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Novel oxidising feed additives reduce *in vitro* methane emissions using the rumen simulation technique

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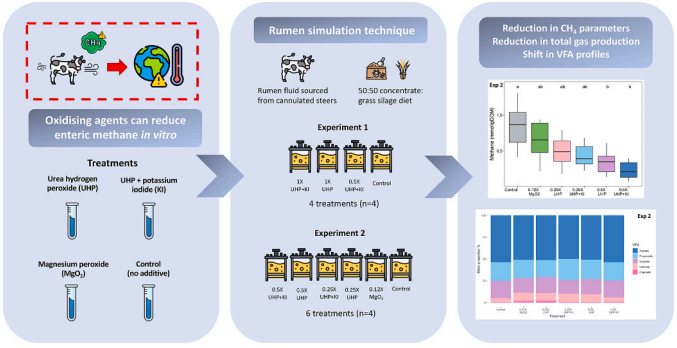
HIGHLIGHTS

- Oxygen-releasing compounds act as anti-methanogenic agents for ruminant feed additives.
- Oxidising agents reduced methane and total gas production *in vitro*.
- No negative effects on volatile fatty acids, digestibility, or pH at low concentrations.
- Identified promising, economical, methane-reducing feed additive components.

GRAPHICAL ABSTRACT



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ABSTRACT

Enteric methane (CH_4) produced by ruminant livestock is a potent greenhouse gas and represents significant energy loss for the animal. The novel application of oxidising compounds as antimethanogenic agents with future potential to be included in ruminant feeds, was assessed across two separate experiments in this study. Low concentrations of oxidising agents, namely urea hydrogen peroxide (UHP) with and without potassium iodide (KI), and magnesium peroxide (MgO_2), were investigated for their effects on CH_4 production, total gas production (TGP), volatile fatty acid (VFA) profiles, and nutrient disappearance *in vitro* using the rumen simulation technique. In both experiments, the *in vitro* diet consisted of 50:50 grass silage:concentrate on a dry matter basis. Treatment concentrations were based on the amount of oxygen delivered and expressed in terms of fold concentration. In Experiment 1, four treatments were tested (Control, 1× UHP + KI, 1× UHP, and 0.5× UHP + KI), and six treatments were assessed in Experiment 2 (Control, 0.5× UHP + KI, 0.5× UHP, 0.25× UHP + KI, 0.25× UHP, and 0.12× MgO_2). All treatments in this study had a reducing effect on CH_4 parameters. A dose-dependent reduction of TGP and CH_4 parameters was observed, where treatments delivering higher levels of oxygen resulted in greater CH_4 suppression. 1× UHP + KI reduced TGP by 28 % ($p = 0.611$), CH_4% by 64 % ($p = 0.075$)

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and CH_4 mmol/g digestible organic matter by 71 % ($p = 0.037$). 0.12× MgO_2 reduced CH_4 volume by 25 % ($p > 0.05$) without affecting any other parameters. Acetate-to-propionate ratios were reduced by treatments in both experiments ($p < 0.01$). Molar proportions of acetate and butyrate were reduced, while propionate and valerate were increased in UHP treatments. High concentrations of UHP affected the degradation of neutral detergent fibre in the forage substrate. Future *in vitro* work should investigate alternative slow-release oxygen sources aimed at prolonging CH_4 suppression.

1. Introduction

Due to population growth, global meat and milk production is predicted to increase by 76 % and 63 %, respectively, from 2005 levels by 2050 (Alexandratos and Bruinsma, 2012). This increase in ruminant production will inevitably lead to higher greenhouse gas emissions (GHG), putting additional pressure on the environment and hindering the achievement of the goals outlined in the Paris Agreement (Huws et al., 2018; UN, 2015). Enteric methane (CH_4) from feed fermentation in ruminant livestock is the largest source of GHG emissions in agriculture, accounting for 6 % of total GHG emissions globally (Beauchemin et al., 2020). As methane is 28 times more effective than carbon dioxide (CO_2) in trapping heat in the atmosphere over a 100-year time frame (Vallero, 2019), yet has a much shorter residence time in the atmosphere, reducing CH_4 emissions now is crucial to limiting the severity of climate change in the short to medium term. Reflecting the urgency of this problem, the Global Methane Pledge led by the United States and the European Union was introduced during COP26, where 111 countries committed to reducing CH_4 emissions by 30 % by 2030 (IEA, 2022).

In addition to its environmental impact, enteric CH_4 production is also an energy-intensive process, representing a loss of 2–12 % of an animal's gross energy intake (Johnson and Johnson, 1995). It has been proposed that decreasing methanogenesis in ruminants can improve their performance by redirecting metabolic hydrogen to more energy-yielding fermentation pathways (Guyader et al., 2017), leading to more efficient livestock.

Feed additives are a particularly attractive strategy for reducing emissions from ruminants as they offer a promising and relatively fast-acting solution (Honan et al., 2021). A recent meta-analysis by Martins et al. (2023) identified chemical inhibitors as the most effective mitigation strategy for *in vitro* enteric CH_4 emissions without negatively affecting fermentation patterns and nutrient digestibility. A chemical inhibitor suitable for incorporation into feed pellets or slow-release boluses that can also be cost-effective, reliable, and durable is required for both confinement and pasture-based grazing systems (Cummins et al., 2022; Waghorn and Clark, 2006).

A chemical amendment incorporating the oxidising agent urea hydrogen peroxide (UHP), combined with potassium iodide (KI), was recently shown to significantly decrease the rates of CH_4 production (−34 %) and the total GHG emissions (−90 %) from stored cattle slurries (Thorn et al., 2022). Similar results were observed in stored cattle slurries using UHP + KI and hydrogen peroxide (H_2O_2) + KI, resulting in a 39 % and 63 % reduction in CH_4 emissions, respectively (Connolly et al., 2023). The CH_4-suppressing effect was mainly attributed to the release of oxygen (O_2) and subsequent production of reactive oxygen species (ROS) upon the decomposition of oxidising agents. ROS can occur as products of the incomplete reduction of O_2, such as superoxide, H_2O_2 and hydroxyl radicals (Thorn et al., 2022; Xu and He, 2021). As methanogens are well known for their O_2 sensitivity, the release of extra O_2 in such an anaerobic environment has the potential to directly inhibit methanogens (Reeve, 1992). It is expected the extra O_2 introduced into the system may also elevate oxidation-reduction potential (ORP) outside of that which is recorded as being favourable for the growth and activity of methanogenesis (Hungate, 1960). In addition to the inhibition attributed to the added O_2 in the system, KI is hypothesised to also aid in CH_4 suppression. Iodine is known to damage microbial cells by disrupting protein and nucleic acid structure and synthesis, cell wall penetration, and oxidation of microbial contents of key rumen microbes (McKeen, 2012), thus it was hypothesised that the incorporation of this component may exert additional CH_4 suppression effects. Considering the similarities in the community composition and origin of the slurry and rumen microbiomes, it was hypothesised that oxidising agents may exert similar or perhaps stronger (considering the low exposure of rumen methanogens to O_2 when compared to those found in deposited slurries) CH_4-suppressing effects in the rumen environment. We hypothesised other oxygen-releasing agents, such as magnesium peroxide (MgO_2), may also exert similar suppressing effects on CH_4 production in the rumen environment. MgO_2 decomposes slower than UHP due to its low solubility (Waite et al., 1999) and thus has the potential as a slow-release feed additive. Anti-methanogenic feed additives with slow-releasing activity are preferred as they can extend the CH_4-mitigation effects. These reagents are also worth testing as alternative feed additives for practical reasons, namely the availability and low cost of such compounds, their environmentally benign nature, and low health risk.

