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# Dietary supplementation with calcium peroxide improves methane mitigation potential of finishing beef cattle

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ABSTRACT

Calcium peroxide (**$CaO_2$**) offers potential as an anti-methanogenic dietary feed material. The compound has been previously assessed *in vitro*, with methane (**$CH_4$**) reductions of > 50% observed. The objective of this study was to assess dietary supplementation of $CaO_2$ at different inclusion levels and physical formats in a finishing beef system on the effects of animal performance, gaseous emissions, rumen fermentation parameters and digestibility. Seventy-two dairy-beef bulls (465 kg; 16 months of age) were randomly allocated to one of four treatments supplemented with $CaO_2$; in a coarse ration (1) **CON** (0% $CaO_2$), (2) **LO** (1.35% $CaO_2$), (3) **HI** (2.25% $CaO_2$), and in a pellet (4) **HP** (2.25% $CaO_2$) (n = 18). Animals received their respective treatments for a 77 d finishing period, during which DM intake (American Calan Inc., Northwood, NH), average daily gain (**ADG**), feed efficiency and enteric emissions (GreenFeed emissions monitoring system; C-Lock Inc., Rapid City, SD) were measured. The finishing diet was isonitrogenous and isoenergetic across the four treatment groups, composed of 60:40 grass silage:concentrate. Silage was offered each morning (0900 h), and concentrates were offered twice daily (0800 and 1500 h). Supplementation of $CaO_2$ had no effect on final weight ($P$ = 0.09), ADG ($P$ = 0.22) or feed efficiency ($P$ = 0.13). Regarding DM intake, the HI treatment group consumed in the order of 1 kg less than CON ($P$ < 0.01), while HP did not affect DM intake compared to CON ($P$ = 0.79). Across treatments, DM intake ranged from 8.43 to 9.57 kg/d, equating to 1.6–1.8% of BW. Daily $CH_4$ values for the control were 240 g/d, while $CaO_2$ supplemented diets ranged from 202 to 170 g/d, resulting in daily $CH_4$ reductions of 16, 29 and 27% for LO, HI and HP, respectively, compared to CON ($P$ < 0.0001). Additionally, hydrogen was reduced in $CaO_2$ supplemented animals by 32–36% relative to CON ($P$ < 0.0001), with a simultaneous reduction in volatile fatty acid production ($P$ < 0.01) and an increase in propionate concentration ($P$ < 0.0001). Across all universally accepted $CH_4$ metrics (yield, intensity, production), the dietary inclusion of $CaO_2$ whether at a low or high rate, or indeed, through a coarse ration or pelleted format reduced $CH_4$ in the order of 16–32%. This study also concluded that $CaO_2$ can successfully endure the pelleting process, therefore, improving ease of delivery if implemented at farm level.


## Implications

Supplementing calcium peroxide to beef cattle at a low and a high inclusion rate reduced daily methane production by 16 and 28%, respectively. Calcium peroxide was found to withstand heat and pressure during the pelleting process. This study reported reductions in palatability and digestibility with high calcium peroxide supplementation, however, not to the extent that it had an effect on animal performance. Supplementation of calcium peroxide offers huge potential for enteric methane reduction, and research needs to be extended to focus on its effects on the rumen microbiome, on differing diet types, stages of production and as an early-life supplement.

## Introduction

Agriculture is a major source of greenhouse gas emissions world-wide, predominantly due to the production of enteric methane (**$CH_4$**) which accounts for 44.3% of global agricultural emissions (FAO, 2022). Globally, there is an obligation to reduce greenhouse gas emissions through legally binding policies such

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as the European Green Deal (Fetting, 2020), and agreements such as the global methane pledge (UNEP, 2022). Due to the significant contribution of the agricultural sector to overall greenhouse gas emissions, and with $CH_4$ being a major contributor to these agri-emissions, there is an urgent requirement for practical solutions to mitigate $CH_4$ emissions from the agricultural sector. In light of this, research into the development of strategies for the mitigation of enteric $CH_4$ has proliferated. Thus, approaches such as breeding lower $CH_4$ emitting animals (Smith et al., 2021), improving digestibility of feed and altering the forage:concentrate ratio of diets (Shibata and Terada, 2010), assessing diverse pastures (Jonker et al., 2019) and forage types (Meo-Filho et al., 2023), and a particular emphasis on dietary supplementation of lipids and anti-methanogenic compounds (Beauchemin et al., 2022) are being explored.

To-date, the role of strategic supplementation of various anti-methanogenic compounds has been assessed in beef cattle, with 3-nitrooxypropanol (Alemu et al., 2021; Gruninger et al., 2022), *Asparagopsis taxiformis* (Roque et al., 2021; Ridoutt et al., 2022) and lipid supplementation (Beauchemin et al., 2007; Gruninger et al., 2022) yielding the most consistent results. Respectively, each of the aforementioned dietary interventions have associated caveats such as transient effectiveness requiring continuous supplementation to evoke a $CH_4$ mitigating response (Van Wesemael et al., 2019), animal health concerns with regard to pathological damage to the rumen wall (Muizelaar et al., 2021) and a noted reduction in DM intake (Arndt et al., 2021).

The development of a $CH_4$ mitigation strategy, such as dietary supplementation of a material that is effective in a twice−daily feeding regime and that is not associated with residue deposition, animal health concerns or reduction in diet digestibility, would be revolutionary in mitigating enteric $CH_4$ emissions. Additionally, heat and pressure associated with the pelleting process can adversely impact volatile anti-methanogenic bioactives (Hegarty et al., 2021) and high lipid concentrations can cause poor pellet quality (Briggs et al., 1999). Therefore, pelletability should be an attribute evaluated for all potential $CH_4$ mitigating compounds.

Calcium peroxide (**$CaO_2$**), through the introduction of oxygen to the rumen, has the potential to reduce enteric $CH_4$ production. Early work has demonstrated the anti-methanogenic potential of $CaO_2$ *in vitro* and in subsequent small−scale sheep studies (Demeyer, 1982). Calcium peroxide is rapidly hydrolysed in aqueous solutions into calcium hydroxide, hydrogen peroxide, oxygen and water, enabling continuous oxygenation (0.222 g oxygen/g $CaO_2$) of the medium into which it is released (Lu et al., 2017), hence, modulation of rumen oxidative reduction potential (**ORP**). Supplementation of $CaO_2$ *in vitro* in the RUSITEC system has resulted in an increase in ORP for 2 h post-feeding and a subsequent 57% reduction in $CH_4$ g/d (Graham et al., 2024b).

In this experiment, the oxidising potential of $CaO_2$ has been exploited and its application as a feed additive to mitigate $CH_4$ in the ruminant feed industry assessed. Our hypothesis is that the introduction and continuous release of oxygen would elevate ruminal ORP, hence, inhibit methanogens and other anaerobic microbes, therefore, reducing $CH_4$ production and altering hydrogen (**$H_2$**) and fermentation pathways. Therefore, the objective of this study was to assess the efficacy of dietary supplementation of $CaO_2$ at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on nutrient digestibility, animal production parameters, gaseous emissions, ORP profiles and animal health parameters in growing bulls.

## Material and methods

### *Animals and experimental design*

Seventy-two early maturing dairy-beef bulls (32 Angus X Holstein-Friesian and 40 Hereford X Holstein-Friesian) with an average BW (± SD) of 465 ± 6.57 kg and age of 16 ± 0.13 months at the beginning of the experiment were selected for this study. The animals were purchased from a commercial farm at < 6 months of age and were reared at the Teagasc Grange Beef Research Centre under normal husbandry conditions until the experiment commenced.

