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Development and *in-vitro* assessment of novel oxygen-releasing feed additives to reduce enteric ruminant methane emissions

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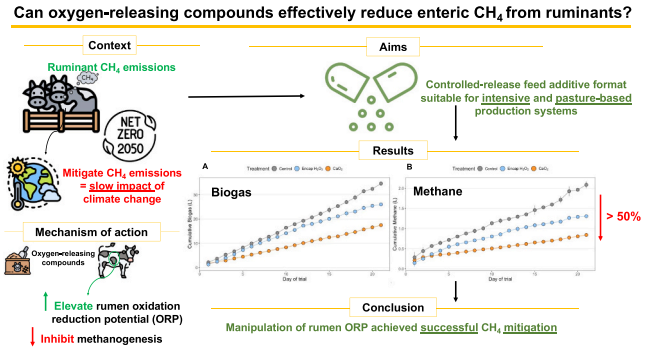
HIGHLIGHTS

- *In-vitro* assessment of novel methane suppressing oxygen-releasing feed additives.
- Oxidative reduction potential is a promising rumen methane modulation strategy.
- Oxygen-releasing compounds achieved consistent methane reductions in excess of 50 %.
- No negative impact on digestibility or VFA content with MgO_2 or Encapsulated H_2O_2.
- Potential cost-effective, slow-release formats identified for *in-vivo* screening.

GRAPHICAL ABSTRACT



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ABSTRACT

Ruminant livestock contribute significantly to global methane production and mitigation of which is of utmost importance. Feed additives represent a cost-effective means of achieving this. A potential target for such additives is rumen Oxidative Reduction Potential (ORP), a parameter which influences CH_4 production rates, with methanogenesis occurring optimally at ORPs below −300 mV. Thus, a controlled elevation of rumen ORP represents a potentially benign means of methanogen suppression. This research involved assessing a range of oxygen-releasing compounds for their ability to increase rumen ORP and inhibit methanogenesis, using the *in-vitro* rumen simulation technique (RUSITEC). Seven potential CH_4 inhibitors were tested in a 21-day trial

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monitoring biogas volume, CH_4 content, ORP, digestibility, ammonia, and volatile fatty acids concentration. The additives evaluated included: liquid peroxide (H_2O_2) and urea hydrogen peroxide (UHP), as well as slower reacting species (calcium and magnesium peroxide), in addition to encapsulated liquid H_2O_2 for controlled, slow release. Consistent CH_4 reductions of >50 % were observed from all additives. Reduced neutral detergent fibre (NDF) digestibility and a reduction in total volatile fatty acids (VFAs) was observed for some treatments, but MgO_2 and encapsulated H_2O_2 reduced CH_4 volume by 62 % and 58 %, respectively, and had no detrimental effects on digestibility ($p > 0.05$) or on VFA production. *Ex-situ* ORP measurements demonstrated significant increases in ORP upon addition of the additives, with MgO_2 and encapsulated H_2O_2 inducing a more moderate effect suggesting a controlled additive release was achieved with the slow-release format of encapsulated liquid H_2O_2. Thus, potential slow-release forms deemed suitable to progress to bolus or pellet format *in-vivo* were identified and could enable a longer-lasting suppression of methanogens within the rumen, facilitating application in both intensive and pasture-based production systems.

1. Introduction

Methane (CH_4) is a potent greenhouse gas (GHG) where agriculture represents a significant source, accounting for 6.3 % of EU total GHG emissions (EEA, 2022). As CH_4 is 28 times more potent than carbon dioxide (CO_2) (Myhre et al., 2014), its reduction has emerged as a key policy response. The Republic of Ireland has committed to reducing agricultural GHG emissions by 25 % by 2030, while other member states have also committed to reducing their agricultural GHG emissions (Leahy et al., 2020). As enteric fermentation is the largest single contributor of agricultural CH_4 emissions, (48 %; EEA, 2023) there is an immediate need for innovative strategies for its reduction.