To our knowledge, the aforementioned oxidising agents have not been applied to the rumen system (both *in vitro* or *in vivo*) as an anti-methanogenic solution previous to this study. Therefore, the objective of the two experiments in this study was to determine the efficacy of low concentrations of oxidising agents, namely UHP, UHP + KI and MgO_2, on ruminal CH_4 production, nutrient disappearance and fermentation patterns *in vitro* using the rumen simulation technique (RUSITEC).

2. Materials & methods

2.1. Experimental licensing

Experiments were conducted at the Teagasc Animal and Bioscience Research Department, Grange, Co. Meath, Ireland, C15 PW93, in January 2021. The experiments utilised cannulated Aberdeen Angus × Friesian steers that were kept on the premises. The animals were cared for in accordance with the Teagasc Animal Ethics Committee guidelines. All procedures were approved by the Health Product Regulatory Authority (AE19132/P113), conducted under the European Directive 2010/63/EU and S.I. No. 543 of 2012.

2.2. Experimental design and apparatus

The study consisted of two separate 21-day experiments. The microbial community was acclimatised to the treatments from day 1 to 14, and the remaining 7 days were considered the main measurement period. Four RUSITEC systems, each equipped with 8 fermentation vessels in total were employed following the experimental procedure described by (Czerkawski and Breckenridge, 1977; Roskam et al., 2022). Each experimental treatment had four biological replicates blocked by RUSITEC system. Treatments were randomly allocated to a vessel within each block and each vessel was considered an experimental unit.

RUSITEC systems housed vessels in 39 °C water baths. The vessels had a working volume of 850 mL and featured separate tubes for continuous buffer infusion, collection of fermentation gases, and effluent removal. Fermentation gases and effluent were collected separately in 3 L reusable polyethylene gas bags and 1 L Schott bottles stored in 2 °C water baths, respectively. A mechanical rod attached to the feed pot containing substrates kept the contents of the vessels under

continuous vertical agitation.

2.3. Diet and treatments

Donor animals were acclimatised to a similar diet applied in the *in vitro* experiments for a period of 3 weeks prior to each experiment, consisting of 50:50 grass silage (GS) (21 % dry matter (DM), 12 % crude protein (CP)) to concentrate (86 % DM, 16 % CP) diet on a DM basis. The basal diet applied to the RUSITEC vessels consisted of 10.00 g of GS (21 % DM, 10 % ash, 12 % CP, 49 % neutral detergent fibre (NDF); 100-μm pore size, 10 × 20 cm) dried at 55 °C for 48 h and 10.00 g of dried and milled concentrate feed (86 % DM, 9 % ash, 16 % CP, 39 % NDF) also dried at 55 °C and ground to pass through a 1 mm sieve. Both the GS and concentrates were added to the vessels in nylon bags daily.

Treatments consisted of a range of concentrations of: UHP in combination with KI (added at 3.5:1, peroxide:KI ratio as per Thorn et al. (2022)); UHP alone; and MgO_2 alone (Table 1). Concentrations were expressed as fold concentrations representing the amount of O_2 delivered (ranging from 0.12× to 1×, doubling at each increment). In Experiment 1, four treatments were tested (Control, 1× UHP + KI, 1× UHP, and 0.5× UHP + KI), and in Experiment 2, six treatments were tested (Control, 0.5× UHP + KI, 0.5× UHP, 0.25× UHP + KI, 0.25× UHP, and 0.12× MgO_2). Experiment 1 served as a proof of concept trial. A starting concentration (1×) of 1.2 mg/mL UHP was based on initial screening using specific methanogenic activity tests of rumen fluid in the presence of the dose of Thorn et al. (2022) (2.4 mg/mL) and incrementally lower doses (data not shown). Experiment 1 results informed treatment concentrations for Experiment 2, which were aimed at testing lower treatment concentrations and reducing the effects on digestibility while maintaining significant CH_4 reductions. For treatment preparations, UHP stocks of each treatment concentration, as well as MgO_2 stock, were prepared fresh each morning, while stocks of KI were prepared weekly. Due to the rapid decomposition of peroxide that occurs upon the addition of KI, for the UHP + KI treatments, the KI was added to the UHP immediately prior to dispensing the mix into the appropriate vessels. The reagents were prepared in distilled water (dH_2O) and made up to 5 mL volumes (Table 1), and delivered into each of the relevant RUSITEC vessels during substrate bag exchange each morning. For UHP + KI treatments, the KI was added to the UHP immediately prior to dispensing the mix into the appropriate vessel.

Table 1
Relative experiment, treatment name, peroxide source, treatment concentrations, amount of oxygen (O_2) delivered and amount of KI added.

Experiment	Treatment name	Peroxide source	Peroxide per vessel (mg/mL)	Amount of O_2 delivered (mg)	KI per vessel (mg)
1	Control	–	–	–	–
1	1× UHP + KI	UHP	0.675	183.7	60
1	1× UHP	UHP	0.675	183.7	–
1	0.5× UHP + KI	UHP	0.338	91.8	30
2	Control	–	–	–	–
2	0.5× UHP + KI	UHP	0.338	91.8	30
2	0.5× UHP	UHP	0.338	91.8	–
2	0.25× UHP + KI	UHP	0.169	45.9	15
2	0.25× UHP	UHP	0.169	45.9	–
2	0.12× MgO_2	MgO_2	0.203	22.1	–

UHP = urea hydrogen peroxide; KI = potassium iodide; MgO_2 = magnesium peroxide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered).

2.4. Rumen simulation technique

Four steers (from a herd of nine) were randomly selected for rumen fluid and digesta collection for each experiment. Approximately 800.00 g of digesta was removed per animal from multiple sites in the rumen and pooled. Rumen fluid was strained through a 1 mm sieve and pooled. On day 0, all vessels were inoculated with a mixture of 450 mL of rumen fluid and 350 mL of freshly prepared anaerobic buffer (McDougall, 1948). Three nylon bags containing one of each; 80.00 g of digesta (fresh weight); 10.00 g GS DM; and 10.00 g concentrates DM were placed inside the feed pot of each vessel. The addition of digesta was to inoculate vessels with microbes attached to the digesta fibre mats.