The finishing period was a randomised complete block design. Animals were blocked on BW at the beginning of the experiment and within block were randomly assigned to one of four dietary treatments (n = 18 animals per treatment); (1) **CON** (0% $CaO_2$), (2) **LO** (1.35% $CaO_2$), (3) **HI** (2.25% $CaO_2$), and (4) **HP** (2.25% $CaO_2$). For blocking, initial BW was standardised by weighing animals on 3 consecutive days prior to the start of the finishing period. The animals were assigned to four pens (n = 18 animals per pen; lying area c. 3.4 $m^2$ per animal) in a concrete-slatted floor finishing facility equipped with electronic Calan Broadbent individual feeding system (American Calan Inc., Northwood, NH) for facilitating allocation and recording of individual DM intake. Each dietary treatment was replicated across pen.

### *Basal diet, dietary treatments and feed intake*

All animals received a standardised finishing diet (60:40 forage:concentrate ratio on a DM basis) for a 77 d finishing period. The forage component of the diet was high quality, > 75% DM digestibility (Patton et al., 2022), second-cut perennial ryegrass silage, ensiled in a silo without the addition of any additives or inoculants. The concentrate portion of the diet consisted of one of the four following dietary treatments: (1) control, barley/soyabean coarse ration with 0% $CaO_2$ inclusion (CON), (2) low, barley/-soyabean coarse ration with $CaO_2$ included at 1.35% of DM intake (LO), 3) high, barley/soyabean coarse ration with $CaO_2$ included at 2.25% of DM intake (HI), and 4) high pellet, barley/soyabean pellet with $CaO_2$ included at 2.25% of DM intake (HP). A full breakdown of concentrate composition and chemical analysis of each dietary component is presented in Table 1.

All finishing diets were formulated to be isonitrogenous and isoenergetic to fulfil the nutritional requirements of finishing bulls. In order to achieve the target inclusion rate of 0, 1.35, 2.25 and 2.25% $CaO_2$ of the animal's total diet for CON, LO, HI and HP, $CaO_2$ was included in the concentrate at 0, 4.35, 7.25 and 7.25%, respectively. The $CaO_2$ was supplied by GlasPort Bio Ltd (Unit 204, Business Innovation Centre, University of Galway, Ireland). Over the 77 d finishing period, individual DM intake was recorded using the Calan Broadbent feeding system. Animals were offered 50% of their dietary concentrate (CON, LO, HI, HP) at 0800 h and the remaining 50% at 1500 h. Grass silage was offered daily at 0900 h. Animals were offered 110% of previous day's silage intake to reduce the possibility of restricting intake. Feed refusals were weighed and recorded on a daily basis.

### *Animal production parameters*

Animals were weighed weekly at 0800 h prior to feeding. Average daily gain (**ADG**) was calculated over a 63 d period, from d 3 until the end of the finishing period. Feed conversion efficiency

**Table 1**
Ingredient composition of concentrates and chemical analysis of dietary ingredients offered during the 77-d supplementation period to finishing beef bulls.

| Item | Grass Silage | GreenFeed | Treatment | | | |
|---|---|---|---|---|---|---|
| | | | CON | LO | HI | HP |
| Ingredient (% DM) | | | | | | |
| Rolled barley | – | 85.7 | 58.3 | 53.16 | 49.5 | 49.5 |
| Soyabean meal | – | 6.00 | 34.0 | 34.8 | 35.6 | 35.6 |
| Molasses | – | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Limestone flour | – | 1.68 | 1.55 | 1.54 | 1.54 | 1.54 |
| Salt | – | 0.62 | 0.65 | 0.65 | 0.65 | 0.65 |
| Vegetable oil | – | 0.50 | 0.00 | 0.00 | 0.00 | 0.00 |
| Min-vit premix | – | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| $CaO_2$ | – | 0.00 | 0.00 | 4.35 | 7.25 | 7.25 |
| Chemical composition (g/kg DM, unless stated) | | | | | | |
| DM | 212 | 867 | 854 | 866 | 868 | 897 |
| CP | 147 | 138 | 247 | 220 | 228 | 240 |
| NDF | 540 | 219 | 171 | 146 | 153 | 145 |
| ADF | 338 | 63.3 | 59.8 | 55.6 | 53.7 | 66.2 |
| Starch | – | 461 | 339 | 361 | 351 | 319 |
| Ether extract | 39.8 | 17.8 | 13.6 | 13.0 | 12.5 | 11.6 |
| Ash | 75.7 | 62.8 | 66.1 | 109.7 | 136.5 | 152.7 |
| GE (MJ/kg) | 16.9 | 16.7 | 17.2 | 16.4 | 16.1 | 16.1 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; Min-vit premix = Mineral-vitamin premix; GE = gross energy.

was calculated as $\frac{ADG}{DMintake}$. Ultrasound measurements were taken at the beginning and end of the experiment to estimate fat and muscle deposition as described by Kelly et al. (2010). Briefly, muscle depth was measured at the third lumbar vertebra and fat depth was measured at three sites on the third lumbar vertebra and on four sites at the rump, an average of the lumbar and rump measurements was calculated as the means of the values recorded.

*Enteric methane, hydrogen and carbon dioxide output*

Enteric $CH_4$, $H_2$ and carbon dioxide measurements were obtained using the GreenFeed emissions monitoring system (C-Lock Inc., Rapid City, SD) during the pre-experimental and finishing period. In-depth workings of the GreenFeed are described by Hammond et al. (2015) and detailed gas calibrations, feed drop calibrations and airflow maintenance are outlined by Smith et al. (2021). Auto callibrations were performed daily, whereas, manual carbon dioxide recoveries were performed d 1 of the pre-experimental period and every month thereafter, as per manufacturers' instructions and averaged 99 ± 2.89%. Air filters were replaced weekly, the average airflow rate was 35.5 ± 0.11 L/s over the finishing period. Feed drops were recorded and weighed weekly; the average drop across all units was 34.63 ± 0.46 g FW.

One GreenFeed unit was installed per pen of 18 animals. A 3 wk GreenFeed acclimatisation period preceded the experiment, during which, a minimum waiting time of 1 h was set between visits, as the animals began to visit the units more frequently, the waiting time was increased to a minimum of 4 h between visits. During the finishing period, animals were allowed a maximum of 6 visits per d to the GreenFeed units, with a maximum of 6 feed drops per visit (35 s interval between feed drops). Daily DM intake per animal from the GreenFeed is presented in Table 2. GreenFeed measurements with a duration of < 3 min were discarded. To account for any potential impact on circadian variation in $CH_4$ emissions, data where the animal visited the GreenFeed < 3 times in that day were discarded, and any animal that had < 30 reliable measurement days ($\geq$ 3 measurements/d of $\geq$ 3 min each) over the finishing period were discarded. Based on the above criteria, 58 animals (CON = 15, LO = 15, HI = 15, HP = 14) had an accurate $CH_4$ phenotype for analysis. The average daily GreenFeed visit frequency was recorded as 4.06 ± 0.05. The above criteria were modified based on findings from Arthur et al. (2017), Smith et al. (2021) and Lahart et al. (2024).

*Rumen fermentation*

*Collection and storage of rumen fluid*

Rumen fluid was harvested using a transoesophageal rumen device (Kirwan et al., 2024), at the end of the pre-experimental period, and at the mid-point and end-point of the finishing period. Animals were fed their morning concentrate allocation 2–4 h prior to sampling and were taken off feed 1 h prior to sampling. Immediately post-harvest, rumen fluid pH was recorded using a pH meter (Orion 3 star pH, Thermo-Scientific, Waltham, MA). A 4 ml sub-sample was mixed with 1 ml 50% (w/v) trichloroacetic acid for subsequent volatile fatty acid (**VFA**), ammonia–nitrogen and lactic acid analyses. All samples were snap frozen in liquid nitrogen and stored at -20 °C.

