ²	
Zn	1.99	1.13	CL55-100S ³	
Total protein	1.20	0.47	18251440 ²	
Albumin	1.04	0.39	18250040 ²	
Ceruloplasmin	2.36	0.50	–	Sunderman and Nomoto, 1970
Haptoglobin	2.74	1.50	–	Skinner et al., 1991
Myeloperoxidase	2.22	0.82	–	Bradley et al., 1982
GOT	1.66	1.55	18257540 ²	
GGT	2.33	0.95	18257640 ²	
Bilirubin	1.75	0.70	18254640 ²	
Alkaline phosphatase	1.76	0.76	18259640 ²	
Paraoxonase	2.25	0.60	–	Ferré et al., 2002
ROM	1.63	1.28	MC003 ⁵	
AOPP	3.72	0.58	–	Hanasand et al., 2012
FRAP	2.79	1.46	–	Benzie and Strain, 1999
Thiol groups	2.35	0.55	MC433 ⁵	

¹ NEFA = nonesterified fatty acids; GOT = glutamate oxaloacetate transferase; GGT = γ -glutamyl transferase; ROM = reactive oxygen metabolites; AOPP = advanced oxidation protein products; FRAP = ferric reducing antioxidant power

² Instrumentation Laboratory s.p.a. Werfen, Milan, Italy

³ Test Medical s.a.s., Zola Pedrosa (BO), Italy

⁴ Randox Laboratories Ltd., Crumlin, Co. Antrim, UK

⁵ Diacron srl, Grosseto, Italy

Supplemental Table S3. Feeding behavior, rumination and lying times in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item, unit	Treatment (Trt)		SEM	P-value ²				
	CTR	3-NOP		Trt	T	Trt×T	P	Trt×P
Eating time, min/d	169.1	168.6	3.53	0.93	<0.01	0.33	<0.01	0.75
Visits to the bins, n/d	28.41	28.72	0.75	0.77	0.15	0.04	0.31	0.10
Meals, n/d	5.64	5.70	0.09	0.65	<0.01	0.16	<0.01	0.93
Meal size, ³ kg DM/meal	4.63 (4.50-4.77)	4.51 (4.38-4.64)	-	0.19	<0.01	0.11	<0.01	0.02
Meal length, min/meal	31.08	30.95	0.62	0.88	0.01	0.06	<0.01	0.69
Rumination time, ⁴ min/d	523	527	11.8	0.81	0.30	0.39	0.07	0.46
Lying time, ³ min/d	707 (684-730)	710 (688-733)	-	0.83	<0.01	0.22	<0.01	0.99

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP.

²P-values of the main effects of the model: Trt = Treatment (CTR vs 3-NOP); T = Timepoint (repeated measures = 21, 42, and 63 d); P = Phase (P1 vs P2)

³Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95% CI

⁴Means without adjustment for the covariate measurement (adjustment period)

Supplemental Table S4. Rumen fluid VFA molar proportions in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹ in phase 1 (P1) or 2 (P2) of the study

Item	CTR	3-NOP	SEM	<i>P</i> -value
P1				
Butyrate	8.72	12.14	0.354	<0.01
Valerate ²	1.17 (1.02-1.34)	1.86 (1.61-2.12)	-	<0.01
Caproate	0.41	0.86	0.068	<0.01
P2				
Butyrate	10.64	11.46	0.315	0.07
Valerate ²	1.10 (0.99-1.22)	1.31 (1.19-1.48)	-	0.02
Caproate	0.49	0.63	0.048	0.05

¹Dose of 3-NOP: 60 mg/kg DM; means adjusted for the covariate measurement (adjustment period) unless otherwise stated; Phase 1 (P1: summer): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 13 for the CTR and n = 14 for the 3-NOP.

²Log10-transformed and presented as back-transformed LSM. Values between parentheses indicate 95%

3 Paper/Manuscript II

Feeding behavior and meal patterns in mid-lactating dairy cows supplemented with 3-nitrooxypropanol in different environmental conditions

E. Fiorbelli¹, L. Cattaneo¹, M. Moschini¹, E. Trevisi^{1,2*}, I. Mueller³, and A. Gallo^{1,2}

¹Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

²Romeo and Enrica Invernizzi Research Center for Sustainable Dairy Production of the Università Cattolica del Sacro Cuore (CREI), 29122 Piacenza, Italy

³dsm-firmenich, 3131 Getzersdorf, Austria

*Corresponding author: Erica Fiorbelli, erica.fiorbelli@unicatt.it

ABSTRACT

This study investigated the impact of 3-nitrooxypropanol (3-NOP) on the feeding behavior of lactating Holstein dairy cows in two phases, one conducted in summer (P1) and the other in fall (P2). In the experiment, thirty mid-lactation multiparous cows were included in a longitudinal study with repeated measurements and a completely randomized design. This included an initial 14-day covariate period, followed by a 63-day treatment period, and a 14-day washout period. Cows were randomly assigned to one of two treatment groups: (i) the control group (CTR), receiving a corn silage-based diet ad libitum with no supplementation once daily, and (ii) the 3-NOP group, receiving the same diet supplemented with 60 mg/kg DM of 3-NOP. Individual feed intake and visits were recorded automatically using electronic feed bins. Meals were defined using a log-transformed method, from which a secondary variable was derived, including eating time (min/meal), meals per day, meal size (kg DM/meal), visits per meal, eating rate (g of DM/min), and time between meals (min). Intraclass correlation coefficients and entropy analyses were used to assess the daily repeatability and daily hour variability of feeding behavior variables. Visits and eating time per day were moderately repeatable within cows, whereas daily meals and DMI were less repeatable, with descriptively lower DMI repeatability under 3-NOP (Table 2) in each phase. The daily hourly distribution of visits and meals was summarized using normalized Shannon entropy. Across both phases, the treatment did not change visit and meal hour distribution, whereas season, particularly in P2, had a significant effect on temporal patterns, reflected by lower entropy.

Keywords: dairy cattle, methane inhibition, meal patterns, eating behavior, entropy

List of nonstandard abbreviations used: 3-NOP = 3-nitrooxypropanol; CTR = control group; ICC = intraclass correlation coefficient; Hnorm = normalized Shannon entropy; MCR = methyl coenzyme M reductase; P1 = phase 1; P2 = phase 2; RIC = roughage intake control; THI = temperature-humidity index.

INTRODUCTION

Enteric methane (CH₄) emissions from ruminants are major contributors to agricultural GHG emissions. Among the available mitigation strategies, 3-nitrooxypropanol (3-NOP) has emerged as a targeted inhibitor of methanogenesis that consistently reduces CH₄ formation in dairy cattle without compromising productivity when properly dosed and delivered (Hristov et al., 2015; Duin et al., 2016; Kebreab et al., 2023). The mechanism of action of 3-NOP involves the rapid inhibition of methyl coenzyme M reductase (MCR, EC 2.8.4.1), an enzyme that catalyzes the final step in methanogenesis carried out only by methanogenic archaea, thereby reducing CH₄ production (Duin et al., 2016). Multiple in vivo and in vitro trials have been conducted to test 3-NOP, and an average reduction of CH₄ of 25-35% has been reported, with dose and diet composition modulating the response (Hristov et al., 2015; Kebreab et al., 2023). In a recent trial that used a corn silage-based diet with a high starch content (29%), we observed a 46% reduction in CH₄ emissions (Moschini et al., 2025). Although mitigation efficacy is well documented, less attention has been paid to 3-NOP effect on DMI and feeding behavior (i.e., meal patterns and bout dynamics).

The DMI is a critical factor in the inhibition of methanogenesis in dairy cows, as the quantity of nutrient intake significantly influences rumen fermentation and CH₄ production. Intake is determined by various dietary factors including nutrient composition, fat and protein levels, carbohydrate and protein degradation rates, and physical characteristics (Albright, 1993; Allen, 2000; Kononoff et al., 2003). Additionally, the feeding behavior of animals influences their daily DMI (Nielsen, 1999).

Feeding is characterized by distinct visits to the feed bunk, which can be collectively defined as a meal. The temporal pattern of these events can be precisely quantified by employing the log transformation of time between visit intervals, which serves to differentiate within-visit from between-visit intervals and establish objective criteria for defining meals (Tolkamp & Kyriazakis,

1999; Tolkamp et al., 1998). In dairy systems, automated intake monitoring and established methods (e.g., log-survivor or log-frequency approaches) enable reproducible characterization of visits, visit length, intervals between visits, meal frequency, and meal size. These metrics can be affected by multiple factors, including diet composition, feed additives, environmental conditions and stressors, animal physiological and health status, herd-level management strategies, and social dynamics within a group (DeVries et al., 2003, 2011; Grant and Albright, 2000). Understanding whether 3-NOP modifies these patterns is important because changes in feeding temporalities can affect rumen substrate flow and hydrogen (H₂) dynamics, with potential implications for both CH₄ production and nutrient partitioning in dairy cows.

Research indicates that responses to 3-NOP intake vary and appear to be influenced by dietary composition, dosage of the additive, and animal and environmental factors. A study conducted to determine the short-term effect of 3-NOP on DMI in lactating cows did not observe a negative effect of 3-NOP on DMI overall, although the highest tested inclusion (120 mg/kg DM) reduced the DMI after a range of ten hours of exposure compared with lower doses (30, 60, and 90 mg/kg DM), but remained similar to the control (Melgar et al., 2020). In beef cattle, some studies have reported a transient preference for 3-NOP supplemented diets, with acclimation occurring within approximately a week and with no long-term effect on intake after adaptation (Lee et al., 2019). Conversely, dose-response and long-term evaluations indicate robust CH₄ abatement without significant effects on DMI or milk performance in dairy cows when 3-NOP is added to the TMR (Hristov et al., 2015; Van Wesemael et al., 2019; Melgar et al., 2020). As demonstrated in an analysis by Hristov and Melgar (2020), the effect of 3-NOP on CH₄ emissions is not constant throughout the day, but fluctuates in relation to feeding activity, being more pronounced shortly after feed delivery and less evident before meals. In a long-term study conducted by Schilde et al. (2021), a gradual loss of persistence of the 3-NOP effect was observed with low-concentrate diets, suggesting that different feed intakes play a

crucial role. This result highlights the importance of integrating feeding behavior measures into CH₄ mitigation studies and interpreting the variability in treatment responses.

These observations motivated a deeper, meal-level assessment of ingestive behavior under 3-NOP treatment rather than relying solely on daily DMI. Recent sensor-based studies have linked ingestive and rumination behaviors with variations in CH₄ levels during different physiological phases, highlighting the importance of high-resolution behavioral metrics in interpreting mitigation responses (Krištolaitytė et al., 2025). Feeding behavior is also regulated by environmental factors. Elevated temperatures and humidity (summarized by the temperature-humidity index [THI]) shift the diurnal distribution of meals, often suppressing daytime feeding and increasing nighttime intake, which can alter rumen fermentation profiles and gaseous outputs (Polsky & von Keyserlingk, 2017). Therefore, evaluating 3-NOP effects on feeding behavior under contrasting environmental conditions is timely and relevant for on-farm implementation.

The present study analyzed data from an *in vivo* trial conducted by Moschini et al. (2025) that investigated the effects of 3-NOP inclusion (60 mg/kg DM) under different environmental conditions. During summer, cows receiving 3-NOP exhibited a reduction in DMI (5.7%) and meal size, whereas feed efficiency increased by 4.8% and dairy efficiency increased by 4% (Moschini et al., 2025). These differences were not observed during fall. Specifically, this study provides a descriptive analysis of feeding behavior and meal structure in mid-lactation dairy cows supplemented with 3-NOP under different environmental conditions using a log-transform method to determine meal and bout parameters. We hypothesized that (1) 3-NOP could shift the visit organization under the meal criterion and (2) environmental heat load would interact with 3-NOP delivery to modulate daily visits and meal patterns.

MATERIALS AND METHODS

All procedures involving animals were performed in accordance with the guidelines of the Committee for the Protection and Welfare of Experimental Animals of the Catholic University of the Sacred Heart. The study was approved by the Italian Ministry of Health (355/2022-PR).

This study is a follow-up of an *in vivo* trial investigating the effects of 3-NOP in dairy cows fed a high-corn-silage diet under different environmental conditions (Moschini et al., 2025), where full details about experimental design and animal management can be found. In the present study, we specifically examined feeding behavior and criteria for meals and visits.

Animal management and experimental design

Briefly, two longitudinal experiments were conducted at CERZOO (Piacenza, Italy) from May 23rd to August 21st, 2022 (Phase 1; **P1**), and from September 20th to December 19th, 2022 (Phase 2; **P2**). The average environmental conditions were 25.7°C, 58.9% relative humidity, and THI 72.8 (P1) and 11.7°C, 86.2% relative humidity, THI 52.8 (P2). The cows were housed in a free-stall barn equipped with a Roughage Intake Control (**RIC**) system (Hokofarm Group, The Netherlands) for individual feed intake and eating behavior monitoring and one GreenFeed unit (C-Lock Inc., Rapid City, SD, USA) for continuous CH₄, carbon dioxide (**CO₂**), and H₂ measurements.

In each phase, 30 multiparous Holstein cows in mid- and late-lactation with no clinically diagnosed disease in the three weeks before enrollment were used. At enrollment, mean ($\pm$ SD) DIM, milk yield, milk fat, and milk protein were 190 ± 72 d, 36.8 ± 7.5 kg/d, $3.73 \pm 0.33\%$, and $3.47 \pm 0.19\%$ (P1) and 158 ± 74 d, 36.8 ± 4.6 kg/d, $3.78 \pm 0.65\%$, and $3.28 \pm 0.28\%$ (P2). Cows were group-housed with free access to water and fed *ad libitum* (target 5–10% refusal) a corn silage-based diet formulated to meet or exceed NRC (2001) requirements for mid-lactation cows. Each phase consisted

of a 10-d adaptation (no data collection), 14-d covariate period (common diet), 63-d treatment period, and 14-d washout.

The enrolled cows were randomly assigned to one of two groups: (i) the treated group (**3-NOP**; n = 15/phase), which received the basal diet supplemented with 600 mg/kg DM of Bovaer®10 (dsm-firmenich Animal Nutrition & Health, Kaiseraugst, Switzerland) containing 100 g/kg of 3-NOP on silicon dioxide + 1,2-propanediol) included in a premix, and (ii) the control group (**CTR**; n = 15/phase), which received the same basal diet supplemented with a placebo (i.e., silicon dioxide + 1,2-propanediol) premix. To avoid cross-contamination, the control TMR was mixed and distributed before preparation of the 3-NOP TMR. The TMR was delivered once daily at 0900 h using a self-moving mixer (Rotomix 5000).

Dry matter intake, Feeding behavior and meal-bout definition

Daily DMI was calculated as the recorded intake between consecutive TMR deliveries corrected for the DM content of the composite samples. Bait-pellet intake, corrected for DM at the GreenFeed unit (automatically recorded by the device), was added to obtain the total DMI.

Daily feeding behavior traits were obtained from electronic bin records at the individual animal level. A “visit” was defined as entry into a gate feeder, followed by a negative weight change. The meal criteria were derived using the Tolkamp and Kyriazakis (1999) log-transform approach, in which the time between visits expressed in seconds was log10-transformed and modeled as a fitted 3-fold density function based on three Gaussian functions (exact maximum likelihood; *mixdist* package of Macdonald and Du, 2018). These three functions represent within-meal, within-meal, including drinking, and between-meal intervals (Tolkamp and Kyriazakis, 1999). The intersection of the second Gaussian and third curve was used to determine the meal criterion. The meal criteria were calculated for each group (Figure 1). The secondary parameters were calculated after the application of meal criteria, specifically meal frequency (meals/day), meal length as a total of visit time

(min/meal), meal size as the sum of visit intake (kg DM/meal), and eating rate (meal size/meal length).

Preprocessing of collected data

Raw data obtained from the RIC system were cleaned before calculating the meal criteria. Specifically, observations with a visit size that was negative or zero and a feeding rate > 500 g/min were excluded from the dataset. Days with RIC malfunction or erratic behavior due to extreme weather were excluded ($n = 5$). Subsequently, data were screened for outliers. The threshold for identifying outliers was established at 1.5 times the interquartile range beyond the 0.25 and 0.75 quantiles, respectively. Prior to conducting statistical analyses, the data were assessed for normality using the Shapiro-Wilk test. Variables that did not conform to a normal distribution were subjected to a logarithmic transformation (meal size).

Statistical analysis

The final dataset comprised of 60 mid-lactating dairy cows ($P1 = 30$; $P2 = 30$). In accordance with the study by Moschini et al. (2025), which aimed to observe and correlate various variables, the three cows excluded due to a lack of persistence in the lactation curve ($n=2$) and foot problems ($n=1$) were retained here because their intake patterns were regular; inclusion did not alter model diagnostics or results. Descriptive statistics are reported as mean $\pm$ SD. The normality of the model residuals was checked using the Shapiro-Wilk test and Q-Q plots; right-skewed variables (e.g., meal size) were analyzed on the log₁₀ scale and, when presented, back-transformed with the corresponding 95% CI.

Two statistical methods were combined: 1) a repeatability framework (intraclass correlation coefficient) to test whether feeding behavior is a cow-specific trait or treatment/season-sensitive response and 2) an information-theoretic metric (entropy) to quantify visit and meal distribution

across 24-h. This allows the evaluation of whether 3-NOP reorganizes feeding behavior and whether the season interacts with 3-NOP to modulate daily patterns.

The repeatability of feeding behavior within individual cows throughout the treatment period was assessed using the intraclass correlation coefficient (ICC) derived from null (intercept only) linear mixed models. These models were fitted separately for each phase (P1 and P2) and treatment (CTR and 3-NOP).

$$y_{ij} = \beta_0 + u_i + \varepsilon_{ij}$$

with random intercept for cow (u_i) and residual error (ε_{ij}). The adjusted ICC was computed as follows:

$$ICC = \frac{\sigma^2_{cow}}{\sigma^2_{cow} + \sigma^2_{residual}}$$

Model singularity was checked prior to estimation, and the 95% CI for ICC was obtained using parametric bootstrapping (2,000 replicates). The results were interpreted as the proportion of total variance attributable to between-cow differences (i.e., repeatability). Analyses were conducted in R (R Foundation for Statistical Computing, Vienna, Austria) using the package *lme4* (Bates et al., 2015) and performance for mixed model fitting and ICC, and *dplyr/lubridate* for data wrangling; mixture modeling used *mixdist*.

To quantify whether treatment and season affected the daily temporal distribution of visits and meals, we computed the Shannon entropy of visits and meals at the cow-day level. For each cow and day, the RIC events were distributed into 24 hourly classes based on entry time, and counts for missing hours were set to zero. Let n_h be the count in hours (h). Entropy was calculated as follows:

$$H = - \sum_{h=0}^{23} p_h \log_2 p_h$$

and normalized to (0,1) as $H_{\text{norm}} = H / \log_2 (24)$, values near 0 indicate activity concentrated over a few hours, and values near 1 indicate activity spread uniformly over the day. We used the Miller-Madow bias-corrected estimator for H (R package *entropy* by Hausser and Strimmer, 2025), which is robust when several hourly bins have zero counts.

Daily H_{norm} values for visits and meals were analyzed using linear mixed models, including treatment (CTR, 3-NOP), phase (P1 summer, P2 fall), and their interaction as fixed effects, and a random intercept for cows was used to account for repeated measures. Because consecutive days within a cow can be autocorrelated, we specified a first-order autoregressive structure, AR(1), on days within a cow. We reported the data as LSM $\pm$ SE according to treatment within each phase. The observation unit was cow/day, and the implementation in R used *nlme* (Pinheiro and Bates, 2025) for the AR(1) models and *emmeans* (Lenth, 2025) for marginal means.

RESULTS

During the treatment period, the RIC recorded were 26,697 (CTR) and 26,477 (3-NOP) visits in P1, and 21,366 (CTR) and 21,496 (3-NOP) visits in P2 (Figure 1).

Feeding behavior and meal criterion

The descriptive statistics of feeding behavior are presented in Table 1, and the average daily DMI between the groups during the entire week of trial at each phase is illustrated in Figure 2. In P1, the visit frequency was similar between the treatments, with an average of 29.0 ± 12.0 visits/d. In P2 cows in the 3-NOP group made 1.5 visits/d than CTR group. Across seasons, visits/d decreased numerically from P1 to P2 in both groups, with average of -13% and -17% for the CTR and 3-NOP groups, respectively.

The eating time per visit was higher for the 3-NOP group in each phase. Eating time per visit numerically increased from P1 to P2 with +1.6 min for the CTR (+28%) and +1.9 min for the 3-NOP group (+32%).

The visit size was smaller during the P1 phase in the 3-NOP group (-100 g DM/visit) but increased in P2 (+ 100 g DM/visit). From P1 to P2, the visit size increased in both groups, with average of +10% and +33% for the CTR and 3-NOP groups, respectively.

For the visit-eating rate, 3-NOP was lower than CTR in P1 (-11 g of DM/min; -6%) and slightly higher in P2 (+5 g of DM/min; +3%). Seasonally, eating rates declined from P1 to P2 in both treatments, with average of -13% and -5% for the CTR and 3-NOP groups, respectively (Table 1). The meal criterion (Figure 1) was the inter-visit interval threshold separating within- from between-meal intervals (CTR: 42.16 min; 3-NOP: 48.31 min).

The daily hour distribution for visits and meals showed clear peaks around the morning feeding (09:00) and late afternoon hours during both phases (Figure 3).

Meal pattern metrics

The descriptive meal pattern metrics are shown in Table 1 and were closely aligned between treatments (Table 1). In P1 compared with CTR, 3-NOP showed a numerically higher meal frequency (n/d) (+1.8%), with no difference in visits per meal; the interval between meals was 6.6 min shorter in 3-NOP. Overall, this reflected a slight shift toward more frequent meals that were slightly closer together.

In P2, the 3-NOP group had fewer meals per day (-5.5%), but meals were slightly larger (+2.2%), longer (+1.0%), and had a longer meal interval (+16.8 min) compared to the CTR group.

In general, the seasonal change from P1 to P2 showed a decline in meal frequency in both groups (CTR -3.5%; 3-NOP -10.3%). Meal size increased by ~300 g/meal and meal duration by ~4.3

min an average. Visits per meal decreased slightly (CTR -7.7%; 3-NOP -8.0%). The interval between the meals slightly increased in CTR (+4.6 min; +2.0%) and more in 3-NOP (+28.0 min; +12.2%) (Table 1).

Repeatability of feeding behavior

The ICC from the random-intercept models showed moderate within-cow repeatability (ICC > 0.40) for visit frequency and eating time, but low repeatability for meal frequency and DMI. Confidence intervals corresponded between treatments within each phase, showing no consistent effect of 3-NOP on repeatability (Table 2).

The daily normalized entropy (0-1) of feeder visits and meals did not differ between cows fed 3-NOP and CTR. Across treatments, Hnorm declined from P1 to P2 for both visits and meals (phase effect, $P < 0.001$), indicating a less uniform daily distribution of feeding events in P2. No treatment per phase interactions were observed for visits or meals ($P = 0.91$ and 0.79 , respectively). The values are expressed as $LSM \pm SE$, as reported in Table 3.

DISCUSSION

This study focused on describing the behavior of dairy cows during in vivo evaluation of the inclusion of 3-NOP in their diet based on corn silage as the main forage in two phases: the first carried out in summer and the second in the fall of 2022.

Visit and meal pattern

In both intensive and extensive production systems, the DMI has been consistently identified as the primary factor influencing enteric CH₄ emissions. However, fermentation of specific nutrients also plays a crucial role in rumen methanogenesis (Hristov et al., 2024). In confined systems, the

amount of feed intake *ad libitum* can be influenced by factors such as taste, feeding behavior, and social dynamics. Since the patterns of CH₄ production are closely associated with the amount and type of feed consumed, it is crucial to clearly define and report any factors affecting an animal's diet, including the quantity, size, and frequency of meals, when evaluating feed additives that reduce CH₄ emissions (Hristov et al., 2025). In this study, the descriptive analysis showed that 3-NOP produced only minor shifts in ingestive behavior relative to CTR, whereas seasonal conditions (P1 vs. P2) were associated with more pronounced differences in visit and meal organization. A study conducted by Maigaard et al. (2024) reported a 13.4% reduction in DMI with the inclusion of 3-NOP at a concentration of 80 mg/kg DM, in which the eating behavior was modified, characterized by more frequent but smaller meals and a slower eating rate. Nevertheless, a few studies have shown that 3-NOP can reduce CH₄ emissions without reducing DMI in lactating cows when added to the TMR (Hristov et al., 2015; Van Wesemael et al., 2019; Kebreab et al., 2023). Short-term intake responses can be variable (e.g., small decreases at certain doses or in early lactation), but prevailing evidence in dairy systems indicates no sustained negative effect on intake or milk yield (Melgar et al., 2020; Hristov et al., 2015; Van Wesemael et al., 2019).

The seasonal patterns observed in this study are consistent with the known effects of heat stress on dairy cattle behavior. Relative to thermoneutral conditions, heat load typically reduces daytime intake and shifts feeding toward cooler hours, thereby altering meal timing and size (Polsky & von Keyserlingk, 2017). In our dataset, the transition from P1 (higher THI) to P2 (lower THI) was accompanied by fewer but larger meals with greater time per meal, consistent with the redistribution of eating effort across the day. The daily hourly distributions for visits and meals exhibited peaks around feed delivery and late afternoon or evening, related to milking time, in both phases (Figure 3), in line with diurnal patterns of feeding in lactating cows (DeVries et al., 2003), and daily rhythms

of feeding and metabolism of dairy cattle without changing total DMI, describing when—not only how much—cows eat (Niu et al., 2014).

In terms of methodology, the log-transform approach used in this study resulted in group-specific meal criteria that were closely aligned between groups (Figure 1). The meal metrics (frequency, size, and duration) remained close between treatments, reinforcing that any 3-NOP effect on feeding microstructure was small compared with the seasonal context.

Repeatability of feeding behavior

Conceptually, the ICC, sometimes called the variance partition coefficient or repeatability, expresses the share of variance attributable to cow identity. It can also be interpreted as the expected correlation between two days from the same cow (Gelman and Hill, 2007; Hox, 2010). Repeatability was assessed using random intercept models for each animal. Gaussian models were employed for the primary analysis of visits and meals (n/d), the DMI, and eating time. In our data, visits/d and eating time showed moderate repeatability (visits/d $\approx$ 0.54-0.68; eating time/d $\approx$ 0.43-0.61), indicating that the temporal routine (how often cows approach the feeder and how much time they spend eating) was a relatively stable individual trait across the 63-d period of treatment between the treatment and phase (Table 2). In contrast, meals/d and, in particular, daily DMI exhibited low to moderate repeatability (meals/d $\approx$ 0.17-0.35; DMI/d $\approx$ 0.15-0.50), implying that most variability in these traits occurred within cows across days rather than between cows. Notably, DMI repeatability was lower under 3-NOP (down to 0.15 in P2), whereas eating time repeatability tended to be higher with 3-NOP, particularly in P2, consistent with a divergence between time and intake volume: two days from the same treatment group 3-NOP, the cow are expected to be quite similar for time spent eating but only weakly similar for DMI. Although several confidence intervals overlap, the differences are

descriptive, and the pattern is consistent with our meal-microstructure results and the seasonal shift from P1 to P2.

This pattern is relevant for emission phenotyping because short GreenFeed tests can be influenced by behavioral state and habituation, and accounting for behavior improves data reliability, even when average CH₄ output appears unchanged across training and testing sessions (Smith et al., 2024).

As reported in the main trial by Moschini et al. (2025) in P1, cows fed 3-NOP had reduced DMI (~5.7%) and meal size, whereas feed and dairy efficiencies increased, and no mean effect on DMI was detected in P2. Taken together, our ICCs indicate that these mean shifts occurred without a reorganization of the daily feeding routine (moderate ICC for visits and eating time) and that under 3-NOP, the amount consumed varied more day-to-day (low ICC for DMI), a pattern compatible with efficiency gains arising from how intake is utilized rather than from time reallocation at the feeder. In P2, the absence of a DMI difference but persistently low DMI repeatability suggests that intake fluctuated around a similar mean, while the routine remained stable, consistent with seasonal (heat-load) modulation rather than the treatment-driven restructuring of behavior. Overall, the combination of the mean responses of Moschini et al. (2025) and ICC value supports the view that 3-NOP can deliver mitigation and efficiency benefits without compromising feeding temporalities, and underscores the need to average DMI over multiple days while using visits and eating time as stable covariates in field evaluations.

Diurnal pattern of feeding events

To assess whether treatment or season altered the timing of activity across the 24-h cycle, we analyzed the Hnorm of visits and meals at the cow-day level, where values near 0 indicate activity concentrated in a few hours and values near 1 indicate activity spread uniformly over the day (Table 3). Treatment had no main effect on entropy for either visits or meals ($P > 0.05$), and no interaction

between treatment and phase was observed. In contrast, the phase was significant for both traits ($P < 0.001$), with a lower H_{norm} in P2 than in P1, indicating a more temporally concentrated pattern in the fall. These entropy results align with the hour-of-day profiles (Figure 3), and both treatments showed clear peaks around the morning TMR delivery and a secondary peak in the late afternoon or evening related to the milking time, with narrower curves in P2 than in P1. The substantial similarity between CTR and 3-NOP within each phase reinforces that the treatment did not retime feeding activity. Instead, the seasonal context (summer vs. fall) governed the degree to which visits and meals were spread across the day. Collectively, Table 3 and Figure 3 indicate that diurnal feeding temporalities were season-dependent but treatment-invariant, with a closer concentration of activity in fall and broadly similar timing under the CTR and 3-NOP conditions.

