The combined opportunities of supplementing varying concentrate-to-forage ratios with 3-nitrooxypropanol in the ration of dairy cows from the periparturient to the mid-lactation period

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Contents

I Abbreviations

AA	amino acids
BCS	body condition score
BHB	ß-hydroxybutyrate
BW	body weight
BWG	body weight gain
CFP	concentrate feed proportion
CL	crude fat
CoM	methyl-coenzyme M
CH_4	methane
CO_2	carbon dioxide
CO_2-eq.	CO_2-equivalent
CP	crude protein
d	day
DE	digestible energy
DM	dry matter
DMI	dry matter intake
EB	energy balance
ECM	energy-corrected milk
EE	ether extract
EEx	energy expenditures
FA	fatty acid
FCM	4% fat-corrected milk
FPCM	fat-and-protein corrected milk
F/P-ratio	fat-to-protein ratio in the milk
FT-MIR	Fourier transform mid-infrared spectrometry
GEI	gross energy intake
GHG	greenhouse gases
[H]	metabolic hydrogen
H_2	dihydrogen, i.e. molecular hydrogen
HC	high-concentrate

HP	heat production
HPA	3-hydroxy-propionic acid
HS-CoB	coenzyme B
HS-CoM	coenzyme M
IPCC	Intergovernmental Panel on Climate Change
LC	low-concentrate
LMD	laser methane detector
mBW	metabolic body weight (kg body weight$^{0.75}$)
MCR	methyl-coenzyme M reductase
ME	metabolizable energy
MFA	milk fatty acids
NAD	reduced form of nicotinamide adenine dinucleotide
NDF	neutral-detergent fiber
NEFA	non-esterified fatty acids
NEL	net energy for lactation
NFC	non-fiber carbohydrates
NH_3-N	ammonia-N
3-NOP	3-nitrooxypropanol
NOPA	3-nitroxy-propionic acid
OM	organic matter
RC	respiration chamber
RNB	rumen nitrogen balance
RSD	residual standard deviation
RUSITEC	rumen simulation technique
SD	standard deviation
SF_6	sulphur hexafluoride tracer technique
TMR	total mixed ration
VFA	volatile fatty acids

II Figures

Background, General discussion

III Tables

Background, General discussion

1. Introduction

Methane (CH_4) formation in dairy cows arising from the enteric fermentation of feed carbohydrates contributes to 44% of global anthropogenic CH_4 emissions (Gerber et al. 2013). In addition, methanogenesis represents a feed inefficiency as 2% to 12% of the gross energy intake is converted to CH_4 during ruminal digestion (Johnson und Johnson 1995). Global efforts have been intensified to develop new technologies (Hill et al. 2016) and models (Engelke et al. 2018, Niu et al. 2018) to quantify CH_4 emissions from cattle. For a low-CH_4 future, current research endeavors to find new CH_4 reducing feeding strategies without compromising social acceptability, animal health and productivity, since ruminants provide significant amounts of high-quality proteins in milk and meat for the growing world population (Beauchemin et al. 2020).

In this context, the promising chemical CH_4 inhibitor 3-nitrooxypropanol (3-NOP) seems to reduce CH_4 production by potentially 20% to 40% in dairy cows acting directly by blocking the enzyme methyl-Coenzyme M reductase which catalyzes the last step during CH_4 synthesis (Hristov et al. 2015, Duin et al. 2016). It appears that the 3-NOP induced CH_4 inhibition shifts fermentation pathways to increased production of propionate for alternative hydrogen removal (van Gastelen et al. 2020), whereby propionate is the most important precursor of hepatic gluconeogenesis in cows (Aschenbach et al. 2010). Feeding starch-rich diets with high concentrate-to-forage ratios in dairy cow rations is a well-known indirect CH_4 abatement strategy which is related to the increased dry matter intake, particulate passage rate and hydrogen-consuming propionate production in favor of an improved animal productivity (Knapp et al. 2014). As a consequence, the amount of CH_4 produced per unit of feed consumed or milk produced decreases. Periparturient cows are challenged with a negative energy balance which occurs when energy demands for lactogenesis exceed the energy supply due to the reduced feed intake, resulting in a substantial adipose tissue mobilization and a higher risk for metabolic diseases (Esposito et al. 2014).

Against this backdrop, the present thesis aimed at investigating potential interactive effects arising from the combined CH_4 mitigation strategy of feeding high-concentrate diets supplemented with 3-NOP on lowering CH_4 emissions and improving energy supply to the periparturient and early-lactation dairy cow.

2. Background

2.1. Methane emissions from cattle livestock

2.1.1. Trade-off between enteric methane emissions and a growing world population

Climate change is one of the most defining challenges of the 21st century. Global warming directly influences all areas of life around the world which is already now experienced due to more frequently occurring ecological disasters, such as droughts. Human population is expected to grow up to 9.8 billion in 2050. Contemporaneously, nutritional requirements of meat and milk are expected to increase by 73% and 58%, respectively (Gerber et al. 2013), due to growing incomes and urbanization (Beauchemin et al. 2020). In particular, milk is the third most important provider of protein and fat for humanity (FAO und GDP 2019). Dairy cattle livestock produces essential nutrients contained in milk and meat (FAO und GDP 2019), while the nutritional spectrum of cattle does not necessarily compete with that of humans (Beauchemin et al. 2020). However, CH_4 formation from enteric fermentation of plant materials in ruminant livestock is a main source of CH_4 emission contributing to 17% and 3.3% of the global budget of atmospheric CH_4 and total GHG emissions, respectively, which implies that there could exist a high potential for mitigation opportunities (Conrad 2009, Knapp et al. 2014). The Global Warming Potential (GWP_{100}) of CH_4 is 28 times greater than that of CO_2 over a 100-yr time horizon (Myhre et al. 2013). Though, the conversion of CH_4 to CO_2-equivalents misrepresents its impact on global temperature (Allen et al. 2018). The newly developed GWP* differentiates between the different climate impacts of long-lived cumulative (CO_2) and short-lived (CH_4) climate pollutants (SLCP) on radiative forcing and temperatures over a wide range of timescales and enables an integrative modeling of mitigation impacts (Allen et al. 2018). According to Allen et al. (2018), radiative forcing scales with the total stock of emissions to date with regard to cumulative pollutants (CO_2). In contrast, SLCPs (CH_4) scale with the current flow (emission rate) multiplied by the SLCP lifetime. As a result, falling CH_4 emissions lead to falling global temperatures (Allen et al. 2018). Expressing CH_4 emissions in CO_2-equivalents incorrectly suggests that CH_4 emissions, which have a half-life of 12.4 years, would cause further global warming after 100 years (Allen et al. 2018). Thus, current CH_4 emissions from enteric fermentation in ruminants do not contribute to further global warming and, as being part of the biogenic carbon circle, they do not cause additional radiative forcing (which is not the case for fossil CH_4). Nevertheless, a constant high level of current CH_4 emissions still represents a major

contribution to global warming which implies that mitigation strategies are needed (Allen et al. 2018). Future efforts to mitigate enteric CH_4 will result in negative methane CO_2-e* emissions and correspond to CO_2 removal from the atmosphere (Allen et al. 2018). In response to rising consumer demands, the absolute global GHG emissions from the dairy sector increased by 18% between 2005 and 2015 which can be related to the 30% increase in milk production and a growing dairy herd population (11%) (FAO und GDP 2019). On the other hand, milk production efficiency substantially increased due to the improved on-farm cow management in combination with optimized feeding practices worldwide (Beauchemin et al. 2020). This trend is expected to continue and has already caused a 11% decline in GHG emission intensities from 2.8 to 2.5 kg CO_2 eq. per kg FPCM. In this regard, the dairy sector is obliged to contribute to the fulfilment of the Climate Agreement adopted at the United Nations Climate Change Conference of Parties in Paris in 2015 which has set itself the objective to limit anthropogenic temperature rise to 1.5 – 2.0 °C. In a global social progress, the dairy sector is challenged to reduce its environmental impact without counteracting improvements in productivity as well as animal welfare (FAO und GDP 2019).

2.1.2. Enteric methane emissions in Germany

Agriculture in Germany contributes 63.6 million tonnes of CO_2 eq. (7.5%) to the total of 793 million tonnes CO_2 eq. of GHG in 2019 (Figure 1). In the German agricultural GHG emission inventory, enteric CH_4 emissions are annually quantified using the national sector-based approach TIER-2 for non-dairy cattle and the more complex TIER-3 for dairy cows according to the IPCC guidelines (IPCC 2006). The CH_4 emissions from enteric fermentation amount to 25 million tonnes of CO_2 eq. (39.4% of the total CO_2 eq. from the agricultural sector) contributing 3% to the total GHG in Germany (Haenel et al. 2020) (Figure 1). The 25 million tonnes of CO_2 eq. can be split into 14.1 million tonnes of CO_2 eq. originating from enteric fermentation in dairy cows and 9.8 million tonnes of CO_2 eq. resulting from fermentation processes in non-dairy cattle (Haenel et al. 2020). Since 1990, the CH_4 emissions from enteric fermentation in German dairy cows declined by 26% due to the reduction in animal numbers and increased feed digestibility (Haenel et al. 2020). The German agriculture targets to reduce its total GHG emissions to 58 million tonnes of CO_2 eq. by 2030.

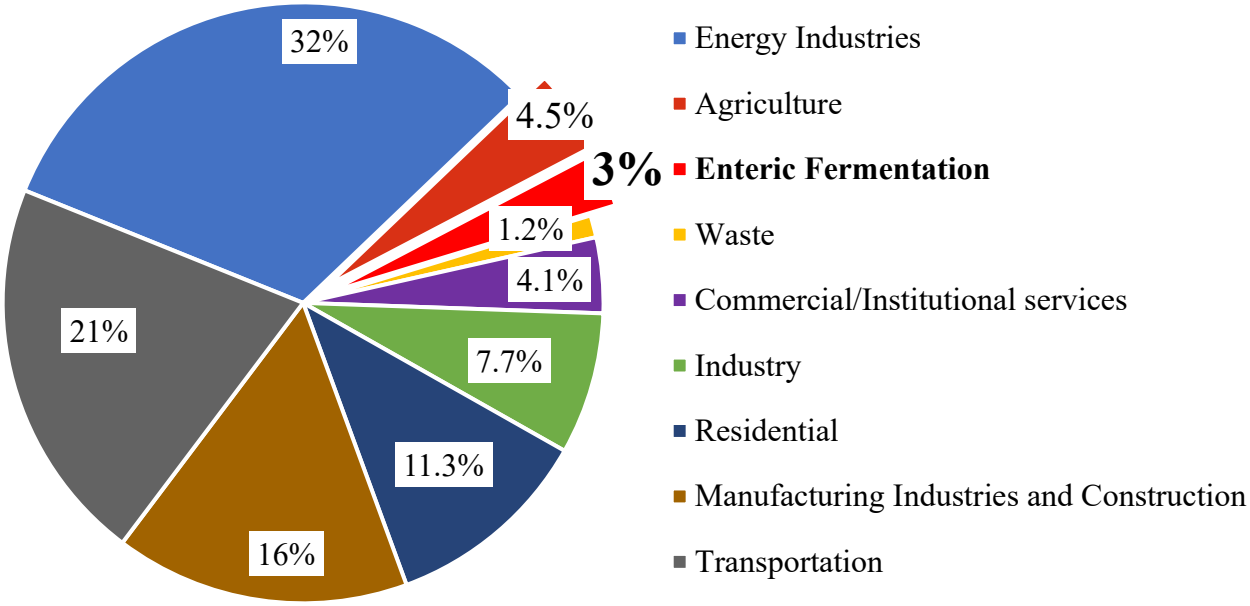

Figure 1: Greenhouse gas emissions from enteric fermentation and further pollutants from German agriculture as a portion of total German greenhouse gas emissions in 2019 (adopted from Umweltbundesamt, National Greenhouse gas inventory from 1990 until 2020.

2.2. Methanogenesis in rumen methanogenic Archaea

The CH_4 mitigation potential in ruminants is limited to be lowered to zero as methanogenesis is a by-product of microbial feed degradation in the rumen. The evolution of ruminants has been directly linked to that of microbes as both of them together fill the ecological niche of converting plant carbohydrates to energy in a symbiotically manner. However, methanogenesis is considered as a digestive inefficiency as up to 12% of the GEI can be lost as CH_4 (Johnson und Johnson 1995). Polysaccharides (mainly starch, cellulose, hemicellulose) are hydrolyzed to 5- and 6-carbon sugars by microbial enzymes in the anaerobic rumen environment and fermented by anaerobic bacteria, protozoa, fungi, and methanogenic Archaea in a trophic chain to generate energy (Moss et al. 2000). As shown in Figure 2, monosaccharides are further fermented to VFA and CO_2 thereby releasing reducing equivalents of metabolic hydrogen [H] and reducing intracellular cofactors, such as NADH (Knapp et al. 2014). Cofactors must be re-oxidized to enable continuation of fermentation processes which is mostly done by transferring electrons to protons (Ungerfeld 2018, Beauchemin et al. 2020). In this context, hydrogenase-expressing bacteria convert [H] to dihydrogen (H_2, i.e. molecular hydrogen) which is transferred to the strictly anaerobic methanogenic Archaea living freely or in an endosymbiotic relationship inside the protozoa.

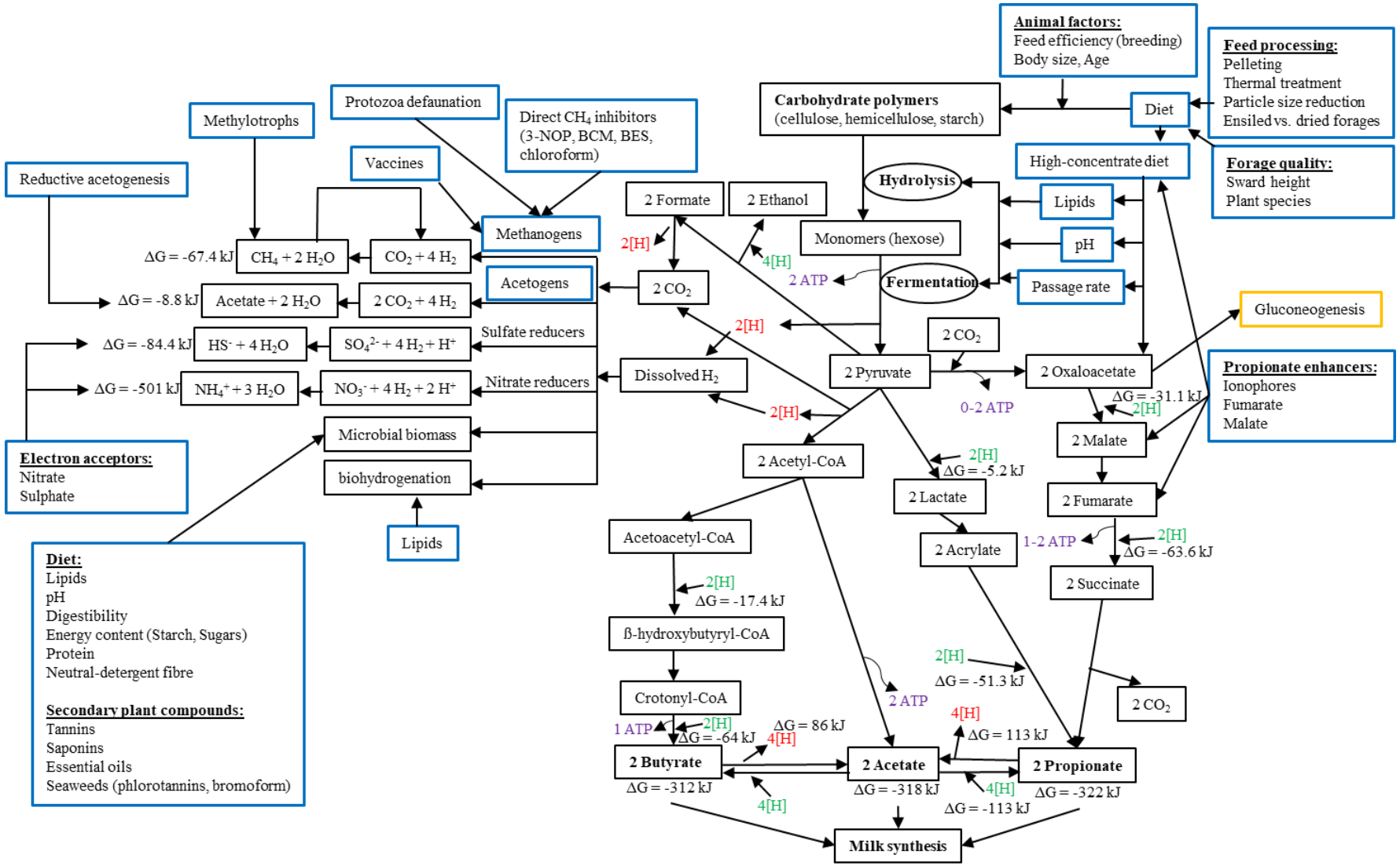

Figure 2: Thermodynamics, release (red) and utilization (green) of metabolic hydrogen in rumen fermentation pathways demonstrated with target points of CH_4 mitigations options (blue colored boxes) and linkage to gluconeogenesis (orange) consolidated from Ungerfeld und Kohn (2006), Ungerfeld (2018), Haque (2018), Beauchemin et al. (2020), Ungerfeld (2020).

However, H_2 can accumulate in the rumen under 3-NOP induced methanogenesis inhibition (Figure 3) which causes an increased H_2 partial pressure being detrimental to the ongoing of microbial fermentation processes and rumen motility. Hydrogen within the rumen exist in three forms: free protons (H^+), dissolved (dH_2) and gaseous H_2 (gH_2) (Wang et al. 2014). Only dissolved H_2 is microbially available and therefore utilized by the methanogens for the reduction of CO_2 to CH_4 in the hydrogenotrophic pathway, i.e. the largest [H] sink in the rumen (Janssen 2010, Beauchemin et al. 2020). About 78% of the methanogens belong to hydrogenotrophic Archaea (*Methanobrevibacter*) and 22% can be assigned to methylotrophic Archaea (*Methanosarcinales, Methanosphaera*, and *Methanomassiliicoccaceae*) (Morgavi et al. 2010, Huws et al. 2018). The H_2-independent methanogenic pathways (e.g., acetoclastic methanogenesis) contribute only 4% to total CH_4 production. However, these methanogens play a major role for detoxification of methanol, amines and mercaptans which result from the protein and AA degradation (Hoedt et al. 2016). It seems that 74% of the ruminal archaeal community around the world belong to the clades of *Methanobrevibacter gottschalkii* and *Mbb. ruminantium* which comprise, together with one *Methanosphaera sp.* and two further groups associated with *Methanomassiliicoccaceae*, about 90% of all rumen Archaea as a core microbiome (Henderson et al. 2015). Recently, it was stated that the composition of the methanogenic community rather than the overall abundance of Archaea was more closely associated to CH_4 production (Tapio et al. 2017). Most of the CH_4 directly escapes the rumen via eructation. CH_4 from the rumen and lower gut can be absorbed into the blood stream and exhaled from the lungs via expiration. Methane produced in the hindgut contributes 13% to the total CH_4 emissions which is mainly absorbed into the blood and eliminated via expiration. Only 2% to 8% of the total CH_4 emissions are emitted in the flatus (Ricci et al. 2014).

In methanogenesis, hydrogenotrophic methanogens generate 0.5 mole of ATP per mole CO_2 reduced to CH_4 (Thauer et al. 2008). Figure 3 illustrates the hydrogenotrophic methanogenic pathway in detail in which the active nickel enzyme MCR plays the central role. The MCR contains a Ni(I) tightly bound in the tetrapyrrole derivative cofactor F_{430} as a prosthetic group (Thauer 2019). The F_{430} in MCR has to be in the Ni(I) oxidation state to be active (Rospert et al. 1991). The last step of methanogenesis in archaeal cells involves MCR which catalyzes the reduction of CoM with coenzyme B and dH_2 to CH_4 and the heterodisulfide CoM-S−S-CoB in an ATP-dependent reaction (Hedderich et al. 1989, Thauer 2019). CH_4 mitigation agents acting as CoM analogues (3-NOP) target the MCR (Figure 3) which is a promising global CH_4 mitigation approach as MCR abundance is worldwide highly specific to the domain of rumen Archaea (Thauer 2019).

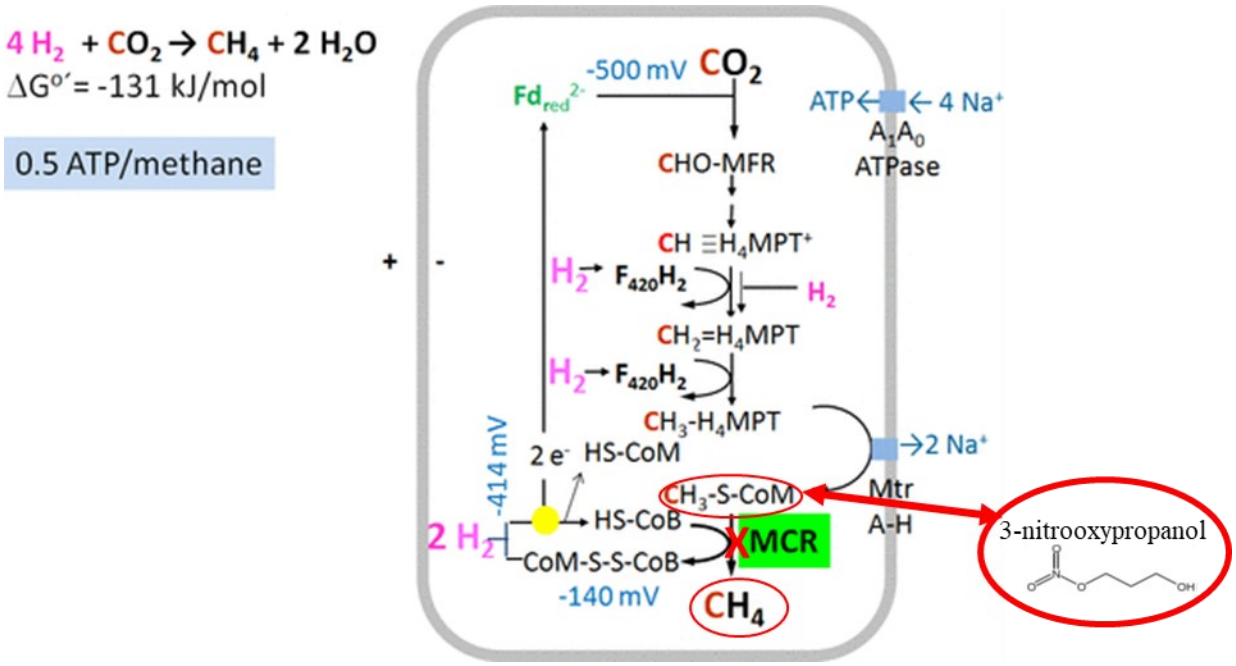

Figure 3: Energy metabolism of *Methanothermobacter marburgensis* and mode of action of 3-nitrooxypropanol (3-NOP). Molecular shape of 3-nitrooxypropanol (molecular formula: $C_3H_7NO_4$; IUPAC: 3-Hydroxypropyl nitrate). Extracted from: (CAS Common Chemistry). Methyl-coenzyme M (HS-CoM) is replaced by its structural analogue 3-NOP causing an inactivation of methyl-coenzyme M reductase (MCR). MFR, methanofuran. H4MPT, tetrahydromethanopterin. F420, coenzyme F420. HS-CoB, coenzyme B. Fd, ferredoxin. Yellow dot, heterodisulfide reductase-hydrogenase complex (HdrABCMvhADG) that couples the exergonic reduction of CoM-S−S-CoB with H_2 with the endergonic reduction of ferredoxin with H_2 via flavin-based electron bifurcation (modified and sourced from Thauer (2019)).

2.3. Dietary interventions to reduce methane emissions in dairy cows

2.3.1. Methane mitigation: success conditions, possibilities and quantification techniques

Methane mitigation strategies in dairy cows must go with social acceptance (e.g., safety of food of animal origin, animal welfare), farm-individual environmental factors, nutritional needs and health of the cow and last but not least farm profitability to be effectively adopted by farmers. Furthermore, the large variability in resources (e.g., availability of feeds, type of feedstuffs) and environmental conditions (e.g., low- or large-scale farms, pasture or indoor-based milk production systems) between dairy production regions around the globe emphasize that there is no single mitigation pathway for a low CH_4 future in dairy production matching all dairy production systems worldwide, but rather a combination of different CH_4 mitigation strategies. However, not all of the CH_4 mitigation options cause additive effects. CH_4 mitigation options become only effective if research results are put to practice, communicated and routinely implemented to farm processes.

A large number of nutritional CH_4 mitigation strategies, presented with their point of attack in Figure 2, has been previously reviewed (Beauchemin et al. 2008, Kumar et al. 2009, Hristov et al. 2013a, Knapp et al. 2014, Patra 2016, Patra et al. 2017, Haque 2018, Beauchemin et al. 2020). However, few of them meet the criteria to be potentially implemented in dairy livestock systems. Thus, numerous CH_4 mitigation agents are not useful due to their transient (ionophores) or low CH_4 inhibiting effects (yeast, essential oils, saponins). Furthermore, some of them are expensive (electron acceptors of malate, fumarate), environmentally harmful (bromochloromethane, bromoform in *Asparagopsis taxiformis*, nitrate), detrimental to animal health (nitrate/nitrite related to methemoglobinemia), impractical to be implemented in farm routines (protozoa defaunation) or the development is still in its early stage (e.g., enzymes as feed additives, vaccination, early life programming of the rumen microbial community). One challenge is common to all anti-methanogenic feed additives: the effects of CH_4 mitigation options vary considerably across the literature which could be due to several interactive effects. Controlling the variability is crucial for an effective and persistent CH_4 mitigation. In this context, substance-related factors such as dosage level, formulation, stability in the rumen, stability during on-farm storage and within a premix or diet, administration technique as well as animal-and-diet-related interactive factors, such as ruminant categorybackground diet, physiological stage of the ruminant and the individuality of the host and microbiome. At present, using those feed additives which are currently regarded as being most promising (e.g., dietary lipids, 3-NOP, tanniferous plants, seaweeds), improving dairy cow management (precision livestock farming), productivity and breeding (genetic selection for long-living and low CH_4-emitting cows), optimizing feeding practices (e.g., concentrate feeding, forage quality and feed processing), and increasing the sector´s overall productive and energetic efficiency (reducing post-harvest losses), appear to represent the currently most promising CH_4 mitigation actions (Yanibada et al. 2020, Beauchemin et al. 2020).

A major prerequisite for the assessment of CH_4 mitigation options relies on the accuracy of CH_4 emissions quantification. However, the agreement between the different CH_4 measurement techniques varies considerably. Variability may result from differences in the underlying technical measurement principles, operating procedures, inherent measurement inaccuracies and, in some instances, the need for converting the generated data (e.g., CH_4 concentration) into other output formats (e.g., CH_4 emission rate) which overall introduces uncertainty and systematic bias (Hristov et al. 2018). In this context, the meta-analysis of a global database, which was built on CH_4 emissions data gained from different CH_4 measurement techniques, revealed that coefficient of

variation (CV) for emission rate (g of CH_4/d) averaged 30% (respiration chamber (RC), n = 3024), 18% (GreenFeed, n = 731) and 28% (SF_6, n = 397) within each CH_4 quantification technique. In particular, the reported variability encompasses all sources of variation which means variation due to animal-related factors (e.g., ration composition and feed intake level) and not only variation due to the method of measurement (Hristov et al. 2018). In essence, the method of choice depends on the experimental purposes and the resources available. The use of methods that are imprecise should not be justified by the need for high throughput methodology, e.g. for breeding purposes (Hammond et al. 2016). The most applied techniques currently in use to record CH_4 emissions include the continuous techniques, namely the SF_6 technique and the RC. The automated head chamber system (GreenFeed system), sniffer and LMD technique represent further widely used techniques and can be categorized as discontinuous methods based on short-term measurements. The sniffer and LMD technique represent indirect CH_4 recording methods which measure the CH_4 concentration in the breath. In comparison to direct measurements of the CH_4 emission rate, the indirect techniques sniffer and LMD are subjected to greater uncertainty because the generated CH_4 concentration data are needed to be converted into daily CH_4 production which is based on assumptions and biased by meteorological (e.g., wind velocity, humidity), methodological (e.g., measuring angle, distance between the LMD device and the animal's nostrils, proximity of other animals) as well as animal-related (e.g., the prevailing animal behavior during measurement) influential factors. Hence, the LMD technique can only provide rough estimates of the average CH_4 production of a (treatment) group being appropriate to phenotype animals according to their emission category (low and high CH_4 emitters) for breeding schemes (Sorg 2022). The agreement between LMD and RC measurements was found to range from low (Denninger et al. 2020) to moderate (r = 0.47) (Chagunda et al. 2013) to high (r = 0.8) (Chagunda und Yan 2011). Larger variability was found with the sniffer method when compared to the GreenFeed system with no agreement between both methods (Huhtanen et al. 2015). In this context, head position and distance from the sampling inlet are the most important factors contributing to the observed variation. Wu et al. (2018) concluded that the sensitivity of the sniffer technique is not sufficient to detect treatment differences in CH_4 emission rates of dairy cows. It is noteworthy, that the RC, SF_6 and handheld LMD techniques are labor intensive and, except for the LMD, not appropriate to screen large animal numbers and may impose restrictions on animal behavior (e.g., feed intake, milk production) which could negatively affect data validity (Hammond et al. 2016). The RC, which is the most accurate measurement technique to date, can be only referred to as the 'gold standard'

when RC gas recovery rates of 100% are achieved before and after each experiment in the calibration routine (Hammond et al. 2016, Hristov et al. 2018). There are several sources of variation in RC measurements, namely the airflow rate through the chamber as well as the dynamics of air mixing (Gardiner et al. 2015, Hammond et al. 2016). The GreenFeed system was assessed to provide similar averaged values when compared to the RC, but lower values than the SF_6 technique (Hammond et al. 2016). The GreenFeed technique was noted to be reliant on the head positioning towards the point of sampling in the chamber hood as well as on the frequency and timing of the animal visitations to the unit which becomes specifically important for ranking purposes of the individual animal (Huhtanen et al. 2015). The GreenFeed short-term spot sampling procedure does not reliably determine the diurnal pattern of CH_4 emissions, which are dependent on the events of feed intake and rumination periods, when compared to RC measurements (Hammond et al. 2016). In this regard, only weak concordance was found between the GreenFeed and RC techniques (Hammond et al. 2015). In essence, an adequate number of GreenFeed visits (more than three per d) and an equal distribution of them throughout the day are crucial to approximately cover the diurnal variation in CH_4 emission which is strongly related to the pattern of DMI. Importantly, the GreenFeed data should be averaged over a week (or a minimum of 30 spot measurements per averaged CH_4 emission value) to obtain reliable CH_4 quantifications comparable to those derived from the RC (Manafiazar et al. 2016). The GreenFeed system is advantageous with regard to the time-saving measurement of large animal numbers (maximum of 40 cows per GreenFeed unit in free stalls), high data accuracy when frequent animal visitations to the unit are facilitated and, apart from the acquisition costs, cost-effectiveness in the mode of operation. The GreenFeed method is specifically suitable for comparing effects of dietary treatments (Hammond et al. 2016).

2.3.2. Concentrate feed proportion

There are several reasons why the scope of feeding HC diets to reduce CH_4 emissions is to some extent limited. A further increase of the currently supplied high dietary CFP in the commercial high-yielding dairy cow rations may adversely affect animal health by increasing the risk for ruminal acidosis (Haque 2018). Implementing HC diets as a CH_4 mitigation strategy is therefore only sustainable when considering adequate concentrate-to-forage ratios in accordance to the nutritional needs and health of the cow during diet formulation. In addition, some concentrate feeds (cereal grain, soybean) directly compete with human nutrition and their import from tropical regions results in increased deforestation and higher total net GHG emissions which are likely to

exceed potential GHG savings due to a decreased CH_4 emissions intensity (FAO und GDP 2019). Hence, all CH_4 mitigation strategies should always be bio-economically evaluated in a life-cycle assessment. It is recommended to use coupled by-products of the regional vegetable cultivation (e.g., sugar beet pulp, and rapeseed meal) and food industry (e.g. brewers and distillers by-products) as concentrate feeds for its conversion into animal proteins (meat, milk) and re-utilize the manure from dairy cows for plant growth in a closed circuit could expand sustainability.

2.3.2.1. Dry matter intake and passage rate in high-concentrate diets affecting methanogenesis

In general, the adjustment of the CFP in the ration on the course of lacation and, therefore, on the nutritional and energy requirements of the cow contribute to lowering performance-related CH_4 emissions intensity (CH4/ECM). The main factors determining the efficiency of dietary CH_4 mitigation strategies concern the feed intake level, digestibility of nutrients, particle retention time in the rumen and the thermodynamically driven prevalence of certain fermentation pathways being interrelated with the concentration and flow of [H] as well as effects on the microbial community structure (Beauchemin et al. 2020). It was shown that DMI and passage rate explain about 52 to 64% and 28% of the variation in CH_4 emissions, respectively (Okine et al. 1989, Knapp et al. 2014). The increase of the CFP in a dairy cow ration represents an indirect CH_4 mitigation action. CH4/ECM can be potentially reduced by 2.5 to 15% when feeding HC diets in conventional dairy livestock (Knapp et al. 2014). Increasing the CFP from 32 to 53% in the ration of early-lactation cows was shown to linearly reduce total CH_4 production, yield and emissions intensity by 17%, 19% and 20%, respectively (Aguerre et al. 2011). However, Hristov et al. (2013a) noted that CH_4 emissions will likely not be affected by small and moderate variations in dietary CFP as significant CH_4 reductions may be expected at concentrate inclusion levels above 40% (Sauvant und Giger-Reverdin 2009). HC diets are characterized by higher proportions of starch relative to fiber. Feeding HC diets composed of highly digestible nutrients to dairy cows is associated with increased total DMI and fermentable OM in the rumen, feed turn-over rates and ruminal digesta passage rates which results in higher CH_4 emission rates (CH_4/d) and milk yields (Boadi et al. 2004).

CH_4 yield (CH_4/DMI), by contrast, decreases in a HC feeding regimen which is mainly due to the higher particulate passage rate, more efficient microbial protein synthesis per unit ingested feed, a reduced DM digestibility and the greater [H]-consuming propionate production from NFC degradation directly competing with methanogenesis for [H] as a substrate (Moe und Tyrrell 1979, Knapp et al. 2014). High particulate passage rates reduce the time being available for microbial

feed particle adhesion and degradation resulting in a reduced fermentation capacity for structural carbohydrates and decreased amounts of fermentation intermediates of [H] acting as a substrate for methanogenic Archaea (Figure 2) (Martin et al. 2010, Knapp et al. 2014, Haque 2018). Increased passage rates may contribute to lowering those CH_4 emissions which particularly originate from rumen fermentation. In consequence of feed carbohydrates that bypass intra-ruminal digestion due to the higher particulate passage rate, some carbohydrate degradation may be translocated towards the small (starch, sugar) and large (NDF, glucans, pectin) intestines and may further take place during manure storage when undigested feed is excreted via feces (Hristov et al. 2013b, Knapp et al. 2014). Both nutrient degradation within the whole digestive system and during on-farm manure storage contribute to the dairy sector´s CH_4 emissions and should be considered when evaluating the CH_4 reduction potentials of feeding HC diets.

Feeding cows with HC diets can be associated with an enhanced conversion of NFC fractions to predominantly propionate which is the most important precursor of gluconeogenesis being primarily utilized for production and immune functions. The desirable higher energy budget of the cow becomes specifically more important during the energy-deficient periparturient period (Aschenbach et al. 2010). High energy-dense diets with increased proportion of digestible nutrients enhance the efficiency of the direct use of nutrients for milk production which reduces the GEI to NEL ratio and CH_4/ECM (Martin et al. 2010, Haque 2018). Correspondingly, the proportion of CH_4 produced from energy intake for maintenance requirements on total CH_4 production from GEI decreases with increasing milk yield and, therefore, CH4/ECM decreases in a curvilinear manner with animal productivity (Beauchemin et al. 2020). Hence, in highly efficient dairy cows with milk yield above 5,000 kg milk per cow per year, as being typical in high-income countries, almost all CH4 emissions arise from feed energy use for productive functions, whereby the proportion of CH4 produced from energy intake for maintenance becomes marginal. As a result, in high-developed dairy systems, further gains in milk production efficiency will result in only minor additional decreases in CH_4 emission intensity (<1%/year) (Beauchemin et al. 2020). Though, higher feed efficiency is always associated with reduced CH_4 emissions intensity.

2.3.2.2. Rumen pH and rumen fermentation pattern influence methanogenesis

One of the most important anti-methanogenic effects of feeding starch-rich diets encompass the reduction in rumen pH when starch is fermented to propionate which hampers the growth of pH-sensitive cellulolytic bacteria, methanogenic Archaea (van Kessel und Russell 1996) and

methanogen-harboring rumen ciliate protozoa (Newbold et al. 2015). As a consequence, the interspecies H_2 transfer between protozoa and methanogens and the overall NDF digestibility may be reduced resulting in decreased ruminal methanogenesis (Newbold et al. 1995).

The extent of CH_4 formation corresponds, among others, to the rumen VFA profile being predominantly reflective of the fermentation pattern and rumen hydrogen balance which result from the dietary NFC to NDF ratio favoring either [H]-generating or [H]-consuming fermentation pathways (Figure 2) (Moe und Tyrrell 1979, Bannink et al. 2006, Knapp et al. 2014). The degradation of the increased NFC fraction in HC diets to propionate and valerate indirectly reduces CH_4 synthesis by redirecting fermentation intermediates of reducing equivalents to alternative [H] removing fermentation pathways (Figure 2) (Ungerfeld 2015). The propionic-metabolic typed pathway is considered to be the second most important [H] sink apart from methanogenesis (Newbold et al. 2005). In contrast, forage-based rations with increased dietary NDF content favor [H]-generating fermentation pathways resulting in microbial end-products of CO_2, acetate and 4 mole H_2 per mole hexose which lead to enhanced CH_4 synthesis (Moss et al. 2000). Sugars are more methanogenic than starch (Haque 2018) which may cause an increased butyrate synthesis and fiber digestibility stimulated by the sugar fraction (Hindrichsen et al. 2005, Knapp et al. 2014). In summary, if animal productivity for milk or meat increases or remains unchanged, feeding NFC-rich diets that shift fermentation processes in favor of alternative [H] sinks will lower both CH_4 yield and CH_4 emissions intensity (Yan et al. 2000, Yan et al. 2010, Hristov et al. 2013a).

2.3.3. Grass silage-to-maize silage ratio in the forage proportion of the ration

Gass and maize silages are among the most important forages in EU dairy feeding systems (and also provided to cows in the present study). The quantitative reduction in CH_4 yield depends further on the forage quality, i.e. the ratio between cellulose and hemicellulose as well as NDF and NFC in the dietary forage fraction. In this light, Moe und Tyrrell (1979) estimated that CH_4 produced from hemicellulose amounts to only 37% when compared to that generated from cellulose. A complete replacement of grass (Hart et al. 2015), alfalfa (Hassanat et al. 2013) or barley (Benchaar et al. 2014) silage with starch-containing maize silage can reduce CH_4 yield by 9% to 14% (Moe und Tyrrell 1979, Mills et al. 2001, Hart et al. 2015). However, the scope for CH_4 inhibition depending on the forage type and quality provided in the diet should be further examined since studies addressing this issue are scarce (Beauchemin et al. 2008). In principle, grain silages include higher starch contents when compared to grass silages which could reduce ruminal pH and

stimulate growth of propionate-enhancing microorganisms and alternative [H] utilization. Furthermore, maize silages contain up to 30% rumen bypass starch (Ørskov 1986) and have a smaller feed particle length when compared with grass silages (Beauchemin et al. 2008). This could result in decreased CH_4 yields due to the reduced retention time of the ingesta within the rumen, while DMI and the energetically more efficient post-ruminal digestion increase (Beauchemin et al. 2008). This could improve milk production efficiency thereby lowering CH_4 emission intensities. Hart et al. (2015) varied the composition of *ad libitum* offered forages with regard to the grass silage-to-maize silage ratio (70:30 and 30:70 DM basis) and added concentrate feed that was either high in starch or fiber to investigate potential effects on CH_4 production. Interestingly, maize silage-based diets significantly reduced CH_4 emissions only when expressed relative to DMI (-9%; 1.7 g CH_4/kg DMI) when compared to the grass silage-based diet which was additionally independent from concentrate type. Total CH_4 production remained unaffected by grass silage-to-maize silage ratio. However, land-use changes of permanent grasslands could offset the CH_4 reduction potential arising from the replacement of grass silages with that of maize (Vellinga und Hoving 2011), even though Rotz et al. (2010) summed up that in the United States 5 kg CO_2 are less produced per tonne DM of maize when compared to grass silage which can be related to higher needs of production factors related to grasslands.

2.3.4. 3-nitrooxypropanol

2.3.4.1. Molecular structure, mode of action, metabolization and toxicity of 3-nitrooxypropanol

The CH_4 inhibitor 3-NOP (DSM Nutritional Products AG, Kaiseraugst, Switzerland) is a novel feed ingredient commercially available under the name BOVAER®. Depending on the manufacturer specification, the powdered compound is typically provided as a mixture of approximately 10% 3-NOP, 54% silica, and 36% propylene glycol. Initially, Ogawa et al. (1990) conducted chemoselective reduction of nitrooxyalkanoates with calcium borohydride to give 3-NOP for its use in the preparation of 1,4-dihydropyridine derivatives possessing nitrooxy groups which were regarded as potential calcium channel antagonists and antihypertensives. Later on, 3D pharmacophore-based virtual screening and molecular docking studies were conducted *in silico* to explore nitrooxy-compounds as potential CH_4 inhibitors for which 3-NOP was found to be the most promising candidate (Duin et al. 2016).

3-NOP is the mononitrate ester of 1,3-propanediol and a structural analogue of CoM with two functional groups, a primary alcohol and organic nitrate ester group (molecular shape of 3-NOP in

Figure 3) (Thiel et al. 2019b). Duin et al. (2016) outlined that the small molecule 3-NOP targets the active site of MCR by molecular docking (Figure 3) and positioning the reducible nitrate-ester group in electron transfer distance to Ni(I) of F_{430}. 3-NOP inactivates MCR by oxidation of its Ni(I) to Ni(II) (Duin et al. 2016, Thauer 2019). Alongside the aforementioned MCR inactivation, a further mode of action of 3-NOP should be mentioned: the nitrate ester is reduced by the Ni(I) in the active MCR to 1,3-propanediol, nitrate and nitrite. The nitrite then oxidizes the Ni(I) once again which results in an additional MCR inactivation, albeit at micromolar concentrations (Duin et al. 2016). Thus, there is a dual mode of action of 3-NOP related to MCR inactivation.

The stability of 3-NOP within the rumen was assessed by Duin et al. (2016) who incubated ^{14}C-labeled 3-NOP in fresh cow rumen fluid. They observed 3-NOP to be almost completely metabolized by rumen bacteria to ^{14}C-labeled products of 1,3-propanediol (a metabolite commonly present in the rumen) after 24 h, whereas a significant enrichment of the other formed products (nitrate, nitrite) was not detected. In methanogenic cultures, it was found that inactivated MCR can be reactivated in an ATP-dependent reduction process (Duin et al. 2016, Thauer 2019) and Duin et al. (2016) concluded that the CH_4 inhibition by 3-NOP is reversible *in vivo*. The uncharged 3-NOP permeates into the cells of methanogens by free diffusion through their cytoplasmic membrane independent from the presence of active membrane-associated carrier systems for CoM transportation which may prevent microbial adaptation to 3-NOP (Thauer 2019). 3-NOP can be absorbed across the rumen wall into the bloodstream, distributed in the animal´s organism and a subsequent denitration may occur in hepatic tissues (Govoni et al. 2013). However, 3-NOP and its potential metabolites of 1,3-propanediol, nitrite and nitrate are suggested to be nontoxic to the animals which could be particularly related to the low inclusion levels of 3-NOP dosed into ruminant diets (Scott et al. 2005). Correspondingly, marginal or virtual residues in milk or meat were reported (Thiel et al. 2019a). 3-NOP and 1,3-propanediol can be quickly oxidized in the circulation to the plasma metabolite of NOPA which is subsequently hydrolyzed yielding HPA and inorganic nitrate (Thiel et al. 2019a). Both NOPA and HPA are naturally occurring metabolites in plasma and mammalian cells resulting from degradation of e.g. AA (Thiel et al. 2019a, Thiel et al. 2019b). HPA can be either metabolized into acetyl-CoA or propanoyl-CoA and CO_2 (Thiel et al. 2019b). The propanoyl-CoA can serve as a substrate for gluconeogenesis being specifically important in transitioning dairy cows, albeit in negligible quantities (Thiel et al. 2019a). Thiel et al. (2019a) reported that lactose is the primary product of 3-NOP degradation processes via HPA in the milk which was tested in lactating goats orally administered with 4.34 mg of 3-NOP/kg BW

(equivalent to 111.7 mg of 3-NOP/kg feed DM). Due to its water-solubility and rapid metabolization, an accumulation of 3-NOP and NOPA residues in milk and edible tissues is not assumed (Thiel et al. 2019a). Plasma kinetic profiles of 3-NOP and its metabolites at different time points were reported in the Thiel et al. (2019b) study. Significant plasma concentrations of 3-NOP were detected in rats treated with very high oral doses of 800 mg 3-NOP per kg BW and d over a 10-days dosing period, whereby differences between single or multiple dosing were not observed (Thiel et al. 2019b). Thiel et al. (2019b) noted that 3-NOP plasma concentration peaked 5 to 15 minutes after dosing followed by a rapid decline and sequential peaks of the 3-NOP metabolites NOPA and HPA 1 h and 2 h after 3-NOP dosing, respectively. Rapid plasma elimination of 3-NOP and its metabolites were suggested as 3-NOP was not detectable 1 h after dosing and the same held true for NOPA and HPA not being detectable 24 h after dosing.

Thiel et al. (2019b) conducted mutagenicity and genotoxicity screening studies and follow-up regulatory compliant experiments of 3-NOP and its metabolites. 3-NOP and its metabolites were stated to have neither genotoxic nor mutagenic potential which was examined in *in silico* predictions for mutagenicity, bacterial reverse mutation (Ames) tests, mouse lymphoma assays, *in vitro* micronucleus tests, and the oral *in vivo* micronucleus tests using rat bone marrow (Thiel et al. 2019b). Furthermore, 3-NOP is suggested to be neither clastogenic nor aneugenic in human lymphocytes (Thiel et al. 2019b).

2.3.4.2. Effectiveness of 3-nitrooxypropanol on CH_4 inhibition - influential factors

In a meta-analysis, Dijkstra et al. (2018) revealed that 3-NOP efficacy depends on the applied dosage level, administration technique (directly dosed into the rumen, mixed in with TMR synchronously to meal event or single dose), animal type (beef or dairy cattle) and diet composition (NDF content). Forest plot analyses across 11 experiments and 38 treatment means indicated greater 3-NOP reduction potential in dairy than in beef cattle (-38.8 ± 5.49% CH_4 yield and -39.0 ± 5.40% CH_4 production for dairy and -17.1 ± 4.23% CH_4 yield and -22.2 ± 3.33% CH_4 production for beef cattle) at a mean 3-NOP dose of 123 mg/kg of DM and mean NDF content of 331 g/kg of DM after adjustment for the effects of 3-NOP dose and NDF content in the diet (Dijkstra et al. 2018). 3-NOP dose notably differed between dairy (mean 81 ± 41.2 mg/kg of DM; range of 27 – 135 mg/kg of DM) and beef (mean 144 ± 82.3 mg/kg of DM; range of 50 – 345 mg/kg of DM) cattle studies (Dijkstra et al. 2018). However, all things considered, large variation in response to 3-NOP effect size has been reported ranging from a CH_4 decrease of 7% (Reynolds et al. 2014) up

to 60% (Haisan et al. 2014) in dairy cattle, whereas a maximum CH_4 decline of 87% was observed in beef cattle provided a finishing diet with 200 mg 3-NOP/kg feed DM (Vyas et al. 2016).