Fresh stocks of buffer were prepared daily, and vessels were continuously infused with buffer at a rate of 27.5 mL/h (dilution rate of 3.2 %/h), using a Watson-Marlow 500 series peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, United Kingdom). Each morning vessels were individually removed from the water baths and substrate bags manually removed, during which vessels were continuously flushed with CO_2 to maintain anaerobic conditions. On day one only, the nylon bag containing digesta was removed and rinsed in 25 mL of buffer, the liquid was then returned to the vessel and the bag was discarded. On day one and each day thereafter, the concentrate and forage bags were removed, rinsed in 25 mL of buffer, and the liquid returned to the vessel. The concentrate bag was replaced daily and the forage bag was returned to the vessel as forage bags were replaced every second day. Thus, at all times, the feed pot contained 3 nylon bags: a 48 h incubation forage bag; a 24 h incubation forage bag; and a 24 h incubation concentrate bag.

2.4.1. Rumen fermentation products, methane production and substrate disappearance

At the time of daily substrate bag exchange, vessel pH, volume and pH of overflow effluent, total gas production (TGP), and CH_4 percentage of TGP were recorded for each vessel. Total gas produced was measured using the dual diaphragm DC-1A dry test gas meter (Sinagawa Corp. Tokyo, Japan). The CH_4 percentage of the TGP was measured using the Guardian NG Gas Monitor (Edinburgh Instruments Ltd., Livingston, UK), which was calibrated using a 10 % CH_4 span gas. The volume of overflow effluent was measured daily. On day 17, 4 mL samples of overflow effluent were collected and acidified using 1 mL of 50 % trichloroacetic acid (TCA). Acidified effluent samples were stored at −20 °C for volatile fatty acid (VFA) and ammonia (NH_3−N) analysis. For substrate degradation, nylon feed bags containing forage and concentrates from each vessel were submerged in an ice bath and then put through a 30-min detergent-free cold rinse to detach any loosely attached microbiota. Individual bags were then dried at 55 °C in a forced air oven for 48 h and weighed (final weight), where DM disappearance was calculated as the initial undigested weight minus the final weight. The dried samples were ground (1 mm sieve) for subsequent nutrient disappearance analysis. Disappearance of organic matter (OM) was determined as the difference between the amount of the components in the substrate before incubation and the amount remaining in the residue after incubation. To determine the degradation of CP and NDF, dried samples from forage and concentrate bags were pooled, and calculated by the difference in components before and after incubation.

2.4.2. Chemical analysis for determination of fermentation products and nutrient disappearance

2.4.2.1. Volatile fatty acids and ammonia. Samples for VFA and NH_3-N analysis were thawed for 16 h at room temperature. Samples were then centrifuged for 10 min (1600*g*; 4 °C) and 100 μL was drawn from the supernatant and added to 900 μL of dH_2O for NH_3-N analysis. Ammonia was determined using the Beckman Coulter AU480 Clinical Analyzer and the Thermo Electron Infinity Ammonia Liquid Stable Reagent

kinetic method as per Owens et al. (2008). For VFA analysis, 250 μL of sample supernatant was mixed with 3.75 mL dH_2O and 1 mL of internal standard (0.5 g 3-metyl-n-valeric acid in 1 L of 0.15 M oxalic acid). This solution was then centrifuged at 260 *g* and 21 °C for 5 min and then filtered using a 0.45 μm filter into three 4 mL gas chromatograph (GC) vials so that each sample was run with three technical replicates. VFA concentrations were then measured in a Varian Saturn 2000 GC (Varian, USA) equipped with a CombiPal autosampler and a flame ionisation detector. GC operating conditions have been described by Nzeteu et al. (2018).

2.4.2.2. Nutrient disappearance. After drying, forage and concentrate from feed bags were ground using a Foss™ CT 293 Cyclotec™ General Purpose Sample Mill fitted with a 1 mm screen for subsequent chemical analysis (Foss, Nils Foss Allé1, DK-3400 Hilleroed, Denmark). Ash content (g/kg DM) was determined upon complete combustion at 550 °C for 5 h using a muffle furnace (Nabertherm, GmbH, Lilienthal, Germany). Crude protein (g/kg DM) was determined by using the nitrogen concentration (g/kg DM) of samples using a LECO FP 528 instrument (Leco Instruments UK ltd., Stockport, UK) and multiplying the nitrogen concentration by a nitrogen-protein conversion factor of 6.25 (Mariotti et al., 2008). Neutral detergent fibre concentration was quantified as per Van Soest et al. (1991) using the ANKOM220 Fibre Analyzer (ANKOM Technology, Macedon, NY, United States).

2.5. Statistical analysis

Data were analysed in the statistical program R (R Team, 2017) and plotted with ggplot2 (Wickham, 2016). Plotted data is presented as the mean of biological replicates and error bars represent the standard deviation from this mean. Where repeated measures were used to assess the effects of additives on rumen function (TGP, CH_4 production, DM, and OM) data were analysed by fitting a linear mixed effect (LME) model using the restricted maximum likelihood (REML) from the NLME package (Pinheiro et al., 2023). If model residuals were not normally distributed, response variables were transformed by raising the variable to the power of lambda, where a Box-Cox transformation analysis was used to identify appropriate lambda values using boxcox function in the MASS package (Venables and Ripley, 2002). The LME model was constructed with treatment, day and RUISITEC system as fixed effects and vessel as a random effect. The resulting model was analysed with an ANOVA and if a treatment effect was observed ($p < 0.05$) then pairwise comparisons were performed using estimated marginal means (emmeans; Lenth et al., 2023). For non-repeated measured data (VFA, NH_3-N, NDF, and CP), data were tested for normality (Shapiro Wilk) and statistically significant mean differences were tested for using parametric ANOVA (with Tukey *post hoc*) or non-parametric Kruskal Wallis (with Dunn) as appropriate. Statistically significant means were considered when $p < 0.05$, while $p < 0.1$ was considered a tendency towards statistical significance.

3. Results & discussion

3.1. Effects of oxidising agents on total gas production

In Experiment 1, the controls produced an average of 1.78 L total gas/day, while UHP treatments reduced TGP to a minimum of 0.82 L/day ($p = 0.176$; Table 2). When testing a lower range of concentrations of UHP and UHP + KI in Experiment 2, the controls produced an average of 2.08 L/d of gas (Table 3), and while all treatments reduced the volume of the total gas produced (by up to 42 % in the case of 0.5× UHP + KI), these differences were not significant ($p = 0.080$). The absence of statistical significance may be attributed to the relatively low number of biological replicates ($n = 4$) and high standard deviation, and as the numerical reductions are notable, they should not be disregarded.

Table 2
Mean total gas and methane production in Experiment 1.