*Volatile fatty acid, ammonia–nitrogen and lactic acid analyses*

Rumen fluid was thawed and centrifuged (1 800 g; 4 °C) for 10 min. For determination of VFA (acetate, propionate, valerate and butyrate), 250 µl supernatant, 3.75 ml distilled water and 1 ml internal standard (0.5 g 3-metyl-n-valeric acid in 1 L of 0.15 M oxalic acid) were transferred to a clean disposable (16 × 100 mm) test tube. The diluted samples were centrifuged for 5 min (260 g; 21 °C) followed by filtration through a 0.45 µm filter (Cronus Syringe filter PTFE 13 mm; SMI-LabHut Ltd., Maisemore, Gloucester, UK) into prelabelled 2 ml GC vials (Thermo Scientific, Langerwehe, Germany). Each sample was prepared in duplicate. The concentration of VFA was measured using an automated Agilent 450-GC (Agilent Technologies, Santa Clara, Canada) fitted with flame ionisation detector (Ranfft, 1973). Acetate, propionate, butyrate and valerate concentrations were divided by total VFA to report the proportion of each individual VFA. For determination of ammonia–nitrogen, a 100 µL sample of supernatant was added to 900 µL distilled water and was analysed using the Beckman Coulter AU480 Clinical Analyser (Beckman Coulter, IN, US) and the Thermo Electron Infinity Ammonia Liquid Stable Reagent (Glenbio Ltd, Co. Antrim, UK) kinetic method as described by Owens et al. (2008), with ammonia–nitrogen reported as mg/L. For the determination of lactic acid, a 1 ml sample of supernatant

**Table 2**
Efficacy of $CaO_2$ supplementation at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on feed intake, performance, feed efficiency and ultrasonically measured muscle and back fat depth.

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| Item | CON | LO | HI | HP | SEM | *P*-value |
| Feed intake (kg/d) | | | | | | |
| DM intake | 9.36[a] | 9.57[a] | 8.43[b] | 9.15[a] | 0.224 | < 0.01 |
| Silage | 6.13[a] | 6.27[a] | 4.79[b] | 5.83[a] | 0.183 | < 0.0001 |
| GreenFeed | 0.764 | 0.799 | 0.785 | 0.736 | 0.0227 | 0.24 |
| Concentrate | 2.62[a] | 2.65[b] | 2.66[c] | 2.74[d] | 0.001 | < 0.0001 |
| CP | 1.66[a] | 1.62[a] | 1.42[b] | 1.61[a] | 0.027 | < 0.0001 |
| NDF | 3.93[a] | 3.95[a] | 3.17[b] | 3.70[a] | 0.101 | < 0.0001 |
| Ether extract | 0.293[ab] | 0.298[b] | 0.238[c] | 0.277[a] | 0.0075 | < 0.0001 |
| $CaO_2$ offered | 0.000[a] | 0.131[b] | 0.219[c] | 0.225[d] | 0.0012 | < 0.0001 |
| Performance and feed efficiency | | | | | | |
| Initial BW[1] (kg) | 488 | 488 | 484 | 482 | 3.0 | 0.42 |
| Mid BW[2] (kg) | 524 | 532 | 526 | 526 | 3.0 | 0.27 |
| End BW[3] (kg) | 566 | 576 | 559 | 565 | 4.4 | 0.09 |
| ADG (kg/d) | 1.24 | 1.39 | 1.21 | 1.31 | 0.071 | 0.22 |
| Feed efficiency[4] | 0.135 | 0.140 | 0.155 | 0.133 | 0.0073 | 0.13 |
| Ultrasound measurements (mm) | | | | | | |
| Lumbar fat | 2.99 | 2.95 | 2.80 | 3.02 | 0.106 | 0.47 |
| Rump fat | 3.99 | 3.95 | 4.38 | 3.62 | 0.222 | 0.12 |
| Muscle depth | 55.0 | 56.5 | 54.0 | 55.8 | 0.77 | 0.14 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; SEM = pooled SEM; ADG = average daily gain
[a-d]Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Weight on d 3 of finishing period.
[2] Weight on d 31 of finishing period.
[3] Weight on d 66 of finishing period.
[4] kg of ADG/kg of DM intake between d 3 and 66.

was analysed using the Beckman Coulter AU480 Clinical Analyser (Beckman Coulter, IN, US) and an Enzytec D-/L- Lactic Acid commercial kit (Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany). Lactic acid was reported as g/L.

*Plasma calcium, haematology and organ pathology*

Blood samples were harvested 2–4 h post-morning feed via jugular venepuncture into 6 ml green top lithium-heparin tubes (BD Vacutainer™ Heparin Plasma Tubes, Franklin Lakes, NJ, USA) and 8.5 ml yellow top SST II Advance tubes (BD Vacutainer™ SSTTM II Advance Tubes, Franklin Lakes, NJ, USA). Green top tubes were centrifuged for 15 min (1 600 g; 4 °C), and plasma was transferred to a clean test tube and stored at -20 °C for haptoglobin and total calcium concentration analysis. Yellow top tubes were centrifuged for 10 min (1 600 g; 21 °C), and serum was poured-off into a clean test tube and stored at -20 °C for subsequent analysis of liver enzymes.

Total calcium concentration was analysed using the *o*-cresolphtalein complexone method (GL102C, Glenbio Ltd, Co. Antrim, UK) (Hartmann and Lewis, 1984), with calcium concentrations reported as mmol calcium/L. Haptoglobin was analysed using the Tridelta PHASE Haptoglobin Assay (TP-801, Tridelta Development Ltd., Co. Kildare, Ireland) and was reported as mg haptoglobin/ml. Liver enzymes, gamma-glutamyl transferase and glutamate dehydrogenase, were analysed from blood serum using a gamma-glutamyl transferase assay (GL705GT, Glenbio Ltd, Co. Antrim, UK) and a glutamate dehydrogenase assay (GL441, Randox Laboratories Ltd., Co. Antrim, UK). Both gamma-glutamyl transferase and glutamate dehydrogenase were reported as units/litre. All blood analyses were conducted using the Beckman Coulter AU480 Clinical Analyser (Beckman Coulter, IN, US) (O'Shaughnessy et al., 2015).

A sub-sample of nine animals per treatment were sent to a local commercial abattoir (Moyvalley Meats, Broadford, Co. Kildare, Ireland) at the end of the finishing period. Visual inspection of the rumen wall and internal organs (kidney, liver, heart) was conducted by the veterinary inspector on duty, using a scoring system ranging from 1 to 5; 1 being badly damaged (abscessation, pathological damage, ulceration) and 5 being healthy.

*Oxidation-reduction potential*

On d 44 of the finishing period, four animals from each treatment group (LO, HI, HP and CON) were selected from the same blocks and administered a novel ORP measuring bolus (being developed by Moonsyst International Ltd.; Kinsale, Co. Cork, Ireland). The ORP measuring boluses were connected to a cloud-based gateway via cellular wifi, and real−time ORP readings were recorded and transmitted to the gateway every 10 min, ORP was expressed in mV. The ORP measuring bolus is a modification of the Moonsyst Smart Rumen Bolus (Han et al., 2022); it has not been validated in peer-reviewed literature previously. Therefore, the ORP measurements obtained are being used to convey a trend as opposed to reporting absolute values.

*Digestibility*

At the end of the 77 d finishing period, four representative animals (n = 16) (Angus X Holstein-Friesian, with an average BW of 568 ± 4.78 kg) per treatment were enrolled to a digestibility study. Animals were housed in a metabolism facility for a 10 d experimental period to measure total tract, organic matter, NDF and calcium digestibility. The animals were offered the same four experimental diets as the finishing period (CON, LO, HI, HP). Silage was offered on an *ad libitum* basis (110% of the previous day's DM intake). Fresh forage and concentrate were offered daily with orts removed and weighed. Silage DM was measured each day to correct for variation in DM and keep the 60:40 forage:concentrate ratio consistent. Concentrate DM was measured on d 2 of the 10 d period.

The 10 d digestibility measurement experiment was conducted as follows: 2 d environmental acclimatisation, followed by 8 d

sampling period. Between d 3 and 10, total faecal collection was conducted every 24 h. Faeces was collected in a pan placed at the rear of the crate. Total faecal production was weighed each morning and homogenised, and a 5% representative sample was dried at 55 °C until it reached a constant weight (~ 60 h). Samples were turned daily in the oven to prevent the build-up of moisture and growth of mould and used for DM determination and subsequently pooled per animal for calcium, ash and NDF analyses.