Significant dose-response relationships of 3-NOP were evidenced. Under *in vitro* conditions, greater 3-NOP effectiveness was consistently reported when compared to *in vivo* studies. However, the dosage levels were notably higher *in vitro* than *in vivo*. In this regard, Romero-Pérez et al. (2015a) observed that CH_4 decreased in a quadratic manner by 76.0%, 84.5%, and 85.6% without compromising DM disappearance when incubating 500, 1,000, and 2,000 mg of 3-NOP/kg of DM with a high-forage diet in a RUSITEC. From a meta-analysis using ten 3-NOP studies (dairy and beef cattle), Jayanegara et al. (2018) revealed that CH_4 yield linearly decreases with increasing 3-NOP doses ranging between 0 and 280 mg 3-NOP/kg of feed DM. The authors calculated a decrease in CH_4 yield by 19.2% when feeding 100 mg of 3-NOP per kg feed DM, whereas supplementing 200 mg 3-NOP/kg feed DM would theoretically result in a 33.4 – 42.1%-decrease in CH_4 emissions (Jayanegara et al. 2018). From the meta-analytical approach conducted by Dijkstra et al. (2018), 3-NOP dose dependency was evidenced as CH_4 yield decreased by 2.48 ± 0.73% per 10 mg/kg DM increase in 3-NOP dose from its mean (123 mg/kg of DM) after adjusting for NDF content and cattle type. Recently, Melgar et al. (2020b) dosed 40, 60, 80, 100, 150, and 200 mg of 3-NOP/kg of feed DM into a TMR of lactating dairy cows. The authors revealed that CH_4 emissions quadratically decreased from 22 to 40% with increasing 3-NOP dose, whereby a maximum 3-NOP effect size but no statistical difference was observed among 3-NOP doses of 100, 150 and 200 mg/kg feed DM (Melgar et al. 2020b). Overall, CH_4 reductions of about 25 – 35% can be expected when supplementing the 3-NOP dose of 60 mg 3-NOP/kg feed DM into the TMR of dairy cows as recommended by manufacturer´s specifications (Hristov et al. 2015, Melgar et al. 2020a, Melgar et al. 2020b). Interestingly, dietary NDF content adversely affected the inhibitory potential of 3-NOP to the extent of a 1.52 ± 0.41% increase in CH_4 yield per 10 g/kg DM increase in NDF content from its mean (331 g NDF/kg of DM) (Dijkstra et al. 2018).

Administration technique of 3-NOP was shown to influence 3-NOP effect size and persistency. It is recommended to deliver 3-NOP synchronously to meal event by mixing in the compound with the TMR, since 3-NOP effects were reported to be transient or non-significant when the substance was delivered as a single dose (‘pulse-dose’) (Reynolds et al. 2014) or infused directly into the rumen in cannulated cattle (Kim et al. 2019). Incorporating 3-NOP into concentrate feed pellets was evidenced to have no adverse effects on 3-NOP effectiveness (Van Wesemael et al. 2019). Vyas et al. (2016) observed that CH_4 reduction efficacy decreased 16 h after feeding 100 mg 3-

NOP/kg feed DM in beef cattle, whereas sustained 3-NOP effects were noted over a 24 h time period when feeding 200 mg 3-NOP/kg of feed DM. Romero-Pérez et al. (2014) stated that the maximum CH_4 inhibiting effect of 3-NOP can be expected within two hours after feeding which can be related to the rapid elution of the highly water-soluble 3-NOP substance out of the rumen. Furthermore, Romero-Pérez et al. (2015b) detected that effects of 3-NOP are reversible since CH_4 emissions recovered within one week to their initial level before 3-NOP administration.

2.3.4.3. Effects of 3-nitrooxypropanol on dry matter intake and digestibility

It appears that 3-NOP does not affect DMI in dairy cows. However, in a meta-analysis, Kim et al. (2020) calculated a tendency for a reduced DMI in beef cattle with increasing 3-NOP dose levels. In this context, the higher starch levels in the beef cattle diets and the use of 3-NOP are known to result in increased ruminal propionate synthesis and absorption. Enhanced propionate could have stimulated hepatic acetyl CoA oxidation which was previously postulated to cause hypophagic effects in feed regulation via stimulation of afferents in the vagus nerve in ruminants (Allen 2000, Allen et al. 2009). Changes in the organoleptic properties of the diet and feeding behavior in beef cattle due to 3-NOP inclusion have not been reported (Kim et al. 2019).

In previous experiments, inconsistent 3-NOP effects on nutrient disappearance were reported. 3-NOP was observed to have no (Melgar et al. 2020a), minor (Romero-Pérez et al. 2014, Jayanegara et al. 2018), negative (Reynolds et al. 2014) or positive (Hristov et al. 2015, Haisan et al. 2017, van Gastelen et al. 2020) effects on apparent total-tract digestibility of DM, OM, CP and fiber content. Zhang et al. (2020) recently addressed the question whether a potential 3-NOP induced H_2 accumulation in the rumen may depress NDF digestibility. The authors substantiated that *in situ* NDF digestibility remained unaffected in 3-NOP fed cannulated beef heifers. In conclusion, it can be summarized that 3-NOP is likely to have no adverse effects on total-tract nutrient digestibility, yet the data on intra-ruminal nutrient degradability related to 3-NOP supplementation are scarce.

2.3.4.4. Rumen fermentation and microbial community structure affected by 3-nitrooxypropanol

Several studies reported either reduced (Reynolds et al. 2014, Melgar et al. 2020a) or unchanged (Haisan et al. 2014, Lopes et al. 2016, Haisan et al. 2017) total VFA concentrations in rumen fluid associated with 3-NOP supplementation in dairy cows. In this regard, it was supposed that elevated pH values in the fermenter fluid detected in a RUSITEC (Guyader et al. 2017) and in 3-NOP fed cattle coincided with the observed reduced total VFA concentration (Jayanegara et al. 2018, Melgar et al. 2020a, Zhang et al. 2021). Previous studies identified that 3-NOP induced a shift from [H]-

generating to [H]-consuming fermentation pathways for alternative [H] disposal (Jayanegara et al. 2018). The thermodynamically controlled pathway selection (Figure 2) was suggested to be driven by the consistently observed increase in dissolved and gaseous H_2 when feeding 3-NOP which could have increased the H_2 partial pressure in the rumen (Hristov et al. 2015, Melgar et al. 2020a, van Gastelen et al. 2020). This may have caused negative feedback mechanisms on the re-oxidation of reduced cofactors (e.g., $NADH_2$) thereby inhibiting growth of rumen microbes (Leng 2014). In detail, the acetate-to-propionate ratio decreased as molar proportions of acetate were reduced whilst that of butyrate and alternative [H] sinks, namely glucogenic propionate and glucogenic/ketogenic valerate, substantially increased under 3-NOP feeding (Jayanegara et al. 2018, Kim et al. 2020). Melgar et al. (2020a) found increased butyrate proportions in 3-NOP fed lactating cows which may coincide to both, the aforementioned increased ruminal pH and the shift in fermentation pattern to alternative [H] sinks. In this regard, the passive diffusion of 1 mole of undissociated butyrate is associated with a concomitant uptake of 1 mole of proton into ruminal epithelial cells and represents the major route of butyrate absorption across the rumen wall (Penner et al. 2009). In addition, stoichiometric equations indicated that reduced quantities of protons and [H] are released during butyrate formation when compared to acetate synthesis (Owens und Goetsch 1988) (Figure 2). Hence, butyrate formation could be preferred over that of acetate in 3-NOP fed cows to prevent excessive [H] accumulation in the rumen. In addition, ruminal interconversion of acetate into butyrate and propionate consumes [H] (Figure 2) which could further explain the previously observed increased molar proportions of the two latter VFA in 3-NOP experiments.

In contrast to findings from *in vivo* experiments, previous *in vitro* experiments (RUSITEC) also observed decreasing acetate-to-propionate ratios due to decreasing molar acetate proportions, albeit molar propionate proportion, total VFA concentration and pH in fermenter fluid remained almost unaffected by 3-NOP inclusion (Romero-Pérez et al. 2015a, Guyader et al. 2017, Romero-Pérez et al. 2017). Interestingly, Guyader et al. (2017) dosed 5 mg of the active 3-NOP substance into RUSITEC fermenters and measured increased concentrations of further alternative [H] sinks, namely formate, heptanoate, caproate, ethanol, n-propanol and ammonium. Increased concentrations of formate, caproate and ethanol were confirmed in 3-NOP fed dairy cows (Reynolds et al. 2014, Melgar et al. 2020a) and beef cattle (Zhang et al. 2021). When calculating a rumen hydrogen balance, it was suggested that all of the aforementioned [H] sinks, the gaseous and dissolved H_2 and the residual CH_4 formation amounted to a proportion of only 53% (Romero-Pérez et al. 2015a) and, respectively, 57% (Melgar et al. 2020a) of the total hydrogen not being

metabolized under 3-NOP methanogenesis inhibition. Thus, further [H]-consuming metabolic pathways may have been upregulated under 3-NOP feeding, such as ruminal biohydrogenation as noted by Zhang et al. (2021) in 3-NOP fed beef cattle, and the formation of lactate and succinate, reductive acetogenesis, and microbial biomass synthesis (Ungerfeld 2015) which, however, has to be examined in future 3-NOP experiments.

Previous results mostly indicated that NH_3-N concentration in ruminal fluid was not affected by 3-NOP (Kim et al. 2019, Melgar et al. 2020a, Zhang et al. 2021), although Guyader et al. (2017) reported increased ammonium concentrations in RUSITEC fermenters. Only a few studies reported decreased NH_3-N concentrations with 3-NOP feeding which was speculated to result from either reduced proteolysis or increased microbial NH_3-N uptake under [H] consumption (Reynolds et al. 2014, Lopes et al. 2016). 3-NOP feeding was consistently reported to increase *iso*-valerate concentrations in rumen fluid *in vitro* (Romero-Pérez et al. 2015a) and *in vivo* (Romero-Pérez et al. 2015b, Lopes et al. 2016, Haisan et al. 2017) which can be related to an increased deamination of leucine commonly known to result in NH_3, CO_2 and *iso*-valerate. Contrastingly, changes in *iso*-butyrate concentrations, resulting from deamination of valine, were not observed in previous 3-NOP experiments (Romero-Pérez et al. 2015b, Romero-Pérez et al. 2015a, Haisan et al. 2017). However, data on intra-ruminal protein turnover and nitrogen incorporation into microbial biomass from *in vivo* 3-NOP experiments are lacking. Guyader et al. (2017) found that neither total microbial nitrogen production nor efficiency of microbial protein synthesis were changed by incubating 3-NOP and a 60%-forage diet substrate in RUSITEC fermenters.

From the extensively evidenced alterations in rumen VFA profile, it can be speculated that the ruminal microbial community adopted to a 3-NOP supplementation. Effects of 3-NOP on protozoa, methanogens and bacteria are presented inconsistently in literature though. Most of the previous literature reported no effects of 3-NOP on total protozoa counts *in vivo* (Haisan et al. 2014, Haisan et al. 2017, Melgar et al. 2020a, Kim et al. 2020, Zhang et al. 2021) and *in vitro* (Romero-Pérez et al. 2015a) which is consistent to the abovementioned unchanged ammonia concentrations when feeding 3-NOP because protozoa are supposed to increase N cycling and ammonia concentration in the rumen (Leng und Nolan 1984). On the contrary, Romero-Pérez et al. (2015b) observed an increased copy number of the rumen protozoal 18S rRNA gene while the copy number of the 16S rRNA gene of methanogens was decreased in 3-NOP supplied beef cattle fed a diet containing 60% barley silage. Hence, both microbial groups did not grow in a uniform manner which underlines the high specificity of 3-NOP towards methanogenic Archaea, yet can be seen as an unexpected

result since rumen methanogens coexist in a symbiotic relationship with protozoa due to the interspecies H_2 transfer (Morgavi et al. 2010) accounting for 9 to 25% of total rumen CH_4 production (Newbold et al. 1995). In addition, it can be hypothesized that protozoal counts decrease under 3-NOP feeding, since protozoa are H_2 producers being potentially disadvantageous when it comes to negative side-effects of intra-ruminal [H] accumulation on rumen fermentation.

Total bacterial 16S rDNA gene copy numbers have been numerously reported to be unaffected by 3-NOP in a RUSITEC (Romero-Pérez et al. 2015a), as well as in sheep (Martínez-Fernández et al. 2014), beef (Romero-Pérez et al. 2015b, Zhang et al. 2020) and dairy (Haisan et al. 2014, Haisan et al. 2017) cattle. By contrast, Lopes et al. (2016) detected a reduced relative abundance of the major bacterial taxa of acetate producing fiber-degrading *Ruminococcus spp.* and an increase of butyrate and propionate producing *Butyrivibrio spp.* and *Selenomonadales*, respectively, which corresponds to the observed 3-NOP induced changes in the VFA profile. However, inconsistent results among 3-NOP studies on the rumen bacterial community may potentially result from the high sensitivity of microbes in response to changes in diet composition and rumen environmental conditions (van Kessel und Russell 1996, Kumar et al. 2015).

In view of its mode of action, 3-NOP specifically targets the enzyme MCR which is unique to methanogenic Archaea. Therefore, effects of 3-NOP may be expected in the metabolic activity and growth of rumen methanogenic Archaea, rather than other rumen microbial consortia. Since methanogens generate their energy from methanogenesis, 3-NOP supplementation may cause energy deprivation on these microbes (Jayanegara et al. 2018). Indeed, it appears that 3-NOP decreases the number of methanogens which was shown in the RUSITEC (Romero-Pérez et al. 2015a, 2017), in beef cattle (Romero-Pérez et al. 2015b, Martínez-Fernández et al. 2018, Zhang et al. 2020) and in 12 lactating dairy cows fed with 2,500 mg of 3-NOP/d mixed into a diet containing 38% forage (Haisan et al. 2014). Lopes et al. (2016) also reported a decreased proportion of methanogens in the total cell counts, whereby genus composition of methanogenic Archaea (*Methanobrevibacter*, *Methanosphaera*, and *Methanomicrobium*) remained uninfluenced in lactating cows fed with 60 mg of 3-NOP/kg DM. Notwithstanding this, Zhang et al. (2020) observed changes in the community structures of methanogens, since the relative abundance of the genus *Methanobrevibacter* decreased when barley silage was incubated *in situ* in 3-NOP fed cattle. Likewise, Martínez-Fernández et al. (2018) observed that feeding 2,500 mg 3-NOP per d to four rumen-cannulated steers decreased the abundance of hydrogenotrophic methanogens *Methanobrevibacter spp.* to a greater extent when compared to that of the methylotrophic

Methanomassiliicoccaceae family. Correspondingly, Pitta et al. (2021) observed that methanogens responded in a different manner to the supplementation of 60 mg 3-NOP/kg of feed DM in dairy cows and diurnal patterns among the individual methanogenic lineages were observed. In this regard, *Methanobrevibacter* was reduced at 2 h after feeding and week 4 after the beginning of the trial, whereas *Methanosphaera* was reduced at 6 and 10 h after feeding and week 8 and 12 after the beginning of the trial (Pitta et al. 2021). The authors further substantiated that 3-NOP reduced the abundance of *Methanobrevibacter ruminantium* in all 3-NOP samples. The abundance of the individual methanogens was driven by a combination of diet composition, DMI with synchronous 3-NOP uptake, intra-ruminal H_2 concentration, and likely structural and functional differences in genes encoding the MCR enzyme (Pitta et al. 2021). Thus, it seems that 3-NOP affects specific genera of rumen methanogenic Archaea in a different manner which was evidenced by the dose-dependent response in 3-NOP sensitivity differing among methanogenic species (Duin et al. 2016). Especially, methanogens that are dependent on external CoM (e.g. *Methanobrevibacter ruminantium*) are more sensitive to methyl CoM analogs compared to Archaea which can synthesize their own CoM. Duin et al. (2016) reported that 3-NOP dosage level required to inhibit different archaeal species ranged between 0.25 to >10 μ*M*, whereby the smallest dose of 0.25 μ*M* 3-NOP was sufficient to inhibit *Methanobrevibacter ruminantium*. Furthermore, decreases in the abundance of methanogens may possibly only occur at a certain extent of CH_4 reduction which could explain discrepancies among studies dealing with 3-NOP effects on methanogens. In this regard, Romero-Pérez et al. (2015b) and Zhang et al. (2020) observed that notably high CH_4 reductions of 53% and 59.2% corresponded to a 37% and 56.6% decrease in the relative abundance of *Euryarchaeota*.

2.4. Effects of CH_4 inhibition on the energy supply of cows with special focus on 3-NOP

Transition cows are challenged with complex metabolic, physiological, immunological and hormonal changes paralleled by the divergence between increasing energy demands but reduced energy intake causing a negative EB, a disproportional energy metabolism (excessive body fat mobilization, fatty liver, ketosis) as well as higher risks for impaired immune response and infectious diseases (mastitis, metritis) (Herdt 2000, Esposito et al. 2014).

The theoretically reduced feed energy conversion losses (Johnson und Johnson 1995) and increased production of [H]-consuming glucogenic VFA (Jayanegara et al. 2018) in 3-NOP fed

methanogenesis-inhibited cows may provide an extra energy supply to the cow which becomes specifically important for metabolic and productive purposes during the transitioning and early-lactation period. Thus, an improved energy availability may counterbalance the negative EB and reduce the excessive adipose tissue mobilization which could otherwise lead to an accumulation of NEFA and ketone bodies in the circulation and a higher risk for ketosis (Herdt 2000).

However, Ungerfeld (2018) noted that inhibiting methanogenesis can result in improved productive performance only if the shifts in fermentation pathways and the H_2 spared from methanogenesis can be energetically utilizable in the cow´s metabolism. It seems that significant parts of the total energy contingent potentially spared from feed conversion due to CH_4 inhibition are simply lost by increased H_2 gas emission in 3-NOP fed cattle (Hristov et al. 2015, van Gastelen et al. 2020) or probably dissipated in an accumulation of energetically not utilizable [H] sinks (e.g. formate). Further proportions of the theoretical energy surplus and their corresponding metabolic pathways of energy utilization remain unaccounted (Ungerfeld 2018, Yanibada et al. 2020). No studies have been published so far that verify flows of altered VFA production rates induced by CH_4 inhibition into metabolic pathways resulting in enhanced net energy allocation toward milk production or accretion of body reserves (Ungerfeld 2018). Moreover, it can be simply speculated that distinctly greater CH_4 reduction effects are needed to discover potential energy gains or that the additional ME spared from methanogenesis is utilized inefficiently. Furthermore, in previous CH_4 inhibition experiments, the feeding regimen were mostly designed to comply with the nutrient requirements of the cows. However, it may be possible that potential effects on productivity gains emerging from the extra supply of glucogenic precursors due to CH_4 inhibition can be revealed only if CH_4 inhibitors are combined with hypocaloric diets (Ungerfeld 2018). It can be illusive to assign the extra energy supply from CH_4 inhibition to specific performance parameters, metabolic pathways and immune responses. Studies dealing with CH_4 inhibition in conjunction with collectively reported net energy partitioning and losses in feces, urine and HP are scarce (Ungerfeld 2018). From a meta-analysis including 44 studies in which CH_4 inhibitors were applied, Ungerfeld (2018) reported no consistent effects on an improved animal productivity exclusively resulting from inhibited CH_4 production. In particular, productivity gains can appear in a number of ways depending on the prevailing and yet complex physiological situation (e.g., increased replenishment of body reserves in late lactation, milk production depending on hormonal prioritization in different lactation states, reduced adipose tissue mobilization, effects of the fetus during gestation).

Experiments investigating potential impacts of 3-NOP on the energy budget of dairy cows particularly during the transition phase are lacking. Previous studies mainly reported that 3-NOP affected neither feed efficiency, ECM, FCM and milk yield (Hristov et al. 2015, Lopes et al. 2016, Haisan et al. 2017, Van Wesemael et al. 2019, Melgar et al. 2020a) nor milk contents of protein, fat, lactose (Hristov et al. 2015, Haisan et al. 2017, Van Wesemael et al. 2019, Melgar et al. 2020a) and urea in early- and mid-lactating cows, whereby changes were found in the MFA profile (Hristov et al. 2015, van Gastelen et al. 2020, Melgar et al. 2020a). However, it is noteworthy that 3-NOP effects on an increased milk protein (van Gastelen et al. 2020) and milk fat content (Lopes et al. 2016, van Gastelen et al. 2020) occasionally occurred in 3-NOP fed cows. Interestingly, BWG was significantly positively associated with CH_4 reduction as being demonstrated in the Ungerfeld (2018) meta-analysis, whereby 3-NOP has been inconsistently reported to influence BWG. It is noteworthy that Haisan et al. (2014) and Hristov et al. (2015) observed BWG to be increased by 63% and, respectively, 80% in mid-lactating Holstein cows a time at which milk production is not energetically prioritized. Herein, the cows were treated with 3-NOP doses of 2,500 (Haisan et al. 2014) and 40, 60 and 80 (Hristov et al. 2015) mg per kg feed DM resulting in methanogenesis inhibition by 60% as well as 25%, 31%, and 32%, respectively. Later on, van Gastelen et al. (2020) noted greater BWG in 3-NOP fed cows during the early-lactation period. By contrast, BWG remained unaffected in most of the 3-NOP studies with dairy cows (Haisan et al. 2017, Melgar et al. 2020a). Besides, BW was also not affected in 3-NOP fed beef cattle (Romero-Pérez et al. 2015b, Vyas et al. 2016, Kim et al. 2019). Both the energy balance and the resumption of ovarian cyclicity were not impaired by 3-NOP in early-lactation cows (Melgar et al. 2020a). Despite of the tendency of an increased plasma glucose (Haisan et al. 2017) and reduced insulin concentration (Melgar et al. 2020a), adding 3-NOP into the ration of early- and mid-lactating cows did not alter further plasma metabolite concentrations of NEFA and BHB being reflective of the cow´s energy balance and body fat mobilization (Haisan et al. 2017, Melgar et al. 2020a). Furthermore, no 3-NOP effects were found with regard to blood cell counts in cows during the early lactation phase (Melgar et al. 2020a).

3. Scope of the thesis

As presented in the background, there is an urgent need to lower climate-relevant enteric CH_4 emissions from dairy cows around the world. The newly developed and direct-acting CH_4 inhibitor 3-NOP appears to be a promising breakthrough in mitigating rumen methanogenesis. However, it seems that the effectiveness of 3-NOP in lowering CH_4 production depends on dose-response relationships and diet composition. Furthermore, 3-NOP has been reported to shift rumen fermentation to propionic-metabolic typed pathways, while H_2 emissions seem to be increased. Feeding high-concentrate diets represents an indirect CH_4 mitigation strategy by affecting several rumen physiological processes, e.g. enhancing the H_2-consuming propionate formation which competes with methanogenesis for H_2 utilization. Periparturient dairy cows are faced with a negative energy balance which may compromise animal performance and lead to metabolic disorders. Theoretically, the increased formation of glucogenic propionate and reduced feed energy losses under the 3-NOP induced CH_4 inhibition could provide an extra energy supply to the cow. There is a lack of knowledge whether both CH_4 mitigation options, feeding 3-NOP together with high-concentrate diets, intertwine as a combined CH_4 mitigation strategy and elicit synergistic effects on the methanogenesis inhibition as well as energy supply in dairy cows during the periparturient and early-lactation period.

Therefore, the present thesis aimed to test the following hypotheses:

I. The combination of feeding 3-NOP with high-concentrate diets reduces CH_4 emissions in an interactive manner and increases the energy supply spared from methanogenesis and increased propionate synthesis which is directed to an improved milk performance and a less negative energy balance (PAPER I).
II. The theoretical extra energy supply from increased glucogenic propionate formation and energy spared from methanogenesis when feeding 3-NOP in combination with high-concentrate diets is utilized to cope with the negative energy balance in periparturient dairy cows which is reflected by decreased lipomobilization from different adipose tissue depots and reduced serum concentrations of NEFA and BHB (PAPER II).
III. The use of the GreenFeed technology for indirect calorimetry is suitable to estimate the dietary effects of 3-NOP and CFP on mechanisms of the ruminal and energetic metabolic processes with special regard to energy retention in body tissues and energy partitioning towards the single energy expenditures (PAPER II).

IV. The efficacy of 3-NOP on CH_4 inhibition responds in a dose-dependent manner and incubating high-concentrate feed proportions causes additive effects on CH_4 reduction *in vitro* (RUSITEC) (PAPER III).

V. Variables of dietary NDF content, rumen VFA and milk fat content, which were observed to correlate with alterations in CH_4 emissions due to 3-NOP supplementation (PAPER I, II, III), can be used as proxies to accurately predict CH_4 emissions in 3-NOP fed cows since the regular quantitative relationship between DMI and CH_4 emission is uncoupled under 3-NOP feeding (GENERAL DISCUSSION).

The aforementioned hypotheses were tested using an animal model which comprised 55 pluriparous German Holstein cows being experimentally studied from d 28 *antepartum* until d 120 *postpartum*. The cows were grouped in a 2×2 factorial design by low or high concentrate feed proportion (CFP) tested without supplements or combined with 3-NOP (50 mg/kg feed DM) (DSM Nutritional Products AG, Kaiseraugst, Switzerland) which was supplied via concentrate feeds from both the partial mixed ration (70% maize silage, 20% grass silage, 10% concentrates on a DM basis) and additional concentrate feeds provided by automatic feeders. The combination of 3-NOP and varying concentrate-to-forage ratios was chosen to reveal potential interactive effects on CH_4 reduction and an improved energy supply. During the *antepartum* period, the cows received a high- (40%) or a low- (15%) CFP in the diet. From parturition until d 21 *postpartum*, the CFP gradually increased from 30 to 55% in the high-concentrate groups, while that of the low-concentrate groups was maintained at 30% from parturition until termination of the experiment. Large-scale measurements of CH_4 emissions and respiratory gas exchanges were carried out using the GreenFeed technology (C-Lock Inc., Rapid City, SD, USA). In vitro dose-response studies were carried out in a 4×2 factorial arrangement with three replications using the RUSITEC. Four doses of 3-NOP (0, 73, 160, and 1200 mg of the active 3-NOP substance/kg of feed DM) were tested with low (30%) or high (60%) CFP in the incubated ration to investigate potential effects on CH_4mitigation, volatile fatty acids as well as substrate disappearance. In the comprehensive discussion, a model development and assessment approach were carried out including the identification of key variables for predicting the CH_4 production with special emphasis on a 3-NOP induced CH_4 mitigation scenario.

4. Paper I

Schilde, M.; von Soosten, D.; Hüther, L.; Meyer, U.; Zeyner, A.; Dänicke, S.

Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows.

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Effects of 3-nitrooxypropanol and varying concentrate feed proportions in the ration on methane emission, rumen fermentation and performance of periparturient dairy cows

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Abstract

The climate-relevant enteric methane (CH_4) formation represents a loss of feed energy that is potentially meaningful for energetically undersupplied peripartal dairy cows. Higher concentrate feed proportions (CFP) are known to reduce CH_4 emissions in cows. The same applies to the feed additive 3-nitrooxypropanol (3-NOP), albeit through different mechanisms. It was hypothesised that the hydrogen not utilised for CH_4 formation through the inhibition by 3-NOP would be sequestered by propionate formation triggered by higher CFP so that it could thereby give rise to a synergistically reduced CH_4 emission. In a 2 × 2-factorial design, low (LC) or high (HC) CFP were either tested without supplements (CONLC, CONHC), or combined with 3-NOP (NOPLC, 48.4 mg/kg dry matter (DM); NOPHC, 51.2 mg 3-NOP/kg DM). These four rations were fed to a total of 55 Holstein cows from d 28 *ante partum* until d 120 *post partum*. DM intake (DMI) was not affected by 3-NOP but increased with CFP (CFP; $p < 0.001$). CH_4/DMI and CH_4/energy-corrected milk (ECM) were mitigated by 3-NOP (23% NOPLC, 33% NOPHC) ($p < 0.001$) and high CFP (12% CON, 22% 3-NOP groups) (CFP × TIME; $p < 0.001$). Under the conditions of the present experiment, the CH_4 emissions of NOPLC increased to the level of the CON groups from week 8 until the end of trial (3-NOP × CFP × TIME; $p < 0.01$). CO_2 yield decreased by 3-NOP and high CFP (3-NOP × CFP; $p < 0.001$). The reduced body weight loss and feed efficiency in HC groups paralleled a more positive energy balance being most obvious in NOPHC (3-NOP × CFP; $p < 0.001$). ECM was lower for NOPHC compared to CONHC (3-NOP × CFP; $p < 0.05$), whereas LC groups did not differ. A decreased fat to protein ratio was observed in HC groups and, until week 6 *post partum*, in NOPLC. Milk lactose and urea increased by 3-NOP (3-NOP; $p < 0.05$). 3-NOP and high CFP changed rumen fermentation to a more propionic-metabolic profile (3-NOP; CFP; $p < 0.01$) but did not affect rumen pH. In conclusion, CH_4 emission was synergistically reduced when high CFP was combined with 3-NOP while the CH_4 mitigating 3-NOP effect decreased with progressing time when the supplement was added to the high-forage ration. The nature of these interactions needs to be clarified.

Key words: 3-nitrooxypropanol; concentrate feed proportion; methane production; GreenFeed; milk production; energy balance; volatile fatty acids

1. Introduction

Methane (CH_4) is a relevant greenhouse gas with a global warming potential 28 times greater than that of carbon dioxide (CO_2) when a 100-year time horizon is assumed (Myhre et al. 2013). Enteric fermentation contributes 44% to anthropogenic CH_4 emissions (Gerber et al. 2013). In ruminants, CH_4 formation represents a major pathway for removal of hydrogen (H_2) but also accounts for a loss of 2 - 12% of gross energy (GE) intake (Johnson and Johnson 1995). Particularly transitional and early-lactating dairy cows are challenged by the energy-requiring onset of lactogenesis paralleled by a reduced energy intake which likely provokes a negative energy balance (EB) detrimentally affecting milk yield and body condition score (BCS) (Dänicke et al. 2018).

Increased concentrate feed proportion (CFP) in the ration was noted to reduce peripartal energy deficit and to decrease CH_4 yield (CH_4/dry matter intake (DMI)) and emission intensity (CH_4/energy-corrected milk (ECM)) by about 0.28 g and 0.17 g, respectively, for every percent increase of CFP (Aguerre et al. 2011). This is commonly attributed to the decreased dietary neutral-detergent fibre (NDF), to non-fibre carbohydrates ratio and corresponding fermentation pathways in favour of propionate synthesis. Propionate competes with methanogenesis for reducing equivalents (McAllister and Newbold 2008) and it is the main precursor for gluconeogenesis in the bovine (Aschenbach et al. 2010). Moreover, increasing CFP is often paralleled by decreasing ruminal pH values which in turn adversely affect methanogenesis due to pH sensitivity of methanogens and methanogen-associated protozoa (Van Kessel and Russell 1996).

Ogawa et al. (1990) synthesised the substance 3-nitrooxypropanol (3-NOP) by chemoselective reduction to improve the efficacy of calcium channel antagonists. Later it was discovered that 3-NOP, a mononitrate ester of 1,3-propanediol and structural analogue of methyl-coenzyme M (CoM), also specifically targets the active site of methyl-coenzyme M reductase (MCR) which catalyses the reduction of CoM and the release of methane during the last step of CH_4 formation in rumen archaea (Duin et al. 2016). For 3-NOP a substantial mean reduction of CH_4 emission by 39.0 ± 5.40% in dairy cattle supplemented at an averaged dose of 123 mg 3-NOP/kg of DM was proved in a recent meta-analysis (Dijkstra et al. 2018). Reportedly, the efficacy of 3-NOP was observed to be variable depending on application technique (Kim et al. 2019), dosage level (Haisan et al. 2017) and diet composition with enhanced inhibitory potential when cows are fed high-concentrate diets (Haisan et al. 2017; Dijkstra et al. 2018). Furthermore, 3-NOP was observed to shift the fermentation pattern towards alternative H_2 sinks, namely propionate (Jayanegara et al. 2018). Body condition and milk performance were reported to be inconsistent in response to potentially additional energy retrieval derived from increased propionate synthesis and CH_4 inhibition by 3-NOP (Hristov et al. 2015b; Kim et al. 2020).

The present study aimed to address missing long-term studies investigating the effects of 3-NOP in combination with varying CFP on CH_4 emissions, rumen fermentation, production, and energetic efficiency in dairy cows including and following the peripartal period. It was hypothesised that 3-NOP and high CFP in the ration (1) synergistically reduce CH_4 yield to a greater extent when

compared to high-forage diets and (2) increase energy recovery, spared from methanogenesis and increased propionate synthesis which is directed to improved efficiency, lactational performance and body tissue retention overall resulting in a less negative EB in peripartal dairy cows.

2. Materials and methods

The experiment was conducted at the experimental station of the Friedrich-Loeffler-Institut (FLI) in Brunswick, Germany in compliance with the German Animal Welfare Act and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany.

2.1. Experimental design and diets

In total, 58 pluriparous German Holstein cows, including 10 cannulated cows, were randomly assigned to four groups according to a 2 × 2 factorial design. Three cannulated cows were allocated to each 3-NOP group and two cannulated cows to each CON group. Low (LC) or high (HC) CFP were either tested without supplements (CONLC, CONHC) or combined with 3-NOP (NOPLC, NOPHC) in the ration. Both the 3-NOP and the placebo supplement (DSM Nutritional Products AG, Kaiseraugst, Switzerland) contained propylene glycol and SiO_2. These substances acted as carriers for 10% of 3-NOP which was only included in the 3-NOP supplement. A target dose of 60 mg 3-NOP/kg of dry matter (DM) was chosen based on dose-response experiments (Hristov et al. 2015b). Before the start of the trial, the experimental groups of CONLC (n = 15), NOPLC (n = 12), CONHC (n = 14), and NOPHC (n = 14) were balanced for calculated date of calving, 4% fat corrected milk yield (FCM) in their previous lactation (6207 ± 1248 kg; mean ± SD), BCS six weeks *ante partum* (a.p.) (3.3 ± 0.4), and number of lactations (3.0 ± 1.1). The trial lasted from d 28 a.p. until d 120 *post partum* (p.p.). The cows were housed at an animal to feeding - place ratio of 1:1 in a single furrow cubicle housing system with slatted floor and high-bed cubicles.

The cows were offered a partial mixed ration (PMR) as a basal diet to comply with the nutrient recommendations for lactating dairy cows of the Society of Nutrition Physiology (GfE 2001). PMR, delivered freshly every day at 0800 h from a mixing waggon, and water were provided *ad libitum* in weighing troughs (type RIC; Insentec B.V., Marknesse, the Netherlands). On a DM basis, the PMR contained 90% silages (78% corn silage, 22% grass silage) and 10% of a pelletised concentrate containing either the 3-NOP compound for 3-NOP groups or a placebo for CON groups to ensure continual consumption of the supplement throughout the day from d 28 a.p. until d 120 p.p. The 3-NOP including concentrate was prepared once a month and stored dry and dark in sealed containers. Concentrate pellets incorporating 3-NOP and placebo were additionally supplied via concentrate feeders (Insentec, B.V., Marknesse, The Netherlands) a.p. for HC groups only, but p.p. for all groups in order to deliver the 3-NOP target concentration. The supplementation of 3-NOP concentrate via the feeding line was performed frequently from the containers throughout the day. To regulate energy density and complete the PMR to the final ration, further pelleted conventional concentrates were provided via the automatic feeders. From d 28 a.p. until the day of calving, LC and HC groups received 15% and 40% concentrates in the whole ration, respectively. After parturition, the CFP was immediately administered to 30% for LC groups and gradually increased

from 30 to 55% until d 21 p.p. for HC groups, where it remained until the end of the study. The chemical composition of feedstuffs and the consumed diets are presented in Tables 1 and 2.

Table 1. Ingredients and chemical composition of concentrates and roughages offered in the experimental diets from d 28 ante partum until d 120 post partum presented as means.

	Concentrates				Roughages	
Item	3NOP[†]	PLA[§]	LAC[+]	MIN[¶]	Corn silage	Grass silage
Ingredients [% DM]						
Rapeseed meal	15	15	22	12		
Soybean meal	12.5	12.5	—	14.5		
Wheat	33	33	21	36		
Barley	—	—	21	—		
Corn	—	—	25.7	—		
Dried sugar beet pulp	29.06	29.06	7.0	27.0		
Soybean oil	1.5	1.5	1.0	1.5		
Calcium carbonate	2.4	2.4	1.3	3.0		
Urea	1	1	—	3		
3-nitrooxypropanol supplement	0.54	—	—	—		
Placebo supplement	—	0.54	—	—		
Vitamin/Mineral premix	5[$]	5[$]	1[#]	3[$]		
Chemical analysis						
DM[‡] [g/kg]	887	886	876	885	321	360
Nutrient [g/kg of DM]						
Crude ash	101	99	54	85	39	104
Crude protein	222	224	165	262	78	141
Utilisable crude protein[◊]	173	173	172	175	130	135
Ether extract	38	38	44	39	28	34
$aNDF_{om}$[♦]	216	216	191	208	444	546
Acid detergent $fibre_{om}$	126	125	98	119	245	318
Starch	306	332	448	323	286	0
Energy[◊] [MJ/kg of DM]						
Net energy lactation	7.4	7.4	8.0	7.5	6.4	6.2

[†]3NOP, name of the concentrate (C) including a supplement of 3-nitrooxypropanol on a carrier of SiO_2 and propylene glycol; [§]PLA, C including a placebo supplement of SiO_2 and propylene glycol; [+]LAC, C for lactation; [¶]MIN, C including mineral premix; [#]Ingredients according to the manufacturer´s specifications in g/kg of premix for lactating dairy cows: 140 Ca; 120 Na; 70 P; 40 Mg; 6 Zn; 5.4 Mn; 1 Cu; 0.1 I; 0.04 Se; 0.025 Co; vitamins in IU: 1,000,000 A; 100,000 D3; 2,235 E; [$]Ingredients in g/kg of premix for dry cows: 10 Ca; 120 Na; 60 P; 60 Mg; 6 Zn; 4 Mn; 1.25 Cu; 0.1 I; 0.05 Se; 0.035 Co; vitamins in IU: 800,000 A; 100,000 D3; 3,725 E; [‡]DM, dry matter; [◊]Calculations for concentrates based on table values according to DLG (1997) and silages according to VDLUFA (2006) analyses used in equations provided by GfE (2001); [♦]aNDFom, α-amylase treated neutral detergent fibre without residual ash.

Table 2. Chemical composition and energy content of the total rations offered during the experimental period from d 28 ante partum until d 120 post partum presented as means.

	CON[†]		3-NOP[§]	
Item	LC	HC	LC	HC
DM[+] [g/kg]	467	582	467	597
Nutrient [g/kg of DM]				
Crude ash	63	61	63	61
Crude protein	130	138	129	140

Utilisable crude protein[¶]	142	150	142	151
Ether extract	32	35	32	36
$aNDF_{om}$[#]	402	344	404	337
Acid detergent $fibre_{om}$	226	191	227	187
$peNDF_{>8mm}$ in the partial mixed ration[$]	268	269	274	273
Starch	249	303	246	307
Energy[¶] [MJ/kg of DM]				
Net energy lactation	6.6	7.0	6.6	7.1

[†]CON, Control groups with low (LC) or high (HC) concentrate feed proportion; [§]3-NOP, 3-nitrooxypropanol groups; [+]DM, dry matter; [¶]Calculations for concentrates based on table values according to DLG (1997) and silages according to VDLUFA (2006) analyses used in equations provided by GfE (2001); [#]aNDFom, α-amylase treated neutral detergent fibre expressed without residual ash; [$]$peNDF_{>8mm}$, physically effective neutral detergent fibre measured as the proportion of particles retained by 19- and 8-mm screens multiplied by dietary NDF content (Lammers et al. 1996).

2.2. Sampling and analyses

2.2.1. Dry matter intake and crude nutrients in feed

During the experiment, the quantitative-individual DMI of PMR and concentrates was continuously registered by the weighing troughs and concentrate feeders, respectively. Feed samples of the PMR components and concentrates were collected twice and once a week, respectively, dried for 72 h at 328.15 K, ground to pass a 1-mm screen (SM 1, Retsch, Haan, Germany) and composited to collective samples of four-week periods. Samples were analysed according to the standard methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA 2006) for DM (3.1), crude ash (8.1), crude protein (Dumas method, 4.1.2), ether extract pre-treated with hydrochloric acid (5.1.1), starch (7.2.1), acid detergent fibre (ADFom; 6.5.2) and α-amylase treated neutral detergent fibre (aNDFom; 6.5.1), both expressed without residual ash. Concentrates from the PMR and automatic feeders were pooled over the entire experimental period to analyse 3-NOP concentration by DSM Nutritional Products AG, Kaiseraugst, Switzerland. PMR was analysed for particle size distribution using the Penn State Particle Separator with 19- and 8-mm sieves according to Heinrichs and Kononoff (2002).

2.2.2. Milk yield and components

Cows were milked twice daily at 0530 and 1530 h in a tandem milking parlour with milk yield recording via automatic milk counters (Lemmer Fullwood GmbH, Lohmar, Germany). Milk samples were taken twice a week, each at the two consecutive morning and afternoon milkings and preserved with bronopol at 277.15 K until further analysis of milk fat, protein, lactose and milk urea (MU) using an infrared milk analyser (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark).

2.2.3. Body condition score and body weight

BCS was monitored weekly by one trained scorer on a 5-point scale of 1 (emaciated) to 5 (obese) according to Edmonson et al. (1989). Body weight (BW) was recorded weekly a.p. and twice daily p.p. after each milking using an automatic electronic scale at the exit of the milking parlour.

2.2.4. GreenFeed measurements

Cow-individual mass fluxes of emitted CH_4 and CO_2 were measured from d 28 a.p. until d 120 p.p. using two GreenFeed (GF) systems (C-Lock Inc., Rapid City, SD, USA) according to Zimmerman and Zimmerman (2012) and Hristov et al. (2015a). The GF systems were installed in a fixed position with side walls in the middle of the row of high-bed cubicles and gave the cows free access all the time. Cows were accustomed to visit the GF prior to starting the trial. During a feeding period, 34 g of concentrate LAC (Table 1) per feed drop were dispensed at 40-s intervals as pelletised bait feed into the GF feeding manger. A daily maximum of eight feed drops per feeding period and a total of six feeding periods with minimum time intervals of 180 min between them were configured. Perforated feed mangers, air filters, and head positioning sensors were cleaned daily. Bait feed dosing was calibrated weekly, whereas CO_2 recovery tests were performed monthly. CH_4 and CO_2 were calibrated daily using a zero (O_2 = 200,000 mg/kg, N_2 = 800,000 mg/kg) and a span gas (CH_4 = 1004 mg/kg, CO_2 = 10,000 mg/kg, O_2 = 210,000 ppm mg/kg H_2 = 9.50 mg/kg, H_2S = 9.80 mg/kg, while the remainder gas was nitrogen). Cannulated cows were excluded from the dataset because of gas leakage through the rumen cannula.

2.2.5. Rumen-fluid variables

Rumen fluid was collected before morning feeding from all experimental cows on d 28 a.p., 49 p.p., and 120 p.p., whereas 10 randomly selected cows of each group were additionally sampled on d 14, and 7 a.p., and d 7, 28, 73, and 98 p.p. The flexible tube of an oro-ruminal probe (Geishauser 1993) was attached to a suction pump (SELEKT Rumen-Fluid Collector, Nimrod Veterinary Products Ltd., Gloucestershire, UK) and introduced to a length of 2.0 m in order to obtain 350 ml fluid from the reticulo-rumen whereby the first 100 ml were rejected to minimise saliva contaminations. The pH of rumen fluid was measured immediately after collection using a glass electrode (pH 525; WTW, Weilheim, Germany). VFA were analysed according to Geissler et al. (1976) using a gas chromatograph (Clarus 680, PerkinElmer LAS GmbH, Rodgau, Germany) equipped with a flame ionisation detector. Ammonia-N concentration (NH_3-N) was measured using steam distillation (DIN38406-E5-2, (Anonymous 1998)). For determination of protozoal density, 15 ml rumen fluid was prepared by mixing with 15 ml of methyl green-formalin solution on d 28 a.p., 49 p.p. and 120 p.p. (Ogimoto and Imai 1981). Rumen ciliates were counted and differentiated between the spirotrich protozoa, summarised as *Entodiniomorpha*, and the holotrich protozoa *Isotrycha* and *Dasytricha* using a Neubauer chamber under an optical microscope.

2.3. Calculations

Equations provided by GfE (2001)were used to calculate GE content [MJ/kg DM] and variables of milk energy concentration and EB which is defined as the difference between net energy intake (NEI) and net energy requirements for maintenance (NE_M), lactation (NE_L), and pregnancy (NE_P):

$$\text{EB [MJ NE}_\text{L}\text{/d]} = \text{NEI [MJ NE}_\text{L}\text{/d]} - \text{NE}_\text{M}\text{[MJ NE}_\text{L}\text{/d]} - \text{NE}_\text{L}\text{[MJ NE}_\text{L}\text{/d]}, \text{ with}$$

$$\text{NE}_\text{M}\ \text{[MJ NE}_\text{L}\text{/d]} = 0.293 \cdot \text{BW}^{0.75}\ \text{[kg]}, \text{ where BW}^{0.75} \text{ is the metabolic live weight,}$$

$$\text{milk energy [MJ NE}_\text{L}\text{/d]} = 0.3 \cdot \text{milk fat [\%]} + 0.21 \cdot \text{milk protein [\%]} + 0.95, \text{ and}$$

$$\mathrm{NE_L\ [MJ\ NE_L/d]} = (\text{milk energy [MJ NE}_\mathrm{L}\text{/d]} + 0.1) \cdot \text{milk yield [kg/d]}.$$

Additionally, gestational energy requirements of late pregnancy were considered by subtracting 13 MJ of NE_L/d in week 4 a.p. and 18 MJ of NE_L/d during week 3 until parturition from EB.

The NEI [MJ of NE_L/d] was calculated by multiplying daily DMI by NE_L concentration of the diet.

Milking performance was expressed as milk yield, fat corrected milk yield (FCM) and ECM.

FCM was calculated according to Gaines (1928):

$$4\%\ \mathrm{FCM\ [kg/d\ ]} = (\text{milk fat [\%]} \cdot 0.5 + 0.4) \cdot \text{milk yield [kg/d]}$$

ECM was estimated as proposed by Sjaunja et al. (1990):

$$\mathrm{ECM\ [kg/d\ ]} = \text{milk yield [kg/d]} \cdot \left(\frac{38.3 \cdot \text{milk fat [g/kg]} + 24.2 \cdot \text{milk protein [g/kg]} + 16.54 \cdot \text{milk lactose [g/kg]} + 20.7}{3140}\right)$$

The feed efficiency (FE; kg/kg) was evaluated by dividing ECM [kg] by DMI [kg]. Energy conversion ratio (ECR), metabolic efficiency (MEff) and residual energy intake (REI) were defined as further energy efficiency parameters according to Hurley et al. (2016):

$$\mathrm{ECR\ [MJ\ NE_L/MJ\ NE_L]} = \mathrm{NEI[MJ\ NE_L]/NE_L[MJ\ NE_L]}$$

$$\mathrm{MEff\ [MJ\ NE_L/kg\ BW^{0.75}]} = \frac{\mathrm{NEI\ [MJ\ NE_L]} - \mathrm{NE_L\ [MJ\ NE_L]}}{\mathrm{BW^{0.75}\ [kg]}}$$

The REI accounts for the part of energy intake not explainable by regression variables. The REI was calculated by subtracting the observed NEI from the expected energy intake (EEI):

$$\mathrm{REI\ [MJ\ NE_L/d\ ]} = \mathrm{NEI\ [MJ\ NE_L/d]} - \mathrm{EEI\ [MJ\ NE_L/d]}$$

Calculations of daily gas emissions were computed by C-Lock Inc. based on the volumetric airflow rate and concentration of captured gas, while correcting for muzzle position, background gas concentration and capture rate as described in Huhtanen et al. (2015). Daily means of emission data were aggregated to weekly means using the arithmetic averaging method according to Manafiazar et al. (2016). CH_4 yield and intensity were defined as CH_4 emission expressed relative to DMI [g CH_4/kg DMI] and ECM [g CH_4/kg ECM], respectively. CH_4 energy [MJ/l] was calculated considering that 1 l of CH_4 corresponds to 39.54 kJ heat of combustion (Brouwer 1965). CH_4 was converted from [g/d] into [l/d] by the use of the CH_4 density which is 0.717 [kg/m^3].

2.4. Statistics

For variables that were recorded more than once a week, means were calculated per cow and week prior to being incorporated into statistical evaluation. Statistics were performed using PROC

MIXED (version 9.4; SAS Institute Inc., Cary, NC) and the following repeated measures mixed model fitted by a restricted maximum likelihood (REML) method according to Littell et al. (1998):

$$Y_{ijk} = \left(\mu + S_i + C_j + T_k + (S \times C)_{ij} + (S \times T)_{ik} + (C \times T)_{jk} + (S \times C \times T)_{ijk} + \varepsilon_{ijk}\right)$$

where Y_{ijk} = response variable; μ = overall mean; S_i = fixed effect of 3-NOP supplementation (i = 3-NOP, CON); C_j = fixed effect of concentrate proportion in the ration (j = LC, HC); T_k = fixed effect of time relative to parturition (k = week 4 a.p., …, 17 p.p.); $(S \times C)_{ij}$, $(S \times T)_{ik}$, $(C \times T)_{jk}$, and $(S \times C \times T)_{ijk}$ = fixed interaction terms; and ε_{ijk} = residual error.

Cow within treatment was implemented as a random effect and the sequence of day or week of sampling as a repeated measure. The variance-covariance structures compound symmetry, autoregressive, variance components, and unstructured were tested using a maximum likelihood method and selected based on the best fit according to the lowest Akaike Information Criterion (AIC). The day of the first baseline measurement before 3-NOP supplementation was regarded as a covariate for rumen fermentation and DMI parameters. Effects were declared statistically significant at p-values ≤ 0.05 and a trend was postulated at p-values between >0.05 and 0.10. Multiple t-tests (PROC PDIFF) with Tukey adjusted p-values were computed to evaluate significant weekly effects. Results are presented as least square means (LS-means) with the pooled standard error of means (PSEM).