	Control	1× UHP + KI	1× UHP	0.5× UHP + KI	p value
TGP, L/d	1.78 (0.85)	1.28 (0.42)	0.82 (0.47)	1.17 (0.65)	0.176
CH_4, %	8.78 (3.64)	3.16 (2.18)	7.37 (3.52)	7.08 (2.79)	0.084
CH_4, L/d	0.16 (0.10) [a]	0.04 (0.04) [b]	0.06 (0.06) [ab]	0.08 (0.04) [ab]	0.047
CH_4, mmol/d	6.19 (3.71) [a]	1.68 (1.73) [b]	2.51 (2.16) [ab]	3.01 (1.53) [ab]	0.047
CH4, mmol/g DOM	0.42 (0.26) [a]	0.12 (0.10) [b]	0.17 (0.14) [ab]	0.21 (0.12) [ab]	0.041
TGP, L/g DOM	0.12 (0.06)	0.10 (0.02)	0.06 (0.03)	0.08 (0.04)	0.101

UHP = urea hydrogen peroxide; KI = potassium iodide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). [a,b,c]: Treatment means without a common superscript differ ($p < 0.05$). TGP, total gas production; CH_4%, percentage CH_4 of total TGP; CH_4 volume; DOM, digestible organic matter. The standard deviation of the mean is presented in parentheses.

Higher doses of UHP treatments resulted in greater TGP suppression, most likely as a consequence of greater O_2 release from the higher concentrations of UHP. In Experiment 1, the addition of KI appeared to reduce the effect of 1× UHP on TGP, leading to a lesser reduction than 1× UHP alone. However the variance between replicates was notable, hence this difference was not statistically significant. In Experiment 2, no discernible differences in TGP with or without KI addition between identical proportions of UHP were observed.

Among the treatments tested in Experiment 2, 0.12× MgO_2 resulted in the same degree of reduction as 0.25× UHP with a 17 % reduction relative to the control. This is a notable impact considering 0.12× MgO_2 treatment contained less than half the amount of deliverable O_2 than 0.25× UHP (Table 1) and that MgO_2 is slower-releasing than UHP by nature (Waite et al., 1999).

Four moles of hydrogen (H_2) are required to produce 1 mol of CH_4, therefore if excess H_2 was being liberated without a H_2 sink, TGP may increase or at the very least, not decrease under such circumstances. An *in vitro* study by Liu et al. (2022) observed that fumarate decreased CH_4 and increased TGP, while Schilde et al. (2021) reported a significant rise in H_2 with 3-NOP, with TGP remaining unaffected. As TGP was decreased with all treatments across both experiments, it is plausible that the extra free H_2 resulting from halting methanogenesis is being contained and utilised within the system. The use of additives such as 3-NOP generally leads to an accumulation of H_2 (Melgar et al., 2021; Romero-Pérez et al., 2015, 2017; Van Gastelen et al., 2020), and in the live animal, some of this excess H_2 can be expelled through eructation, resulting in a net loss of energy that escapes to the atmosphere. As CH_4 production is the primary H_2 sink in the rumen, inhibition of this process may allow more beneficial alternative H_2 sinks to utilise the available energy source (Beauchemin et al., 2008), such as chain elongation of fatty acids (Baleeiro et al., 2021) or an increase in microbial protein (Guyader et al., 2017). Indeed (Martinez-Fernandez et al., 2017) demonstrated how addition of phloroglucinol as a precursor to acetate, could promote capture of excess H_2 resulting from a CH_4 suppressing additive.

In terms of effects upon the cumulative TGP in Experiment 2 (Fig. 1), the three low-dose treatments (0.25× UHP + KI, 0.25× UHP, and 0.12× MgO_2) had a similar effect, showing a small but consistent reduction ($p = 0.080$). The two higher treatments (0.5× UHP + KI and 0.5× UHP) were more effective, particularly 0.5× UHP + KI ($p = 0.09$), but they were again indistinguishable as a function of the presence or absence of KI (Fig. 1).

Table 3
Mean total gas and methane production in Experiment 2.

	Control	0.5× UHP + KI	0.5× UHP	0.25× UHP + KI	0.25× UHP	0.12× MgO2	p value
TGP, L/d	2.08 (0.56)	1.20 (0.46)	1.25 (0.48)	1.79 (0.42)	1.72 (0.34)	1.73 (0.65)	0.080
CH_4, %	13.20 (1.87) [a]	6.17 (1.21) [b]	8.37 (1.32) [bc]	8.05 (2.08) [bc]	8.95 (3.13) [bc]	12.08 (1.50) [ac]	0.001
CH_4, L/d	0.28 (0.10) [a]	0.07 (0.03) [b]	0.10 (0.04) [bc]	0.15 (0.06) [ab]	0.15 (0.06) [ab]	0.21 (0.08) [ac]	0.002
CH_4, mmol/d	10.90 (3.78) [a]	2.88 (1.32) [b]	4.02 (1.56) [bc]	5.67 (2.13) [ab]	5.98 (2.21) [ab]	8.15 (3.23) [ac]	0.002
CH_4, mmol/g DOM	0.85 (0.26) [a]	0.23 (0.12) [b]	0.34 (0.14) [b]	0.43 (0.14) [ab]	0.49 (0.17) [ab]	0.65 (0.24) [ab]	0.007
TGP, L/g DOM	0.16 (0.04)	0.09 (0.04)	0.10 (0.04)	0.14 (0.02)	0.14 (0.02)	0.14 (0.05)	0.094

UHP = urea hydrogen peroxide; KI = potassium iodide; MgO_2 = magnesium peroxide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). [a,b,c]: Treatment means without a common superscript differ ($p < 0.05$). TGP, total gas production; CH_4%, percentage CH_4 within TGP; CH_4 volume; DOM, digestible organic matter. The standard deviation of the mean is presented in parentheses.

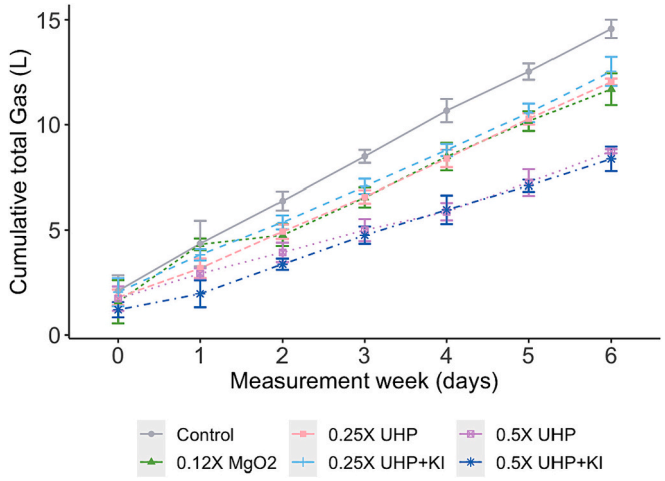

Fig. 1. Cumulative total gas (L/day).

3.2. Effects of oxidising agents on methane production

All treatments in this study reduced CH_4 (CH_4%, CH_4 mmol/day, and CH_4 L/day) in a dose-dependent manner, where higher concentrations of treatments resulted in greater CH_4 suppression, predominantly within the same category of treatment (*i.e.* UHP + KI, UHP alone, MgO_2) and experiment (*i.e.* Experiment 1 and Experiment 2). In Experiment 1, the average CH_4 content of TGP from controls was 8.78 %, and all UHP-based treatments reduced this content, with 1× UHP + KI lowering CH_4 content to 3.16 % ($p = 0.075$), equating to a 64 % reduction. Large standard deviations in CH_4 content meant this numerically large reduction was not statistically significant. Absolute CH_4 emissions (CH_4 L/d and CH_4 mmol/d) further reveal the mitigation effects of the additives tested, as evident by 1× UHP + KI treatment lowering CH_4 volume (L/d) by 75 % ($p = 0.043$), CH_4 mmol/day by 73 % ($p = 0.043$), and CH_4 mmol/g DOM by 71 % ($p = 0.037$; Table 2).