Several approaches exist for ruminant CH_4 reduction (Palangi et al., 2022), including altered feeding regimes; the use of feed additives (Alemu et al., 2021; Jayanegara et al., 2018); breeding programs for low emissions and high feed efficiency traits, and early life studies involving microbiome modulation (Meale et al., 2021). However, as breeding programs and early life studies could take years to achieve their goals (Króliczewska et al., 2023), feed additives therefore represent a valuable tool for more rapidly achieving mitigation targets. A multitude of feed additives have been tested and report reductions in CH_4, including oils, algae, seaweeds, tannins and saponins, as reviewed by Króliczewska et al. (2023). Seaweeds are among the most widely researched of these (Roskam et al., 2022), but results are variable, with animal trials failing to provide the reductions observed *in-vitro* (MLA, 2023). The anti-methanogenic feed additive 3-nitrooxypropanol (3-NOP; Bovaer), shows promising results of >30 % CH_4 reduction in rigorous animal trials (Romero-Perez et al., 2014) and has been approved for commercial use in the dairy sector within the EU, New Zealand, Brazil, and Chile. It remains somewhat cost-prohibitive and thus, has yet to reach many farmers (Yu et al., 2021). As with 3-NOP, many additives are developed for confined feedlot systems which vary dramatically to the pasture-based systems that dominate the dairy and beef sector in Ireland and New Zealand, and beef sectors elsewhere (Greenwood, 2021). Such systems provide challenges in terms of additive formulation (Cummins et al., 2022) and necessitate an additive that is active in the rumen throughout the day, and thus needs to be intrinsically long lasting or able to undergo heat and pressure treatment for compression into either pellet format, a bolus or other slow-release technology. The format of 3-NOP is thus not currently suitable for pasture-based production systems due to issues related to long-term efficacy in the rumen and practical complexity of application on-farm (Leahy et al., 2020; Romero-Perez et al., 2015).

Hence, there is growing demand for alternative feed additive technologies which are simple to administer to animals, can maintain efficacy during long-term storage and use, are long-acting in the rumen, are cost-effective, and do not leave meat or milk residues. As well as reducing CH_4, improving ruminant animal performance and increasing rumen function would also be advantageous qualities of a potential CH_4 inhibitor. It is reported that ruminant CH_4 production represents a 12 % loss of the gross energy intake of animal feed, leading to a direct reduction in the energy available for animal growth and milk production (Ellis et al., 2008; Johnson and Johnson, 1995). Thus, if alternative hydrogen (H_2) sinks are found, CH_4 reduction may increase feed efficiency (Ungerfeld, 2020).

The ORP, a parameter which indicates the oxidation-reduction reactions occurring in a system (Grimalt-Alemany et al., 2021; Huang et al., 2018), has recently emerged as a tool for monitoring and manipulation of the rumen environment (O'Donnell et al., 2024). Understanding electron flow, and thus ORP, has been proposed as key to mitigating ruminant methanogenesis (Leahy et al., 2022). The ORP of the rumen, similarly to pH, both influences and is influenced by microbial activity, where microbes are adapted to specific redox conditions (Husson, 2013). Methanogens are adapted to strongly reducing conditions, where their growth, and thus methanogenesis, are inhibited at ORP values above −300 mV (Hungate, 1969; Hungate, 1960). Thus, periodically elevating the ORP slightly above this threshold represents a potentially benign means of suppression of methanogenic microorganisms, without impacting on other important rumen processes such as hydrolysis or fermentation. This research proposes extending this period of elevated ORP using low doses of slow-release oxygen releasing compounds, in order to inhibit the growth and activity of methanogens (Karekar et al., 2022; Ungerfeld, 2020). O'Donnell et al. (2024) demonstrated the efficacy of urea-hydrogen peroxide in reducing rumen CH_4 emissions where reactive oxygen species were identified as a potential inhibitory effect. However, catalase activity in the rumen is reportedly rapid (93.20 nmol/min/mL; Bartel et al., 2022) and as such the peroxide in these additives would rapidly be detoxified to water and oxygen. O'Donnell et al. (2024) also proposed a mode of action whereby the provision of a more energetically favourable electron acceptor (oxygen) diverts electrons away from methanogenesis. Manipulating redox has been used to improve efficiency of industrial fermentation and represents a potential means of redirecting electrons away from undesired products and towards those of added value (Liu et al., 2017).