*Chemical analysis of feed and faecal samples*

Silage samples were collected three times per week, and concentrates were collected weekly, samples were dried in a forced air oven at 55 °C for 48 h for DM determination using the following formula $\left(\frac{dryweight}{wetweight}\right) * 100$. Dried feed and faecal samples were ground in a Foss™ CT 293 Cyclotec™ General Purpose Sample Mill fitted with a 1 mm screen for subsequent chemical analysis (Foss, Nils Foss Allé 1, DK-3400 Hilleroed, Denmark). Starch, ADF and NDF concentrations were determined as described by O'Kiely (2011). Briefly, the determination of ADF and NDF was conducted using the ANKOM220 Fibre Analyzer (ANKOM Technology, Macedon, NY, United States), ADF and NDF were corrected for ash content and reported as g/kg DM. Enzymatic starch determination was carried out using the Megazyme total starch analysis procedure (Amyloglucosidase / α-Amylase Method). Petroleum ether was used for the extraction of ether extract using the Soxtec ST 243 (Foss, Hilleroed, Denmark). No acid hydrolysis was performed prior to extraction.

Gross energy content was determined using an adiabatic bomb calorimeter (Parr 6300 isoperibol calorimeter; Parr Instruments, IL USA). Concentration of CP (g/kg DM) was determined by obtaining the nitrogen concentration (g/kg DM) of the feed samples using a LECO FP 528 instrument (Leco Instruments UK ltd., Stockport, UK) and then multiplying this figure by a nitrogen-protein conversion factor of 6.25 (Mariotti et al., 2008). Ether extract (Oil A) was measured as determined by Lenehan et al. (2017). Ash concentration (g/kg DM) was determined by complete combustion in a muffle furnace (Nabertherm, GmbH, Lilienthal, Germany) at 550 °C for 5 h. Proximate analysis of each dietary component is reported in Table 1. Pooled faecal samples were sent to Analab (Box 208, 18246 Waller Road, Fulton, Illinois, Unites States) for determination of calcium concentration. Briefly, calcium was analysed using inductively coupled plasma-optical emission spectrometry using a wet digest procedure (Method 985.01) (AOAC, 2000), and reported as % calcium/kg DM.

*Statistical analysis*

Data were checked for normality and homogeneity of variance using qqplots, histograms and formal statistical tests as part of the UNIVARIATE procedure of SAS (Version 9.4; SAS Institute Inc., Cary, NC). A mixed model (MIXED procedure of SAS) ANOVA was used to analyse animal production, feed efficiency and gaseous emissions. The statistical model used included dietary treatment (CON, LO, HI, HP) and pen as fixed effects. Pre-experimental $CH_4$ production was included in the model as a linear covariate for $CH_4$ traits; however, this was statistically non-significant and was subsequently removed from the final model. A random block effect was included in the final model for all traits. For variables with multiple observations per subject (muscle and fat depth, rumen fermentation parameters and animal health indicators), a repeated measures ANOVA (Proc MIXED) was used, with dietary treatment, time, treatment*time interaction and pen included as fixed effects in the statistical model. Block was included in the model as a random effect. Non-statistically significant ($P > 0.10$) interactions were subsequently excluded from the final model. Diurnal pattern (24 h) of $CH_4$, $H_2$ and ORP was profiled using a repeated measures ANOVA (Proc MIXED) with dietary treatment, time of day (h), treatment*time of day interaction and pen included as fixed effects in the statistical model. For all repeated measures analysis, the type of variance–covariance structure used was chosen depending on the magnitude of the Akaike information criterion for models run under compound symmetry, unstructured, autoregressive, heterogeneous, 1st order autoregressive, or Toeplitz variance–covariance structures. The model with the lowest Akaike information criterion co-efficient was selected. The digestibility experiment was analysed using a MIXED model ANOVA, fixed effects in the model included dietary treatment, day and treatment*day interaction. Differences in treatment group were determined by F-tests using type III sums of squares. The Tukey-Kramer adjustment factor was applied to evaluate pairwise comparisons between treatment means. For all analyses, $P < 0.05$ was considered significant and $P < 0.1$ was a tendency towards statistical significance.

## Results

*Animal intake, production and efficiency variables*

A breakdown of DM intake, animal BW, ADG, feed efficiency and ultrasound measurements are reported in Table 2. Total DM intake ranged from 8.43 to 9.57 kg/d. There was a reduction in intake in the group consuming the HI treatment ($P < 0.01$) in the order of 1 kg/d compared to other treatments. Silage intake was also lower for HI compared to CON, LO and HP, and the quantity of experimental concentrate offered throughout the experiment was highest for PE, followed by HI, LO and CON, respectively. Actual inclusion of $CaO_2$ in the diet equated to 0, 1.35, 2.69 and 2.42% of DM intake for CON, LO, HI and HP, respectively. There were no treatment*time interactions detected for any production or efficiency variables ($P > 0.10$). Animal BW at the beginning ($P = 0.42$) and end of the experiment ($P = 0.09$) and ADG ($P = 0.22$) did not differ between treatment groups, ADG ranged from 1.21 to 1.39 kg/d. There was no difference between dietary treatments for either muscle depth or fat deposition over the duration of the experiment ($P > 0.10$).

*Methane*

The control treatment group produced on average 240 g $CH_4$/d, whereas, $CaO_2$ supplementation resulted in the production of 170–202 g $CH_4$/d (Table 3), resulting in reductions of 16, 29 and 27% for LO, HI and HP compared to CON ($P < 0.0001$). Across the supplemented groups, there was no difference between HI and HP ($P = 0.99$), producing 170 and 175 g $CH_4$/d, respectively. When expressed per kg of DM intake, animals supplemented with $CaO_2$ produced 20–27% less $CH_4$ compared to the control ($P < 0.0001$), ranging from 26.1 g/kg DM intake for the control to 19.0–20.7 g/kg DM intake for the supplemented animals. With regard to $CH_4$ intensity (g $CH_4$/kg ADG), $CaO_2$ reduced emissions by 22–32% ($P < 0.0001$), ranging from 192 g/kg ADG for the control group and 130–150 g/kg ADG for the treatment groups. Hourly production of $CH_4$ is presented in Fig. 1. A significant treatment*time of day interaction was observed for $CH_4$ ($P < 0.0001$), $H_2$ ($P < 0.0001$) and ORP ($P = 0.003$). Across all universally accepted

**Table 3**
Efficacy of $CaO_2$ supplementation at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on gaseous emissions.

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| Item | CON | LO | HI | HP | SEM | *P*-value |
| GreenFeed visits/d[1] | 3.93 | 4.18 | 4.15 | 3.99 | 0.103 | 0.24 |
| DM intake[1] | 9.27[a] | 9.73[a] | 8.23[b] | 9.17[a] | 0.206 | < 0.0001 |
| Methane | | | | | | |
| g/d | 239.9[a] | 201.7[b] | 169.6[c] | 174.8[c] | 4.46 | < 0.0001 |
| g/kg DM intake | 26.08[a] | 20.70[b] | 20.84[b] | 18.99[b] | 0.583 | < 0.0001 |
| g/kg BW | 0.467[a] | 0.383[b] | 0.332[c] | 0.336[c] | 0.0062 | < 0.0001 |
| g/kg ADG | 192.0[a] | 149.7[b] | 129.6[b] | 146.3[b] | 8.33 | < 0.0001 |
| Hydrogen (g/d) | 0.590[a] | 0.380[b] | 0.382[b] | 0.404[b] | 0.0176 | < 0.0001 |
| Carbon dioxide (g/d) | 8 232[a] | 7 896[ab] | 7 309[c] | 7 664[bc] | 147.4 | < 0.01 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; SEM = pooled SEM; ADG = average daily gain.
[a-c]Means within a row with different superscripts differ significantly ($P < 0.05$).
[1] Calculated from animals (n = 58) used for emissions data.