Calculation of the REI was conducted using the R software package (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria). A non-linear regression model was applied to find the best model fit for EEI. Significant independent variables and related regression coefficients were estimated in a forward stepwise manner and accounted for the lowest AIC. Outliers were excluded from the model which was also proved for multicollinearity.

3. Results

3.1. Feed intake, energy balance, body condition and efficiency

Fifty-five cows out of the initial 58 completed the trial. In the NOPLC group, three cows were excluded p.p. because of two cases of abomasal displacement and a necrotising endometritis.

Parameters of feed intake, body mass and energy efficiency are presented in Table 3. An actual dose of 48.4 ± 2.7 (NOPLC) and 51.1 ± 2.9 (NOPHC) mg 3-NOP/kg of DM (means ± SD) was obtained from the feed analyses which resulted in a total uptake of 882 ± 175 and 1031 ± 184 mg of 3-NOP/d in NOPLC and NOPHC, respectively. The intake of DM (kg/d; % of $BW^{0.75}$), roughage, aNDFom and NE_L were influenced by CFP ($p < 0.001$) and CFP × TIME ($p < 0.01$) but unaffected by 3-NOP supplementation. DMI was approximately 10% lower in LC compared to HC groups during the experiment. DMI and NE_L intake significantly decreased by approximately 28% until the day of calving followed by a marked increase until week 5 p.p. which amounts to 38% in LC and, more pronounced, 48% in HC groups (Table 3; Fig. 1a; CFP × TIME; $p < 0.001$). Roughage intake was significantly lower in HC and increased more slightly after parturition when

compared to LC groups (CFP × TIME; $p < 0.01$). From week 5 p.p. until the end of the trial, concentrate and NE_L intake were reduced by 50% and 15% in LC groups. During the a.p. period as well as from week 10 p.p. until the end of the experiment, daily concentrate intake in NOPHC was approximately 1 kg DM higher when compared to CONHC (3-NOP × CFP = 0.038).

The 3-NOP × CFP combination had an impact on calculated EB ($p = 0.001$), whereas a trend was noted for CFP × TIME ($p = 0.072$). 3-NOP × CFP interaction was driven by the NOPHC group whose EB was in a significantly more positive range and reached in week 8 p.p. an earlier balanced status when compared to the other groups. In addition, LC groups were distinguished from HC groups by significantly more pronounced negative EB which were, in contrast to HC groups, not compensated until the end of the trial (Fig. 1d; $p = 0.072$ for CFP × TIME).

CFP × TIME interaction was significant for body weight gain (BWG) ($p = 0.043$; Table 3). BW was lowest in NOPHC but a slight increase was observed in BWG from 625 up to 658 kg starting in week 8 p.p. correspondingly to the balanced EB. All groups indicated a loss in BW and BCS from week 2 a.p. until week 6 p.p. being most obvious in NOPHC. A more rapid but temporary decline in BCS was identified in HC groups closely around calving, whereas BCS loss was higher in LC groups over the entire experimental period. Initial BCS of 3.7 ± 0.1 decreased by 1.0 ± 0.14 from week 2 a.p. until 6 p.p. regarding CONLC and NOPHC, whereas NOPLC and CONHC lost 1.06 ± 0.15 and 1.16 ± 0.14 BCS points, respectively. From week 6 p.p. until the end of the trial, BCS gain, starting time-delayed in CONLC, averaged 0.41 ± 0.07 ending up to BCS of 3.0 ± 0.1.

In all groups feed efficiency decreased continuously from the onset of lactogenesis due to an increasing DMI paralleled by an inversely shaped time course in ECM (Table 3; $p < 0.001$). HC groups exhibited a lower FE ($p = 0.010$) which remained uninfluenced by 3-NOP. ECR, MEff and REI were affected by CFP × TIME ($p = 0.025$) and generally lower in LC groups indicating higher efficiency. The 3-NOP × CFP interaction ($p = 0.008$) was driven by a considerably elevated ECR, MEff and REI in NOPHC which implies a reduced efficiency in this group (Table 3). The forward stepwise regression resulted in the following variables and coefficients to calculate the EEI (R^2 = 0.74; residual standard deviation = 11.86 MJ NE_L/d):

$$\begin{aligned}\text{EEI [MJ NE}_\text{L}\text{/d]} &= -6.8424 + 3.2418 \cdot \text{BCS} + 0.0053055 \cdot \text{CO}_2\text{ emissions [g/d]} - 1.9067 \\ &\cdot \text{CH}_4\text{ yield [g/d]} + 11.8144 \cdot \text{week} - 1.0140 \cdot \text{week}^2 + 0.027 \cdot \text{week}^3 \\ &- 0.0974 \cdot \text{milk urea [mg/l]} + 10.1010 \cdot \text{milk protein [\%]} + 1.1386 \\ &\cdot \text{milk yield [kg/d]}\end{aligned}$$

Table 3. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion (CFP), time relative to parturition (TIME) and interactions between them on feed intake, energy balance, body condition and efficiency traits in experimental cows from d 28 ante partum until d 120 post partum.

Variable	Treatments[†]				PSEM[+]	*p*-values[§]					
	CONHC (*n* = 15)	CONLC (*n* = 14)	NOPHC (*n* = 14)	NOPLC (*n* = 12)		3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME
Feed intake[¶] [kg DM[#]/d]											
DM [kg/d]	20.1	18.2	20.2	18.2	0.14	0.907	<0.001	<0.001	0.970	0.869	0.160
DM [% of BW[$]]	2.97	2.80	3.05	2.78	0.12	0.525	<0.001	<0.001	0.312	0.759	0.008
Roughage	10.6	12.5	10.1	12.7	0.13	0.419	<0.001	<0.001	0.186	0.876	0.005
Concentrate	9.7[b]	5.5[c]	10.4[a]	5.6[c]	0.11	0.003	<0.001	<0.001	0.038	0.403	<0.001
Neutral detergent fibre	6.8	7.0	6.7	7.1	0.13	0.685	0.020	<0.001	0.574	0.927	0.635
GEI[‡] [MJ/d]	365	318	369	324	1.26	0.250	<0.001	<0.001	0.811	0.895	<0.001
MEI[◊] [MJ/d]	231	201	233	202	0.86	0.787	<0.001	<0.001	0.923	0.834	0.036
Energy balance [MJ NE_L[♦]/d]											
Energy intake	143	121	145	122	0.49	0.396	<0.001	<0.001	0.817	0.572	<0.001
Energy requirement	139	141	133	136	1.42	0.070	0.526	0.052	0.794	0.944	0.032
Energy balance	-4.3[b]	-17.1[c]	8.4[a]	-17.1[c]	0.53	0.001	<0.001	<0.001	0.001	0.888	0.072
Body condition											
BW [kg]	694	662	662	681	2.03	0.714	0.700	<0.001	0.149	0.814	0.074
BW gain [kg/week]	-0.54	-0.67	-0.43	-0.61	0.22	0.383	0.132	<0.001	0.794	0.427	0.043
Body condition score	2.93	2.81	3.00	2.92	0.13	0.283	0.211	<0.001	0.857	0.734	0.514
Efficiency parameters											
FE[‡] [kg ECM/kg DM intake]	1.89	1.94	1.77	1.94	0.11	0.173	0.010	<0.001	0.186	0.827	0.516
ECR[‖] [MJ NE_L/MJ NE_L]	1.19[b]	1.09[c]	1.31[a]	1.10[c]	0.11	0.001	<0.001	<0.001	0.008	0.789	0.043
MEff[∏] [MJ NE_L/kg $BW^{0.75}$]	0.17[b]	0.07[c]	0.28[a]	0.08[c]	0.11	0.002	<0.001	<0.001	0.007	0.796	0.025
REI[△] [MJ NE_L]	2.65[ab]	-2.22[bc]	6.32[a]	-6.88[c]	0.40	0.750	<0.001	0.024	0.008	0.078	0.007
MEI:GEI ratio	0.65[ab]	0.64[bc]	0.66[a]	0.63[c]	0.02	0.837	<0.001	<0.001	0.036	0.722	0.292

[†]CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-NOP high concentrate; NOPLC, 3-NOP low concentrate. Values presented as LS-means. [a-c]LS-means with different superscripts within a row differ significantly ($p < 0.05$); [§]3-NOP×CFP×TIME with *p*-values >0.05 for all variables; [+]PSEM, pooled standard error of the means; [¶]Statistics with first measured value as covariate; [#]DM, dry matter; [$]BW, body weight; [‡]GEI, gross energy intake; [◊]MEI, metabolisable energy intake; [♦]NE_L, net energy lactation; [‡]FE, feed efficiency = energy corrected milk yield [kg ECM]/DM intake [kg DMI]; [‖]ECR, energy conversion ratio = energy intake [MJ NE_L]/energy excretion with milk [MJ NE_L]; [∏]MEff, metabolic efficiency = (energy intake [MJ NE_L] – energy in milk [MJ NE_L])/$BW^{0.75}$ [kg]; [△]REI, residual energy intake = energy intake [MJ NE_L] – expected energy intake [MJ NE_L].

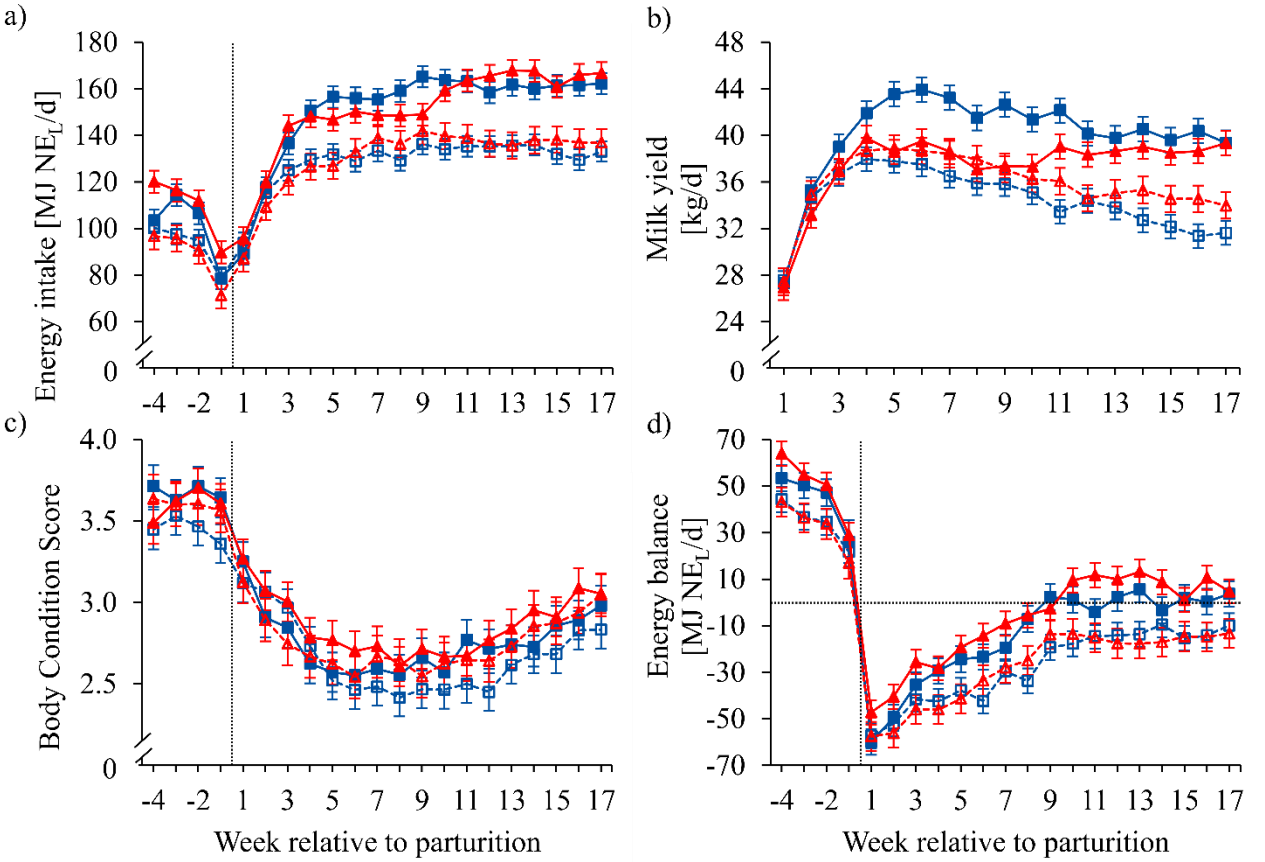

Figure 1. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion and time relative to parturition on energy partitioning in peripartal dairy cows (LS-means). ■, solid line = control high concentrate group (CONHC); □, dashed line = control low concentrate group (CONLC); ▲, solid line = 3-NOP high concentrate group (NOPHC); Δ, dashed line = 3-NOP low concentrate group (NOPLC).

3.2. Milk performance

Parameters of milk performance are presented in Table 4. For all groups, milk yield increased until week 6 p.p. and slightly decreased thereafter which became more apparent in LC groups. However, an increase in milk yield was observed only in NOPHC from week 8 p.p. until the end of the experiment (Fig. 1b; 3-NOP × CFP × TIME; p = 0.068). The 3-NOP × CFP interaction was significant for FCM, ECM, milk energy output, lactose and fat yield, whereas a trend was observed for protein yield (p = 0.066). Thus, ECM and FCM were significantly reduced by 3.5 and 3.9 kg, respectively, in NOPHC compared to CONHC. The decrease in milk fat yield of NOPHC amounted to 0.14 kg/d compared to CON groups. NOPLC neither differed from CONLC for milk performance and composition nor NOPHC concerning parameters of milk yield. A time-dependent variation between HC and LC groups was ascertained for milk protein content (p = 0.019). Milk energy concentration, fat content and fat to protein ratio were affected by 3-NOP × TIME and CFP × TIME interactions and all of them remained higher in LC groups. Elevated milk lactose contents were found in 3-NOP groups (p = 0.049). Both CFP and 3-NOP × TIME interaction were considered significant for MU which was generally higher in LC groups and furthermore increased in 3-NOP treated cows during the course of the experiment.

Table 4. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion (CFP), time relative to parturition (TIME) and interactions between them on lactational performance of the experimental cows from parturition until d 120 post partum.

Variable	Treatments[†]				PSEM[+]	*p*-values[§]					
	CONHC (*n* = 15)	CONLC (*n* = 14)	NOPHC (*n* = 14)	NOPLC (*n* = 12)		3-NOP	CFP	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
Milk yield [kg/d]											
Milk	40.1[a]	35.0[b]	36.6[ab]	36.1[b]	0.21	0.270	0.008	0.029	0.551	<0.001	0.068
4% FCM[¶]	40.0[a]	37.5[ab]	36.1[b]	37.7[ab]	0.22	0.039	0.616	0.027	0.459	0.335	0.581
ECM[#]	39.7[a]	36.6[ab]	36.2[b]	36.8[ab]	0.21	0.072	0.179	0.039	0.509	0.137	0.399
Fat	1.60[a]	1.57[a]	1.43[b]	1.55[ab]	0.11	0.009	0.225	0.040	0.252	0.572	0.825
Protein	1.28	1.09	1.19	1.11	0.13	0.212	<0.001	0.066	0.749	<0.001	0.272
Lactose	1.91[a]	1.66[b]	1.76[ab]	1.73[ab]	0.13	0.477	0.012	0.047	0.582	0.001	0.072
Milk composition [%]											
Fat	4.04	4.50	3.95	4.34	0.05	0.190	<0.001	0.681	0.005	0.005	0.456
Protein	3.23	3.15	3.28	3.11	0.13	0.806	0.002	0.256	0.984	0.019	0.184
Lactose	4.74	4.76	4.80	4.79	0.12	0.049	0.951	0.573	0.987	0.162	0.883
Milk fat:protein ratio	1.25	1.43	1.21	1.40	0.02	0.233	<0.001	0.871	0.022	<0.001	0.792
Milk urea [mg/kg]	96	134	117	148	3.31	0.011	<0.001	0.545	0.014	0.092	0.278
Milk energy											
concentration [MJ/kg]	3.16	3.32	3.14	3.25	0.02	0.267	<0.001	0.542	0.005	0.018	0.335
output [MJ/d]	129.8[a]	119.4[ab]	117.8[b]	120.2[ab]	0.58	0.060	0.173	0.033	0.542	0.162	0.418

[†]CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-NOP high concentrate; NOPLC, 3-NOP low concentrate. Values presented as LS-means. [a-c]LS-means with different superscripts within a row differ significantly ($p < 0.05$); [§]effect of TIME with *p*-values <0.001 for all variables; [+]PSEM, pooled standard error of the means; [¶]FCM, 4% fat corrected milk yield; [#]ECM, energy corrected milk yield.

Table 5. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion (CFP), time relative to parturition (TIME) and interactions between them on methane (CH4) and carbon dioxide (CO2) emissions measured in experimental cows from d 28 ante partum until d 120 post partum using the GreenFeed system.

Variable[+]	Treatments[†]				PSEM[¶]	*p*-values[§]					
	CONHC (*n* = 12)	CONLC (*n* = 13)	NOPHC (*n* = 11)	NOPLC (*n* = 9)		3-NOP	CFP	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
GreenFeed visits [frequency/d]	3.9	4.9	2.8	3.4	0.12	<0.001	<0.001	0.191	<0.001	0.608	0.840
Gas emissions											
CH_4 production [g/d]	365	373	246	291	1.92	<0.001	0.005	0.051	0.003	0.055	0.006
CH_4 yield [g CH_4/kg DMI[#]]	18.7[b]	21.0[a]	12.2[d]	16.2[c]	0.16	<0.001	<0.001	0.048	0.259	<0.001	0.940
CH_4 intensity [g CH_4/kg ECM[$]]	9.2	10.4	6.5	8.0	0.19	<0.001	<0.001	0.640	0.037	<0.001	0.002
CH_4 energy [% of MJ GEI[‡]]	5.8	6.7	3.8	5.1	0.12	<0.001	<0.001	0.123	0.578	<0.001	0.703
CH_4 energy [MJ/d]	20.1	20.6	13.6	16.0	0.17	<0.001	0.005	<0.001	0.051	0.003	0.055
CO_2 production [g/d]	13811	12991	12791	12593	44.27	<0.001	0.017	0.138	<0.001	0.080	0.244
CO_2 yield [g CO_2/kg DMI]	709[a]	735[a]	633[b]	711[a]	3.09	<0.001	<0.001	0.011	0.258	0.151	0.377
CO_2 intensity [g CO_2/kg ECM]	352	361	345	347	5.34	0.333	0.590	0.750	0.005	<0.001	0.297
CH_4/CO_2 [g/g]	0.0262	0.0286	0.0193	0.0229	3.7×10^{-4}	<0.001	<0.001	0.411	0.003	<0.001	<0.001

[†]CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-NOP high concentrate; NOPLC, 3-NOP low concentrate. Values presented as LS-means. [a-d]LS-means with different superscripts within a row differ significantly ($p < 0.05$); [§]TIME with *p*-values <0.001 for all variables; [+]only non-cannulated cows measured; [¶]PSEM, pooled standard error of the means; [#]DMI, dry matter intake; [$]ECM, energy corrected milk yield; [‡]GEI, gross energy intake.

3.3. Gas emissions

CH_4 production (g/d) and intensity (g/kg ECM) were affected by 3-NOP × CFP × TIME ($p < 0.006$), whereas 3-NOP × CFP ($p < 0.048$) and CFP × TIME ($p < 0.001$) interactions were significant concerning CH_4 yield (g/kg DMI) (Table 5).

In all groups, CH_4 production decreased by about 58 ± 10 g/d from initiating the trial until parturition. Afterwards, a more pronounced increase in CH_4 emissions was noted for CON groups until week 7 to 388 ± 58 g/d (Fig. 2a). Over the trial period, 3-NOP treatment significantly reduced CH_4 production, CH_4 yield, CH_4 intensity and CH_4 energy by approximately 33% in NOPHC and 22% in NOPLC as compared to the respective CON group. Nevertheless, 3-NOP inhibitory effects remained persistent only in NOPHC, as CH_4 emissions (g/d; g/kg DMI; g/kg ECM) in NOPLC gradually increased to the level of the CON groups from week 8 until the end of the experiment (Fig. 2a). CH_4 production was by 16% significantly lower in NOPHC compared to NOPLC, whereas differences were stated to be almost similar between CONHC and CONLC. Effects of CFP on CH_4 yield and intensity were, however, more distinctive and both were reduced by about 12% in CONHC and 22% in NOPHC when compared to CONLC and NOPLC. Energy spared from methanogenesis accounted for 2% (6.5 MJ/d) and 1.6 % (4.6 MJ/d) of GE intake in NOPHC and NOPLC, respectively (Table 5). CO_2 emission, yield and intensity were reduced by 3-NOP. CO_2 yield was also lower in HC groups (Fig. 2d-f). In 3-NOP groups, CO_2 yield increased to the level of the respective CON group from week 9 p.p. until termination of the trial (Fig. 2e).

CH_4 and CO_2 yield decreased with increasing EB (Fig. 3a and 3b). The increase in CH_4 yield per g increase in aNDFom content of the total ration was more pronounced in NOPLC as compared to NOPHC group (Fig. 3c). The CH_4/CO_2 ratio was lower for 3-NOP and HC groups but increased for NOPLC from parturition until the end of the trial (3-NOP × CFP × TIME; $p < 0.001$). As illustrated in Fig. 3d, the CH_4/CO_2 ratio decreased with increasing REI or reduced efficiency.

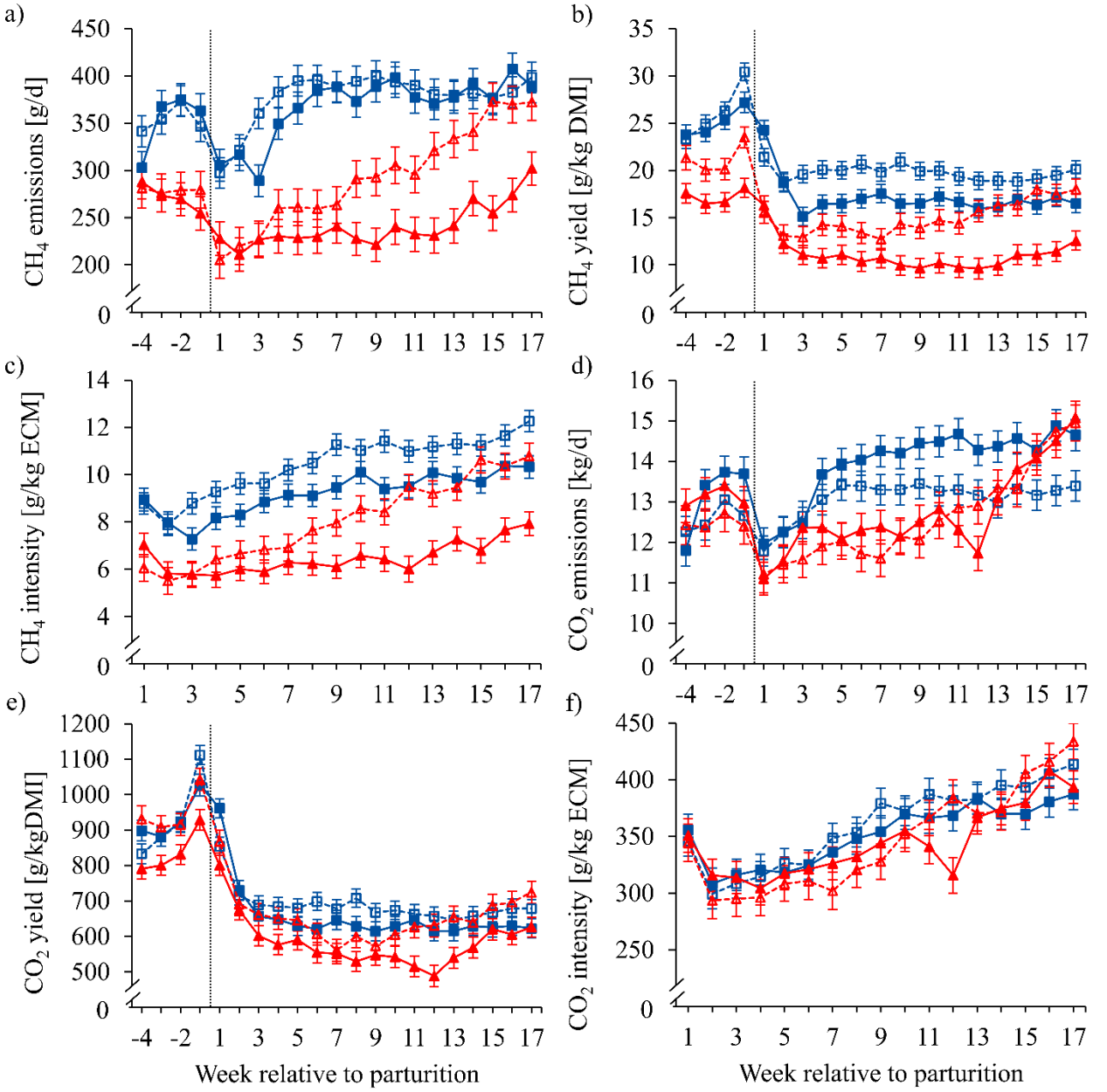

Figure 2. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion and time relative to parturition on total emissions [g/d], yield [g/kg dry matter intake (DMI)] and emission intensity [g/kg energy-corrected milk yield (ECM)] of CH_4 and CO_2 in peripartal dairy cows (LS-means). ■, solid line = control high concentrate group (CONHC); □, dashed line = control low concentrate group (CONLC); ▲, solid line = 3-NOP high concentrate group (NOPHC); Δ, dashed line = 3-NOP low concentrate group (NOPLC).

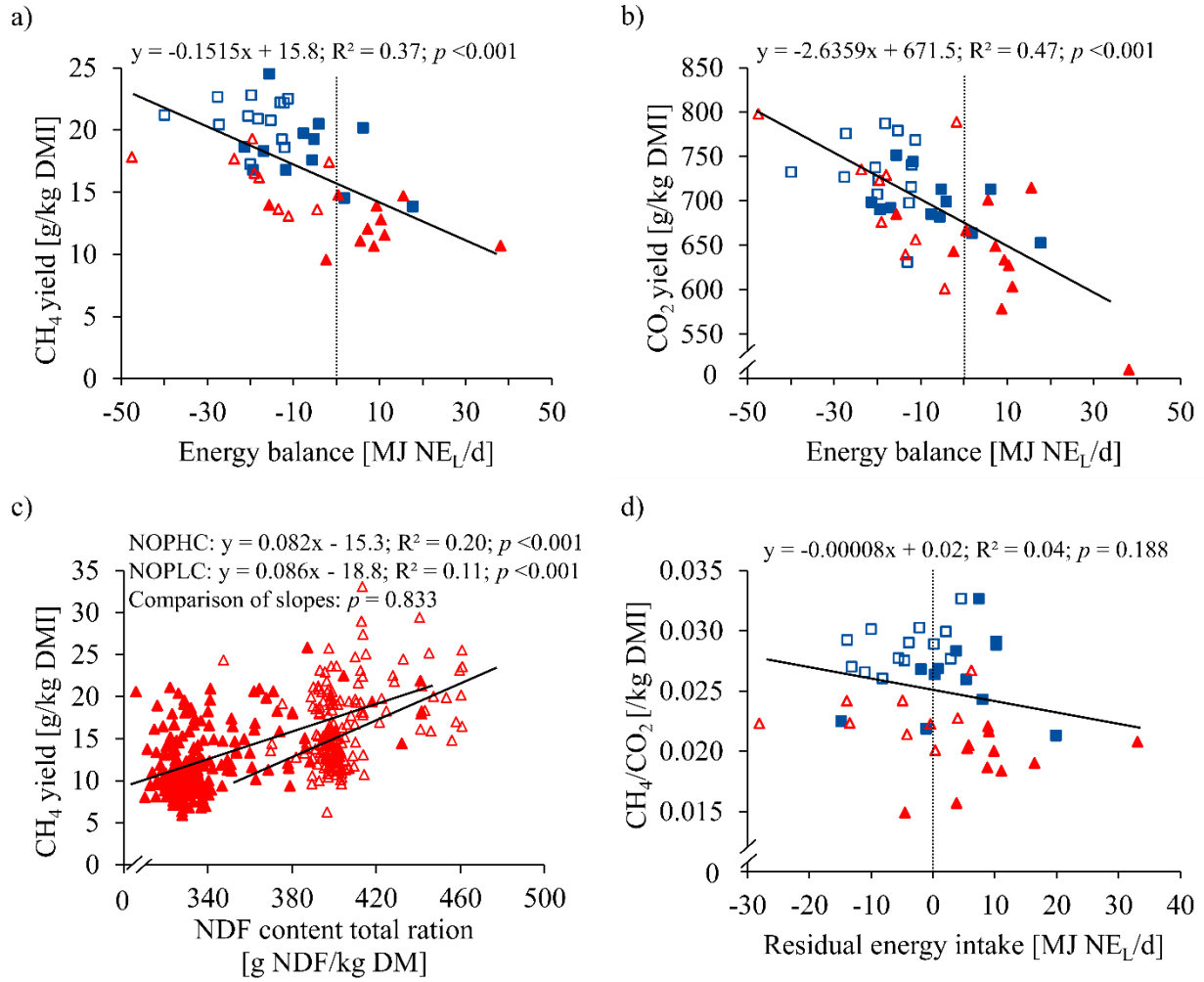

Figure 3. Effects of 3-nitrooxypropanol (3-NOP) and concentrate feed proportion on relationships between CH_4 and CO_2 yield [g/kg dry matter intake (DMI)], energy balance, neutral detergent fibre (NDF) and residual energy intake (LS-means) of peripartal dairy cows. ■ = control high concentrate group (CONHC); □ = control low concentrate group (CONLC); ▲ = 3-NOP high concentrate group (NOPHC); Δ = 3-NOP low concentrate group (NOPLC).

3.4. Rumen fermentation

LS-means and *p*-values of rumen fermentation parameters and abundance of protozoa are presented in Fig. 4 and Tables 6 and 7. Changes in fermentation pattern of individual and total VFA primarily occurred from d 28 a.p. until d 28 p.p. including all the changes in diet composition. From d 28 p.p. until the end of the experiment, molar proportions of the individual VFA (Fig. 4) and total VFA concentration (Table 7) remained almost constant, except for butyrate which decreased continuously (Fig. 4d). Supplementation of 3-NOP and high CFP increased the molar proportion of propionate by approximately 8% and also that of butyrate and valerate (Fig. 4c, 4d, 4e; 3-NOP; $p < 0.01$; CFP × TIME; $p < 0.01$; Table 6). *Iso*-valerate proportions also increased in 3-NOP groups (3-NOP × TIME; $p = 0.007$), whereas higher molar proportions were only found in CONLC compared to CONHC (3-NOP × CFP; $p = 0.043$). In contrast, acetate was reduced by approximately 7% due to 3-NOP (3-NOP × TIME; $p < 0.01$) and a high CFP (CFP × TIME; $p <$

0.01). *Iso*-butyrate proportion decreased in HC groups (CFP × TIME; $p < 0.01$) but remained unaffected by 3-NOP. The acetate to propionate ratio (A:P-ratio) markedly decreased until d 28 p.p. and remained lower in 3-NOP groups (3-NOP × TIME; $p = 0.006$; Table 6). The A:P-ratio was higher in HC groups until d 28 p.p. but changed thereafter to lower values when compared to LC groups (Fig. 4a; CFP × TIME; $p < 0.01$; Table 6). However, total VFA concentration was only affected by TIME ($p < 0.001$; Table 7). On average, NH_3-N concentration highly fluctuated by 27% around a mean value of 2.63 mmol/l; however, significantly lower NH_3-N concentrations were found in 3-NOP and HC groups (Fig. 4h; 3-NOP; $p < 0.01$; CFP × TIME; $p = 0.043$; Table 6).

Neither CFP nor 3-NOP exerted influence on ruminal pH value and rumen protozoal counts which were only significantly affected by TIME. Irrespective of treatments, rumen pH decreased within the first four experimental weeks from 7.7 to 6.9 where it remained more or less until the completion of the trial (Table 7). Counts of total protozoa and *Entodiniomorpha* were reduced by 12% from d 28 a.p. until d 49 p.p. but subsequently increased by 16% to 188 [10^3/ml] on d 120 p.p. (Table 7). Neither treatments nor TIME affected the abundance of *Holotricha* (Table 7).

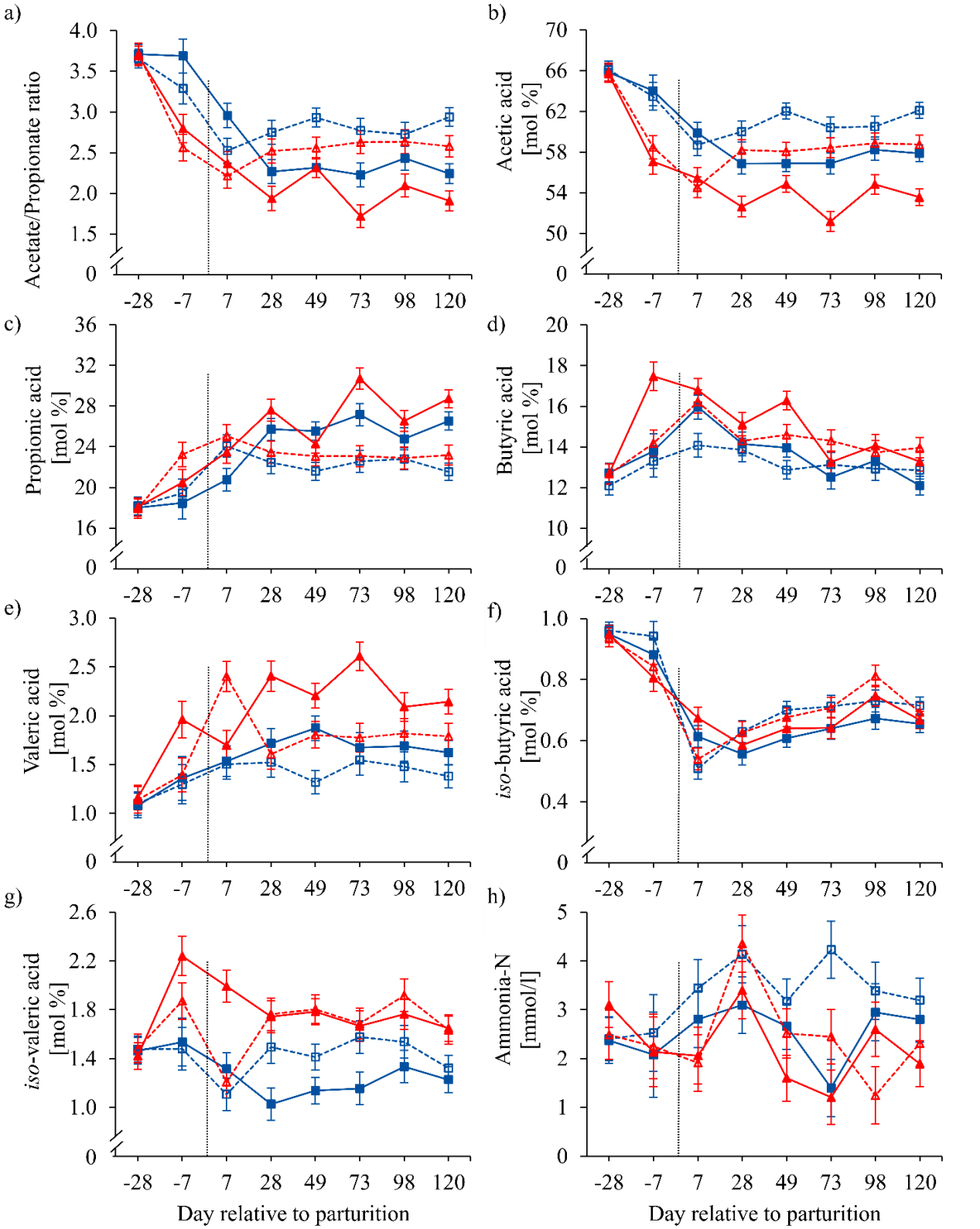

Figure 4. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion and time relative to parturition on molar proportions of volatile fatty acids in rumen fluid of peripartal dairy cows (LS-means). ■, solid line = control high concentrate group (CONHC; *n* =14); □, dashed line = control low concentrate group (CONLC; *n* =15); ▲, solid line = 3-NOP high concentrate group (NOPHC; *n* =14); Δ, dashed line = 3-NOP low concentrate group (NOPLC; *n* =12). Statistics with first measured value as covariate. *p*-values are presented in Table 6.

Table 6. P-values of the effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion (CFP), time relative to parturition (TIME) and interactions between them on volatile fatty acids (VFA) and ammonia-N concentration in rumen fluid of the experimental cows from d 28 ante partum until d 120 post partum.

		p-values[†]						
Variable	PSEM[§]	3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP×TIME
Molar proportions of total VFA [mol %]								
Acetic acid	0.253	<0.001	<0.001	<0.001	0.265	0.003	<0.001	0.413
Propionic acid	0.203	0.005	<0.001	<0.001	0.641	0.260	<0.001	0.328
Butyric acid	0.122	<0.001	0.031	<0.001	0.683	0.081	0.002	0.360
Valeric acid	0.040	<0.001	0.002	<0.001	0.355	0.110	<0.001	0.008
iso-butyric acid	0.017	0.724	0.024	<0.001	0.319	0.193	0.002	0.999
iso-valeric acid	0.032	<0.001	0.751	0.006	0.043	0.007	<0.001	0.534
Ratio acetate/propionate	0.097	<0.001	<0.001	<0.001	0.440	0.006	<0.001	0.395
Ammonia-N [mmol/l]	0.108	0.010	0.027	0.014	0.168	0.171	0.043	0.821

[†]Corresponding LS-means are presented in Figure 4; Statistics with first measured value as covariate; [§]PSEM, pooled standard error of the means.

Table 7. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion (CFP) in the ration, time relative to parturition (TIME) and interactions between them on total volatile fatty acids (VFA) [mmol/l], pH and the abundance of protozoa [103/ml] in rumen fluid of the experimental cows from d 28 ante partum (a.p.) until d 120 post partum (p.p.).

	Day relative to parturition[†]									*p*-values						
Variable[§]	28 a.p.	7 a.p.	7 p.p.	28 p.p.	49 p.p.	73 p.p.	98 p.p.	120 p.p.	PSEM[+]	3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
Total VFA	73[b]	72[b]	93[a]	94[a]	97[a]	97[a]	95[a]	95[a]	1.06	0.577	0.280	<0.001	0.253	0.162	0.205	0.564
pH	7.7[a]	7.7[a]	7.0[b]	6.9[b]	6.9[b]	7.0[b]	7.1[b]	7.0[b]	0.06	0.989	0.311	<0.001	0.334	0.218	0.339	0.803
Protozoa																
Total protozoa	177[ab]				158[b]			188[a]	18	0.588	0.518	0.026	0.268	0.983	0.576	0.819
Entodiniomorpha	173[ab]				155[b]			184[a]	18	0.580	0.520	0.030	0.277	0.988	0.536	0.825
Holotricha	4.2				3.0			3.8	1.12	0.876	0.486	0.414	0.407	0.679	0.658	0.913

[†]Emerged groups: CONHC: $n = 15$; CONLC: $n = 14$; NOPHC = 14; NOPLC: $n = 12$. Values presented as LS-means. [a-b]LS-means with different superscripts within a row differ significantly ($p < 0.05$); [§]Statistics with first measured value as covariate; [+]PSEM, pooled standard error of the means.

4. Discussion

4.1. Effects of diet composition and 3-NOP on methane emission

Methane production is positively correlated to feed intake level and ingested amounts of plant cell carbohydrates and fermentable OM (Dijkstra et al. 2011) which explains the reduced CH_4 emission closely around parturition and the almost similar total CH_4 emission between CON groups of the present trial. However, CH_4 yield was reduced in HC groups as degradation of readily fermentable carbohydrates to propionate via acrylate and succinate pathways competes for the methanogenic use of H_2 (McAllister and Newbold 2008). This finding gives rise to the observed negative correlation between propionate proportion and CH_4 yield (r = -0.318; $p < 0.001$). In addition, the reduced CH_4 yield could be related to an increased digesta passage of the HC rations followed by reduced microbial access to OM and extent of fermentation per unit of ingested feed. Thus, Knapp et al. (2014) amounted the CH_4 reduction potential of an increased CFP to a maximum of 15% which is comparable to the 12%-decrease observed between CONHC and CONLC group in our trial.

In the present combined approach, the investigations on the opposing interrelationships between increased CFP and CH_4 yield were widened by adding 3-NOP into the diet and an improved extent of the CH_4 inhibitor effect was hypothesised in high-concentrate fed cows. With regard to NOPHC group, our results of approximately 33% lower CH_4 emissions (g/d; g/kg DMI; g/kg ECM) verified the recently postulated 30%-reduction potential of 3-NOP. To investigate this, both Hristov et al. (2015b) and Melgar et al. (2020) supplemented 60 mg of 3-NOP/kg of DM mixed into a forage-based diet (60% forages) for 48 and 56 lactating cows and reported a persistent CH_4 inhibition by 31% and 26% over a 12- and 15-week trial period, respectively. In contrast, in our trial, antimethanogenic effects of 3-NOP were identified to be transient in NOPLC group (70% forages). This could be related to the notably higher dietary aNDFom content of approximately 400 g/kg in NOPLC and 340 g in NOPHC group, respectively (Table 2) when compared to 276 (Hristov et al. 2015b) and 318 g of NDF/kg of DM (Melgar et al. 2020). In a meta-analytical approach, Dijkstra et al. (2018) identified that the 3-NOP effect on CH_4 yield decline was impaired by 1.52 ± 0.41% per 10 g/kg DM increase of dietary NDF content in dairy cattle. Correspondingly, the higher aNDFom content in the NOPLC diet could have potentially reduced 3-NOP efficacy by approximately 15% which, however, exceeds the observed 10%-reduction (Table 4). Furthermore, the CH_4 yield of NOPLC was observed to increase to a larger extent per increase in aNDFom content when compared to NOPHC which was, however, only numerical (Fig. 3c; $p = 0.833$). Due to the inverse relationship between dietary NDF content and DMI (Krizsan et al. 2010), a reduced DMI was revealed in LC groups and an increased ruminal digesta retention time and NDF degradation can be assumed in NOPLC which could have resulted in elevated concentrations of rumen H_2 serving as a substrate for the growth of methanogenic archaea. As a consequence, higher amounts/activity of archaeal MCR may have been present in the rumen of NOPLC cows and, besides, archaeal MCR activity is generally considered to temporarily peak immediately after feed intake (Vyas et al. 2016). Furthermore, the highly water-soluble 3-NOP has been shown to be

metabolised in the rumen into two natural fragments (1,3-propanediol and nitrate) by its specific interaction with MCR (Duin et al. 2016). Therefore, 3-NOP has to be supplemented at adequate dosage levels contemporaneously to meal event in order to establish the equilibration between amounts of archaeal MCR and 3-NOP. This was ensured in the present experiment by conducting a two-way supplementation method of 3-NOP via concentrate feeders and mixing in with the basal diet. Both the incorporation of 3-NOP into concentrate pellets (Van Wesemael et al. 2019) and the mixing in with the TMR (Haisan et al. 2014) were considered appropriate whereas 3-NOP application as a pulse-dose (Reynolds et al. 2014) or infusion (Kim et al. 2019) directly into the rumen did not assure its sustained inhibitory potential. Dose–response interrelations were reported by Dijkstra et al. (2018) and indicated a decreased CH_4 yield by 2.48 ± 0.73% per increase of 10 mg of 3-NOP/kg of DM. However, 3-NOP doses only marginally differed between NOPLC and NOPHC in the present study. In addition, Duin et al. (2016) reported that 3-NOP dosage levels, required to inhibit MCR, vary widely between methanogenic archaeal species which may be indicative for differences in their sensitivity towards the inhibitor (Dijkstra et al. 2018). In the present study significant changes concerning fermentation end-products induced by the offered ration type and 3-NOP were exhibited. Hook et al. (2011) showed that species of rumen methanogens differed between high-concentrate and high-forage diets. Changes in the abundance of archaeal species and a diet-dependent 3-NOP efficacy can therefore not be excluded in the present study. Dijkstra et al. (2018) concluded that supplementing higher doses of 3-NOP may affect a greater number of methanogenic archaeal species and hence result in a more substantial CH_4 mitigation.

The combination of 3-NOP with a fibre-rich but hydrogen-releasing diet in NOPLC could have caused an increased ruminal H_2 partial pressure paralleled by a decreased re-oxidation of reduced cofactors and therefore an inhibition of H_2 producing protozoa, fungi and fibrolytic bacteria (Morgavi et al. 2010; Martinez-Fernandez et al. 2014). Accordingly, numerically lower CO_2 emissions were identified in 3-NOP groups which, however, increased contemporaneously to the observed changes in CH_4 emission up to the level of the CON groups (Fig. 2d-f). Therefore, microbial adaptations as well as metabolic changes due to 3-NOP supply and CFP could have occurred, in particular since effects of 3-NOP on bacterial communities in lactating dairy cows were reported to be inconsistent (Lopes et al. 2016; Haisan et al. 2017; Jayanegara et al. 2018). However, the abundance of protozoa living in symbiosis with methanogenic archaea due to interspecies H_2 transfer (Morgavi et al. 2010) remained unaffected in the present trial.

4.2. Changes in rumen fermentation variables

The combined strategy of supplementing a high-concentrate diet with 3-NOP caused an improved 3-NOP efficacy in NOPHC. This was explained by the decreased A:P-ratio reflecting a shift from H_2 liberating to H_2 consuming fermentation pathways and the potential redirection of excessive H_2 from inhibited methanogenesis to alternative sinks. The present experiment confirmed that both 3-NOP and the readily degradable carbohydrates in HC diets decreased the molar proportions of acetate (Van Kessel and Russell 1996; Lopes et al. 2016) but increased that of propionate, butyrate and valerate (Haisan et al. 2014; Lopes et al. 2016; Melgar et al. 2020) which are

thermodynamically favourable for H_2 disposal (Morgavi et al. 2010). Additionally, the deamination of branched-chain amino acids could have been affected as *iso*-valerate increased due to 3-NOP supplementation. Still, *iso*-butyrate remained unaffected as also described previously by Romero-Pérez et al. (2015). Both branched-chain fatty acids originate from the deamination of leucine and valine, respectively (Andries et al. 1987).

NH_3-N concentration was reduced in HC and 3-NOP groups supporting results from a 3-NOP meta-analysis by Jayanegara et al. (2018). 3-NOP supplementation could have inhibited microbial activity due to increased H_2 partial pressure resulting in side-effects, namely a decreased CP (Kohn et al. 2002; Lopes et al. 2016) and NDF digestibility. This may explain the observed increased MU content while NH_3-N concentration, CO_2 emission and acetate proportion were reduced in 3-NOP groups (Morgavi et al. 2010). Increased MU contents could be reflective of decreased milk N efficiency and use of rumen degradable protein even though milk protein was not affected by 3-NOP in the present experiment. Hence, the assignment of individual factors to the observed NH_3-N and MU kinetics is difficult. One can only suggest either a negatively affected recirculation or a more efficient removal of NH_3-N out of the rumen being converted to urea by the liver and excreted via milk in 3-NOP compared to CON groups. The decreased CO_2 emissions in 3-NOP groups confirmed similar results to those stated by Melgar et al. (2020) and may also lead to the assumption of a decreased DM digestibility (Reynolds et al. 2014). Thus, CO_2 emission is directly related to microbial fermentation capacity and was therefore positively correlated to CH_4 emission ($r = 0.767$; $p < 0.001$). Conversely, Hristov et al. (2015b) reported no detrimental 3-NOP effects on CP and total tract digestibility. Accordingly, total VFA and, correspondently, rumen pH remained unaffected in the present study (Table 6). Van Kessel and Russell (1996) assumed a lower CH_4 production and number of methanogens in concentrate-fed than in forage-fed cows when ruminal pH decreased below 6.0. Sampling rumen fluids using an oro-ruminal probe leads to higher pH values because of unavoidable saliva contamination (Duffield et al. 2004). Therefore, it cannot be excluded that at least a temporarily decline in rumen pH could have restricted the pH-sensitive fibrolytic and methanogenic activity resulting in decreased H_2 and CH_4 formation thereby explaining the increased 3-NOP efficacy in NOPHC.

4.3. Animal productivity and energy efficiency

The energy-dense diet in HC groups caused an increased NE_L intake and thereby improved milk performance, milk protein content and BWG when compared to LC groups. Reduced CH_4 intensity was observed with increasing animal productivity which is due to the proportionally lower NE_M at high productivity levels (Beauchemin et al. 2009). The increased milk fat concentration in LC groups may have responded to both the high-forage diet and an increased energy release from depot fat mobilisation which was assumed to be due to a more negative EB when compared to HC groups. As a consequence of reduced energy retention in adipose tissues, LC groups appeared to be more efficient which was illustrated by the lower REI and MEff, whereas FE, not including body mass parameters, was almost similar between HC and LC groups. Haisan et al. (2017) detected FE not being affected by 3-NOP in dairy cows which was confirmed in the present study. However, a reduced efficiency of converting feed energy into milk energy output and an earlier balanced EB

corresponding to the enhanced REI and MEff were observed in NOPHC when compared to CONHC. The less negative EB in NOPHC was attributed to the combination of an overall numerically lower BW throughout the study and a significantly reduced ECM and FCM, whilst DMI expressed as a percentage of BW did not differ between NOPHC and CONHC. The significantly reduced ECM and FCM in NOPHC could be ascribed to the numerically lower NE intake by approximately 12 MJ NE_L/d from week 5 until week 9 p.p. (Fig. 1a).