In Experiment 2, the average CH_4 content of TGP from controls was numerically higher (13.20 ± 1.87 %) than from controls in Experiment 1 (8.76 ± 3.64 %). This difference is primarily a consequence of the larger variation between replicate vessels in Experiment 1, in addition to the variable nature of these *in vitro* systems compared to *in vivo* systems (Hristov et al., 2012) as well as the strong effects of host animal on microbial community structure (Li et al., 2009) where 4 of 9 animals were randomly selected for each experiment. In Experiment 2, all UHP-based treatments significantly reduced CH_4% relative to the control ($p < 0.01$; Table 3). In terms of absolute CH_4 emissions, both 0.5× UHP ± KI treatments reduced CH_4 in terms of volume (L/d), CH_4 mmol/d, and CH4 mmol/g DOM ($p < 0.01$), while 0.25× doses had a lesser but still notable effect ($p < 0.1$) on the same CH_4 parameters. Similar to Experiment 1, a clear dose-dependent response was observed, with CH_4 suppression increasing synchronously with increasing treatment concentration. This dose-dependent effect is highlighted when considering the average CH_4 production per gram DOM over the entire measurement week (Fig. 3), with 0.5× UHP treatments providing statistically significant reductions compared to the controls. In Experiment 2, the average CH_4 reduction in terms of mmol/g DOM across 0.5× UHP treatments was 66 %, a reduction of 46 % across 0.25× UHP treatments, and 24 % in the 0.12× MgO_2 treatment.

The strong relationship between the amount of O_2 delivered and the relative CH_4 reductions suggests the decomposition of peroxides and the subsequent release of O_2 into the rumen environment is the main driver for CH_4 inhibition. Methanogens are known for their O_2 sensitivity (Reeve, 1992), therefore, upon the decomposition of peroxides to water and O_2, there is the potential direct inhibition from the extra O_2 in the environment. Considering that O_2 is the most energetically favourable electron acceptor, the added O_2 may also act as such, oxidising the rumen environment and redirecting electrons away from methanogenesis. Although not measured in this study, this would elevate the oxidation-reduction potential (ORP) within the rumen environment, potentially shifting the redox level outside of that which is recorded as being favourable for the growth and activity of methanogenesis (approx.. −300 mV; Hungate, 1960). An important aspect of utilising electron flow as a tool to manipulate methanogenesis is identifying key routes of electron flow and the key microbes involved (Leahy et al., 2022) and future work should consider this. Thorn et al. (2022) attributed much of the antimethanogenic effects of the UHP + KI chemical amendment in cattle slurries to the production of ROS. The decomposition of H_2O_2 released from UHP may produce ROS and oxidative free radicals (Ofoedu et al., 2021; Xu and He, 2021), which may contribute to the suppression of methanogenesis. Similarly, the incomplete reduction of O_2 can lead to the generation of cell-damaging products such as superoxide and hydroxyl radicals (Xu and He, 2021), which may play a general inhibitory role.

3.3. Potassium iodide aids in methane suppression

Potassium iodide has been included as a component in recently investigated slurry additives (Connolly et al., 2023; Nolan et al., 2024; Thorn et al., 2022) and was included in this study to determine whether it could enhance CH_4 suppression in an *in vitro* rumen model. Potassium iodide had varying effects on TGP between treatments in Experiment 1 and Experiment 2. In Experiment 1, 1× UHP without KI reduced TGP by 48 % >1× UHP with KI. Whereas no discernible difference between UHP with or without KI could be observed in Experiment 2. However, when considering CH_4 as an individual component of the total gas produced, the effect was more apparent, indicating KI has a specific impact on CH_4. In Experiment 1, 1× UHP + KI reduced CH_4% by 64 %, while UHP alone reduced it by 16 %, the opposite effect of KI on TGP levels. Similar yet less prominent results were observed in Experiment 2, where the 0.5× and 0.25× concentrations of UHP + KI reduced CH_4% by 53 % and 39 %, respectively, while the same concentrations of UHP alone reduced CH_4% by 37 % and 32 %, respectively. This effect was further evidenced when observing in terms of cumulative CH_4 production (Fig. 2) where a clear CH_4 inhibition response was observed with KI slightly enhancing CH_4 inhibition compared to UHP alone.

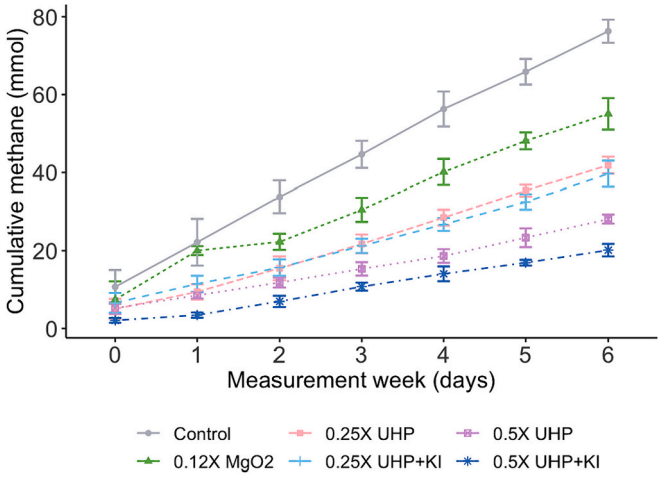

Fig. 2. Cumulative methane production (mmol/d).

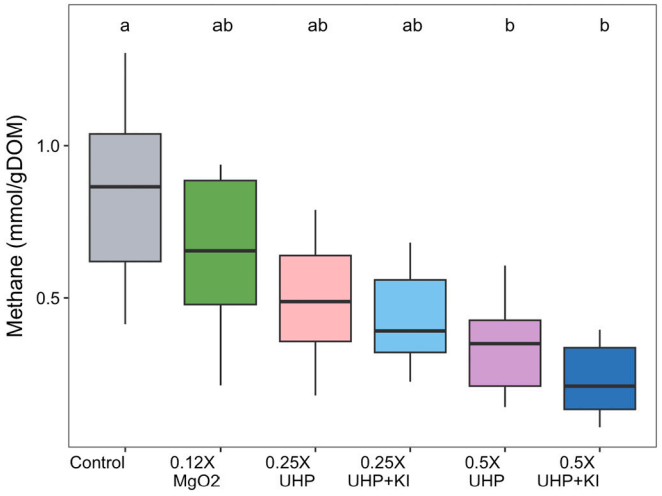

Fig. 3. Methane production (mmol/g DOM).