CONCLUSIONS

This study provides an integrated description of the feeding behavior of mid-lactation dairy cows supplemented with 3-NOP under different seasonal conditions. Across both phases, 3-NOP produced only minor changes in visit and meal distribution, whereas the season consistently shaped feeding temporalities: from summer to fall, cows performed fewer, longer meals with slightly longer intervals, and more concentrated hour-of-day profiles. The repeatability results showed that daily visits and eating time were moderately stable within cows, whereas meals and DMI were less repeatable. Notably, intake exhibited lower repeatability under the additive, suggesting greater day-to-day variability in volume consumed despite stable routines. Entropy analyses confirmed a concentrated distribution in the fall, with no treatment or treatment-by-phase effects, indicating that the additive did not change feeding activities. Overall, mitigation with 3-NOP did not affect the temporal organization of feeding, whereas seasonal context had a major impact on the daily hourly intake distribution.

NOTES

This study was funded by DSM-Firmenich (Kaiseraugst, Switzerland). Research activities in the Department of Animal Science, Food, and Nutrition of the Università Cattolica del Sacro Cuore were supported by the Romeo and Enrica Invernizzi Foundation. I. Mueller is an employee of the dsm-firmenich. The authors declare that they have no conflicts of interest. E. Fiorbelli was supported by an Agrisystem Ph.D. fellowship (UNICATT, Piacenza, Italy). During manuscript preparation, the first author used Jenni AI to improve readability, and subsequently reviewed and edited the content, assuming full responsibility.

REFERENCES

- Albright, J. L. 1993. Feeding Behavior of Dairy Cattle. *J. Dairy Sci.* 76:485–498. [https://doi.org/10.3168/jds.S0022-0302\(93\)77369-5](https://doi.org/10.3168/jds.S0022-0302(93)77369-5).
- Allen, M. S. 2000. Effects of diet on short-term regulation of feed intake by lactating dairy cattle. *J. Dairy Sci.* 83:1598–1624. [https://doi.org/10.3168/jds.S0022-0302\(00\)75030-2](https://doi.org/10.3168/jds.S0022-0302(00)75030-2).
- Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* 67:1–48. <https://doi.org/10.18637/jss.v067.i01>
- de Mol, R., A. Bannink, J. Dijkstra, N. Walker, and S. van Gastelen. 2024. The effect of feeding and visiting behavior on methane and hydrogen emissions of dairy cattle measured with the GreenFeed system under different dietary conditions. *J. Dairy Sci.* 107:7769–7785. <https://doi.org/10.3168/jds.2024-24673>

- DeVries, T. J., M. A. G. von Keyserlingk, and K. A. Beauchemin. 2003. Diurnal feeding pattern of lactating dairy cows. *J. Dairy Sci.* 86:4079–4082.
- DeVries, T. J., L. Holtshausen, M. Oba, and K. A. Beauchemin. 2011. Effect of parity and stage of lactation on feed sorting behavior of lactating dairy cows. *J. Dairy Sci.* 94:4039–4045. <https://doi.org/10.3168/jds.2011-4264>.
- Duin, E. C., T. Wagner, S. Shima, D. Prakash, B. Cronin, D. R. Yáñez-Ruiz, S. Duval, R. Rümblei, R. T. Stemmler, R. K. Thauer, and M. Kindermann. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proc. Natl. Acad. Sci. USA* 113:6172–6177. <https://doi.org/10.1073/pnas.1600298113>
- Grant, R. J., and J. L. Albright. 2000. Feeding Behaviour. Pages 365-382 in *Farm animal metabolism and nutrition*. Ed. J.P.F. D'Mello. CABI Publishing, New York
- Gelman, A., and J. Hill. 2006. *Data Analysis Using Regression and Multilevel/Hierarchical Models*. Cambridge University Press, Cambridge, UK.
- Hausser, J., and K. Strimmer. 2025. entropy: Estimation of entropy, mutual information and related quantities. R package version 1.3.2. <https://doi.org/10.32614/CRAN.package.entropy>
- Hox, J. J. 2010. *Multilevel analysis: Techniques and applications*. 2nd ed. Routledge, New York, NY. <https://doi.org/10.4324/9780203852279>

- Hristov, A. N., J. Oh, F. Giallongo, T. W. Frederick, M. T. Harper, H. L. Weeks, A. F. Branco, P. J. Moate, M. H. Deighton, S. R. O. Williams, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc. Natl. Acad. Sci. USA* 112:10663–10668. <https://doi.org/10.1073/pnas.1504124112>
- Hristov, A.N., and A. Melgar, 2020. Short communication: Relationship of dry matter intake with enteric methane emission measured with the GreenFeed system in dairy cows receiving a diet without or with 3-nitrooxypropanol. *Animal*. Volume 14. Supplement 3. Pages s484-s490, ISSN 1751-7311, <https://doi.org/10.1017/S1751731120001731>
- Hristov, A. N., A. Bannink, M. Battelli, A. Belanche, M. C. Cajarville Sanz, G. Fernandez-Turren, F. Garcia, A. Jonker, D. A. Kenny, V. Lind, S. J. Meale, D. Meo Zilio, C. Muñoz, D. Pacheco, N. Peiren, M. Ramin, L. Rapetti, A. Schwarm, S. Stergiadis, K. Theodoridou, E. M. Ungerfeld, S. Van Gastelen, D. R. Yáñez-Ruiz, S. M. Waters, and P. Lund. 2025. Feed additives for methane mitigation: Recommendations for testing enteric methane-mitigating feed additives in ruminant studies. *J. Dairy Sci.* 108:322–355. <https://doi.org/10.3168/jds.2024-25050>
- Kebreab, E., A. Bannink, E. M. Pressman, N. Walker, A. Karagiannis, S. van Gastelen, and J. Dijkstra. 2023. A meta-analysis of effects of 3-nitrooxypropanol on methane production, yield, and intensity in dairy cattle. *J. Dairy Sci.* 106:927–936. <https://doi.org/10.3168/jds.2022-22211>

- Kononoff, P. J., A. J. Heinrichs, and H. A. Lehman. 2003. The effect of corn silage particle size on eating behavior, chewing activities, and rumen fermentation in lactating dairy cows. *J. Dairy Sci.* 86:3343–3353. [https://doi.org/10.3168/jds.S0022-0302\(03\)73937-X](https://doi.org/10.3168/jds.S0022-0302(03)73937-X).
- Krištolaitytė, J., K. Džermeikaitė, A. Rutkauskas, G. Šertvytytė, G. Lembovičiūtė, S. Arlauskaitė, A. Girdauskaitė, V. Juškienė, R. Juška, W. Baumgartner, and R. Antanaitis. 2025. Impact of blood metabolic profile and ingestive behaviours registered with noseband sensor on methane emission during transition period in dairy cows. *Life* 15:760. <https://doi.org/10.3390/life15050760>
- Lee, C., S. H. Kim, K. Beauchemin, P. Celi, and S. Duval. 2019. Short-term eating preference of beef cattle fed high-forage or high-grain diets supplemented with 3-nitrooxypropanol. *Animals (Basel)* 10:64. <https://doi.org/10.3390/ani10010064>
- Lenth, R. V. 2025. emmeans: Estimated marginal means, aka least-squares means. R package. <https://CRAN.R-project.org/package=emmeans>
- Lüdecke, D., M. S. Ben-Shachar, I. Patil, P. Waggoner, and D. Makowski. 2021. performance: An R package for assessment, comparison and testing of statistical models. *J. Open Source Softw.* 6:3139. <https://doi.org/10.21105/joss.03139>
- Macdonald, P., and J. Du. 2018. mixdist: Finite Mixture Distribution Models. R package version 0.5–5.

- Maigaard, M., Weisbjerg, M. R., Johansen, M., Walker, N., Ohlsson, C., and P. Lund. 2024. Effects of dietary fat, nitrate, and 3-nitrooxypropanol and their combinations on methane emission, feed intake, and milk production in dairy cows. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2023-23420>.
- Melgar, A., K. Nedelkov, C. M. M. R. Martins, K. C. Welter, X. Chen, M. T. Harper, S. Duval, and A. N. Hristov. 2020b. Short communication: Short-term effect of 3-nitrooxypropanol on feed dry matter intake in lactating dairy cows. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2020-18331>
- Miller, G. A. 1955. Note on the bias of information estimates. In: *Information theory in psychology: Problems and methods II-B*, p. 95–100.
- Moschini M., Frolidi F., Piccioli-Cappelli F., P. Bani, Fiorbelli E., Gallo A., Mueller I., Tamassia L.F.M., Zihlmann R., Bulgarelli P., Agazzi S., Trevisi E., and L. Cattaneo. 2025. Effects of 3-nitrooxypropanol on enteric methane emissions and milk production characteristics in dairy cows fed a high corn-silage diet in different environmental conditions. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2025-26640> (in press)
- National Research Council (NRC). 2001. *Nutrient requirements of dairy cattle*. 7th rev. ed. National Academies Press, Washington, DC. <https://doi.org/10.17226/9825>
- Nielsen, B. L. 1999. On the interpretation of feeding behaviour measures and the use of feeding rate as an indicator of social constraint. *Appl. Anim. Behav. Sci.* 63:79-91. [https://doi.org/10.1016/S0168-1591\(99\)00003-9](https://doi.org/10.1016/S0168-1591(99)00003-9).

- Niu, M., Y. Ying, P. A. Bartell, and K. J. Harvatine. 2014. The effects of feeding time on milk production, total-tract digestibility, and daily rhythms of feeding behavior and plasma metabolites and hormones in dairy cows. *J. Dairy Sci.* 97:7764–7776. <https://doi.org/10.3168/jds.2014-8261>
- Pinheiro, J., D. Bates, and R Core Team. 2025. nlme: Linear and nonlinear mixed effects models. R package version 3.1-168. <https://doi.org/10.32614/CRAN.package.nlme>
- Polsky, L., and M. A. G. von Keyserlingk. 2017. Invited review: Effects of heat stress on dairy cattle welfare. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2017-12651>
- R Core Team. 2024. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Schilde, M., von Soosten, D., Hüther, L., Meyer, U., Zeyner, A., & Dänicke, S. (2021). Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows. *Archives of Animal Nutrition*, 75(2), 79–104. <https://doi.org/10.1080/1745039X.2021.1877986>
- Shannon, C. E. 1948. A mathematical theory of communication. *Bell Syst. Tech. J.* 27:379–423 (and 623–656). <https://doi.org/10.1002/j.1538-7305.1948.tb01338.x>
- Shapiro, S. S., and M. B. Wilk. 1965. An analysis of variance test for normality (complete samples). *Biometrika* 52:591–611. <https://doi.org/10.2307/2333709>

- Smith, O. A., C. M. Rochus, C. F. Baes, and N. van Staaveren. 2024. A note on dairy cow behavior when measuring enteric methane emissions with the GreenFeed emission monitoring system in tiestalls. *JDS Commun.* 5:395–399. <https://doi.org/10.3168/jdsc.2023-0451>
- Tolkamp, B. J., D. J. Allcroft, E. J. Austin, B. L. Nielsen, and I. Kyriazakis. 1998. Satiety splits feeding behaviour into bouts. *J. Theor. Biol.* 194:235–250. <https://doi.org/10.1006/jtbi.1998.0759>
- Tolkamp, B. J., and I. Kyriazakis. 1999. To split behaviour into bouts, log-transform the intervals. *Anim. Behav.* 57:807–817. <https://doi.org/10.1006/anbe.1998.1022>
- van Wesemael, D., L. Vandaele, B. Ampe, H. Cattrysse, S. Duval, M. Kindermann, V. Fievez, S. De Campeneere, and N. Peiren. 2019. Reducing enteric methane emissions from dairy cattle: Two ways to supplement 3-nitrooxypropanol. *J. Dairy Sci.* 102:1780–1787. <https://doi.org/10.3168/jds.2018-14534>

Table 1. Descriptive statistics of feeding visiting behavior and meals in mid-lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹

Item , unit	P1								P2							
	CTR				3-NOP				CTR				3-NOP			
	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th
Visit to the bin (n/d)	29.0	12.0	20.0	37.0	28.5	12.0	20.0	34.0	25.2	9.6	18.0	30.0	23.7	10.3	17.0	29.0
Eating time of visit (min/visit)	5.8	5.4	1.7	6.8	5.9	5.1	2.0	7.4	7.4	7.0	2.2	9.4	7.8	7.2	2.1	9.8
Visit size (kg of DM/visit)	1.0	0.9	0.3	1.2	0.9	0.8	0.3	1.1	1.1	1.0	0.3	1.3	1.2	1.1	0.3	1.4
Eating rate of visit (g of DM/min)	191.4	56.7	157.1	209.3	180.2	49.7	141.4	188.1	166.1	50.6	130.0	178.3	171.1	55.9	130.2	183.2
Eating time of meal (min/meal)	37.39	22.62	23.61	46.88	37.90	24.58	22.82	47.47	41.70	24.10	27.43	51.12	42.12	24.50	28.04	51.24
Meals (number)																
Full 24-h period	5.7	1.3	5.0	6.3	5.8	1.2	5.0	7.0	5.5	1.2	5.0	6.0	5.2	1.3	4.0	6.0
Between 0000 h and 07:00	1.1	0.8	1.0	2.0	1.3	0.7	1.0	2.0	1.1	0.7	1.0	2.0	1.0	0.7	1.0	1.0
Between 07:00 h and 16:00	2.5	0.7	2.0	3.0	2.4	0.6	2.0	3.0	2.5	0.7	2.0	3.0	2.3	0.7	2.0	3.0
Between 16:00 h and 00:00	2.4	0.8	2.0	3.0	2.3	0.8	2.0	3.0	2.2	0.7	2.0	3.0	2.3	0.8	2.0	3.0
Meal size (kg of DM/meal)	4.4	2.1	3.2	5.4	4.2	2.0	3.0	5.0	4.6	2.2	3.3	5.6	4.7	2.3	3.5	5.7
Visits per meal	5.2	3.4	3.2	6.3	5.0	3.2	3.1	6.1	4.8	3.1	2.9	5.9	4.6	3.0	2.8	5.7
Eating rate of meal (g of DM/min)	175.7	26.3	162.2	185.8	168.9	20.6	157.3	177.9	150.2	22.2	138.6	159.4	155.6	23.6	143.1	165.3
Time between the meal (min)	235.4	149.4	145.7	273.2	228.8	130.4	151.1	268.9	240.0	143.3	154.8	279.6	256.8	158.0	167.6	294.3

¹Dose of 3-NOP: 60 mg/kg DM; Phase 1 (P1: summer): sample sizes were n = 15 for CTR and n = 15 for 3-NOP. Phase 2 (P2: fall): The sample sizes were n = 15 for CTR and n = 15 for 3-NO.

Table 2. Intraclass correlation coefficient (ICC) for dry matter intake, eating time, visit (no./d) and meal (no./d) in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹, in phase 1 (P1, summer) or 2 (P2, fall) of the study

Item, unit	ICC (95% CI) ²	
	CTR	3-NOP
P1		
Visit/day , n/d	0.68 (0.45 to 0.80)	0.59 (0.36 to 0.73)
Meal/day , n/d	0.30 (0.14 to 0.45)	0.17 (0.06 to 0.28)
DMI/day, kg	0.49 (0.27 to 0.65)	0.26 (0.11 to 0.40)
Eating Time/day , min	0.58 (0.34 to 0.72)	0.61 (0.38 to 0.74)
P2		
Visit/day , n/d	0.54 (0.31 to 0.69)	0.55 (0.31 to 0.69)
Meal/day , n/d	0.31 (0.14 to 0.46)	0.35 (0.17 to 0.51)
DMI/day , kg	0.41 (0.20 to 0.57)	0.15 (0.05 to 0.25)
Eating Time/day , min	0.43 (0.21 to 0.59)	0.59 (0.36 to 0.73)

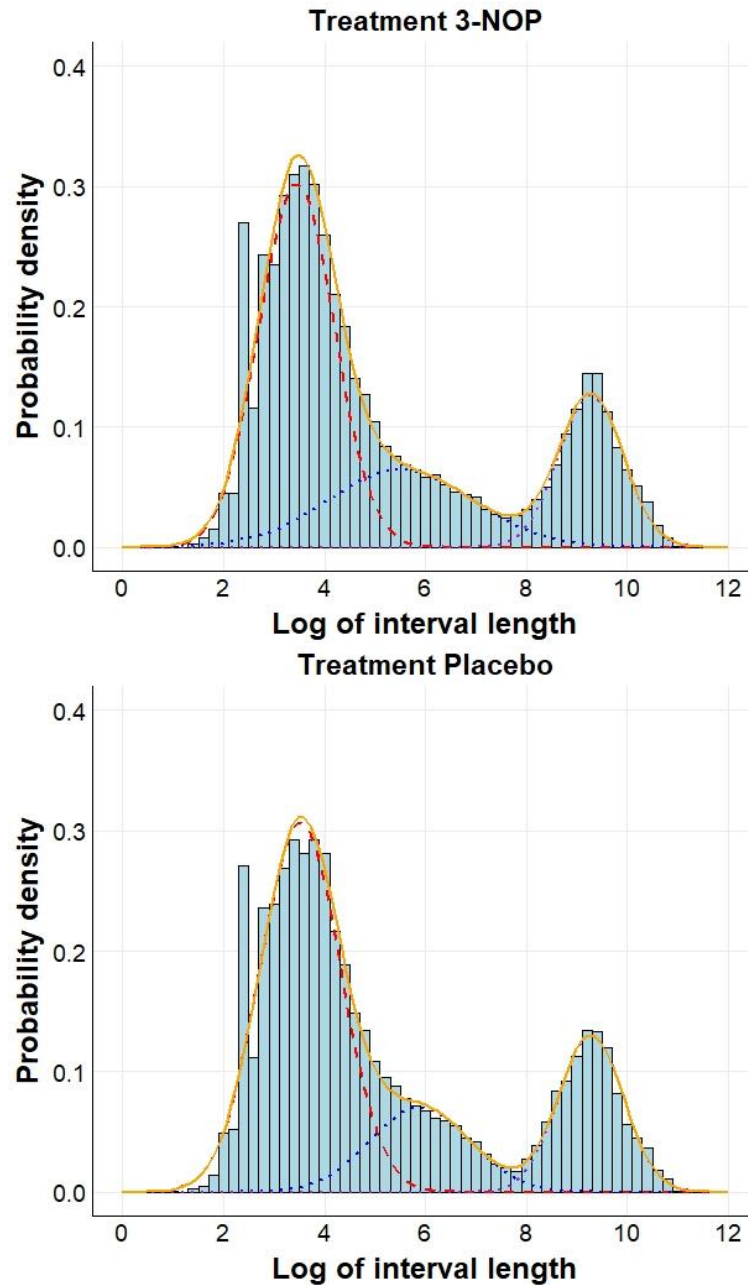
¹Dose of 3-NOP: 60 mg/kg DM; Phase 1 (P1: summer): sample sizes were n = 15 for CTR and n = 15 for 3-NOP. Phase 2 (P2: fall): the sample sizes were n = 15 for the CTR and n = 15 for the 3-NOP; ² The ICC from random-intercept models; 95% CI by parametric bootstrap (2,000 replicates). Observation unit: Cowday aggregated across the treatment period; models fitted separately by phase and treatment.

Table 3. Daily entropy (Hnorm) of visits and meals in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹, in phase 1 (P1, summer) or 2 (P2, fall) of the study

Item	P1		P2		Trt	p-value	
	CTR	3-NOP	CTR	3-NOP		Phase	Trt x P
Daily entropy of visits (Hnorm, 0-1) ²	0.655 ± 0.008	0.653 ± 0.009	0.632 ± 0.009	0.629 ± 0.009	0.63	<0.001	0.91
Daily entropy of meals (Hnorm, 0-1)	0.680 ± 0.009	0.662 ± 0.008	0.646 ± 0.009	0.631 ± 0.008	0.59	<0.001	0.79

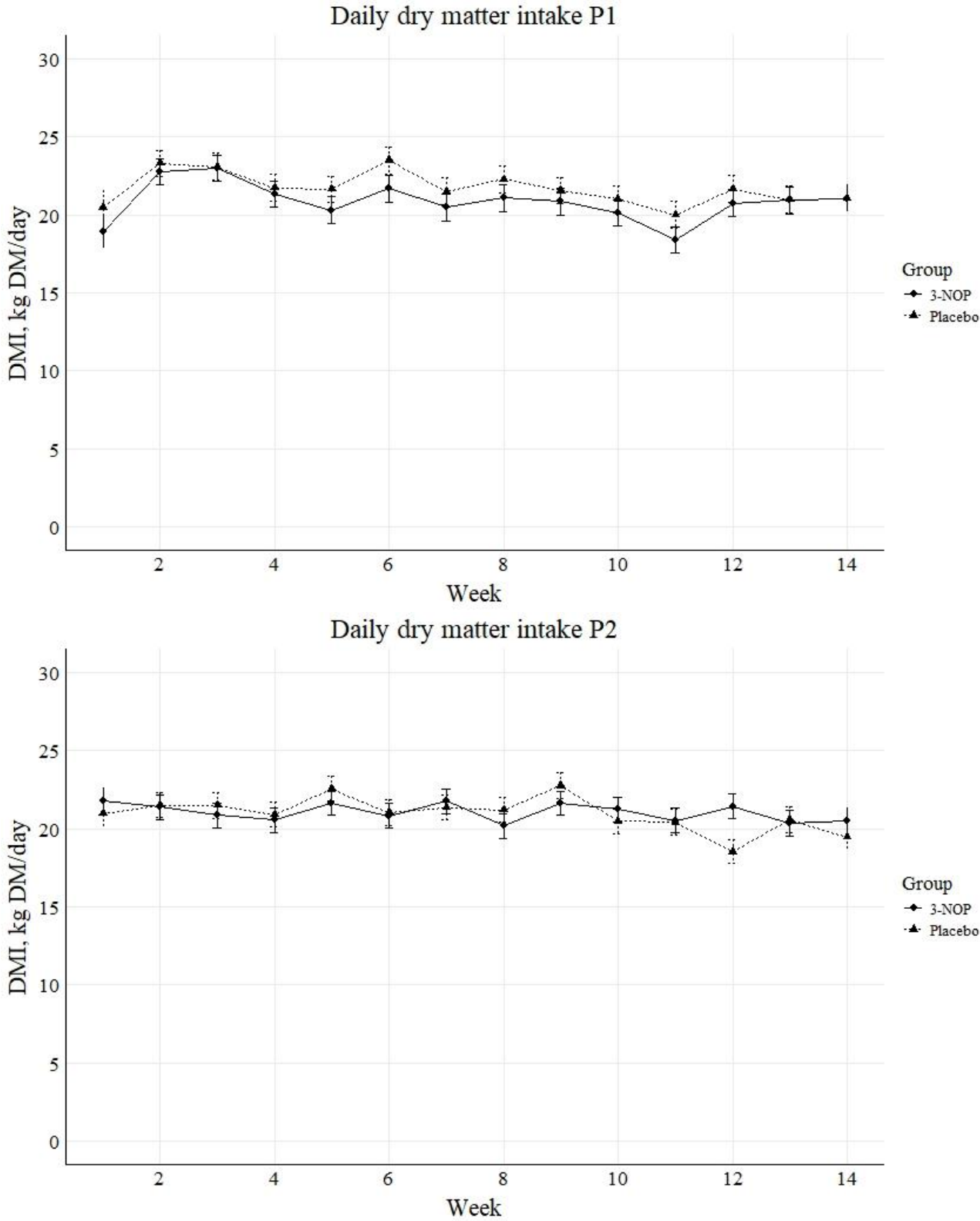
¹Dose of 3-NOP: 60 mg/kg DM; Phase 1 (P1: summer): sample sizes were n = 15 for CTR and n = 15 for 3-NOP. Phase 2 (P2: fall): The sample size was 15 for the CTR group and 15 for the 3-NOP group. ²H_norm = Shannon entropy computed over 24 hourly bins and normalized to [0,1]. Values are LSM ± SE from linear mixed models with fixed effects of treatment (Trt), phase (P1 summer; P2 fall), and Trt× phase. The observational unit was a cow day. p-values refer to fixed-effects tests from the mixed model.

Figure 1. Histograms per treatment of log-transformed lengths of intervals between successive visits of the bins in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP¹ of the study; the solid orange curve is a fitted 3-fold density function based on three Gaussian functions (dashed red line, dotted purple lines, and dotted blue line). The intersection of the second Gaussian and third curves was used to determine the meal criterion.



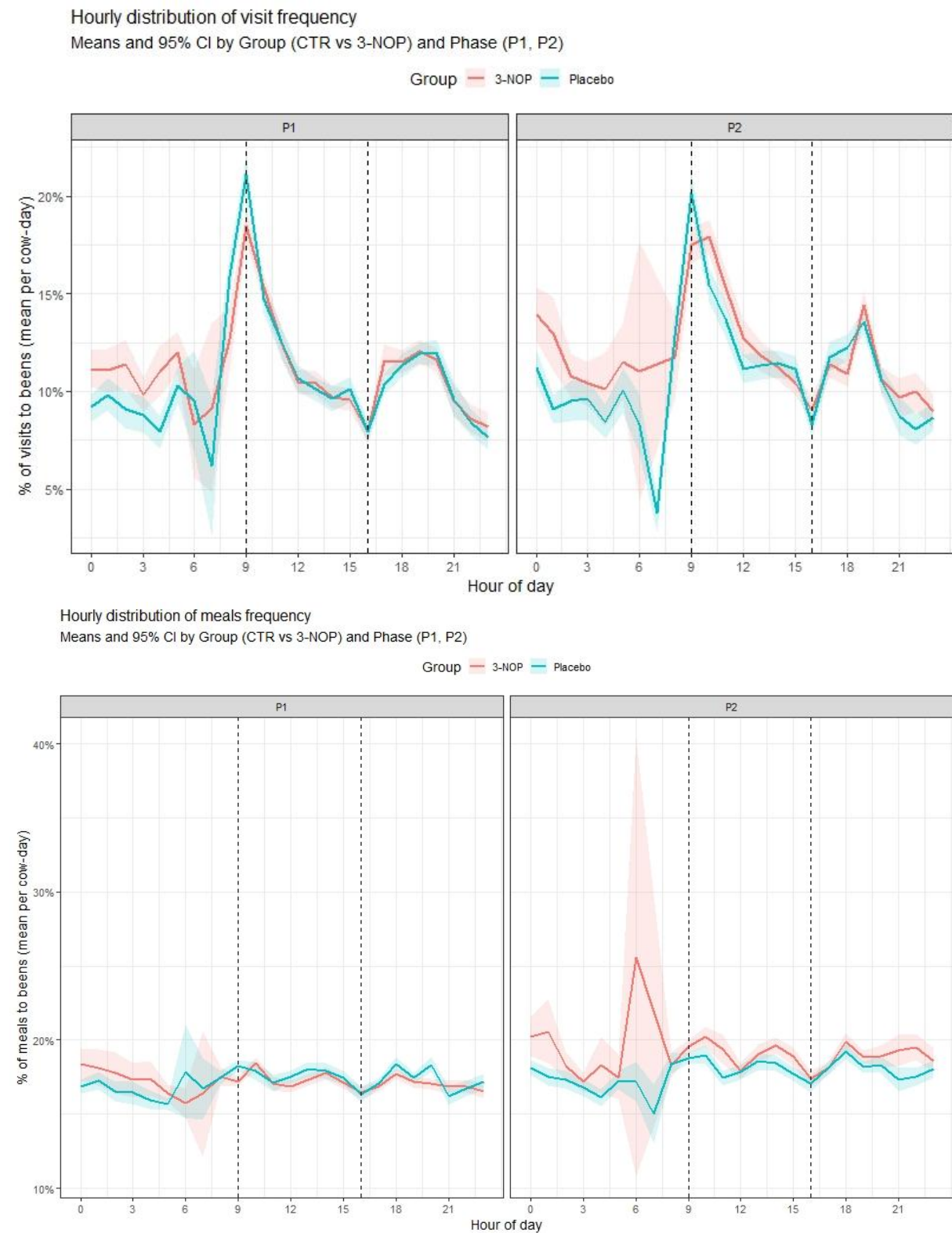
¹Dose of 3-NOP: 60 mg/kg DM; The sample sizes were $n = 48,063$ for the CTR and $n = 47,973$ for the 3-NOP.

Figure 2. Daily dry matter intake (DMI) (kg DM/day) in lactating Holstein cows fed a diet supplemented with placebo (CTR) or 3-NOP¹ in phase 1 (P1, summer) or 2 (P2, fall) of the study. Pre-treatment period: weeks 1–3; treatment period: weeks 4–12; washout period: weeks 13–14.



¹Dose of 3-NOP: 60 mg/kg DM; Phase 1 (P1: summer) and phase 2 (P2: fall). Values are expressed as mean ± SE.

Figure 3. Hourly distribution of visit and meal frequency in lactating Holstein cows fed a diet supplemented with a placebo (CTR) or 3-NOP1, in phase 1 (P1, summer) or 2 (P2, fall) of the study



1Dose of 3-NOP: 60 mg/kg DM; Phase 1 (P1: summer) and phase 2 (P2: fall). Values are expressed as the mean $\pm$ 95% CI across cow-days. Vertical dashed lines mark the TMR delivery at 9 am and 16 pm for milking.