All of the investigated parameters of body mass, milk and efficiency of energy use were not different between NOPLC and CONLC. Although precursors for gluconeogenesis (propionate) increased due to 3-NOP treatment, BCS and BWG were not significantly affected which confirmed similar results reported by Melgar et al. (2020). To the contrary, Hristov et al. (2015b) assumed that the reduced GE loss from the 3-NOP-induced CH_4 mitigation by 31% was recovered in the observed remarkably increased BWG in mid-lactating cows. It was expected that the transitional and early lactation cows of the present study prioritised energy partitioning towards milk production rather than BWG which could explain the lack of 3-NOP effects on body mass parameters (Kirkland and Gordon 2001). Furthermore, decreases in CH_4 formation do not generally lead to additional energy availability being expended for productive purposes or effects are rather marginal (van Zijderveld et al. 2011). The additional GE spared from 3-NOP inhibited CH_4 formation amounted to 6.5 MJ/d in NOPHC and 4.6 MJ/d in NOPLC (Table 5). An efficiency of 0.65 (MEI:GEI ratio; Table 3) was assumed for the conversion of GE into ME and 0.64 and 0.75 (NRC 2001) for the redirection of the ME spared from CH_4 formation to net energy for milk and BWG, respectively. With regard to energy requirements of 25.5 MJ NE_L/kg of BWG (GfE 2001) and milk energy concentrations of 3.25 MJ/kg in NOPLC and 3.14 MJ/kg in NOPHC (Table 4), the energy spared from methanogenesis would theoretically increase daily milk yield by 0.59 kg and 0.86 kg and BWG by 88 g and 124 g in NOPLC and NOPHC, respectively. However, energy spared from methanogenesis is only useable if the energy-dense rumen H_2, which is not eliminated in methanogenesis, is redirected towards energetically useful fermentation pathways and when GE intake and DM digestibility are not lowered (van Zijderveld et al. 2011).

The present experiment confirmed milk protein content not being affected by 3-NOP (Hristov et al. 2015b; Haisan et al. 2017). The temporarily decreased milk fat content in NOPLC group until week 9 could be attributed to the lowered A:P-ratio. On the one hand, acetate serves as a main carbon source for milk fat synthesis and the enhanced propionate proportion could have reduced circulating non-esterified fatty acids originating from peripartal body fat mobilisation being otherwise converted into milk fat (Bauman and Griinari 2003). On the other hand, increased milk fat could be expected as the proportion of butyrate, a further precursor of the *de novo* milk fat synthesis (Bauman and Griinari 2003), increased due to 3-NOP. Milk lactose concentration was significantly increased in 3-NOP groups and Thiel et al. (2019) proved lactose to be the major metabolite of 3-NOP in the aqueous phase of the milk. 3-NOP is supposed to be metabolised to 3-hydroxypropionic acid which is further metabolised into propionyl-CoA (Thiel et al. 2019), the main carbon source of gluconeogenesis (Aschenbach et al. 2010). The increased propionyl-CoA supply in conjunction with the shift to a more propionic-typed fermentation pathway could have

increased substrate availability for the synthesis of glucose which in turn could have been directed to milk lactose synthesis (Thiel et al. 2019).

5. Conclusions

Our study provides the first long-term experiment including the peripartal period of high-yielding dairy cows in which the CH_4 inhibitor 3-NOP was combined with varying CFP. The hypothesis that feeding high CFP in combination with 3-NOP synergistically decreases CH_4 emissions was verified. However, 3-NOP efficacy in high-forage diets needs to be further investigated in long-term dose-response experiments with dairy cows at different lactation states. The hypothesis that energy spared from CH_4 formation and increased amounts of glucogenic VFA induced by 3-NOP treatment are utilised by peripartal dairy cows for an improved energy conversion and recovery in productive parameters was rejected.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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5. Paper II

Schilde, M.; von Soosten, D.; Frahm, J.; Kersten, S.; Meyer, U.; Zeyner, A.; Dänicke, S.

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.

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Article

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

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Abstract: Methanogenesis in ruminants contributes to both greenhouse gas emissions and feed energy losses whereby the latter becomes specifically important in energy-deficient periparturient cows. It was hypothesized that increased concentrate feed proportions (CFP) and feeding with the methane inhibitor 3-nitrooxypropanol (3-NOP), as well as their potential synergism, improve the energy status of peripartal cows. Periparturient dairy cows were fed low or high dietary CFP either tested without or combined with 3-NOP. The GreenFeed system was used to calculate the metabolic respiration quotient ($RQ_{metabolic}$) and tissue energy retention (ER_{tissue}) by methods of indirect calorimetry. The calorimetrically estimated ER_{tissue} coincided with a conventionally calculated energy balance except for the antepartal period. Neither CFP nor 3-NOP affected the ultrasonographically assessed lipomobilization in adipose depots. In the group fed 3-NOP and a high concentrate feed proportion, the $RQ_{metabolic}$ significantly rose over the course of the experiment and the ER_{tissue} was also increased. Serum non-esterified fatty acid concentrations were lower in the 3-NOP groups albeit ß-hydroxybutyrate (BHB) remained unaffected. Higher CFP reduced BHB and increased blood glucose levels. In conclusion, 3-NOP and high CFP improved the energy budget of the cows in an interactive manner, which was, however, not apparent in all of the examined parameters. The application of the GreenFeed system for indirect calorimetry is a promising approach, which needs further validation in the future.

Keywords: dairy cows; methane production; 3-nitrooxypropanol; GreenFeed; indirect calorimetry; energy metabolism

1. Introduction

In ruminants, feed is mainly converted to volatile fatty acids (**VFA**) by the rumen microbiota, thereby yielding hydrogen (H_2) and carbon-dioxide (CO_2), which are redirected to methane (CH_4) formation in methanogenic archaea [1]. 3-nitrooxypropanol (**3-NOP**), a structural analogue of methyl-coenzyme M, is currently supposed to be one of the most potent CH4 inhibitors in cattle [2,3]. The CH4-mitigating effect of 3-NOP potentially amounts to 39.0 ± 5.40% in dairy cows [4] but ranges widely from 7 [5] to 60% [6] depending on the provided ration type (neutral-detergent fibre (**NDF**) content), administration technique (mixing in with the total-mixed ration (**TMR**), infusion, pulse-dose) and dosage level [4].

Besides the ecological benefits of reducing greenhouse gas emissions from ruminant livestock [7], CH4 mitigation is assumed to improve feed energy efficiency as up to 12% of the gross energy intake (GEI) can be lost by methanogenesis in the bovine rumen [8]. Furthermore, both increased dietary concentrate feed proportion (**CFP**) [9] and 3-NOP [4] were observed to shift rumen fermentation to H2-consuming propionic-

metabolic typed pathways [4,9] which could increase the hepatic supply of glucogenic precursors [10], with this being specifically advantageous in periparturient cows. Hence, transitional dairy cows metabolically adapt to the negative energy balance (**EB**), which is the disparity between energy intake and requirements for maintenance and lactogenesis, by induction of an accelerated lipolysis in adipose tissue (**AT**) depots [11]. Subsequently, the massive hepatic influx of non-esterified fatty acids (**NEFA**) risks a metabolic overload of the hepatic capacity for NEFA oxidation, which results in increased ketone body synthesis and predisposition of the cow to hyperketonaemia and hepatic lipidosis [11,12].

Reynolds et al. [5] identified a decreased total EB and increased heat production (**HP**) when energy metabolism was expressed as a percentage of digested energy in dairy cows supplemented with 2500 mg of 3-NOP per day. In contrast, van Gastelen et al. [13] reported that HP and energy retention in body fat and protein remained unaffected in 16 early-lactation dairy cows supplemented with 51 mg of 3-NOP/kg dry matter (**DM**). Correspondingly, energy allocation to body weight gain (**BWG**), representing a positive EB at the tissue level, was observed to be either increased [2,13] or not affected in 3-NOP-fed dairy cows, as reported by Haisan et al. [14] and in the accompanying manuscript [15]. 3-NOP was comprehensively reported to exert no influence on energy expenditure (EE) for milk production [16] even though varying 3-NOP effects on milk composition were published [13,16,17]. However, the effects on milk ingredients were attributed to the aforementioned 3-NOP-induced shift in the rumen fermentation pattern toward a decreased acetate–propionate ratio [18] rather than being associated with alterations in post-ruminal energy metabolism [17]. Accordingly, excessive accumulation of NEFA and ß-hydroxybutyrate (**BHB**) in blood, being indicative of a severe negative EB [19], were not affected in early- [20] and mid-lactating [14] 3-NOP-supplemented cows.

However, there is a gap in the knowledge of the energy turnover at the tissue level and intermediary pools of correspondingly regulated blood metabolites in cows provided varying CFP combined with 3-NOP in their rations during the periparturient period. Gas exchange measurements of CO_2 production and O_2 consumption in dairy cows are commonly measured in the respiration chamber (**RC**), which is often referred to as the 'gold-standard' technique. These gas measurements allow for an indirect calorimetric estimation of the HP and the total ($\mathbf{RQ_{total}}$) and metabolic (rumen fermentation corrected) respiration quotient ($\mathbf{RQ_{metabolic}}$). The RQ_{total} mirrors the whole animal metabolism including feed nutrient degradation in the rumen, whereby the $RQ_{metabolic}$ rather reflects the intermediary oxidation of specific macronutrients and, therefore, dynamics in physiological and nutritional adaptations [21]. Accordingly, $RQ_{metabolic}$ values of 1.0 mirror a prevailing carbohydrate oxidation whereas those of fat oxidation and deposition amount to 0.71 and above 1.0, respectively. Protein oxidation is associated with $RQ_{metabolic}$ values of 0.81 [22]. However, the costly gas quantification in RC restricts the cow's normal behaviour and only allows the measurement of small animal numbers over short-term periods [23]. Therefore, the present approach aimed to use spot gas flux measurements of CH_4, CO_2 and O_2 from the open-circuit GreenFeed (**GF**) system (C-Lock Inc., Rapid City, SD, USA) for the indirect calorimetric estimation of EE for maintenance, production and energy retention in body tissues ($\mathbf{ER_{tissue}}$) in periparturient dairy cows provided 3-NOP and varying CFP in the ration. This assessment of cow energetics was accompanied by an ultrasonic-based estimation of lipomobilization from AT depots in concert with frequent blood sampling for analyses of energy-related metabolites.

It was hypothesized that increased glucogenic propionate (Schilde et al. 2021) and energy spared from methanogenesis due to feeding 3-NOP in combination with high CFP caused a surplus of energy being utilized to cope with the negative EB in periparturient dairy cows, which was reflected by the decreased lipomobilization from AT depots and serum concentrations of NEFA and BHB.

2. Materials and Methods

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) in Brunswick, Germany in accordance with the German Animal Welfare Act and approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Oldenburg, Germany) (33.19-42502-04-15/1858).

2.1. Experimental Design

The presented investigations are part of a comprehensive experiment: the fundamental experimental design, CH_4 and CO_2 emissions, dry matter intake (**DMI**), rumen VFA, BW measures, EB according to the 'Gesellschaft für Ernährungsphysiologie' (**GFE**) [24] (**EB**$_{GFE}$), milk performance parameters and feed efficiency are presented in Schilde et al. [15]. During the present experiment, 55 pluriparous German Holstein cows were assigned to four groups according to a 2 × 2-factorial design. In this context, treatments of low (**LC**) or high (**HC**) CFP were tested without supplements (**CONLC** (*n* = 14), **CONHC** (*n* = 15)), or combined with 3-NOP (DSM Nutritional Products AG, Kaiseraugst, Switzerland) (**NOPLC**, 48.4 mg/kg dry matter (**DM**) (*n* = 14); **NOPHC**, 51.2 mg 3-NOP/kg DM (*n* = 12)) in the ration from d 28 ante-partum (**a.p.**) until d 120 post-partum (**p.p.**). The whole experimental period (d 28 a.p. until d 120 p.p.) was split into three periods, namely the ante-partum (Per 1: d 28 a.p. until parturition) and post-partum (Per 2: d 1 until d 28 p.p.) phase of the transition period and the early-lactation period (Per 3: d 29 until d 120 p.p.) in order to compare metabolic and respiratory changes assessed by indirect calorimetry with energy expenditures and supply between the different periods. The experimental groups were balanced for calculated date of calving, 4% fat-corrected milk yield (FCM) in their previous lactation (6207 ± 1248 kg; mean ± SD), body condition score six weeks ante-partum (a.p.) (3.3 ± 0.4) and number of lactations (3.0 ± 1.1). The cows were housed in a free stall barn with high bed cubicles and slatted floor. Three cows out of the initial 58 did not complete the trial (two cases of abomasal displacement and one case of necrotising endometritis in the NOPLC group). Ten out of the 55 cows were cannulated (three cows in each of the 3-NOP and two cows in each of the CON groups). During the a.p. period, CFP amounted to 15% for LC and 40% for HC groups. Starting from the day of parturition until d 21 p.p., a gradual increase in CFP from 30 to 55%, where it remained until the end of the experiment, was scheduled in HC groups. In LC groups, CFP was fixed at 30% from the day of parturition until termination of the trial. The target CFP was administered by computerized self-feeding stations.

Cows were offered a partial mixed ration (**PMR**) for ad libitum intake in weighing troughs (type RIC, Insentec B.V., Marknesse, the Netherlands) which was composed of 70% maize silage, 20% grass silage and 10% of a pelletized concentrate including either 3-NOP or the placebo (DM basis). Further 3-NOP compound was incorporated into pelletized concentrates, which was provided by the concentrate feeders to adjust the aforementioned 3-NOP target concentration. This two-way method of supplementing concentrate pellets, including 3-NOP via mixing with the PMR and the concentrate feeders, facilitated the regulation of 3-NOP target consumption and the 3-NOP supply synchronously to the meal event [15]. The CONLC and CONHC groups received a placebo in the concentrate feed pellets that contained propylene glycol, with SiO_2 also being part of the 3-NOP supplement.

2.2. Sample Collection

The DMI of the PMR and concentrates was continuously monitored by the computerized weighing troughs and concentrate feeders. Concentrates and PMR were sampled once and twice a week and pooled to collective samples of four-week periods, respectively.

Continuously from d 28 a.p. until d 120 p.p., gas samples were collected from the exhaust air pipe to measure gas mass fluxes (g/d) of O_2 consumption and CH_4 and CO_2 emissions using the GF system (C-Lock Inc., Rapid City, SD, USA) as described previously [15].

Rectal temperature was measured each time before blood sampling. Blood samples were taken by puncturing a Vena jugularis externa at d 28, 14, 7, 3 a.p. and d 1, 3, 7, 14, 21, 28, 35, 49, 73, 98, and 120 p.p. after morning milking using heparinized sample syringes (Werfen GmbH, Kirchheim, Germany) and serum tubes. Serum tubes were allowed to clot for 30 min at 303 K, were subsequently centrifuged (Heraeus Varifuge 3.0R Heraeus Instruments GmbH, Hanau, Germany; 2123× *g*, 288 K, 15 min), and separated serum was stored at −80 °C until further analyses were conducted.

According to Raschka et al. [25], ultrasonic measurements (**UM**) of fat layer thickness in millimetres were conducted in duplicate at the seven topographic points on the right side of the cow at d 3 and 28 p.p. with the use of a Mindray M5 Vet (Mindray, Shenzhen, China) diagnostic ultrasound system equipped with

a linear (6 MHz, Mindray 6LE5Vs) and a convex probe (3 MHz, Mindray 3C5s). The description of the seven topographic measurement points was detailed in Schäfers et al. [26].

2.3. Analyses

Samples of concentrates and PMR components were dried for 72 h at 55 °C, ground to pass a 1-mm screen (SM 1, Retsch GmbH, Haan, Germany) and analysed for chemical composition according to the standard methods published by the Association of German Agricultural Analysis and Research Centers [27]. The chemical composition of the experimental diets is illustrated in Table 1. The 3-NOP contents in concentrate feed samples were analysed by DSM Nutritional Products AG, Kaiseraugst, Switzerland.

Table 1. Chemical composition, peNDF and energy (means) of the total rations offered during the experimental period from d 28 ante-partum until d 120 post-partum (reproduced from and with permission from Schilde et al. [15] at Taylor & Francis Group https://www.tandfonline.com/).

Item	CON †		3-NOP §	
	LC	HC	LC	HC
DM + (g/kg)	467	582	467	597
Nutrient (g/kg of DM)				
Crude ash	63	61	63	61
Crude protein	130	138	129	140
Utilizable crude protein	142	150	142	151
Ether extract	32	35	32	36
$aNDF_{om}$ ¶	402	344	404	337
Acid detergent $fibre_{om}$	226	191	227	187
$peNDF_{>8\ mm}$ #	268	269	274	273
Starch	249	303	246	307
Sugar	17	25	17	27
Energy $ (MJ/kg of DM)				
GE	18.4	18.5	18.4	18.4
ME	11.0	11.5	11.0	11.5
NE_L	6.6	7.0	6.6	7.1

† Control (CON) groups were provided a placebo and low (LC) or high (HC) concentrate feed proportion in the ration. § 3-nitrooxypropanol (3-NOP) groups were supplemented with 48.4 and 51.2 mg of 3-NOP/kg of DM and LC and HC in the ration. +DM, dry matter. ¶ $aNDF_{om}$, α-amylase treated neutral detergent fibre without residual ash. # $peNDF_{>8\ mm}$, physically effective NDF in the partial mixed ration defined as the proportion of DM retained by a 8-mm screen multiplied by the dietary NDF content [28]. $ Calculations for concentrates based on table values according to DLG [29], silages according to VDLUFA [27] analyses and GE calculated according to GfE [30].

Gas samples and background gases were analysed by the already installed GF sensors. CH_4 and CO_2 concentrations were analysed by non-dispersive infrared absorption sensors and O_2 was analysed using a paramagnetic sensor. Sensor calibration was performed automatically on a daily basis using a zero (O_2 = 200,000 mg/kg, N_2 = 800,000 mg/kg) and a span gas (CH_4 = 1004 mg/kg, CO_2 = 10,000 mg/kg, O_2 = 210,000 mg/kg H_2 = 9.50 mg/kg, H_2S = 9.80 mg/kg, while the remainder of the gas was nitrogen). The air velocity in the pipe was measured by an anemometer to determine total mass flow of all gases. CO_2 recovery tests were conducted once a month (recovery rate ± SD: 101% ± 5.7). The amount of bait feed delivered per feed drop was calibrated on a weekly basis.

Serum samples were photometrically analysed (Indiko Plus, Thermo Scientific GmbH, Dreieich, Germany) for concentrations of BHB, NEFA, triacylglycerides (**TAG**) and glucose. An automated blood gas and electrolyte analyser (GEM Premier 4000, Werfen GmbH, Kirchheim, Germany) was used to determine the temperature-corrected pH, hydrogen carbonate ions, haemoglobin and lactate concentrations immediately after sample collection.

2.4. Calculation of Energy Metabolism Parameters by Indirect Calorimetry and Ultrasonography

Processing and validation of gas exchange data were conducted by C-Lock Inc. Gas measurements were converted from g/d to L/d according to the gas density of 0.717 kg/m3 for CH4, 1.977 kg/m3 for CO2 and 1.729 g/m3 for O2 under standard conditions (1013.25 hPa). The cow´s visiting time and head position in the GF were used to check for data plausibility [31]. Daily means of GF data were averaged to weekly means using the previously described arithmetic averaging method [32]. Due to technical reasons, O2 consumption in CON groups was estimated from weekly means of CO2 production and DMI using the following regression equation:

$$O_2\,(g/d) = 2056 - 72.5 \times \text{dry matter intake (kg/d)} + 0.62 \times CO_2\,(g/d) \quad (1)$$

with $R^2 = 0.90$ and a residual standard error (**RSE**) of 371 g/d on 337 degrees of freedom.

In ruminants, total CO_2 production (**VCO_2**) is the sum of fermentative (**$VCO_{2fermentative}$**) and metabolic (**$VCO_{2metabolic}$**) CO_2 derived from microbial fermentation in the rumen and the intermediary metabolism, respectively [33]. A differentiation between the two is essential in order to refer to the intermediary substrate oxidation [34]. As proposed by Chwalibog et al. [33], VCO2fermentative was calculated by applying the stoichiometrically derived factor of 1.7, which was confirmed to be applicable for a variety of ration compositions [35].

$$VCO_{2fermentative}\,(L/d) = 1.7 \times VCH_4\,(L/d) \quad (2)$$

Then, $VCO_{2fermentative}$ was subtracted from VCO_2 to obtain $VCO_{2metabolic}$, which was used to calculate the $RQ_{metabolic}$ mirroring the intermediary oxidation of the macronutrients of carbohydrates, fat and protein [36]:

$$RQ_{metabolic} = VCO_{2metabolic}\,(L/d) \div VO_2\,(L/d) \quad (3)$$

The total RQ (RQ_{total} = total VCO_2 production (L/d) ÷ VO_2 consumption (L/d)) reflected the cow´s nutritional plane. Gross energy (**GE**) content of the feedstuffs was calculated according to GfE [24]. The metabolizable energy (**ME**) content of the concentrates was derived from tabular values according to DLG [29] and that of silages was derived according to VDLUFA [27] analyses.

HP was quantified according to the Brouwer [22] formula:

$$HP\,(kJ) = 16.18 \times VO_2\,(L/d) + 5.02 \times VCO_2\,(L/d) - 2.17 \times VCH_4\,(L/d) - 5.99 \times N_U\,(g/d), \quad (4)$$

whereby urinary nitrogen excretion (**N_U**) was set to 50 g/d [37] even though the real NU in dairy cows varies between 75 and 150 g/d [38]. However, the NU contribution to HP is negligible and an error of about 0.3% in the absolute HP values was accepted [39].

Methane energy (**CH_4E**; MJ/d) was derived from the multiplication of the energy equivalent value of 39.54 kJ/L of CH4 [22] and the daily CH_4 production (L/d).

The partitioning of EE for energy retention (**ER**) was computed as follows:

$$\text{ER in body tissues and milk } (ER_{total})\,(MJ/d) = \text{ME intake} - HP \quad (5)$$

$$\text{ER in body tissues } (ER_{tissue})\,(MJ/d) = \text{ME intake} - HP - ME_E - NE_P \quad (6)$$

Analyses and calculations of milk energy excretion (**ME_E**; MJ/d) according to GfE [24] were used from Schilde et al. [15]. Net energy demand for pregnancy (NEP; MJ/d) was averaged for period 1 according to constants proposed by GfE [24] with 13 MJ NEL/d for week 4 a.p. and 18 MJ NEL/d for week 3 a.p. until calving resulting in an average of 17 MJ NE_L/d for period 1. The **EB_{GFE}** data were extracted from Schilde et al. [15] in which EBGFE was calculated according to GfE [24].

The residual ER in body protein and intramuscular fat was assessed as:

$$ER_{residual}\,(MJ/d) = ER_{tissue} - ER_{fat\ depot} \quad (7)$$

$ER_{fat\ depot}$ was calculated from UM as described in the following:

$$ER_{fat\ depot}\,(MJ/d) = \text{daily mobilized fat depot masses (kg/d)} \times 39.8\,(MJ/kg) \times 0.84 \quad (8)$$

The daily mobilization of fat depot masses from each AT depot was described by the difference in AT masses between d 3 p.p. and d 28 p.p. divided by the number of days. The energy release from mobilized fat depots being used for milk production was calculated based on the assumption that 1 g of body fat corresponds to 39.8 kJ of GE [22], whereby 16% is lost as heat energy when body tissue energy is used for milk synthesis [40].

Depot masses of each AT, namely the retroperitoneal (**RAT**), mesenteric (**MAT**) and omental (**OAT**), collectively referred as the visceral AT (**VAT**), and the subcutaneous AT (**SAT**) were estimated in kg from ultrasonographically measured distances of the different sites as described in Schäfers et al. [26] according to the following regression equations established by Raschka et al. [25]:

$$SAT = -6.66 + 0.72 \times R12 + 0.31 \times AW3c \quad (9)$$

$$RAT = -9.55 + 0.62 \times R12 + 0.06 \times KD3b \quad 5$$

$$OAT = -2.32 + 0.55 \times BFT + 0.37 \times AW3b \quad (11)$$

$$MAT = -12.8 + 0.38 \times AW1b + 1.73 \times AW3b - 1.45 \times AW3c + 0.07 \times KD2c \quad (12)$$

$$VAT = RAT + OAT + MAT \quad (13)$$

The efficiency of utilization of ME for lactation (k_l) was calculated according to AFRC [41]:

$$k_l = (ME_E + a \times ER_{tissue})/(ME\ intake - ME_m), \quad (14)$$

where ME_E is adjusted to zero energy balance with a coefficient of a = 0.84 for negative ER_{tissue} or a = 1/0.95 for positive ER_{tissue}. ER_{tissue} is the energy balance obtained by indirect calorimetry using the GreenFeed system. The maintenance requirement (**ME_m**) was estimated using the equation from GfE [24]:

$$ME_m\ (MJ/d) = 0.488 \times BW^{0.75}\ (kg), \quad (15)$$

where $BW^{0.75}$ is the metabolic body weight.

2.5. Statistical Analyses

Prior to statistical evaluation, means were calculated per cow and week for variables used in indirect calorimetry. A.p. blood samples were retrospectively assigned to the actual day relative to parturition by tolerating a deviation of 24 h for the d -3 sample and a deviation of 2 days for the d −7 and d −14 samples. Due to gas leakage through the fistula, cannulated cows were excluded from statistics except for blood and ultrasonic variables. The statistical analyses were conducted using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA) and a repeated measures mixed model (PROC MIXED) fitted by a restricted maximum likelihood [42]. The sequence of day, week of sampling or period (PER) was a repeated measure. 3-NOP, CFP, time relative to parturition and the interaction between them were set as fixed effects and each cow within treatment was set as a random effect. Data on indirect calorimetry and gas measurements were evaluated according to periods fixed at d 28 a.p. until parturition (period 1), d 1 until d 28 p.p. (period 2) and d 29 until d 120 p.p. (period 3). For clinical chemistry parameters, the autoregressive variance–covariance structure was selected based on the best fit according to the lowest Akaike Information Criterion and the result of the first measurement at d 28 before 3-NOP supplementation was regarded as a covariate. Parameters of indirect calorimetry and ultrasonic measurements were tested using a compound symmetry structure. Effects were regarded as statistically significant at p-values ≤ 0.05 and a trend was implied at p-values between 0.05 and 0.10. Multiple t-tests (PROC PDIFF) with Tukey-adjusted p-values were computed to evaluate significant means.

The R software package (version 3.6.1, R Foundation for Statistical Computing, Vienna, Austria) was used to calculate Pearson correlation coefficients and to perform linear regression of EB_{GFE} and ER_{tissue} data. Further, R was applied to estimate the O_2 consumption in CON groups in a linear regression model,

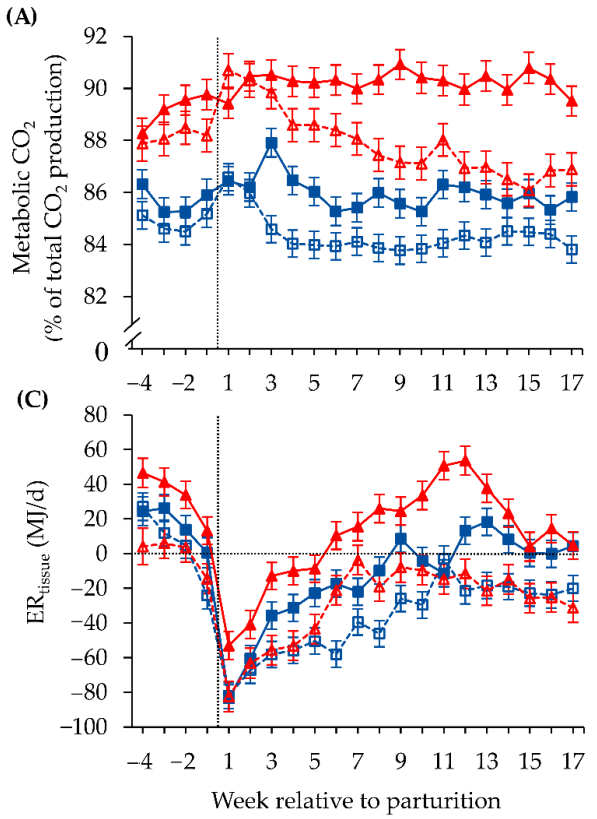
whereby significant independent variables and related regression coefficients were estimated in a forward stepwise manner.

The degree of agreement between both methods to estimate the energy balance (ER_{tissue} and EB_{GFE}) was assessed according to Bland and Altman [43]. The differences between EB_{GFE} and ER_{tissue} were plotted against the arithmetic mean of these pairs at each cow and period. The bias as the mean difference including a 95% confidence interval and the standard deviation (**SD**) of the differences were calculated. The lower and upper limits of agreement (**LoA**) were calculated (bias ± 1.96 SD) and used to define the range within which 95% of the differences lay. A regression line was plotted through the differences to detect changes in the bias depending on the magnitudes of the measurements themselves. The normality of differences was tested using the Shapiro–Wilk test in R.

3. Results

3.1. Methane Emission and Respiratory Gas Exchange Measured with the GreenFeed system

Data on the emitted fermentation gases and metabolic respiratory gas exchange are presented in Table 2 while detailed dynamics of VCH_4 and VCO_2 emissions on a weekly basis are presented in Schilde et al. [15]. The $VCO_{2metabolic}$ expressed as a percentage of total VCO_2 decreased after parturition until week 4 p.p. in the LC groups, as depicted in Figure 1A. $VCO_{2metabolic}$ in NOPLC continued to decline over the course of the experiment (Figure 1A; 3-NOP × CFP × PER; $p < 0.001$). The mean VCH_4 over the three periods was reduced by 24.2% in NOPLC and 29% in NOPHC when compared to the respective CON group (Table 2). VCH_4 increased from an average of 315 L/d in period 2 by 27.7% to 438 L/d in NOPLC and by 8% to 341 L/d in NOPHC in period 3 and, therefore, to a greater extent in the NOPLC than in the NOPHC group. VCH_4 (L/d) positively correlated to VCO_2 (L/d) (r = 0.67; $p < 0.001$; N = 917). Both VCH_4 and VCO_2 production (total and metabolic VCO_2) decreased from the a.p. period to parturition but increased thereafter over the p.p. period, which was also the case for VO_2 consumption (Table 2). In period 3, VCO_2 and VCH_4 emissions were affected by 3-NOP and the high CFP (Table 2; 3-NOP × CFP × PER; $p < 0.001$). Hence, VCO_2 was significantly higher in CONHC, whereas VCH_4 decreased, which was most apparent for NOPHC. VCO_2 production was positively correlated to VO_2 consumption (r = 0.92; $p < 0.001$; N = 915). VO_2 consumption decreased in the 3-NOP groups over the course of the experiment (Table 2; 3-NOP × PER; $p < 0.001$), whereas CFP did not exert an influence. Both the RQ_{total} and $RQ_{metabolic}$ were affected by the 3-NOP × CFP × TIME interaction (Table 3; $p < 0.001$). The $RQ_{metabolic}$ markedly dropped from approximately 0.92 ± 0.03 during the a.p. period to its lowest point of 0.90 ± 0.007 in week 1 p.p. (Figure 1B; Table 2). Afterwards, the $RQ_{metabolic}$ increased to 0.99 in NOPLC and 0.94 in CONLC until week 4 p.p. and remained more or less constant. In contrast, the $RQ_{metabolic}$ continued to slightly increase to 1.01 in NOPHC and 0.98 in CONHC until week 9 p.p., respectively (Table 2; Figure 1B; 3-NOP × CFP × TIME; $p = 0.024$).



Variable	PSEM[§]	*p*-values[†] 3-NOP × CFP	3-NOP × TIME	CFP × TIME	3-NOP × CFP × TIME
Metabol. CO_2	0.222	0.476	0.003	<0.001	<0.001
Metabol. RQ	0.003	0.781	<0.001	0.004	0.024
$ER_{tissues}$	0.959	0.006	0.055	0.097	0.283

[†]effects of 3-NOP, CFP and TIME with p <0.001 for all variables.
[§]PSEM, pooled standard error of the means.

Figure 1. Effects of 3-nitrooxypropanol (3-NOP), dietary concentrate feed proportion (CFP) and time relative to parturition (TIME) on (**A**) metabolic CO_2 production, (**B**) metabolic respiration quotient (Metabolic RQ) and (**C**) energy retention in body tissues (ER_{tissue}) in peripartal dairy cows. ■, solid line = control high CFP (CONHC, n = 12); □, dashed line = control low CFP (CONLC, n = 13); ▲, solid line = 3-NOP high CFP (NOPHC, n = 11); Δ, dashed line = 3-NOP low CFP (NOPLC, n = 9). Values are presented as LS-means.

Table 2. Fermentation and respiration gases (L/d; measured using the GreenFeed system), total (RQ_{total}) and metabolic respiration quotient ($RQ_{metabolic}$) of the experimental groups during period (Per) 1 (d 28 ante-partum until day of calving), 2 (d 1 until d 28 post-partum (p.p.)) and 3 (d 29 until d 120 p.p.).

	Treatments [†]					*p*-Values [§]				
Item	**CONHC (*n* = 12)**	**CONLC (*n* = 13)**	**NOPHC (*n* = 11)**	**NOPLC (*n* = 9)**	**SEM**	**3-NOP**	**CFP**	**3-NOP ×PER**	**CFP ×PER**	**3-NOP ×CFP ×PER**
VCH_4 production										
Per 1	490	495	381	397	20	<0.001	0.156	<0.001	<0.001	<0.001
Per 2	440	475	314	316						
Per 3	535	542	341	438						
Total VCO_2 production										
Per 1	6632	6378	6662	6369	160	0.064	0.132	<0.001	0.282	<0.001
Per 2	6368	6289	6002	5823						
Per 3	7278	6719	6557	6553						
$VCO_{2metabolic}$ [+]										
Per 1	5798	5537	6014	5695	140	0.653	0.041	<0.001	0.043	0.003
Per 2	5621	5481	5468	5285						
Per 3	6368	5797	5978	5809						
VO_2 consumption										
Per 1	6348	6190	6267	6205	138	0.073	0.436	<0.001	0.342	0.166
Per 2	5988	5933	5709	5693						
Per 3	6480	6167	5910	5911						
$RQ_{metabolic}$ [¶]										
Per 1	0.91	0.90	0.96	0.92	0.007	<0.001	<0.001	<0.001	0.014	<0.001
Per 2	0.94	0.92	0.95	0.93						
Per 3	0.98	0.94	1.01	0.99						
RQ_{total} [#]										
Per 1	1.04	1.03	1.06	1.03	0.008	0.638	0.004	0.001	0.602	<0.001
Per 2	1.06	1.06	1.05	1.02						
Per 3	1.12	1.09	1.11	1.11						

[†] Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), time period relative to parturition (PER), and interactions between them; effect of PER with $p < 0.001$ and 3-NOP×CFP with $p > 0.1$ for all variables. [+] $VCO_{2metabolic}$ (L/d) = Total VCO_2 production (L/d) − $VCO_{2fermentative}$ (L/d), whereby $VCO_{2fermentative}$ (L/d) = 1.7 × VCH_4 production (L/d). [¶] $RQ_{metabolic}$ = $VCO_{2metabolic}$ (L/d) ÷ VO_2 (L/d), corrected for fermentative VCO_2. [#] RQ_{total} = VCO_2 (L/d) ÷ VO_2 (L/d).

3.2. Energy Turnover Estimated by Indirect Calorimetric and Ultrasonic Methods

The parameters of $BW^{0.75}$, GEI, metabolizable energy intake (**MEI**), EE and ER are presented in Table 3. GEI and MEI significantly increased in the HC groups by an average of 28% from period 1 to period 2 and by 31% up to period 3, whereas in the LC groups, GEI and MEI increased, on average, by 35% from period 1 to period 2 and by 23% up to period 3 (CFP × PER; $p < 0.001$). The experimentally intended gradual increase in energy intake resulted, during period 3, in significantly higher daily GE and ME uptakes in the HC groups, by about 0.4 MJ and 0.32 MJ/kg $BW^{0.75}$ and d, respectively, when compared to the LC groups.

Table 3. Energy intake, expenditure and retention of the experimental groups during period (Per) 1 (d 28 ante-partum until day of calving), Per 2 (d 1 until d 28 post-partum (p.p.)) and Per 3 (d 29 until d 120 p.p.) estimated according to gas measurements presented in Table 4.

Item	Treatments †				SEM	*p*-Values §					
	CONHC (*n* = 12)	CONLC (*n* = 13)	NOPHC (*n* = 11)	NOPLC (*n* = 9)		3-NOP	CFP	3-NOP ×CFP	3-NOP ×PER	CFP ×PER	3-NOP ×CFP ×PER
Metabolic body weight (kg $BW^{0.75}$)											
Per 1	146	144	144	146	2.9	0.780	0.868	0.517	0.227	<0.001	0.417
Per 2	132	131	129	131							
Per 3	131	127	128	128							
Energy intake (kJ/kg $BW^{0.75}$ and d)											
Gross energy intake (GEI)											
Per 1	1963	1812	2022	1738	64	0.546	<0.001	0.210	0.146	<0.001	0.092
Per 2	2468	2434	2620	2335							
Per 3	3272	2900	3379	2961							
Metabolizable energy intake (MEI)											
Per 1	1193	1076	1288	1031	40	0.325	<0.001	0.110	0.379	<0.001	0.084
Per 2	1529	1458	1631	1402							
Per 3	2032	1734	2111	1769							
Energy expenditures (kJ/kg $BW^{0.75}$ and d)											
Net energy demand for pregnancy (NE_P)											
Per 1	115	117	118	116	2.9	0.702	0.951	0.575			
Milk energy excretion (ME_E) ¶											
Per 2	978	991	945	960	25	0.195	0.492	0.476	0.852	<0.001	0.056
Per 3	1010	931	948	935							
Heat production (HP) #											
Per 1	916	905	930	896	17	0.028	0.211	0.738	<0.001	0.750	0.267
Per 2	967	966	940	918							
Per 3	1071	1044	1001	987							
Methane energy (CH_4E) ‡											
Per 1	134	137	106	106	5.3	<0.001	0.099	0.913	<0.001	<0.001	<0.001
Per 2	132	144	96	95							
Per 3	161	170	106	134							
Energy retention (kJ/kg $BW^{0.75}$ and d)											
Energy retention in body tissues and milk (ER_{total}) ◊											
Per 1	295	183	372	125	41	0.084	<0.001	0.137	0.002	<0.001	0.176
Per 2	562	492	697	485							
Per 3	961	690	1103	783							
Energy retention in body tissues (ER_{tissue}) ◆											
Per 1	170	65	254	6	45	0.036	<0.001	0.101	0.003	0.022	0.715
Per 2	−396	−499	−222	−474							
Per 3	−23	−230	167	−139							
Energy balance calculated according to GfE [24] (EB_{GFE}) ‡											
Per 1	323	245	379	216	28	0.104	<0.001	0.077	0.066	0.059	0.982
Per 2	−323	−397	−229	−398							
Per 3	−58	−171	54	−154							
Energy retention in fat depots ($ER_{fat\ depot}$) ‖											
Per 2	−185	−175	−169	−181	24	0.841	0.974	0.637			
Residual energy retention ($ER_{residual}$) Π											
Per 2	−211	−324	−55	−292	48	0.058	0.001	0.205			

† Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. § Effects of 3-NOP, concentrate feed proportion (CFP), time period relative to parturition (PER), and interactions between them; effect of PER with $p < 0.001$ for all variables. ⁺NE_P (MJ/kg) = mean of 13 MJ of NE_L/d in week 4 a.p. and 18 MJ of NE_L/d during week 3 until parturition according to GfE [24]. ¶ ME_E (MJ/kg) data from Schilde et al. [15]. # HP (MJ/d) = 16.18 × O_2 (L/d) + 5.02 × CO_2 (L/d) − 2.17 × CH_4 (L/d) − 5.99 × 50 (g of urine nitrogen excretion/d) [22], gas volumes used from Table 4. ‡ CH_4E (MJ/kg) = CH_4 (L/d) × 0.03954 (MJ/L) [22]. ◊ ER_{total} (MJ/d) = MEI − HP. ◆ ER_{tissue} = MEI − HP − NE_P and ME_E, resp. ⁑ EB_{GFE} (MJ/d) = energy balance data from Schilde et al. [15] and calculated according to GfE [24] (footnote Figure 2). ‖ $ER_{fat\ depot}$ (MJ/d) = loss of fat depot masses (kg/d) from d 3 until d 28 p.p. from ultrasonic measurements × 39.8 (MJ/kg) × 0.84, 1 kg of body fat corresponds to 39.8 MJ of GE [22], whereby 16% is lost as heat when body tissue energy is converted into milk [40], 1 kg of body fat corresponds to 39.8 MJ of GE [22]. ⊓ $ER_{residual}$ (MJ/d) = ER_{tissue} − $ER_{fat\ depot}$.

The 3-NOP × PER interaction ($p < 0.001$) of HP was driven by a decreasing HP from 3-NOP when compared to the CON groups in period 3 (Table 3). HP was positively correlated with MEI, which was not different between treatment groups (r = 0.37; $p < 0.001$; N = 895. During the course of the experiment, ME_E decreased in the LC groups, whereas that of the HC groups increased (CFP × PER; $p < 0.001$). With regard to period 3, CH_4E was lowest in NOPHC, in contrast with the NOPLC and the CON groups (3-NOP × CFP × PER; $p < 0.001$).

During the course of the experiment ER_{total} and ER_{tissue} increased with elevated CFP in the diet (Table 3; CFP × PER; $p < 0.05$). ER_{tissue} was more positive in the NOPHC group over the experimental periods (Table 3; Figure 1C: 3-NOP × CFP; $p = 0.006$). ER_{tissue} is shown on a weekly basis in Figure 1C and a sharp drop can be seen in ER_{tissue} starting from the initiation of the trial until week 1 p.p., when the tissue energy balance was the most negative, independent of the experimental group (Figure 1C; TIME; $p < 0.001$). In all of the treatment groups, a continuous rise in ER_{tissue} was observed from week 1 p.p. onwards, with this being the most distinctive in the HC groups (CFP; $p < 0.001$). In the NOPHC group, ER_{tissue} reached a positive range in week 4 p.p. which was earlier when compared to CONHC (positive ER_{tissue} from week 8 p.p.). In contrast, ER_{tissue} in the LC groups remained in a negative range until termination of the trial.

The described group differences concerning the extent of energy retained in body tissues were, however, not recovered in the $ER_{fat\ depot}$ (Table 3; 3-NOP × CFP; $p = 0.637$) which was estimated ultrasonographically during period 2. The effect of time (Table A1; TIME; $p < 0.001$) was reflected by a decrease in each AT depot (Table 4). Irrespective of treatment group, the average lipomobilization from the visceral and subcutaneous AT of 0.69 kg of fat depot masses per day contributed to a daily energy release of about 177.5 kJ/kg $BW^{0.75}$ and d being potentially utilizable for milk synthesis (Table 3). Correspondingly, back fat and rib fat thickness decreased, on average, by 0.14 and 0.15 cm/d, respectively (Table 4). In addition, the visceral fat deposit was mobilized to a larger extent when compared to the subcutaneous one (0.53 kg/d vs. 0.17 kg/d; Table 4). Due to the described differences in ER_{tissue} between groups but the missing effects of 3-NOP and CFP on depot fat mobilization from ultrasonic measurements, $ER_{residual}$ was higher in the 3-NOP and HC groups (Table 3; 3-NOP; $p = 0.058$; CFP; $p = 0.001$).

Table 4. Changes in fat layer thickness (mm/d) and adipose tissue (AT) depot mass (kg/d) estimated from ultrasonic measurements of the experimental cows from d 3 until d 28 post-partum.

Item	Treatments †				SEM	*p*-values §		
	CONHC (*n* = 14)	CONLC (*n* = 15)	NOPHC (*n* = 14)	NOPLC (*n* = 12)		3-NOP	CFP	3-NOP ×CFP
Change in fat layer thickness								
Back fat thickness	−0.15	−0.15	−0.14	−0.12	0.03	0.709	0.847	0.786
Rib fat thickness	−0.16	−0.15	−0.17	−0.13	0.03	0.814	0.493	0.699
Change in AT depot mass								
Mesenteric	−0.26	−0.22	−0.20	−0.31	0.05	0.775	0.479	0.136
Omental	−0.18	−0.18	−0.16	−0.15	0.02	0.285	0.956	0.952
Retroperitoneal	−0.12	−0.10	−0.13	−0.11	0.02	0.780	0.367	0.894

Subcutaneous	−0.17	−0.18	−0.17	−0.14	0.02	0.410	0.616	0.399
Visceral [+]	−0.56	−0.50	−0.48	−0.57	0.07	0.998	0.996	0.330
Visceral and subcutaneous	−0.73	−0.68	−0.65	−0.70	0.08	0.758	0.994	0.627

[†] Values are presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. [§] Effects of 3-NOP, concentrate feed proportion (CFP), and interactions between them. [+] PSEM, pooled standard error of the means. [+] Visceral AT depot mass (kg/d) = mesenteric + omental + retroperitoneal AT depot mass (kg/d).

3.3. Validation of the ERtissue Outcome of the GreenFeed Indirect Calorimetry Method

The EB_{GFE} varied between experimental periods, which was similar to ER_{tissue} (Table 3; PER; $p < 0.001$). In contrast to ER_{tissue}, the EB_{GFE} of the NOPHC group was more positive during each of the experimental periods when compared to the other treatment groups (Table 3; 3-NOP × CFP; $p = 0.082$). The Bland–Altman analysis (Figure 2A; mean bias of 70 kJ NE_L/kg $BW^{0.75}$ and d; $p < 0.001$ over all of the experimental periods) and the slope of the regression line of the linear relationship indicated that the EB_{GFE} was estimated to be approximately 33% (Figure 2B) higher when compared to ER_{tissue}. The slope of the regression line through the data points of differences was not significant ($p = 0.756$), indicating a constant bias over the experimental periods (Figure 2A). Nevertheless, greater differences between both methods with increasing magnitude of a positive energy balance can be visually identified regarding the a.p. period 1 (Figure 2A,B). Furthermore, the agreement between the EB_{GFE} and the calorimetrically obtained ER_{tissue} was most accurate concerning period 2 and 3 (Figure 2A,B). Hence, a non-significant mean bias of 21 kJ NE_L/kg $BW^{0.75}$ and d ($p = 0.051$) was calculated for the agreement between both methods for period 2 and 3. In contrast, greater differences between EB_{GFE} and ER_{tissue} were found in period 1, with a mean bias of 167 kJ NE_L/kg $BW^{0.75}$ and d ($p < 0.001$). The average k_l over the experimental groups and periods totalled 0.61 (data not shown).