Additional effects from KI may be attributed to an iodophor effect resulting from the release of free iodine upon the decomposition of individual constituents (McKeen, 2012). The decomposition of H_2O_2, aided by KI, can also produce a variety of oxidative free radicals in the presence of metal ions (Ofoedu et al., 2021) which could also be a contributing factor.

Iodine is a necessary trace element that animals and microbes in the rumen require for growth and development. However, consuming high amounts of iodine can pose a toxicity risk to humans. Consequently, there has been a rise in legislation and consumer requirements for iodine levels in feed to be regulated (van der Reijden et al., 2017). European law permits a maximum of 5 mg/kg diet with 88 % DM (≈ 5.7 mg/kg DM; (National Research Council, 2001)). Therefore, while the inclusion of KI in this study demonstrated its ability to enhance CH_4 suppression, the concentrations used are impractical and cannot be directly translated to real-world conditions. Due to the lack of significant numerical differences observed with the addition of KI in CH_4%, and considering that we cannot surpass the KI levels tested in this study, along with the added expense and complexity, it is not advisable to conduct further testing of KI as a CH_4-suppressing component in feed additives.

3.4. Magnesium peroxide as an alternative oxygen source

MgO_2 was included in Experiment 2 to investigate if the antimethanogenic effects observed with UHP would be observed using an alternative peroxide source. MgO_2 was selected due to its slow-release rates (Waite et al., 1999), stability and environmentally benign character (Ropp, 2013). MgO_2 is a fine white powder with low solubility in water, making it more stable than UHP, which is a water-soluble crystalline solid (Ropp, 2013). This allows MgO_2 to release O_2 content at a controlled and relatively slow rate, unlike UHP, which begins to decompose immediately when exposed to hydrous fluids. Due to this slow-release property, the utilization of MgO_2 has potential in addressing the urgent need for an effective antimethanogenic additive that has prolonged antimethanogenic effects. This is of particular interest for pasture-based grazing systems, where animals remain outdoors for extended periods. MgO_2 was included in Experiment 2 at a low concentration of almost half that of the lowest UHP concentration, in terms of total O_2 delivered.

MgO_2 had the least pronounced effects among the treatments tested in Experiment 2, with maximum reductions of 25 % (CH_4 L/d and mmol/d; $p > 0.05$). Although not statistically significant, a 25 % reduction in CH_4 volume while having no negative impact on VFA production or nutrient disappearance at low concentrations is an encouraging indication for alternative O_2 sources. Indeed, MgO_2 results for fermentation and digestibility profiles were indistinguishable from the controls. In addition to the lower concentration (0.12×), the aforementioned slow decomposition rates of MgO_2 when compared to UHP, are thought to be a contributing factor to the level of CH_4 suppression recorded here. Further *in vitro* experimentation with the inclusion of balanced concentrations of UHP and MgO_2 (in terms of deliverable O_2) is necessary to confirm the impact of slower release rates on CH_4 suppression and rumen function. Additionally, screening of other slow-release oxidising agents, such as calcium peroxide (CaO_2) is recommended.

3.5. Impact of oxidising agents on nutrient disappearance

The application of UHP treatments slightly reduced total OM and DM disappearance relative to the control at the higher doses used in Experiment 1 (Table 4), with DM disappearance decreasing from 72.10 ± 5.09 % in controls to 65.3 ± 9.17 % in 1× UHP + KI ($p = 0.490$). The reduction in OM digestibility was observed only in forage bags, where forage OM disappearance was 72.11 ± 8.03 % in the control, 57.1 ± 16.80 % in 1× UHP + KI ($p = 0.271$) and 64 % in the other two treatments ($p > 0.1$). Meanwhile, concentrate OM disappearance was the same (or marginally higher) in concentrate bags. However, these differences were not statistically significant ($p > 0.1$), which is thought to be due to the low number of replicates and high variation.

The effects of treatments on nutrient disappearance in Experiment 2 showed a similar trend, with UHP lowering forage destruction ($p = 0.069$) while having minimal or no effects on concentrate digestion

Table 4
Effect of UHP (±KI) on feed disappearance in Experiment 1. Expressed as % of nutrient disappeared.

	Control	1× UHPKI	1× UHP	0.5× UHPKI	p value
Total DM	72.10 (5.09)	65.31 (9.17)	69.84 (5.22)	68.82 (5.87)	0.553
Total OM	70.20 (5.64)	63.59 (9.28)	68.44 (5.44)	67.15 (6.57)	0.557
OM concentrates	69.14 (3.59)	69.61 (3.58)	71.53 (3.46)	69.71 (3.34)	0.752
OM forage	72.11 (8.03)	57.10 (16.80)	64.86 (8.91)	64.54 (10.25)	0.338

UHP = urea hydrogen peroxide; KI = potassium iodide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). DM, dry matter; OM, organic matter. The standard deviation of the mean is presented in parentheses.

Table 5
Effect of UHP (±KI) and MgO2 on feed disappearance in Experiment 2. Expressed as % of nutrient disappeared.

	Control	0.5× UHPKI	0.5× UHP	0.25× UHPKI	0.25× UHP	0.12× MgO_2	p value
Total DM	64.77 (4.80)	62.02 (3.68)	59.51 (2.36)	63.49 (3.69)	60.90 (4.05)	65.23 (3.50)	0.312
Total OM	63.49 (5.17)	60.72 (4.02)	57.80 (2.54)	62.29 (3.87)	59.43 (4.24)	64.16 (3.76)	0.210
OM concentrates	52.50 (3.29)	55.18 (3.78)	51.30 (2.30)	54.12 (4.44)	51.72 (3.86)	52.72 (3.07)	0.737
OM forage	74.49 (7.83)	66.25 (5.35)	64.30 (3.53)	70.45 (4.70)	67.15 (5.27)	75.60 (5.00)	0.069
Total CP*	76.54 (2.23)	76.21 (3.07)	74.65 (2.57)	76.38 (3.03)	74.72 (3.20)	76.41 (1.62)	0.814
CP concentrates*	68.27 (2.33)	70.37 (2.03)	67.47 (3.98)	68.35 (4.76)	66.38 (4.44)	67.34 (1.98)	0.903
CP forage*	85.72 (2.17)	83.76 (2.88)	82.63 (1.45)	85.32 (2.01)	83.99 (2.21)	86.66 (1.54)	0.129
Total NDF*	56.73 (8.06)	50.11 (5.56)	46.27 (2.17)	52.19 (5.47)	49.59 (6.35)	57.54 (5.30)	0.099
NDF concentrates*	55.23 (1.22)	56.81 (5.57)	54.64 (3.41)	53.97 (3.99)	55.66 (3.58)	54.15 (1.81)	0.892
NDF forage*	57.87 (13.88) [ab]	45.00 (7.13) [ab]	39.89 (2.48) [a]	50.84 (9.35) [ab]	44.96 (9.29) [ab]	60.17 (8.22) [b]	0.043

UHP = urea hydrogen peroxide; KI = potassium iodide; MgO_2 = magnesium peroxide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). [a,b,c]: Treatment means without a common superscript differ ($p < 0.05$). DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre. The standard deviation of the mean is presented in parentheses.
* CP & NDF parameters are non-repeated measures.