4 Paper/Manuscript III

Kinetics of in vitro gas and methane production in presence of 3-nitrooxypropanol in rumen fluid of lactating dairy cows

E. Fiorbelli,¹ P. Bani,¹ M. Moschini,¹ F. Frolidi,¹ F. Piccioli-Cappelli,¹ E. Trevisi,^{1,3} I. Mueller,²
L. Tamassia,² and A. Gallo^{1,3*}

¹Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

²DSM Nutritional Products, Animal Nutrition and Health, PO Box 2676, 4002 Basel, Switzerland

³Romeo and Enrica Invernizzi Research Center for Sustainable Dairy Production of the Università Cattolica del Sacro Cuore (CREI), 29122 Piacenza, Italy

*Corresponding author: Antonio Gallo, antonio.gallo@unicatt.it

Article submitted: Journal Dairy Science Communications

ABSTRACT

This study aimed to investigate the short-term effects of dietary supplementation with 3-nitrooxypropanol (3-NOP) on rumen fermentation kinetics, VFA, and gas production in dairy cows using an in vitro batch fermentation method. Rumen inocula were individually collected from twenty lactating Holstein cows fed either a basal diet supplemented with 3-NOP or a placebo with no 3-NOP. Total gas production, its composition (i.e., methane, hydrogen, and carbon dioxide), and VFA concentrations were measured at different rumen incubation time points (i.e., 2, 5, 9, 24, 36, 48, and 72 h). Supplementation with 3-NOP resulted in an initial decrease in gas production and carbon dioxide emissions, particularly during the first 9 h of rumen incubation. Similarly, a reduction in acetate concentration was observed during early fermentation. Conversely, both butyrate and iso-acid proportions increased with 3-NOP inoculum during the first incubation period. Despite these initial changes in kinetic fermentation, no significant differences in cumulative gas production or total VFA concentrations were observed at the end of incubation (i.e., 72 h). These results suggest that 3-NOP supplementation can influence early fermentation dynamics and microbial activity in the rumen, highlighting the importance of monitoring fermentation over short-term intervals to understand the temporal impacts of methane-inhibiting additives. These findings contribute to the refinement of dietary strategies for reducing methane emissions from dairy cattle.

Abbreviations: CTR = control group, DDM = digestible dry matter, GLM = general linear model, MCR = methyl coenzyme M reductase, PMR = partial mixed ration, TRT = treatment group, 3-NOP = 3-nitrooxypropanol.

MAIN BODY

The use of feed additives to reduce enteric methane (CH₄) production in cattle has been an intensively investigated research area in recent decades. In particular, 3-nitrooxypropanol (3-NOP) has been demonstrated to be an effective inhibitor of CH₄ in the rumen, both *in vitro* and *in vivo*, in dairy and beef cattle (Yu et al., 2021). The mode of action of this molecule was specifically studied by Duin et al. (2016), who reported that it is capable of inhibiting the function of methyl-coenzyme M reductase (MCR), which is involved in methanogenesis in the rumen. Due to its mode of action, increased hydrogen (H₂) emissions have been observed in different *in vivo* studies in which 3-NOP was fed to dairy cows (Yu et al., 2021), which is a logical consequence of the direct enzymatic inhibition of methanogenesis. Based on the literature, 0.1 µM 3-NOP completely inactivates MCR within several minutes of exposure in an *in vitro* study (10 min) (Duin et al., 2016). The timing of the action of 3-NOP suggests it is sensitive to the feed intake of the animals. Additionally, a closer examination of the short-term effects of 3-NOP on rumen fermentation kinetics could provide valuable insights into its mechanism of action (Pressman and Kebreab, 2024). Therefore, we investigated a detailed temporal approach aimed at capturing the dynamic changes in gas production, as well as the VFA concentrations and proportions more accurately, adopting an *in vitro* rumen-simulating method. We hypothesized a differential change in total VFA production and profile between the control and 3-NOP inocula, coupled with changes in carbon dioxide, methane and hydrogen production, as a metabolic consequence of methanogenesis inhibition by 3-NOP and a reversible dynamic time-dependent pathway. The experiment was conducted at the Research Center for Livestock and Environment (CERZOO s.r.l., Piacenza, Italy). Animal care procedures were observed according to the guidelines of the Committee for the Protection and Welfare of Experimental Animals (OPBA – Organismo Preposto al Benessere degli Animali)

of the Catholic University of the Sacred Heart and were authorized by the Italian Ministry of Health (number 355/2022-PR). The current study was part of a larger *in vivo* experiment (Moschini et al., 2025); however, only data based on *in vitro* rumen fermentation are presented here to integrate earlier results. Twenty mid- to late-lactation Holsteins cows were randomly selected as donors: CTR (placebo, n = 10) and TRT (3-NOP 60 mg/kg DM, n = 10) per group. The cows were housed in the same pen of a free stall barn and fed a partial mixed ratio (PMR) composed, as a percentage of DM, of corn silage (32%), alfalfa hay (21%), barley straw (1.9%), soybean meal (9%), barley (9.3%), sunflower (3.5%) and corn meal (14%). A pelleted concentrate mixture (ME = 2.78 Mcal/kg; CP = 184 g/kg DM; starch = 316 g/kg DM; NDF = 259 g/kg DM) completed the PMR offered as a bait via a Greenfeed system (C-Lock Inc., Rapid City, SD). The PMR nutrient composition on a DM basis was 29.5% starch, 15.8% CP and 32.2% NDF. After nine weeks of 3-NOP inclusion in the TRT group's diet and of placebo for the CTR group, rumen fluid samples were individually collected from the cows before morning feeding (one per day) via an oral stomach probe connected to a manual vacuum pump (Ruminator; profs-products.com). Immediately after sampling, rumen pH was measured using a pH meter (GLP 21, Crison Strumenti SpA, Modena, IT). Samples were filtered through four layers of cheesecloth to remove large feed particles, and 100 mL aliquots of each sample were stored anaerobically in a thermal bath at 39 °C until processing. The time between rumen fluid collection and the *in vitro* process was kept to a maximum of 60 min to prevent any effects on microbial activity and fermentation. The samples were processed individually. The substrate used in the *in vitro* trial was the control PMR that had been previously collected and dried at 65 °C in an oven until it reached a constant weight. The dried PMR was then ground to a particle size of 1 mm using a laboratory mill (Fritsch pulverisette 19 universal cutting mill, Idar-Oberstein, Deutschland). *In vitro* gas production was set up using a 100 mL glass bottle filled with approximately 220 mg of air dry substrate, 10 mL of individual rumen fluid, and 20

mL of buffered medium (Menke and Steingass, 1988). Seven replicates *per* cow were used, and incubation times of 2, 5, 9, 24, 36, 48 and 72 h were adopted. Additionally, six bottles with only a pool of buffered rumen fluid were used as blanks, and another six bottles with the inclusion of 220 mg of starch (Gelose 80 maize starch; Penford Food Ingredients Co., Florenceville-Bristol, Canada) as substrate were used as internal standards. The procedures for processing the buffered rumen fluid was previously described by Pirondini et al. (2012) and Serment et al. (2016). The buffered rumen fluids were kept at 39 °C under continuous agitation, and carbon dioxide (CO₂) flushing was ensured to keep anaerobic conditions. A volume of 30 mL was then added to each bottle, and the headspace was flushed with CO₂ and hermetically closed with rubber caps. Finally, the bottles were incubated in a thermal water bath at 39°C. Headspace gas pressure was measured at each time points using a digital test gauge (XP2i, Crystal Engineering Corp). After the measurement, the glass bottles associated with the respective time point were removed and stored on ice to stop the fermentation. The headspaces and aliquots of buffered rumen fluid were sampled from these bottles to measure gas composition and VFA profile. The remaining bottles were then degassed with a sterile needle to return them to atmospheric pressure levels. To maintain regular microbial activity, the headspace pressure never exceeded 48 kPa, following the recommendation of Theodorou et al. (1994). The observed absolute pressure was converted into cumulative gas mL (López et al., 2007), as follows: the data were then used to fit the gas production data and to obtain curve parameters, such as final volume (mL/g of OM), rate of gas production (kd, h⁻¹), and lag time (h) (Wang et al., 2013). At each time point, the gas (1 mL) of the sacrificed bottles was sampled in the headspace using a 2-mL gas-tight glass syringe with a pressure lock (VICI, Precision Sampling Inc.) and immediately injected into a gas chromatograph (Agilent 7820A, Agilent Technologies) to determining the CH₄, H₂ and oxygen (O₂) concentration with nitrogen (N₂) as a carrier. Instrument calibration was performed with using an external standard mixture of

CO₂ and CH₄ prepared by SIAD S.p.A. (Bergamo, Italy). Peak areas generated by the GC were calculated by automatic integration. Subsequently, 10 mL aliquots of the fermented rumen fluid were withdrawn from the bottle and stored in tubes at -20 °C for VFA determination. On the analysis day, the method of Ghilardelli et al. (2022) was applied with slightly variation as described: the tubes were thawed for 10 min at 39 °C and centrifuged at 3,000 rpm for 15 min at 4 °C. An aliquot of 2-mL of the supernatant, 1-mL of solution of pivalic acid (1 g/L, as internal standard) and formic acids (50 ml/L), and 1-mL of oxalic acid (0.12 M) were transferred into a 10-mL tube, vortexed and centrifuged at 3,000 rpm for 15 min. Consequently, an aliquot was directly micro-filtered on Minisart GF-prefilters syringe filter 0.45 µm pore size (Sartorius, AG, Germany) and transferred to a vial and analyzed by GC. The GC device had an autosampler (AOC-20i Shimadzu S.r.l.), flame-ionization detector, and capillary column (DB-WAX UI (60 m × 0.250 mm; 0.25 µm; Agilent Technologies, Inc.). The injector run at 200 °C and the detector at 220 °C. The injection volume was 1 µL and the split ratio was 100:1. The carrier gas was H₂ at constant flow of 1.5 mL/min. The column oven temperature was programmed as follows: 90 °C for 1min, from 90 to 160 °C at 5 °C/min and from 160 to 190 °C at 10 °C/min for 7min. All statistical analyses were conducted using SAS statistical software (2004). For all variables displayed in tables and figures, the observations were averaged with each cow being the experimental unit. The kinetic of gas production and the VFA concentration measured at the end of the in vitro test were evaluated using the general linear model (GLM) procedure of SAS (SAS Institute, 2004), according to the following model:

$$Y_{ij} = \mu + Ri + \epsilon_{ij}$$

where Y_{ij} is the response variable (i.e., Vf, kd, lag, or VFA concentrations); μ is the overall mean; Ri is the fixed effect of treatment (i = 2: CTR and TRT) and ϵ_{ij} is the random

residual error. The composition of the gas and molar VFA production measured at each time point were evaluated using the GLM with the following model:

$$Y_{ijk} = \mu + Ri + Tj + \epsilon_{ijk}$$

where Y_{ijk} is the response variable; μ is the overall mean; Ri is the fixed effect of treatment ($i = 2$: CTR and TRT), Tj is the fixed block effect of the hours and ϵ_{ijk} is the random residual error. The data are presented in the figures as LSM and related SE. Differences between LSM were compared post hoc using Tukey's method. Tendency was declared when $0.05 < P \leq 0.10$, and statistical significances were reported at $P \leq 0.05$. To our knowledge, this is the first *in vitro* batch study to evaluate the dynamics of gas production from individual rumen inocula sampled from lactating dairy cows supplemented with 3-NOP. This study did not evaluate the effect of the direct addition of 3-NOP *in vitro*, previously reported in *in vitro* experiments (Romero-Pérez et al., 2015, 2017; Schilde et al., 2021). This study aimed to assess the dynamic production of gases and VFA production in lactating dairy cows under methanogenesis inhibition and to evaluate the short-term effects of 3-NOP on the fermentation patterns of rumen microbial inocula. We chose to measure both the total cumulative production (Table 1) and the hourly kinetics production *in vitro* (Figures 1 and 2) to obtain a more comprehensive understanding of the temporal dynamics promoted by 3-NOP inclusion in the *in vivo* diet. While cumulative CH₄ or VFA production is often used as a general indicator of treatment, it may overlook transient or early-stage effects that are critical for elucidating the mechanistic action of 3-NOP. Collecting samples at multiple time points, we aimed to capture the outcomes and the fermentation pathway over time, including possible temporary shifts in microbial activity or redirection of H₂ flux. These kinetic data are particularly relevant in the context of 3-NOP, considering its rapid inhibitory effect on methanogenesis in the rumen environment and its short lifespan before inactivation. Previous *in vitro* continuous rumen-

simulating studies, carried out at different doses of 3-NOP, did not report effects on total gas production, expressed as mL/d or mL/g of digestible dry matter (DDM) (Romero-Pérez et al., 2015; Schilde et al., 2021). Overall, in our study the total cumulative production of gas (mL/g of OM), CH₄, H₂ and CO₂ production, and the proportion of VFA at the end of 72 h of incubation revealed no significant difference between CTR and TRT (Table 1), but the initial lag phase was significantly higher in TRT compared to CTR (0.47 h vs. negligible, respectively; $P < 0.01$), suggesting a slower initial fermentation, probably due to lower microbial activity. The gas production (mL h⁻¹ g⁻¹ of OM) was lower at 2 h and 5 h in TRT compared to CTR, with values of 16.6 vs. 28.4 mL/g OM ($P = 0.07$) and 44.9 vs. 68.4 ($P = 0.02$), respectively (Figure 1). This suggested a short-term inhibition of microbial rumen activity, with the inclusion of 3-NOP. Similar trends were observed for CO₂ production at different incubation times. In particular, the CO₂ production was lower in TRT vs. CTR tendentially ($P = 0.08$) at 2 h (i.e., 69.1 vs. 71.5 mL), and significantly ($P = 0.01$ and 0.02) at 5 and 9 h, with values of 74.3 vs. 79.0 mL and 84.7 vs. 88.9 mL, respectively (Figure 1). The dynamics of VFA production in rumen fluid were expressed as absolute concentrations (mmol/L) and as molar proportions (% of total VFA) for each rumen incubation time (Figure 2). The absolute concentration of VFA provides essential information on the total fermentative activity and the energetic contribution of ruminal fermentation. On the other hand, the VFA molar proportions are essential for capturing qualitative changes in rumen fermentation patterns that can depend on the substrate composition and digestion characteristics of the single nutrients (Nozière et al., 2011a; b). The VFA proportions can also reflect adaptations in microbial metabolism resulting from alterations of some ruminal conditions, such as pH fluctuations and changes in microbial populations. In the current context, comparing the absolute production of VFA and its proportion among treatments could provide a comprehensive shift in single VFA as an alternative H₂ sink to compensate for the reduced

methanogenesis (Muñoz-Tamayo et al., 2016). The total VFA concentrations (mmol/L) at the end of the incubation showed no differences between treatments (Table 1). However, evaluating the total VFA production at different times of incubation (Figure 1), it tended to be lower in TRT than in CTR at both 5 h (34.4 vs. 42.4 mmol/L; $P=0.05$) and 9 h (41.8 vs. 49.5 mmol/L; $P=0.08$). Similar reductions were observed in rumen fluid sampled at different time points (i.e., 0.5, 1, 1.5, 2, and 4 h) after feeding fistulated dairy cows with 2.50 g/d 3-NOP (Reynolds et al., 2014). The acetate concentrations (mmol/L) tended to be lower at the initial incubation times in TRT, i.e. at 2 h (20.2 vs. 24.3 mmol/L, $P=0.08$), 5 h (20.3 vs. 26.0 mmol/L, $P=0.02$), and 9 h of incubation (24.2 vs. 29.7 mmol/L, $P=0.06$). Similarly, propionate concentrations tended to be lower in the TRT inocula at 9 h of incubation than in CTR (10.2 vs. 11.5 mmol/L; $P=0.07$) (Figure 1). The production of butyrate, valerate, caproate, isobutyrate, and isovalerate (mmol/L) did not differ between the treatments at any time, although numerically lower values were measured in TRT than in CTR. The ratios of acetate to propionate ($C2/C3$) and acetate plus butyrate to propionate ($((C2+C4)/C3)$) were similar between the treatments at the end of incubation (Table 1). The change in VFA production and its proportions can be attributed to the impact of 3-NOP inclusion on the microbial population. In a previous study on dairy cows (Pitta et al., 2021), 3-NOP inclusion (60 mg/kg DM) selectively reduced the abundance of *Methanobrevibacter* populations after four weeks of exposure and significantly lower *Methanosphaera* by weeks 8 and 12, highlighting the differential sensitivity and adaptive responses among methanogenic lineages due to prolonged 3-NOP administration. Moreover, H_2 metabolism changes by enhancing the expression of the hydrogenase enzyme and increasing the gene expression involved in the butyrate synthesis pathway (Pitta et al., 2022). In addition, Hristov et al., (2015) and Melgar et al., (2020) observed a different pattern of ruminal H_2 during 3-NOP inclusion. Initially, the H_2 levels increased until week 9 followed by a significant decrease by week 12. Pitta et al. (2022) hypothesized that the

accumulation of H₂ stimulated hydrogen-sensing hydrogenases, activating a bacterial feedback mechanism that eventually reduced ruminal H₂ emissions. Significant shifts in ruminal VFA proportions were observed, characterized by decreased acetate and increased butyrate concentrations (Yu et al., 2021). In our experiment, hourly production dynamics showed significant differences when comparing VFA concentrations (mmol/L) and proportions (% of total VFA) (Figure 2). Acetate proportions tended to be lower with TRT than in CRT at 2 h (59.3% vs. 62.0%, P=0.09) and 5 h (59.0% vs. 62.0%, P=0.08). Congruently, propionate proportions were numerically higher at all incubation times, and in agreement with previous studies (Haisan et al., 2014; Kim et al., 2019) that reported reduced acetate and increased propionate proportions with 3-NOP addiction. Butyrate reached 12.1 % vs 11.2 % at 2 h (P= 0.06) and remained higher in TRT at 5 h (12.5 % vs 11.3 %, P=0.03) and 9 h (12.9 % vs 11.9 %, P=0.05). Isobutyrate (1.2 % vs 1.0 % at 2h, P= 0.05; 2.1 % vs 1.7 % at 5 h, P=0.02) and isovalerate (2.1 % vs 1.7 % at 2 h, P= 0.02; 1.8 % vs 1.5 % at 5 h, P= 0.04) followed the same pattern. These results align with *in vitro* findings (Romero-Pérez et al., 2015; Schilde et al., 2021), but differ from *in vivo* data (Reynolds et al., 2014) for isovalerate. Notably, transient early elevations in isoacid levels, although not statistically significant, may indicate short-term microbial adaptation to fermentation shifts induced by 3-NOP. Such dynamic changes highlight the importance of temporal sampling, as endpoint measurements alone would overlook these variations. The future integration of metagenomic or metabolomic methods could further reveal microbial and regulatory mechanisms underlying these responses, especially with regard to sustained CH₄ inhibition. Further research is necessary to elucidate the pathways of rumen fermentation, address the variability in CH₄ production responses to different feed additives, and evaluate the enhancement of mechanistic models of rumen dynamics under CH₄ production inhibition by these additives.

NOTES

This study was partially funded by dsm-firmenich (Kaiseraugst, Switzerland). Research in the Department of Animal Science, Food and Nutrition (UNICATT) was supported by the Romeo and Enrica Invernizzi Foundation. E. Fiorbelli is supported by an Agrisystem Ph.D. fellowship (UNICATT, Piacenza, Italy). During manuscript preparation, the first author used Jenni AI to improve readability and subsequently reviewed and edited the content, assuming full responsibility. I. Mueller and L. Tamassia are employees of dsm-firmenich; the authors declare no other conflicts of interest. All smart images used in the graphical abstract were provided by Servier Medical Art, licensed under CC-BY 4.0. The 3-NOP molecule was obtained free to PubChem Identifier: CID 10011893

REFERENCES

- Duin, E.C., T. Wagner, S. Shima, D. Prakash, B. Cronin, D.R. Yáñez-Ruiz, S. Duval, R. Rumbeli, R.T. Stemmler, R.K. Thauer, and M. Kindermann. 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proc. Natl. Acad. Sci.* 113:6172–6177. doi:10.1073/pnas.1600298113.
- Ghilardelli, F., G. Ferronato, and A. Gallo. 2022. Near-infrared calibration models for estimating volatile fatty acids and methane production from in vitro rumen fermentation of different total mixed rations. *JDS Commun.* 3:19–25. doi:10.3168/jdsc.2021-0156.
- Haisan, J., Y. Sun, L.L. Guan, K.A. Beauchemin, A. Iwaasa, S. Duval, D.R. Barreda, and M. Oba. 2014. The effects of feeding 3-nitrooxypropanol on methane emissions and productivity of Holstein cows in mid lactation. *J. Dairy Sci.* 97:3110–3119. doi:10.3168/jds.2013-7834.

- Hristov, A.N., J. Oh, F. Giallongo, T.W. Frederick, M.T. Harper, H.L. Weeks, A.F. Branco, P.J. Moate, M.H. Deighton, S.R.O. Williams, M. Kindermann, and S. Duval. 2015. An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc. Natl. Acad. Sci.* 112:10663–10668. doi:10.1073/pnas.1504124112.
- Kim, S.-H., C. Lee, H.A. Pechtl, J.M. Hettick, M.R. Campler, M.D. Pairis-Garcia, K.A. Beauchemin, P. Celi, and S.M. Duval. 2019. Effects of 3-nitrooxypropanol on enteric methane production, rumen fermentation, and feeding behavior in beef cattle fed a high-forage or high-grain diet¹. *J. Anim. Sci.* 97:2687–2699. doi:10.1093/jas/skz140.
- López, S., M.S. Dhanoa, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. *Anim. Feed Sci. Technol.* 135:139–156. doi:10.1016/j.anifeedsci.2006.06.005.
- Melgar, A., M.T. Harper, J. Oh, F. Giallongo, M.E. Young, T.L. Ott, S. Duval, and A.N. Hristov. 2020. Effects of 3-nitrooxypropanol on rumen fermentation, lactational performance, and resumption of ovarian cyclicity in dairy cows. *J. Dairy Sci.* 103:410–432. doi:10.3168/jds.2019-17085.
- Menke, K., and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Pages 7–55. *Animal Research and Development*.
- Moschini M., Frolidi F., Piccioli-Cappelli F., P. Bani, Fiorbelli E., Gallo A., Mueller I., Tamassia L.F.M., Zihlmann R., Bulgarelli P., Agazzi S., Trevisi E., and L. Cattaneo. 2025. Effects of 3-nitrooxypropanol on enteric methane emissions and milk production characteristics in dairy cows fed a high corn–silage diet in different environmental conditions. *J. Dairy Sci.* <https://doi.org/10.3168/jds.2025-26640>

- Muñoz-Tamayo, R., S. Giger-Reverdin, and D. Sauvant. 2016. Mechanistic modelling of in vitro fermentation and methane production by rumen microbiota. *Anim. Feed Sci. Technol.* 220:1–21. doi:10.1016/j.anifeedsci.2016.07.005.
- Nozière, P., F. Glasser, C. Loncke, I.O. Marty, J. Vernet, and D. Sauvant. 2011a. Modelling rumen volatile fatty acids and its evaluation on net portal fluxes in ruminants. D. Sauvant, J. Van Milgen, P. Faverdin, and N. Friggens, ed. Academic Publishers, Wageningen.
- Nozière, P., F. Glasser, and D. Sauvant. 2011b. In vivo production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. *Animal* 5:403–414. doi:10.1017/S1751731110002016.
- Pirondini, M., L. Malagutti, S. Colombini, P. Amodeo, and G.M. Crovetto. 2012. Methane yield from dry and lactating cows diets in the Po Plain (Italy) using an in vitro gas production technique. *Ital. J. Anim. Sci.* 11:e61. doi:10.4081/ijas.2012.e61.
- Pitta, D., N. Indugu, K. Narayan, and M. Hennessy. 2022a. Symposium review: Understanding the role of the rumen microbiome in enteric methane mitigation and productivity in dairy cows. *J. Dairy Sci.* 105:8569–8585. doi:10.3168/jds.2021-21466.
- Pitta, D.W., N. Indugu, A. Melgar, A. Hristov, K. Challa, B. Vecchiarelli, M. Hennessy, K. Narayan, S. Duval, M. Kindermann, and N. Walker. 2022b. The effect of 3-nitrooxypropanol, a potent methane inhibitor, on ruminal microbial gene expression profiles in dairy cows. *Microbiome* 10:146. doi:10.1186/s40168-022-01341-9.
- Pitta, D.W., A. Melgar, A.N. Hristov, N. Indugu, K.S. Narayan, C. Pappalardo, M.L. Hennessy, B. Vecchiarelli, V. Kaplan-Shabtai, M. Kindermann, and N. Walker. 2021. Temporal changes in total and metabolically active ruminal methanogens in dairy cows supplemented with 3-nitrooxypropanol. *J. Dairy Sci.* 104:8721–8735. doi:10.3168/jds.2020-19862.

- Pressman, E.M., and E. Kebreab. 2024. A review of key microbial and nutritional elements for mechanistic modeling of rumen fermentation in cattle under methane-inhibition. *Front. Microbiol.* 15:1488370. doi:10.3389/fmicb.2024.1488370.
- Reynolds, C.K., D.J. Humphries, P. Kirton, M. Kindermann, S. Duval, and W. Steinberg. 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. *J. Dairy Sci.* 97:3777–3789. doi:10.3168/jds.2013-7397.
- Romero-Pérez, A., E.K. Okine, L.L. Guan, S.M. Duval, M. Kindermann, and K.A. Beauchemin. 2015. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (Rusitec). *Anim. Feed Sci. Technol.* 209:98–109. doi:10.1016/j.anifeedsci.2015.09.002.
- Romero-Pérez, A., E.K. Okine, L.L. Guan, S.M. Duval, M. Kindermann, and K.A. Beauchemin. 2017. Rapid Communication: Evaluation of methane inhibitor 3-nitrooxypropanol and monensin in a high-grain diet using the rumen simulation technique (Rusitec). *J. Anim. Sci.* 95:4072. doi:10.2527/jas2017.1896.
- Schilde, M., D. Von Soosten, L. Hüther, S. Kersten, U. Meyer, A. Zeyner, and S. Dänicke. 2021. Dose–Response Effects of 3-Nitrooxypropanol Combined with Low- and High-Concentrate Feed Proportions in the Dairy Cow Ration on Fermentation Parameters in a Rumen Simulation Technique. *Animals* 11:1784. doi:10.3390/ani11061784.
- Serment, A., S. Giger-Reverdin, P. Schmidely, O. Dhumez, L.P. Broudiscou, and D. Sauvant. 2016. In vitro fermentation of total mixed diets differing in concentrate proportion: relative effects of inocula and substrates. *J. Sci. Food Agric.* 96:160–168. doi:10.1002/jsfa.7076.
- Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation

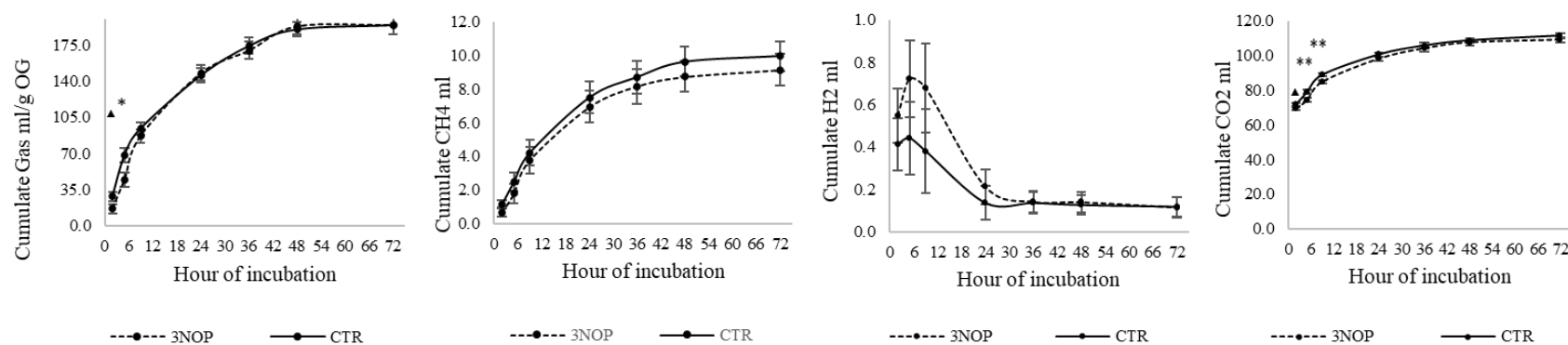
- kinetics of ruminant feeds. *Anim. Feed Sci. Technol.* 48:185–197. doi:10.1016/0377-8401(94)90171-6.
- Wang, M., X.Z. Sun, S.X. Tang, Z.L. Tan, and D. Pacheco. 2013. Deriving fractional rate of degradation of logistic-exponential (LE) model to evaluate early in vitro fermentation. *Animal* 7:920–929. doi:10.1017/S1751731112002443.
- Yu, G., K.A. Beauchemin, and R. Dong. 2021. A Review of 3-Nitrooxypropanol for Enteric Methane Mitigation from Ruminant Livestock. *Animals* 11:3540. doi:10.3390/ani11123540.