Here, a range of other oxygen-releasing compounds, including calcium and magnesium peroxide, were screened *in-vitro* for their ability to modulate the rumen ORP and inhibit methanogenesis, using the RUSITEC system (Abbott et al., 2020). This system mimics the rumen environment and allows for highly replicated testing of feed additives in anaerobic vessels, prior to *in-vivo* experiments. Effects on digestibility, volatile fatty acid production and ammonia concentrations were also assessed. The 6 treatments were chosen to encompass a range of oxygen delivery rates in order to establish the timeframe in which these additives could suppress methanogenesis before CH_4 production resumed (O'Donnell et al., 2024).

The overall goal of this research was to identify a controlled- or slow-release feed additive, to facilitate a longer lasting suppression of methanogenesis within the rumen, while being easily implementable into pasture-based production systems. For example, consumption of a pelleted nut ration fed following milking of pasture based dairy cows when indoors twice daily, may facilitate the suppression of CH_4 emissions between milkings.

2. Materials and methods

2.1. Experimental licensing

Research was conducted at the Teagasc Grange Animal Bioscience Research Centre, Co. Meath, C15 PW93, Ireland (Longitude 6° 40′ W; Latitude 53° 30′ N), in September 2021. Rumen fluid was harvested from five cannulated Aberdeen Angus × Friesian steers residing at Teagasc Grange Animal Bioscience Research Centre, Co. Meath on day zero of the trial. The animals utilised for the collection of rumen fluid and digesta 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. System design, apparatus, and operation

Four RUSITEC systems (Sanshin Industrial Co. Ltd., Yokohama, Japan) were used to mimic the rumen environment, each of which incorporated eight 1 L fermentation vessels with a working volume of 850 mL and whose operation was performed as described by Czerkawski and Breckenridge (1977). Vessels were incubated in a water bath maintained at 39 °C using a water heater (Thermal Robo TR-1 a, Axel Global). Each vessel was continuously infused with artificial saliva (McDougall's Buffer; McDougall, 1948), prepared daily as detailed in Guyader et al. (2017), using a Watson-Marlow 500 series peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, United Kingdom), at a rate of 27.5 mL/h (dilution rate of 3.2 %/h) for the duration of the trial. Vessel contents were under continuous vertical agitation using mechanical agitators, operating at a rate of six revolutions per minute. The displaced effluent was collected in 1 L effluent Schott flasks (maintained at 2 °C) and fermentation gasses collected in 3 L reusable polyethylene gas bags. A total of thirty-two vessels were used, permitting four biological replicates per treatment when testing a total of six treatments, plus untreated controls. Treatments were randomly allocated within the four RUSITEC systems, with one biological replicate per treatment per RUSITEC system (randomised block design). Each individual vessel was deemed an experimental unit.

2.3. Experimental diets, procedure, and rumen inoculum

Rumen fluid and digesta from each of the five donor cows was collected on day 0. Fluid and digesta were placed together in 2 L insulated flasks then sieved (1 mm), to remove digesta, and the fluid from all five donor cows was pooled together. Each RUSITEC vessel was inoculated with 450 mL of this rumen fluid, and 350 mL of anaerobically prepared artificial saliva (McDougall, 1948). Digesta (80 g fresh weight) was placed in nylon bags (100 × 200 mm, 50 +/− 10-μm porosity (pore size) bag, ANKOM TM Technology, Macedon, NY, USA), and each vessel received one digesta bag as a means of inoculating the vessel with digesta-attached microbes. This bag was discarded after 24 h.