(Table 5). The NDF of concentrates were unaffected by the addition of all treatments ($p = 0.892$). In contrast, supplementation with UHP treatments suppressed the disappearance of forage NDF ($p = 0.043$).

For all digestibility parameters measured, the low-dose (0.12×) MgO_2 treatment was indistinguishable from the control. In both experiments higher doses of UHP ± KI treatments had greater impact on digestibility parameters than lower dose UHP ± KI treatments. The lower OM and NDF in the forage of UHP-treated samples may suggest potential inhibition of fibre digestibility. Protozoa, among other groups are known to play a role in NDF degradation. A meta-analysis of *in vivo* defaunation studies found that removing protozoa reduced OM and NDF digestibility by 7 % and 20 %, respectively (Newbold et al., 2015). However, as RUSITEC systems are not conducive to thriving protozoal populations due to the lack of floating digesta mat, among other factors (Hristov et al., 2012), this effect could be exacerbated and thus future work should examine the potential impact of treatments on NDF degrading microbes. In addition, cellulolytic bacteria have also been revealed to be particularly oxygen sensitive (Newbold et al., 1996), where Rigobelo (2012) notes that this may be due to the absence of ROS detoxifying enzymes in many of these organisms.

3.6. Impact of oxidising agents on fermentation profiles

UHP treatments had no effect on ruminal pH levels ($p = 0.408$) or daily levels of total VFA (mmol/day; $p = 0.779$) in Experiment 1 (Table 6). This was also the case for all treatments in Experiment 2 where again no effect on ruminal pH levels ($p = 0.587$) or daily levels of total VFA (mmol/day; $p = 0.113$) were observed (Table 7).

All three UHP treatments in Experiment 1 reduced the ratio of acetate to propionate (A:P; $p < 0.001$), from 2.49 in the control to between 1.60 and 1.86. Similarly, in Experiment 2, A:P levels in the 0.5× UHP ± KI were lowered ($p = 0.009$). The A:P ratio in rumen methanogenesis reflects the balance between energy-yielding and energy-consuming pathways of the microbial community. Antimethanogenic additives, such as seaweeds, have been shown to decrease A:P levels *in vitro* and *in vivo* (Li et al., 2016). Propionate production is strongly associated with decreased CH_4 production (Janssen, 2010) and is considered an effective sink for sequestering excess H_2 (Rocque et al., 2021). Increasing propionate, or at the very least decreasing the A:P ratio, results in less substrate for acetoclastic methanogens.

Consistent trends were observed in UHP treatments relative to the control in Experiment 1 (Table 6). Decreases in molar proportions of acetate (7–11 %), butyrate (15–29 %), and caproate (2–36 %) occurred in all three UHP treatments, while increases in molar proportions of propionate (23–36 %) and valerate (21–40 %) were observed ($p > 0.05$). A dose-dependent reduction in butyrate was evident as 1× UHP + KI decreased molar proportions by 29 % and 0.5× UHP + KI by 15 %. Despite the notable shifts in fermentation profiles, the lack of statistical

Table 6
Effects of UHP (±KI) on fermentation patterns in Experiment 1.

	Control	1× UHPKI	1× UHP	0.5× UHPKI	p value
Fermentation parameters					
NH_3-N* (mg/L)	162.29 (11.44) [a]	341.15 (28.91) [b]	324.48 (24.99) [b]	240.69 (21.03) [c]	<0.001
Vessel pH	6.48 (0.14)	6.61 (0.18)	6.46 (0.18)	6.52 (0.13)	0.408
VFA production					
Total VFA (mM/d)	29.82 (13.25)	23.66 (14.31)	31.05 (8.30)	25.07 (8.39)	0.779
Acetate: propionate ratio					
A:P ratio	2.49 (0.12) [a]	1.65 (0.14) [b]	1.60 (0.19) [b]	1.86 (0.43) [b]	<0.001
VFA molar proportion (%)					
Acetate	47.53	43.99	43.08	42.29	0.679
Propionate	19.33	26.20	27.45	23.70	0.546
Butyrate	19.24	13.58	15.08	16.36	0.450
Valerate	9.72	12.41	11.73	13.57	0.882
Caproate	4.18	3.83	2.67	4.08	0.254

UHP = urea hydrogen peroxide; KI = potassium iodide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). [a,b,c]: Treatment means without a common superscript differ ($p < 0.05$). Total VFA (TVFA) expressed in mmol per day (mM/d). NH_3-N, Ammonia nitrogen expressed as mg/L. The standard deviation of the mean is presented in parentheses.
* Maximum theoretical NH_3-N derived from the urea fraction of 1× and 0.5× UHP (±KI) treatments are 860 mg/L and 430 mg/L respectively.

significance may be attributed to the low number of replicates ($n = 4$) and the relatively high standard deviation among VFA samples (Supplementary Table 1).

In Experiment 2, both 0.5× UHP ± KI treatments followed the same trends as Experiment 1, where again a decrease in molar proportions of acetate (5–7 %), butyrate (11–20 %), and caproate (20–42 %) was observed, while proportions of propionate and valerate were increased (5–16 % and 92–99 %, respectively). Both 0.25× UHP ± KI treatments varied slightly in that the molar proportion of propionate was marginally reduced relative to the control (by up to 9 %), and unlike all higher concentrations of UHP treatments (1× and 0.5×), caproate was substantially increased (76–101 %) rather than decreased.

The production of butyrate and acetate are positively correlated with CH_4 production (Williams et al., 2019), and production of these VFA produce +2H and +4H respectively (Wang et al., 2023). Meanwhile, propionate competes with methanogenesis for H_2, and as such is negatively correlated to CH_4 production. The production of propionate

Table 7
Effects of UHP (±KI) and MgO_2 on fermentation patterns Experiment 2.

	Control	0.5× UHP + KI	0.5× UHP	0.25× UHP + KI	0.25× UHP	0.12× MgO_2	p value
Fermentation parameters							
NH_3-N* (mg/L)	102.19 (27.92)	142.60 (9.69)	140.52 (45.67)	122.22 (11.89)	123.44 (24.27)	96.15 (12.17)	0.104
Vessel pH	6.49 (0.17)	6.57 (0.14)	6.59 (0.10)	6.53 (0.11)	6.59 (0.11)	6.50 (0.14)	0.587
VFA production							
Total VFA, mM/d	126.07 (37.19)	84.05 (16.60)	88.88 (4.94)	104.64 (20.16)	97.62 (12.15)	121.70 (20.16)	0.113
Acetate: propionate ratio							
A:P ratio	2.54 (0.17) [a]	2.31 (0.13) [ab]	2.06 (0.14) [b]	2.72 (0.25) [a]	2.44 (0.09) [ab]	2.50 (0.18) [ab]	0.009
VFA molar proportion (%)							
Acetate	53.80	50.95	49.94	51.41	50.99	53.89	0.064
Propionate	20.93	22.03	24.24	19.03	20.88	21.41	0.095
Butyrate	19.59 [a]	17.45 [ab]	15.70 [b]	18.71 [ab]	16.82 [ab]	19.88 [a]	0.022
Valerate	4.67 [a]	8.98 [ab]	9.30 [ab]	8.82 [b]	9.54 [b]	4.40 [a]	0.004
Caproate	1.01 [ab]	0.59 [a]	0.81 [ab]	2.03 [b]	1.78 [ab]	0.41 [a]	0.008

UHP = urea hydrogen peroxide; KI = potassium iodide; MgO_2 = magnesium peroxide. Treatment concentrations are expressed in terms of fold concentration (based on the amount of oxygen delivered). [a,b,c]: Treatment means without a common superscript differ ($p < 0.05$). Total VFA (TVFA) expressed in mmol per day (mM/d). NH_3-N, Ammonia nitrogen expressed as mg/L. The standard deviation of the mean is presented in parentheses.