Table 1. Gas production, composition, and VFA proportion after 72 h of incubation of rumen inoculum of control (CTR) and treatment (TRT) groups.

Item	Treatment		SEM	P-value ¹
	CTR	3-NOP		
Final volume gas, mL/g OM	197.84	198.63	9.50	0.94
kd gas, h ⁻¹	0.06	0.06	0.004	0.94
Lag phase gas, h	0.00	0.47	0.40	0.01
Cumulate CH ₄ , mL	9.97	9.14	0.90	0.53
Cumulate H ₂ , mL	0.25	0.36	0.04	0.14
Cumulate CO ₂ , mL	111.46	109.06	1.50	0.27
Total VFA in %:				
Acetate	58.41	57.91	0.60	0.60
Propionate	23.19	23.82	1.10	0.69
Butyrate	11.29	11.65	0.30	0.49
Valerate	2.06	1.91	0.12	0.41
Caproate	0.55	0.44	0.05	0.22
Isobutyrate	1.59	1.54	0.07	0.63
Isovalerate	2.88	2.70	0.20	0.56
C2/C3 ratio	2.61	2.44	0.14	0.43
(C2+C4)/C3 ratio	3.12	2.94	0.18	0.49

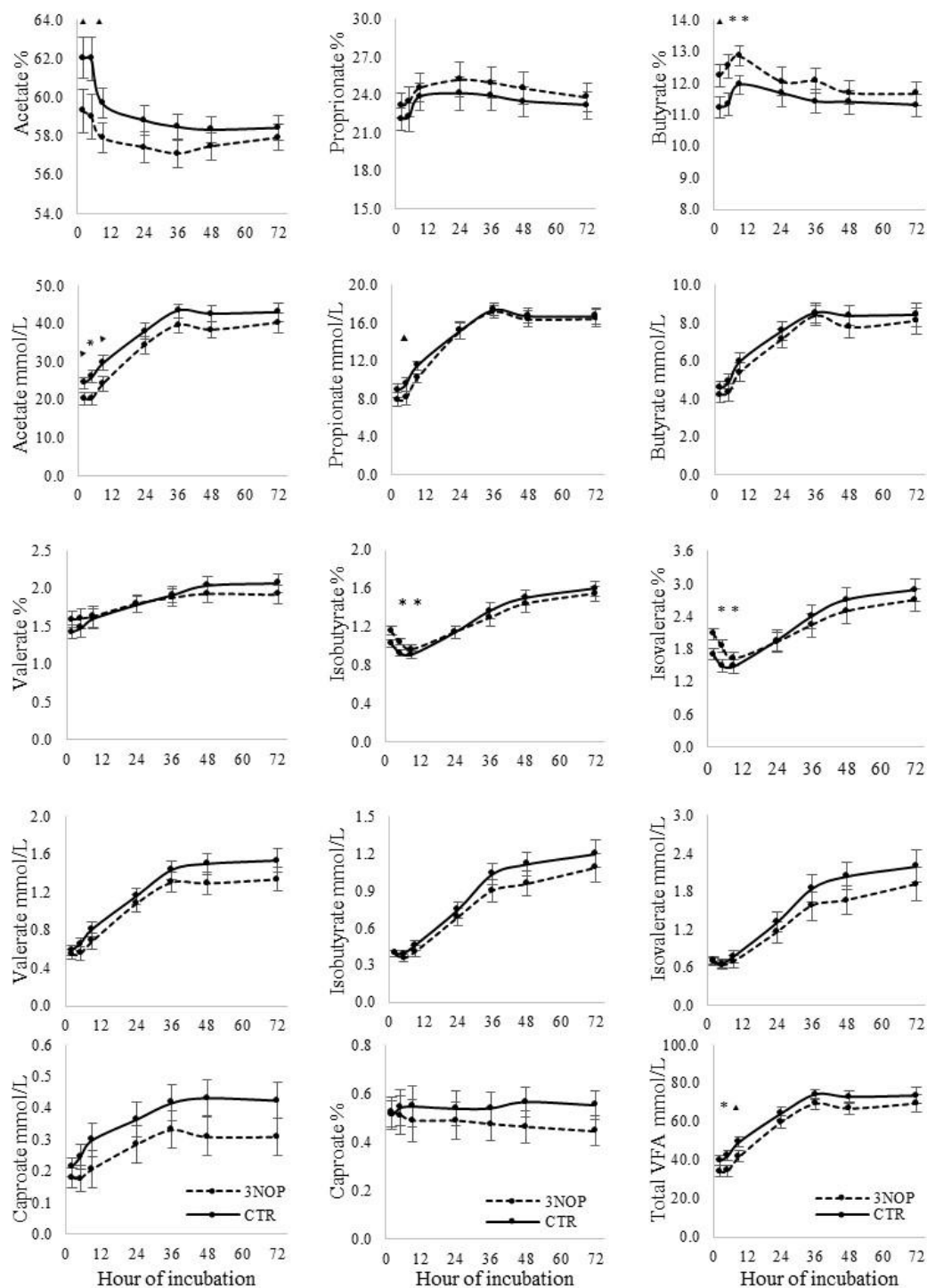
¹Statistical significances, $P \leq 0.05$ and tendencies = $0.05 < P \leq 0.10$.
Data expresses as LSM $\pm$ SE.

Figure 1. Dynamic gas production composition in fermented rumen fluid of donor cows fed the control (CTR) diet or the 3-nitrooxypropanol (TRT) diet.



¹Statistical significances, $0.1 \leq P \leq 0.05$ ▲; $P \leq 0.05$ (*); $P \leq 0.01$ (**). Data expresses as LSM $\pm$ SE.

Figures 2. Dynamic of VFA concentration and proportion in fermented rumen fluid of donor cows fed the control (CTR) diet or the 3-nitrooxypropanol (TRT) diet.



¹Statistical significances, $0.1 \leq P \leq 0.05$ (▲); $P \leq 0.05$ (*). Data expresses as LSM $\pm$ SE.

5 Paper/Manuscript IV

Evaluating *vitro* nutritional strategies clusters for mitigating methane emissions in intensive dairy farms by a mechanistic model of in *vitro* rumen fermentation

Erica Fiorbelli^a, Alberto Stanislao Atzori^b, Rafael Muñoz-Tamayo^c, Antonio Gallo^{a,*}

^a Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122, Piacenza, Italy.

^b Department of Agricultural Science, University of Sassari, 07100 Sassari, Italy

^c Université Paris-Saclay, INRAE, AgroParisTech, UMR Modélisation Systémique Appliquée aux Ruminants, 91120, Palaiseau, France.

* Corresponding author: antonio.gallo@unicatt.it (A. Gallo)

ABSTRACT

Mitigating enteric methane emissions is a key challenge in intensive dairy farming. This study evaluated 39 corn silage-based total mixed rations (TMRs) grouped into four dietary clusters representative of Italian commercial systems, using an *in vitro* gas production technique. Fermentation data were interpreted through a dynamic mechanistic model of rumen fermentation to estimate biological parameters related to sugar utilization pathways and hydrogen metabolism. While methane production did not differ significantly among diets, the model parameters revealed shifts in volatile fatty acid profiles and hydrogen utilization that were associated with nutrient digestibility and farm performance indicators. In particular, the hydrogen utilization rate was positively correlated with crude protein and fiber digestibility, as well as with dry matter intake and residual energy intake, suggesting a link between increased microbial activity and methane potential. These findings support the use of mechanistic modeling as a decision-support tool to identify nutritional strategies that balance methane mitigation with animal productivity and economic performance in intensive dairy systems.

Keywords: ruminant, methane, emission, greenhouse gas, modelling, rumen fermentation

Abbreviations: ADF, acid detergent fibre; ADL, acid detergent lignin; CH₄, methane; CL, cluster; CO₂, carbon dioxide; CP, crude protein; DEE, digestible energy use efficiency; DMI, dry matter intake; EE, ether extract; ECM, energy-corrected milk; FCM, fat corrected milk; FE, feed efficiency; GC, gas chromatography; GHG, greenhouse gas; GWP, global warming potential; H₂, hydrogen; IEFC, income energy feed cost; IOFC, income over feed cost; MEE, metabolizable energy use efficiency; NDF, neutral detergent fibre; DNDF 24h, NDF degradability evaluated after 24 h of rumen incubation; NEE, net energy use efficiency; O₂, oxygen; Su, sugar; REI, residual energy intake; TMR, total mixed ration; VFA, volatile fatty acids.

INTRODUCTION

Mitigating greenhouse gas (GHG) emissions has become a central focus in dairy cattle research, with the main challenge being the reduction of methane (CH₄) emissions to promote environmental sustainability. According to the European Environment Agency database (EEA), CH₄ emissions from the agriculture sector in the EU member states (EU-27) account for 230,297 kt of carbon dioxide equivalents base (CO₂e), which represents 6.8% of the total GHG emissions in 2022. The major source of these emissions is enteric fermentation from ruminants, although a decrease in emission levels was reported in 2020 compared to 1990, -56 kt CO₂e as -23.8 % of total GHG. In Agreement with intergovernmental panel climate change fifth assessment report (IPCC - AR5), the global warming potential (GWP) of CH₄ is twenty-eight times higher than that of carbon dioxide (CO₂). However, it is a short-lived GHG compared to CO₂, and its mitigation has a unique position in the global efforts to address climate change. Recent reviews were focused on nutritional strategies to reduce CH₄ emissions without compromising animal productivity (Beauchemin et al., 2022; Hristov, 2024). However, predicting the impact of these strategies on rumen fermentation dynamics remains a significant challenge due to the complex of measurement and the interaction between diet composition, microbial activity, and gas production (GP).

A promising approach to understanding this complexity involves the use of mechanistic models (Hristov et al., 2018; Tedeschi et al., 2022b). Mechanistic models allow researchers to simulate the flux of nutritional feed source, and the biochemical processes involved in rumen microbial metabolism. Models have the potential to be used to provide insights into how different dietary strategies influence volatile fatty acids (VFA) production, hydrogen (H₂) balance and its potential conversion to CH₄.

Over the years, fundamental knowledge was established by research on the mechanistic rumen function by using different modelling approaches (Baldwin et al., 1977; Dijkstra et al., 1992; Mills et al., 2001; Bannink et al., 2006; Danfær et al., 2006; Serment and Sauvant, 2011).

Muñoz-Tamayo et al., (2016) developed a dynamic mechanistic model of in vitro rumen fermentation capable of representing rumen fermentation, which has shown promise in predicting the dynamics of VFA, CH₄, H₂ and CO₂ in fermentations using rumen inoculum from goats and cattle (Muñoz-Tamayo et al., 2019). The model was further extended to represent the impact of *Asparagopsis taxiformis* on rumen fermentation and CH₄ production under inhibition condition (Muñoz-Tamayo et al., 2021).

In the context of intensive dairy farming systems with corn silage representing the base of the rations, the model developed by Muñoz-Tamayo et al. (2016) was used to provide a comprehensive mechanistic representation of in vitro rumen fermentation and enteric CH₄ production. The objective of this study was to assess the impact of different rations on potential emission impacts and to evaluate the effectiveness of the mechanistic model developed by Muñoz-Tamayo et al. (2016) to represent the dynamics of rumen fermentation in vitro, including the VFA pathways and CH₄ production. We hypothesized that different inclusion of feed and nutritional strategies can modulate the rumen fermentation resulting in different patterns of VFA formation and consequently change CH₄ emissions. Animal performance are the reflection of fermentation processes and these performance outcomes can subsequently be assessed through economic indexes, which we previously calculated (Gallo et al., 2022). Therefore, this study includes the biological rumen variables, estimated by the model, not only as a major comprehensive understanding of the effect of nutritional strategies on rumen function and their environmental impact, but also integrated them into an economic evaluation to capture their economic implications.

The findings of this research are expected to provide valuable insights into the relationship between diet composition and rumen fermentation dynamics, thereby contributing to the development of sustainable nutritional strategies for intensive dairy farming.

MATERIALS AND METHODS

The 39 total mixed rations (TMRs) based on corn silage were selected from a previously study (Gallo et al., 2022). They were sampled from commercial dairy farms in a specific region of Italy (i.e., the Po Valley). The TMRs were considered representative of the intensive dairy farm system of Italy, which raised Holstein-Friesian cows housed in free-stall barns and fed TMR without access to pasture (Bellingeri et al., 2019, 2020). Details regarding locations of dairy farms and TMR sampling technique were previously reported (Atzori et al., 2021). As reported (Gallo et al., 2022), the TMRs were categorized into six distinct nutritional strategy clusters (CL) based on the type and quantity of different feed ingredients used in lactating dairy cow rations. In this study the examined TMRs represented the CL 3, 4, 5, and 6 of the study of (Gallo et al., 2022). For easiness of presentation, we renamed the CL as Diet 1 (6 TMRs) , Diet 2 (18 TMRs), Diet 3 (7 TMRs), and Diet 4 (8 TMRs). Farm indices were determined and associated with the respective Clusters; the methods applied during the determination were largely described in the study of Gallo et al., (2022).

The analyses in a close bath in vitro rumen fermentation was carried out to evaluate the kinetics of GP, VFA concentrations and CH₄ production, as described in a previous report (Ghilardelli et al., 2022).

The data produced were used to determine biological parameters by the mathematical mechanistic model developed by Muñoz-Tamayo et al. (2016) using data from an in vitro batch study with rumen fluid of goats.

Therefore, only a summary of the materials and methods are provided here.

In vitro gas production method

The 39 TMRs were collected and dried at 65 °C in an oven until it reached a constant weight. Then, they were ground to a particle size of 1 mm and a 0.5 mm using a laboratory mill with adjustable speed (Fritsch pulverisette 19 universal cutting mill, Idar-Oberstein, Deutschland). These samples at 0.5 mm were analyzed by near-infra-red spectroscopy (NIR) analysis with a Foss (Hillerød, Denmark) NIR systems 5000 spectrophotometer equipped with a monochromator and transport module, scanning over the wavelength range 400–2500 nm, every 2 nm. The calibrations used to obtain TMRs and forage characterizations were produced by a commercial forage testing laboratory (CRPA Lab, Reggio Emilia, Italy) and samples were analyzed for ash, crude protein (CP), soluble CP, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), NDF degradability evaluated after 24 h of rumen incubation (DNDF 24h), ether extract (EE), starch, sugar (Su), neutral detergent insoluble CP (NDICP).

Rumen fluid was collected before the morning feeding individually from two cannulated dry Holstein dairy cows fed at maintenance level ($BW 625 \pm 10$ kg) and were maintained at the CERZOO experimental station (San Bonico, Piacenza, Italy). Immediately after collection, the fluid's pH was determined using a pH meter, filtered through four layers of cheesecloth, and stored in thermal bottles in anaerobically condition during the transportation into laboratory. The time between rumen fluid collection and inoculation was kept to a minimum to prevent any changes in microbial activity and fermentation. At the same day of inoculation, the buffer-mineral solution was prepared and maintained at 39 °C in a water bath, under

anaerobic conditions with continuous CO₂ flushing and adjusted pH around 6.5 to 6.6 (Menke and Steingass, 1988).

The in vitro GP technique was involved placing 220 mg (as fed basis) of TMR samples into 100 mL glass bottles with 30 mL buffered rumen fluid (ration 1:2, rumen-buffer) (Gallo et al., 2016). For each TMRs there were six replicates glass bottles related to the 6-incubation time (2, 5, 9, 24, 36 and 48-h). The dynamics of GP were verified by adding two bottles for each thermal bath with only buffered rumen fluid (blanks) and 2 bottles with a starch sample for internal standard (Gelose 80 maize starch; Penford Food Ingredients Co.). The cumulative GP from blanks and internal standards was averaged, and it was considered satisfactory if it fell within ± 1 standard deviation.

The adjustment procedure used to process the buffered rumen fluid was described by Pirondini et al. (2012) and Serment et al. (2016). A volume of 30 mL of buffered rumen fluid was added to each bottle. Initially, the headspace of each bottle was flushed with CO₂ and then hermetically sealed with rubber caps for degassing. All the bottles were incubated in a thermal water bath, and the pressure in the headspace was measured at intervals of 2, 5, 9, 24, 36, and 48 hours using a digital test gauge XP2i by Crystal Engineering Corp. After the measurements, the replicated glass bottles associated with the respective incubation times were removed from the thermal bath for GC determination. The remaining bottles were degassed with a sterile needle, and the pressure was brought back to normal levels. To maintain regular microbial activity, the headspace pressure never exceeded 48 kPa, in accordance with the recommendation by Theodorou et al. (1994). The absolute pressures observed were summed to determine the cumulative GP and then converted to moles of gas using the formula reported by López et al. (2007).

Gas composition and ruminal volatile fatty acids concentration

Two different GC procedures were performed after removing the bottles from the thermal bath. For the gas quality determination (CH_4 , H_2 and O_2), 1 mL of the headspace gas was collected using a 2-mL gas-tight glass syringe with a pressure lock (VICI, Precision Sampling Inc.). This gas sample was then analyzed using a GC system with nitrogen as carrier gas. An external standard mixture of CO_2 and CH_4 prepared by SIAD S.p.A. (Bergamo, Italy) was used to calibrate the instrument, and the peak areas were automatically integrated and express the concentration of each gas as percentage. The amount of mmol CH_4 , H_2 and O_2 determined between the time points and the final amount were calculated as describe by López et al. (2007), regarding the CO_2 the amount was obtained per difference of total gas and the percentage of CH_4 , H_2 and O_2 calculated by the GC system..

For the VFA concentration at each time point were collected an aliquot of 10 mL of fermented rumen fluids and determined using a GC system (2025 Shimadzu GC, Shimadzu S.r.l.) as described by Ghilardelli et al. (2022) and Gallo et al. (2022).

Rumen model parameters estimation

The observed data on GP, CH_4 , H_2 , CO_2 and VFA production from the *in vitro* experiments were used to estimate the parameters of a mechanistic dynamic model of rumen fermentation described in a previous study (Muñoz-Tamayo et al., 2016b). The model integrates metabolic transformations, liquid-gas exchange, and acid-base reactions. It consists of eighteen ordinary differential equations derived from mass balance equations for a batch system, along with algebraic equations representing the acid-base reactions. The microbial functional groups in the model are represented by three theoretical functional groups based on their utilization of specific substrates. These groups are:

Sugars Utilizers (X_{su}): Microbes that utilize soluble sugars represented by glucose.

Amino Acids Utilizers (X_{aa}): Microbes that utilize soluble amino acids.

Hydrogen Utilizers (X_{H_2}): Microbes that utilize soluble hydrogen.

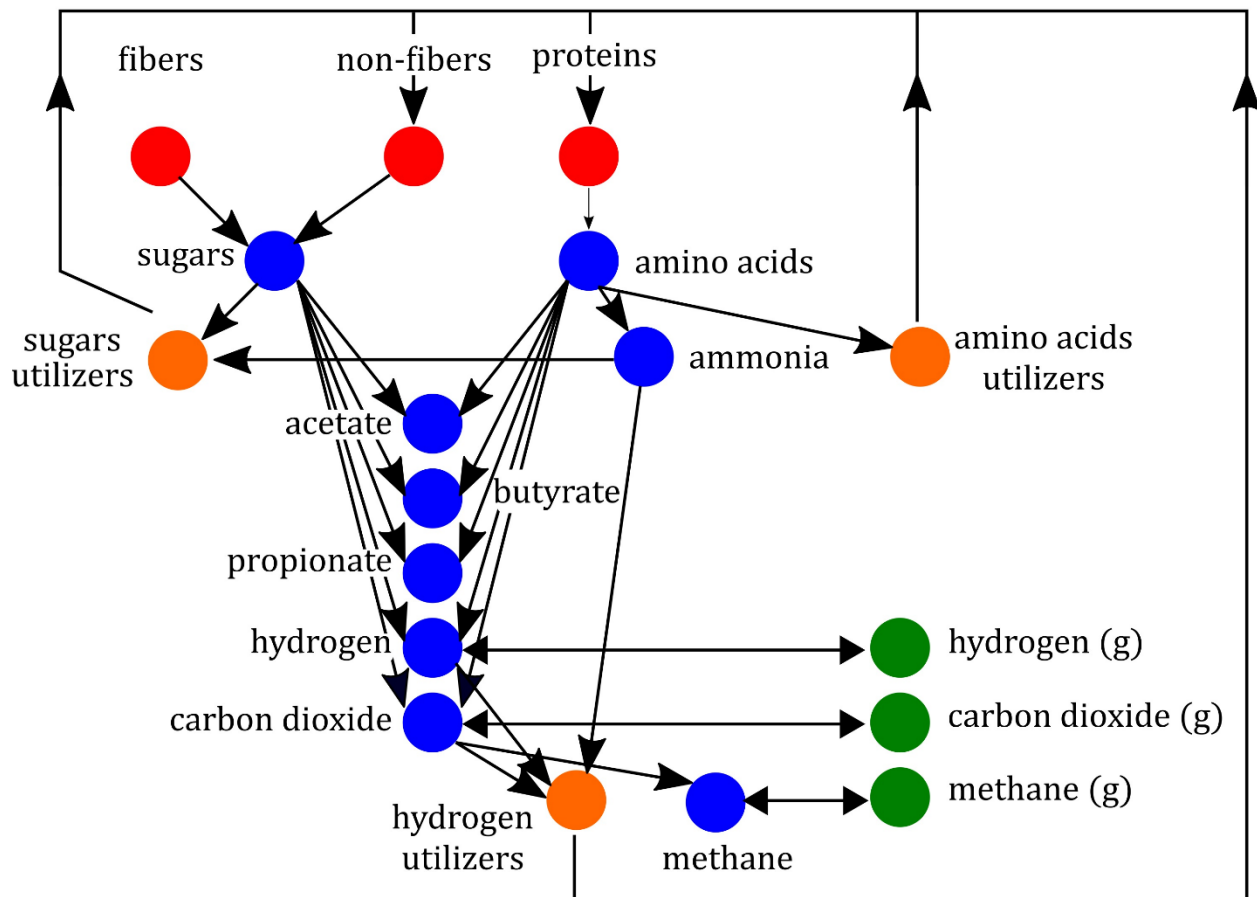


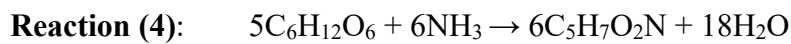
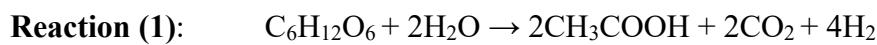
Figure 1. Schematics of the rumen fermentation model developed by Muñoz-Tamayo et al. 2016. Figure taken from (Muñoz-Tamayo and Tedeschi, 2023).

A preliminary investigation was conducted by Muñoz-Tamayo et al. (2019) to assess the robustness of the model challenging it with an independent data set of TMRs fermented using cattle rumen fluid. The model demonstrated effectiveness in representing VFA profile and CH₄ production.

In this study, the dynamic model was further evaluated. The parameter estimation process involved minimizing the number of parameters that required estimation, with the

parameters being set as known and consistent across all the TMRs. The values of these parameters were obtained from the existing literature, as reported in the study of Muñoz-Tamayo et al. (2016). The estimation was centered on the following model parameters λ_1 , λ_2 e km_{H_2} which will be explained below.

A set of parameters correspond to the flux distribution in the process of Su utilization. In the model, Su utilization is represented by one anabolic reaction for microbial biomass formation and 3 catabolic reactions for VFA formation.



The parameter lambda λ_1 represents the moles of glucoses utilized in reaction 1. The parameter lambda λ_2 represents the moles of glucose utilized in reaction 2.

Another parameter to be estimated is the maximum specific utilization rate constant of H_2 (km_{H_2}). This parameter is particularly relevant in the Monod equation used into the model for describe microbial growth rate:

$$\rho_j = km_j \frac{s_j}{Ks_j + s_j} x_j$$

where ρ_j represents the kinetic rate of substrate utilization, s_j the substrate concentration, x_j the microbial biomass concentration, km_j the maximum specific utilization rate constant of the substrate (j) and Ks_j the Monod constant. The kinetic expression is characterized by the km_j and Ks_j . The maximum specific utilization rate constant reflects the

activity of the microbes, while the Monod constant indicates the substrate affinity of the microbes.

The chemical composition of the TMRs (NDF, ADF, ADL, Starch, Ash, FA) were used for setting the initial condition and the hydrolysis of NDF and starch degradability rates were determined by enzymatic based methods (Gallo et al., 2018). These values were used as known in the model. The liquid-gas transfer coefficient k_{La} in the first evaluation was set it as an unknowns parameters and estimated by the model, consequently we decided to average the result and set to 0.33 h^{-1}

The parameters estimation was done for each TMRs using the IDEAS MATLAB toolbox (Munoz-Tamayo et al., 2009) following a maximum likelihood implementation.

Farm index determination

The 39 commercial dairy farms associated with the TMRs evaluated in vitro were selected from a major survey, conducted between January 2018 and June 2018. The farms were visited to represent different ranges in milk yield, herd management and feeding strategies. The data were given by the farmers during a large interview in person regarding information on herd characteristics and nutritional management strategies, dietary information and regarding the home-produced forages, the costs was considered fixed and typical for specific area in which dairy farms were located (Gallo et al., 2022). Based on the collected data, technical and economic indexes were calculated for each farm. CP, NDF and starch digestibility were obtained using ADL as internal marker on TMR and feces sample as reported by Gallo et al. (2022); the average milk yield per day was determine the day of the visit in the farm and the composition express as a percentage of fat and protein.

The observed dry matter intake (real DMI) was calculated as the difference between the amount of feed delivered to the lactating dairy cow groups and the estimated amount of residualorts in the feeder, as reported by the farmer on the day following the interview; the feed efficiency (FE) was expressed as the ratio between the kilograms of fat corrected milk at 4% (4% FCM; NRC, 2001) produced in respect to the amount of DMI in kilograms. The digestible energy use efficiency (DEE), metabolizable energy use (MEE), and net energy use (NEE) were calculated based on the fraction of energy supply by the diet converted to the gross energy milk. The residual energy intake (REI) was determined by the difference between the actual DMI and the estimated DMI.

The economic performance indicators, including income over feed costs (IOFC) income equal to feed costs (IEFC), and feed cost per unit of milk production, were also calculated as described by (Atzori et al., 2021). The parameters were calculated based on the data from lactating animals. The feed costs used were based on the values declared by the farmers or local market prices (Bellingeri et al., 2019)

Statistical analysis

The data were then analyzed using a completely randomized design, with the main fixed effect represented by different nutritional strategies CL. The General Linear Model procedure within the SAS statistical software (2004) was employed, and the least-squares means were compared post-hoc using the LSMEANS option. Differences among the means were declared significant at a p-value of 0.05, while important tendencies were noted for a p-value range of 0.05 to 0.10.

Furthermore, the relationships among the biological variable estimated and farm indexes were quantified using a simple linear Pearson correlation analysis, which was conducted using the CORR procedure in SAS (2004). The software MATLAB

(<https://it.mathworks.com/>) and Rstudio (<https://www.r-project.org/>) were used to plot the figures.

RESULTS

Dietary ingredients and nutritional composition

Four different nutritional strategies were identified, each representing different feed composition to represent the principal ration commonly used in Italian dairy lactating farms. They were grouped respectively in Diet 1 (n=6), Diet 2 (n=18), Diet 3 (n=7), Diet 4 (n=8) TMRs.

A briefly description of characteristics of the Diets, previously reported on Gallo et al. (2022), is reported below and in the supplemental Table S1. Regarding the forage inclusion level, high moisture ear corn (HMC) was not used in the TMR of Diet 3, and 5, whereas Diet 2 and 4 used the highest amount of this ingredient (i.e. on average 17.9% DM). The TMR of the Diet 1, 2, 3, and 4 had an inclusion level of alfalfa silage (LS) lower than 3.0% DM. Concerning protein sources, the Diet 1 and 2 used solvent-extracted soybean meal and dehulled soybean meal (HVProteinFeed) by an inclusion level of about 10% DM, whereas Diet 3, and 4 included less than 2% DM of this CP source. Similarly, the dairy TMR of Diet 1 and 2 were characterized by the use of steam-flaked, expeller or extruded soybean (TRTProteinFeed), with the greatest ($p < 0.05$) inclusion observed in Diet 2 (i.e. 1.3% DM). The compound feeds, used in more than 3.5 kg/cow per day, with a CP concentration higher than 30% as fed (ProteinCompoundFeed) had an inclusion level of about 23.0% DM on Diet 3 and 4. The use of this ingredient in other Diet was negligible. Lastly, the compound feeds, used in less than 3.5 kg/cow per day, with a CP concentration between 21% and 30% as fed (ProteinRestrictedFeed) was adopted only in Diet 1 and 2, at an average inclusion level of 3.3%

DM. The inclusion level of corn meal (YellowStarchFeed) differed among Diet, with Diet 1 and 3 characterized by the greatest values (i.e. >20.0% DM).

The chemical composition and in vitro digestibility of NDF and starch were reported in Table 1.

The rations' parameters differed numerically among the Diets in terms of the content of NDF, CP and starch. The Diet 1 had the highest NDF content, 35.5 ± 5.9 % DM with the lowest digestibility, 47.1 ± 2.9 % NDF, and the CP and starch level with the lowest values, 13.7 ± 1.6 % DM, and 22.6 ± 4.5 % DM, respectively. However, this diet had the highest starch digestibility at 73.6 ± 3.4 % starch.