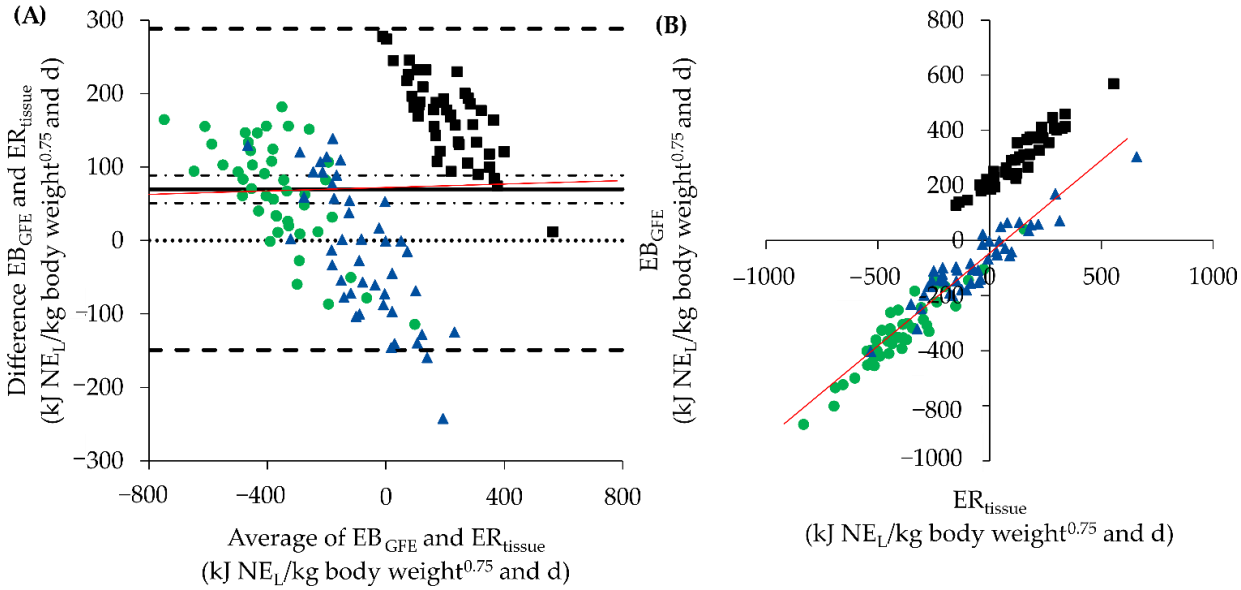
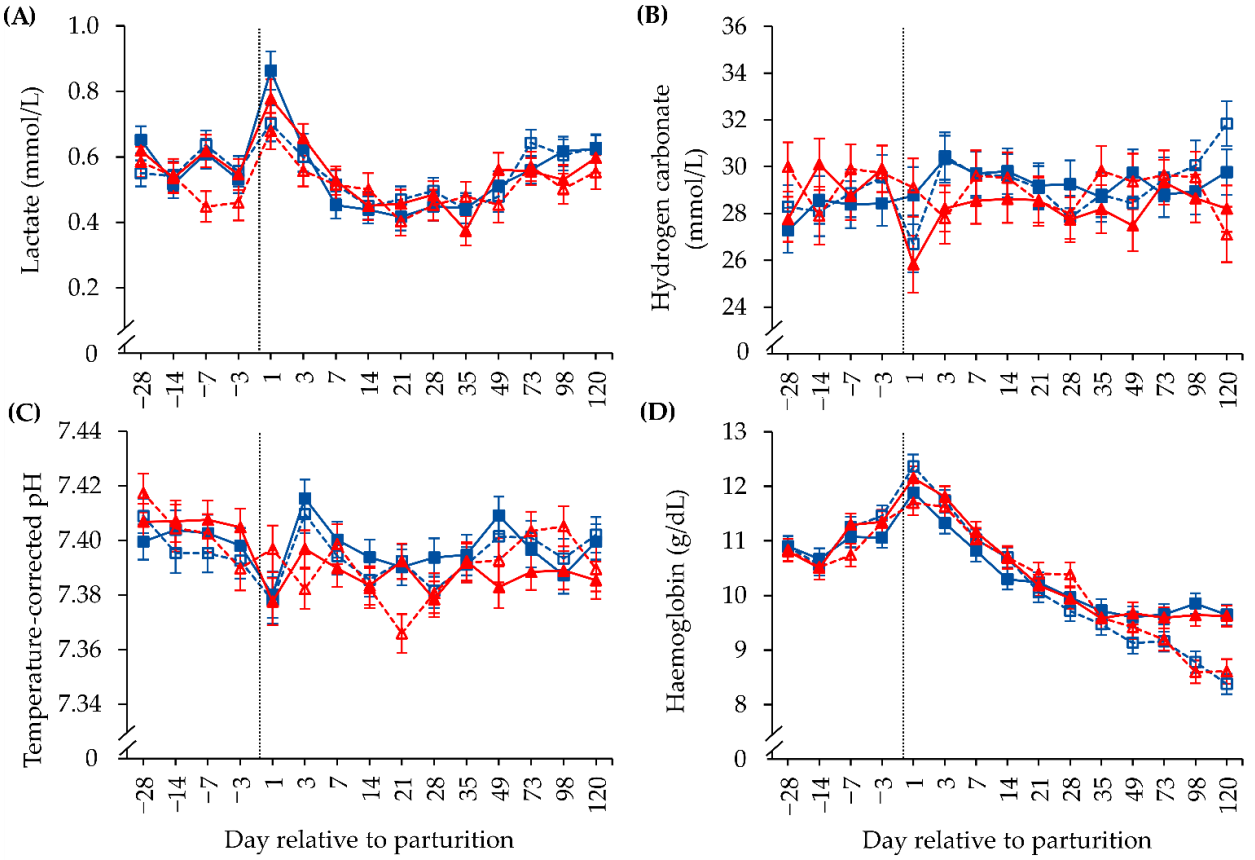


Figure 2. Bland–Altman plot (**A**) and linear relationship (**B**) of energy balance (EB_{GFE}) and energy retention in body tissues (ER_{tissue}) during ■ = period 1 (d 28 ante-partum − d 1 post-partum (p.p.)), ● = period 2 (d 1 p.p. − d 28 p.p.) and ▲ = period 3 (d 28 p.p. − d 120 p.p.). ER_{tissue} was calculated by indirect calorimetry using the GreenFeed system and EB_{GFE} according to GfE [24]. **Formula EB_{GFE}**: EB (kJ NE_L/kg body $weight^{0.75}$ ($BW^{0.75}$) and d) = net energy intake − net energy requirements for maintenance (NE_M) − net energy for pregnancy (NE_P) − net energy for lactation (NEL) with NE_M (kJ NE_L/d) = 0.293 × $BW^{0.75}$ (kg), milk energy (kJ NE_L/d) = 0.3 × milk fat % + 0.21 × milk protein % + 0.95, and NEL (kJ NE_L/d) = milk energy (kJ NE_L/d) + 0.1 × milk yield (kg/d). **Formula ER_{tissue}**: ER in body tissues (kJ NE_L/kg $BW^{0.75}$ and d) = metabolizable energy intake − heat production − NEL − NE_P (period 1). **Statistics 2A**: Bias (———): 70 kJ NE_L/kg $BW^{0.75}$ and d; $p < 0.001$ with confidence interval (– · – ·); lower limits of agreement (LoA) (– – –): −149 kJ NE_L/kg $BW^{0.75}$ and d; upper LoA: 288 kJ NE_L/kg $BW^{0.75}$ and d; regression line (———): y

= 0.01x + 71 (RSE = 112 kJ/kg $BW^{0.75}$ and d on 133 degrees of freedom, R^2 = 0.0007, p = 0.756). **Statistics 2B:** regression line (——; period 2 and 3): y = 0.67(0.02) x − 51(7); (RSE = 51 kJ NE_L/kg $BW^{0.75}$ and d on 88 degrees of freedom, R^2 = 0.92, p < 0.001).

3.4. Biochemical Blood Parameters

Lactate peaked on the day of calving (Figure 3A; TIME; p < 0.001). Hydrogen carbonate and the temperature-corrected blood pH marginally fluctuated around their mean of 28.9 mmol/L (TIME; p = 0.208; Figure 3B) and 7.39, respectively. However, a slight drop in blood pH values was observed at d 1 p.p. (TIME; p < 0.001; Figure 3C). Antepartal haemoglobin levels slightly increased, on average, from 10.6 to 12.0 g/dL on the day of parturition but continuously decreased by approximately 24% afterwards. From d 49 until termination of the experiment, haemoglobin diverged to constant levels of 9.5 g/dL in the HC groups but still decreased to approximately 8.5 g/dL in the LC groups (Figure 3D; CFP × TIME; p < 0.001).

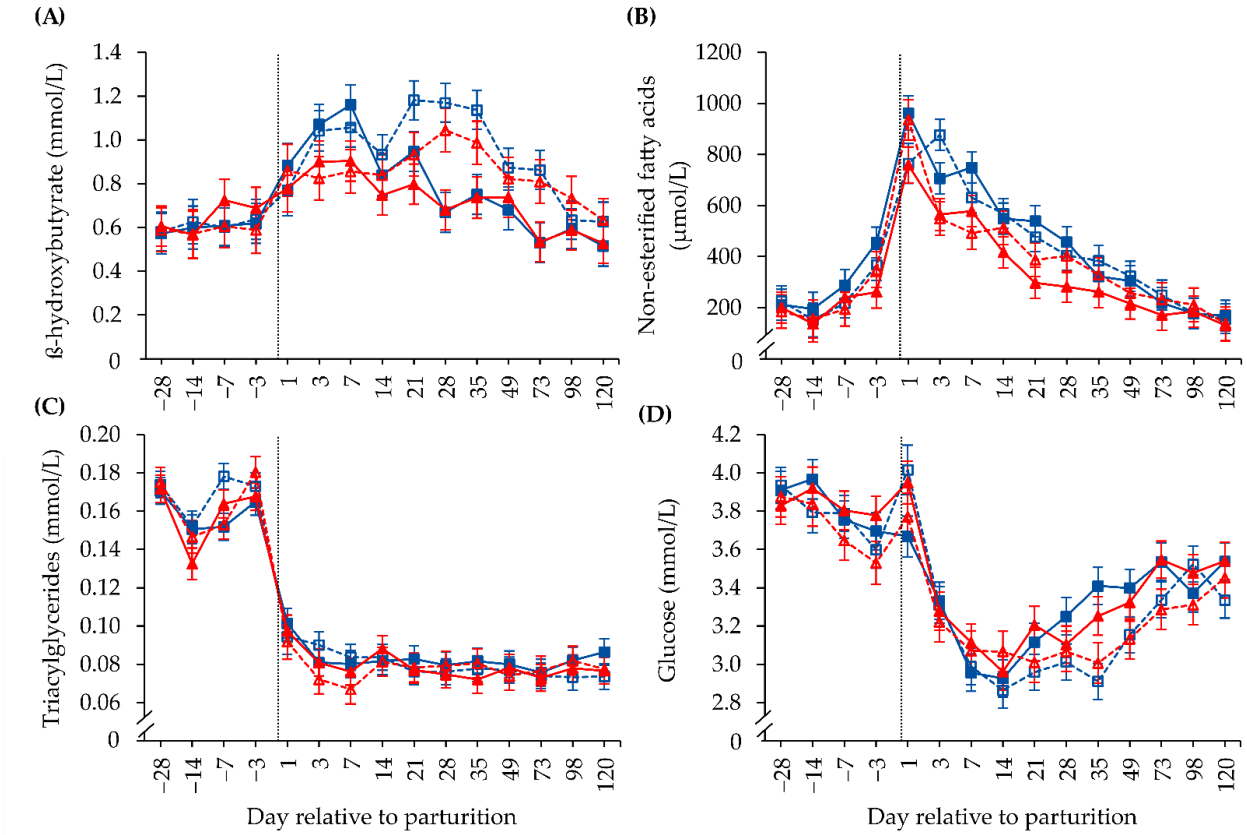


Variable	SEM	*p*-Values						
		3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP×CFP ×TIME
Lactate	0.012	0.077	0.102	<0.001	0.102	0.714	0.261	0.699
Hydrogen carbonate	0.53	0.587	0.315	0.208	0.398	0.062	0.906	0.424
Temperature-corrected pH	0.002	0.191	0.726	<0.001	0.263	0.014	0.334	0.611
Haemoglobin	0.10	0.513	0.083	<0.001	0.700	0.399	<0.001	0.257

Figure 3. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion in the ration (CFP) and time relative to parturition (TIME) on blood chemical parameters of (**A**) lactate, (**B**) hydrogen carbonate, (**C**) temperature-corrected pH, and (**D**) haemoglobin in peripartal dairy cows. ■, solid line = control high CFP (CONHC, n = 14);

□, dashed line = control low CFP (CONLC, n = 15); ▲, solid line = 3-NOP high CFP (NOPHC, n = 14); △, dashed line = 3-NOP low CFP (NOPLC, n = 12). Values are presented as LS-means. SEM, standard error of the means. Statistics with first measured value as covariate.

Blood serum concentrations of BHB, NEFA, TAG and glucose are presented in Figure 4. During the transitional period, the characteristic changes of BHB, NEFA, TAG and glucose were observed in all treatment groups (TIME; $p < 0.001$; Figure 4). In all experimental groups, TAG and glucose decreased by 68% from d 3 a.p. until d 3 p.p. and by 11% from d 1 p.p. to d 7 p.p., respectively. Starting from an initial value of 0.205 mmol/L, the NEFA concentration peaked to 0.856 mmol/L at d 1 p.p. followed by a decline to the a.p. baseline level until d 98 p.p. BHB increased from 0.63 mmol/L at d 3 a.p. to 1.11 mmol/L at d 7 p.p. in the CON groups, whereas a numerically lower peak of 0.88 mmol/L was observed in the 3-NOP groups. 3-NOP treatment did not impact the BHB, TAG and glucose concentrations but lowered that of NEFA by approximately 19.5% in the 3-NOP compared to the CON groups (3-NOP; $p < 0.001$). CFP affected neither NEFA nor TAG but did affect BHB (CFP × TIME; $p = 0.009$). Thus, a more pronounced decrease in BHB serum concentrations was observed in the HC compared to the LC groups from d 7 p.p. until termination of the experiment. Elevated blood glucose levels in the HC groups were considered significant from d 21 p.p. until d 73 p.p., in contrast with the LC groups (CFP × TIME; $p = 0.073$; CFP; $p = 0.006$). NEFA concentration was correlated with TAG after parturition (r = 0.47; $p < 0.001$; N = 350). Blood glucose was positively related to TAG (r = 0.49; $p < 0.001$; N = 532) and HP (r = 0.24; $p < 0.001$; N = 512) but negatively associated with both serum NEFA (r = −0.28; $p < 0.001$; N = 532) and BHB (r = −0.47; $p < 0.001$; N = 532). NEFA and BHB were significantly interrelated (r = 0.42; $p < 0.001$; N = 532) and decreased with elevated ER_{tissue} (r = −0.52; $p < 0.001$ for NEFA and r = −0.29; $p < 0.001$ for BHB; N = 511) and MEI (r = −0.29; $p < 0.001$; N = 525 for NEFA and r = −0.18; $p < 0.001$; N = 514 for BHB). Accordingly, increased MEI went along with increased ER_{tissue} (r = 0.39; $p < 0.001$; N = 914) and CO_2 yield (g CO_2/kg DMI) (r = 0.41; $p < 0.001$; N = 914). CO_2 yield had a strongly positive correlation with TAG (r = 0.56; $p < 0.001$; N = 511) and postpartal NEFA levels (r = 0.44; $p = 0.001$; N = 350) but had a negative relationship with ER_{tissue} (r = −0.35; $p < 0.001$; N = 914).



Variable	PSEM	*p*-Values						
		3-NOP	CFP	TIME	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
ß-hydroxybutyrate	0.075	0.111	0.003	<0.001	0.595	0.634	0.009	0.998
Non-esterified fatty acids	8.091	<0.001	0.633	<0.001	0.138	0.334	0.703	0.437
Triacylglycerides	0.001	0.254	0.842	<0.001	0.991	0.932	0.914	0.344
Glucose	0.086	0.989	0.006	<0.001	0.665	0.949	0.073	0.395

Figure 4. Effects of 3-nitrooxypropanol (3-NOP), concentrate feed proportion in the ration (CFP) and time relative to parturition (TIME) on energy-related biochemical blood parameters of (**A**) ß-hydroxybutyrate, (**B**) non-esterified fatty acids, (**C**) triacylglycerides, and (**D**) glucose in peripartal dairy cows. ■, solid line = control high CFP (CONHC, n = 14); □, dashed line = control low CFP (CONLC, n = 15); ▲, solid line = 3-NOP high CFP (NOPHC, n = 14); Δ, dashed line = 3-NOP low CFP (NOPLC, n = 12). Values are presented as LS-means. PSEM, pooled standard error of the means. Statistics with first measured value as covariate.

3.5. Interrelations between Metabolic RQ, Energy Metabolism and Methane Emission

Figure 5A shows that $RQ_{metabolic}$ was positively correlated with ER_{tissue} (r = 0.37; $p < 0.001$; N = 912) and negatively with serum NEFA (r = −0.29; $p < 0.001$; N = 510) (multiple $R^2 = 0.37$; $p = 0.015$). ER_{tissue} and serum NEFA levels were adversely interrelated. Lower serum NEFA concentrations could be identified for 3-NOP groups (Figure 5A) and the NOPHC group showed increased ER_{tissue} (Figure 5A). Figure 5B shows that CH_4 yield was negatively related to molar propionate proportions in rumen fluid (r = −0.46; $p < 0.001$; N = 165; data from Schilde et al. [15]), whereby the opposite holds true concerning p.p. NEFA concentration (multiple $R^2 = 0.59$, $p < 0.001$). Serum NEFA and propionate were inversely related (r = −0.22; $p = 0.007$; N = 165) and affected CH_4 yield in an interactive manner (Figure 5B). Approximately, 59% of the variation of the CH_4 yield can be explained by the explanatory variables (multiple $R^2 = 0.59$, $p < 0.001$). The CH_4 yield was decreased by 3-NOP supplementation (Figure 5B).

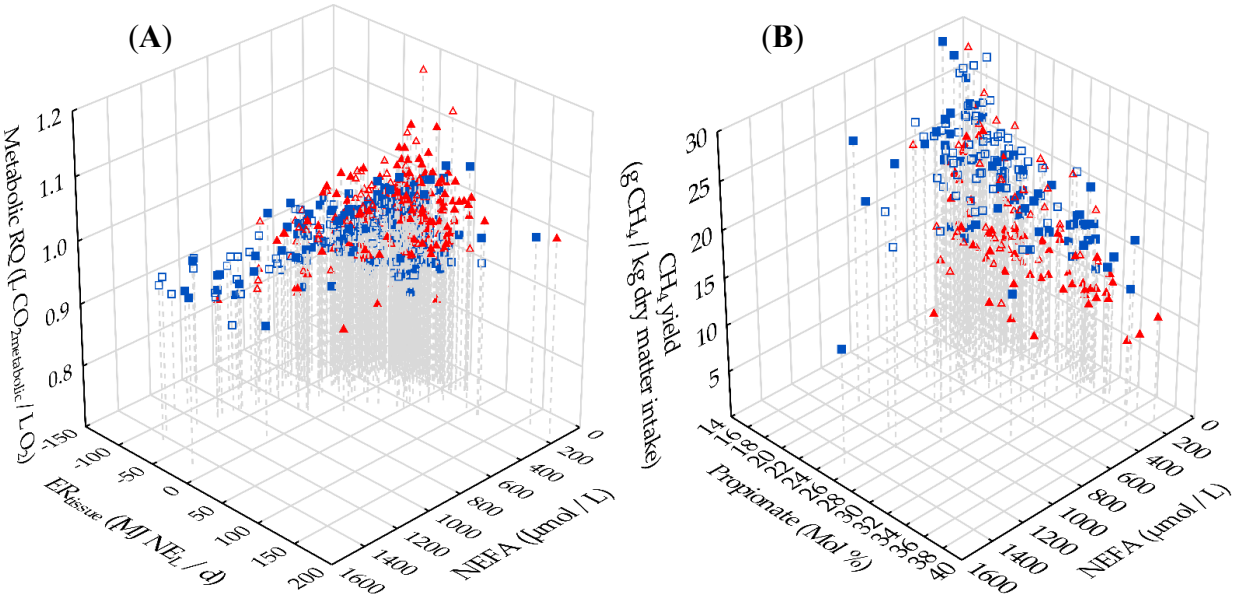


Figure 5. (**A**) Relationship between (**A**) metabolic respiratory quotient (metabolic RQ), energy retention in body tissues (ER_{tissue} (MJ/d)) and non-esterified fatty acids (NEFA (µmol/L)) as well as (**B**) Relationship between CH_4 yield (g CH_4/kg dry matter intake), molar propionate proportion in rumen fluid (Mol %) (data are shown in Schilde et al. [15]) and NEFA (µmol/L) from d 28 ante-partum until d 120 post-partum in experimental dairy cows supplied with 3-nitrooxypropanol (3-NOP) and varying concentrate feed proportion (CFP) in the ration. ■ = control high CFP (CONHC); □ = control low CFP (CONLC); ▲ = 3-NOP high CFP (NOPHC); Δ = 3-NOP low CFP (NOPLC). **Statistics (A):** multiple $R^2 = 0.38$, $p < 0.001$. **Statistics (B):** multiple $R^2 = 0.59$, $p < 0.001$.

4. Discussion

4.1. Limitations of the GreenFeed Technology for its Use in Indirect Calorimetry

The accurate indirect calorimetric calculation of the HP and RQ depends on precise gas respiration measurements [44]. Due to technical reasons, the VO_2 consumption of CON groups needed to be regressively predicted from VCO_2 and DMI. Even though slightly increased VO_2 consumption rates were temporarily observed in the CON groups, the highly predictive performance of the applied model (R^2 = 0.90; RSE = 371 g/d) confirmed its validity. In contrast to RC, a reliable within-day gas exchange pattern could not be obtained from GF measurements, which precluded investigations on intraday HP and RQ kinetics [23] and potentially explained some of the variations, as shown for $RQ_{metabolic}$ and HP. Over the present trial period, the coefficients of variation for the within-day GF spot measurements of VCH_4, VO_2 and VCO_2 were, on a weekly average, 22.1, 10.0 and 11.1%, respectively. Correspondingly, this could have resulted in deviations of HP of approximately ± 1.3 MJ (1.02% of total HP), ± 9.7 MJ (7.65% of total HP) and ± 3.5 MJ (2.76% of total HP), respectively, indicating that HP estimation is most sensitive towards variations in O_2 consumption. In conclusion, the overall variability of the present GF gas mass flux measurements was stated to be low. This was related to an accurate data acquisition, which was realized by a high-sampling frequency being evenly distributed throughout the day. In addition, GF data were averaged over seven days and validated for visiting time and head positioning of the cow in the GF hood. Hence, the measurement procedure applied herein (detailed in Schilde et al. [15]) was previously noted to produce comparable results to those obtained from RC [23,32]. In particular, both RC and GF used the same equations and sensor types for O_2 (para-magnetic), CH_4 and CO_2 (non-dispersive infrared) respiration measurements. However, in particular, further validation of the GF algorithm principles is needed as O_2 sensor validation data from RC measurements are lacking. In the present study, the $VCO_{2metabolic}$ was differentiated from the fermentative VCO_2 to calculate $RQ_{metabolic}$ at the intermediary level. Indeed, this fractionation can be visually conducted for each cow visit from the VCO_2 gas-measurement trajectory depicted in the GF graphical online interface. In this way, a "baseline" CO_2 level reflects the amount of expired lung-derived CO_2 ($VCO_{2metabolic}$) that needs to be corrected for background CO_2 gas concentration. The "baseline" CO_2 level is temporarily interrupted by CO_2 eructation peaks ($VCO_{2fermentative}$) [45]. However, this visual evaluation is impractical for large datasets and, therefore, algorithms for an automatized graphical assessment should be developed in the future. As a consequence, the commonly applied factor of 1.7 [34,36] was used, resulting in $VCO_{2fermentative}$ proportions of 12 ± 0.5% in CONHC, 9 ± 0.5% in NOPHC and, more incrementally, 13 ± 0.4% in CONLC and 10 ± 1.1% in NOPLC (mean ± SD) (Figure 1A; Table 2). Comparatively, Caetano et al. [45] visually estimated the $VCO_{2fermentative}$ from the GF online interface to be between 6 and 20% of the total VCO_2 production in beef cattle offered diets of varying energy density for ad libitum and restricted intake.

4.2. Validation of the Energy Partitioning estimated by Indirect Calorimetry and Ultrasonography

The present GF method of indirect calorimetry resulted in ER_{tissue} values that strongly corresponded to the EB_{GFE} values measured for period 2 and 3. However, both methods significantly differed with regard to the a.p. period (period 1; Figure 2A; compare ER_{tissue} and EB_{GFE} in Table 3). Erdmann et al. [39] compared the EB_{GFE} with that calculated from indirect calorimetric RC measurements over the same antepartal period as the present period 1 and also reported higher EB_{GFE} values (by about 33 MJ/d) when compared to the RC energy balance. The higher EB_{GFE} could have been a result of an underestimation of EE during the ante-partum period 1 when compared to the calorimetrically derived ER_{tissue}. Thus, the dynamically increasing antepartal energy requirements for the onset of lactogenesis and foetal growth could have been captured more accurately by continuous calorimetric measurements in contrast with the constants applied in EB_{GFE} calculations. Furthermore, the impact of maintenance requirements on the EB outcome was proportionally higher during the dry period when compared to the lactation period. The factors applied in the German NE system for calculating ME_m were derived from 40-year-old data. Meanwhile, the breeding of higher genetic merit cows resulted in generally increased body sizes of cows and a greater proportion of liveweight as

body protein mass while back fat thickness decreased [46]. As a consequence, the increased feed intake resulted in greater digestive loads and blood flow-rates in the total splanchnic tissues being paralleled by increased metabolic rates, internal organ masses and O_2 consumption [47]. These metabolic changes are related to higher energy demands for maintenance metabolism, which implies an underestimation of maintenance energy requirements in the German NE feeding system and a further explanation of the higher EB_{GFE} when compared to the ER_{tissue} values.

The mean k_l value of 0.61 is within the range of k_l values (0.60 to 0.67) summarized in a literature review by Agnew and Yan [47] and close to the k_l value of 0.60 reported by Van Es [48], which confirms the suitability of the GreenFeed system as an indirect calorimeter.

The estimated energy released from the ultrasonographically assessed lipolysis in AT depots ($ER_{fat\ depot}$; Equation 8) was subtracted from the negative ER_{tissue} in period 2 (Table 3) yielding the remaining fraction of glycogen, triglycerides and proteins deposited in skeletal muscles and organs ($ER_{residual}$). It was supposed that protein and lipid breakdown in skeletal muscles around parturition partially compensated for the observed negative ER_{tissue} (Figure 1C; Table 3) and contributed to the decreased $RQ_{metabolic}$ (Figure 1B) [11]. Thus, gluconeogenesis from the oxidation of alanine, one of the most important glucogenic amino acids (**AA**) [11], and intramuscular lipids result in very low $RQ_{metabolic}$ values of 0.13 and 0.7, respectively. Tamminga et al. [49] estimated the fractional rate of skeletal muscle protein breakdown in dairy cows to be 0.38, 0.22, 0.04 and 0.02 kg per day in week 1, 2, 3 and 4 p.p. From a rough calculation, this would correspond to a total of 4.6 kg mobilized body protein (92 MJ NE_L) during the complete period 2 and an energy equivalent of 3.3 MJ NE_L/d (92 MJ NE_L from proteolysis divided by 28 days in period 2 = 3.3 MJ NE_L/d; 1 g of body protein = 23.8 kJ [22]; energy efficiency of 84% [50]). Comparatively, von Soosten et al. [51] reported a lower energy yield from body protein mobilization, which amounted to an average of 2.1 MJ/d over the period from d 1 until d 42 p.p. in primiparous cows measured by the comparative slaughter technique. However, those results are not directly comparable to the present periparturient pluriparous dairy cows and observation period (d 1 until d 28). Von Soosten et al. [51] assumed protein accretion in the growing primiparous cows (BW of approximately 490 kg) and protein mobilization is generally supposed to change to repletion from d 35 p.p. onwards [52]. The non-explained remainder of the difference between $ER_{residual}$ and the estimated energy supply from skeletal muscle proteolysis can be partially assigned to energy mobilized from inter- and intramuscular and organ tissues. Furthermore, the corresponding models estimating the fat depot masses are, to some extent, prone to error. Although ER_{tissue} was more positive in the HC groups (Figure 1C), the ultrasonographically assessed lipolysis from AT and serum NEFA levels (indicative for negative EB) were not different between the HC and LC groups (Table 3; Figure 4B). Raschka et al. [25] validated the ultrasonographic-based multiple regression model for the predicted weights of the SAT and VAT depots as highly accurate with R^2 values of 0.88 and 0.94 and root mean square errors of 3.4 and 6.1 kg, respectively. In the present experiment, an assumed ± 10% variation between the predicted and actual daily changes in SAT and VAT would result in $ER_{fat\ depot}$ variations of approximately ± 2.3 MJ NE_L/d.

4.3. Effects of 3-NOP, CFP and Parturition on Energy Metabolism Parameters

Both RQ and HP notably depend on the magnitude of MEI (r = 0.69 and r = 0.22 resp.; $p < 0.001$; N = 914), its utilization for maintenance and productive purposes [53], whether substrates are either deposited or mobilized in tissues (Figure 5A) and, finally, on the type of the metabolized substrate itself [22,34]. In contrast to LC groups, the higher $RQ_{metabolic}$ (Figure 1B) and ER_{tissue} (Figure 1C) in HC groups reflected their increased GEI:EE ratio (Table 3) and dietary content of non-fibre carbohydrates (Table 1) being microbially degraded into gluconeogenic substrates. Correspondingly, increased blood glucose (Figure 4C) and reduced BHB (Figure 4A) concentrations were observed in the HC groups.

During the a.p. period, the pro-lipogenic effect of the dietetically designed energetic oversupply was manifested in the positive ER_{tissue} (Figure 1C) and $RQ_{metabolic}$ values of 0.92 (Figure 1B). In principle, lipid deposition in AT would result in $RQ_{metabolic}$ values above 1.0 [44] but $RQ_{metabolic}$ reflects the net oxidation rates of a mixture of substrates irrespective of the metabolic interconversions of the substrate [44]. Hence, flowing transitions between oxidation and de novo synthesis of lipids were assumed, which became apparent in the steady decrease in ER_{tissue} since the beginning of the trial in spite of the energetic oversupply (Figure 1C).

The present energy-deficient transition from gestation to lactation was accompanied by significant metabolic adaptations (Figure 4). The decreased $RQ_{metabolic}$ corresponded to the negative ER_{tissue} and accumulation of serum NEFA (Figure 5A), collectively indicating excessive fat oxidation from AT resulting in increased O_2 consumption (NEFA vs. O_2 consumption (g/d); r = 0.18; $p < 0.001$; N = 511) and ketogenesis from acetyl-CoA and NEFA (NEFA vs. BHB; r = 0.42; $p < 0.001$; N = 532). The observed increased circulating BHB (Figure 4A) likely originated from an oxaloacetate deficiency [10] and a concomitant hepatic overload to completely oxidize the excessively flooding NEFA (Figure 4B), released by lipolysis in AT (Tables A1 and 4), into ATP and CO_2 [11](Drackley et al. 2001). It can be summarized that the decrease in $RQ_{metabolic}$ could be partially explained by the increased O_2 consumption due to lipolysis in AT, whereby CO_2 did not increase due to the aforementioned incomplete metabolization of NEFA into BHB but not into CO_2. Correspondingly, the $RQ_{metabolic}$ did not behave in the same manner as the RQ_{total} because the latter also reflected CO_2 production arising from rumen fermentation. Hence, in the early-lactation period, increased fermentative CO_2 production from high-forage diets led to higher RQ_{total} values, whereby $RQ_{metabolic}$ decreased due to the abovementioned increased but incomplete fat oxidation resulting in less intermediary CO_2 formation when NEFA were converted to BHB, rather than CO_2 and ATP. Accordingly, as previously published for the present experiment, the CO_2 yield (g CO_2 production/kg DMI) was significantly higher in LC when compared to the HC groups, but the opposite was the case when it came to total CO_2 production (g CO_2/d) over the complete experimental period [15].

In the present experiment, the tendency for an increased ER_{total} in the NOPHC group (Table 3; Figure 1C) confirmed similar results reported by van Gastelen et al. [13]. The increased ER_{tissue}, $ER_{residual}$ and $RQ_{metabolic}$ of 1.01 in the NOPHC group (Figure 1B; Table 2) could be explained by an improved energy budget in that group. Hence, decreased NEFA levels were associated with increased ruminal propionate concentrations, and both were inversely related to CH_4 production (Figure 5B). Recently, it was observed that supplementing 3-NOP combined with high CFP in the ration shifted rumen fermentation pathways to hydrogen-consuming glucogenic propionate and decreased loss of CH_4 energy in a synergistic manner (Tables 2 and 3) (details in Schilde et al. [15]). Correspondingly, lower serum NEFA concentrations were observed in the 3-NOP cows (Figure 4B) although neither 3-NOP nor CFP affected $ER_{fat\ depot}$ and lipomobilization in AT depots (Tables 3 and 4). This could indicate that the increased glucogenic propionate proportions in the 3-NOP groups improved the intramitochondrial oxaloacetate availability and, therefore, the hepatic capacity for NEFA oxidation. Interestingly, neither blood glucose (NEFA oxidation and conversion of elevated propionate levels to glucose and CO_2) nor TAG (re-esterification of NEFA) and BHB (reduced incomplete NEFA oxidation) were affected by 3-NOP, which confirms previous findings [20]. This opens the question as to whether the direct extrapolation of NEFA concentrations to circulating BHB levels is appropriate in the present CH4 mitigation experiment. Accordingly, in the companion study, Schilde et al. [15] observed that butyrate formation was preferred to that of acetate in the 3-NOP-treated cows, which can be explained by the reduced hydrogen release when carbohydrates are degraded into butyrate and not into acetate [54]. Butyrate also serves as a carbon source for ketone body synthesis in the rumen epithelium [55]; therefore, increased circulating BHB originating from enhanced intraepithelial ketogenesis could have masked the assumed causal relationship that decreased serum NEFA concentrations in the 3-NOP-treated cows, which would necessarily have led to reduced BHB in the blood stream. Besides the intraepithelial butyrate metabolization, propionate can be metabolized to lactate in the rumen epithelium, which could also have reduced the propionate flux to the liver, thereby eliminating the energetic advantage of the 3-NOP-mediated increased propionate formation in the rumen. The observed accumulation of ketoacids (BHB) and the blood lactate peak at d 1 p.p. (Figure 3A) could have increased the risk for metabolic acidosis [44]. Indeed, blood pH was observed to slightly drop from 7.41 to 7.38 at parturition contemporaneously to the lactate peak at d 1 p.p. (Figure 3C; TIME; $p < 0.001$). In this context, the temporal decrease in the 3-NOP groups (Figure 3C; 3-NOP × TIME; $p = 0.014$) is, however, difficult to explain as blood lactate and BHB were not affected by 3-NOP treatment. The pH decrease at d 1 p.p. possibly caused buffering reactions via the largest CO_2 body pool, hydrogen carbonate, which could have led to an overestimation of HP and $RQ_{metabolic}$ [44]. Indirect calorimetry is stated to be accurate as long as body pool sizes of energy-related metabolites (ketone bodies, lactate) and intermediary products (O_2 and CO_2, N_U) remain stable [44]. However, the

potential effects of intermediary pool sizes on HP from nutrient oxidation and $RQ_{metabolic}$ were considered negligible because the bicarbonate and pH values remained within their physiological area [56,57] (Figure 3B, C). In general, the CO_2 pool size is supposed to be subjected to greater fluctuations when compared to the O_2 body pool [44]. Accordingly, blood concentrations of haemoglobin, the main O_2 body pool, remained stable within the physiological range [57] although a slight divergence was observed between the LC and HC groups at the end of the experiment (Figure 3D; CFP × TIME; $p < 0.001$).

5. Conclusions

The present study revealed that using the GF system as an indirect calorimetry chamber for the assessment of cows' energy metabolisms is a promising approach, although further validations of the O_2 sensor and algorithm principles are needed. The ER_{tissue} determined by indirect calorimetry coincided with that calculated from GfE [24], except for the antepartal period. The hypothesis that feeding 3-NOP in combination with high CFP synergistically improves the cows' energy budgets was partially confirmed because effects were not apparent in all of the examined parameters. 3-NOP combined with high CFP increased $RQ_{metabolic}$ and ER_{tissue} and decreased serum NEFA. In contrast, lipomobilization from fat depots and blood lactate were neither affected by 3-NOP nor CFP and 3-NOP did not affect blood glucose, TAG and BHB levels. Blood pH and bicarbonate remained within their physiological range and metabolic adaptations to energy-related changes via the CO_2 body pool were not observed. High CFP decreased BHB but increased blood glucose and, at the end of the trial, haemoglobin levels, which possibly indicates that the cows adapted differently to metabolic changes. Future research will be focused on the relationship between the 3-NOP-induced changes in the rumen VFA profile and gene expression in the liver.

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

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the present article and in the previously published manuscript of the comprehensive experiment by Schilde et al. [15] (article number: 1877986).

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

Appendix A

Table A1. Thickness and absolute masses of adipose tissue (AT) depots of the experimental groups at d 3 and d 28 post-partum (p.p.).

Item	Treatments †				SEM	*p*-Values §					
	CONHC (*n* = 14)	CONLC (*n* = 15)	NOPHC (*n* = 14)	NOPLC (*n* = 12)		3-NOP	CFP	3-NOP ×CFP	3-NOP ×TIME	CFP ×TIME	3-NOP ×CFP ×TIME
Back fat thickness (cm)											
d 3 p.p.	1.49	1.34	1.43	1.49	0.08	0.341	0.538	0.152	0.551	0.938	0.924
d 28 p.p.	1.16	1.01	1.15	1.21							
Rib fat thickness (cm)											
d 3 p.p.	1.6	1.5	1.6	1.6	0.08	0.424	0.671	0.395	0.739	0.395	0.355
d 28 p.p.	1.2	1.1	1.2	1.3							
Absolute masses of AT depot (kg)											
Mesenteric AT											
d 3 p.p.	13.5	11.7	13.9	13.8	0.84	0.197	0.300	0.415	0.452	0.551	0.486
d 28 p.p.	7.3	7.0	7.6	7.4							
Omental AT											
d 3 p.p.	14.8	13.3	14.4	14.3	0.66	0.395	0.320	0.305	0.353	0.588	0.806
d 28 p.p.	10.4	9.3	10.5	10.6							
Retroperitoneal AT											
d 3 p.p.	9.4	8.8	9.7	9.5	0.53	0.422	0.837	0.539	0.603	0.184	0.552
d 28 p.p.	6.8	6.6	6.6	7.3							
Subcutaneous AT											
d 3 p.p.	13.6	12.8	13.9	13.3	0.73	0.453	0.499	0.594	0.559	0.468	0.336
d 28 p.p.	9.6	8.7	9.6	10.0							
Visceral AT +											
d 3 p.p.	37.7	33.8	37.9	37.5	1.72	0.276	0.387	0.359	0.683	0.324	0.679
d 28 p.p.	24.5	22.9	24.8	25.3							

† Values presented as LS-means; CONHC, control high concentrate; CONLC, control low concentrate; NOPHC, 3-nitrooxypropanol (3-NOP) high concentrate; NOPLC, 3-NOP low concentrate. § Effects of 3-NOP, concentrate feed proportion (CFP), time relative to parturition (TIME), and interactions between them; effect of TIME with $p < 0.001$ for all variables. + Visceral AT = mesenteric + omental + retroperitoneal AT.

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6. Paper III

Schilde, M.; von Soosten, D.; Hüther, L.; Kersten, S.; Meyer, U.; Zeyner, A.; Dänicke, S.

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.

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Article

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

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Simple Summary: Feeding strategies which aim at mitigating ruminal methane formation, a significant contributor to total greenhouse gas emissions, are being continuously developed, yet they need to be investigated in relation to their effectiveness and the mechanisms behind their effects in vitro before they undergo further assessment in vivo. In this context, the present study investigated the dose–response relationships of the methane inhibitor 3-nitrooxypropanol supplemented to varying concentrate feed proportions in a rumen simulation technique. Methane production was effectively reduced with an increasing dose of 3-nitrooxypropanol, which was, however, independent of concentrate feed proportion. Total gas production and fibre degradability were not affected by 3-nitrooxypropanol, indicating no negative side effects on fermentative capability. However, the hydrogen-liberating acetate production was reduced, whilst hydrogen gas was notably increased in a dose-dependent manner. The present in vitro study provides a deeper insight into a combined (3-nitrooxypropanol and high-concentrate feed proportions) methane abatement strategy under controlled conditions. The present combined approach reveals neither negative side effects nor additive effects between 3-nitrooxypropanol and varying concentrate feed proportions, which should be further investigated in future experiments in vivo.

Abstract: Methane (CH_4) from ruminal feed degradation is a major pollutant from ruminant livestock, which calls for mitigation strategies. The purpose of the present 4 × 2 factorial arrangement was to investigate the dose–response relationships between four doses of the CH_4 inhibitor 3-nitrooxypropanol (3-NOP) and potential synergistic effects with low (LC) or high (HC) concentrate feed proportions (CFP) on CH_4 reduction as both mitigation approaches differ in their mode of action (direct 3-NOP vs. indirect CFP effects). Diet substrates and 3-NOP were incubated in a rumen simulation technique to measure the concentration and production of volatile fatty acids (VFA), fermentation gases as well as substrate disappearance. Negative side effects on fermentation regarding total VFA and gas production as well as nutrient degradability were observed for neither CFP nor 3-NOP. CH_4 production decreased from 10% up to 97% in a dose-dependent manner with increasing 3-NOP inclusion rate (dose: $p < 0.001$) but irrespective of CFP (CFP × dose: $p = 0.094$). Hydrogen gas accumulated correspondingly with increased 3-NOP dose (dose: $p < 0.001$). In vitro pH ($p = 0.019$) and redox potential ($p = 0.066$) varied by CFP, whereas the latter fluctuated with 3-NOP dose ($p = 0.01$). Acetate and *iso*-butyrate (mol %) decreased with 3-NOP dose, whereas *iso*-valerate increased (dose: $p < 0.001$). Propionate and valerate varied inconsistently due to 3-NOP supplementation. The feed additive 3-NOP was proven to be a dose-dependent yet effective CH_4 inhibitor under conditions in vitro. The observed lack of additivity of increased CFP on the

CH_4 inhibition potential of 3-NOP needs to be verified in future research testing further diet types both in vitro and in vivo.

Keywords: 3-nitrooxypropanol; concentrate feed proportion; RUSITEC; methane inhibitor; methane production

1. Introduction

Methane (CH_4) is a climate-relevant greenhouse gas with a direct environmental impact insofar as its global warming potential exceeds 28 times that of carbon dioxide (**CO_2**) on a 100-year time horizon [1]. In particular, enteric CH_4 from feed fermentation contributes to 46% of the total emissions from the dairy supply chain worldwide [2]. Accordingly, the development and implementation of CH_4 abatement strategies in ruminant livestock production systems can be expected to gain in importance [3].

The rumen simulation technique (RUSITEC) was introduced by Czerkawski and Breckenridge [4] as a semi-continuous-flow system to facilitate investigations on rumen fermentation processes, such as CH_4 production, and its manipulation under strictly controlled conditions. In parallel, dose–response relationships can be examined in the RUSITEC by incubating different dosage levels of CH_4 inhibitor substances on diet substrates in the juxtaposed reaction vessels.

Methane formation in ruminants, being catalysed by methyl Coenzyme M reductase (MCR) in hydrogenotrophic methanogenic Archaea, is the major pathway of removing metabolic hydrogen by reduction of CO_2 [5]. Apart from intraruminal volatile fatty acid (VFA) synthesis, CO_2 and hydrogen (H_2) result from microbial degradation of fibre as well as non-fibre carbohydrates (NFC) supplied by the feed ration.

The synthetic substance 3-nitrooxypropanol (3-NOP) is a direct CH_4 inhibitor and structural analogue of methyl-coenzyme M (CoM). Thus, 3-NOP binds to the active site of the nickel enzyme methyl-coenzyme M reductase (MCR), causing its inactivation by oxidising the Ni(I) to Ni(II) in the cofactor F_{430}. As a consequence, the MCR catalysed the reduction of CoM with coenzyme B to CH_4 and the heterodisulphide is intermitted during the last step of methanogenesis [5]. In contrast, increasing concentrate feed proportions (CFP) in the feed ration were previously proven as an indirect CH_4 abatement strategy [6], which can be related to diet-dependent effects on microbial community structures [7], reduced rumen pH values being detrimental to the growth of pH-sensitive methanogens and fibrolytic bacteria [8], and alterations in fermentation pathways. Thus, higher contents of NFC in high-concentrate diets are mainly degraded by propionate enhancers and, therefore, redirected to H_2-consuming fermentation pathways, which results in substrate competition with methanogenesis [9].

Significant dose–response relationships of 3-NOP on CH_4 mitigation were observed in vitro [10] and in vivo [11–13]. Romero-Pérez et al. [10] tested 500, 1000, and 2000 mg of 3-NOP/kg of feed DM incubated with a high-forage diet substrate in a RUSITEC and observed quadratic effects of 3-NOP dose on CH_4 reduction (76.0%, 84.5%, and 85.6%). However, little information has been reported to reveal the dose–response relationships of 3-NOP in consideration of the potential additive effects with low and high CFP. Romero-Pérez et al. [14,15] supplemented 3-NOP in combination with the ionophore monensin to either high-forage [14] or high-grain [15] diets in a RUSITEC and reported additive effects of neither monensin nor high-grain diets on CH_4 reduction.

Regarding in vivo experiments, Vyas et al. [13] supplemented 50, 75, 100, 150, and 200 mg of 3-NOP/kg of feed dry matter (DM) to beef cattle provided high-forage and high-grain diets. The authors observed a significant dose response with regard to the higher dosage levels of 100, 150, and 200 mg of 3-NOP and a significant effect of the ration type. Thus, 3-NOP efficacy was greater in high-grain (26, 33, and 45% CH_4 reduction, resp.) when compared to high-forage (16, 21, and 23% CH_4 reduction, resp.) diets. However, CH_4 emissions at 50 and 75 mg 3-NOP dose/kg feed DM were not significantly different from the control. Correspondingly, in a meta-analysis of 3-NOP experiments including dairy and beef cattle, Dijkstra et al. [11] confirmed the dose-dependent 3-NOP effect on CH_4 yield, which was modelled to -2.48 ± 0.0734% CH_4 yield per 10 mg/kg DM increase in 3-NOP dose from its mean (123 mg 3-NOP/kg of feed DM). Melgar et al. [12] mixed 3-NOP into a forage-based total-mixed ration (TMR) for dairy cows and reported that CH_4 yield quadratically decreased by 24.3, 26.5, 22.5, 33.5, 35.9, and 31.8% for 40, 60, 80, 100, 150, and 200 mg of 3-

NOP/kg feed DM, respectively, with no statistical difference among 40, 60, and 80 as well as between 100, 150, and 200 mg 3-NOP/kg DM.

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

It was hypothesised that CH_4 production decreases with increasing 3-NOP dosage level and that supplementing high-concentrate feed proportions causes additive effects on CH_4 reduction in vitro.

2. Materials and Methods

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

2.1. Experimental Design and Diets

The experiment was conducted using the RUSITEC according to the general incubation procedure described by Czerkawski and Breckenridge [4]. The study was arranged as a 2 × 4 factorial design with low- (LC) and high- (HC) concentrate feed proportion (CFP) in the incubated diet and the methane inhibitor 3-nitrooxypropanol (3-NOP; DSM Nutritional Products AG, Kaiseraugst, Switzerland) supplied at four doses of 0 (placebo, PLA), 73 (LOW), 160 (MED), and 1200 (HIGH) mg of the active 3-NOP substance/kg of feed DM. Both the placebo and the 3-NOP supplement contained propylene glycol and SiO_2 acting as carriers for 10% of the active 3-NOP substance (1,3-propanediol mononitrate) in product DM, which was included in 3-NOP treatments only. On a DM basis, the experimental diet substrates were formulated according to the forage:concentrate ratio of 70:30 (LC) and 40:60 (HC). The forage proportion of the LC and HC diet was composed of 70% maize silage and 30% grass silage. The 3-NOP supplement was mixed into the ground concentrate feed and homogenised for 10 min (M4.REI; Gebr. Lödige Maschinenbau GmbH, Paderborn, Germany).

2.2. Experimental Procedure

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

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

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

	Experimental Diet †	
Item	**LC**	**HC**
Ingredients (g/kg of diet DM §)		
Maize silage	495	286
Grass silage	212	122
Rapeseed meal	44.4	90.1
Soybean meal	37.2	74.7

Wheat	97	195.5
Dried sugar beet pulp	85	172
Soybean oil	4.5	9.2
Calcium carbonate	7	14.2
Urea	2.9	6.1
Vitamin/Mineral premix [+]	15	30.2
Chemical analysis of the ration		
DM (g/kg)	908	897
Nutrient (g/kg of DM)		
Organic matter	923	938
Crude protein	131	171
Ether extract	33	33
aNDFom [¶]	382	308
ADFom [#]	217	178
Starch	257	284

[†] Experimental diets with low- (LC) and high- (HC) concentrate feed proportion supplied at four doses of 0, 0.073, 0.16, and 1.2 mg of the active 3-nitrooxypropanol substance/g feed DM. [§] DM, dry matter. [+] Ingredients according to the manufacturer's specifications: minerals (g/kg of premix): Ca, 140; Na, 120; P, 70; Mg, 40; Zn, 6; Mn, 5.4; Cu, 1; I, 0.1; Se, 0.04; Co, 0.025; vitamins (IU/kg of premix): A, 1,000,000; D3, 100,000; E, 2,235. [¶] aNDFom; α-amylase treated neutral detergent fiber expressed without residual ash; [#] ADFom; acid detergent fibre expressed without residual ash.

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

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

2.3. Sampling and Analyses

Feed samples of the pre-incubated and fermented diet were analysed according to the standard methods of the Association of German Agricultural Analytic and Research Institutes [19] for DM (3.1), crude ash (CA; 8.1), crude protein (CP; Dumas method, 4.1.2), ether extract (EE) pre-treated with hydrochloric

acid (5.1.1), starch (7.2.1), acid detergent fibre (ADFom; 6.5.2), and α-amylase treated neutral detergent fibre (aNDFom; 6.5.1), both expressed without residual ash.

During the four-day sampling period, all the samples were taken contemporaneously for one daily feed bag exchange.

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

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

The effluent was collected in 1-litre volumetric flasks placed on ice and effluent volume was noted daily. VFA were analysed from daily collected effluent samples (80 mL) according to Geissler et al. [20] using a gas chromatograph (Clarus 680; PerkinElmer LAS GmbH, Rodgau, Germany) equipped with a flame ionisation detector. Ammonia-N concentration (NH_3-N) was measured using steam distillation (DIN38406-E5-2, [21]).