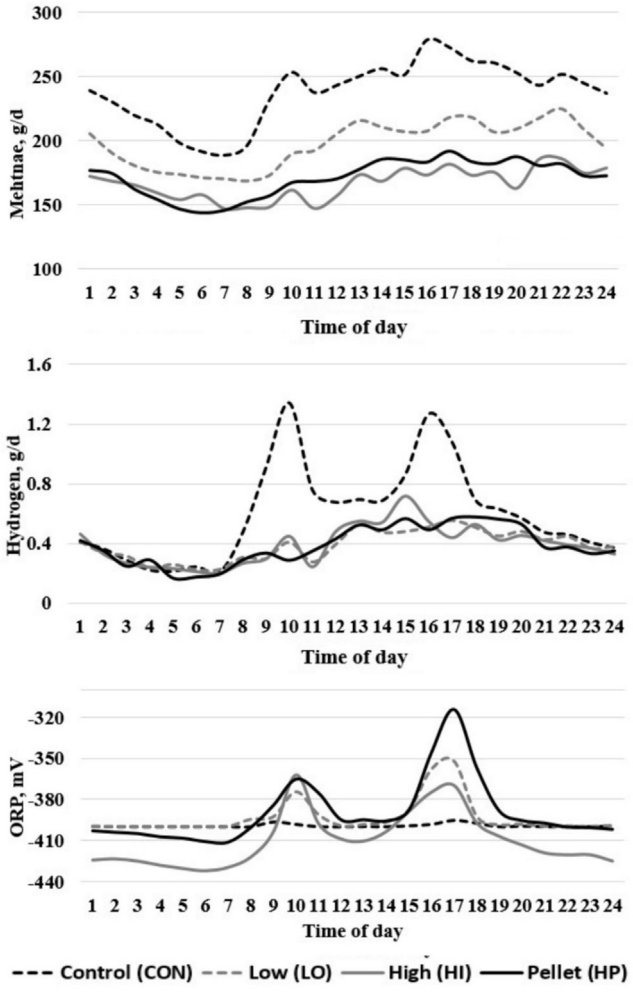


**Fig. 1.** Diurnal pattern of daily methane (g/d), hydrogen (g/d) and oxidative reduction potential (mV) of finishing beef bulls offered calcium peroxide twice daily mixed into concentrate feed. Respective dietary treatments were offered at 0800 h and 1500 h. Least squares means of hourly methane g (pooled SE = 14.2; $P < 0.0001$), hourly hydrogen g (pooled SE = 0.05; $P < 0.0001$) and hourly oxidative reduction potential mV (pooled SE = 9.55; $P < 0.0001$) for all treatment groups (control, low, high and high pellet). Abbreviation: ORP = oxidative reduction potential. Created in BioRender. Roskam, E. (2024) BioRender.com/i44e691.

metrics for $CH_4$ production, the HI and HP treatments did not differ significantly from each other; $CH_4$ g/d (170 and 175) ($P = 0.99$), $CH_4$ g/kg DM intake (20.8 and 19.0) ($P = 0.11$), $CH_4$ g/kg BW (0.332 and 0.336) ($P = 0.96$) and $CH_4$ g/kg ADG (130 and 146) ($P = 0.53$).

*Hydrogen and carbon dioxide*

Mean values for $H_2$ and carbon dioxide production over the 77 d finishing period are presented in Table 3. Supplementation of $CaO_2$ significantly reduced $H_2$ production by 32–36% relative to the control group ($P < 0.0001$). Inclusion level and physical format of $CaO_2$ had no effect on $H_2$ production ($P = 0.74$). Mean $H_2$ production for $CaO_2$ supplemented groups was 0.380–0.404 g/d compared to 0.590 g/d for the control group. This reduction in $H_2$ is evident through the observed hourly change post-feeding, whereby $H_2$ production was 0.331 g/h for 2 h post-feeding for the control group and ± 0.06 g/h for 2 h post-feeding for the $CaO_2$ supplemented animals ($P < 0.01$). Hourly production of $H_2$ is presented in Fig. 1. Mean carbon dioxide production for the control group was 8 232 g/d. Inclusion level of $CaO_2$ affected carbon dioxide production, whereby the LO group did not differ to CON (8 323 and 7 896 g/d) ($P = 0.34$) but the HI and HP groups were lower than CON ($P < 0.05$). Delivery format of $CaO_2$ had no effect on carbon dioxide production whereby HI and HP produced 7 309 and 7 664 g/d ($P = 0.31$), respectively.

*Fermentation parameters and oxidative reduction potential*

Ruminal fermentation parameters are presented in Table 4. There were no treatment*time interactions detected for any rumen fermentation parameters ($P > 0.10$). Irrespective of inclusion rate or physical format, supplementation with $CaO_2$ increased rumen pH (7.12–7.14) compared to CON (6.89) ($P < 0.0001$). Ammonia-nitrogen concentration was consistently highest in CON followed by LO, HI and HP, which were all significantly different from CON but not different from each other ($P < 0.05$). Supplementation of $CaO_2$ had a tendency to effect lactic acid production ($P < 0.10$); however, there were no significant differences between any of the treatment groups ($P > 0.10$), with the exception of HI*HP ($P < 0.10$). The concentration of total VFA was affected by $CaO_2$ supplementation ($P < 0.01$), CON resulted in the greatest total VFA concentration (119 mmol/L), followed by HP, LO and HI (105, 99, 93 mmol/L, respectively). Inclusion of dietary $CaO_2$ had no effect on acetate proportion ($P > 0.10$). With regard to propionate, there was a treatment effect ($P < 0.0001$) whereby $CaO_2$ supplementation increased proportions compared to CON ($P < 0.05$). Within $CaO_2$ groups, LO had reduced propionate proportions compared to HI ($P < 0.05$). Supplementation of $CaO_2$ reduced butyrate proportion ($P < 0.0001$); LO, HI and HP had lower butyrate than CON ($P < 0.0001$), while, HP did not differ from HI ($P = 0.72$) but was reduced compared to LO ($P = 0.03$). An increase in ORP was observed for the $CaO_2$ containing treatments for 2 h post-feeding ($P < 0.0001$), and subsequently returned to presupplementation levels (Fig. 1). The ORP probes have not been validated *in vivo*;

**Table 4**
Efficacy of $CaO_2$ supplementation at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on rumen fermentation parameters.

| | Treatment | | | | | Day | | | *P*-value | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | CON | LO | HI | HP | SEM | D32 | D73 | SEM | Trt | Day |
| pH | 6.89[a] | 7.14[b] | 7.17[b] | 7.12[b] | 0.044 | 7.06 | 7.10 | 0.030 | < 0.0001 | 0.27 |
| $NH_3$-N (mg/L) | 98.0[a] | 81.6[b] | 78.0[b] | 70.9[b] | 4.99 | 96.4 | 67.8 | 3.40 | < 0.01 | < 0.0001 |
| Lactic acid (g/L) | 0.185 | 0.160 | 0.221 | 0.158 | 0.0176 | 0.190 | 0.172 | 0.0121 | 0.05 | 0.30 |
| Total VFA (mmol) | 118.9[a] | 99.2[b] | 93.3[b] | 105.3[ab] | 4.88 | 115.6 | 92.7 | 3.34 | < 0.01 | < 0.0001 |
| Acetate (%) | 76.0 | 74.9 | 72.4 | 74.6 | 0.98 | 76.8 | 72.4 | 0.67 | 0.19 | < 0.0001 |
| Propionate (%) | 12.94[a] | 15.71[b] | 19.13[c] | 16.88[bc] | 0.672 | 15.68 | 16.75 | 0.461 | < 0.0001 | 0.08 |
| Butyrate (%) | 10.99[a] | 8.58[b] | 7.70[bc] | 7.21[c] | 0.330 | 7.47 | 9.80 | 0.226 | < 0.0001 | < 0.0001 |
| Valerate (%) | 1.00 | 1.08 | 1.14 | 1.21 | 0.059 | 1.23 | 1.00 | 0.041 | 0.08 | < 0.0001 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; SEM = pooled SEM; Trt = treatment; $NH_3$-N = ammonia–nitrogen; VFA = volatile fatty acid.
[a-c]Means within a row with different superscripts differ significantly ($P < 0.05$).