Diet 2 had the lowest value of NDF at 30.0 ± 2.7 % DM with highest level of digestibility at 49.0 ± 2.6 % NDF and the highest starch level at 26.4 ± 2.7 % DM. Diet 3 had the highest CP level, 16.5 ± 1.0 % DM. Diet 4 had a moderate level of NDF, CP and starch 31.6 ± 2.0 % DM, 15.8 ± 0.9 % DM and 25.3 ± 2.2 % DM respectively. Moreover, it had the lowest level of starch digestibility at 55.2 ± 16.6 % starch.

Total gas production, composition and VFAs profile

The in vitro rumen fermentation dynamics showed distinct patterns of GP across different CL (Table 2). Diet 2 produced the highest volume rate of gas (123.1 mL/g OM), while Diets 1 produced the lowest (111.8 mL/g OM). The CH₄ production was similar across CL, with no statistically significant differences.

The production of VFA was dominated by acetate with an important statistical tendency (p-value<0.09), followed by propionate and butyrate. Diets 2 had the highest acetate concentration (48.3 mmol/L), while Diet 4 exhibited the lowest (42.6 mmol/L).

Propionate production varied, with Diets 4 exhibiting the lowest levels and Diets 1 the highest but remaining within the expected ranges for intensive dairy cow diets. The ratios of

acetate/propionate for Diets 1, 2, 3, and 4 were 1.89, 2.23, 2.30, respectively. The butyrate concentration was similar across diet 2 and 3 (12.2 mmol/l and 12.1 mmol/L), whereas Diet 1 showed the highest level (13.7 mmol/L) and diet 4 the lowest (9.0 mmol/L).

Dynamic prediction of rumen fermentation

Figure 2 shows the dynamic data of fermentation variables of a TMR compared against the model predicted variables. Figure 3 displays the comparison of all observations against model predictions. Figure 4 shows the residuals for all variables against centered predicted values. Table 3, shows the calculated standard statistical indicators of model performance. These statistic indicators provide an indication of the precision and accuracy of the model structure to represent the fermentation dynamics of Italian TMR.

For the VFAs the coefficients of determination ranged from $R^2 = 0.77$ (butyrate) to 0.93 (acetate) while the methane showed the higher value ($R^2 = 0.91$). The coefficient of variation of the RMSE (CV(RMSE)) remained below 30% for all compounds with the highest for butyrate (29%). Concordance correlation coefficients (CCC) confirmed a good agreement between observed and predicted values for acetate, butyrate, propionate (0.85 – 0.89) and an excellent agreement for methane (0.95).

Residual analysis in Table 3 revealed a numeric negative intercept (α) for all metabolites, even if the values were very near to 0. Furthermore, slopes of the residuals on centered predictions (β) were negligible to identify important trends for systematic errors, suggesting the model properly fits acetate, propionate and methane data independently by the prediction times.

Rumen model parameters and correlations among farms' indexes

The average values of the estimated rumen parameters for the different nutritional strategies are reported in Table 4. These parameters did not differ significantly across the diets. Diet 3 had numerically the highest value of λ_1 (0.38 mol/mol), indicating that a larger proportion of glucose was fermented through the acetate pathway in reaction R1 compared to the other diets, suggesting an abundance of substrate available for acetogenic microbes. Diet 1 with a lower value of 0.25 mol/mol, reflected a more balanced fermentation between acetate and propionate, which may contribute to a reduced availability of H_2 for methanogenesis.

The parameter λ_2 , as reported, exhibited similar average values for Diets 2 and 3, as well as for Diets 1 and 4. Regarding km_{H_2} , a key parameter in determining microbial efficiency in utilizing H_2 , Diet 3 reported the highest value (19.1 mol/(mol h)), indicating elevated microbial activity.

The correlation analysis between the biological parameters of the model and farm indices, shown in Table 5, provides further insights. λ_1 and λ_2 did not show significant correlations. However, λ_2 exhibited negative correlations with milk yield, real DMI, FE, IOFC and IEFC, and the efficiency of energy utilization.

A significant correlation was observed between km_{H_2} and different farms' indexes farms ($p < 0.05$). A positive correlation between the km_{H_2} and the real DMI ($r = 0.410$) and REI ($r = 0.346$), was observed. Additionally, the digestibility of NDF and CP showed significant positive correlations with km_{H_2} ($r = 0.387$ and $r = 0.380$, respectively), suggesting that nutritional strategies that improve H_2 utilization also promote efficient ruminal degradation, whereas significant negative correlation was reported for the Milk fat ($r = -0.323$).

DISCUSSION

The use of mathematical models has been used for the last decades as a valid instrument for understand the complex rumen system and provided more information how to predicting enteric CH₄ emission (Hristov et al., 2018; Tedeschi et al., 2022b).

Previous work demonstrated that in vitro method can useful predict the in vivo trend of CH₄ emission from different combinations of feed ingredients (Hatew et al., 2015). The 55 TMRs used in the model satisfactory represented the ration formulated in intensive farm system, nevertheless the amount of TMRs on nutritional strategy cluster could be improved for a major representation. The Diet 2 presented the highest number of TMRs, suggesting that the feed used and their level of inclusion in rations probably are those most commonly adopted in Northern Italy.

Evaluation of fermentability of total mixed ration

The results of this study provide convincing evidence that the mechanistic model accurately predicted the VFAs profiles and CH₄ production under the tested nutritional strategies. The dietary compositions presented in Table 1 were consistent with other studies that utilized corn silage-based diets (Pirondini et al., 2012, 2015; Boivin et al., 2013). The total gas and CH₄ production in Table 2 showed slight variation among the diets. The average cumulated production of gas per ml/220 mg OM was 118.8 and an average CH₄ production expressed per g/OM was 6.1, ranging from 5.7 to 6.4 across the four dietary treatments without significant differences ($p>0.05$). Similar values were reported (Pirondini et al., 2012; Maccarana et al., 2016; Serment et al., 2016) Diet 2 showed the highest GP rate (123.1 mL/g OM), while Diet 1 had the lowest (111.8 mL/g OM). As expected, these differences might be explained by the differences of NDF levels (+5% DM) for Diet 1, whereas opposite situations were reported for the starch concentration (+4.2 % DM) for the Diet 2.

The impact of dietary modifications and the ratio of forage to concentrate on the regulation of VFAs and CH₄ production has been extensively studied and documented (Beauchemin et al., 2020). The CH₄ production was largely dependent on H₂ availability, due to the methanogenic archaea role in maintaining redox balance within the rumen by utilizing excess H₂ (Pitta et al., 2022a). Previous studies highlighted the complexity of H₂ flows and their influence on methanogenesis, particularly when inhibitors or other antimethanogenic strategies are employed (Ungerfeld and Pitta, 2024). The study by Muñoz-Tamayo et al., (2016) demonstrated the effective influence of the inoculum on VFA variation, which can be attributed to the role of the ruminal microflora and its capacity to adapt to different diets. Our results support these findings, demonstrating that rations such as Diet 1 with moderate inclusion of corn silage and corn meals produced a greater proportion of propionate, which acts as a H₂ sink and subsequently reduces CH₄ production. A recently *in vivo* study demonstrated the effective CH₄ reduction of corn silage compared to other inclusion and silage (Giagnoni et al., 2024). Additionally, the effect of silage and concentrate types on dry matter intake (DMI) was additive. Regarding production efficiency, energy-corrected milk (ECM) yield was not affected by silage type in first-lactation cows, but it was higher in second-lactation cows fed corn silage compared to those fed grass-clover silage.

Evidence suggests that animals at different growth stages exhibit varying patterns of feed utilization, indicating the need for more studies on the impacts of diverse nutritional strategies on CH₄ emissions.

Rumen fermentation model performance and limitations

The Su utilization flow had a central role in this study to explain the impact of different nutritional strategies on VFAs and CH₄ production. The estimated values are reported in Table 4. The λ_1 and λ_2 were coherent with our expectations regarding their impact on acetate and

propionate production, and the subsequent influence of these VFAs on GP and CH₄ emissions. Diet 2 and 3, with a higher proportion of acetate production ($\lambda_1 = 0.31$ and 0.38 mol/mol), also presented high CH₄ production; this is consistent with previous studies showing that acetate production is concomitant with high production of H₂, which methanogenic archaea use to reduce CO₂ to CH₄. Our results showed numerical variation in the km_{H_2} reflecting differences in microbial activity and fermentation efficiency.

Conversely, promoting the propionate production reduces H₂ concentration and their availability for methanogens. In Diet 1 $\lambda_1 = 0.25$ mol/mol was the numerical lowest value. There was a tendency for reduced CH₄ emissions with a lower value for km_{H_2} although these differences were not statistically significant. This underscores the importance of promoting propionate pathways in nutritional strategies aimed at CH₄ mitigation. Further exploration into dietary additives or adjustments that enhance propionate formation could be a valuable area for future research.

Implications for Sustainable Nutritional Strategies

The study conducted evaluated different nutritional strategies clusters and their impact on CH₄ emissions. Mitigating CH₄ emissions while maintaining or improving animal productivity is a critical challenge for sustainable dairy farming. To address this, the technical and economic parameters of each farm were calculated and used to further evaluate the impact of the nutritional strategies clusters.

Strategies such as dietary manipulation, the use of feed additives like 3-nitrooxypropanol (3-NOP), and improving forage quality have shown to be promising in reducing CH₄ emissions without compromising animal performance (Hristov, 2024). Our study allowed to identify biological parameters (k_{m,H_2} , λ_1 , λ_2) estimated by the model for explaining

the variation of rumen fermentation pattern and CH₄ production between 4 types of formulation diets.

The positive correlation, observed in Table 5, between km_{H_2} and the apparent digestibility of CP and NDF might suggest that more digestible rations lead to a major rumen microbial activity and production, consequently increasing H₂ accumulation and CH₄ potential emissions. Additional positive correlations were observed between actual DMI, REI observed in vivo, and km_{H_2} estimated by the model in vitro condition; these correlations probably are explained with the in vivo condition where the animal that increased the feed consumption provided more substrate for the rumen microbiota, consequently resulting in greater H₂ availability for methanogenesis. REI calculated with difference between measured and predicted energy intake, have been used as a nutritional index of FE on dairy cattle (Becker et al., 2021). A recent study evaluated the impact of the REI on nutrient use and CH₄ emissions (Ahvenjärvi et al., 2024). The results showed that cows with low REI, considered more feed-efficient, had high CH₄ yields and mobilized more energy and protein from body reserves to maintain similar milk production levels compared to the high-REI group. This theory aligns with our results, which show a positive correlation between km_{H_2} and REI. This suggests that as feed inefficiency increases, there is a corresponding rise in the maximum utilization rate of H₂, which leads to increased production of CH₄ emissions.

Morgavi et al. (2023), argued that CH₄ mitigation may not always lead to significant improvements in FE due to the accumulation of H₂ when methanogenesis is inhibited.

Significant negative correlation was observed between km_{H_2} and milk fat percentage ($r = -0.323$) suggests that increased the km_{H_2} negatively impact on milk fat concentration. In ruminant the acetate is a principal source for the milk fat synthesis, where short- and medium-chain fatty acids, and a portion of the 16-carbon fatty acids, originate from de novo synthesis (Harvatine et al., 2009). Recently, a study was conducted on milk fat acids variation as an index

to determine CH₄ emissions (De Souza et al., 2020). The results showed a positive relationship between de novo milk fatty acids, particularly C4:0, and CH₄ production per mole of VFA. In contrast, anteiso C15:0 and C15:0 were negatively related to CH₄ production. This consideration suggests investigating more deeply in our results regarding the impact of km_{H_2} on the specific milk fat acids profiles and their relationships with parameters of rumen fermentation and CH₄ production. Other parameters of the model can also be integrated in the future to explore the connection between rumen fermentation parameters and economic indicators.

CONCLUSIONS

This study confirms the potential use of the mechanistic model developed by Muñoz-Tamayo et al., (2016,2021) to represent the rumen fermentation pattern and CH₄ production for different nutritional strategies applied to Italian intensive dairy farm systems. The nutritional strategies did not have a direct impact on CH₄ production under *in vitro* conditions. However, the model-estimated parameters provided insights into the relationships between substrate utilization, VFAs, and CH₄ production. The findings indicate the need for a balanced approach that considers CH₄ mitigation, animal performance, and farm index. The study emphasizes the value of mechanistic modeling in evaluating the sustainability of nutritional strategies in intensive dairy farming, and the need for further research on the effect of dietary components and additives on rumen fermentation and milk composition. To achieve this goal, model extensions are needed to account for *in vivo* conditions.

Acknowledgments

This study was supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

REFERENCES

- Ahvenjärvi, S., Bayat, A.-R., Toivanen, M., Mäntysaari, P., & Tapio, I. (2024). The effects of residual energy intake on nutrient use, methane emissions and microbial composition in dairy cows. *Scientific Reports*, 14(1), 613. <https://doi.org/10.1038/s41598-024-51300-7>
- Atzori, A. S., Valsecchi, C., Manca, E., Masoero, F., Cannas, A., & Gallo, A. (2021). Assessment of feed and economic efficiency of dairy farms based on multivariate aggregation of partial indicators measured on field. *Journal of Dairy Science*, 104(12), 12679–12692. <https://doi.org/10.3168/jds.2020-19764>
- Baldwin, R. L., Koong, L. J., & Ulyatt, M. J. (1977). A dynamic model of ruminant digestion for evaluation of factors affecting nutritive value. *Agricultural Systems*, 2(4), 255–288. [https://doi.org/10.1016/0308-521X\(77\)90020-8](https://doi.org/10.1016/0308-521X(77)90020-8)
- Bannink, A., Kogut, J., Dijkstra, J., France, J., Kebreab, E., Van Vuuren, A. M., & Tamminga, S. (2006). Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. *Journal of Theoretical Biology*, 238(1), 36–51. <https://doi.org/10.1016/j.jtbi.2005.05.026>
- Beauchemin, K. A., Ungerfeld, E. M., Abdalla, A. L., Alvarez, C., Arndt, C., Becquet, P., Benchaar, C., Berndt, A., Mauricio, R. M., McAllister, T. A., Oyhantçabal, W., Salami, S. A., Shalloo, L., Sun, Y., Tricarico, J., Uwizeye, A., De Camillis, C., Bernoux, M., Robinson, T., & Kebreab, E. (2022). Invited review: Current enteric methane mitigation options. *Journal of Dairy Science*, 105(12), 9297–9326. <https://doi.org/10.3168/jds.2022-22091>
- Beauchemin, K. A., Ungerfeld, E. M., Eckard, R. J., & Wang, M. (2020). Review: Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal*, 14, s2–s16. <https://doi.org/10.1017/S1751731119003100>

- Becker, V. a. E., Stamer, E., Spiekers, H., & Thaller, G. (2021). Residual energy intake, energy balance, and liability to diseases: Genetic parameters and relationships in German Holstein dairy cows. *Journal of Dairy Science*, 104(10), 10970–10978. <https://doi.org/10.3168/jds.2021-20382>
- Bellingeri, A., Cabrera, V., Gallo, A., Liang, D., & Masoero, F. (2019). A survey of dairy cattle management, crop planning, and forages cost of production in Northern Italy. *Italian Journal of Animal Science*. <https://www.tandfonline.com/doi/abs/10.1080/1828051X.2019.1580153>
- Bellingeri, A., Gallo, A., Liang, D., Masoero, F., & Cabrera, V. E. (2020). Development of a linear programming model for the optimal allocation of nutritional resources in a dairy herd. *Journal of Dairy Science*, 103(11), 10898–10916. <https://doi.org/10.3168/jds.2020-18157>
- Boivin, M., Gervais, R., & Chouinard, P. Y. (2013). Effect of grain and forage fractions of corn silage on milk production and composition in dairy cows. *Animal*, 7(2), 245–254. <https://doi.org/10.1017/S1751731112001486>
- Danfær, A., Huhtanen, P., Udén, P., Sveinbjörnsson, J., & Volden, H. (2006). The Nordic dairy cow model, Karoline—Description. In *Nutrient digestion and utilization in farm animals: Modelling approaches* (pp. 383–406). <https://doi.org/10.1079/9781845930059.0383>
- De Souza, J., Leskinen, H., Lock, A. L., Shingfield, K. J., & Huhtanen, P. (2020). Between-cow variation in milk fatty acids associated with methane production. *PLOS ONE*, 15(8), e0235357. <https://doi.org/10.1371/journal.pone.0235357>
- Dijkstra, J., Neal, H. D. St. C., Beever, D. E., & France, J. (1992). Simulation of Nutrient Digestion, Absorption and Outflow in the Rumen: Model Description1,2. *The Journal of Nutrition*, 122(11), 2239–2256. <https://doi.org/10.1093/jn/122.11.2239>

- Gallo, A., Giuberti, G., & Masoero, F. (2016). Gas production and starch degradability of corn and barley meals differing in mean particle size. *Journal of Dairy Science*, 99(6), 4347–4359. <https://doi.org/10.3168/jds.2015-10779>
- Gallo, A., Valsecchi, C., Masseroni, M., Cannas, A., Ghilardelli, F., Masoero, F., & Atzori, A. S. (2022). An observational study to verify the influence of different nutritional corn silage-based strategies on efficient use of dietary nutrients, faecal fermentation profile, and profitability in a cohort of intensive dairy farms. *Italian Journal of Animal Science*, 21(1), 228–243. <https://doi.org/10.1080/1828051X.2022.2025932>
- Ghilardelli, F., Ferronato, G., & Gallo, A. (2022). Near-infrared calibration models for estimating volatile fatty acids and methane production from in vitro rumen fermentation of different total mixed rations. *JDS Communications*, 3(1), 19–25. <https://doi.org/10.3168/jdsc.2021-0156>
- Giagnoni, G., Lund, P., Johansen, M., Hellwing, A. L. F., Noel, S. J., Thomsen, J. P. S., Poulsen, N. A., & Weisbjerg, M. R. (2024). Effect of carbohydrate type in silages and concentrates on feed intake, enteric methane, and milk yield from dairy cows. *Journal of Dairy Science*, 107(10), 7851–7866. <https://doi.org/10.3168/jds.2024-24642>
- Harvatine, K. J., Boisclair, Y. R., & Bauman, D. E. (2009). Recent advances in the regulation of milk fat synthesis. *Animal*, 3(1), 40–54. <https://doi.org/10.1017/S1751731108003133>
- Hatew, B., Cone, J. W., Pellikaan, W. F., Podesta, S. C., Bannink, A., Hendriks, W. H., & Dijkstra, J. (2015). Relationship between in vitro and in vivo methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle. *Animal Feed Science and Technology*, 202, 20–31. <https://doi.org/10.1016/j.anifeedsci.2015.01.012>

- Hristov, A. N. (2024). Invited review: Advances in nutrition and feed additives to mitigate enteric methane emissions. *Journal of Dairy Science*, 107(7), 4129–4146. <https://doi.org/10.3168/jds.2023-24440>
- Hristov, A. N., Kebreab, E., Niu, M., Oh, J., Bannink, A., Bayat, A. R., Boland, T. M., Brito, A. F., Casper, D. P., Crompton, L. A., Dijkstra, J., Eugène, M., Garnsworthy, P. C., Haque, N., Hellwing, A. L. F., Huhtanen, P., Kreuzer, M., Kuhla, B., Lund, P., ... Yu, Z. (2018). Symposium review: Uncertainties in enteric methane inventories, measurement techniques, and prediction models. *Journal of Dairy Science*, 101(7), 6655–6674. <https://doi.org/10.3168/jds.2017-13536>
- López, S., Dhanoa, M. S., Dijkstra, J., Bannink, A., Kebreab, E., & France, J. (2007). Some methodological and analytical considerations regarding application of the gas production technique. *Animal Feed Science and Technology*, 135(1–2), 139–156. <https://doi.org/10.1016/j.anifeedsci.2006.06.005>
- Maccarana, L., Cattani, M., Tagliapietra, F., Bailoni, L., & Schiavon, S. (2016). Influence of main dietary chemical constituents on the in vitro gas and methane production in diets for dairy cows. *Journal of Animal Science and Biotechnology*, 7(1), 54. <https://doi.org/10.1186/s40104-016-0109-5>
- Menke, K. (1988). Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. <https://www.semanticscholar.org/paper/Estimation-of-the-energetic-feed-value-obtained-and-Menke/d3ac6b5a6ac47bb07b0e9055c818dc785b440d18>
- Mills, J. A. N., Dijkstra, J., Bannink, A., Cammell, S. B., Kebreab, E., & France, J. (2001). A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: Model development, evaluation, and application1. *Journal of Animal Science*, 79(6), 1584–1597. <https://doi.org/10.2527/2001.7961584x>

- Morgavi, D. P., Cantalapiedra-Hijar, G., Eugène, M., Martin, C., Nozière, P., Popova, M., Ortigues-Marty, I., Muñoz-Tamayo, R., & Ungerfeld, E. M. (2023). Review: Reducing enteric methane emissions improves energy metabolism in livestock: is the tenet right? *Animal*, 17, 100830. <https://doi.org/10.1016/j.animal.2023.100830>
- Muñoz-Tamayo, R., Atzori, A. S., Cannas, A., Masoero, F., Giger-Reverdin, S., Sauvant, D., & Gallo, A. (2019). Assessing the predictive capabilities of a dynamic mechanistic model of in vitro rumen fermentation and methane production.
- Muñoz-Tamayo, R., Giger-Reverdin, S., & Sauvant, D. (2016). Mechanistic modelling of in vitro fermentation and methane production by rumen microbiota. *Animal Feed Science and Technology*, 220, 1–21. <https://doi.org/10.1016/j.anifeedsci.2016.07.005>
- Munoz-Tamayo, R., Laroche, B., Leclerc, M., & Walter, E. (2009). IDEAS: a Parameter Identification Toolbox with Symbolic Analysis of Uncertainty and its Application to Biological Modelling.
- Muñoz-Tamayo, R., & Tedeschi, L. O. (2023). ASAS-NANP symposium: Mathematical Modeling in Animal Nutrition: The power of identifiability analysis for dynamic modeling in animal science:a practitioner approach. *Journal of Animal Science*, 101, skad320. <https://doi.org/10.1093/jas/skad320>
- National Research Council. (2001). *Nutrient Requirements of Dairy Cattle* (Seventh Revised Edition,). National Academies Press. <https://doi.org/10.17226/9825>
- Pirondini, M., Colombini, S., Mele, M., Malagutti, L., Rapetti, L., Galassi, G., & Crovetto, G. M. (2015). Effect of dietary starch concentration and fish oil supplementation on milk yield and composition, diet digestibility, and methane emissions in lactating dairy cows. *Journal of Dairy Science*, 98(1), 357–372. <https://doi.org/10.3168/jds.2014-8092>
- Pirondini, M., Malagutti, L., Colombini, S., Amodeo, P., & Crovetto, G. M. (2012). Methane yield from dry and lactating cows diets in the Po Plain (Italy) using an in vitro gas

- production technique. *Italian Journal of Animal Science*, 11(3), e61.
<https://doi.org/10.4081/ijas.2012.e61>
- Pitta, D., Indugu, N., Narayan, K., & Hennessy, M. (2022). Symposium review: Understanding the role of the rumen microbiome in enteric methane mitigation and productivity in dairy cows. *Journal of Dairy Science*, 105(10), 8569–8585.
<https://doi.org/10.3168/jds.2021-21466>
- Serment, A., Giger-Reverdin, S., Schmidely, P., Dhumez, O., Broudiscou, L. P., & Sauvant, D. (2016). In vitro fermentation of total mixed diets differing in concentrate proportion: Relative effects of inocula and substrates. *Journal of the Science of Food and Agriculture*, 96(1), 160–168. <https://doi.org/10.1002/jsfa.7076>
- Serment, A., & Sauvant, D. (2011). A mechanistic model of pH and gas exchanges in the rumen and its in vitro application. In D. Sauvant, J. Van Milgen, P. Faverdin, & N. Friggens (Eds.), *Modelling nutrient digestion and utilisation in farm animals* (pp. 148–157). Academic Publishers. https://doi.org/10.3920/978-90-8686-712-7_16
- Tedeschi, L. O., Abdalla, A. L., Álvarez, C., Anuga, S. W., Arango, J., Beauchemin, K. A., Becquet, P., Berndt, A., Burns, R., De Camillis, C., Chará, J., Echazarreta, J. M., Hassouna, M., Kenny, D., Mathot, M., Mauricio, R. M., McClelland, S. C., Niu, M., Onyango, A. A., ... Kebreab, E. (2022). Quantification of methane emitted by ruminants: A review of methods. *Journal of Animal Science*, 100(7), skac197.
<https://doi.org/10.1093/jas/skac197>
- Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllan, A. B., & France, J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, 48(3), 185–197.
[https://doi.org/10.1016/0377-8401\(94\)90171-6](https://doi.org/10.1016/0377-8401(94)90171-6)

Ungerfeld, E. M., & Pitta, D. (2024). Review: Biological consequences of the inhibition of rumen methanogenesis. *Animal*, 101170. <https://doi.org/10.1016/j.animal.2024.101170>

Table 1. Chemical composition of different nutritional strategies clusters Diet 1 (6 TMRs) , Diet 2 (18 TMRs), Diet 3 (7 TMRs), and Diet 4(8 TMRs)

<i>Chemical Composition</i>	Units	Diet				Diet				Diet				Diet			
		1				2				3				4			
		mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th
NDF	g/ 100 g DM	35.5	5.9	32.6	33.8	30.0	2.7	28.2	31.5	32.2	3.0	31.0	32.9	31.6	2.0	31.3	32.2
ADF	g/ 100 g DM	21.3	2.5	18.5	18.5	18.5	2.0	15.9	15.9	20.1	2.3	18.0	18.0	20.6	1.6	18.4	18.4
ADL	g/ 100 g DM	3.1	1.0	2.3	3.8	2.6	0.6	2.1	2.9	2.8	0.2	2.7	2.9	3.0	0.6	2.8	3.2
NDICP	g/ 100 g DM	1.8	0.7	1.8	2.1	1.9	0.5	1.7	2.2	2.3	0.3	2.1	2.5	2.2	0.2	2.0	2.3
CP	g/ 100 g DM	13.7	1.6	13.6	14.7	15.7	0.8	15.4	15.9	16.5	1.0	15.9	17.2	15.8	0.9	15.5	16.3
RDP	g/ 100 g DM	52.6	8.5	45.9	61.3	52.0	5.8	48.9	55.5	46.2	2.7	44.8	47.8	53.6	4.1	52.4	55.1
Starch	g/ 100 g DM	22.6	4.5	22.0	25.2	26.4	2.7	25.3	27.4	24.1	1.4	23.5	24.4	25.3	2.2	24.2	27.4
Sugar	g/ 100 g DM	5.7	1.1	4.9	6.8	5.3	0.8	4.9	5.6	5.8	0.5	5.7	6.1	5.0	0.7	4.3	5.4
EE	g/ 100 g DM	3.0	0.6	2.7	3.5	3.6	0.7	3.2	4.1	3.6	0.4	3.3	3.9	3.1	0.2	3.1	3.3
Ash	g/ 100 g DM	9.5	0.8	8.8	10.3	9.3	1.0	8.4	9.9	9.1	0.6	8.7	9.3	9.2	0.8	9.0	10.0
DNDF 24h	g/ 100 g NDF	47.1	2.9	45.2	48.9	49.0	2.6	47.5	51.2	47.8	1.2	46.8	48.7	47.9	2.3	47.5	49.1
dStarch	g/ 100 g Starch	73.6	3.4	70.7	76.3	64.4	14.4	52.6	75.1	63.0	12.4	53.9	72.8	55.2	16.6	48.8	69.9

NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent fiber; NDICP, neutral detergent insoluble CP; CP, crude protein; EE, ether extract; RDP, ruminal degradable protein; OM, organic matter; DNDF 24h, digestibility of NDF at 24h of rumen incubation; dStarch; digestibility of starch; SD, standard deviation; 25th quartile; 75th quartile.

Table 2. Levels of *in vitro* rumen fermentation products of different nutritional strategy clusters Diet 1 (6 TMRs) , Diet 2 (18 TMRs), Diet 3 (7 TMRs), and Diet 4(8 TMRs)

		Diet				Diet				Diet				Diet				√MSE	P-Value
		1		2		3		4											
Items	Units	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th		
Cum GP	ml/220 gr OM	111.8	85.5	29.7	182.2	123.1	85.8	39.3	200.5	122.3	83.0	44.8	196.5	117.9	84.6	36.5	192.9	23.77	0.76
CH ₄	ml	5.7	3.9	1.9	8.7	6.1	4.0	2.4	9.4	6.4	4.0	2.5	9.7	6.2	4.0	2.5	10.0	0.73	0.33
Ac	mmol/L	46.6	10.7	39.0	55.2	48.3	10.9	39.1	58.0	47.5	11.3	38.4	55.3	42.6	10.9	36.1	51.8	5.02	0.09
Pr	mmol/L	24.7	7.8	20.5	30.2	21.6	8.4	14.3	28.9	20.7	8.7	13.4	26.9	15.7	8.0	9.7	21.9	7.84	0.21
Bu	mmol/L	13.7	5.2	11.2	15.7	12.2	5.2	9.4	15.2	12.1	5.4	9.0	14.4	9.0	3.7	5.4	11.6	3.92	0.16
Va	mmol/L	1.9	0.9	1.3	2.6	1.7	0.9	0.9	2.4	1.7	0.9	0.9	2.4	1.2	0.9	0.5	2.0	0.53	0.49
IsoBu	mmol/L	1.2	0.5	0.7	3.9	1.0	0.6	0.6	3.7	1.0	0.6	0.5	3.5	0.9	0.5	0.4	3.1	0.45	0.61
IsoVa	mmol/L	2.7	1.5	1.1	3.9	2.3	1.5	0.9	3.7	2.3	1.6	0.8	3.5	1.8	1.4	0.5	3.1	1.38	0.72

Ac, Acetic Acid; Pr, Propionic Acid; Bu, Butirric Acid; Va, Valerianic Acid; IsoBu, IsoButirric Acid; IsoVa, IsoValerianic Acid; GP, Cumulate gas production; CH₄, Methane.