The quantity and composition of feed source was based on the donor animals' diet and consisted of 50:50 forage (grass silage; 21 % dry matter (DM), 12 % crude protein (CP)) to concentrate (86 % DM, 16 % CP including maize, barley and soya bean). Feed was weighed into nylon bags and two bags were delivered daily containing (a) 10 g DM forage (100 × 200 mm, 50 +/− 10-μm porosity (pore size) bag) and (b) 10 g DM concentrate (50 × 100 mm, 50 +/− 10 μm porosity (pore size) bag). Concentrate bags were collected after 24 h, and forage bags after 48 h fermentation, where this difference in residence time reflect that *in-vivo* (Mambrini and Peyraud, 1997).

2.4. Experimental treatments

Six potential CH_4 inhibitors were tested where doses were based off the 0.5× dose of O'Donnell et al. (2024) and normalised by reactive oxygen content, and these were compared to an untreated control. Previous work by (Thorn et al., 2022), where slurry was treated with peroxide-based additives to reduce CH_4 emissions also informed the selection process of the potential CH_4 inhibitors examined this study. The additives investigated included (in weights per vessel): four powder formats i) 293.4 mg of calcium peroxide (CaO_2; 75 % purity Sigma); ii) 342 mg of (MgO_2 complex, Sigma); iii) 270 mg of urea-hydrogen peroxide ($CO(NH_2)_2{\cdot}H_2O_2$ Sigma; referred to as UHP); iv) 325.5 mg of sodium percarbonate ($Na_2CO_3{\cdot}1.5H_2O_2$ Sigma, referred hereon as SodP;); in addition to one liquid format consisting of v) 296 μL of hydrogen peroxide (H_2O_2; 30 % *w/v* Sigma), and a controlled-release format consisting of vi) 296 μL of encapsulated liquid H_2O_2 (Encap H_2O_2). Encap H_2O_2 was prepared by mixing 296 μL liquid H_2O_2 (30 % w/v Sigma) with 296 μL of distilled water (dH_2O), and 592 mg of 10 % sodium alginate (Sigma) to create small beads. The beads were placed in a solution of calcium carbonate ($CaCO_3$) to harden for approximately 45 min. Treatments were delivered daily at the same time and location within each vessel as the feed bags.

2.5. Experimental sampling and analytical methods

The experiment was structured as a 21-day trial, consisting of a two-week biomass acclimation period (to allow for microbial adaptation and fermentation stabilisation) followed by one week of measurements, where the measurement week was comprised of days 15 to 21. On days 0, 1, 3, 7, 9, 14, 15, 16, 17, 18, 19, 20, 21, liquid samples for fermentation profiles were extracted pre-feeding from each vessel *via* a glass sampling port, and total gas volume, CH_4%, pH of fermentation fluid and the overflow, as well as overflow volume, were measured. All sampling of the vessels was performed while flushing with CO_2. pH was tested using a static Thermoscientific Orien pH meter and probe. Total gas volume was measured *via* manual expulsion through the dual diaphragm DC-1 A dry test gas meter (Sinagawa Corp. Tokyo, Japan). The Guardian NG Gas Monitor (Edinburgh Instruments Ltd., Livingston, UK) was used to estimate CH_4%, where daily calibration was conducted with a 10 % CH_4 span gas. ORP from all vessels was recorded using a static Thermoscientific Orien ORP meter on day 13, and a continuous *in-situ* ORP probe (Moonsyst) located within individual vessels on days 13, 14, 15 and 16. Due to the time intensive method of taking static ORP samples, this feasibly could not be performed during the measurement week, thus static ORP readings were taken two days prior when biogas and CH_4 data was evidenced to be stable. To complement the static ORP data, a single, continuous ORP probe was available, and this was placed in a vessel receiving Encap H_2O_2, on days 13 to 16 inclusive.