**Table 5**
Efficacy of $CaO_2$ supplementation at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on animal performance and diet digestibility in metabolism crates.

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| Item | CON | LO | HI | HP | SEM | *P*-value |
| Feed intake (kg/d) | | | | | | |
| DM intake | 8.36 | 7.95 | 7.90 | 7.96 | 0.370 | 0.80 |
| Silage | 5.02 | 4.70 | 4.75 | 4.78 | 0.223 | 0.75 |
| GreenFeed | 0.973 | 0.945 | 0.880 | 0.916 | 0.029 | 0.18 |
| Concentrate | 2.37 | 2.30 | 2.28 | 2.27 | 0.141 | 0.96 |
| CP | 1.45 | 1.33 | 1.34 | 1.37 | 0.067 | 0.53 |
| NDF | 3.24 | 3.05 | 2.97 | 2.90 | 0.15 | 0.44 |
| Ether extract | 0.249 | 0.234 | 0.233 | 0.233 | 0.0113 | 0.68 |
| $CaO_2$ offered | 0.000[a] | 0.113[b] | 0.187[c] | 0.187[c] | 0.0076 | < 0.0001 |
| BW (kg) | 568 | 572 | 566 | 569 | 6.5 | 0.93 |
| Faeces (kg/d) | 1.84 | 2.13 | 2.10 | 2.16 | 0.128 | 0.31 |
| Faecal calcium (% DM) | 2.96[a] | 4.61[b] | 5.41[c] | 5.67[c] | 0.116 | < 0.0001 |
| Digestibility (%) | | | | | | |
| DM | 78.1[a] | 73.4[b] | 73.6[b] | 73.0[b] | 0.67 | < 0.01 |
| Organic matter | 80.4[a] | 77.0[b] | 77.7[b] | 77.3[b] | 0.62 | < 0.01 |
| NDF | 75.9[a] | 71.9[ab] | 72.0[ab] | 69.7[b] | 0.95 | < 0.01 |
| Calcium | 17.0[a] | 17.2[a] | 26.5[b] | 20.7[ab] | 2.66 | 0.09 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; SEM = pooled SEM.
[a-c]Means within a row with different superscripts differ significantly ($P < 0.05$).

therefore, their application in this experiment is to convey trends rather than absolute changes in ORP.

*Diet digestibility*

Feed intake and faecal output, faecal calcium output and nutrient digestibility (DM, organic matter, NDF, calcium) from the digestibility study are reported in Table 5. Mean BW/treatment was equal for all treatments ($P = 0.93$), and there was no effect on DM intake ($P = 0.80$), silage intake, concentrate intake or faecal output ($P = 0.31$) between treatments. Quantity of $CaO_2$ offered was 0, 113, 187 and 187 g/d for CON, LO, HI and HP, respectively ($P < 0.0001$); this equated to 0, 1.42, 2.36 and 2.34% of DM intake, respectively ($P < 0.0001$). Dietary supplementation with $CaO_2$ significantly increased faecal calcium concentration ($P < 0.0001$) in a dose–dependent manner. Within $CaO_2$ supplemented groups, faeces produced by the LO group contained 56% greater calcium than CON ($P < 0.0001$). Additionally, the HI and HP supplemented groups contained 83 and 92% more calcium in faeces than CON ($P < 0.0001$). Delivery format did not have an effect, whereby faecal calcium concentration of HI and HP did not differ ($P = 0.40$). Supplementation of $CaO_2$ had a negative impact on diet digestibility. Dry matter digestibility was consistent for all $CaO_2$ supplemented groups (73–74% DM digestibility) ($P > 0.90$), but was reduced relative to CON (78% DM digestibility) ($P < 0.01$). Digestibility of organic matter followed the same trend, diets supplemented with $CaO_2$ had a reduced organic matter digestibility compared to CON ($P < 0.01$). Digestbility of NDF was also reduced ($P < 0.01$), CON had the highest NDF digestibility of 75.9%, followed by 71.9, 72.0 and 69.7% for LO, HI and HP, respectively. Regarding calcium digestibility, LO ($P = 0.97$) and HP ($P = 0.35$) did not differ significantly from CON; however, HI was increased relative to CON ($P = 0.03$) but did not differ from HP ($P = 0.15$).

*Animal health indicators*

All blood parameters assessed (glutamate dehydrogenase, gamma-glutamyl transferase, haptoglobin, calcium) were within the normal acceptable range for the bovine species (Table 6). Supplementation of $CaO_2$ had no effect on glutamate dehydrogenase ($P = 0.55$) or gamma-glutamyl transferase ($P = 0.25$) compared to CON. Similarly, haptoglobin levels were not affected by $CaO_2$ supplementation ($P = 0.68$). Total blood calcium concentration was also unaffected by $CaO_2$ concentration in the diet ($P = 0.28$). All animals scored 5 for organ health at slaughter during preliminary organ observation.

## Discussion

The supplementation of $CaO_2$ has previously shown antimethanogenic potential *in vitro* (Demeyer, 1982; Graham et al., 2024b). Other peroxide-based compounds such as magnesium per-

**Table 6**
Efficacy of $CaO_2$ supplementation at differing inclusion levels (low vs high) and physical formats (coarse ration vs pellet) in an intensive beef finishing diet on haematology and serum calcium.

| | Treatment | | | | | Day | | | *P*-value | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | CON | LO | HI | HP | SEM | D32 | D73 | SEM | Trt | Day |
| GLDH (U/L) | 17.0 | 17.9 | 15.0 | 14.6 | 1.84 | 17.0 | 15.3 | 1.26 | 0.55 | 0.33 |
| GGT (U/L) | 17.9 | 16.6 | 16.6 | 17.3 | 0.58 | 17.9 | 16.3 | 0.40 | 0.25 | < 0.01 |
| Haptoglobin (mg/ml) | 1.77 | 1.63 | 1.77 | 1.74 | 0.093 | 1.73 | 1.72 | 0.064 | 0.69 | 0.96 |
| Blood calcium (mmol/L) | 2.39 | 2.39 | 2.28 | 2.37 | 0.044 | 2.36 | 2.35 | 0.030 | 0.28 | 0.82 |

Abbreviations: CON = control; LO = low; HI = high; HP = high pellet; SEM = pooled SEM; Trt = treatment; GLDH = glutamate dehydrogenase; GGT = gamma-glutamyl transferase.

oxide and urea hydrogen peroxide have been assessed in the RUSITEC system (O'Donnell et al., 2024), resulting in less potent reductions in $CH_4$ than $CaO_2$.

*Animal production and efficiency*

In order to be readily adoptable at farm level, an effective dietary supplementation strategy must have no negative effects on animal performance. In the current study, supplementation of $CaO_2$ had no impact on animal performance, bulls consistently achieved a targeted daily growth rate ranging from 1.2 to 1.4 kg per day. A reduction in DM, organic matter and NDF digestibility was reported for $CaO_2$ supplemented animals in the digestibility study. Findings from the production study reported a reduction in total VFA and carbon dioxide production, which could be explained due to a reduction in rumen fermentation associated with the observed reduction in digestibility. However, future work should analyse rumen fermentation parameters in conjunction with digestibility to confirm these findings. The reduction in digestibility is in accordance with Graham et al. (2024b) and was plausibly due to the introduction of oxygen negatively impacting anaerobic microbes, including fibre digesting bacteria; therefore, refinement of the release rate of oxygen is necessary going forward.