SD, standard deviation; 25th quartile; 75th quartile.

Table 3. Statistical indicators of model performance

	Acetate	Butyrate	Propionate	Methane
R ²	0.83	0.77	0.80	0.91
RMSE ^a	0.0057	0.003	0.0036	5.33E-05
100xCV _{RMSE} ^b	13	29	24	21
CCC ^c	0.89	0.86	0.85	0.95
Residual analysis				
$residual = \alpha + \beta * (predicted - mean\ predicted\ value)$				
	Acetate	Butyrate	Propionate	Methane
α	-0.0026	-0.0010	-0.0020	-2.25E-05
β	-0.0637	-0.0220	-0.0362	-0.0745

^a Root mean squared error (RMSE)^b Coefficient of variation of the RMSE (CV(RMSE))^c Concordance correlation coefficient (CCC)

Table 4. Average estimated molar fraction of the sugars utilized via reaction acetate (R1) and acetate-propionate (R2) of nutritional strategy clusters Diet 1 (6 TMRs) , Diet 2 (18 TMRs), Diet 3 (7 TMRs), and Diet 4(8 TMRs)

		Diet				Diet				Diet				Diet				√MSE	P-Value
		1		2		3		4		5		6		7		8			
Items	Units	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th	mean	SD	25th	75th		
Lambda 1 ^a	mol/mol	0.25	0.09	0.18	0.31	0.31	0.13	0.25	0.36	0.38	0.12	0.27	0.55	0.29	0.09	0.21	0.37	0.11	0.21
Lambda 2 ^b	mol/mol	0.30	0.13	0.28	0.39	0.33	0.13	0.21	0.40	0.33	0.11	0.24	0.41	0.30	0.14	0.18	0.42	0.12	0.92
Km_H2 ^c	mol/(mol h)	13.29	6.55	10.84	12.82	17.33	6.70	12.49	20.92	19.10	5.06	14.62	24.87	18.83	8.51	13.28	23.20	6.84	0.41

^a Reaction 1 (R1) sugar utilization associated with acetate production.

^b Reaction 2 (R2) sugar utilization associated with acetate and propionate production.

^c Maximum specific utilization rate constant of hydrogen.

SD, standard deviation; 25th quartile; 75th quartile.

The Effects of the model were significant at a P-value < 0.05.

Table 5. Coefficients correlation of Pearson between biological parameters estimated by the model Muñoz-Tamayo et al. (2016) and diet digestibility, economic indexes, milk yield and composition

Items	Units	Lambda 1 ^a	Lambda 2 ^b	Km_H ₂ ^c
Crude protein digestibility	% CP	0.170	0.106	0.380*
NDF digestibility	% NDF	0.201	0.046	0.387*
Starch digestibility	% Starch	0.042	0.064	0.140
Milk yield	kg/d per milking cow	0.190	-0.108	0.268
Milk fat	%	0.043	0.311	-0.323*
Milk protein	%	0.036	0.160	0.060
Real Dry matter intake	kg DM/d per cow	0.296	-0.024	0.410*
Feed efficiency	kg of milkFCM4.0/kg of DMI	0.231	-0.141	0.304
Digestible energy use efficiency	dmnl	0.066	-0.056	-0.026
Metabolizable energy efficiency	dmnl	0.076	-0.155	0.009
Net energy efficiency	dmnl	0.072	-0.157	0.012
Residual energy intake	Mcal/day	0.248	0.097	0.346*
Income over feed costs	€/cow/day	0.140	-0.079	0.188
Income equal feed costs	liter/cow/day	0.179	-0.096	0.295
Feed Costs for hundred weights of milk	€/kg of milk	0.010	0.037	0.012

^a Reaction1 (R1) sugars utilization associated with acetate production.

^b Reaction2 (R2) sugar utilization associated with acetate and propionate production.

^c Maximum specific utilization rate constant of hydrogen.

*If $p < 0.05$ an asterisk was reported

CP, crude protein; DM, dry matter; DMI, dry matter intake; milkFCM4.0, fat corrected milk at 4%; NDF, Neutral Detergent Fiber; dmnl, dimensionless

Figure 2. Example of model fitting. Experimental data (●) of the sample variable are compared with the model predicted response in solid black

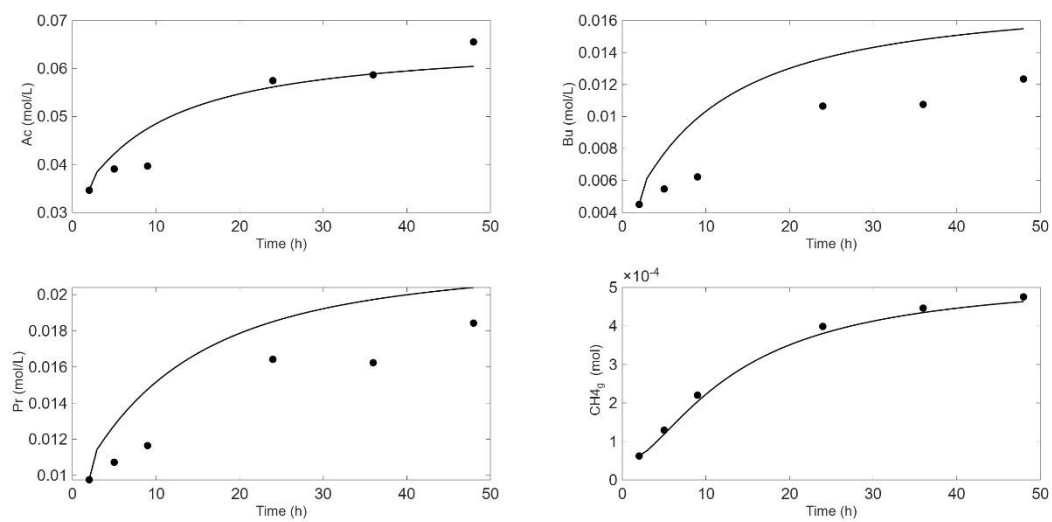


Figure 3. Summary of the model performance calibration integrating data of all treatments.

Experimental data are plotted against the model predicted variables

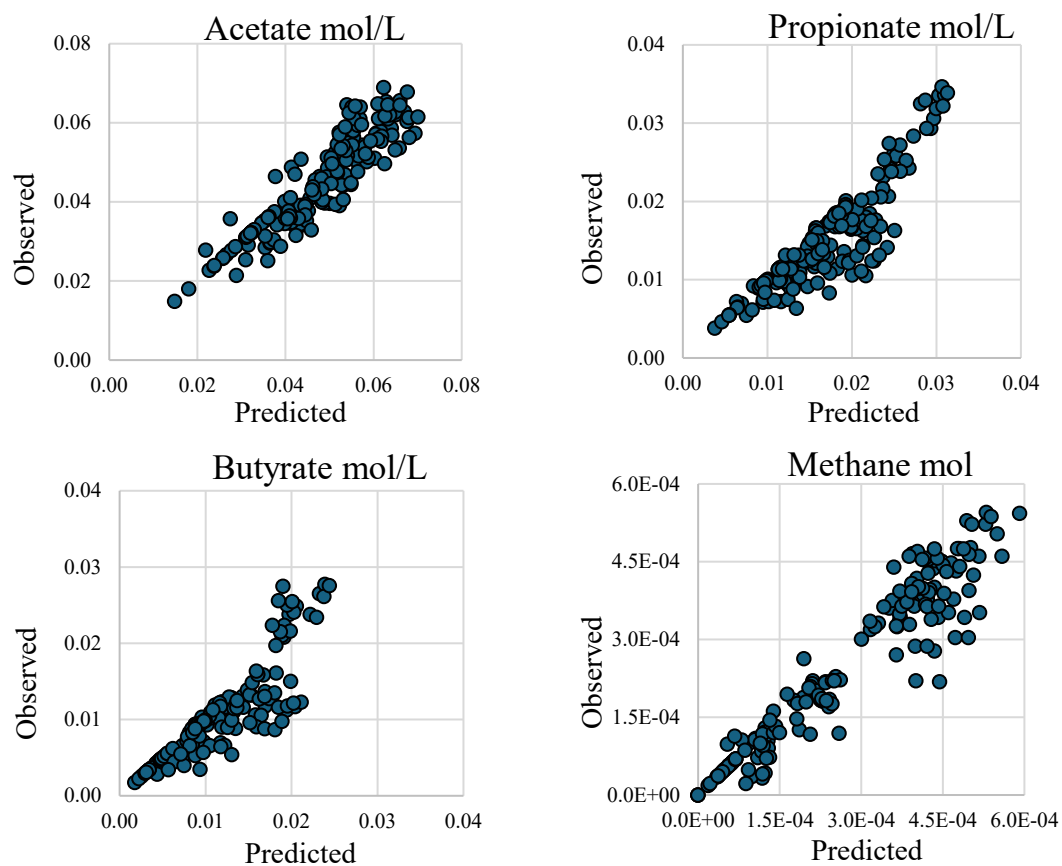


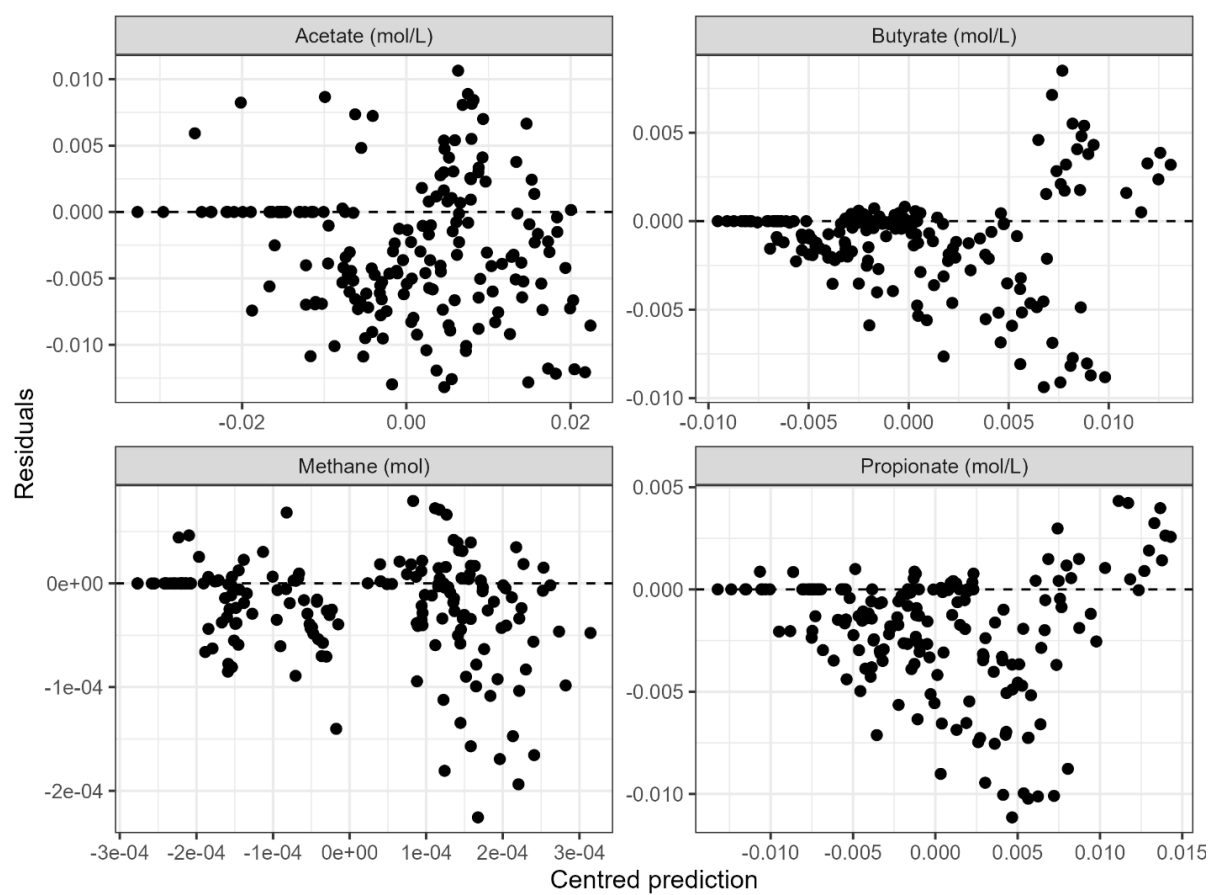
Figure 4. Residual values of observed variables against centered predicted variables

Table S1 Diet ingredient formulation in different nutritional strategy clusters

	Diet1	Diet2	Diet3	Diet4
Corn silage	33.10	31.20	26.70	33.10
High moisture corn		17.60		18.30
Alfalfa silage	1.40	1.20	1.00	2.80
Grass Silage	12.20	6.10	7.90	5.70
Alfalfa hay	2.90	2.50	0.60	3.80
Grass Hay	7.00	6.60	9.10	4.60
Straw	0.20	0.80	0.70	0.60
By products feeds		0.80	0.60	
High Value Protein Rich Feed	10.60	11.00	0.80	1.40
Low Value Protein Rich Feed	0.30	0.90		
TRT Protein Rich Feed	0.20	1.30		
Corn meals	21.00	8.00	20.70	4.70
White starch meals	0.70	0.10	1.10	
TRT starch feeds			1.90	
Full fat seeds	1.70	1.10		
Fat feeds	0.20	0.30	0.20	0.10
Molasses	0.50	1.70	1.60	0.50
Cotton seeds	0.30	1.60	1.50	
Mineral-Vitamins and Additives1	1.40	1.80	1.30	0.70
Starch Compound Feed	0.80	1.10	0.50	0.10
Protein Compound Feed	2.20	0.80	23.90	23.70
Restricted Compound Feed	3.50	3.00		

6 Paper/Manuscript V

A in vitro rumen screening of Italian mediterranean macroalgae on potential reduction of methane emission and its phenolic and flavonoid profile

Erica Fiorbelli,¹ Davide Moccia,² Gabriele Rocchetti,¹ Alberto Stanislao Atzori,³
Antonio Pusceddu,² Pierantonio Addis,² and Antonio Gallo,^{1*}

¹Department of Animal Science, Food and Nutrition (DIANA), Faculty of Agricultural, Food and Environmental Sciences, Università Cattolica del Sacro Cuore, 29122 Piacenza, Italy

²Department of Life and Environmental Sciences, University of Cagliari, Via T. Fiorelli 1, 09126 Cagliari, Italy

³Department of Agricultural Science, University of Sassari, Viale Italia, 07100 Sassari, Italy

*Corresponding author: antonio.gallo@unicatt.

ABSTRACT

This study evaluated the potential of 28 Mediterranean macroalgae, collected along the Italian coastline, to reduce methane production during *in vitro* rumen fermentation. Each macroalga was included at 2% of the diet's dry matter and incubated with rumen fluid from cannulated dairy cows. Total gas production, methane, hydrogen, and volatile fatty acid profiles were measured at 2, 5, 9, 24, 36, 48, and 72 hours. Several species, including green, red, and brown algae, reduced methane production compared with the untreated control, with *Halimeda tuna*, *Flabellia petiolata*, *Codium decorticatum*, *Phyllophora crispa*, and *Cystoseira compressa* showing the strongest effects. No statistical difference was observed on volatile fatty acid profile across the treatment group compared to the control. These findings highlight the potential use of locally available macroalgae to modulate rumen fermentation and reduce methane emissions, supporting their application as sustainable feed additives in ruminant nutrition. The overall impact of the treatments can be considered null to low. Therefore, it is recommended to investigate the timing of harvest, processing techniques, and varying doses of incubation to enhance the existing body of knowledge.

Key words: seaweed, cattle, *in vitro*

Nonstandard abbreviations: CH₄ = methane, CO₂ = carbon dioxide, CO_{2eq} = CO₂ equivalent, CTR = control, H₂ = hydrogen, GLM = general linear model, Mx = Macroalga x, N₂O = nitrous oxide, PMR = partial mixed ration, TPC = total phenolic content; TFC = total flavonoid content, Vf = final gas volume

INTRODUCTION

Cattle, a prominent livestock species, are significant contributors to global greenhouse gas emissions, generating approximately 3.8 Gt CO₂eq annually, which accounts for about 62% of total livestock emissions. Direct emissions from the livestock sector worldwide, including methane (CH₄) from enteric fermentation and CH₄ and nitrous oxide (N₂O) from manure management, amount to 3.7 Gt CO₂eq, representing roughly 60% of the total livestock emissions (FAO, 2023b). Although CH₄ has a relatively short atmospheric lifespan (9-12 years), its immediate warming potential is substantial, making enteric CH₄ mitigation a priority to achieve the Paris Agreement's near-term climate targets.

In the rumen, *methanogenic archaea* facilitate the reduction of hydrogen (H₂) and carbon dioxide (CO₂), which are byproducts of hexose catabolism by protozoa, fungi, and bacteria. This process results in the production of CH₄ and the generation of ATP. The *archaea* provide an electron sink for reducing equivalents, thereby lowering the partial pressure of H₂ inside the rumen, which is advantageous to the microbial community (Puniya et al., 2015). Redirecting H₂ toward alternative sinks (e.g., propionogenesis or bio-hydrogenation) therefore remains a promising mitigation strategy that does not compromise, and may even enhance, animal productivity (Hristov, 2024).

There is an urgent need to find new sources of animal feed additives that promote environmental sustainability. Marine macro algae or seaweed have been used to feed livestock from immemorial time in coastal regions during periods of feed scarcity (Makkar et al., 2016). It was demonstrated they are a potential and valid alternative option as feed additives, revealing synergistic effects of algal biomass on CH₄ reduction and animal productivity (McCauley et al., 2020). They are classified into three major phyla based on their photosynthetic pigments: red algae (*Rhodophyta*), green algae (*Chlorophyta*), and brown algae (*Ochrophyta*). The potential of macroalgae to reduce CH₄ emissions in ruminants is rooted in their distinctive

biochemical composition, which includes a variety of bioactive compounds known for their anti-methanogenic properties (McCauley et al., 2020). Red and green algae are rich sources of bromophenols, phenolic acids, and flavonoids, whereas brown algae have a high phlorotannin content (Adarshan et al., 2024). The nutritional value of seaweed for ruminants is highly variable, depending on the species, algal composition, and the animal's adaptation to the specific feed. The high mineral content of seaweed generally limits their gross energy content, consequently affecting their digestible, metabolizable, and net energy values (Makkar et al., 2016).

Research over the past decade, however, has been dominated by the tropical red alga *Asparagopsis taxiformis*, native to Australia, whose bromoform-rich biomass can suppress enteric CH₄ by up to 99% in vitro and in vivo (Liu et al., 2025). Yet large-scale adoption of *Asparagopsis* in Europe faces two critical difficulties: (1) the ecological and economic cost of importing a non-indigenous, warm-water species, and (2) growing regulatory concerns surrounding bromoform persistence and occupational exposure.

Consequently, attention is turning to temperate macroalgae that can be cultivated locally. A search conducted on SCOPUS (<https://www.scopus.com>) on June 18, 2025, with the keys “seaweed,” “cattle,” and “methane” and limit for the area “veterinary” and “agricultural and biological science” returned a total of 58 articles, including in the last 12 years and fewer than 25% examined European taxa; within the Italian context, studies were collaborative screenings rather than evaluations of endemic species.

Comparative in vitro screens of Nordic or North Atlantic algae reveal substantial inter- and intra-specific variability, underscoring the need for region-specific assessments (Abbott et al., 2020; Pandey et al., 2022). The absence of Italian Mediterranean candidates thus represents a clear knowledge gap.

The objective of the present study was to screen a panel of Italian macro algae collected along the Mediterranean coasts for their capacity to reduce CH₄ production in an in vitro rumen fermentation system and to characterize additional shifts in total gas production and VFA profiles.

In conclusion, a chemical characterization by omics approaches will be carried out to give a comprehensive and holistic overview of the biochemical compounds responsible for the reduction of methanogenesis. We hypothesized that local species could reduce the CH₄ inhibition at an inclusion rate of 2% of substrate DM and identifying home-grown seaweed resources with verifiable anti-methanogenic potential. This work aims to advance scalable, circular bio-solutions for reducing the climate impact of European dairy production.

MATERIALS AND METHODS

The cannulate cows involved in the experiment at the Research Center for Livestock and Environment (CERZOO s.r.l., Piacenza, Italy) were cared for according to the guidelines of the Committee for the Protection and Welfare of Experimental Animals of the Catholic University of the Sacred Heart. The study was authorized by the Italian Ministry of Health.

Sample collection and preparation

28 macroalgae were harvested from wild populations along the different coastal zone of the Mediterranean Sea on Sardinian region (Italy) from June to November of 2023. Detailed information about harvested zone and taxonomy are reported in Table 1. Immediately after the collections, algae were cleaned with deionized water and then were dried at 65 °C in an oven until they reached a constant weight. The samples were then ground to a particle size of 1 mm using a laboratory mill with an adjustable speed (Fritsch pulverisette 19 universal cutting mill, Idar-Oberstein, Deutschland).

The substrate for the in vitro procedure was collected in the farm facility of CERZOO, dried and ground at 1 mm and analyzed for wet chemistry. The substrate was a partial mixed ratio (PMR) composed, as a percentage of DM, of corn silage (32%), alfalfa hay (21%), barley straw (1.9%), soybean meal (9%), barley (9.3%), sunflower (3.5%), and corn meal (14%). The PMR nutrient composition on a DM basis was 29.46% starch (2014.10), 15.8% CP (990.03) (AOAC, 2016), and 32.2% NDF (Mertens, 2002).

In vitro experimental design

Each macroalgae was tested in vitro individually with an inclusion on the PMR at 2% DM. The in vitro gas production methods were set up using 100 mL glass bottle that contained ~220 mg PMR, 10 mL of rumen fluid, and 20 mL of a buffered medium solution that simulates the electrolyte composition of rumen fluid (Gallo et al., 2016). The treatment used in the in vitro experiment were:

CTR: substrate of PMR without macroalgae, consider the control;

Mx: substrate of PMR with macroalgae x (**Mx**) (n=28);

For each treatment, there were two analytical replicates associated at the incubation time (2, 5, 9, 24, 48 and 72-hours post-incubation) and two experimental runs, 29 treatments x 6 incubation times x 2 analytical replicates with the run x 2 incubation runs (as an experimental replicate), for a total of 696 samples. The dynamics of gas production were verified to add 6 bottles with only buffered rumen fluid (blanks) and 6 bottles with a starch sample for internal standard (Gelose 80 maize starch; Penford Food Ingredients Co.) in each experimental run. Cumulative gas production from blanks and internal standards was averaged under the experimental run, and it was considered satisfactory if it fell within ± 1 standard deviation.

Rumen fluid collection

Rumen fluid samples were obtained through manual collection from two canulate Holstein dry cows, with a body weight averaging 625 ± 10 kg, farmed at the CERZOO. The cows were fed with maintenance diets as part of their daily regimen, and samples of rumen content were meticulously gathered after the morning feeding session, in accordance with the guidelines outlined by the National Research Council (NRC, 2001).

The time between rumen fluid collection and the inoculum in vitro was kept to a maximum of 40 minutes to prevent any changes in microbial activity and fermentation. This was guaranteed with the use of thermos during the transportation in the laboratory, and a rapid filtered through two layers of cheesecloth.

Inoculum procedure

The rumen fluid was diluted in a 1:2 volume ratio of rumen fluid to buffer-mineral solution. Continuous agitation and CO₂ flushing were ensured to maintain anaerobic conditions at 39 °C. The procedure to prepare the buffer was described by Menke and Steingass, 1988. On the same day of fermentation, it was prepared and maintained at 39 °C in a water bath under anaerobic conditions with continuous CO₂ flushing and adjusted pH around 6.5 to 6.6. Adjustment procedure use to process the buffered rumen fluid was described by Pirondini et al., 2012 and Serment et al., 2016, where a volume of 30 mL of diluted rumen fluid was added to each glass bottle. Then, the headspace of the bottle was flushed with CO₂ and hermetically closed with rubber caps, degassing with a needle to set atmospheric pressure. All the bottles were incubated in a thermal water bath and the pressure in the headspace was measured at each time of incubation (2, 5, 9, 24, 36, 48 and 72 h) using a digital test gauge XP2i by Crystal Engineering Corp. The analytical replicate associated with the hour of incubation were removed from the thermal batch and stored in the ice to stop the fermentation for the gas chromatography analysis. The remaining glass bottles in the bath were degassed with needles

to reset the pressure to atmospheric levels. To maintain regular microbial activity, the headspace pressure never went beyond 48 kPa, in accordance with Theodorou et al., 1994 recommendation. The absolute pressure collected was converted to estimate the total gas volume (mL) produced during the fermentation (López et al., 2007). The kinetics of gas production were computed using a singlepool exponential model (Wang et al., 2013), in agreement with the following differential equation:

$$V(t) = [1 - e^{-k_d} \times (t - lag)]$$

where $V(t)$ is the volume of gas accumulated (mL/g of OM) at time t ; V_f is the final gas volume (mL/g of OM); k_d is the kinetic constant (h^{-1}); t is time (h); and lag is the lag time (h).

Analysis of gas chromatography

The gas was directly collected from the headspace, after the measure of gas pressure to determine gas composition. Briefly, an aliquot of 2.5 mL of gas was collected with gas-tight glass syringe with a pressure lock (VICI, Precision Sampling Inc.) and manually injected on the gas chromatography GC system (Agilent 7820A, Agilent Technologies). The analysis's run time is 5 minutes per sample using N_2 as a carrier. The instrument was calibrated with an external standard mixture of CO_2 and CH_4 prepared by SIAD S.p.A. (Bergamo, Italy). For each sample a final report on PDF was generated at the end of the analysis where the concentration of CH_4 , H_2 , and oxygen (O_2) were expressed in percentage determined with an automatic integration of peak areas on the gas chromatogram. The percentage of CO_2 was obtained per difference respect the percentage of the other gas previously analyzed. The volume of CH_4 and H_2 (mL) produced at different time intervals was calculated using the formula suggested by López et al., 2007. This formula is used when the gas is subsequently released after the gas

pressure measurement. The production expresses as mmol of CH₄ and H₂ were calculated applying the ideal gas law.

Ruminal volatile fatty acids determination

Subsequently for the gas composition analysis the glass bottles were opened and an aliquot of 10 mL of fermented rumen fluid were stored in tubes for VFA determination. The tubes were centrifuged at 3,000 rpm for 15 min at 4 °C. An aliquot of 2-mL of the supernatant was directly injected to MPA II Multi-Purpose FT-NIR Analyzer (Bruker Italia S.r.l. Unipersonale). The concentration of the VFA profile, expressed in mmol/L, was predicted using a Bruker FT-NIR calibration that has been previously validated (Ghilardelli et al., 2022).

In vitro screening of total phenolics and total flavonoids in macroalgae

Three replicates (0.5 g) of each dried macroalgae sample were extracted in 100 mL of methanol/water (80:20 v/v), acidified with 0.1% of formic acid, by using a Polytron homogenizer (PT1200E). The extracts were then centrifuged at 6,000 ×g for 15 min at 4 °C and filtered through 0.22 µm cellulose syringe filters into UHPLC vials, until further spectrophotometric analysis. The total phenolic content (TPC) and total flavonoid content (TFC) were determined colorimetrically, as previously reported by Zhang et al. (2020). The results were expressed as mg of standard compounds (gallic acid for TPC; and quercetin for TFC) per 100 g of dried extract.

Statistical analysis

All the statistical analyses were conducted using the SAS statistical software (2004). The sample (n=2) at each incubation time (n=6) for each treatment (n=29) within the run were considered analytical repetitions, and sample between runs (n=2) were considered

experimental replicates. For all the variables displayed in tables and figures, the experimental replicates were averaged within the treatment group.

The gas production and composition, and VFA proportion measured at the end of in vitro incubation (72h) were evaluated using the general linear model (GLM) procedure of SAS (SAS Institute, 2004), according to the following model:

$$Y_{ij} = \mu + Ri + \epsilon_{ij}$$

where Y_{ij} is the response variable (i.e., final volume of gas (Vf), kd, lag, or VFA concentrations); μ is the overall mean; Ri is the fixed effect of treatment ($i = 29$: CTR and Mx) and ϵ_{ij} is the random residual error.

The hourly composition of the gas measured at each time point was evaluated using the GLM with the following model:

$$Y_{ijk} = \mu + Ri + Tj + \epsilon_{ijk}$$

Where Y_{ijk} is the response variable; μ is the overall mean; Ri is the fixed effect of treatment ($i = 29$: CTR and Mx), Tj is the fixed effect of the hours and ϵ_{ijk} is the random residual error.

The data are presented in the figures as LSM. Differences between LSM were compared post hoc using Tukey's method. Tendency was declared when $0.05 < P \leq 0.10$, and statistical significances were reported at $P \leq 0.05$.