On days 13 to 21 inclusive, nutrient disappearance was measured, where extra bags from two extra days (13 and 14) were needed for sufficient sample size to allow for chemical feed analysis. Following removal from each vessel, each bag was gently rinsed with approximately 25 mL of artificial saliva which was returned to the vessel and the feed bags were immediately submerged in ice, then underwent a 30-min, detergent free, cold rinse cycle in a washing machine, to remove any remaining loosely attached microbiota. The DM content (g/kg) of the principal diet (forage, concentrates) and feed residue samples were determined after drying samples at 55 °C for 48 h in a forced-air oven and hot-weighed to determine DM disappearance (DMD). Dried feed samples and feed residue samples were crushed and ground using a FossTM CT 293 CyclotecTM General Purpose Sample Mill fitted with a 1 mm screen, after which feed residues from each vessel (days 13 to 21) were pooled. These underwent subsequent chemical analysis (Foss analytical, Nils Foss Allé 1, DK-3400 Hilleroed, Denmark) for organic matter (OM), CP, neutral detergent fibre (NDF) and starch (concentrate only).

Ash concentrations (g/kg DM) were determined by complete combustion in a muffle furnace (Nabertherm, GmbH, Lilienthal, Germany) at 550 °C for ~5 h. Concentration of CP (g/kg DM) for all samples was determined by obtaining the nitrogen concentration (g/kg DM) of the

feed and residue samples using a LECO FP 528 instrument (Leco Instruments UK ltd., Stockport, UK) and then multiplying this value by a nitrogen-protein conversion factor of 6.25 (Mariotti et al., 2008). Determination of NDF was conducted using the ANKOM220 Fibre Analyzer (ANKOM Technology, Macedon, NY, United States) as described by Van Soest et al. (1991). The forage and concentrate residue samples were analyzed for NDF using sodium sulphite, with a heat stable amylase included with the concentrate samples to aid in the removal of starch. NDF was expressed exclusive of residual ash. Disappearance of nutrients was determined as the difference between the amount of the components in the feed prior to incubation, and the amount remaining in the residue following incubation.

Samples for VFA and NH_3 quantification were acidified with 3 to 4 drops of 80 % orthophosphoric acid (Nzeteu et al., 2018) and stored at −20 °C for subsequent analysis. The concentration of VFAs was determined using an automated Agilent 450-GC (Agilent Technologies, Santa Clara, Canada) fitted with a flame ionisation detector (Ranfft, 1973) run using the conditions in Nzeteu et al. (2018) and compared against a range of standards. VFA production in mmol per day was calculated by multiplying concentration of VFAs by volume of outflow (Duarte et al., 2017). Ammoniacal nitrogen (N) content was determined spectrophotometrically from syringe filtered (0.2 ⌈m) samples using the indophenol method of Kandeler and Gerber (1988). The 48-well procedure of Bollmann et al. (2011) was followed where plates were read at 660 nm (Bio Tek PowerWave XS2 spectrophotometer) and samples were compared against a range of standards.

2.6. Statistical analysis

Data were analyzed in R (R Core Team, 2017) and visualised using ggplot2 (Wickham, 2011). Data that resulted from repeated measures (total biogas, CH_4, DMD, VFAs, ORP, pH and NH_3) were analyzed by fitting linear mixed effect models using restricted maximum likelihood (REML) from the NLME package (Pinheiro et al., 2007). If model residuals were not normally distributed, the response variable was transformed using Box-Cox transformation (MASS package; Venables and Ripley, 2002). Models were constructed with treatment and day as fixed effects and vessel and machine as random effects. The resulting models were analyzed with an ANOVA and when a treatment effect was seen ($p < 0.05$), estimated marginal means tests (Emmeans package; Lenth et al., 2023) were performed for pairwise comparisons with p-adjustment. For all other data (NDF, OM, protein and starch digestibility), statistically significant differences in mean were tested for using the non-parametric Kruskal Wallis test and Dunn post-hoc tests. Statistically significant means were considered when $p < 0.05$.

3. Results and discussion

To dually address the 13th UN 2030 Sustainable Development Goal of climate action and national targets to reduce agricultural GHG emissions, this research aimed to reduce enteric CH_4 emissions from cattle *via* dietary manipulation, by developing effective ruminant feed additives, which are both affordable and widely accessible.