Fermentation gases were collected over the whole sampling period in 10-litre gas bags (Plastigas; Linde GmbH, Pullach, Germany). After termination of the run, 10 mL of fermentation gases were withdrawn via the septum of the gas bag using a gas-tight syringe. The gas samples were injected on a chromatography column (Porapak QS; 80/100 mesh, 3 m × 3 mm, Agilent Technologies, Inc., Santa Clara, CA, USA) of a gas chromatograph equipped with a thermal conductivity detector (GC-14B; Shimadzu, Kyoto, Japan) and argon as carrier gas. Gas samples were determined for percentage of CH_4, CO_2, and H_2. The gas volume in the gas bags was measured using a drum-type gas meter (TG05; Ritter Apparatebau GmbH & Co. KG, Bochum, Germany) and added to the gas volume of the gas space in the effluent bottle.

2.4. Calculations and Statistical Analyses

Total gas volume was corrected for temperature (0 °C) and pressure (101.325 kPa) conditions. The daily production of VFA and NH_3-N resulted from multiplication of the measured concentrations by the effluent and gas volume, respectively. Apparent disappearance of organic matter (OMAD) and the degradability of DM (DMD) and aNDFom (NDFD) after 48 h of incubation were calculated by subtracting the pre- and post-incubated nutrient contents and substrate masses.

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

To fit the nested polynomial regression model and convert 3-NOP doses to equally spaced dosage levels, the linear LOGDOSE = LOG2(DOSE) and quadratic LOGDOSE_2 = LOGDOSE^2 regression parameters were created for 3-NOP doses within each CFP in the DATA step. As fixed regressive components were considered the effects of CFP, increasing 3-NOP dose (LOGDOSE) within the treatment (LC or HC) "LOGDOSE(CFP)" (linear regression term) and, for calculating the quadratic regression term, additionally its square "LOGDOSE _2 (CFP)". RUN×DOSE was set in the RANDOM statement to define the whole-plot error. The HTYPE option was set = 1 to enter and test the model terms (linear, quadratic) in sequential order.

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

PROCEDURE MIXED METHOD = REML;

CLASS CFP RUN DOSE;
MODEL Y = CFP LOGDOSE(CFP) LOGDOSE_2(CFP)/NOINT DDFM = KENWARDROGER SOLUTION HTYPE = 1;
RANDOM RUN RUN × DOSE;
CONTRAST ' LINEAR: coefficients equal' LOGDOSE(CFP) 1 −1;
CONTRAST ' QUADRATIC: coefficients equal' LOGDOSE _2 (CFP) 1 −1;
RUN.

Effects were declared statistically significant at p-values ≤ 0.05 and a trend was postulated at p-values between >0.05 and 0.10. Results are presented as least square means (LS means) with the standard error of means (SEM). Pearson correlation coefficients were calculated with N = 24 observations.

3. Results

3.1. Diet Composition and Substrate Degradability

Ingredients and chemical composition of the incubated diets are presented in Table 1.

The DMD (dose; $p = 0.041$) and OMAD (dose: $p = 0.052$) varied by 3-NOP dosage level (Table 2). In LC diets, DMD and OMAD increased from PLA to LOW by 11% and decreased by 7% in HIGH when compared to PLA. The DMD and OMAD were comparable between PLA and MED. In HC, DMD and OMAD were highest in diets with LOW and HIGH 3-NOP dosage levels but lower in PLA and MED. Percentage of DMD (%) was positively related to percentage proportion of CH_4 (Vol.-%) ($r = 0.471$; $p = 0.020$) and CO_2 (Vol.-%) ($r = 0.487$; $p = 0.016$) but negatively to H_2 (Vol.-%) ($r = -0.368$; $p = 0.077$) in total fermentation gas.

Degradability of NDF tended to be higher in LC diets (CFP: $p = 0.091$; Table 2) irrespective of 3-NOP dose. The NDFD correlated negatively with pH in fermenter fluid ($r = -0.665$; $p < 0.001$) and NH_3-N (mg/g of DMD) ($r = -0.632$; $p = 0.001$) but positively to Eh ($r = 0.495$; $p = 0.014$) and acetic acid concentration (mmol/L) ($r = 0.734$; $p < 0.001$).

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

Item		Treatments †				SEM §	*p*-Values ⁺						
	CFP	PLA	LOW	MED	HIGH		CFP	dose	CFP × dose	L	Q	$ß_L > F$	$ß_Q > F$
DMD	LC	640	718	638	596	26	0.640	**0.041**	0.335	0.464	0.229	0.364	0.216
	HC	655	687	627	658					0.703	0.743		
OMAD	LC	643	723	642	601	26	0.654	0.052	0.254	0.395	0.170	0.275	0.136
	HC	658	687	628	669					0.621	0.620		
NDFD	LC	358	505	362	293	49	0.091	0.246	0.145	0.346	0.149	0.349	0.162
	HC	310	319	288	350					0.699	0.585		

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

3.2. Gas Production and Gas Composition

Total GP (mL/d and mL/g of DMD) and CO_2 (% and mL/g of DMD) were affected by neither 3-NOP dose nor CFP (Table 3) and both were negatively correlated to pH in the fermenter fluid (GP: $r = -0.436$; $p = 0.033$; CO_2: $r = -0.460$; $p = 0.024$). A trend was observed for a quadratic effect of 3-NOP dose on CO_2 (Vol.-%) regarding LC diets (Q: $p = 0.066$; Table 3) and the difference in quadratic regression coefficients between LC and HC diets ($ß_Q > F$: $p = 0.086$).

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

Item	CFP	Treatments †				SEM §	*p*-Values +						
		PLA	LOW	MED	HIGH		CFP	dose	CFP × dose	L	Q	$ß_L > F$	$ß_Q > F$
Total GP ¶	LC	1902	1904	1664	1818	18	0.506	0.600	0.978	0.411	0.479	0.823	0.732
(mL/d)	HC	1844	1797	1648	1654					0.543	0.705		
Gas production (mL/g of DMD ‡)													
Total GP	LC	1091	977	938	1152	110	0.494	0.711	0.698	0.181	0.121	0.423	0.272
	HC	1042	970	982	944					0.703	0.825		
CH_4	LC	15.8	12.2	5.2	0.5	1.2	0.419	**<0.001**	0.241	**0.001**	0.145	**0.028**	**0.045**
	HC	14.5	12.1	8.7	1.1					0.133	0.407		
CO_2	LC	100.2	94.2	92.7	98.4	13.8	0.538	0.962	0.981	0.642	0.637	0.835	0.745
	HC	93.4	94.1	86.6	86.8					0.812	0.907		
H_2	LC	0.44	2.11	6.03	11.89	1.92	0.579	**0.001**	0.623	0.290	0.766	0.595	0.859
	HC	1.42	3.21	3.80	8.96					0.749	0.584		
Gas composition (Vol.-%)													
CH_4	LC	5.8	5.1	2.2	0.2	0.3	0.138	**<0.001**	0.094	**0.002**	0.566	0.082	0.124
	HC	5.5	5.0	3.6	0.5					0.241	0.119		
CO_2	LC	36.6	38.5	39.6	33.5	1.9	0.567	0.430	0.302	0.146	0.066	0.173	0.086
	HC	35.6	37.8	35.0	36.6					0.979	0.959		
H_2	LC	0.2	0.9	2.6	3.9	0.5	0.755	**<0.001**	0.458	**0.046**	0.579	0.233	0.307
	HC	0.5	1.2	1.5	3.7					0.699	0.368		
CH_4/CO_2	LC	0.157	0.131	0.055	0.004	0.008	0.024	<0.001	**0.026**	**<0.001**	0.220	**0.035**	**0.048**
	HC	0.155	0.133	0.102	0.014					0.177	0.082		
CO_2/CH_4	LC	6.4	7.8	18.9	915.6	193.2	0.185	**0.036**	0.179	0.398	0.066	0.592	0.238
	HC	6.5	7.6	10.0	163.6					0.887	0.741		
CH_4/H_2	LC	39.45	5.84	0.86	0.04	2.65	0.002	<0.001	**0.001**	**<0.001**	**0.001**	**0.001**	**0.002**
	HC	10.53	4.32	3.00	0.22					0.183	0.422		

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

The greatest CH_4 proportion (5.8%) was recorded for the control (PLA), followed in descending order by LOW, MED, and HIGH 3-NOP treatment down to 0.2% as in the case referring to CH_4 production (from 15.8 to 0.5 mL/g of DMD) in LC diets and 5.5% (PLA) to 0.5% (HIGH) and 14.5 (PLA) to 1.1 mL/g of DMD (HIGH) in HC diets, respectively (Table 3; dose: $p < 0.001$). Increasing 3-NOP dosage levels reduced CH_4 (% and mL/g of DMD) in a linear manner in LC diets only (L: $p < 0.01$; Table 3) and, with regard to CH_4 (Vol.-%), 3-NOP efficacy tended to be less pronounced in HC substrates (CFP × dose: $p = 0.094$; Table 3). The linear ($ß_L > F$: $p = 0.028$) and quadratic ($ß_Q > F$: $p = 0.045$) components of the regression were significantly different between LC and HC, indicating a variation in 3-NOP mitigation efficiency depending on the provided CFP, whereas the CFP main effect was not significant. In LC diets, 3-NOP supplementation reduced CH_4 (Vol.-%) by 12% (LOW), 61% (MED), and 97% (HIGH) relative to CH_4 (Vol.-%) analysed in the fermentation gas of the PLA treatment. Comparatively, CH_4 (Vol.-%) was mitigated to a lower extent in HC treatments, namely by 10% (LOW), 35% (MED), and 90% (HIGH) in relation to PLA. Methane proportion (Vol.-%) and production (mL/g of DMD) significantly differed among 3-NOP doses, except in the PLA versus LOW treatments (CH_4 (Vol.-%): LC: $p = 0.147$; HC: $p = 0.258$ and CH_4 (mL/g of DMD): LC: $p = 0.045$; HC: $p = 0.166$).

Positive correlations ($p < 0.05$) were found between CH_4 (Vol.-%) and *iso*-butyrate (mol %) ($r = 0.695$), acetate (mol %) ($r = 0.427$), and propionate (mol %) ($r = 0.420$), whereas CH_4 (Vol.-%) was negatively linearly related ($p < 0.05$) to H_2 (Vol.-%) ($r = -0.872$), *iso*-valerate (mol %) ($r = -0.796$), and total VFA production (mmol/g of DMD) ($r = -0.450$).

The ratio of CH_4/CO_2 was significantly affected by the CFP × dose interaction (CFP × dose: $p = 0.026$). In HC diets, the CH_4/CO_2 ratio tended to be quadratically influenced by 3-NOP dose level (Q: $p = 0.082$), whereas that of LC substrates was affected in a linear dose-dependent manner (L: $p < 0.001$) (Table 3; Figure 1A). The CFP treatment caused significantly different courses of the 3-NOP dose-related CH_4/CO_2 ratio as the linear and quadratic regression coefficients significantly differed ($ß_L > F$: $p = 0.035$; $ß_Q > F$: $p = 0.048$). Regarding 3-NOP dose MED, CH_4 was mitigated more effectively in LC compared to HC diets (CH_4 (Vol.-%) and CH_4/CO_2 ratio: contrast LC versus HC for dose MED: $p < 0.01$) (Table 3; Figure 1A). In an inverse ratio, the CO_2/CH_4 ratio increased with increasing 3-NOP dose ($p = 0.036$; Table 3). A considerably wider CH_4/H_2 ratio was found in the PLA treatment, which was most apparent in the LC diet (CFP × dose: $p = 0.001$). Increasing 3-NOP inclusion levels caused a linear and quadratic decrease in the CH_4/H_2 ratio for LC diets (L: $p < 0.001$; Q: $p = 0.001$) and the dose–response curves significantly differed by CFP ($ß_L > F$: $p = 0.001$; $ß_Q > F$: $p = 0.002$).

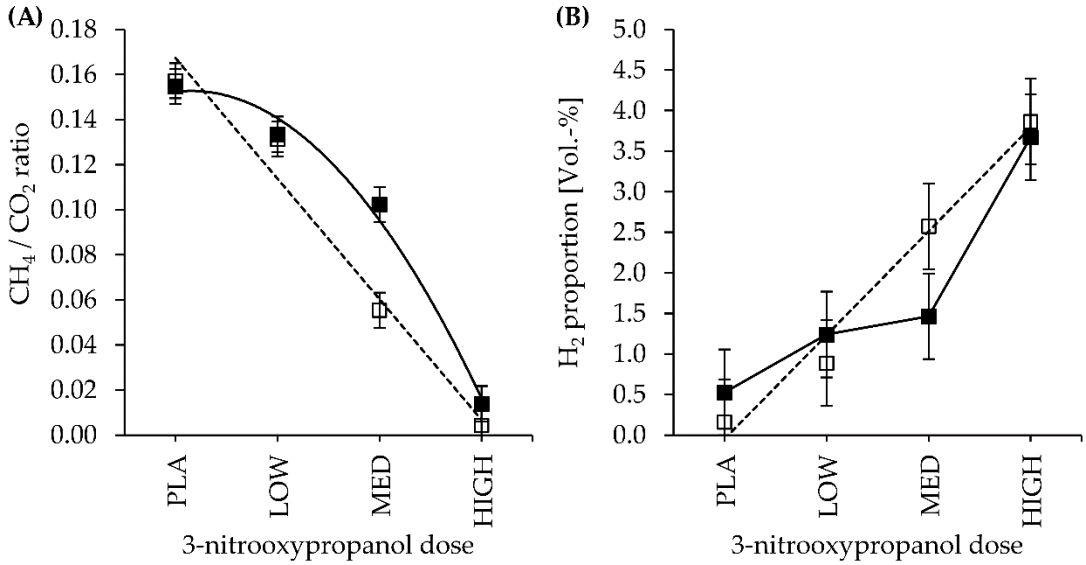

Figure 1. Effect of 3-nitrooxypropanol dose (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (□, dashed line) or high- (■, solid line) concentrate proportion in the incubated diet on (**A**) methane (CH_4) to carbon dioxide (CO_2) ratio and (**B**) hydrogen (H_2) proportion (Vol.-%) in fermentation gases; curve fitting according to (non)significant L and Q effects (Table 3).

Figure 1B illustrates a 27-fold increase in H_2 (% and mL/g of DMD) in LC and, to a lesser extent, a 6.2-fold increase in HC diets with increasing 3-NOP dose relative to PLA (Table 3; dose: $p = 0.001$). However, H_2 (% and mL/g of DMD) was not different between PLA and LOW. Both linear and quadratic effects of 3-NOP dose on H_2 (Vol.-%) remained not significant concerning the HC diet. In contrast, the linear regression coefficient was significant for the LC diet (L: $p = 0.046$), indicating that the slope of the curve increased more steadily when compared to HC (Figure 1B). In HC diets, H_2 (% and mL/g of DMD) was not significantly higher for 3-NOP dose MED in comparison to PLA, whereas contrast analysis revealed a significant variation between PLA and MED for LC diets ($p < 0.05$). Furthermore, H_2 (Vol.-%) significantly correlated to CH_4 (Vol.-%) ($r = -0.872$), acetate (mol %) ($r = -0.553$), propionate (mol %) ($r = -0.379$; $p = 0.068$), and iso-butyrate (mol %) ($r = -0.570$) in a negative manner, whereas positive relationships ($p < 0.05$) were found for *iso*-valerate (mol %) ($r = 0.601$) and the production of butyrate (mmol/g of DMD) ($r = 0.606$).

3.3. Fermentation Parameters and End-Products

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

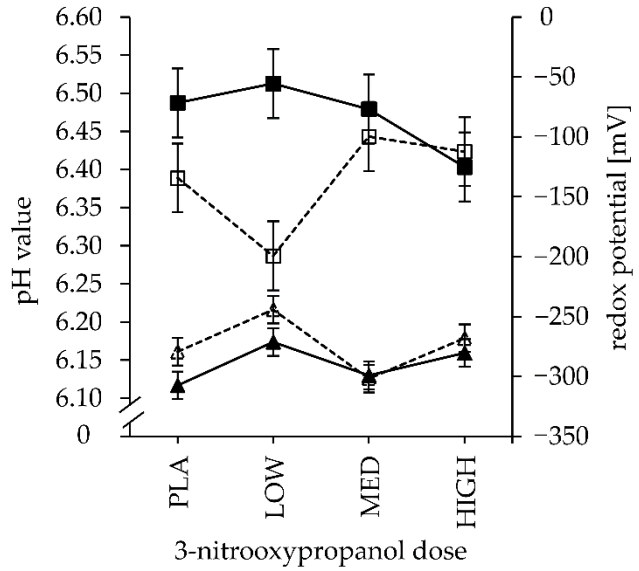

Figure 2. Effect of 3-nitrooxypropanol dose (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (□,△, dashed line) or high- (■,▲, solid line) concentrate proportion in the incubated diet on pH values (■,□) and redox potential (▲,△) in fermenter fluid; curve fitting according to (non)significant L and Q effects (see Table 4).

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

Table 4. Effects of 3-nitrooxypropanol (3-NOP) dosage levels (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (LC) or high- (HC) concentrate feed proportion in the diet (CFP) on fermentation characteristics and production of volatile fatty acids (VFA).

Item		Treatments †				SEM §	*p*-Values †						
	CFP	PLA	LOW	MED	HIGH		CFP	dose	CFP × dose	L	Q	$ß_L > F$	$ß_Q > F$
pH	LC	6.39	6.29	6.44	6.42	0.05	**0.019**	0.552	0.083	0.912	0.830	0.880	0.495
	HC	6.49	6.51	6.48	6.40					0.753	0.470		
Eh (mV) ¶	LC	−279	−244	−302	−268	12	0.066	**0.010**	0.517	0.623	0.645	0.100	0.178
	HC	−308	−271	−299	−281					0.621	0.745		
Effluent (mL/d)	LC	656	588	650	655	25	0.950	0.789	0.065	0.442	0.328	0.433	0.361
	HC	622	666	625	642					0.731	0.752		
NH_3-N (mg/L)	LC	157	165	136	142	8	**0.009**	**0.006**	0.499	0.344	0.611	0.187	0.125
	HC	214	235	207	191					0.608	0.228		
NH_3-N(mg/g DMD #)	LC	14.9	12.4	12.5	14.7	1.1	**<0.001**	0.861	0.081	0.065	**0.049**	**0.040**	**0.020**
	HC	18.3	20.9	19.1	17.7					0.260	0.151		
Total VFA (mmol/L)	LC	76.3	83.4	78.0	71.1	5.1	0.250	0.849	0.203	0.466	0.287	0.547	0.229

	HC	69.0	70.6	71.3	80.5					0.955	0.587		
Fermentation pattern (mol % of VFA)													
Acetate	LC	53.4	53.1	49.4	51.0	1.14	0.548	**0.044**	0.705	0.097	0.193	0.180	0.267
	HC	51.4	52.3	49.8	50.7					0.594	0.730		
Propionate	LC	17.9	18.4	18.1	13.8	0.73	0.326	0.012	**0.029**	0.197	**0.017**	0.683	0.135
	HC	16.2	15.9	17.2	16.0					0.387	0.385		
Butyrate	LC	13.6	13.5	15.5	15.3	1.13	0.190	0.864	0.281	0.414	0.629	0.495	0.988
	HC	16.7	16.7	16.1	14.6					0.964	0.641		
Iso-butyrate	LC	0.91	0.82	0.83	0.79	0.03	0.003	<0.001	**0.014**	0.134	0.371	0.170	**0.034**
	HC	0.98	1.00	0.92	0.74					0.821	0.052		
Valerate	LC	4.9	4.3	5.8	4.5	0.39	0.122	**0.005**	0.255	0.238	0.209	0.772	0.872
	HC	6.2	5.9	6.5	4.8					0.396	0.152		
Iso-valerate	LC	9.3	9.8	10.4	14.7	0.83	**0.044**	**<0.001**	0.893	0.828	0.102	0.797	0.867
	HC	8.6	7.9	9.4	13.1					0.569	0.066		
C_2/C_3 ratio [$]	LC	3.03	2.92	2.77	3.80	0.18	0.832	0.011	**0.032**	0.068	**0.008**	0.414	0.090
	HC	3.23	3.36	2.91	3.19					0.335	0.392		
VFA production (mmol/g of DMD)													
Total VFA	LC	7.18	6.86	7.11	7.36	0.264	**0.012**	**0.050**	0.409	0.569	0.416	0.468	0.997
	HC	6.22	6.26	6.56	7.32					0.927	0.418		
Acetate	LC	3.85	3.64	3.52	3.73	0.131	**0.004**	0.105	0.143	0.079	0.090	0.194	0.583
	HC	3.20	3.27	3.27	3.73					0.799	0.266		
Propionate	LC	1.30	1.27	1.29	1.02	0.073	0.022	0.531	0.031	0.566	0.191	0.950	0.155
	HC	1.02	1.00	1.14	1.19					0.604	0.997		
Butyrate	LC	0.96	0.93	1.09	1.13	0.087	0.777	0.551	0.640	0.554	0.931	0.645	0.994
	HC	1.04	1.05	1.05	1.05					0.898	0.925		
Iso-butyrate	LC	0.065	0.060	0.059	0.058	0.003	0.421	0.134	0.605	0.198	0.384	0.149	0.126
	HC	0.060	0.062	0.060	0.055					0.725	0.366		
Valerate	LC	0.34	0.30	0.41	0.33	0.029	0.256	**0.035**	0.469	0.245	0.265	0.848	0.944
	HC	0.39	0.38	0.42	0.35					0.346	0.232		
Iso-valerate	LC	0.68	0.66	0.74	1.09	0.071	**0.012**	**<0.001**	0.991	0.539	**0.048**	0.858	0.894
	HC	0.52	0.49	0.62	0.95					0.716	0.069		

[†] Values presented as LS means. [§] SEM, standard error of the means. [+] Effects of CFP, 3-NOP dose, and interactions between them; L, Q, *p*-values for linear and quadratic effects of 3-NOP; $ß_L > F$, $ß_Q > F$, probability under H_0 that an F-distributed random variable exceeds observed *F*, for the difference in the linear and quadratic regression coefficients between LC and HC. [¶] *Eh*, redox potential. [#] DMD, dry matter degradation. [$] C_2/C_3 ratio, acetate/propionate ratio. Significant values ($p \leq 0.05$ are highlighted in bold.

Irrespective of 3-NOP dose, NH_3-N concentration (mg/L) was 41% lower in LC compared with HC diets (CFP: $p = 0.009$; Table 4). In addition, NH_3-N concentration increased when supplementing 3-NOP dose LOW but decreased at higher 3-NOP doses of MED and HIGH independently of the CFP in the incubated ration (dose: $p = 0.006$).

The NH_3-N production ranged from 12.4 to 14.9 mg/g of DMD within LC and between 17.7 and 20.9 mg/g of DMD within HC treatment (Table 4). Moreover, NH_3-N production significantly differed by 5.4 mg/g of DMD between LC and HC (CFP: $p < 0.001$) and tended to be influenced by the CFP × dose interaction ($p = 0.081$). In LC treatments, NH_3-N production varied in a quadratic manner with increasing 3-NOP dose (Q: $p = 0.049$) (Figure 3A). The curves of NH_3-N production were shaped in an inverse manner between the incubated LC (convex) and HC (concave) substrates, and the quadratic regression coefficients differed significantly ($ß_Q > F$: $p = 0.020$).

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

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

The molar percentage of propionate (mol %) and its production (mmol/g of DMD) were numerically increased in LC diets and affected by a CFP × dose interaction ($p < 0.05$), which was related to the notable drop at 3-NOP dose HIGH in the LC treatment when compared to the relatively constant fluctuations observed in the HC treatment. Accordingly, a quadratic effect of 3-NOP dose on propionate (mol %) was noted in LC diets (Q: $p = 0.017$) (Figure 3C).

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

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

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

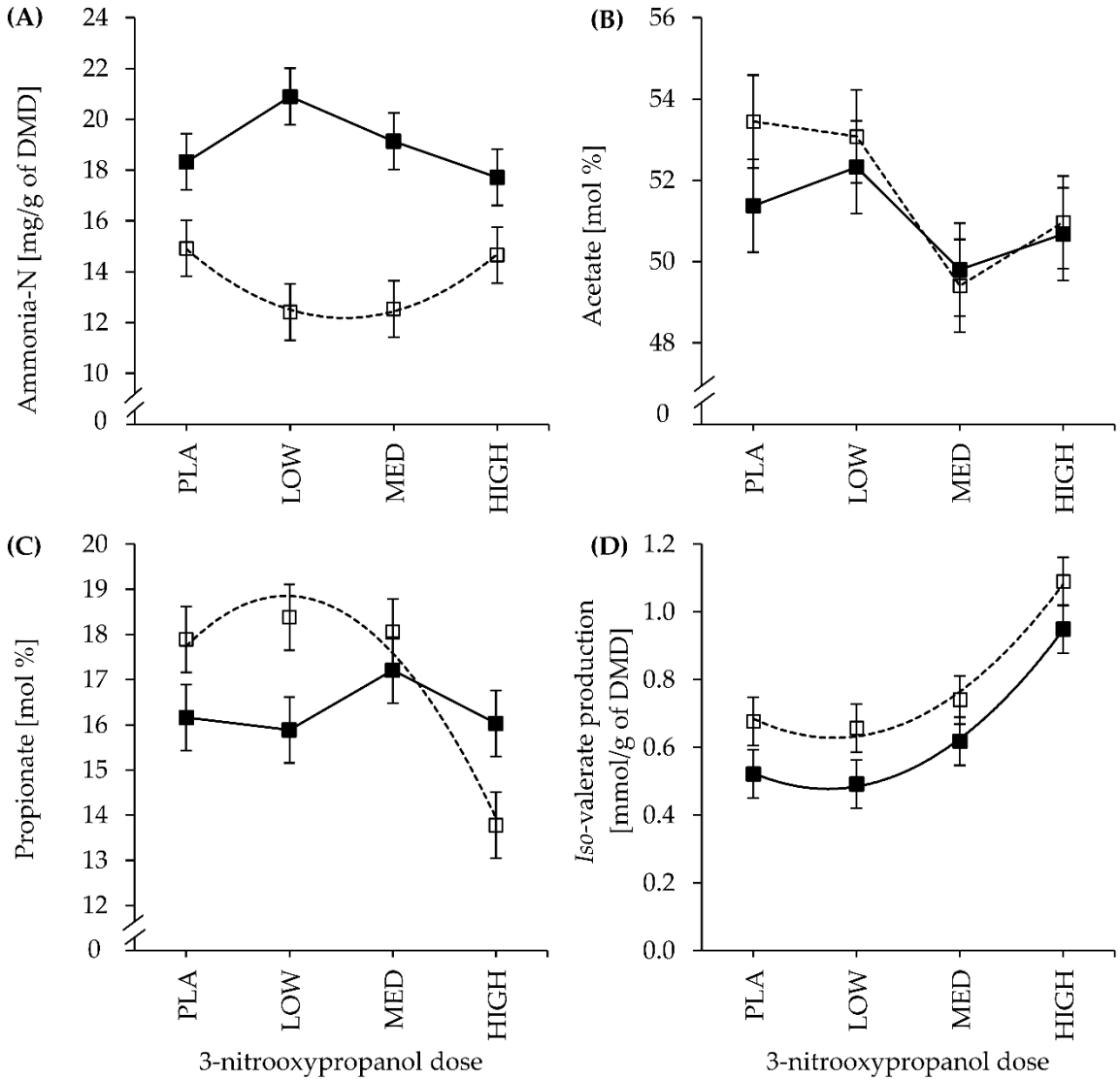

Figure 3. Effect of 3-nitrooxypropanol dose (PLA: 0, LOW: 73, MED: 160, and HIGH: 1200 mg of 3-NOP/kg of feed DM) and low- (□, dashed line) or high- (■, solid line) concentrate proportion in the incubated diet on (**A**) ammonia-N production (mg/g of dry matter degradation (DMD)), (**B**) acetate (mol %), (**C**) propionate (mol %), and (**D**) *iso*-valerate production (mmol/g of DMD) measured in the effluent; curve fitting according to (non)significant L and Q effects (see Table 4).

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

4. Discussion

In the present in vitro experiment, it was hypothesised that CH_4 production would decrease with increasing inclusion levels of 3-NOP and concentrate feeds in the incubated diet in an interactive manner.

4.1. 3-NOP Dosage Level

The magnitude of CH_4 reduction was highly affected by 3-NOP inclusion level, but this occurred independently of concentrate proportion in the diet substrate. A wider range of CH_4 inhibition was covered by the presently applied 3-NOP doses and diet substrates (Table 3) when compared to previous in vitro studies. Comparatively, Romero-Pérez et al. [10,14] incubated a forage-based substrate with 200, 500, 1000, and 2000 mg of 3-NOP/kg of feed DM in RUSITEC apparatuses. In the course of a saturation curve, they observed a high 3-NOP efficacy of reduction of 71.5, 76.0, and 84.5% at 200, 500, and 1000 mg of 3-NOP/kg of feed DM but no further CH_4 reduction with 3-NOP dose increment from 1000 to 2000 mg of 3-NOP/kg of feed DM (84.5 and 85.6%, resp.). Interestingly, in the present study, higher CH_4 mitigation maxima of 97 and 90% in LC and HC diets, respectively, were observed for the highest 3-NOP inclusion rate of 1200 mg/kg of feed DM applied. These different dose–response relationships may result from inherent sources of variation in the RUSITEC experiments, such as the use of different apparatuses and experimental protocols between laboratories. In the present experiment, the CH_4 reduction (Vol.-%) increased in a linear (LC) and convex parabolic (HC) shaped manner (Figure 1A) but not as a saturation curve, as had been previously reported in vitro [10,14] and in vivo [12]. Hence, 3-NOP inhibited CH_4 production at even lower doses (73 mg 3-NOP/kg feed DM: 12 and 10%; 160 mg 3-NOP/kg feed DM: 61 and 35% CH_4 reduction in LC and HC diets, resp.) when compared to the 71.5% CH_4 reduction at the minimum 3-NOP dose of 200 mg/kg of feed DM reported previously [10,23]. The differences in CH_4 production between LOW 3-NOP dose and the PLA treatment were, however, not significant (Figure 1A). This may indicate a compensatory response by the archaeal community attempting to counterbalance the 3-NOP inhibiting effect, which was likely metabolically feasible only at the lowest 3-NOP dose. Accordingly, methanogens can reactivate MCR through internal repair systems. In fact, Duin et al. [5] concluded that CH_4 inhibition is reversible.

Interestingly, 3-NOP dose LOW (73 mg of 3-NOP/kg of feed DM) seems to cause, in relative terms, a lower CH_4 reduction potential under in vitro conditions (10–12%; Table 3) when compared to supplementing comparable 3-NOP dose levels to dairy cows in vivo (23% with 68 mg of 3-NOP/kg DM [24]; 26.5% and 22.5% with 60 and 80 mg of 3-NOP/kg DM, resp. [12]). Conversely, a considerably high CH_4 reduction of more than 77.7% with 200 mg of 3-NOP/kg of feed DM can apparently only be achieved in vitro [15,23]. However, the maximum CH_4 reduction potential seems to be limited to 40% under in vivo conditions when 3-NOP is continuously supplied at an equal dose of 200 mg/kg of feed DM to dairy cows by mixing in the compound with the TMR [12]. Thus, Melgar et al. [12] quantified the maximum CH_4 mitigation effect to 40% at a 3-NOP dose of 100 mg/kg of feed DM without any statistical improvement in 3-NOP efficacy when supplementing higher doses of 150 and 200 mg of 3-NOP/kg of feed DM into the TMR of lactating cows. In conclusion, when compared to 3-NOP supplementation in vivo, the 3-NOP efficacy seems to be reduced at low but increased at high 3-NOP dose levels in vitro. This leads to the assumption that the dose–response relationships and 3-NOP effect mechanisms find expression in a different manner depending on whether 3-NOP is supplemented in vitro or in vivo and corresponding technical as well as rumen physiological factors affecting the mode of action of 3-NOP.

In the present experiment, the 3-NOP compound was mixed into the concentrate feed and therefore supplemented once per day as a ‘single dose’ with the feed bag into the fermenter but not as a continuous infusion. The 3-NOP compound is supposed to be water-soluble and rapidly metabolised in rumen liquid [25] and, therefore, recommended to be dosed at sufficient amounts synchronously to the MCR activity stimulating feed degradation [11]. In the present experiment, it is likely that the compound was rapidly disaggregated into 1,3-propanediol and nitrate [5] and further washed out of the vessel with the liquid outflow due to the high dilution rate of 3%/h, which could, conclusively, explain the general need for higher 3-NOP inclusion rates under conditions in vitro. In correspondence, Vyas et al. [26] observed that 3-NOP efficacy decreased 16 h after feeding when supplementing only 100 mg of 3-NOP/kg of feed DM to beef cattle, whereas a persistent CH_4 inhibition over 24 h was achieved at higher 3-NOP inclusion levels of 200 mg/kg DM. Thus, the highest 3-NOP dose applied in the present study could have prevented the complete washing out of the 3-NOP supplement from the fermenter, which could have resulted in sufficient amounts of the feed additive remaining in the fermenter fluid for targeting archaeal MCR over the whole 24 h incubation time horizon until the next feed bag exchange. This would become even more important during the course of the incubation with regard to inactivating the MCR activity arising time-delayed from slow

fermentable fibre fractions in the LC diets. Thus, rates of fermentation of NDF are significantly lower as compared to that of rapidly fermentable NFC [27]. However, the CFP × dose interaction was not significant. Moreover, RUSITEC experiments are limited to investigating the short-term gas production kinetics of fast and slow fermentable fractions between feeding bag exchange. Therefore, 3-NOP effects on 24 h fermentation kinetics should be the focus in future experiments, e.g., using the Hohenheim Gas Test, according to Menke et al. [28].

Interestingly, Duin et al. [5] found that a 100-fold increase in 3-NOP concentration is required to suppress the growth of the methanogenic Archaea *Methanomicrobium mobile* and *Methanosarcina barkeri* when compared to the required 3-NOP amounts for inhibiting the growth of the predominant species in the bovine rumen, i.e., *Methanobrevibacter ruminantium* [29]. The reasons for the different degrees of sensitivity of methanogenic species towards 3-NOP remain to be elucidated, yet 3-NOP's effects on individual methanogenic lineages were recently observed in vivo [30,31]. However, the possibility that not all of the methanogenic species were captured by the lower 3-NOP dose could have favoured those methanogenic Archaea being less sensitive to 3-NOP, causing a shift in the methanogenic community structure to those occupying this ecological niche. As a consequence, the 3-NOP dose HIGH could have targeted a greater number and wider range of methanogenic archaeal species, causing a more comprehensive and effective blocking of those MCR amounts arising from immediate feed fermentation processes directly after feed bag exchange. This could have led to a more sustained suppressive effect on methanogenic activity until the next feed bag exchange and could further explain the high CH_4 reduction of more than 90%. In conclusion, the 3-NOP stability and its CH_4 inhibiting persistency in vitro should be investigated in future experiments by conducting continual 3-NOP infusion into the fermenter paralleled with frequent gas sampling from the gas bag for CH_4 analyses between feeding events.

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

In the present in vitro study, the nonsignificant combination effect of 3-NOP and CFP on CH_4 reduction contrasts findings from in vivo experiments [13,16]. This leads to the assumption that diet type per se does not contribute to synergistic effects but, rather, specific diet-induced rumen physiological factors and, more importantly, those being controlled in a RUSITEC. Thus, feeding HC diets may cause additive indirect effects on CH_4 inhibition that are related to the increased production of propionate from H_2-consuming fermentation pathways, passage rate (thereby limiting the time available for degradation of slowly fermentable carbohydrates), and reduced pH values (thereby inhibiting pH-sensitive methanogens) [7,32], affecting fermentation kinetics and microbial community structures [11,16,33]). As is typical for RUSITEC experiments, the fermentation conditions (e.g., particle retention time, flow rate of the (artificial) saliva, size of feed particles, motility, temperature, ratio of feed to liquid content, and liquid outflow rate) were standardised and strictly controlled in the present study. This could have equalised the abovementioned potential concentrate feed effects on fermentation characteristics and, therefore, explain the lack of synergistic effects between high CFP and 3-NOP on CH_4 inhibition.

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

End-products of microbial fermentation, i.e., VFA, act as electron acceptors, which maintain the strongly reductive rumen milieu and can, therefore, directly be linked to microbial activity [34]. Correspondingly, the *Eh* in the fluid, reflecting the redox homeostasis and electron transfer, is hypothesised to be a control of enzymatic processes in rumen microorganisms [34,35]. However, *Eh* positively correlated to total VFA ($r = 0.563$; $N = 24$; $p = 0.004$) in the present investigation, which contrasts findings of an inverse

relationship [36]. From a literature review, Huang et al. [36] indicated that *Eh* increased with CFP (r = 0.497; $p = 0.015$) and negatively correlated to pH, whereby the latter was also observed in the study at hand (*Eh* vs. pH: r = −0.57; N = 24; $p = 0.004$). Hydrogen produced from microbial fermentation preserves reducing conditions in the rumen. In the present experiment, though, negative correlations between *Eh* and H_2 concentrations in fermentation gases of 3-NOP treatments were not observed and *Eh* fluctuated inconsistently over the 3-NOP dose levels. However, interpretation should be made with caution as *Eh* was measured during feed bag exchange so that oxygen entrance, notably affecting oxidation-reduction conditions in the fermenter fluid, was unavoidable.

The present RUSITEC experiment confirmed the previous studies of Guayder et al. [23] and Romero-Peréz et al. [10] reporting H_2 accumulation in fermentation gases and decreased molar acetate proportions with increasing 3-NOP dose and CH_4 mitigation (H_2 (Vol.-%): dose: $p < 0.001$ (Table 3); acetate (mol %): dose: $p = 0.044$ (Table 4)). As an H_2-liberating fermentation process, acetate production could have been downregulated to prevent a further increase in H_2 partial pressure in the fermenter fluid, which would be detrimental to the growth of cellulolytic bacteria. However, negative side effects of reduced NDF degradability (Table 2) or total gas production (Table 3) were not consistently observed in the present experiment. Starch fermentation in the HC diets and the 3-NOP-induced H_2 increase (Figure 1B) were both assumed to promote a shift in fermentation balance from acetate to alternative H_2 sinks of valerate and propionate, as previously observed under conditions in vivo [16,37]. However, contrary to these expectations, the propionate proportion (mol %) was lower in HC when compared to LC diet, whereas the opposite held true concerning valerate proportions (Table 4). However, valerate formation can be traced back not only to NFC fermentation but also to the deamination of proline. In addition, a consistent increase in propionate and valerate proportions due to the 3-NOP supply was, interestingly, observed in neither the present study nor in previous in vitro experiments [10,23], except for valerate, which was previously found to be increased with 3-NOP inclusion in vitro [23]. These observations could be explained by the unphysiological longer retention time of small-sized feed particles in a RUSITEC (fixed time of 48 h) when compared to rumen conditions in vivo. For instance, Prigge et al. [38] reported that the retention time of 3 mm particles amounts to 20 h in vivo. Martinez et al. [39] incubated a 30:70 alfalfa hay:concentrate diet in RUSITEC fermenters and observed that reducing the retention time of concentrates from 48 to 24 h and increasing the dilution rate from 3.78 to 5.42%/h increased the production and molar proportions of propionate. Furthermore, retention time depends on dry matter intake, stratification of the rumen content, and the size and density of feed particles [40]. These influencing factors were, however, standardised in the reaction vessels, which could have negatively affected the adaptation of propionate enhancers to the environmental conditions in the RUSITEC [41]. The question about a possible redirection of H_2 spared from methanogenesis to further alternative H_2 utilising pathways other than propionate synthesis becomes even more interesting as it can be assumed that H_2 accumulation does not exclusively occur in fermentation gas but also in the liquid phase. Thus, in the performed experiment, the calculative amounts of the H_2 excess from CH_4 inhibition (assuming that 4 moles of metabolic hydrogen are spared from the inhibition of 1 mole of CH_4) were not completely recovered in alternative H_2 removals (H_2 emission via fermentation gas, H_2 incorporation into propionate and valerate), which can be deduced from the decreasing CH_4/H_2 ratio with increasing 3-NOP dose (Table 3). In conclusion, a rechannelling of the spared H_2 to further alternative metabolic routes not analysed in the present experiment may have occurred. Regarding this, Guyader et al. [23] observed increased concentrations of atypical H_2 sinks of ethanol, formate, caproate, and heptanoate in the fermenter liquid at a 200 mg inclusion rate of 3-NOP/kg of feed DM.

The convex parabolic curve of the NH_3-N production in LC diets was inversely shaped to that of the HC diets (Figure 3A). These interrelations might be of multifactorial origin, such as reduced proteolysis or an increased microbial NH_3-N uptake in LC fermenter fluid [42], but also with regard to the lower CP content in LC diets (Table 1). Molar proportions of *iso*-butyrate decreased with increasing 3-NOP dose, which could indicate a decreased deamination of amino acids (AA). In contrast, as reported previously [10], *iso*-valerate production increased in a quadratic manner with 3-NOP dose, which contradicts the hypothesis of a decreased AA deamination as BCVFA results from both deamination and decarboxylation of valine and leucine, respectively [43]. There are possibly different metabolic processes of these BCVFA under CH_4

inhibition and H_2 accumulation which need to be clarified in future (Figure 3D). Microbial uptake and release of BCVFA thus are the main determinants of BCVFA concentrations and microbial protein synthesis is regarded as a H_2 sink apart from methanogenesis [43,44].

5. Conclusions

The hypothesis of synergistic effects between 3-NOP and increased CFP on CH_4 inhibition was rejected for the applied in vitro conditions. The present RUSITEC experiment evidenced that 3-NOP effectively inhibited methanogenesis in a dose-dependent manner irrespective of CFP in the incubated diet. Negative side effects on nutrient degradability and, correspondingly, total VFA and gas production were not consistently observed for 3-NOP or CFP. However, 3-NOP dose increment was paralleled by H_2 gas accumulation, whereas alternative H_2 sinks of propionate and valerate remained unaffected. Increasing 3-NOP dosage decreased H_2-liberating acetate formation, whereas butyrate proportion remained unchanged. Conclusions from in vitro experiments cannot be fully transferred to the rumen environment in vivo. This study and others suggest that extrapolating findings from dose-dependent dynamics of the 3-NOP efficacy under conditions in vitro should be treated with caution for planning 3-NOP application in vivo. The present research should be broadened by focusing on potential changes in microbial community structures when 3-NOP is supplemented to different dietary concentrate:forage ratios.

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

Informed Consent Statement:

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

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7. General discussion

7.1. Methane prediction models

7.1.1. Introduction to the needs and challenges of CH_4 prediction model development

Intensive global efforts are going on to quantify and predict enteric CH_4 emissions from ruminant livestock systems in conjunction with the detection of explanatory key variables of methanogenesis for the development of effective CH_4 abatement strategies. The development and implementation of CH_4 inhibitors in practice, e.g. 3-NOP, require new CH_4 prediction models and the identification of its relevant key variables in a CH_4 mitigation scenario. Ongoing CH_4 modeling approaches aim at permanently optimizing the accuracy of e.g. GHG emission inventories and assessing the effectiveness of different on-farm CH_4 mitigation strategies for a low-carbon future in milk production (chapter 2.1.2.). Direct measurements of enteric CH_4 emissions from ruminants for on-farm use are currently not available for application in the farm routine (chapter 2.3.1.) (Hill et al. 2016). As a consequence, there is an urgent need for accurate and robust CH_4 prediction models being usable for the abovementioned purposes and areas of application (Appuhamy et al. 2016). The development of robust CH_4 prediction models requires comprehensive country-specific reference datasets including a high variability and accuracy of the actually measured target variable (CH_4/d) and corresponding explanatory variables (Negussie et al. 2017). In this context, the GreenFeed technique provides an opportunity to generate large reference datasets and to explore relevant proxies for the prediction of CH_4 emissions (chapter 2.3.1.). Major prerequisites for the suitability of proxies in predicting CH_4 emissions in the farm routine encompass the cost-effective, preferably automatized generation of accurate proxy data with strong correlation to the CH_4 production on a group as well as animal-individual level.

However, there is a trade-off between the on-farm availability of powerful proxies being usable as predictor variables in CH_4 prediction models and the desirable high prediction accuracy of the models. In this regard, CH_4 prediction model accuracy is expected to increase with model complexity. More complex mechanistic CH_4 prediction models may reflect the underlying biochemical mechanisms of methanogenesis, but they depend on input variables which cannot be monitored in practice (e.g., rumen VFA pattern) (Alemu et al. 2011). At the moment, there is no single on-farm proxy available for an accurate prediction of CH_4 emissions. As a consequence, the combination of phenotypical and diet-related proxies can be used to describe their stochastic

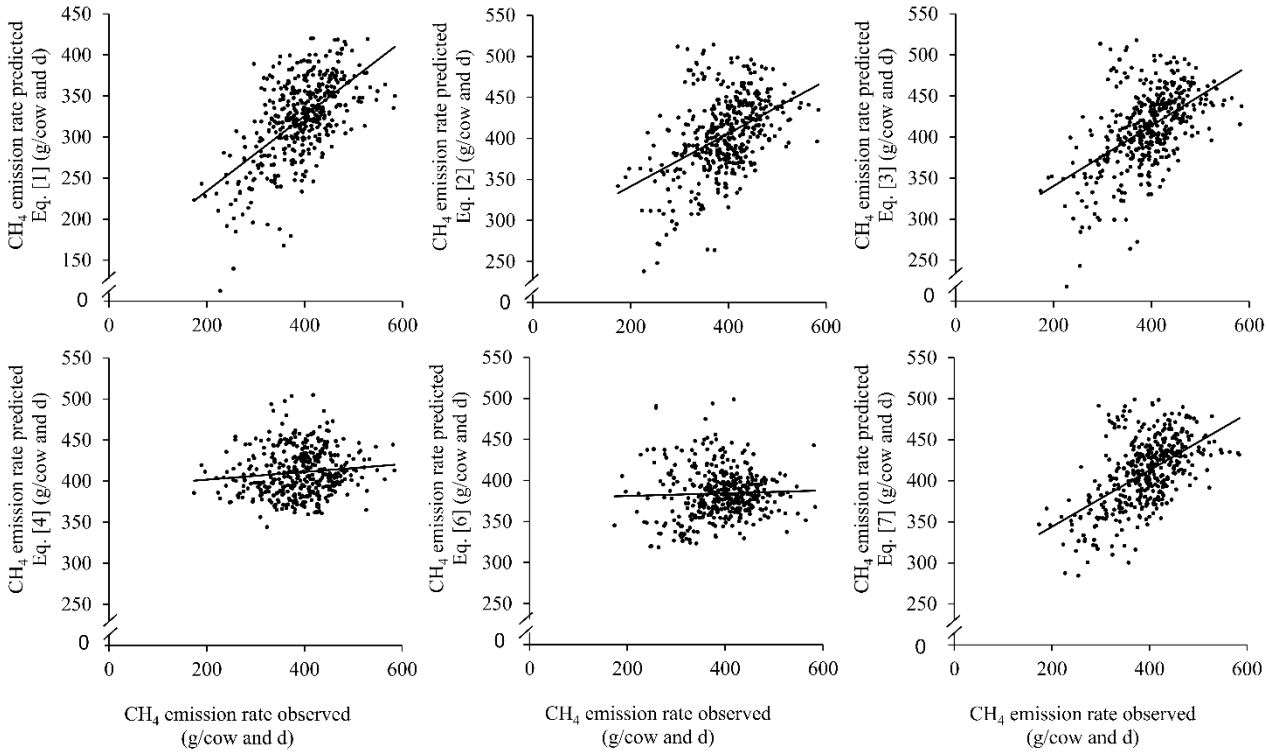
relationship to the CH_4 production in more easily applicable empirical CH_4 prediction models (Negussie et al. 2017, Niu et al. 2018, van Lingen et al. 2019). The development and use of different CH_4 prediction models in conjunction with the targeted application (e.g., on-farm use, scientific purposes, GHG emissions inventory), ruminant category (beef or dairy cattle, small ruminants) and region (German or US feeding systems) is becoming more and more required (Negussie et al. 2017). In this regard, Niu et al. (2018) revealed a further trade-off concerning the robustness of CH_4 prediction models in their general applicability in different countries and regions around the world, since country-specific models mostly perform better than intercontinental models which implicates that regional models are needed, in particular for national GHG inventories. As outlined in chapter 2.1.1., CH_4 production appears to be strongly dependent on characteristics of the prevailing milk production system which differs between regions due to differences in ration formulation, nutrient composition, animal genetics and performance.

A further challenge in developing CH_4 prediction models will prospectively gain in importance, which is the consideration of the effect size of CH_4 mitigation resulting from an effective use of CH_4 inhibitors in a CH_4 prediction model. Hence, the conventional physiological legalities and proxies on which extant CH_4 prediction models have been built up so far may not apply for rumen conditions when 3-NOP is fed to ruminants (PAPER I-III; Background 2.3.4.). Importantly, DMI represents the most important proxy for CH_4 production (Niu et al. 2018), but several studies detailed that the reduction in CH_4 emissions in consequence of supplementing 3-NOP was not paralleled by changes in DMI (2.3.4.; PAPER I). The model evaluation metrics of Figure 4 and 5 demonstrate the challenge of the limited adequacy of extant CH_4 prediction models presented in Table 1, which were recently developed from the EU dairy CH_4 database in the 'GLOBAL NETWORK' project by Niu et al. (2018), when applied to the datasets of the CH_4 inhibited 3-NOP fed cows (Fig. 5) in comparison to the non-CH_4 inhibited (CON) cows (Fig. 4) of the present study. Statistics revealed a considerably higher RMSPE and mean bias paralleled by a reduced model fit (R^2) and CCC when applying the CH_4 prediction models to the 3-NOP fed cows (explanation of model evaluation metrics presented in the footnote of Table 1). Future model development approaches should focus on the detection and implementation of those proxies for CH_4 emissions

Table 1: Overview of extant CH_4 prediction models (g CH_4/cow and d) with internal model evaluation assessment for different target applications cross-validated using EU reference data.