* Maximum theoretical NH_3-N derived from the urea fraction of 1× and 0.5× UHP (±KI) treatments are 430 mg/L and 215 mg/L respectively.

results in −2H (Wang et al., 2023). The formation of propionate is thermodynamically favoured when H_2 concentrations are high (Wang et al., 2023), which is the case when methanogenesis is inhibited, such as with UHP treatments in this study. Conversely, the formation of acetate and butyrate is thermodynamically favourable when H_2 concentrations are low, which is reflected in the decline of acetate and butyrate with UHP treatments.

The increase in the medium-chain fatty acid (MCFA) valerate in all UHP treatments, and the increase of the MCFA caproate in 0.25× UHP ± KI treatments may indicate a move towards chain elongation. Chain elongation occurs where a rich and diverse microbial community persists (Wang et al., 2022), without which such a process could unlikely occur. The chain elongation of fatty acids has been identified as a beneficial alternative H_2 sink in the absence of methanogenesis (Baleeiro et al., 2021), as it is hypothesised that increased rumen H_2 favours fermentation pathways that consume H_2, such as valerate and caproate (Terry et al., 2023).

Concentrations of the branched-chain fatty acids iso-valerate and iso-butyrate for both experiments are presented in the supplementary material. Iso-valerate was decreased by all three UHP treatments in Experiment 1 ($p = 0.003$), while iso-butyrate concentrations remained similar across all treatments. In Experiment 2 concentrations of iso-valerate was not detected in 0.5× UHP ± KI and greatly reduced in 0.25× UHP ± KI (Supplementary Table 2). Concentrations of iso-butyrate were depleted entirely in all UHP treatments in Experiment 2.

0.12× MgO_2 was virtually indistinguishable from the control in all VFA groups, with the exception of caproate where a 59 % decrease was recorded. Although it should be noted the coefficient of variation (CV%) for caproate with 0.12× MgO_2 was extremely high at 70 %.

Higher levels of peroxides may lead to direct oxidation of VFA, and exceeding optimal concentrations have been reported to dampen VFA accumulation due to inhibition of hydrolysis and acidogenesis (Xu and He, 2021). Therefore lower concentrations of peroxide or perhaps one with a slower release rate, the inhibition is thought to be kept to the methanogenesis process and thus limits the consumption of VFA, and the hydrolytic and acidogenic bacteria are less affected and can better adapt to the presence of peroxide agents.

High doses of UHP (1×) in Experiment 1 increased NH_3-N levels by at least twofold ($p < 0.001$), while 0.5× UHP + KI increased NH_3-N by 48 % ($p < 0.001$). In Experiment 2, 0.5× UHP ± KI treatments increased NH_3-N by an average of 39 %, while 0.25× UHP ± KI treatments increased NH_3-N by an average of 20 % ($p > 0.05$). A clear linear dose-dependent increase in NH_3-N can be seen across both experiments. It should be noted that the UHP treatments themselves are a source of the urea. Therefore, as NH_3-N concentrations were elevated in all urea-containing treatments, and no difference in NH_3-N was observed in the MgO_2 treatment, this increase is most likely attributed to the presence of urea in the treatment, which is a nitrogen source and can serve as a precursor to ammonia. Nevertheless, it is important to note that the increase in NH_3-N was still lower than the maximum theoretical NH_3-N that could be produced from the amount of urea added to the system by each treatment (Tables 6 and 7).

4. Conclusion

The utilization of oxidising agents as methane-suppressing components has been demonstrated as a novel approach to effectively reduce CH_4 *in vitro*. High concentrations of UHP ± KI were most effective in reducing CH_4 emissions, however these treatments also impacted digestibility. Considering the overall impact on all process parameters, it is suggested that low concentrations of UHP ± KI (0.25×) are more suitable as they reduced CH_4 levels with a lesser impact on nutrient disappearance and VFA concentrations.

Due to residue concerns and regulations around limitations with KI, it is not recommended to include it in future work. MgO_2 is very slow to react, potentially taking >24 h to release all its O_2 (Waite et al., 1999), yet despite this and its use at a very low dose (0.12×), reductions ($p > 0.05$) in methane were observed with MgO_2 addition. Based on this, it is recommended that other slow-release oxidising agents, such as CaO_2, be evaluated *in vitro* alongside lower doses of UHP coated for slow-release, with doses normalised based on O_2 delivery rate.

Urea is already used as a feed additive, to enhance microbial protein synthesis, particularly in low-quality forages (Currier et al., 2004). To prevent toxicity, it has a strict safety limit (1 % of total DM intake or 30 g urea/100 kg body weight/day; Aquilina et al., 2012) and thus is not used in quantities outside the realm of estimated *in vivo* concentrations of a 2.5× UHP dose (~0.8 % DMI), meaning dietary urea could be mostly replaced with UHP. However, this would ideally be coated for slow release, where slow-release urea has been demonstrated to increase N-use efficiency, lower CP intake and reduce N emissions in faeces (Salami et al., 2021). Furthermore, since the activity of these additives is transient and their breakdown products are mainly O_2 and H_2O, they do not represent a potential risk as environmental contaminants.

In closing, we present a novel means of reducing enteric CH_4

emissions from *in vitro* fermentation systems through the addition of oxygen-releasing additives that have potential to be included in ruminant feed additives as a GHG mitigation solution.

CRediT authorship contribution statement

Caroline O'Donnell: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Camilla Thorn:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Emily Roskam:** Data curation, Investigation. **Ruairi Friel:** Conceptualization, Writing – review & editing. **Stuart F. Kirwan:** Conceptualization, Writing – review & editing, Investigation. **Sinéad M. Waters:** Conceptualization, Funding acquisition, Writing – review & editing. **Vincent O'Flaherty:** Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of competing interest

GlasPort Bio Ltd. has applied for patent protection of aspects of the treatments described in this paper. VOF and RF are shareholders 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 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 public-good funding awards, which funded the study (RSF 2019R479 and 19/FFP/6746). The other authors declare no real or perceived conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171808.

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