3.1. Effects of oxygen-releasing compounds on total biogas and CH_4

Untreated controls produced an average of 1.56 L of biogas per day (Fig. 1a; Table 1), and all treatments reduced biogas volume (L) *versus* the control, where liquid H_2O_2 and CaO_2 treated vessels produced the least amount of biogas during the measurement week, at 0.71 and 0.82 L/day, equating to an average reduction of 50 % ($p < 0.05$). SodP and UHP vessels produced similar volumes of biogas to each other (0.88 and 0.9 L/day respectively) while MgO_2 and encapsulated H_2O_2 had a lesser effect on biogas, producing on average 1.16 L/day which represents a 25 % reduction ($p > 0.05$). While rates of biogas production for most treatments followed a similar trajectory during both the acclimation and measurement period (Fig. 1a), the encapsulated H_2O_2 treated vessels did not follow this trend and produced the same as the control (1.4 L/day) until after day 13, when production slowed. This could suggest this slow-release version of the additive required a longer time to affect the microbial community.

Control vessels produced an average of 0.07 L/day of CH_4 during the measurement period (Fig. 1b; Table 1). All treatments reduced CH_4 volume as a result of both the lower volume of biogas, and a lower CH_4% in the biogas. The reduction in CH_4% was most notable for SodP and MgO_2 which decreased the CH_4 content of biogas by 25 % and 21 %. Both liquid and Encap H_2O_2 had smaller effects on CH_4%. When CH_4 production was assessed in terms of dry matter digested, SodP and CaO_2 treated vessels produced the least amount of CH_4 (0.2 mmol) per gram of DMD, which equates to a 57 % reduction ($p < 0.05$). Overall, the MgO_2 treatment appeared to demonstrate the most CH_4-specific suppression, where it resulted in the least reduction in biogas and the largest reduction in CH_4 percent.

Total gas production is typically associated with the rate of feed degradation, and as such the digestibility of feed was then assessed to further investigate these gas data and determine to what extent gas

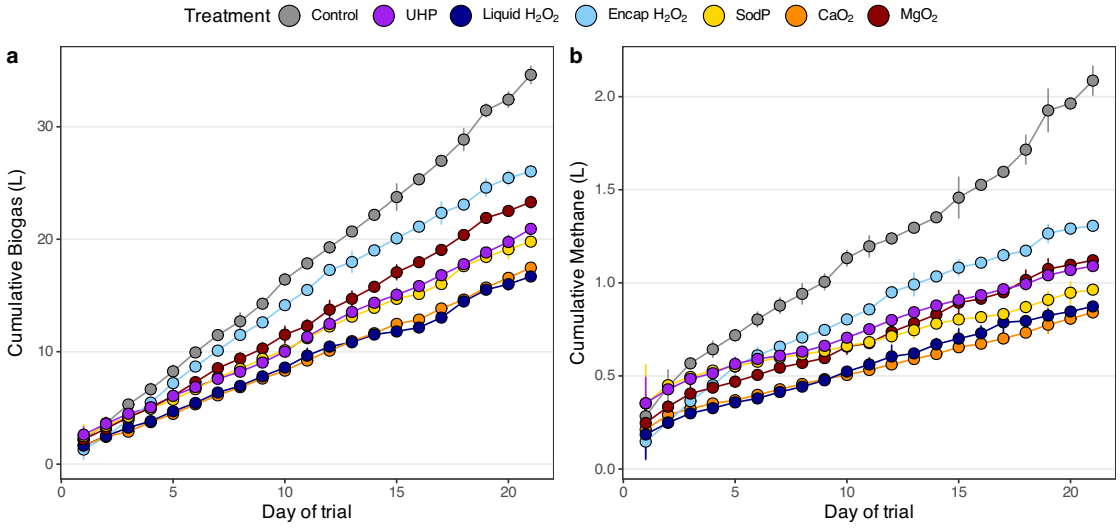

Fig. 1. Cumulative (a) total biogas volume and (b) CH_4 volume during the RUSITEC 21-day trial for control and additive treated vessels. Points represent the mean of four biological replicates and error bars represent standard deviation of the mean.