Model specification			Model development		Model	Model evaluation metrics[Ω]					
Reference[†]	Equation	Model name	CH_4 prediction model[‡]	n_1[⁂]	application[Π]	RMSPE, %	RSR	MB, %	SB, %	CCC	n_2[§]
[A]	[1]	TIER-3	CH_4 (g/cow and d) = 0.063 + CF intake (g/cow and d) × 0.079 + NfE intake (g/cow and d) × 0.01 + CP intake (g/cow and d) × 0.026 − EE intake (g/cow and d) × 0.212		emissions inventory in Germany	-	-	-	-	-	-
[B]	[2]	DMI_C	CH_4 (g/cow and d) = 107 (12.6) + DMI × 14.5 (0.39)	2,022	SCIENCE	15.0	0.66	3.72	1.27	0.71	1,423
[B]	[3]	DMI + NDF_C	CH_4 (g/cow and d) = −26.0 (16.7) + DMI × 15.3 (0.41) + NDF × 3.42 (0.309)	1,779	SCIENCE	14.7	0.65	1.63	1.05	0.72	1,423
[B]	[4]	ECM + Com_C	CH_4 (g/cow and d) = 141 (18.9) + ECM × 4.75 (0.22) + MP × 27.4 (3.70)	2,022	INVENTORY, ON-FARM	16.6	0.73	0.99	4.64	0.58	1,423
[B]	[5]	MY_C	CH_4 (g/cow and d) = 287 (14.1) + MY × 3.16 (0.224)	2,022	ON-FARM	18.4	0.81	1.15	3.67	0.45	1,423
[C]	[6]	Diet + Milk	CH_4 (MJ/cow and d) = 8.967 + MY (kg/d) × 0.141 + MP × 1.514 + MF × 1.919 + NDF × 0.054 − ME × 0.707	489	ON-FARM	20.7	0.94	5.6	1.5	0.21	215
[B]	[7]	Animal_C	CH_4 (g/cow and d) = −52.2 (21.7) + DMI × 13.0 (0.49) − EE × 10.9 (1.50) + NDF × 2.80 (0.349) + MF × 7.26 (1.59) + BW × 0.154 (0.0167)	1,423	SCIENCE	14.6	0.64	2.58	2.60	0.72	1,423
[D]	[8]	MSMYPB	CH_4 (g/cow and d) = FT-MIR spectra, parity, breed, test-day MY	1,089	INVENTORY, ON-FARM	14.1	-	-	-	0.81	1,089

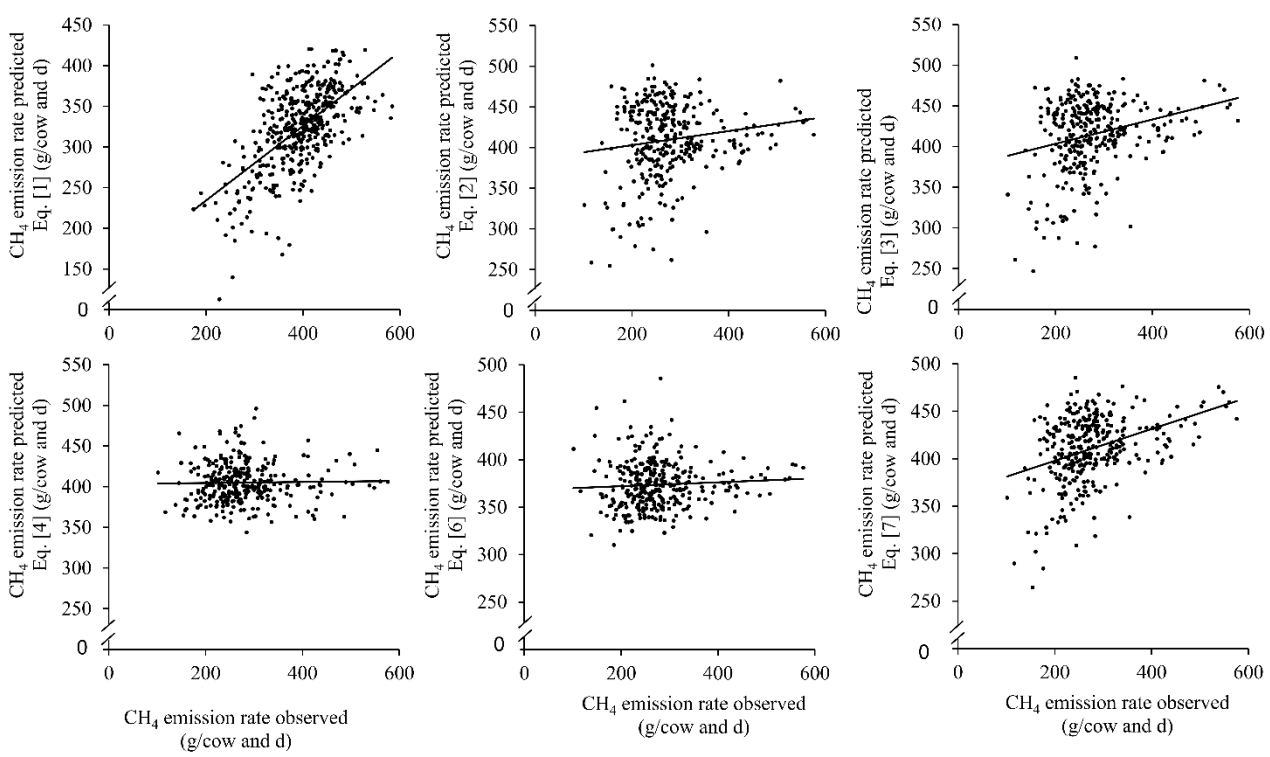
[†][A] Kirchgessner et al. (1994), [B] Niu et al. (2018), [C] Santiago-Juarez et al. (2016), [D] Vanlierde et al. (2020).
[‡]CH_4 prediction models with estimate and standard error; BW = body weight (kg), CF = crude fibre, EE = ether extract (% of DM), CP = crude protein, DMI = dry matter intake (kg/d), ECM = energy-corrected milk yield (kg/d) according to Tyrrell und Reid (1965), FT-MIR = Fourier-Transform mid-infrared spectroscopy, MF = milk fat content (%), ME = metabolizable energy (MJ/kg DM), MP = milk protein content (%), MY = milk yield (kg/cow and d), NDF = neutral detergent fibre (% of DM), NfE = N-free extracts.
[⁂]n_1 = number of observations for CH_4 prediction model development. [Ω]Model evaluation metrics: description is detailed in chapter 7.1.2. n_2 = number of observations for cross-validation of the CH_4 prediction models.



Model evaluation†	Eq. [1]	Eq. [2]	Eq. [3]	Eq. [4]	Eq. [5]	Eq. [6]	Eq. [7]
Intercept	143	277	267	393	383	378	275
Slope	0.457	0.322	0.367	0.047	0.06	0.017	0.344
Mean ± SD	322 ± 50	403 ± 47	410 ± 47	411 ± 28	406 ± 19	384 ± 29	410 ± 41
R^2	0.37	0.21	0.27	0.01	0.05	0.001	0.30
Residual SD	54	62	58	69	65	72	56
RMSPE, %	22.5	16.0	15.6	18.4	17.1	18.4	15.0
MB, %	62.8	3.7	9.9	7.6	5.2	0.9	10.0
SB, %	1.2	7.3	3.9	8.1	0.5	14.0	0.6
CCC	0.56	0.42	0.89	0.66	0.11	0.73	0.85

†Description of model evaluation metrics is presented in the footnote of Table 1.

Figure 4: External validation of extant CH_4-prediction models (Tab. 1) developed from the 'GLOBAL NETWORK' CH_4 database using the GreenFeed-System and weekly means of 25 dairy cows from d 1 until d 120 *postpartum* of the underlying experiment of the thesis (Schilde et al. 2021). Cows were fed without (CON; n = 418; average CH_4 emission rate: 391 g/cow and d) 3-nitrooxypropanol.



Model evaluation†	Eq. [1]	Eq. [2]	Eq. [3]	Eq. [4]	Eq. [5]	Eq. [6]	Eq. [7]
Intercept	241	386	374	403	393	368	364
Slope	0.287	0.087	0.149	0.006	0.047	0.021	0.168
Mean ± SD	321 ± 48	410 ± 45	415 ± 43	405 ± 24	406 ± 19	374 ± 24	411 ± 35
R^2	0.24	0.02	0.07	0.0004	0.039	0.004	0.014
Residual SD	68	84	78	81	76	80	73
RMSPE, %	29.2	56.4	57.1	54.5	53.8	45.1	54.7
MB, %	29.1	71.6	75.7	71.5	74.0	59.4	77.3
SB, %	0.9	4.4	2.1	2.2	0.1	2.4	0.2
CCC	0.71	0.04	0.25	0.16	0.02	0.24	0.22

†Description of model evaluation metrics is presented in the footnote of Table 1.

Figure 5: External validation of extant CH_4-prediction models (Tab. 1) developed from the 'GLOBAL NETWORK' CH_4 database using the GreenFeed-System and weekly means of 20 dairy cows from d 1 until d 120 *postpartum* of the underlying experiment of the thesis (Schilde et al. 2021). Cows were fed with (n = 320; average CH_4 emission rate: 277 g/cow and d) 3-nitrooxypropanol (3-NOP; 50 mg/kg of feed DM).

which perform at a high accuracy and applicability under different CH_4 mitigation scenarios and milk production systems. In essence, the abovementioned trade-offs are needed to be considered in CH_4 modeling approaches in regard to the target application of the CH_4 prediction model to avoid systematic biases, since there is no exclusive CH_4 prediction model being universally applicable among regions, cattle types, CH_4 mitigation strategies and feeding systems so far (Niu et al. 2018). The following part of the thesis aims at detecting potential proxies for CH_4 production in order to evaluate extant and to develop new CH_4 prediction models, which are categorized by their applicability (ON-FARM, SCIENCE) in periparturient and early-lactation cows, for its application in CH_4 mitigation scenarios with 3-NOP (CH_4 MITIGATION) using experimental data of the present study. The following section aims at testing hypothesis V (chapter 3).

7.1.2. CH_4 prediction model development

CH_4 prediction models (g/cow and d) were developed on the data of the underlying feeding experiment (PAPER I and PAPER II) (Table S1). Daily measurements were summarized to weekly means to improve the robustness of the GreenFeed data (PAPER I; averaging method according to Manafiazar et al. (2016)). The refined complete dataset contained a total of 45 cows and 914 weekly observations. The *antepartum* period was excluded from the dataset when milk parameters were used as input variables in modeling. The overall dataset was split into a training and a test dataset and their descriptive statistics are presented in Table S1:

In a first step, the performances of previously published CH_4 prediction models (Tab. 1; Eq. [1] – [8]) were assessed for their robustness and accuracy in an external validation as they were applied to the experimental cows of the CON (Fig. 4) and, respectively, 3-NOP groups (Fig. 5) of this study. This comparison facilitated an assessment of the adequacy of extant CH_4 prediction models under a CH_4 mitigation scenario with 3-NOP.

In a second step, the experimental data of the CON groups were used as a training dataset for the development of the CH_4 prediction models Eq. [9] – [14] (Tab. 2). Both CON and 3-NOP data were used as a training set for the development of Eq. [16] using rumen VFA variables only. This was done in order to directly compare Eq. [15] (CON data) and [16] (CON plus 3-NOP data) and to assess the improvement in model validity due to the inclusion of CH_4 data from a 3-NOP-based CH_4 mitigation strategy into the database for model development procedures. Moreover, a dataset including only 3-NOP data was used, on the one hand, to train the model Eq. [17] and, on the other hand, to assess the performance of the self-developed CH_4 prediction models from CON data (Tab.

2; Eq. [9] – [15]) under the 3-NOP driven CH_4 mitigation (Tab. 3). Overall, the model development approach and 10-fold cross-validation enabled variable selection (identification of relevant proxies of CH_4 emissions) and an assessment of the predictive performance of own CH_4 prediction models under different feeding strategies with and without 3-NOP.

CH_4 emission rate (g/d) was predicted by fitting a multiple linear regression model. The potential explanatory variables were selected prior to entering the model computation with regard to their availability in commercial dairy farms (ON-FARM; diet and milk composition, milk production and body mass measures), in scientific animal experiments (SCIENCE; rumen VFA and blood variables in addition to the on-farm variables) or in a CH_4 mitigation scenario with 3-NOP (CH_4 MITIGATION; rumen VFA and milk variables due to their relationship to FT-MIR spectra), respectively (selection shown in Tab. S1). All the CH_4 prediction models were categorized according to their field of application (Tab. 1 and 2: ON-FARM, SCIENCE, CH_4 MITIGATION). Models were constructed using the '*olsrr*' package (R, v. 4.0.5). In a stepwise regression, candidate variables were selected (forward and backward) according to the lowest Akaike´s Information Criterion considering the best trade-off between the explanatory power and an overfitting of the model (model complexity). Multicollinearity between the selected explanatory variables was proved using the variance inflation factor (VIF) ('*car*' package). A VIF greater than 5 indicates that the predictor variables are highly linearly interrelated in the model and, accordingly, the respective variable(s) were removed (O'Brien 2007).

Model validity was graphically assessed by residual analyses (Fig. 6) with regard to the assumptions for linear regression of linearity between the predictor and response variable, normality and homoscedasticity of the residuals and a test for extreme values. Outliers were eliminated when exceeding the influential threshold of 0.04 (Cook 1977). A scatterplot matrix was constructed to explore the linearity, the normality of the data and the Bravais-Pearson correlation coefficients between the key variables and CH_4 production (Fig. 7). Log-transformation was applied to skewed data to conform them to normality.

The predictive accuracy of the models was evaluated by a 10-fold cross-validation using the '*caret*' package and '*trainControl*' function. The complete dataset was split into ten subsets. In a sequential manner, each of the subsets was used as a validation dataset while the remaining k-1 subsets were used to train the model. Indices of the explanatory power of the model were calculated ('*caret*'). Resultantly, the 10-fold cross-validated model evaluation metrics were tabulated (Tab. 1 and 2).

The CH_4 prediction models developed from the CON dataset (Tab. 2) were regressed against the observed CH_4 production under 3-NOP treatment (Tab. 3).

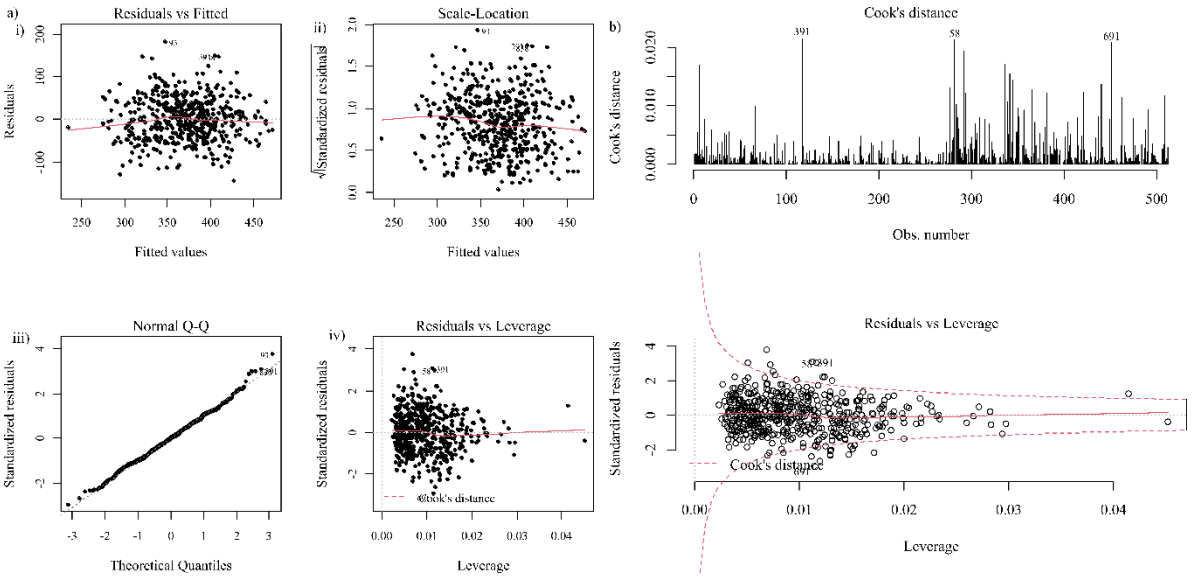


Figure 6: Example of a) diagnostic plots and b) the spike plot of the Cook´s distance of Eq. [12].

The residuals were plotted against the fitted values to check for non-linear patterns of the residuals. The nearly horizontal line in Fig.6 a) i) supports that residuals are not dependent on fitted values. The Scale-location plot (Fig. 6 a) ii)) illustrates a nearly horizontal line with equally spread points identifying homogeneity of variance of the residuals (homoscedasticity). Normality of the standardized residuals was assessed using the Normal Q-Q plot (Fig. 6 a) iii)). The standardized residuals were strongly related to their theoretical quantiles supporting the view that the residuals were normally distributed. The Residuals vs Leverage plot (Fig. 6 a) iv)) detects extreme values with potentially influential impact on the outcome of the regression. Extreme values were eliminated after looking at Cook´s distance in the spike plot (Fig. 6 b)).

The predictive performance and robustness of the developed CH_4 models were assessed using evaluation metrics of RSD (standard deviation of the residuals, g/d) and further RMSPE, MB, SB, RSR, and CCC as follows:

$$RMSPE, \% = \frac{\sqrt{\frac{1}{n}\sum_{i=1}^{n}(O_i - P_i)^2}}{\frac{1}{n}\sum_{i=1}^{n} O_i} \times 100$$

, whereby RMSPE denotes the root mean square prediction error expressed as a percentage of observed CH_4 production means, n = number of observations, O_i = observed value and P_i =

predicted value of the response variable of the i^{th} observation. The RMSPE enables an assessment of the overall model prediction error (Bibby und Toutenburg 1977).

The MSPE can be decomposed into error terms of the random bias, MB and SB which are measures of systematic biases and expressed as a percentage of MSPE:

$$MB, \% = (\bar{P} - \bar{O})^2$$

, MB denotes the mean bias or error in central tendency with $\bar{P}$ and $\bar{O}$ as predicted and observed means. The MB is used to assess model precision and lower MB estimates indicate that data points are uniformly scattered around the y = x line.

$$SB, \% = (SD_P - r \times SD_O)^2$$

, SB denotes the slope bias which is the error due to the regression and a measure of model accuracy, whereby r, SD_P und SD_O denote the Pearson correlation coefficient and the SD of the predicted and observed values, respectively (Bibby und Toutenburg 1977).

$$RSR = \frac{RMSPE}{SD_O}$$

, RSR denotes the RMSPE-to-SD_O values ratio and ranges from 0 (optimum) to large positive. Smaller RSR estimates indicate less prediction error compared to the SD_O with RSR = 1 indicating the RMSPE is equal to observed data variance. RSR accounts for the specific variability of the data used for model evaluation and can be used to compare the performance of models based on data of different subsets (Moriasi et al. 2007).

$$CCC = r \times C_b$$

, CCC denotes the Lin´s concordance correlation coefficient (dimensionless). CCC simultaneously accounts for both accuracy and precision based on the bias correction factor (C_b) which indicates how far the observed data deviate from the line of unity (perfect concordance: y = x). Pearson´s *r* is a measure of the model precision. The CCC ranges between 0 and the optimum of 1. The closer the CCC of a model to 1, the better the model performance which means that when CCC is equal to 1, no deviation from the line of unity had occurred (Lin 1989).

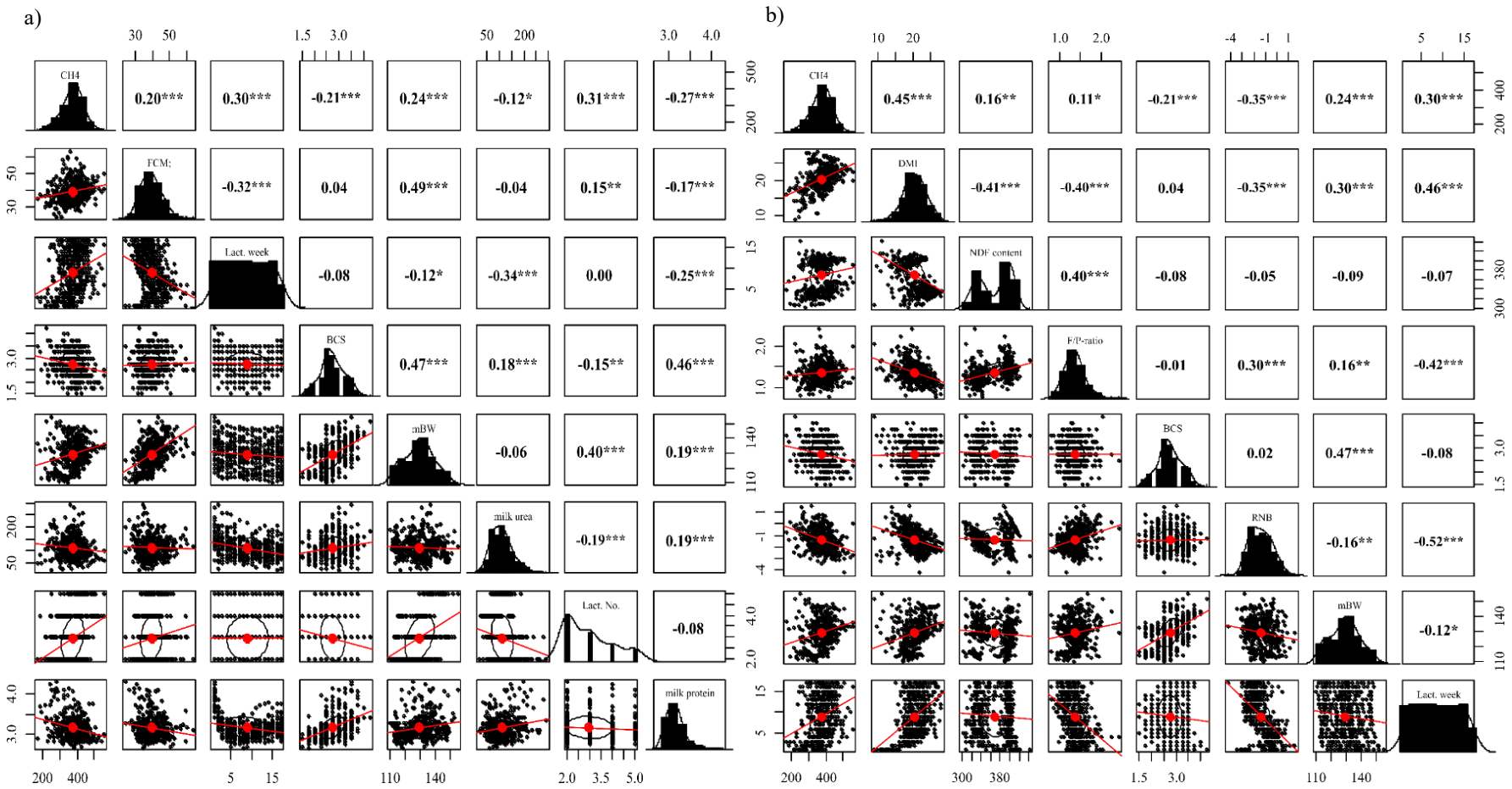


Figure 7: Scatterplot matrix of a) Eq. [11] and b) Eq. [12] (Tab. 2) with data distribution, linear relationships between response and explanatory variables, and the significance of Pearson correlation coefficients marked with *** asterisks. Abbreviated variables are explained in the footnote of Tab. 2.

7.1.3. Discussion of the key variables and performance of CH_4 prediction models

7.1.3.1. Feed intake level

Methane production primarily depends on the key parameters of feed intake (Hristov et al. 2018, Niu et al. 2018), digestibility of the OM and dietary EE content (Moraes et al. 2014, Benaouda et al. 2019). DMI explains most of the variation in CH_4 emissions and is positively related to CH_4 production which is due to the greater availability of fermentable OM for ruminal digestion ($r = 0.45$; $p < 0.001$; Figure 7b). The present modeling approach confirmed that DMI is the most important proxy of the CH_4 emission rate ($r = 0.45$; Fig. 7b) and extant as well as self-developed CH_4 equations including DMI as a predictor variable mostly outperformed those models without DMI. Especially, the lower and upper range of CH_4 emissions can be estimated more accurately if DMI is included as a predictor variable (higher SB in non-DMI models Eq. [4] and [5] compared to Eq. [2] and [3]) which was also reflected by non-significant slopes of the regression in those models without including DMI (Fig. 4 Eq. [4]). As a consequence, DMI, whether measured or estimated in groups or on an animal-individual level in the farm routine, is highly recommended to be included as a predictor variable in CH_4 prediction models. Actual cow-individual DMI measurements are not available in practice but commonly estimated for cow groups by weighing the amounts of feed offered and refused using the scale of the mixer wagon (Sova et al. 2013). The performance of CH_4 prediction models primarily depends on the type of predictor variables used in the model and not on whether animal-individual or group means of CH_4 emissions are estimated (Benaouda et al. 2019). The less explanatory power of on-farm proxies and the non-availability of DMI in practice can be only partially compensated by integrating further routinely available proxies (e.g. BCS, week of lactation, milk yield) in CH_4 prediction equations. In this connection, the more complex on-farm CH_4 equation [11] (RMSPE: 15.6%) outperformed the most simplified DMI-based equation [9] (RMSPE: 18.3%). Most of the sensors applied in commercial dairy farms and experimental facilities are able to measure parameters of animal activity, rumination and eating behavior (e.g. ear tags, neck collars) (Beauchemin 2018) which are currently under investigation for the estimation of the DMI (Clément et al. 2014). Eating time measured from the weighing troughs was included into the development of Eq. [4] and [5] (Table S1), yet this variable had no significant explanatory power. Rumination activity and rumen motility affect breakdown and colonization of feed particles which enhances CH_4 formation per unit of ingested feed. In this connection, Watt et al. (2015) investigated the potential of rumination time as a predictor variable

for CH_4 production in grazing dairy cows but found that rumination time is not appropriate as a single proxy for CH_4 production. It is noteworthy that relationships between rumination activity and CH_4 production are not well explored until now.

In light of the need for improving the accuracy of the TIER-3 model currently used for the German GHG emission inventory, it is promising to develop CH_4 prediction models with on-farm proxies obtained from data-generating technologies presently used in the farm routines (e.g., cow sensors, group-based DMI estimation using the mixer wagon) to report farm-specific GHG emissions to national authorities. Although the TIER-3 model (Eq. [1]) has been externally validated to predict Min- and Max-values more precisely (Fig. 4; R^2: 0.37) when compared to most of the other extant models (Tab. 2), the model was shown to systematically underpredict actual CH_4 emissions (Fig. 4: 322 ± 50 g predicted CH_4/d and Tab. S1: 369 ± 63 g observed CH_4 emission/d) measured in the present experiment which introduces inaccuracy (Fig. 4; RMSPE: 22.5%). In the TIER-3 model, the CH_4 emission factor is calculated according to Kirchgessner et al. (1994) as a function of the intake of different feed nutrients (Eq. [1]) and amounts to 137.8 kg CH_4 per cow and year (average EU value: 136.6 kg CH_4 per cow and year ± 11.9 SD). The DMI is estimated from regional data of the assumed energy requirements (lactation, maintenance, reproduction) and the energy content of the feeds under the general assumption that the cows are fed in accordance with their energy requirements. In a second step, the CH_4 emission factor is inverted into the equation for the CH_4 conversion rate which indicates the proportion of the cows´ GEI converted to enteric CH_4. As shown in Eq. [1], the CH_4 conversion rate for German dairy cows amounts to 6.3% (average EU value: 6.3% ± 0.3 SD) (Dämmgen et al. 2012). However, both the enteric CH_4 conversion rate and the emission factor are no constants because they depend on dynamic changes in DMI and digestibility of the feedstuffs which is, apart from the derivation of model input variables from assumptions, a main uncertainty in the TIER-3 model. The application of constants introduces higher errors of a central tendency which was evidenced by the high mean bias (Eq. [1]; Fig. 4; MB: 62.8%). Therefore, the input data for GHG inventories must be obtained and reported as accurately as possible to continuously re-assess the currently applied CH_4 conversion rate of 6.3% in the GHG emission inventory. The superiority of farm-specific advanced CH_4 prediction models including actually measured DMI variables over the TIER-3 model for on-farm GHG inventories was demonstrated by the outcome of the present model development procedure (Eq. [12]; Tab. 2) and further by external validation of extant DMI-based CH_4 prediction models (Eq. [3]; Fig. 4).

7.1.3.2. Chemical composition of the diet

The prediction accuracy of extant (Tab. 1) and developed (Tab. 2) CH_4 prediction models improved with increasing model complexity. Especially, the combination of DMI with further phenotypical (milk performance, BW, BCS, lactation stage) and diet-related proxies increases the power of CH_4 prediction models mainly because different explanatory variables describe independent sources of variation in CH_4 and one predictor variable may compensate for deficiencies in the others (Negussie et al. 2017, Vanlierde et al. 2020). Extensive CH_4 prediction models developed on those proxies being deducible from scientific experiments outperform the more simplified on-farm models which is due to the less explanatory power and accuracy of data collection related to on-farm proxies (e.g., BCS as a subjective variable). When adding all the experimental parameters of the underlying feeding trial (PAPER I, II) to the model development procedure, the resulting complex Eq. [13] performed best among the other models (RMSPE: 9.9%; CCC: 0.73; Tab. 2).

On the other hand, the present modeling approach confirmed previous findings from Niu et al. (2018) that the accuracy of less complex CH_4 prediction models (Eq. [3] and [10]; RMSPE: 14.7% and 14.6%, resp.) could be comparable to that of more complex models (Eq. [7] and [12]; RMSPE: 14.6% and 12%, resp.) given that the simplified CH_4 model includes DMI and dietary NDF content as predictor variables. This result emphasizes that integrating DMI and NDF variables into CH_4 prediction models is imperative and, under the condition that DMI is available as a proxy, it can be recommended to prefer the less complex model (Eq. [3]) over more complex models (Eq. [7]) for on-farm use due to the lower need for other proxies.

Interestingly, adding variables of the chemical composition of the diet in DMI-based CH_4 prediction models was shown to improve the model performance compared to using DMI alone (Eq. [10]; RMSPE: 14.6% compared to Eq. [9]; RMSPE: 18.3%), although internal cross-validation of the CH_4 prediction equations [2] and [3] from Niu et al. (2018) revealed that both models are characterized by a comparably low RMSPE of 15.0% and 14.7%, respectively. One could think that DMI alone may be adequate to accurately predict CH_4 emissions, but integrating the NDF content improved the predictive power of these models. The higher model performance becomes apparent in reduced systematic biases (lower MB and SB in Eq. [3] compared to Eq. [2]) as revealed from the external validation using the CON data of the underlying feeding trial (Fig. 4). Thus, a higher robustness of the Eq. [3], which integrated both DMI and NDF content (CCC: 0.89; SB of 3.9%), was stressed out when compared to that of Eq. [2] modeled on DMI only (CCC: 0.42; SB: 7.3%; R^2 = 0.21). Besides, the present modeling approach revealed that the intake of

NDF seems to cause higher predictive power than the intake of DM (Eq. [2] and [5]) which was evident by a stronger relationship between NDF intake and CH_4 production ($r = 0.53$; $p < 0.05$) compared to DMI ($r = 0.45$; $p < 0.001$). In this regard, the combination of DMI and NDF content reflects the intake level of dietary fiber which is the main driver of total CH_4 production referred to as CH_4 emission rate (g/d). It can be stated that, among other feed- and animal-related properties, the relationship between CH_4 production and feed consumption is moderated by feed digestibility. Among those predictor variables being related to the nutrient composition of the diet, the NDF content was proven to be the most powerful one (followed by the CL content) (Hristov et al. 2018). Structural carbohydrates in the diet are primarily fermented to acetate under H_2 release which notably enhances methanogenesis (Johnson und Johnson 1995). In general, it should be noted that the scope of the relationship between CH_4 emissions and the proportion of a group of nutrients (e.g., NDF) in the ration depends on the inherent composition of the nutrient group with regard to its carbohydrate fractions (cellulose, hemicellulose, linin) (Hindrichsen et al. 2005). In this regard, the NDF content is positively related to CH_4 production but only on the condition that proportions of lignin are low, i.e. at a high digestibility of the NDF (Warner et al. 2016, van Lingen et al. 2019). Moreover, the scope of the relationship depends on the proportion of other nutrient groups. High dietary starch contents negatively correlate with CH_4 emissions, whereby the anti-methanogenic effect of the starch seems to be reduced in forage-rich diets and further depends on a critical minimum concentration of starch within the whole ration (van Gastelen et al. 2015, van Lingen et al. 2019). In conclusion, the interrelationship between single nutrient fractions in a diet and the variability in CH_4 emissions might be causative for the greater explanatory impact of NDF instead of the starch content in the presented CH_4 models (Tab. 1, 2).

The present study revealed that extant CH_4 prediction models (Niu et al. 2018) performed worse than self-developed models which can be related to the lower variability and size of the training and test dataset generated under the same and strictly controlled environmental conditions (no changes in animals/herd, husbandry, and CH_4 quantification technique (chapter 2.3.1)). The varying CFP in the present study beneficially contributed to the variability of the reference dataset (Tab. S1), but the rations did not vary in the type of forage or concentrate itself. In contrast, diets of the GLOBAL NETWORK database included different forage types (e.g. corn silage, alfalfa, grass, hay) (and CH_4 quantification techniques) leading to more variability in CH_4 emissions. In addition, the variation in CH_4 emissions due to the feeding management (e.g., feeding either partial mixed ration or a total mixed ration, feeding technique) and physical characteristics (e.g., particle

length) of the diet are not captured adequately in most of the extant CH_4 models at the moment. As a consequence, the validity of the self-developed models is to an extent restricted to the ration type provided in the study (PAPER I-III) and a greater robustness of the Niu et al. (2018) models, being therefore more applicable in different regions and dairy production systems, was concluded from the low RSR (Tab. 1). The CFP was not selected as a relevant proxy in the present modeling approach, and besides, not referred to as a meaningful proxy in CH_4 prediction models in so far as information on the composition of the concentrate feeds are often lacking and a clear assignment of certain feed materials (e.g. corn-corb-mix) to the term "concentrate feed" is not always feasible. Except for Eq. [17], the dietary CL content was not detected as a key predictor variable which contrasts to previous findings that CL content was the third most important proxy following DMI and NDF content (Niu et al. 2018, van Lingen et al. 2019, Benaouda et al. 2019). Enhancing the CL content is a well-accepted CH_4 mitigation strategy potentially reducing CH_4 yield by 3.8% with each 1% dietary addition of lipid supplements, but to some extent limited (Beauchemin et al. 2008). Thus, total CL content is recommended to be limited to 7% in the DM of dairy cow rations to avoid negative effects on DMI, OM digestibility and milk fat synthesis (NRC 2001). Dietary lipids (especially medium chain and unsaturated FA) reduce CH_4 by replacing rumen fermentable OM, suppressing the activity of ciliate protozoa and methanogens, reducing fiber degradability and providing an alternative [H] sink during biohydrogenation of unsaturated FA (Patra 2013). However, in the present experiment, the diets fed to the cows did not contain any extra lipid additive except for the 1.0% to 1.5% soybean oil inclusion into the concentrate feeds. Table S1 demonstrates an only narrow range in CL concentrations (2.9 – 3.8% of DM) of the experimental diets when compared to that of the Niu et al. (2018) database (1.5 – 7.7% of DM) in which no lipid supplements were included. Thus, the lower variability in CL content in diets of the present study could explain that variations in CH_4 emissions were not explained by variations in dietary CL content.

7.1.3.3. Lactation period and body mass parameters

The number of lactations, an on-farm available proxy, was found to have a positive relationship to CH_4 production (Figure 7b); $r = 0.31$; $p < 0.001$) and was a key predictor variable included in most of the models (Eq. [9], [10], [11], [13], [14], [17]). The dataset used in the present modelling approach contained only pluriparous cows and, therefore, conclusions on the explanatory power of this variable when considering primiparous cows cannot be drawn. However, the positive relationship could be explained by findings from Watt et al. (2015) who reported that older cows

Table 2: CH_4 prediction models (g CH_4/cow and d) developed and 10-fold cross-validated on the experimental data of the underlying study (Schilde et al. 2021). The training dataset for Eq. [9] – [15] included only data of the 25 experimental cows fed the control ration without 3-nitrooxypropanol (CON data), whereby Eq. [16] was developed on both CON and 3-NOP data (20 cows fed 3-NOP) and Eq. [17] on 3-NOP data only.

Model specification		Model development		Model	Model evaluation metrics[Ω]					
Equation	**Training dataset and model type†**	**CH_4 prediction model[‡]**	***n*[*]**	**application[Π]**	**RMSPE, %**	**RSD**	**RSR**	**MB, %**	**SB, %**	**CCC**
[9]	CON *DMI; without milk*	CH_4 = 213.3 + DMI × 5.8 (0.6) + lactation number × 16.3 (2.2)	503	SCIENCE	18.3	52	0.87	1.88	1.55	0.37
[10]	CON *DMI, diet composition; without milk*	CH_4 = 391.6 (32.6) + NDF intake (kg DM/d) × 24 (1) − CP × 1.38 (0.18) + lactation number × 16.3 (2.2) − BCS × 10.8 (4.1)	512	SCIENCE	14.6	49	0.85	17.7	0.01	0.53
[11]	CON *milk; without DMI*	CH_4 = 115.6 (50.3) + FCM × 1.6 (0.55) + week of lactation × 5 (0.63) − BCS × 37.8 (7.1) + metabolic BW × 2.1 (0.43) + MU × 0.18 (0.06) + lactation number × 8.4 (3) − MP × 19.4 (11.6)	412	ON-FARM	15.6	52	0.82	0.29	0.01	0.50
[12]	CON *advanced model*	CH_4 = −161.2 (44.5) + DMI × 8.9 (0.96) + NDF (g/kg) × 0.36 (0.08) + fat-protein ratio × 79.5 (12) − BCS × 30.7 (5) − RNB × 11.3 (2.87) + metabolic BW × 1.29 (0.3) + week of lactation × 1.3 (0.58)	403	SCIENCE	12	42	0.75	3.8	0.08	0.61
[13]	CON *all possible experimental variables*	CH_4 = −133.6 (49.6) + NDF intake (kg DM/d) × 23 (5) + MP yield (kg/d) × 145.7 (32.6) + acetate-to-propionate ratio × 51.1 (9.1) + lactation number × 20.2 (4.4) + EB × 0.58 (0.25)	88	SCIENCE	9.9	35	0.65	5.4	0.49	0.73

Continuation of Table 2:

Model specification		Model development		Model	Model evaluation metrics[Ω]					
Equation	**Training dataset and model type[†]**	**CH_4 prediction model[‡]**	***n*1[⁂]**	**application[Π]**	**RMSPE, %**	**RSD**	**RSR**	**MB, %**	**SB, %**	**CCC**
[14]	CON *only milk and VFA data*	CH_4 = −515.9 (121.2) + lactation number × 19.6 (4.7) + MP yield (kg/d) × 204 (31) + acetate (%) × 9.6 (1.6) + week of lactation × 3.1 (0.78)	88	SCIENCE CH_4 MITIGATION (3-NOP)	11.5	41	0.71	2.76	0.06	0.63
[15]	CON *only VFA data*	CH_4 = 275.4 (32.8) + acetate (mmol/L) × 0.85 (0.57) + iso-butyrate (mmol/L) × 72.1 (44.6)	132	SCIENCE CH_4 MITIGATION (3-NOP)	19	62	0.92	0.20	0.91	0.16
[16]	CON+3-NOP *only VFA data*	CH_4 = 171.3 (24.5) − valerate (mmol/L) × 20.9 (8.8) + acetate (mmol/L) × 6.2 (0.6) − butyrate (mmol/L) × 9.0 (2.3) − iso-valerate (mmol/L) × 22.2 (10.8)	238	SCIENCE CH_4 MITIGATION (3-NOP)	21.3	63	1.15	13.5	14.1	0.18
[17]	3-NOP *milk, diet composition, animal-related variables*	CH_4 = 12.4 (88.3) + week of lactation × 8.5 (0.66) − CL × 9.2 (1.6) + BW × 0.265 (0.05) + fat-protein ratio × 86.7 (14.9) + lactation number × 21.8 (5.5) + BCS × 16.5 (7.3) + $peNDF_{>8mm}$ × 3.6 (1.6)	320	ON-FARM CH_4 MITIGATION (3-NOP)	48.7	49	0.98	8.2	1.2	0.72

[†]model type and dataset, a list of all the candidate variables which were examined in the stepwise regression approach can be found in Table S1. VFA, volatile fatty acids; FT-MIR. [‡]CH_4 prediction models with regression coefficients and standard error; BCS = body condition score according to Edmonson et al. (1989), BW = body weight (kg), CL = crude fat content (g/kg of feed DM), CP = crude protein content (g/kg of feed DM), DMI = dry matter intake (kg/d), EB = energy balance (MJ NEL/d) (GfE, 2001), ECM = energy-corrected milk yield (kg/d) according to Tyrrell und Reid (1965), FCM = 4% fat-correct milk yield (kg/d), MP = milk protein content (%), MU = milk urea content mg/L), MY = milk yield (kg/cow and d), NDF = neutral detergent fibre (% of DM), peNDF>8mm = physically effective neutral detergent fibre measured as the proportion of particles retained by 19- and 8-mm screens multiplied by dietary NDF content (Lammers et al. 1996), RNB = ruminal N balance = [(crude protein – utilizable crude protein)/6.25].

[⁂]n_1 = number of observations in the training dataset (CON groups) for CH_4 prediction model development. Eq. [16] was developed from both CON and 3-NOP experimental data.

[Ω]Model evaluation metrics: Descriptions are detailed in chapter 7.1.2

[§]n_2 = number of observations for cross-validation of the CH_4 prediction models.

are heavier which results in greater DMI (due to higher maintenance requirements), longer rumination time due to a larger rumen size and, consequently, higher CH_4 production when compared to younger cows. Moreover, the present modeling approach stressed out that "week of lactation" was a significant source of variation in CH_4 emissions which can be explained by the close relationship between DMI level and lactation stage. However, multi-collinearity between week of lactation and DMI has not been found in the present modeling approach (variance inflation factor) indicating that, to some extent, week of lactation affects CH_4 emissions independently from DMI. In this context, changes in diet composition (increased CFP) with ongoing lactation period in the present experiment (PAPER I) could have altered microbial community structures in the rumen and adversely affected H_2-producing fibrolytic bacteria and pH-sensitive methanogenic Archaea. Much of the variation in CH_4 production has been shown to be attributable to factors other than DMI (Hegarty et al. 2007).

The combination of DMI and diet-related variables with phenotypical body mass (and milk production) parameters increased the performance of CH_4 prediction models indicating further sources of variation not directly related to DMI (Eq. [7], [12]) (Negussie et al. 2017). Both BCS and mBW were, as being indirect traits of the DMI, significant input variables in the CH_4 prediction models (Eq. [10], [11], [12], [17]). Rumen size and weight are positively correlated with BW and, when DMI is kept constant, a higher rumen volume results in a lower passage rate and longer ruminal retention time of the fermenta leading to increased CH_4 production and CH_4 yield (Demment und Van Soest 1985, Goopy et al. 2014). It is assumed that dairy cows with higher mBW ingest more feed due to elevated maintenance energy requirements which leads to increased CH_4 production (Hristov et al. 2013a, Niu et al. 2018). In contrast to BW, BCS is an easily available proxy in commercial dairy farms by using either BCS cameras (Mullins et al. 2019) or visual monitoring in the management routine (Edmonson et al. 1989). Variation in BCS reflects changes in energy expenditure and energy intake, whereby the latter is positively related to CH_4 production due to the greater uptake of substrates stimulating H_2 production. In the present periparturient dairy cows, the BCS was strongly negatively related to the response variable CH_4 (Tab. 2; Fig. 7) which can be explained by the diverging changes between pre-and post-calving BCS and DMI (PAPER I and II) (Roche et al. 2009). Adipose tissue reserves were mobilized in response to the periparturient energy deficit of the experimental cows (PAPER I and II). The BCS loss occurred in the periparturient animals (PAPER I and II) possibly due to the homeorhetic adaptations associated with lower plasma insulin concentrations and reduced insulin sensitivity of the peripheral tissues

causing an upregulation of fat mobilization in the form of NEFA (PAPER II) which adversely affects DMI and, consequently, the DMI-dependent CH_4 production (Roche et al. 2009, Wankhade et al. 2017). Furthermore, obese cows may emit less CH_4 than lean cows at the same BW and offered diet since, when compared to body fat tissues, maintenance requirements and turnover of body protein mass requires more energy resulting in higher DMI (Agnew und Yan 2000).

7.1.3.4. Milk yield and composition

It has become common practice to routinely measure milk yield and analyze milk composition once a month during milk testing in commercial dairy farms. A growing number of milking robots enables more frequent and automatized measurements of milk parameters which is beneficial to predict CH_4 production (Eq. [11], [12], [13], [17]). Milk yield and milk composition parameters were evidenced to be significant determinants of CH_4 emissions (Eq. [11], [14], [17]) as long as DMI is not part of the CH_4 prediction model. Nevertheless, there is generally only a weak correlation between CH_4 emissions and parameters of milk yield and composition (Negussie et al. 2017). Milk yield was observed to strongly correlate with DMI ($r = 0.56$; $p < 0.001$; data not shown) and it can be used as a proxy for DMI in CH_4 prediction models. However, models with milk input variables have been shown to predict CH_4 production always less accurate than those including DMI (Eq. [4], [5], [6], Fig. 4). In particular, non-DMI CH_4 prediction models based on variables of milk production systematically fail to predict lower and upper means of CH_4 emissions as indicated by higher SB values (Tab. 1; Eq. [4], [5]) and non-significant slopes of the regression (Fig. 4). Transition cows are challenged with metabolic changes and a negative EB which becomes apparent in a DMI not increasing with the same intensity as milk yield does p.p., whereby the increase in milk yield is enabled by an exaggerated release of mobilized energy from body fat tissues (PAPER I, II). As a consequence, the positive relationship between DMI and milk yield, leading to higher CH_4 emission rates, is uncoupled during the early-lactation period in high-yielding cows. Interestingly, milk protein content was negatively related to CH_4 emission rate, whereas the opposite held true for milk protein yield in the CH_4 prediction models (Eq. [11], [13], [14]) which confirms previous findings (Vanlierde et al. 2015). Accordingly, milk protein yield and content appeared to constitute a higher explanatory power than other milk yield variables which is surprising since the magnitude of changes in milk protein content in response to changes in diet composition and DMI is much smaller than that presumed for milk fat (DePeters und Cant 1992). In this context, both the hydrogenotrophic and acetoclastic methanogenesis as well as *de novo* milk

fat synthesis sensitively respond to the supply of its substrates, i.e. H_2 and acetic acid, and higher NDF contents are strongly associated with increased H_2-generating acetate synthesis in the rumen. However, the greater explanatory power of variables of milk protein in CH_4 prediction models instead of other milk constituents highlights the importance of the uptake of readily fermentable carbohydrates as a positively related proxy not only of the CH_4 emission rate (g/d), but also of increased milk yield as well as ruminal microbial protein synthesis and, therefore, milk protein synthesis. The finding of a negative correlation between CH_4 emission rate and milk protein content in the present modelling approach (Eq. [11]) could be explained by divergent changes of a rapid decrease in milk protein content being paralleled by increasing DMI and, therefore, CH_4 production during the early-lactation period (PAPER I).

7.1.3.5. Rumen fermentation variables, biochemical and hematological blood parameters

Biochemical as well as hematological blood parameters did not explain significant variations in CH_4 emissions which is in line with previous findings (Negussie et al. 2017). Those CH_4 prediction models being solely developed on rumen VFA variables, in order to explore potential candidate proxies under a CH_4 mitigation scenario with altered VFA profile, were characterized by moderate model accuracy (Eq. [15], [16]; RMSPE: 19% and 21.3%). Ruminal acetate was observed to be the most important predictor variable among rumen VFA variables (Eq. [13], [14], [15], [16]) and positively associated with CH_4 production. This can be related to fiber degradation yielding acetate and H_2 which serve as substrates for methanogenesis (Fig. 2). Inclusion of milk variables to those models based on the VFA profile (Eq. [14]; RMSPE: 11.5%) notably increased model performance compared to models based on rumen VFA variables only (Eq. [15, [16]; RMSPE: 19%, 21.3%).