Despite the 10% reduction in DM intake noted for the HI treatment, animals were still consuming 1.6% of BW which is consistent with animals of similar phenotype reported by Keane and Moloney (2010). Additionally, no reduction in animal performance was reported for the HI treatment group, similar findings have been reported by Roque et al. (2021) who observed a 14% reduction in DM intake when supplementing a high dose of *Asparagopsis* species, with no negative effects on ADG. The aforementioned study does not report rumen fermentation or microbiome data, but suggests that the lack of effect on ADG is due to improved feed efficiency. In the current study, feed efficiency for HI was only numerically improved compared CON. However, the authors hypothesise that the discrepancy between DM intake and ADG is attributable to the reported increase in propionate production, and potentially increased microbial biomass, providing additional energy to the animal. To reach the target inclusion rate of 2.25% $CaO_2$ in the overall diet, there was 7.25% $CaO_2$ in the concentrate portion of the diet. However, switching to a pelleted format while retaining the same dietary $CaO_2$ level resulted in no reduction in DM intake compared to CON. Therefore, the 10% reduction in DM intake for HI, may be attributable to palatability. Although no concentrate refusals were present, it is possible that small quantities of $CaO_2$ powder precipitated through the silage, rendering it less palatable. Future studies should analyse silage refusals for $CaO_2$ content. Our inference is that we may have reached the upper $CaO_2$ inclusion limit within a coarse ration. This highlights the intricate relationship between dietary composition, format, and animal preferences. Notably, the pelleted format (HP) effectively reduced $CH_4$ production, indicating that $CaO_2$ can successfully withstand the high temperature and pressure associated with the pelleting process, which would aid in ease of delivery and application at farm level.

As mentioned above, a 6–7% reduction in DM digestibility, a 4% reduction in organic matter digestibility and a 5–8% reduction in NDF digestibility were observed for $CaO_2$ supplemented animals compared to the control. However, even the lowest reported digestibility figures are consistent with a highly digestible 60:40 forage:concentrate diet as reported by Brask-Pedersen et al. (2022). The reduction in DM intake reported in the production study for HI coupled with a reduction in digestibility could plausibly have reduced animal performance, which was not the case in the current study. Future studies with increased animal numbers per treatment may detect statistical differences.

It has been reported that oxygen is released as $CaO_2$ decomposes (Lu et al., 2017), it was likely that the compound would have a suppressing effect on the anaerobic microbial ecosystem, including fibre–degrading bacteria. Graham et al. (2024b) reported reductions in forage digestibility but no effect on concentrate digestibility when supplementing $CaO_2$ in a RUSITEC study. It is conceivable that in a more fibrous diet with a higher degree of acetic-based fermentation, greater concerns around digestibility may arise with elevated oxygen levels in the rumen, which warrants further investigation. Varying degrees of protozoa reduction have been reported with $CaO_2$ supplementation *in vivo* (Demeyer, 1982); however, no characterisation of protozoa or analysis of other kingdoms was conducted in the study. Therefore, it remains challenging to quantify the extent of the influence of the supplementary oxygen on the rumen microbiome. Further investigations are essential to gain a thorough understanding of how the elevated oxygen levels affect the ruminal microbial ecosystem, including microbial fermentation.

*Methane emissions*

This study builds upon early findings from Demeyer (1982) and subsequently, Graham et al. (2024b) who reported $CH_4$ reductions of > 50% *in vitro* in batch incubations and the RUSITEC system, respectively. Subsequently, Graham et al. (2024a) assessed the *in vivo* effectiveness of $CaO_2$, delving into various inclusion rates. By using ORP profiles as a proxy for $CH_4$ emissions, the study revealed that $CaO_2$ supplemented at 1.35 and 2.25% of DM intake would have significant anti-methanogenic effects.

The current experiment began with a consistent $CH_4$ production performance within each dietary treatment. During the pre-experimental period, CON, LO, HI and HP produced 229, 230, 222 and 228 g $CH_4$/d, respectively. As $CaO_2$ supplementation commenced, a distinct shift in the $CH_4$ emissions profile became evident after d 1 of supplementation, clearly setting LO, HI and HP apart from CON. Notably, during the finishing period, CON exhibited an average daily $CH_4$ production of 240 g, equivalent to 26 g of $CH_4$ per kg DM intake. This aligns closely with the $CH_4$ emissions observed in beef bulls of comparable weight and age, consuming a

total DM intake of 9 kg with a similar basal diet, as reported by Foley et al. (2009).

The supplementation of dairy-beef bulls with $CaO_2$ consistently reduced $CH_4$ from a low to high inclusion rate in the order of 16–32% across all universally accepted $CH_4$ measuring metrics (production, g/d; yield, g/kg DM intake; intensity, g/kg ADG). Aside from this study, research assessing periodic supplementation of anti-methanogenic dietary compounds is becoming more prevalent. Supplementing dairy cows with 3-nitrooxypropanol mixed into a compound feed (similar to the HP treatment in the current study) reduced $CH_4$ g/d by 23%. However, the 3-nitrooxypropanol compound feed was offered through an automatic feeder where cows had an average of 2.4 h between visits (Van Wesemael et al., 2019). It is hypothesised that if cows had less frequent access, the reduction in $CH_4$ would be less significant, due to the transient effectiveness of 3-nitrooxypropanol. As reported by Costigan et al. (2024), twice daily supplementation of 3-nitrooxypropanol reduced $CH_4$ production by 5%. Recently, a study was conducted by Alvarez-Hess et al. (2023), offering *Asparagopsis* species−infused canola oil to dairy cows, twice a day, combined in a coarse ration (similar to LO and HI), resulting in $CH_4$ reductions of 44%. Methane was measured using $SF_6$; therefore, no diurnal pattern of $CH_4$ production was reported.

In the current study, data captured from the GreenFeed demonstrates that $CaO_2$ supplementation consistently suppressed $CH_4$ production throughout the day in a dose−dependant manner compared to CON (Fig. 1). Due to the diurnal nature of enteric emissions and the feeding regime of the dietary treatments, an accurate $CH_4$ phenotype was captured for 58 out of 72 animals in the study based on the set criteria. Data were analysed for 14–15 representative animals per treatment group. Hammond et al. (2016) recommends 12–14 animals per treatment to detect a significant difference in $CH_4$. Therefore, the authors are confident that $CH_4$ emissions have been accurately captured with a robust methodology. It can be argued that the consistently lower $CH_4$ may be partly due to the observed reduction in digestibility, however, digestibility was equally impacted by the three $CaO_2$ treatments, whereas, the effects on $CH_4$ production were dose−dependent.

*Hydrogen emissions and rumen fermentation parameters*

Methanogenesis is considered to be the largest $H_2$ sink in the rumen (Ellis et al., 2008); hence, many effective anti-methanogenic compounds assessed to-date have resulted in increased $H_2$ emissions. For example, *A. taxiformis* increased $H_2$ g/d by 318% in beef steers (Roque et al., 2021), 3-nitrooxypropanol increased $H_2$ g/d by 227% in young beef bulls (Kirwan et al., 2024) and nitrate increased $H_2$ L/d by 140% in dairy cows (Olijhoek et al., 2016). However, in the current study, $CaO_2$ supplementation reduced $H_2$ production by 32–36%, irrespective of inclusion level. Olijhoek et al. (2016) observed that $H_2$ production followed DM intake, increasing 1 h after feeding and declining after 3 h, which is comparable to $H_2$ production from CON in the current study (Fig. 1). However, $H_2$ production for $CaO_2$ supplemented animals did not spike after feeding, and it remained consistent throughout the day.