Therefore, the CTR mean was compared with the other treatments using Dunnett's multiple comparison test to determine which treatments differ significantly from the CTR. The difference was considered significant if the P value was below 0.05.

The data of total phenolics and total flavonoids in macroalgae were analyzed by one-way ANOVA followed by Tukey's HSD post hoc test. Significance was declared at $P < 0.05$.

RESULTS

Total Gas production and composition

After 72 h of in vitro incubation, the cumulated gas production was compared with the CTR (209.3 ± 5.1 mL/g OM). The individual inclusion of specific macroalgae belonging to the green algae significantly reduced ($p < 0.05$) the gas volume for *Halimeda tuna*, *Codium decorticatum*, and *Flabellia petiolata*, which produced -30.90, -29.43, and -29.91 mL/g OM, respectively (Table 2). The *Phyllophora crispa*, a Red algae, significantly reduced ($p < 0.05$) the gas volume to -28.25 mL/g OM (Table 2).

The *Halimeda tuna* (-12.8%), *Flabellia petiolata* (-12.1%), and *Codium decorticatum* (-11.8%) showed the greatest CH₄ reductions ($p < 0.05$) in respect to the CTR (Table 2). The *Phyllophora crispa* and the Brown algae *Cystoseira compressa* significantly reduced ($p < 0.05$) the CH₄ production (mL) an average of -11.45% respect to the CTR (Table 2). The red algae, *Asparagopsis taxiformis*, produced -2.0% of CH₄ (mL) which did not differ significantly from the CTR. In addition, no treatment reports significant differences in the proportion of CH₄ in total gas production (Table 2).

The Red algae, *Corallina elongata* and *Gracilaria gracilaris* significant increased +15% and +14.3% respectively; ($p < 0.05$) the production of H₂ (mL) in respect to the CTR, (Table 2).

Volatile Fatty Acid Profiles

At 72 h, the inclusion of macroalgae, at 2% DM the treatments, did not change significantly - around the three species - the VFA profile. Specie such as *Halopteris scoparia*, and *Jania rubens* showed the highest levels of acetate (≥ 60 mmol/L), compared with the CTR (52.1 ± 5.1 mmol/L) in Table 3. Propionate value had an average range from 21.6 to 37.2 mmol/L (Table 3), with *Halopteris scoparia* showing the highest values. Butyrate and minor

VFAs (isobutyrate, isovalerate, valerate) also followed similar trends, with numerical but nonsignificant increases.

Screening of total phenolics and total flavonoids

Significant variation ($P < 0.001$) in both TPC and TFC was observed among macroalgae species (Table 5). The Brown algae *Phyllophora crispa* exhibited the highest TPC values, exceeding 150 mg/100 g GAE, whereas red algae like *Jania rubens* showed the lowest 9 mg/100 g GAE. Similarly, TFC was greatest in *Flabellia petiolata* and *Caulerpa cylindracea* (≥ 150 mg/100 g QE), while values in *Jania rubens* were below 5 mg/100 g QE.

DISCUSSION

Dynamic Total Gas Production and CH₄ Composition

This study represents the first screening of Mediterranean macroalgae from Italian coasts for their effects on rumen fermentation kinetics and CH₄ reduction. The inclusion of macroalgae at 2% DM significantly affected total gas production, CH₄, and H₂ yield (mL).

The most promising were the green algae *Halimeda tuna*, *Codium decorticatum*, and *Flabellia petiolata*, which led to significant reductions in both cumulative gas volume in a range of -29 to -31 mL/g OM and CH₄ production at -11.8% to -12.8% compared to the CTR. This apparent mitigation of CH₄ may partly reflect a general suppression of total gas production, underscoring the need to confirm in follow-up studies that *Halimeda tuna*, *Codium decorticatum*, and *Flabellia petiolata* do not inhibit the rumen microbial community or compromise intake, digestibility, or animal performance in vivo. Similarly, the red alga *Phyllophora crispa* and the brown alga *Cystoseira compressa* significantly reduced CH₄ production (mL) by an average of -11.5%. These results place these locally sourced algae in the same efficacy range as *Bonnemaisonia hamifera*, a cultivated Red alga in Denmark, that

reduced CH₄ emissions by 13-16% in dairy cows with a lower bromoform content than *Asparagopsis* (Thorsteinsson et al., 2025).

Based on literature, no previous study has tested the antimethanogenic potential of *Halimeda tuna*, *Flabellia petiolata*, *Codium decorticatum*, or *Phyllophora crispa*. These findings, therefore, provide the first experimental evidence for their role in modulating rumen fermentation and mitigating CH₄. Only *Dictyota spp.* and *Cystoseira spp.* had been previously reported (Machado et al., 2014; Roskam et al., 2022), yet with different efficacy likely due to species-specific chemical composition.

Interestingly, *Asparagopsis taxiformis* did not significantly differ from the CTR in this study (-2.0%), in contrast to past reports where reductions exceeded 80% (Liu et al., 2025). This divergence could be justified to a probable low concentration of bromoform, due the period of harvest, as highlighted in previous literature (Thorsteinsson et al., 2025). A hypothesis that requires confirmation through specific gas chromatography analysis.

In contrast, the red algae *Gracilaria gracilaris* and *Corallina elongata* significantly increased H₂ production (by +14.5%), a possible indicator of inhibited methanogenesis without effective redirection of metabolic H₂. The same findings were observed from *Bonnemaisonia hamifera*, where CH₄ reduction was accompanied by elevated H₂ excretion, suggesting partial inhibition without redirection into reductive pathways (Thorsteinsson et al., 2025).

A in vitro study which compared the effect of multiple red algae on CH₄ mitigation found that an inclusion level of *Asparagopsis armata* at 2% OM completely inhibited CH₄ production and significantly increased H₂ production (Mihaila et al., 2022). In addition, *Bonnemaisonia hamifera* (10% DM) demonstrated the highest significant reduction in CH₄ (98.9%) and an increase in the production of H₂.

The data suggests that multiple Mediterranean macroalgae rich in bioactive compounds could modulate rumen fermentation, although the exact mechanisms remain to be elucidated. The relatively modest effect of this local species underscores the importance of region-specific screening and the potential of temperate algae as scalable, low-risk alternatives to tropical taxa.

VFA profiles

The inclusion of macroalgae at 2% DM did not result in significant differences in the proportion of individual VFAs among the treatments. Although a numerical increase was observed for the acetate on *Halopteris scoparia* and *Jania rubens*, where the acetate concentrations was above 60 mmol/L, compared to the CTR (52.1 ± 5.1 mmol/L), suggesting a potential stimulation of fibrolytic fermentation.

Halopteris scoparia also displayed the highest recorded propionate value (37.2 mmol/L), which may indicate an increased flux of metabolic H₂ toward propionogenesis. High acetate values observed in certain brown and red algae treatments in previous studies explained by the nutritional value of the seaweed characterized by low concentration of starch and cellulose, and high content of cell wall polysaccharides (Maia et al., 2019).

The shift between CH₄ to propionate is generally considered beneficial for energy efficiency and corresponds to the redirection mechanism proposed by Hristov, 2024 and Liu et al., 2025, whereby propionate serves as a competitive H₂ sink to methanogenesis. However, due to the absence of a significant overall treatment effect on propionate or butyrate ($P > 0.05$), these findings remain exploratory and require further validation.

Interestingly, *Asparagopsis taxiformis* did not exhibit superior performance in terms of VFA modulation (Table 3). A similar result was observed on total VFA, which were not significantly affected with 2% inclusion; nevertheless, the acetate levels were reduced respect

a linear increase dose depend of propionate and butyrate. (Kinley et al., 2016). This reinforces the hypothesis that its antimethanogenic efficacy is highly dependent on bromoform content and may not be paralleled by broader fermentation benefits at low bioactive concentrations.

In this context, the Mediterranean species in the present study did not show selective and species-specific effects on VFA profiles. The response variability across algae types underscores the complexity of interactions between algal metabolites and rumen microbial populations and highlights the importance of chemical characterization of algal biomass, particularly for flavonoids, phlorotannins, and polyphenols, to better understand their mechanistic role in fermentation modulation (Patwary et al., 2021; Adarshan et al., 2024).

Total phenolics and total flavonoids profile

Polyphenols belong to a broad category of plant secondary metabolites characterized by a phenolic structure with at least one hydroxyl group attached. Based on their degree of polymerization, they can be categorized into simple phenolics like ellagic and gallic acids, dimeric or oligomeric compounds such as procyanidins and lignans, or high molecular weight polymeric compounds like tannins. Polyphenols are further classified into flavonoids and nonflavonoids (Lind et al., 2023).

The present study highlights a significant interspecific variation in TPC and total TFC among Mediterranean macroalgae. Notably, *Phyllophora crispa* exhibited the highest TPC values (199.2 mg GAE/100 g DM), while *Flabellia petiolata* and *Caulerpa cylindracea* recorded TFC levels as an average of 171 mg QE/100 g DM. Post hoc Tukey analysis confirmed multiple statistically distinct groups, indicating species-specific polyphenolic profiles. These differences could reflect metabolic specialization and ecological adaptation, potentially linked to secondary metabolite production for grazing deterrence, or microbial interaction.

These results underscore the species-specific accumulation of secondary metabolites, likely driven by ecological and physiological adaptations. Such biochemical diversity supports the hypothesis that certain macroalgae may serve as reservoirs of bioactive compounds with functional relevance in ruminant nutrition.

At a 2% DM inclusion rate, several species such as *Halimeda tuna*, *Flabellia petiolata*, *Codium decorticatum*, *Phyllophora crispa*, and *Cystoseira compressa*, significantly reduced total gas (mL/g OM) and CH₄ mL production under in vitro fermentation conditions. Additionally, *Jania rubens* associated an increase level of H₂ mL production showed the lowest TFA concentration 3.24 mg QE/100 g DM.

Previous studies demonstrated that polyphenols and flavonoids can modulate ruminal fermentation and reduce CH₄ emissions. Jo et al. (2022) reported CH₄ reductions of 34-41% using five oriental medicine plant extracts with polyphenol contents ranging from 30 to 266 mg GAE/g and flavonoids from 5 to 125 mg CHE/g under a batch culture system (48 h). Similarly, Choi et al. (2021) showed that seaweed extracts from *Undaria pinnatifida*, *Sargassum fusiforme*, and *Sargassum fulvellum* reduced CH₄ production by approximately 25% at 0.25 mg/mL inclusion, correlating with their TPC and TFC content. The *Sargassum fulvellum* showed the higher content of TFA (25.21 ± 1.72 mgCE/ g) and TPC (5.77 ± 0.07 mg GAE/g).

The mechanisms underlying these effects are multifaceted. A natural polyphenol such as (+)-catechin tested under in vitro incubation batch at different amounts of inclusion (from 0.016 to 0.125 mmol), showed a CH₄ reduction associated to a H₂ sinks, diverting reducing equivalents away from methanogenesis (Becker et al., 2014). A study in vitro and in vivo tested a tea polyphenol and demonstrated a significantly reduced CH₄ production and the acetate/propionate (Teng et al., 2024). Additionally, tea polyphenols have also been shown to suppress methanogenic archaea and protozoa populations, further contributing to methane reduction (Teng et al., 2024). Kinley et al. (2016) described a fermentation shift toward

propionate in the presence of *Asparagopsis taxiformis*, reducing H₂ availability for methanogens. While the methane-lowering effect of *A. taxiformis* is primarily linked to its bromoform content, with reductions exceeding 90% (Roque et al., 2019; Wasson et al., 2022) polyphenol-rich macroalgae provide an alternative, potentially safer mitigation strategy without relying on halogenated compounds.

The concentrations of phenolic compounds identified in the present study are within or above the range tested in prior research, supporting the potential of these Mediterranean macroalgae as natural feed additives for CH₄ mitigation. Nevertheless, it is important to note that these findings are based on in vitro conditions. Further in vivo trials are necessary to assess the bioavailability, palatability, and safety of these compounds, as well as their long-term effects on animal health and productivity.

CONCLUSIONS

This study provides the first screening of antimethanogenic activity from Mediterranean macroalgae collected along the Italian coasts. At a 2% DM inclusion rate, several species, including *Halimeda tuna*, *Flabellia petiolata*, *Codium decorticatum*, *Phyllophora crispa*, and *Cystoseira compressa*, significantly reduced total gas and CH₄ production in vitro. Additionally, certain species showed tendencies to influence VFA profiles, notably *Halopteris scoparia* and *Jania rubens*, suggesting modulation of fibrolytic fermentation or redirection of H₂ toward propionogenesis. These findings highlight the potential of endemic seaweeds as scalable, low-risk alternatives for CH₄ mitigation in ruminant nutrition. Further research is needed to assess their efficacy and safety under in vivo conditions.

NOTES

This study was conducted at the Research Center for Livestock and Environment (CERZOO s.r.l., Piacenza, Italy). The experimental procedures involving animals were conducted in accordance with the guidelines of the Committee for the Protection and Welfare of Experimental Animals of the Catholic University of the Sacred Heart and were authorized by the Italian Ministry of Health (authorization no. 355/2022-PR). The authors declare no conflicts of interest. During the preparation of this work, the author Erica Fiorbelli used Jenny AI in order to improve the readability of the manuscript. After using Jenny AI, the author reviewed and edited the content as needed and take full responsibility for the content of the publication.

REFERENCES

- Abbott, D.W., I.M. Aasen, K.A. Beauchemin, F. Grondahl, R. Gruninger, M. Hayes, S. Huws, D.A. Kenny, S.J. Krizsan, S.F. Kirwan, V. Lind, U. Meyer, M. Ramin, K. Theodoridou, D. Von Soosten, P.J. Walsh, S. Waters, and X. Xing. 2020. Seaweed and Seaweed Bioactives for Mitigation of Enteric Methane: Challenges and Opportunities. *Animals* 10:2432. doi:10.3390/ani10122432.
- Adarshan, S., V.S.S. Sree, P. Muthuramalingam, K.S. Nambiar, M. Sevanan, L. Satish, B. Venkidasamy, P.G. Jeelani, and H. Shin. 2024. Understanding Macroalgae: A Comprehensive Exploration of Nutraceutical, Pharmaceutical, and Omics Dimensions. *Plants* 13:113. doi:10.3390/plants13010113.
- AOAC, I. 2016. *Official Methods of Analysis*. 20th ed. AOAC int., Gaithersburg, MD.
- Becker, P.M., P.G. van Wikselaar, M.C.R. Franssen, R.C.H. de Vos, R.D. Hall, and J. Beekwilder. 2014. Evidence for a hydrogen-sink mechanism of (+)catechin-mediated emission reduction of the ruminant greenhouse gas methane. *Metabolomics* 10:179–189. doi:10.1007/s11306-013-0554-5.

- Choi, Y., S.J. Lee, H.S. Kim, J.S. Eom, S.U. Jo, L.L. Guan, J. Seo, H. Kim, S.S. Lee, and S.S. Lee. 2021. Effects of seaweed extracts on in vitro rumen fermentation characteristics, methane production, and microbial abundance. *Sci. Rep.* 11:24092. doi:10.1038/s41598-021-03356-y.
- FAO. 2023. Pathways towards Lower Emissions. FAO ;
- Gallo, A., G. Giuberti, and F. Masoero. 2016. Gas production and starch degradability of corn and barley meals differing in mean particle size. *J. Dairy Sci.* 99:4347–4359. doi:10.3168/jds.2015-10779.
- Ghilardelli, F., G. Ferronato, and A. Gallo. 2022. Near-infrared calibration models for estimating volatile fatty acids and methane production from in vitro rumen fermentation of different total mixed rations. *JDS Commun.* 3:19–25. doi:10.3168/jdsc.2021-0156.
- Hristov, A.N. 2024. Invited review: Advances in nutrition and feed additives to mitigate enteric methane emissions. *J. Dairy Sci.* 107:4129–4146. doi:10.3168/jds.2023-24440.
- Jo, S.U., S.J. Lee, H.S. Kim, J.S. Eom, Y. Choi, D.S. Oh, D. Bae, and S.S. Lee. 2022. Effects of oriental medicinal plants on the reduction of methane production mediated by microbial population. *Ital. J. Anim. Sci.* 21:522–531. doi:10.1080/1828051X.2022.2046192.
- Kinley, R.D., R. De Nys, M.J. Vucko, L. Machado, and N.W. Tomkins. 2016. The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Anim. Prod. Sci.* 56:282. doi:10.1071/an15576.
- Lind, V., A. Schwarm, M. Mele, A. Cappucci, G. Foggi, Ö. Sizmaz, E. Tsiplakou, A.S. Atzori, J. Van Mullem, and N. Peiren. 2023. Nutritional Approaches to Reduce Enteric Methane Emission from Ruminants. T. Bartzanas, ed. Springer International Publishing, Cham.

- Liu, Y., M. Zhou, Q. Diao, T. Ma, and Y. Tu. 2025. Seaweed as a feed additive to mitigate enteric methane emissions in ruminants: Opportunities and challenges. *J. Integr. Agric.* 24:1327–1341. doi:10.1016/j.jia.2024.09.036.
- López, S., M.S. Dhanoa, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. *Anim. Feed Sci. Technol.* 135:139–156. doi:10.1016/j.anifeedsci.2006.06.005.
- Machado, L., M. Magnusson, N.A. Paul, R. De Nys, and N. Tomkins. 2014. Effects of Marine and Freshwater Macroalgae on In Vitro Total Gas and Methane Production. *PLoS ONE* 9:e85289. doi:10.1371/journal.pone.0085289.
- Maia, M.R.G., A.J.M. Fonseca, P.P. Cortez, and A.R.J. Cabrita. 2019. In vitro evaluation of macroalgae as unconventional ingredients in ruminant animal feeds. *Algal Res.* 40:101481. doi:10.1016/j.algal.2019.101481.
- Makkar, H.P.S., G. Tran, V. Heuzé, S. Giger-Reverdin, M. Lessire, F. Lebas, and P. Ankers. 2016. Seaweeds for livestock diets: A review. *Anim. Feed Sci. Technol.* 212:1–17. doi:10.1016/j.anifeedsci.2015.09.018.
- McCauley, J.I., L. Labeeuw, A.C. Jaramillo-Madrid, L.N. Nguyen, L.D. Nghiem, A.V. Chaves, and P.J. Ralph. 2020. Management of Enteric Methanogenesis in Ruminants by Algal-Derived Feed Additives. *Curr. Pollut. Rep.* 6:188–205. doi:10.1007/s40726-020-00151-7.
- Menke, K., and H. Steingass. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Pages 7–55. *Animal Research and Development*.

- Mertens, D.R. 2002. Gravimetric Determination of Amylase-Treated Neutral Detergent Fiber in Feeds with Refluxing in Beakers or Crucibles: Collaborative Study. *J. AOAC Int.* 85:1217–1240. doi:10.1093/jaoac/85.6.1217.
- Mihaila A A, Glasson C R K, Lawton R, Muetzel S, Molano G, Magnusson M. 2022. New temperate seaweed targets for mitigation of ruminant methane emissions: An *in vitro* assessment. *Applied Phycology*, **3**, 274–284.
- Nutrient Requirements of Dairy Cattle. 2001. Seventh revised edition. National Academies Press, Washington, D.C.
- Pandey, D., H.H. Hansen, R. Dhakal, N. Aryal, S.P. Rai, R. Sapkota, M.O. Nielsen, M. Novoa-Garrido, and P. Khanal. 2022. Interspecies and seasonal variations in macroalgae from the Nordic region: Chemical composition and impacts on rumen fermentation and microbiome assembly. *J. Clean. Prod.* 363:132456. doi:10.1016/j.jclepro.2022.132456.
- Patwary, Z.P., N.A. Paul, K. Nishitsuji, A.H. Campbell, E. Shoguchi, M. Zhao, and S.F. Cummins. 2021. Application of omics research in seaweeds with a focus on red seaweeds. *Brief. Funct. Genomics* 20:148–161. doi:10.1093/bfpg/elab023.
- Pirondini, M., L. Malagutti, S. Colombini, P. Amodeo, and G.M. Crovetto. 2012. Methane yield from dry and lactating cows diets in the Po Plain (Italy) using an *in vitro* gas production technique. *Ital. J. Anim. Sci.* 11:e61. doi:10.4081/ijas.2012.e61.
- Puniya, A.K., R. Singh, and D.N. Kamra. 2015. *Rumen Microbiology: From Evolution to Revolution*. 1st ed. Springer New Delhi.
- Roque, B.M., J.K. Salwen, R. Kinley, and E. Kebreab. 2019. Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *J. Clean. Prod.* 234:132–138. doi:10.1016/j.jclepro.2019.06.193.
- Roskam, E., S.F. Kirwan, D.A. Kenny, C. O'Donnell, V. O'Flaherty, M. Hayes, and S.M. Waters. 2022. Effect of brown and green seaweeds on diet digestibility, ruminal

- fermentation patterns and enteric methane emissions using the rumen simulation technique. *Front. Anim. Sci.* 3. doi:10.3389/fanim.2022.1021631.
- Serment, A., S. Giger-Reverdin, P. Schmidely, O. Dhumez, L.P. Broudiscou, and D. Sauvant. 2016. In vitro fermentation of total mixed diets differing in concentrate proportion: relative effects of inocula and substrates. *J. Sci. Food Agric.* 96:160–168. doi:10.1002/jsfa.7076.
- Teng, Z., S. Liu, L. Zhang, L. Zhang, S. Liu, T. Fu, N. Zhang, and T. Gao. 2024. Tea Polyphenols Inhibit Methanogenesis and Improve Rumen Epithelial Transport in Dairy Cows. *Animals* 14:2569. doi:10.3390/ani14172569.
- Theodorou, M.K., B.A. Williams, M.S. Dhanoa, A.B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Technol.* 48:185–197. doi:10.1016/0377-8401(94)90171-6.
- Thorsteinsson, M., A.A. Schönherz, S.J. Noel, Z. Cai, Z. Niu, A.L.F. Hellwing, P. Lund, M.R. Weisbjerg, and M.O. Nielsen. 2025. A preliminary study on the effects of red *Bonnemaisonia hamifera* seaweed on methane emissions from dairy cows. *JDS Commun.* 6:217–222. doi:10.3168/jdsc.2024-0670.
- Wang, M., X. Z. Sun, S. X. Tang, Z. L. Tan, and D. Pacheco. 2013. Deriving fractional rate of degradation of logistic-exponential (LE) model to evaluate early in vitro fermentation. *Animal* 7:920–929. <https://doi.org/10.1017/S1751731112002443>.
- Wasson, D.E., C. Yarish, and A.N. Hristov. 2022. Enteric methane mitigation through *Asparagopsis taxiformis* supplementation and potential algal alternatives. *Front. Anim. Sci.* 3. doi:10.3389/fanim.2022.999338

Table 1. Seaweed species and sampling details from the Sardinian Coast, Italy

Class	Species Name	Taxonomy	Site 1	Site 2	Depth
Brown Algae	Cladostephus spongiosus (Hudson) C.Agardh, 1817	Ph, Phaeophyceae, Sphacelariales, Cladostephaceae	Lido D'Orri ¹		1-3 m
	Cystoseira compressa				
	Cystoseira crinita				
	Dictyota dicotoma (Hudson) J.V.Lamouroux, 1809	Ph, Phaeophyceae, Dictyotales, Dictyotaceae	Lido D'Orri ¹		1-3 m
	Dictyota sp.				
Green algae	Ericaria amentacea var. stricta (Montagne) Sauvageau, 1911	Ph, Phaeophyceae, Fucales, Sargassaceae	Lido D'Orri ²		0-1 m
	Halopteris scoparia (Linnaeus) Sauvageau, 1904	Ph, Phaeophyceae, Sphacelariales, Stypocaulaceae	Lido D'Orri ¹		1-3 m
	Padina pavonica (Linnaeus) Thivy, 1960	Ph, Phaeophyceae, Dictyotales, Dictyotaceae	Lido D'Orri ¹	Sa Rocca Tunda ⁵	0-1 m
	Caulerpa cylindracea Sonder, 1845	Chl, Ulvophyceae, Bryopsidales, Caulerpaceae	Lido D'Orri ³	Sa Rocca Tunda ⁵	0-1 m
	Caulerpa prolifera (Forsskål) J.V.Lamouroux, 1809	Chl, Ulvophyceae, Bryopsidales, Caulerpaceae	Lido D'Orri ⁴		0-1 m
	Cladophora prolifera				
	Codium Bulsa				
	Codium decorticatum (Woodward) M.A.Howe, 1911	Chl, Ulvophyceae, Bryopsidales, Codiaceae	Lido D'Orri ⁴		0-1 m
	Flabellia petiolata (Turra) Nizamuddin, 1987	Chl, Ulvophyceae, Bryopsidales, Udoteaceae	Lido D'Orri ⁴		1-3 m
	Halimeda tuna (J.Ellis & Solander) J.V.Lamouroux, 1816	Chl, Ulvophyceae, Bryopsidales, Halimedaceae	Lido D'Orri ⁴		1-3 m
	Penicillus capitatus Lamarck, 1813	Chl, Ulvophyceae, Bryopsidales, Udoteaceae	Lido D'Orri ⁴		0-1 m
	Peyssonelia rubra				
	Ulva rigida C.Agardh, 1823	Chl, Ulvophyceae, Ulvales, Ulvaceae	Santa Gilla ⁶		0-1 m
Red Algae	Asparagopsis taxiformis				
	Corallina elongata J.Ellis & Solander, 1786	Rh, Florideophyceae, Corallinales, Corallinaceae	Lido D'Orri ²		1-3 m
	Digenea simplicex				
	Gracilaria gracilis				
	Gracilaria sp.	Rh, Florideophyceae, Gracilariales, Gracilariaceae	Santa Gilla ⁶		0-1 m
	Jania rubens (Linnaeus) J.V.Lamouroux, 1816	Rh, Florideophyceae, Corallinales, Corallinaceae	Lido D'Orri ²		1-3 m
	Phyllophora crispa				
	Sphaerococcus coronopifolius				
	Wrangelia penicillata				

¹ (39°06'35.9"N 9°00'55.6"E). ² (39°06'25.6"N 9°00'58.1"E) ³ (39°06'38.5"N 9°00'54.9"E) ⁴ (39°06'28.3"N 9°00'56.9"E) ⁵ (40°02'53.5"N 8°25'42.4"E) ⁶ (39°13'53.6"N 9°04'27.7"E)

Table 2. Levels of in vitro rumen fermentation products at 72h of incubation at 2% DM inclusion of macroalgae

	Treatment	Cum Gas mL/g OM	Cum CH ₄ mL	Cum CO ₂ mL	Cum H ₂ mL	CH ₄ % total GP
Control	CTR	209.25 ± 5.08	13.42 ± 0.24	101.35 ± 1.75	0.12 ± 0	28.84 ± 0.75
Brown Algae	Halopteris scoparia	195.54 ± 8.02	13.18 ± 0.39	102.68 ± 2.77	0.13 ± 0.01	28.31 ± 0.75
	Padina pavonica	199.63 ± 5.67	13.44 ± 0.27	100.54 ± 1.96	0.13 ± 0	32.04 ± 0.96
	Cystoseira crinita	205.22 ± 5.67	13.6 ± 0.27	100.65 ± 1.96	0.12 ± 0	30.11 ± 1.18
	Cladostephus spongiosus	196.58 ± 5.67	13.2 ± 0.27	99.15 ± 1.96	0.12 ± 0	30.53 ± 0.83
	Cystoseira compressa	186.63 ± 5.67	11.88 ± 0.27 ^a	97.39 ± 1.96	0.11 ± 0	30.23 ± 0.83
	Ericaria amentacea var. stricta	200.83 ± 6.55	13.16 ± 0.32	99.87 ± 2.26	0.13 ± 0	29.92 ± 0.83
	Dictyota sp.	192.72 ± 11.35	12.43 ± 0.55	99.58 ± 3.92	0.13 ± 0.01	30.51 ± 0.83
Green algae	Peyssonelia rubra	200.65 ± 5.67	13.42 ± 0.27	99.53 ± 1.96	0.12 ± 0	30.43 ± 0.83
	Penicillus capitatus	194.92 ± 5.67	13.34 ± 0.27	98.82 ± 1.96	0.12 ± 0	30.78 ± 0.83
	Ulva rigida	191.8 ± 5.67	13.15 ± 0.27	99.14 ± 1.96	0.12 ± 0	30.26 ± 0.83
	Caulerpa cylindracea	200.82 ± 5.67	13.71 ± 0.27	101.72 ± 1.96	0.12 ± 0	30.54 ± 0.83
	Cladophora prolifera	194.13 ± 5.67	13.24 ± 0.27	99.48 ± 1.96	0.12 ± 0	31.01 ± 0.83
	Halimeda tuna	178.27 ± 5.67 ^a	11.7 ± 0.27 ^a	96.66 ± 1.96	0.12 ± 0	31.24 ± 1.67
	Codium decorticatum	179.81 ± 5.67 ^a	11.83 ± 0.27 ^a	99.04 ± 1.96	0.12 ± 0	30.61 ± 0.83
	Flabellia petiolata	179.34 ± 5.67 ^a	11.8 ± 0.27 ^a	99.08 ± 1.96	0.12 ± 0	30.32 ± 0.83
	Codium Bursa	199.73 ± 6.55	13.12 ± 0.32	101.51 ± 2.26	0.13 ± 0	29.29 ± 0.83
	Caulerpa prolifera	191.46 ± 6.55	13.07 ± 0.32	101.7 ± 2.26	0.13 ± 0	29.43 ± 0.83
Red Algae	Asparagopsis taxiformis	206.34 ± 5.08	13.06 ± 0.24	100.52 ± 1.75	0.12 ± 0	29.5 ± 0.83
	Gracilaria gracilis	193.4 ± 6.55	13.71 ± 0.32	100.55 ± 2.26	0.14 ± 0 ^b	29.63 ± 0.83
	Wrangelia penicillata	195.81 ± 5.67	13.46 ± 0.27	100.54 ± 1.96	0.12 ± 0	30.94 ± 1.18
	Digenea simplicex	194.78 ± 11.35	13.56 ± 0.55	98.12 ± 3.92	0.13 ± 0.01	30.17 ± 0.96
	Phyllophora crispa	180.99 ± 5.67 ^a	11.87 ± 0.27 ^a	98.25 ± 1.96	0.12 ± 0	29.8 ± 0.96
	Jania rubens	198.6 ± 8.02	13.64 ± 0.39	101.65 ± 2.77	0.14 ± 0.01	30.89 ± 0.96
	Sphaerococcus coronopifolius	194.97 ± 6.55	13.03 ± 0.32	101.76 ± 2.26	0.13 ± 0	29.71 ± 0.96
	Corallina elongata	201.89 ± 6.55	13.53 ± 0.32	103.1 ± 2.26	0.14 ± 0 ^b	29.25 ± 0.96
√MSE		11.34	0.54	3.91	0.0074	0.016

Superscripts indicated that the specific mean was lower (i.e., a) or higher (i.e., b) than CTR at a P < 0.05, as evaluated with Dunnett's multiple comparison test. The data expressed as LSM ± standard error.