7.1.3.6. Evaluation of the model performance under a CH_4 mitigation scenario with 3-NOP

The upcoming use of CH_4 inhibitors underlines the need for new CH_4 prediction models for the assessment of CH_4 mitigation strategies. The present modeling approach revealed the inadequacy of empirical models to accurately predict CH_4 emissions under a CH_4 mitigation scenario. The present empirical models do not account for the (thermodynamic) kinetics of the rumen metabolic physiology, whereby the latter was proven to be changed by 3-NOP (PAPER I; Jayanegara et al. 2018). Thus, functional and causal rumen metabolic processes still remain as a black-box in the present empirical modeling approach. The empirical models were built on the quantitative and stochastic relationship between input and output variables and depend, to some extent, on a probability distribution which makes the use of large reference datasets imperative. Dynamic

mechanistic modeling could overcome the main disadvantages of the present empirical models but requires input variables not available in commercial dairy farms. However, a more integrated and process-based mathematical rumen modeling is needed to assess the impact of mitigation strategies to reduce CH_4 emissions. The present study revealed that, depending on the CFP, 3-NOP reduced CH_4 production by 23% to 33% and further induced changes in milk fat content and rumen VFA pattern (PAPER I). Varying effects of 3-NOP were observed on the energy metabolism (reduced NEFA levels while TAG, glucose, BHB, body fat mobilization and BCS were not affected; PAPER I, II).

Extant CH_4 models (Tab. 1) and those developed in the present modeling approach (Eq. [9] – [13]) have been validated to adequately explain sources of variation in CH_4 production due to dietary changes but do not allow for exploring nutritional mitigation options (Fig. 5 and Tab. 3). In the 3-NOP-based CH_4 mitigation scenario, the hitherto quantitative relationship between CH_4 production and its key determinant DMI is still positive but obsolete since 3-NOP reduces CH_4 production without suppressing DMI (PAPER I, II). The TIER-3 model outperformed all the other extant CH_4 prediction models when applied to 3-NOP fed cows which is due to its abovementioned systematic underprediction of CH_4 emission rates (Fig. 4 and 5). Interestingly, the extant CH_4 prediction models [3], [6], [7] and the self-developed model [12] performed well when applied to 3-NOP fed cows (Tab. 3; Fig. 5). All the four models have in common, that they include milk fat (except for Eq. [3]) and NDF content as predictor variables. The relationship between these variables and CH_4 emissions under 3-NOP feeding were confirmed in the model development of Eq. [17] using 3-NOP data only. Thus, milk fat and NDF were selected as significant predictor variables in the modeling approach. In a meta-analysis, Dijkstra et al. (2018) demonstrated that the efficacy of 3-NOP on CH_4 mitigation negatively correlates with the NDF content which was confirmed in the present experiment (PAPER I). Furthermore, a reduced molar acetate proportion has been consistently observed in 3-NOP fed cows (PAPER I; Jayanegara et al. 2018). This is probably due to preventing an exceeding intra-ruminal [H] accumulation under methanogenesis inhibition which can be assumed from the observed higher H_2 emissions whilst NDF digestibility remained unchanged in 3-NOP supplemented diets *in vitro* (PAPER III). In literature (van Gastelen et al. 2020, Melgar et al. 2020b) and in the present experiment (PAPER I), lower milk fat contents have been reported in 3-NOP fed cows. The *de novo* synthesis of MFA in the mammary gland, being reflected by milk fat content and the MFA profile, is known to be directly related to the synthesis of ruminal acetic acid which in turn is mainly formed from NDF fermentation. In more detail, Eq.

[15] of the present modeling approach was constructed by using rumen VFA variables only and molar acetate proportion was verified as the key variable in the CH_4 prediction model which was further confirmed in Eq. [13], [14], [15], [16]. Interestingly, Hristov et al. (2015) observed that most of the short-chain MFA (C6:0 through C12:0) and the C15:0 and C17:0 medium-chain MFA were increased in 3-NOP fed cows, while monounsaturated MFA decreased. Ruminal propionate is the major substrate for the *de novo* synthesis of C15:0 and C17:0 and was consistently observed to increase with 3-NOP feeding because of acting as an alternative [H] sink (chapter 2.3.4.4.; PAPER I). In addition, van Gastelen et al. (2020) found that the group of saturated MFA tended to increase and the group of unsaturated MFA tended to decrease in 3-NOP fed cows. These findings may indicate an upregulation of ruminal biohydrogenation in 3-NOP fed cows for alternative [H] removal. The prediction of CH_4 emissions using milk FT-MIR spectra is based on the direct biochemical relationship between MFA composition as a signature of ruminal microbial digestion and VFA pattern and, therefore, CH_4 production. In this light, the relationship between changes in ruminal microbial community structures, CH_4 production and the rumen VFA pattern driven by 3-NOP feeding in periparturient cows was evidenced from recently conducted 16S sequencing in rumen samples of the present study (Schilde et al. 2022). It needs to be investigated in future studies whether the relationships between 3-NOP feeding and effects on ruminal microbial community structures can be reflected indirectly by milk FT-MIR spectra for its use in CH_4 prediction models.

Table 3: Evaluation of the self-developed CH_4 prediction models presented in Table 2 when being regressed on the observed CH_4 emission rates of cows fed with 3-nitrooxypropanol (3-NOP).

Model evaluation[†]	Eq. [9]	Eq. [10]	Eq. [11]	Eq. [12]	Eq. [13]	Eq. [14]	Eq. [15]	Eq. [16]
n	320	320	320	320	89	89	89	89
Intercept	359	324	294	271	290	237	342	188
Slope	0.083	0.258[*]	0.292[*]	0.184[*]	0.305[*]	0.396[*]	0.084	0.342[*]
Mean ± SD	380 ± 18	392 ± 35	371 ± 38	319 ± 34	373 ± 51	344 ± 49	365 ± 15	281 ± 42
R^2	0.11	0.30	0.32	0.16	0.18	0.33	0.16	0.34
Residual SD	70	62	61	68	68	59	67	58
RMSPE, %	51.3	53.7	46.6	33.1	46.4	35.8	43.8	22.3
MB, %	73.4	80.9	75.4	39.6	78.2	71.5	75.4	7.9
SB, %	0.21	0.18	0.09	0.34	2.42	0.50	1.67	0.02
CCC	0.05	0.12	0.17	0.21	0.17	0.31	0.06	0.50

[†]n, number of observations in the test dataset (3-NOP). SD, standard deviation. R^2, goodness-of-fit. RMSPE, MB, SB, CCC, explanation of model evaluation metrics presented in chapter 7.1.2.
[*]Significance ($p < 0.05$) of the slopes of the regression is marked with an asterisk.

However, this biochemical relationship between CH_4 production, ruminal VFA pattern, milk fat content and dietary NDF content was indicated in the present modeling approach as shown in variable selection of Eq. [14]. The Eq. [14] was developed on milk and rumen VFA variables only,

but performed best in predicting CH_4 production in the 3-NOP fed experimental cows among all models (Tab. 3; CCC: 0.31; RMSPE: 35.8%). The CH_4 prediction model [16] not surprisingly outperformed the Eq. [9]-[15] when applied to 3-NOP fed cows (Tab. 3; [16]; CCC: 0.50) because it was developed on both the CON and 3-NOP datasets. Furthermore, Eq. [17] has been evaluated as being the most accurate one which can be explained by the fact that only 3-NOP data were used to train the model (Tab. 2; CCC: 0.72). Therefore, global efforts are needed to develop inhibitor-specific reference datasets and CH_4 prediction models (because CH_4 inhibitors differ in their mode of action within the rumen (Fig. 2)) with high data variability and inclusion of animal- and diet-related parameters in combination with information on dosage, administration technique, and formulation of the CH_4-inhibiting substance to improve the robustness and accuracy of future CH_4 prediction models. In conclusion, the present mdeling approach verifies that the dietary NDF and milk fat content (and composition) perform as on-farm and, in addition to the molar acetate proportion, as scientific proxies of the CH_4 emission in 3-NOP-based CH_4 mitigation strategies in future (hypothesis V). The closer the model reflects rumen functions the higher is the model performance to predict the abatement of CH_4 emissions in a 3-NOP mitigation scenario.

8. Conclusion and recommendations for political action

Methane emissions from enteric fermentation contribute to climate change. Therefore, there is an urgent need for ambitious action. The present thesis revealed that the combined approach of feeding high CFP and 3-NOP reduced CH_4 emissions in an interactive manner in periparturient and early-lactation cows (HYPOTHESIS I) which was, though, not confirmed under *in vitro* conditions (HYPOTHESIS IV). The CH_4 abatement strategy demonstrated in the present thesis provides a perspective of how the development of future CH_4 mitigation approaches can succeed, i.e. the combination of varying diet compositions supplemented with an effective CH_4 inhibitor due to their complementary effect mechanisms. 3-NOP reduced CH_4 emissions by 23 to 33% and can be evaluated as a promising CH_4 inhibitor. However, the present study evidenced a dose-, diet- and time-dependent effect size of 3-NOP, especially 3-NOP effects were transient in cows provided a diet with low CFP. This result substantiates the continuing need for further long-term *in vivo* 3-NOP studies in future before licensing 3-NOP for its widespread on-farm use.

Feeding high CFP as an indirect CH_4 mitigation strategy is limited: certain concentrate feeds compete with their use in human nutrition and may cause environmental-related costs which leads to the conclusion that life-cycle assessments of the whole feed supply chain are imperative; decreased feed digestibility associated with increased CFP reduces feed conversion efficiency into milk and increases CH_4 emissions from manure; and, last but not least, feeding excessive dietary concentrate levels may cause detrimental effects on animal health depending on its dietary inclusion level. In conclusion, it is recommended that future research on potential CH_4 mitigation strategies in dairy cows should primarily focus on formulating the adequate diet according to nutritional needs of the cow in conglomerate with improving productivity gains through optimized cow health and management practices. In this context, the use of by-products from crops and human nutrition as concentrate feeds is expected to improve the sustainability of the feed supply chain.

As shown in the present thesis, feeding high-concentrate diets reduced CH_4 yield and emissions intensity, whereas CH_4 emission rate was similar to that of the low-concentrate diets. The reasons for this can be explained by increased starch levels being degraded to propionate which is the most important competitor of methanogenesis for H_2 removal, lower pH-values which inhibit growth of fibrolytic and methanogenic microorganisms and a reduced fermentation capacity per unit fermentable organic matter due to the increased ruminal passage rate of the ingesta as feed intake increases with higher concentrate proportion. The present thesis indicates that feeding 3-NOP and

varying concentrate-to-forage ratios induced significant shifts in rumen fermentation pathways to a reduced acetate-to-propionate ratio which needs to be addressed in future research in relation to potential changes in rumen microbial community structures.

In the present thesis, the hypothesized energy gains from the 3-NOP induced CH_4 inhibition in terms of a reduced feed inefficiency and increased glucogenic propionate production in the rumen were not consistently rediscovered in an improved energy budget and animal performance (HYPOTHESES I and II). The GreenFeed system was proven to adequately estimate the dietary effects of 3-NOP and CFP on mechanisms of ruminal and energetic metabolic processes with special focus on energy retention in body tissues and energy partitioning to productive functions by indirect calorimetry (HYPOTHESIS III). The reasons for the lack of effects of the CH_4 inhibition on a potential extra energy supply to the cow remain unclear but may be attributed to the observed accumulation of hydrogen *in vitro* and presumably *in vivo*, which is regarded as energetically inefficient. Hence, gene expression analyses and metabolomics may bring light into darkness with regard to the identification of the pathways and utilization of energy gains from CH_4 inhibition and alterations in ruminal volatile fatty acids.

The present study indicates that 0.67% to 0.96% of the total GHG emissions in Germany can be potentially reduced if 3-NOP is persistently effective and routinely implemented in the on-farm feeding practice. In this regard, the reduced GHG emissions have to be quantified and reported in the national GHG emission inventories in view of the Kyoto Agreement.

In the present study, feed intake and diet-related factors were identified to be the most important prediction variables of the CH_4 emission rate. In particular, the combination of proxies was evidenced to be advantageous for model performance. However, the present thesis revealed that the conventional relationships between feed intake and CH_4 emissions are uncoupled in a 3-NOP mediated CH_4 mitigation scenario. The present thesis indicates that rumen VFA variables, dietary NDF and milk fat content may function as proxies in 3-NOP-specific CH_4 prediction models since they were affected by 3-NOP (HYPOTHESIS V). In this light, future research should focus on milk fatty acids which may reflect the observed 3-NOP induced alterations in rumen fermentation pattern, ruminal microbial community and reduced CH_4 production in more process-based models.

In conclusion, a global effort to combine striving for gains in production efficiency at lowest environmental costs with achieving breakthroughs in the development of innovative CH_4 mitigation strategies without compromising animal health and economical profit will be needed to tackle the aim of a sustainable milk production.

9. Summary

Methane from enteric fermentation in ruminants contributes 39% to the total agricultural GHG emissions in Germany. Methanogenesis constitutes a loss of up to 12% of the gross energy intake, yet represents the major hydrogen-eliminating pathway in the rumen. The chemical CH_4 inhibitor 3-nitrooxypropanol (3-NOP), a structural analogue of methyl-coenzyme M, inactivates methyl-coenzyme M reductase which catalyzes the last step in methanogenesis. It was shown that 3-NOP induces a shift in fermentation pathways favoring hydrogen-consuming propionate synthesis. Feeding high concentrate feed proportions (CFP) in the ration is known as an indirect CH_4 mitigation strategy being primarily based on the degradation of the increased starch levels to propionate and thereby competing with methanogenesis for H_2 removal. Periparturient dairy cows are subjected to drastic endocrinological and metabolic changes being flanked by a negative energy balance when energy requirements for milk synthesis exceed the energy supply due to a reduced feed intake. As a consequence, an excessive mobilization of body energy reserves occurs in form of non-esterified fatty acids (NEFA) leading to an accumulation of ß-hydroxybutyric acid (BHB) in the blood circulation associated with metabolic and hepatic disorders.

It was hypothesized that the combined approach of feeding 3-NOP and a high-concentrate diet caused additive effects on methanogenesis inhibition, since the abovementioned mode of action on CH_4 inhibition differs between both experimental factors. Further synergistic effects of the combined CH_4 mitigation strategy on an improved 3-NOP efficacy were assumed in so far as the elevated hydrogen-eliminating propionate formation from the starch-rich diet may compensate for a 3-NOP induced surplus of rumen hydrogen. The increased synthesis of glucogenic propionate from both the high-concentrate diet and the 3-NOP induced shift in fermentation pathways taken together with the reduced feed inefficiency under methanogenesis inhibition were hypothesized to improve the energy supply to the periparturient cow. The GreenFeed system was hypothesized to be usable as an indirect calorimetric chamber to estimate the energy retention in body tissues and to differentiate the energy partitioning towards the single energy expenditure. Moreover, it was hypothesized that the efficacy of 3-NOP on CH_4 inhibition responds in a dose-dependent manner and incubating high-concentrate feed proportions causes additive effects on CH_4 reduction *in vitro*.

For the abovementioned purposes, a study was conducted using 55 pluriparous German Holstein cows in the periparturient and early-lactation period which lasted from d 28 *antepartum* to d 120 *postpartum*. The cows were grouped in a 2×2 factorial design characterized by a low (LC) or high

(HC) CFP tested without supplements or combined with 50 mg of 3-NOP per kg of feed dry matter (DM) in the ration. During the *antepartum* period, CFP was set to 15% and 40% in the LC and HC groups, respectively. From parturition until d 21 *postpartum*, CFP gradually increased from 30 to 55% in the HC groups, whereas that of the LC groups was fixed at 30%. The CH_4 emissions were quantified using the GreenFeed system (C-Lock Inc., Rapid City, SD, USA) and feed intake was measured in weighing troughs. Milk yield at each milking was recorded in the milking parlor. Samples of the diet, rumen fluid, blood and milk were collected at selected time points. Ultrasonographic measurements of fat depot masses were conducted to examine potential effects on energy mobilization from adipose tissues. In the rumen simulation technique (RUSITEC), dose-response relationships of 3-NOP, dosed at inclusion levels of 0, 73, 160, and 1,200 mg per kg of feed DM combined with low or high CFP, were investigated in a 4×2 factorial arrangement.

The present *in vivo* study revealed that feeding 3-NOP in combination with HC diets reduced CH_4 emissions (g/d, g/DMI, g/ECM) by 33% in an interactive manner. However, in LC diets, the 3-NOP effects were observed to be less persistent and pronounced (23%-decrease in CH_4). Given that the aforementioned 3-NOP efficacy can be achieved in cattle over a complete production cycle, 3-NOP could potentially decrease GHG emissions by 5.75 (LC) to 8.25 (HC) million tonnes of CO_2 eq. corresponding to a reduction of 0.67% to 0.96% in the total GHG emissions in Germany. The effect of feeding high CFP reduced CH_4 (g/DMI, g/ECM) by 12 to 17% and, to a lesser extent, the absolute CH_4 emissions in the CON groups (4%). The interactive effects between 3-NOP and high CFP were not confirmed *in vitro*. However, 3-NOP was shown to linearly reduce CH_4 production in a dose-dependent manner by 12%, 61%, and 97% in LC diets and by 10%, 35%, and 90% in HC diets. The CH_4 reduction was accompanied by a 27-fold increase in H_2 release *in vitro*, whereby negative side-effects on nutrient degradability were not observed. Fermentation variables of total VFA production, protozoal counts and pH in the fermenter liquid were not altered *in vitro* and only affected by the event of parturition in the *in vivo* study. However, significant effects of 3-NOP, CFP and calving were observed on the molar proportions of VFA in the rumen fluid. Feeding 3-NOP caused a shift in rumen fermentation pathways towards increased hydrogen-consuming production of propionate, butyrate, valerate and *iso*-valerate, whereas the hydrogen-generating acetate formation decreased *in vivo*. In the RUSITEC, alternative H_2 sinks of propionate and valerate remained unaffected, whereas 3-NOP dosage decreased acetate formation.

Irrespective of 3-NOP treatment, greater DMI was found in HC groups but temporarily declined at the event of parturition in all groups. Milk fat content and 4% FCM were reduced in 3-NOP groups,

respectively, whereas milk lactose content increased by 3-NOP. The energy balance was slightly more positive in 3-NOP-HC groups. The use of GreenFeed gas measurements of CO_2, O_2 and CH_4 for indirect calorimetry was shown to be promising for estimating the energy turnover in body tissues as well as the energy partitioning to several energy expenditures in large numbers of cows. Energy losses from the conversion of the feed gross energy into CH_4 contributed 5.8% and 6.7% to the total gross energy intake in the CON-HC and CON-LC groups, respectively. 3-NOP reduced this feed inefficiency by 2% and 1.6% which theoretically constitutes an extra energy supply sufficient to increase milk production by 0.86 and 0.59 kg in the HC and LC groups, respectively. The potential effect of an improved feed efficiency on either an increased milk production or body weight gain was not confirmed in the study. However, NEFA were significantly reduced by 3-NOP, although BHB, glucose and triacylglycerides in serum, the BCS and the ultrasonographically assessed adipose tissue mobilization remained unaffected by 3-NOP, yet variously influenced by CFP and calving. Hence, the observed additive effects of feeding 3-NOP and HC diets on CH_4 inhibition were not evidenced to consistently result in an improved energy supply to the cows.

The high correlation between DMI and CH_4 emissions can be used in empirical CH_4 prediction models to adequately predict CH_4 emissions if CH_4 quantification techniques are lacking. The prediction of changes in CH_4 emissions due to 3-NOP is challenging since the conventional relationship between DMI and CH_4 production is uncoupled under 3-NOP feeding. The present thesis revealed that variables of rumen VFA, milk fat and dietary NDF content provide alternative proxies to predict CH_4 emissions in a 3-NOP-based CH_4 mitigation scenario. Thus, models based on these variables as predictors can be used to predict CH_4 emissions affected by 3-NOP.

In conclusion, 3-NOP can be regarded as a promising CH_4 inhibitor with dose-, diet- and time-dependent efficacy. Beneficial effects on the energy budget of the cow were not evidenced in the present experiment. Further long-term investigations on the diet and dose dependency of 3-NOP in cows at different lactational stages are needed before its widespread on-farm use. Additional research is warranted in order to examine whether the 3-NOP induced shifts in the fermentation pattern of rumen volatile fatty acids directly respond to changes in the community of methanogenic Archaea and whether these relationships can be reflected by milk mid-infrared analysis to develop CH_4 prediction models for 3-NOP based CH_4 mitigation scenarios. Investigations on the gene expression in the liver as well as potential changes in the plasma metabolome and functional parameters of the immune system may provide a deeper look into potential effects of the 3-NOP induced CH_4 inhibition on the energy metabolism of the cow.

10. Zusammenfassung

Die enterischen Methanemissionen (CH_4) von Wiederkäuern tragen mit einem Anteil von 39.4 % zu den gesamten anthropogen verursachten Treibhausgasemissionen der Landwirtschaft in Deutschland bei. Die Methanogenese führt zu einem Verlust von bis zu 12 % der aufgenommenen Futter-Bruttoenergie, stellt jedoch den bedeutendsten Stoffwechselweg zur Elimination des während der Fermentation anfallenden Wasserstoffs (H_2) dar. Der chemische CH_4-Inhibitor 3-Nitrooxypropanol (3-NOP) ist ein Strukturanalogon zu Methyl-Coenzym M und inaktiviert das Enzym Methyl-Coenzym M Reduktase im letzten Schritt der Methanogenese. Es konnte gezeigt werden, dass 3-NOP zu einer Verschiebung des ruminalen Fermentationsmusters in Richtung erhöhter Propionat- und niedrigerer Azetatanteile führt. Die Fütterung hoher Kraftfutteranteile (KFA) ist als eine indirekte CH_4-Minderungsstrategie bekannt. Dabei führen die erhöhten Anteile an Nicht-Faserkohlenhydraten zu einer vermehrten H_2-verbrauchenden Propiontabildung und damit zu einer Substratkonkurrenz mit der Methanogenese. Im peripartalen Zeitraum unterliegen Milchkühe drastischen endokrinologischen und stoffwechselbedingten Veränderungen. Diese werden von einer negativen Energiebilanz begleitet, wenn der Energiebedarf für die Milchsynthese die Energiezufuhr auf Grund der verminderten Futteraufnahme übersteigt. Infolge dessen kommt es zu einer massiven Mobilisierung von Köperenergiereserven in Form von nicht-veresterten freien Fettsäuren (NEFA), was zu einer Akkumulation von ß-Hydroxybuttersäure (BHB) in der Blutzirkulation führt und mit Erkrankungen des Stoffwechsels und der Leber verbunden ist.

Daraus ergeben sich folgende Fragestellungen: Der kombinierte Ansatz der Fütterung von 3-NOP und hohen KFA in der Ration führt zu additiven Effekten auf eine verminderte Methanogenese, insofern als dass sich die oben beschriebenen Wirkmechanismen der Faktoren 3-NOP und KFA auf die Methaninhibierung unterscheiden. Zudem werden weitere synergistische Effekte der kombinierten Methanminderungsstrategie erwartet, da die begünstigte H_2-verbrauchende Propionatbildung aus der stärkereichen Ration die von 3-NOP induzierte erhöhte H_2-Akkumulation im Pansen kompensieren könnte, wodurch die 3-NOP Effizienz gesteigert werden würde. Des Weiteren könnte die erhöhte Synthese von glukogenem Propionat als Resultat der Fütterung einer kraftfuttereichen Ration sowie der von 3-NOP induzierten Verschiebung der Fermentationsprozesse zusammen mit der erhöhten Futtereffizienz durch die verminderte Methanogenese zu einer verbesserten Energieversorgung der Milchkuh im perinatalen Zeitraum beitragen. Es wurde hypothetisiert, dass das GreenFeed-System zur indirekten kalorimetrischen

Schätzung der Energieretention im Körpergewebe und der Energieaufteilung zwischen einzelnen energieverbrauchenden Stoffwechselprozessen genutzt werden kann. Darüber hinaus wurde die Hypothese aufgestellt, dass die Effektivität von 3-NOP in Bezug auf die Senkung der CH_4-Produktion Dosis-Wirkungs-Beziehungen unterliegt und die Inkubation von 3-NOP mit niedrigen und hohen KFA zu additiven Effekten auf die Minderung der Methanogenese *in vitro* führt.

Zur Beantwortung dieser Hypothesen wurden 55 pluripare Milchkühe der Rasse Deutsche Holstein während der peripartalen und frühlaktierenden Phase im Zeitraum von Tag 28 *antepartum* bis Tag 120 *postpartum* in einem Fütterungsversuch verwendet. Die Kühe wurden in einem 2×2 faktoriellen Versuchsdesign nach hohem (KFH) und niedrigem (KFN) KFA sowie einer Supplementierung mit (3-NOP) oder ohne (KON) 50 mg 3-NOP pro kg Futter-Trockenmasse (TM) in der Ration gruppiert. Der KFA *antepartum* betrug in der KFN-Gruppe 15 %, der der KFH-Gruppe 40 %. Von der Kalbung bis Tag 21 *postpartum* wurde der KFA in der KFH-Gruppe schrittweise von 30 % auf 55 % angehoben, wohingegen der KFA der KFN-Gruppe auf 30 % festgelegt wurde. Die CH_4-Emissionen wurden mit dem GreenFeed System (C-Lock Inc., Rapid City, SD, USA) und die Futteraufnahme über Wiegetröge quantifiziert. Die Milchmenge wurde morgens und abends im Melkstand erfasst. Die Probennahme von Pansenchymus, Blut, Milch und Futter erfolgte zu definierten Zeitpunkten. Ultraschallmessungen der Fettauflagen zur Abschätzung der Fettdepotmassen sowie die Körperkonditionsbeurteilung wurden durchgeführt. Zusätzlich wurden Dosis-Wirkungs-Beziehungen zwischen 3-NOP Dosierungen von 0, 73, 160 und 1200 mg 3-NOP pro kg Futter-Trockenmasse kombiniert mit hohem und niedrigem KFA in einem 4×2 faktoriellen Design mit Hilfe der Pansensimulationstechnik (RUSITEC) untersucht.

Die in der vorliegenden *in vivo* Studie gezeigten interaktiven Effekte, resultierend aus der Supplementierung einer kraftfutterreichen Ration mit 50 mg 3-NOP pro kg Futter-TM, reduzierten die CH_4-Emissionen (g/Tag, g/kg TM-Aufnahme, g/kg energiekorrigierte Milchmenge) um 33 %. In der mit 3-NOP supplementierten KFN-Gruppe wurde eine weniger persistente und ausgeprägte Minderung der CH_4-Emission (23 %) beobachtet. Unter der Annahme, dass diese Wirkung von 3-NOP bei allen Rindern in Deutschland erreicht werden würde und anhaltend wäre, könnte 3-NOP die Treibhausgasemissionen um 5,75 (KFN) bis 8,25 (KFH) Millionen Tonnen CO_2eq reduzieren, was einem Anteil von 0,67% bis 0,96% an den gesamten deutschen Treibhausgasemissionen (858 Millionen Tonnen CO_2-Äquivalente in 2018) entspräche. Die Fütterung hoher KFA in der Ration reduzierte die CH_4-Emissionen pro kg TM-Aufnahme sowie pro kg energiekorrigierter Milchmenge um 12 % bis 17 %, wobei die Reduktion der absoluten CH_4-Emissionen (g/Tag) mit

4 % in den Kontrollgruppen geringer ausfiel. Die *in vivo* beobachteten interaktiven Effekte zwischen 3-NOP und dem hohen KFA konnten *in vitro* nicht bestätigt werden. Es zeigten sich Dosis-Wirkungsbeziehungen in Form einer linearen Abnahme der CH_4-Produktion um 12 %, 61 % und 97 % in den inkubierten KFN-Rationen und um 10 %, 35 % und 90 % in den KFH-Rationen. Die Reduktion der CH_4-Emissionen war begleitet von einem 27-fachen Anstieg der H_2-Emission *in vitro*, wobei eine geringere TM-Abbaubarkeit nicht beobachtet wurde. Zudem zeigten sich keine Behandlungseffekte auf die Gesamtproduktion an flüchtigen Fettsäuren, die Abundanz der Protozoen sowie den pH-Wert im Pansenchymus unter *in vitro* und, mit Ausnahme des Effekts der Kalbung, unter *in vivo* Bedingungen. Allerdings wurden signifikante Effekte von 3-NOP, dem KFA und der Kalbung auf die molaren Anteile der flüchtigen Fettsäuren im Pansenchymus festgestellt. 3-NOP führte *in vivo* zu einer Verschiebung der Fermentationsprozesse in Richtung einer erhöhten H_2-verbrauchenden Bildung von Propion-, Valerian- und *iso*-Valeriansäure, wohingegen die der H_2-freisetzenden Essigsäure reduziert war. Im RUSITEC-Experiment verblieben die alternativen H_2-Senken Propion- und Valeriansäure unverändert, während die molaren Anteile an Essigsäure ebenfalls mit zunehmender 3-NOP Dosierung abnahmen.

Unabhängig von der 3-NOP Zulage wurde eine höhere TM-Aufnahme bei den KFH-Gruppen beobachtet, die zudem zum Zeitpunkt der Kalbung in allen Gruppen deutlich abnahm. Der Milchlaktosegehalt war bei den 3-NOP supplementierten Kühen höher, während ein niedrigerer Milchfettgehalt in der 3-NOP-KFN-Gruppe sowie eine reduzierte 4 % fettkorrigierte Milchmenge in der 3-NOP-KFH-Gruppe beobachtet wurden. Die Energiebilanz der 3-NOP-KFH-Gruppe war positiver als die der anderen Gruppen. Die Messung des Gasaustausches von CO_2, O_2 und CH_4 mit Hilfe des GreenFeed Systems ermöglichte eine moderat valide indirekte kalorimetrische Schätzung des Energieumsatzes im Körpergewebe und der Energieaufteilung zwischen verschiedenen Energieaufwendungen bei einer großen Anzahl von Milchkühen. Die mit der Konvertierung der Futter-Bruttoenergie assoziierten Energieverluste in Form von CH_4 betrugen 5,8 % und 6,7 % der Gesamt-Bruttoenergieaufnahme in den KFN- bzw. KFH-Gruppen. Diese Futterineffizienz wurde kalkulatorisch durch 3-NOP um 2 % bzw. 1,6 % reduziert, was theoretisch zu einer zusätzlichen Energiezufuhr geführt haben könnte, die für einen Anstieg der Milchleistung um 0,86 kg in den KFH- bzw. 0,59 kg in den KFN-Gruppen ausreichen würde. Der Effekt einer potentiell verbesserten Futtereffizienz wurde in der vorliegenden Studie jedoch nicht in Form einer erhöhten Milchleistung oder Körpermassezunahme wiedergefunden. Allerdings zeigte sich ein signifikant reduzierter NEFA-Spiegel in den 3-NOP-Gruppen. Demgegenüber wurden keine Effekte von 3-

NOP auf die Konzentration an BHB, Glukose und Triglyzeriden im Serum sowie den BCS und die Mobilisierung von Köperenergiereserven aus Fettdepots beobachtet. Hierbei wurden teilweise Effekte des KFA und der Kalbung festgestellt. Damit ist festzuhalten, dass die beobachteten additiven Effekte zwischen 3-NOP und hohem KFA auf die Minderung der CH_4-Emission zu keiner konsistent beobachteten Verbesserung der Energieversorgung der Milchkuh beitrugen.

Für die Bewertung von CH_4-Minderungsstrategien zeichnen sich, bei Nichtverfügbarkeit von CH_4-Erfassungstechniken, empirische Schätzgleichungen aufgrund der engen Beziehung zwischen Futteraufnahme und CH_4-Emission als Mittel der Wahl ab. Die Schätzung der CH_4-Minderung durch 3-NOP stellt hierbei eine Herausforderung dar, weil die direkte Beziehung zwischen der Futteraufnahme und CH_4-Emission bei Fütterung von 3-NOP entkoppelt ist. Die vorliegende Studie zeigt auf, dass die Schätzung der CH_4-Emission zukünftig jedoch über die Kombination von Variablen des Milchfett- und NDF-Gehalts sowie des Fermentationsmusters und deren Verwendung als Hilfsmerkmale erfolgen könnte.

Schlussfolgernd lässt sich sagen, dass 3-NOP als ein vielversprechender Methaninhibitor mit dosis- und rationsabhängiger Wirksamkeit angesehen werden kann, wobei positive Effekte von 3-NOP auf das Energiebudget der Kuh in den vorliegenden Experimenten nicht belegt werden konnten. Die Ergebnisse der vorliegenden Studie implizieren, dass weitere Experimente hinsichtlich der rations- und dosisabhängigen Wirksamkeit von 3-NOP bei Milchkühen in unterschiedlichen Laktationsstadien durchgeführt werden sollten, bevor der Zusatzstoff weitverbreitete Anwendung in Milchviehbetrieben findet. Zusätzlicher Forschungsbedarf besteht bezüglich der Fragestellung, inwiefern die beobachteten 3-NOP induzierten Verschiebungen im Fermentationsmuster der flüchtigen Fettsäuren mit Veränderungen der Gemeinschaft der methanogenen Archaeen im Pansen korrespondieren. Zudem schließt sich die Frage an, ob diese potentiellen Zusammenhänge mittels Infrarotspektroskopie der Milch reflektiert werden können und anschließend in der Entwicklung von Modellen zur Vorhersage der Methanemissionen in einem 3-NOP basierten Methanminderungsszenario für die Treibhausgasinventarisierung Anwendung finden könnten. Untersuchungen zur Genexpression in der Leber genauso wie zu potentiellen Veränderungen im Metabolom im Plasma sowie funktionalen Parametern des Immunsystems könnten einen tieferen Einblick bezüglich eines potentiellen vorteilhaften Effekts der 3-NOP vermittelten Methanminderung auf den Energiestoffwechsel der Kuh geben.

11. Supplementary data

Table S1: Descriptive statistics and allocation of candidate predictor variables to the different methane prediction models.

	CON groups						3-NOP groups						Candidate variable included in Eq. (Tab. 2):								
Variable	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Response variable																					
CH_4 production, g/d	515	369	166	556	391	63	399	266	97	549	452	70									
Candidate explanatory variables																					
Animal variables																					
Week of lactation	515	7	-4	17	21	6.3	399	6.8	-4	17	21	6.3	1	2	3	4	5	6			9
Lactation number	515	2.8	1	5	4	1.1	399	2.6	1	4	3	0.7	1	2	3	4	5	6			9
Body condition score	515	2.9	1.5	4.5	3.0	0.6	399	2.9	1.5	4.8	3.3	0.5	1	2	3	4	5				9
Metabolic body weight, kg	501	132	110	169	58.7	11.3	394	132	111	172	60.6	11.2			3	4	5				9
Feed intake variables																					
Eating time, min	515	166	16.7	292	275	49.8	399	164	61.7	306	245	48.3			3	4	5				9
Dry matter (DM), kg/d	515	19.4	7.1	28.1	21.0	3.9	399	19.7	9.4	27.2	17.8	3.8	1	2		4	5				
Neutral detergent fibre, kg/d	515	7.0	2.7	9.4	6.7	1.3	399	7.0	3.5	9.8	6.3	1.2		2		4	5				
Forage, kg DM/d	515	11.8	3.4	17.5	14.1	2.5	399	11.5	5.0	16.9	12.0	2.4					5				
Concentrate, kg DM/d	515	7.6	0.9	14.8	13.9	3.2	399	8.4	1.7	14.4	12.7	3.4					5				
Gross energy, MJ/d	515	359	130	522	392	73.2	399	366	172	505	333	71.1		2		4	5				
Energy balance, MJ NEL/d	494	-13.2	-114	85.3	200	32.4	389	-3.9	-122	84.1	206	34.8				4	5				
Diet composition, g/kg DM																					
Crude ash	515	62.0	55.9	72.5	16.7	4.0	399	62.0	57.3	73.5	16.3	3.8		2	3	4	5				9
Crude protein	515	134	102	159	57.1	12.8	399	135	102	157	54.7	11.7		2	3	4	5				9
Crude fat	515	33.5	29.2	37.7	8.6	2.2	399	33.9	29.0	37.9	8.9	2.3		2	3	4	5				9
Crude fibre	515	182	135	227	91.9	21.6	399	178	141	227	85.5	23.2		2	3	4	5				9
Neutral detergent fibre	515	374	301	462	160	36.4	399	367	306	461	155	39.2		2	3	4	5				9
Acid detergent fibre	515	209	163	251	88.4	21.3	399	205	169	251	82.0	22.9		2	3	4	5				9
Starch	515	275	189	362	172	38.8	399	280	189	369	180	42.1		2	3	4	5				9
Metabolic Energy, MJ/kg DM	515	11.2	10.7	11.8	1.1	0.3	399	11.3	10.7	11.8	1.1	0.3		2	3	4	5				9
Net energy lactation, MJ/kg DM	515	6.8	6.4	7.3	0.9	0.2	399	6.9	6.4	7.3	0.9	0.2		2	3	4	5				9
Utilizable crude protein	515	146	136	158	22.1	5.4	399	147	136	157	20.9	5.6		2	3	4	5				9
Ruminal N balance	515	-1.9	-5.4	1.6	7.0	1.4	399	-1.9	-5.4	1.5	6.9	1.3		2	3	4	5				9

Continuation of Table S1.

	CON groups						3-NOP groups						Candidate variable included in Eq. (Tab. 2):								
Variable	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Milk performance, kg/d																					
Milk yield	425	37.8	20.6	51.5	30.9	6.0	325	37.5	21.3	53.3	32.0	5.9			3	4	5	6			9
Fat, %	418	4.3	2.5	7.5	5.0	0.8	320	4.1	2.2	6.9	4.7	0.7			3	4	5	6			9
Protein, %	418	3.2	2.7	4.3	1.6	0.3	320	3.2	2.5	4.5	2.0	0.3			3	4	5	6			9
Urea	417	113	19.5	291	271	44.7	320	128	28.5	237	208	35.6			3	4	5	6			9
Lactose, %	418	4.8	4.2	5.1	0.9	0.1	320	4.8	4.2	5.2	1.0	0.1			3	4	5	6			9
Milk fat synthesis	418	1.6	0.9	3.1	2.2	0.3	320	1.5	0.9	2.7	1.8	0.3			3	4	5	6			9
Milk protein synthesis	418	1.2	0.8	1.6	0.8	0.2	320	1.2	0.8	1.6	0.8	0.2			3	4	5	6			9
Lactose synthesis	418	1.8	0.9	2.5	1.6	0.3	320	1.8	0.9	2.6	1.6	0.3			3	4	5	6			9
4%-fat corrected milk	418	39.3	24.0	63.4	39.5	6.3	320	37.7	22.5	61.6	39.1	5.7			3	4	5	6			9
Energy corrected milk	418	38.5	23.9	58.5	34.6	5.8	320	37.3	22.7	58.4	35.7	5.3			3	4	5	6			9
Fat-milk protein ratio	418	1.4	0.8	2.4	1.6	0.2	320	1.3	0.7	2.1	1.4	0.2			3	4	5	6			9
Rumen fermentation variables																					
Ammonia, mmol/L	132	2.9	0.1	11.7	11.6	2.0	115	2.3	0.2	10.5	10.3	1.6					5				
Acetate, mmol/L	132	55.8	16.8	81.2	64.4	10.4	115	51.1	30.0	70.9	40.9	8.7					5	6	7	8	
Propionate, mmol/L	132	21.1	5.0	41.7	36.8	6.7	115	22.1	7.9	43.5	35.5	7.0					5	6	7	8	
Isobutyrate, mmol/L	132	0.6	0.3	1.1	0.8	0.1	115	0.6	0.3	1.4	1.1	0.1					5	6	7	8	
Butyrate, mmol/L	132	12.2	3.4	26.0	22.6	3.1	115	13.1	6.6	21.4	14.8	3.4					5	6	7	8	
Isovalerate, mmol/L	132	1.2	0.3	2.3	2.0	0.4	115	1.5	0.5	2.9	2.4	0.5					5	6	7	8	
Valerate, mmol/L	132	1.3	0.3	4.4	4.1	0.5	115	1.8	0.5	4.4	3.9	0.8					5	6	7	8	
Acetate-to-propionate ratio, mmol/L	132	2.8	1.4	4.4	3.0	0.7	115	2.5	1.3	4.5	3.2	0.7					5	6	7	8	
Total volatile fatty acids, mmol/L	132	92.2	25.8	130	104	18.0	115	90.3	49.5	134	84.0	17.0					5	6	7	8	
Acetate, %	132	60.8	49.0	69.0	20.0	4.2	115	57.0	47.6	68.0	20.4	5.2					5	6	7	8	
Propionate, %	132	22.6	15.1	37.0	21.8	4.6	115	24.2	15.0	38.0	22.9	4.6					5	6	7	8	
Isobutyrate, %	132	0.7	0.4	1.3	0.9	0.2	115	0.7	0.4	1.2	0.8	0.2					5	6	7	8	
Butyrate, %	132	13.1	8.4	24.7	16.3	1.8	115	14.5	8.5	26.4	17.9	2.4					5	6	7	8	
Isovalerate, %	132	1.3	0.4	2.3	1.9	0.4	115	1.7	0.7	2.9	2.2	0.4					5	6	7	8	
Valerate, %	132	1.4	0.8	4.2	3.4	0.4	115	1.9	0.9	4.9	4.0	0.7					5	6	7	8	

Continuation of Table S1.

	CON groups						3-NOP groups						Candidate variable included in Eq. (Tab. 2):								
Variable	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Biochemical blood variables, mmol/L																					
O2 capacity, mL O2/mL blood	266	137	88.4	171	82.4	14.6	207	134	101	170	69.7	13.6					5				
Na	271	140	130	145	10.0	1.9	212	141	136	147	11.0	2.0					5				
K	271	3.8	2.9	6.2	3.3	0.4	212	3.8	2.9	5.6	2.7	0.4					5				
Cl	271	104	98.0	112	14.0	2.8	212	105	99.0	115	16.0	2.4					5				
Ca	271	1.3	1.0	1.5	0.5	0.1	212	1.3	1.0	1.5	0.5	0.1					5				
Hematocrit, %	271	25.2	19.0	30.5	11.5	2.3	212	24.6	18.0	30.0	12.0	2.3					5				
Lactate	270	0.6	0.3	2.7	2.4	0.2	212	0.5	0.3	1.3	1.0	0.1					5				
Hemoglobin, g/dL	266	10.2	6.6	12.8	6.2	1.1	207	10.0	7.5	12.7	5.2	1.0					5				
Oxyhemoglobin, %	266	60.2	39.1	83.1	44.0	8.7	207	60.9	27.5	79.5	52.0	8.2					5				
Carbaminohemoglobin, %	266	0.1	0.0	1.7	1.7	0.3	207	0.2	0.0	1.3	1.3	0.2					5				
Methemoglobin, %	266	2.7	1.7	3.5	1.8	0.3	207	2.7	1.3	3.3	2.0	0.4					5				
Desoxyg. hemogl., %	266	36.9	14.8	58.1	43.3	8.7	207	36.1	17.9	69.2	51.3	8.2					5				
Venous O_2 saturation, %	266	62.0	40.2	84.9	44.7	8.9	207	62.8	28.4	81.6	53.2	8.4					5				
pHT	271	7.4	7.3	7.5	0.1	0.0	212	7.4	7.3	7.5	0.1	0.0					5				
pCO2T, mmHg	270	48.4	30.0	62.0	32.0	5.6	212	48.1	36.0	64.0	28.0	4.9					5				
pO2T, mmHg	271	35.2	20.0	49.0	29.0	4.6	212	35.8	18.0	47.0	29.0	4.1					5				
Total CO2	270	30.8	18.9	42.8	23.9	4.0	212	30.5	22.2	39.0	16.8	3.1					5				
Base excess extracell. fluid	270	4.8	-7.4	17.0	24.4	4.0	212	4.6	-4.6	12.5	17.1	3.2					5				
Base excess	270	3.5	-6.7	14.6	21.3	3.5	212	3.3	-5.1	9.9	15.0	2.7					5				
Bicarbonate	270	29.4	17.9	41.0	23.1	3.8	212	29.1	21.0	37.2	16.2	3.0					5				
Hematocrit, %	266	30.7	20.0	38.5	18.5	3.3	207	30.0	23.0	38.0	15.0	3.1					5				
Temperature, °C	271	38.4	36.6	40.8	4.2	0.5	212	38.4	37.0	40.6	3.6	0.5					5				
Anion-gap	270	10.8	0.4	19.6	19.2	2.5	212	10.6	5.2	15.3	10.1	2.0					5				

Continuation of Table S1.

	CON groups						3-NOP groups						Candidate variable included in Eq. (Tab. 2):								
Variable	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**n**	**Mean**	**Min**	**Max**	**Range**	**SD**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Hematological blood variables, mmol/L																					
Leucocytes, G/L	285	7.7	3.9	12.4	8.5	1.6	227	8.1	2.6	15.4	12.8	1.9					5				
Lymphocytes, %	285	38.3	20.1	87.7	67.6	7.1	227	36.1	15.5	54.9	39.4	7.1					5				
Monocytes, %	285	0.8	0.0	14.7	14.7	1.1	227	0.8	0.0	3.2	3.2	0.6					5				
Eosinophils, %	285	10.9	0.0	49.3	49.3	7.5	227	10.4	0.0	52.8	52.8	7.7					5				
Granulocytes, %	285	49.9	10.7	74.1	63.4	10.6	227	52.7	10.0	77.0	67.0	11.5					5				
Lymphocytes, G/L	285	2.9	1.6	8.3	6.7	0.6	227	2.8	1.2	6.0	4.8	0.6					5				
Monocytes, G/L	285	0.1	0.0	1.3	1.3	0.1	227	0.1	0.0	0.3	0.3	0.1					5				
Eosinophils, G/L	285	0.8	0.0	3.5	3.5	0.5	227	0.8	0.0	3.2	3.2	0.5					5				
Granulocytes, G/L	285	3.9	0.6	8.8	8.2	1.4	227	4.4	0.5	11.9	11.4	1.8					5				
Erythrocytes, G/L	285	5.7	4.0	7.4	3.4	0.6	227	5.5	4.1	7.3	3.3	0.6					5				
Hemoglobin, g/dL	285	8.6	5.7	10.8	5.1	1.0	227	8.4	6.1	10.3	4.2	0.9					5				
Hematocrit, %	285	28.0	18.2	36.5	18.3	3.3	227	27.4	19.3	34.4	15.1	2.9					5				
Mean corpuscular vol., fL	285	49.0	40.4	57.0	16.6	3.2	227	49.8	42.3	56.9	14.6	2.7					5				
Mean corp. hemogl., pg/cell	285	15.0	11.9	17.6	5.7	1.0	227	15.2	12.5	17.4	4.9	0.8					5				
Mean corp. hemogl., g/dL	285	30.6	27.8	35.9	8.1	0.8	227	30.6	25.8	33.5	7.7	0.9					5				
Platelets, G/L	285	364	33	856	823	97	227	367	48	667	619	88					5				
Plateletcrit, %	285	0.1	0.0	0.3	0.3	0.0	227	0.1	0.0	0.2	0.2	0.0					5				
Mean platelet vol., fL	285	3.3	2.2	4.6	2.4	0.5	227	3.3	2.4	4.6	2.2	0.4					5				
Platelet distrib. Width, %	285	17.5	14.2	23.3	9.1	1.2	227	17.6	15.4	23.3	7.9	1.0					5				
Triacylglycerides	285	0.1	0.1	0.3	0.2	0.0	226	0.1	0.1	0.3	0.2	0.0					5				
Cholesterol	285	3.8	0.9	8.8	7.9	1.7	226	3.8	1.2	9.2	8.0	1.6					5				
Albumin, g/L	285	32.4	25.5	37.5	12.0	1.7	226	31.4	26.9	35.0	8.0	1.7					5				
Bilirubin, µmol/L	285	2.9	1.3	12.2	10.9	1.8	226	2.4	1.4	8.5	7.1	1.2					5				
Blood urea	285	2.2	0.9	4.7	3.8	0.7	226	2.3	1.0	4.8	3.8	0.6					5				
γ-glutamyltransferase, µkat/L	285	0.5	0.2	1.5	1.3	0.2	226	0.5	0.2	1.8	1.5	0.2					5				
ß-hydroxybutyrate	285	0.8	0.3	4.5	4.2	0.5	226	0.7	0.3	3.1	2.9	0.3					5				
Total protein plus, g/L	285	71.6	60.0	96.5	36.4	6.0	226	70.4	56.0	91.9	35.9	6.4					5				
Alanine transaminase, µkat/L	285	0.6	0.3	1.2	0.9	0.2	226	0.6	0.3	1.4	1.1	0.2					5				
Aspartate transaminase, µkat/L	285	1.5	0.8	4.5	3.7	0.5	226	1.3	0.8	3.3	2.5	0.4					5				
Glucose	285	3.4	1.1	5.6	4.5	0.5	226	3.4	2.3	4.6	2.3	0.4					5				
Non-esterified fatty acids, µmol/L	285	370	63	2622	2560	299	226	283	45	951	905	179					5				
Glutamate dehydrogenase, µkat/L	285	0.3	0.0	4.3	4.3	0.4	226	0.3	0.1	3.0	2.9	0.4					5				
Ionizing Cl	285	103	96.1	112	15.8	2.9	226	104	96.6	111	14.1	2.2					5				
Ionizing K	285	4.0	2.7	6.9	4.3	0.5	226	4.0	3.1	10.4	7.3	0.6					5				
Ionizing Na	285	141	134	152	18.9	2.6	226	141	135	147	11.8	2.2					5				

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Eidesstattliche Erklärung / *Declaration Under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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