The reduction in $CH_4$ coupled with a reduction in $H_2$ indicates that there are alternative $H_2$ sinks at play to counteract the reduction in hydrogenotrophic methanogenesis. Our initial hypothesis postulated that a reduction in $CH_4$ production would entail a rechannelling of energy resources towards enhanced propionate production, as was similarly observed by Graham et al. (2024b) *in vitro*. As anticipated, our findings reveal a discernible shift in VFA profiles, characterised by a substantial increase in propionate proportion which is a well-documented and widely accepted alternative $H_2$ sink to $CH_4$ production, as substantiated by a body of research, including the work of Wang et al. (2023). In depth analysis on the effects of $CaO_2$ on the rumen microbial community is necessary. Demeyer (1982) reported a reduction in total protozoa counts; however, authors theorise that for remaining protozoa, there may be a shift to less efficient $H_2$ producers, which would further support the reduction in $H_2$ production. Protozoa are tolerant to higher levels of oxygen than methanogens (Ellis et al., 1989). The introduction of oxygen to the rumen has previously resulted in the cessation of $H_2$ production from *Dasytricha ruminantium*, a prolific $H_2$−producing ciliated protozoan (Yarlett et al., 1983). The rate of release of oxygen and the levels reached in the rumen will determine the effects, if any, on microbial populations.

Finally, authors hypothesise that dietary supplementation with $CaO_2$ may result in an increase in microbial biomass and the growth of facultative anaerobes, hydrogen oxidising bacteria, which can oxidise oxygen to water, using $H_2$ as an electron source, therefore, mopping up oxygen and $H_2$ in the rumen. In addition, hydrogen oxidising bacteria are efficient biomass producers (Pander et al., 2020), which can increase the production of rumen microbial protein and yield more energy for the animal. This supports the finding of reduced digestibility in the $CaO_2$ supplemented treatments with no reduction in animal performance. This hypothesis will have to be explored further using rumen microbiome sequencing analysis.

*Oxidative reduction potential*

As $CaO_2$ decomposes in the rumen, it breaks down into oxygen, water, hydrogen peroxide and calcium hydroxide. The release of oxygen alters the redox potential of the rumen by elevating the ORP which would likely inhibit methanogenic activity. Ruminal redox status is an indirect measure of microbial activity in the rumen. Increased ORP is associated with a reduction in VFA production, pH and diversity of rumen bacterial communities (Huang et al., 2018), likely due to the unfavourable environment for anaerobic microorganisms. The ORP data generated from this study is in line with Huang et al. (2018), ranging from − 385 to − 430 mV, however, a challenge with orally administered boluses is that they typically settle in the reticulum, rather than the rumen (Han et al., 2022). Therefore, the extent of the ORP changes (13–27 mV over a 2 h period post-feeding) in the current study would be expected to be significantly higher if the ORP measurements were obtained directly from rumen fluid or the rumen of cannulated animals, nonetheless, the ORP profile and trends observed in the current study are evident. Aside from small amounts of oxygen being introduced through the ingestion of feed, no distinctive shift in ORP in the unsupplemented animals was observed. However, for $CaO_2$ supplemented animals, in accordance with Graham et al. (2024b), rumen ORP spiked for 2 h post-feeding. In the current study, this was coupled with a reduction in $H_2$ production and a concurrent but longer−lasting reduction in $CH_4$ (Fig. 1).

*Calcium*

Target inclusion rate of $CaO_2$ was 0, 1.35, 2.25 and 2.25% of DM intake; however, actual inclusion during the digestibility study was 0, 1.42, 2.36 and 2.34%. Calcium peroxide is hydrolysed to calcium hydroxide, which is an alkali. Therefore, the associated increase in rumen pH with $CaO_2$ was anticipated. Calcium metabolism is a highly regulated system, calcium enters the blood primarily through absorption from the intestines and excess calcium is excreted through the form of metabolic or endogenous faecal calcium (Boda and Cole, 1956). Therefore, it was hypothesised that $CaO_2$ supplementation would have no effect on blood calcium levels and excess calcium would be excreted through the faeces

in a dose−dependent manner. This was confirmed through haematological analysis, with blood calcium mmol/l reported to be 2.28–2.39 for both the supplemented and unsupplemented groups. Calcium digestibility was highest for HI (26.5%), compared to HP (20.7%), LO (17.2%) and CON (17.0). More calcium was digested in HI than HP, even though the same amount of $CaO_2$ was offered, which may be attributed to delivery format i.e. coarse ration vs pellet. Conversely, faecal calcium (% of DM) was 3.0 for CON compared to 4.6, 5.4 and 5.7 for LO, HI and HP. This translates to an increase of faecal calcium in the order of 56, 83 and 92% for LO, HI and HP, respectively, compared to CON, supporting the hypothesis that excess calcium is excreted through the faeces. Additionally, it has been stated that digestate treated with $CaO_2$ during anaerobic digestion resulted in a high calcium carbonate content, improving its potential as a soil conditioner for acidic soils (Zhang et al., 2020), therefore, faeces from animals supplemented with $CaO_2$ may also carry similar properties, yielding additional benefits from the slurry produced, further research is required to support this assumption.

*Animal health indicators*

Liver enzyme and acute phase protein (haptoglobin) concentrations were all within the normal range. An elevated level of tissue-specific serum enzymes is typically indicative of damage to bile ducts (cellular hyperplasia), renal tubular damage and liver disease, due to the release of the enzymes through damaged cells (Kessell, 2015). Haptoglobin is a sensitive indicator of inflammation which would indicate any organ damage (Kessell, 2015). All animals scored 5 for organ health at slaughter, indicating that there was no observable ulceration, pathological damage, tissue adhesion or flattening of rumen villi caused by $CaO_2$ supplementation. A more in-depth slaughter assessment with a robust scoring system and histology of the organ tissues is necessary to confirm the above findings. The authors are confident that $CaO_2$ has no negative effects on animal health; haematological analysis and the observations at the abattoir following slaughter yielded no adverse results. Additionally, animals were monitored closely throughout the experiment with no ill thrift reported. None of the aforementioned health indicators were expected to be elevated due to the mild and non-toxic nature of $CaO_2$ (Abedi and Pourmohammadi, 2020).

Across all universally accepted metrics of $CH_4$ quantification, the supplementation of $CaO_2$ from a low to a high inclusion rate reduced $CH_4$ emissions in the order of 16–28% with no effects on animal performance or health. An upper limit of 7.25% $CaO_2$ within a coarse ration was established, due to a resulting reduction in DM intake; however, this was overcome when combining 7.25% $CaO_2$ in a pelleted feed. Additionally, it was established that $CaO_2$ can successfully withstand the pelleting process without negatively impacting the efficacy of the $CH_4$ mitigating component. Supplementation of $CaO_2$ offers potential for enteric $CH_4$ mitigation. However, research needs to be extended to focus on its efficacy on differing diet types, stages of production and as an early−life supplement. Additionally, its effects on the rumen microbiome and an in-depth analysis of protein, energy and fat metabolism warrant further investigation.

## Ethics approval

The experiment was conducted under the European Directive 2010/63 EU and S.I. No. 543 of 2012 at the Teagasc Grange Animal and Bioscience Research Department, Co. Meath, Ireland. All animal procedures used in this experiment were approved by the Teagasc Animal Ethics Committee (TAEC2020-279) and the Health Products Regulatory Authority (AE19132/P136).

## Data and model availability statement

None of the data were deposited in an official repository. Data and further details regarding statistical analysis from this experiment can be obtained by contacting the corresponding author.

## Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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**E. Roskam:** Writing – original draft, Visualization, Project administration, Methodology, Investigation, Data curation, Conceptualization. **D.A. Kenny:** Resources, Project administration, Methodology, Conceptualization. **A.K. Kelly:** Writing – review & editing, Validation, Formal analysis, Data curation. **V. O'Flaherty:** Supervision, Resources, Methodology, Conceptualization. **S.M. Waters:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of interest

VOF is a shareholder in GlasPort Bio Limited. GlasPort Bio Ltd collaborated with Teagasc and the University of Galway on this study by providing information on preliminary *in vitro* studies, by supplying $CaO_2$ formulation and by providing advice on inclusion rates and, safety and toxicity data. The company formally agreed *a priori* that all data generated from the study, positive or negative, would be published on an open−access basis as a condition of a public-good funding award, which funded the study (RSF 2019R479). The other authors declare no real or perceived conflicts of interest.

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