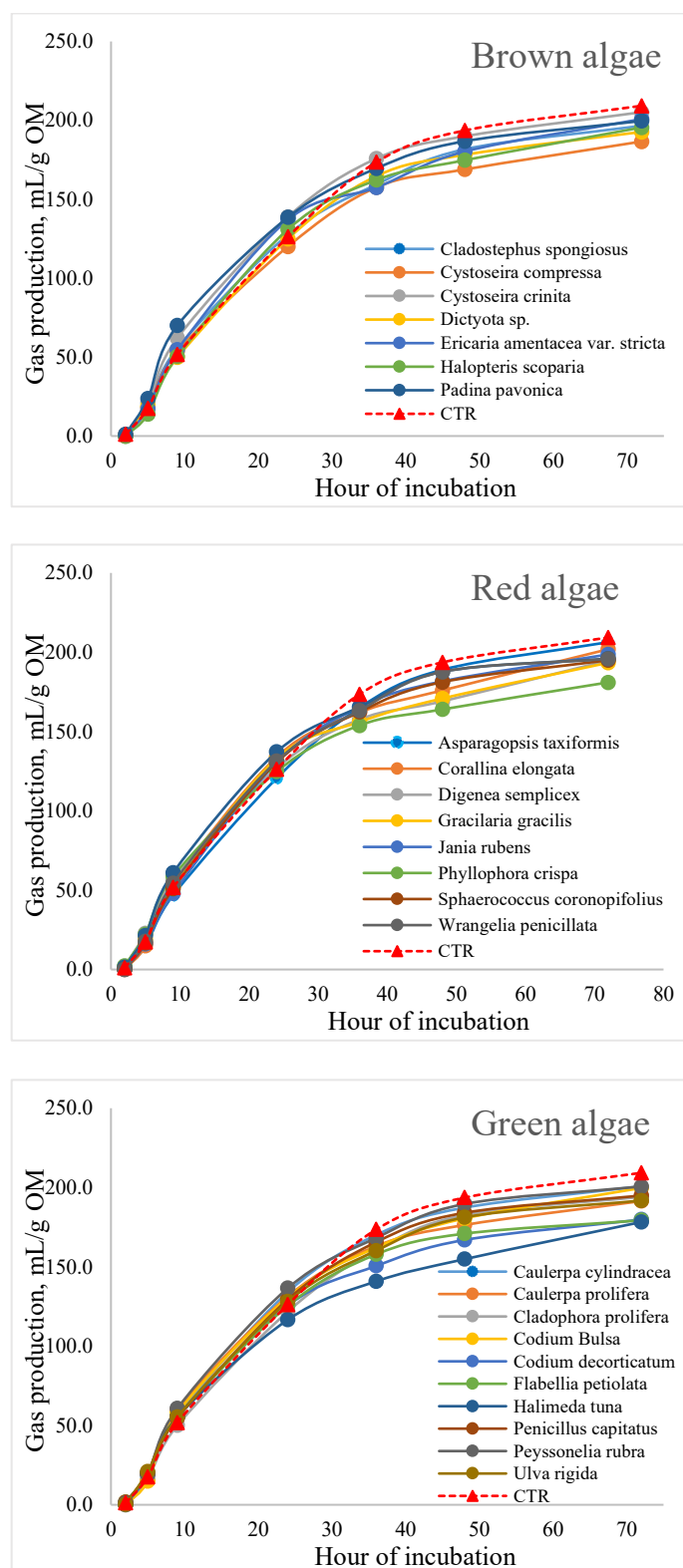
Table 3. Levels of volatile fatty acid *in vitro* rumen fermentation at 72h of incubation at 2% DM inclusion of macroalgae

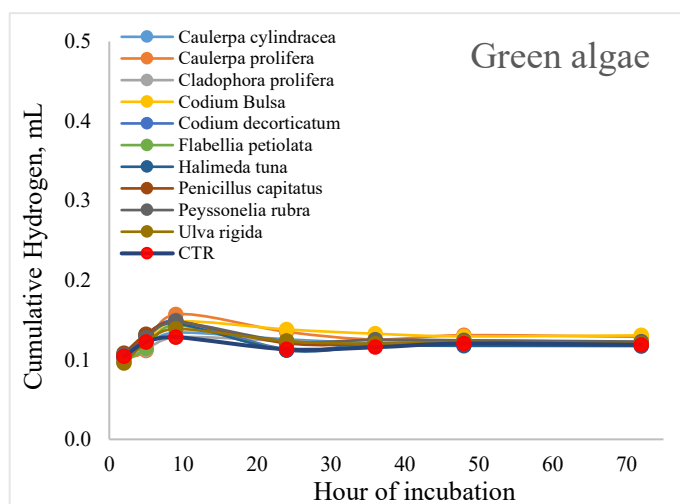
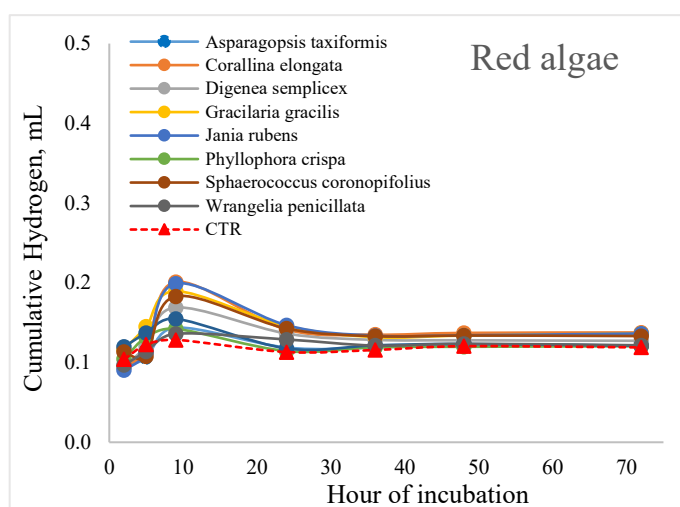
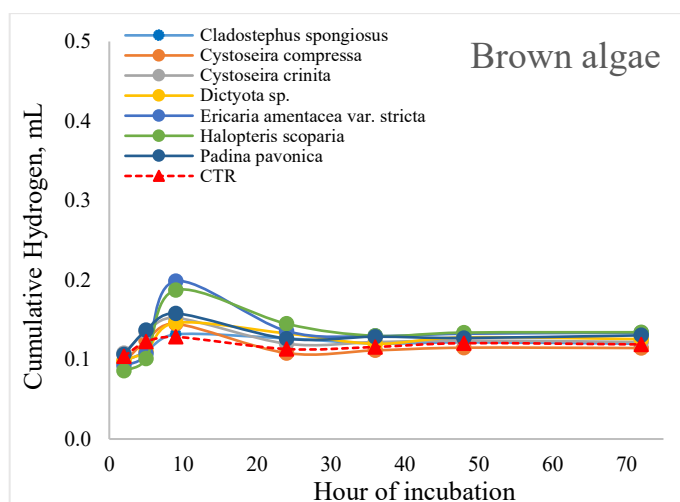
	Treatment	Acetate mmol/L	Butyrate mmol/L	Isobutyrate mmol/L	Isovalerate mmol/L	Propionate mmol/L	Valerate mmol/L
Control	CTR	52.07 ± 5.09	13.76 ± 1.31	1.64 ± 0.19	3.99 ± 0.53	26.25 ± 3.34	2.46 ± 0.4
Brown Algae	Halopteris scoparia	60.01 ± 5.09	17.9 ± 1.31	2.2 ± 0.19	5.4 ± 0.53	37.23 ± 3.34	3.98 ± 0.4
	Padina pavonica	57.97 ± 5.09	14.41 ± 1.31	1.95 ± 0.19	4.79 ± 0.53	30.4 ± 3.34	2.96 ± 0.4
	Cystoseira crinita	56.24 ± 5.09	14.67 ± 1.31	1.93 ± 0.19	4.73 ± 0.53	29.7 ± 3.34	2.97 ± 0.4
	Cladostephus spongiosus	55.28 ± 5.09	14.01 ± 1.31	1.89 ± 0.19	4.35 ± 0.53	27.22 ± 3.34	2.71 ± 0.4
	Cystoseira compressa	54.01 ± 5.09	13.26 ± 1.31	1.6 ± 0.19	3.53 ± 0.53	22.81 ± 3.34	2.21 ± 0.4
	Ericaria amentacea var. stricta	55.04 ± 5.09	18.34 ± 1.31	1.79 ± 0.19	5.14 ± 0.53	35.15 ± 3.34	3.68 ± 0.4
	Dictyota sp.	47.6 ± 7.19	16.85 ± 1.86	2.14 ± 0.27	5.21 ± 0.75	34.95 ± 4.72	3.63 ± 0.57
Green algae	Peyssonelia rubra	57.47 ± 5.09	14.67 ± 1.31	1.99 ± 0.19	4.86 ± 0.53	30.6 ± 3.34	3.09 ± 0.4
	Penicillus capitatus	57.23 ± 5.09	14.87 ± 1.31	1.95 ± 0.19	4.74 ± 0.53	29.36 ± 3.34	3.0 ± 0.4
	Ulva rigida	57.26 ± 5.09	13.99 ± 1.31	1.8 ± 0.19	4.33 ± 0.53	27.66 ± 3.34	2.91 ± 0.4
	Caulerpa cylindracea	55.96 ± 5.09	14.16 ± 1.31	1.82 ± 0.19	4.31 ± 0.53	28.07 ± 3.34	2.87 ± 0.4
	Cladophora prolifera	55.89 ± 5.09	13.86 ± 1.31	1.86 ± 0.19	4.28 ± 0.53	26.93 ± 3.34	2.75 ± 0.4
	Halimeda tuna	45.22 ± 5.09	11.89 ± 1.31	1.54 ± 0.19	3.41 ± 0.53	21.94 ± 3.34	2.21 ± 0.4
	Codium decorticatum	50.75 ± 5.09	13.29 ± 1.31	1.61 ± 0.19	3.82 ± 0.53	23.94 ± 3.34	2.33 ± 0.4
	Flabellia petiolata	46.86 ± 5.09	12.2 ± 1.31	1.52 ± 0.19	3.56 ± 0.53	21.69 ± 3.34	2.03 ± 0.4
	Codium Bursa	35.93 ± 5.09	12.79 ± 1.31	1.47 ± 0.19	3.79 ± 0.53	26.15 ± 3.34	2.54 ± 0.4
	Caulerpa prolifera	41.02 ± 5.09	15.62 ± 1.31	1.96 ± 0.19	5.02 ± 0.53	34.29 ± 3.34	3.59 ± 0.4
Red Algae	Asparagopsis taxiformis	55.07 ± 4.15	14.62 ± 1.07	1.81 ± 0.15	4.14 ± 0.43	27.75 ± 2.73	2.89 ± 0.33
	Gracilaria gracilis	55.74 ± 5.09	15.38 ± 1.31	1.94 ± 0.19	4.51 ± 0.53	30.62 ± 3.34	3.26 ± 0.4
	Wrangelia penicillata	51.63 ± 5.09	13.55 ± 1.31	1.67 ± 0.19	4.33 ± 0.53	26.22 ± 3.34	2.81 ± 0.4
	Digenea simplicex	49.71 ± 7.19	15.34 ± 1.86	1.9 ± 0.27	4.28 ± 0.75	28.45 ± 4.72	3.12 ± 0.57
	Phyllophora crispa	46.98 ± 5.09	12.06 ± 1.31	1.51 ± 0.19	3.41 ± 0.53	21.63 ± 3.34	2.0 ± 0.4
	Jania rubens	62.65 ± 7.19	16.01 ± 1.86	1.78 ± 0.27	4.4 ± 0.75	32.79 ± 4.72	3.21 ± 0.57
	Sphaerococcus coronopifolius	39.27 ± 5.09	12.79 ± 1.31	1.5 ± 0.19	3.48 ± 0.53	24.48 ± 3.34	2.33 ± 0.4
	Corallina elongata	39.2 ± 5.09	15.12 ± 1.31	1.88 ± 0.19	4.62 ± 0.53	31.84 ± 3.34	3.23 ± 0.4

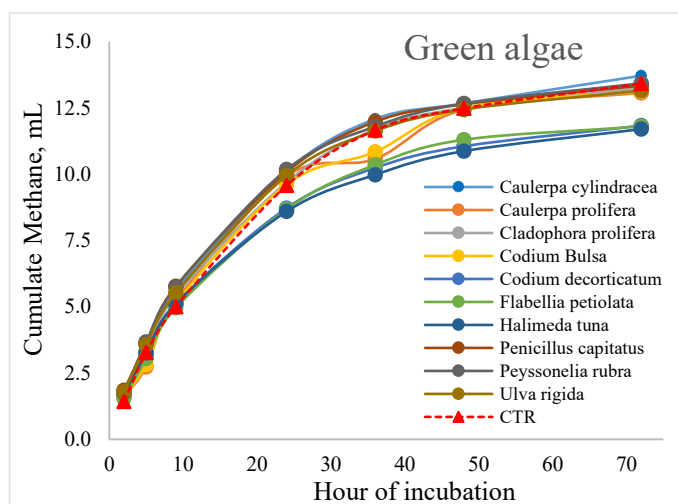
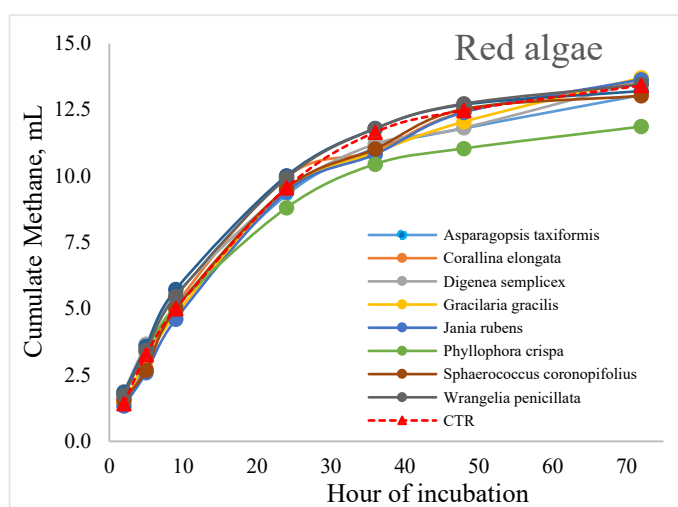
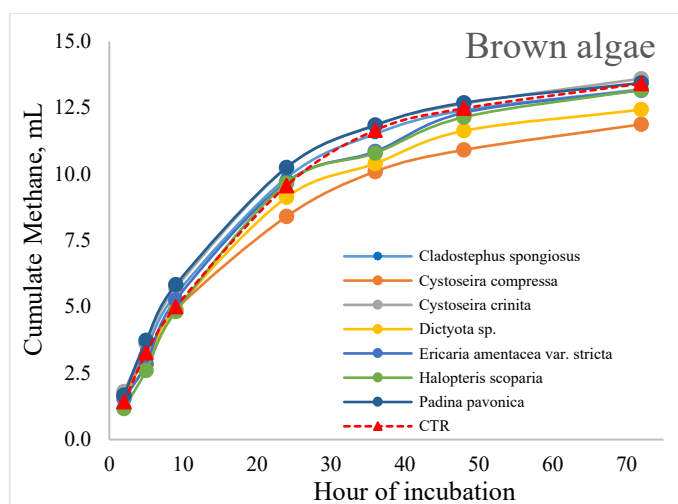
Superscripts indicated that the specific mean was lower (i.e., a) or higher (i.e., b) than CTR at a $P < 0.05$, as evaluated with Dunnett's multiple comparison test.

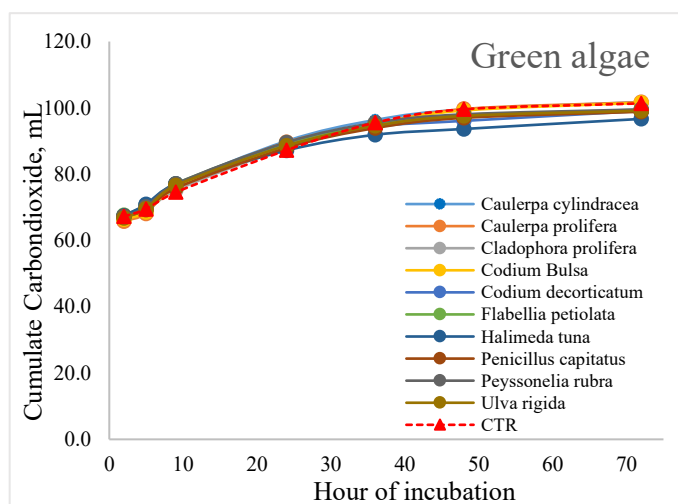
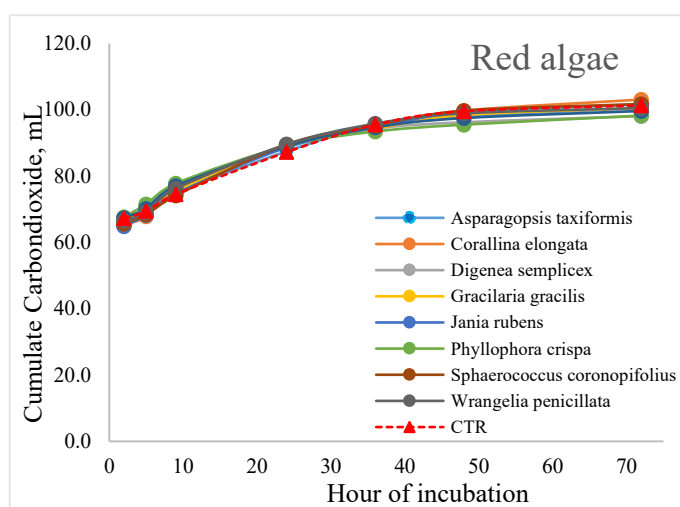
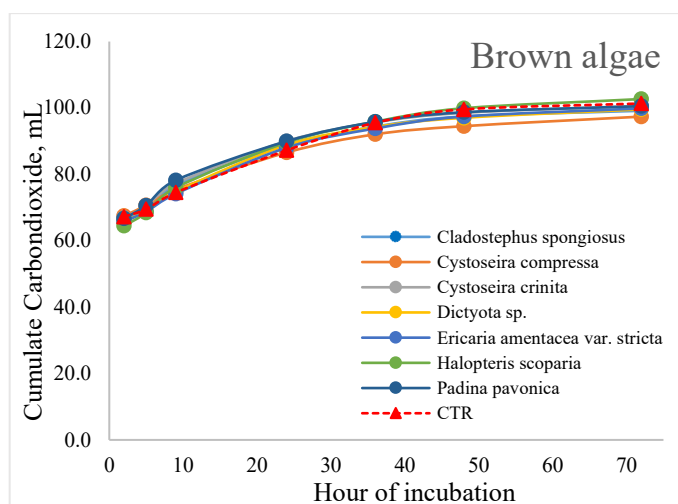
The data was expressed as LSM ± standard error.

Figure 1. Dynamic production of cumulated total gas, methane, carbon dioxide and hydrogen production under *in vitro* rumen fermentation of macroalgae species over the 72h incubation time.









CTR, control.

The data expressed as LSMEAN.

Table 5. Total phenolic and total flavonoid content in selected Mediterranean macroalgae species

	Treatment	Total phenolic content (mg/100 g GAE)	Total flavonoid content (mg/100 g QE)
Brown Algae	<i>Cladostephus spongiosus</i>	61.38 ± 2 ^g	29.58 ± 0.34 ^g
	<i>Cystoseira compressa</i>	46.74 ± 1.58 ^f	33.71 ± 0.42 ^h
	<i>Cystoseira crinite</i>	30.97 ± 0.13 ^d	17.06 ± 0.28 ^e
	<i>Dictyota</i> sp.	108.22 ± 0.47 ⁿ	29.58 ± 1.5 ^g
	<i>Ericaria amentacea</i> var. <i>stricta</i>	92.14 ± 0.57 ^{kl}	41.22 ± 0.88 ⁱ
	<i>Halopteris scoparia</i>	18.98 ± 0.77 ^b	18.01 ± 0.22 ^e
	<i>Padina pavonica</i>	21.08 ± 0.79 ^b	7.47 ± 0.18 ^b
Green Algae	<i>Caulerpa cylindracea</i>	126.44 ± 0.54 ^p	188.93 ± 2.34 ^q
	<i>Caulerpa prolifera</i>	108.75 ± 1.2 ⁿ	116.57 ± 0.42 ^m
	<i>Cladophora prolifera</i>	94.28 ± 0.27 ^{lm}	130.69 ± 0.36 ^o
	<i>Codium Bursa</i>	90.88 ± 1.0 ^k	32.37 ± 0.5 ^h
	<i>Codium decorticatum</i>	74.02 ± 1.83 ^h	120.68 ± 0.3 ⁿ
	<i>Flabellia petiolate</i>	118.85 ± 0.27 ^o	153.57 ± 1.14 ^p
	<i>Halimeda tuna</i>	78.03 ± 0.73 ⁱ	108.53 ± 0.53 ^l
	<i>Penicillus capitatus</i>	48.75 ± 0.6 ^f	32.67 ± 0.25 ^h
	<i>Peyssonelia rubra</i>	43.39 ± 0.39 ^e	13.62 ± 0.26 ^d
	<i>Ulva rigida</i>	91.84 ± 0.96 ^{kl}	90.35 ± 0.21 ^k
Red Algae	<i>Asparagopsis taxiformis</i>	97.33 ± 1.46 ^m	21.96 ± 0.38 ^f
	<i>Corallina elongata</i>	26.65 ± 0.35 ^c	10.86 ± 0.25 ^c
	<i>Digenea simplicex</i>	27.31 ± 0.35 ^c	13.41 ± 0.11 ^{cd}
	<i>Gracilaria gracilis</i>	130.36 ± 0.59 ^q	28.12 ± 0.99 ^g
	<i>Jania rubens</i>	9.01 ± 0.35 ^a	3.24 ± 1.06 ^a
	<i>Phyllophora crispa</i>	199.2 ± 0.94 ^s	39.8 ± 1.26 ⁱ
	<i>Sphaerococcus coronopifolius</i>	73.02 ± 1.0 ^h	20.87 ± 0.88 ^f
	<i>Wrangelia penicillata</i>	84 ± 1.4 ^j	18.1 ± 1.39 ^e

The data was express as means ± SEM (n = 3). GAE = gallic acid equivalents; QE = quercetin equivalents. Different superscript letters within a column indicate significant differences (Tukey HSD, P < 0.05)

7 General discussion

This PhD thesis explored the complexity of enteric CH₄ emissions in dairy systems using three complementary experimental approaches: *in vivo*, *in vitro*, and *in silico*. Through five scientific studies, the research provided mechanistic insights and practical evaluations of nutritional strategies aimed at reducing CH₄ production while preserving or improving animal performance.

An integrative framework for methane mitigation

In vivo trials (Paper I) demonstrated that 3-nitrooxypropanol (3-NOP) effectively inhibited CH₄ emissions in mid-lactating dairy cows, consistent with previous literature. Notably, in summer the effects on reduction of DMI and increased feed efficiency in summer and at metabolic level as milk composition. These varied responses lend support to the idea that strategies for reducing CH₄ might lead to systemic changes involving energy metabolism.

In vivo trials (Papers II) in our longitudinal *in vivo* study across summer (P1) and fall (P2), 3-nitrooxypropanol did not modify core behavioral metrics (daily eating time, number of bin visits, meal frequency, or mean meal length) indicating no re-timing of feeding at the day scale. Instead, effects were season-contingent and quantitative: in summer, cows fed 3-NOP ate smaller meals and showed lower DMI, whereas no intake differences emerged in fall. This pattern coincided with improved feed efficiency in summer only, suggesting that methanogenesis inhibition altered the amount consumed per bout rather than the structure of feeding behavior. Overall, these findings support a dissociating between time spent at the feeder and intake volume under CH₄ inhibition, consistent with a metabolic rather than behavioral retiming response.

In vitro rumen trials (Paper III) confirmed the short-term inhibition effect of 3-NOP, using rumen fluid collected from treated animals. The fermentation profile revealed temporal shifts in total gas and VFA composition, by increased butyrate and isoacids. This suggests that methanogenesis

inhibition of 3-NOP at microbial level promoted alternative H₂ sinks, such as reductive acetogenesis and branched-chain VFA synthesis at early incubation time, demonstrated the faster reversible rumen condition.

The simulation study (Paper IV) demonstrated the predictive capacity of a mechanistic rumen model (Muñoz-Tamayo et al., 2016) to capture the in vitro rumen fermentation of 39 Italian TMR formulations. Biological parameters estimated by the model like λ_1 , λ_2 , and km_H_2 did not varied significantly across nutritional clusters but were strongly associated with digestibility indices. The model allowed to estimate the maximum H₂ utilization rate, revealing potential trade-offs between feed efficiency and CH₄ yield in high intake scenarios.

Finally, the screening of 28 Italian Mediterranean macroalgae species (Paper V) offered an initial assessment of their antimethanogenic potential. Though modest in CH₄ reduction compared to tropical species like *Asparagopsis taxiformis*, some native algae (e.g., *Flabellia petiolata*, *Codium decorticatum*) showed favorable polyphenolic profiles that justify further investigation. The integration of omics data could identify candidate bioactives for future additive development.

Limitation of the PhD study

H₂ flow emerged as a merging mechanism across approaches. Both modeling and experimental results pointed to the centrality of H₂ dynamics in regulating CH₄ formation and VFA profiles. The in vitro study utilizing rumen fluid from treated animals unequivocally underscores the critical importance of conducting time-resolved measurements and comprehensive microbial analyses. This approach is essential to accurately determine the shifts in fermentation pathways following 3-NOP exposure. By prioritizing these detailed assessments, we can gain invaluable insights into the dynamic changes occurring within the rumen environment, ultimately advancing our understanding and application of 3-NOP in animal health and nutrition (e.g., metataxonomics,

metatranscriptomics). The in silico model translated kinetic parameters into farm-level sustainability metrics (e.g., REI, FE, milk fat), enabling a deeper interpretation of the biological signals observed.

However, limitations exist. The macroalgae study lacked replication over seasons and did not isolate specific bioactive compounds. The in vitro trials may have underestimated microbial resilience due to short incubation times.

8 Conclusion and Perspective

This PhD work underscores the value of integrating multiple methodological approaches to address the global challenge of enteric methane mitigation in ruminants.

Major findings include:

- 3-NOP consistently reduced CH₄ emissions in vivo, while inducing secondary effects on fermentation profiles and milk composition.
- 3-NOP did not alter daily eating time, visit rate, meal frequency, in summer it reduced meal size and DMI and increased feed efficiency, with no intake effect in fall.
- Macroalgae species from the Mediterranean Sea showed variable effects on gas production and methane reduction, supplemental study needs to supporting their role as regionally adapted, low-impact mitigation options.
- Mechanistic modeling of fermentation kinetics enabled the identification of dynamic CH₄ predictors and their correlation with productivity and efficiency indicators

9 Future perspectives

Refining feed additive strategies

Further trials should assess synergistic effects between 3-NOP and other additives (e.g., lipids, tannins, algae extracts) on H₂ partitioning and CH₄ abatement.

Expanding the model framework

Future model versions should integrate microbial modules, metabolic health indicators, and host energy partitioning, with validation under variable diets and climates.

Omic screening

Untargeted metabolomics and transcriptomics could uncover the bioactive compounds responsible for antimethanogenic effects in seaweeds, particularly polyphenols and halogenated metabolites.

From experimental to practical applications

Collaborations with industry and policy-makers should translate findings into scalable recommendations for methane inventories (Tier 3), carbon credit schemes, and climate-smart feeding guidelines.