Table 1
Effect of oxygen-releasing compounds on total gas production and CH_4 parameters.

	Treatment								
	Control	UHP	Liquid H_2O_2	Encap H_2O_2	SodP	CaO_2	MgO_2	*p* value	SEM
Total gas (L/day)	1.56 ^{a}	0.90 ^{b}	0.71 ^{b}	1.16 ^{ab}	0.88 ^{b}	0.82 ^{b}	1.16 ^{ab}	0.004	0.0403
CH_4 (%)	4.34 ^{a}	3.76 ^{ab}	4.28 ^{ab}	4.15 ^{ab}	3.26 ^{b}	4.03 ^{ab}	3.41 ^{ab}	0.053	0.1527
CH_4 (L/day)	0.068 ^{a}	0.034 ^{bc}	0.030 ^{bc}	0.048^{bc}	0.029 ^{c}	0.033 ^{b}	0.039 ^{b}	<0.001	0.0031
CH_4 (mmol/g DMD)	0.48 ^{a}	0.24 ^{ab}	0.23 ^{ab}	0.33 ^{ab}	0.20 ^{b}	0.20 ^{b}	0.26 ^{ab}	0.0044	0.0204

a,b,c,d: for each variable, shared letters denote no difference in mean while unshared letters denote statistically significant differences in mean at the p value detailed for each row.
DMD, dry matter digested.

reduction was due to depression of fermentation.

3.2. Impact of oxygen-releasing compounds on feed digestibility

As the primary role of the rumen microbiota is feed digestion, it is imperative that no negative effects on animal health and performance occurs *in-vivo*. The assessment of substrate digestibility *in-vitro*, while not always a predictor of *in-vivo* impacts, can provide important information on potential CH_4-inhibiting feed additives,

When assessing the effects of treatments upon nutrient disappearance (Table 2), relative to the untreated control, no significant negative treatment effect was observed on concentrate DM, OM, protein or starch destruction from Encap H_2O_2, CaO_2 and MgO_2. In fact, CaO_2 and MgO_2 marginally increased DMD and OMD ($p > 0.05$). This reflects findings of Chang et al. (2008), who noted an increase in OM utilisation when spiking an anaerobic system with MgO_2, which they hypothesised was due to dominance of facultative heterotrophs resulting from O_2 release. In contrast, the remaining treatments (liquid H_2O_2, SodP and UHP) decreased concentrate DM and NDF destruction ($p > 0,05$). In terms of forage digestibility, no statistically significant negative impacts were observed from Encap H_2O_2 or MgO_2 addition, relative to the control diet, for DM, OM, NDF or protein, as also seen by O'Donnell et al. (2024) when using MgO_2. All other treatments, at the doses tested, reduced the destruction of all forage fractions, with the largest effect upon NDF (~50 % decreases) seen with SodP, UHP and CaO_2 ($p < 0.05$). As with methanogens, the presence of oxygen within the rumen has been demonstrated to negatively affect fibre degrading organisms, which are less abundant when oxygen is present (Newbold et al., 1996). This was proposed by Chaucheyras-Durand et al. (2012) to be related to a lack of detoxification enzymes necessary to remove reactive oxygen species, and perhaps accounts for these forage-specific detrimental effects. This effect is exacerbated in forage bags, not only because they contain higher proportions of NDF, but additionally they were exposed to the treatments for 48 h as opposed to 24 h for the concentrate bags. A slight reduction in the 48-h residence time used in RUSITEC systems has been proposed (Deitmers et al., 2022) and would be of interest, particularly for Encap H_2O_2 and MgO_2 where minor reductions in forage NDF destruction were observed and perhaps would be alleviated at residence times that more accurately reflect *in-vivo* conditions of high production animals.

The lack of detrimental impacts on forage digestibility with the controlled-release Encap H_2O_2 and the slowly reacting MgO_2 suggest that a slower evolution of lower doses of oxygen would be more effective in reducing CH_4 without adversely affecting forage digestion.

3.3. Fermentation patterns

3.3.1. Slow oxygen-releasing compounds outperform quick release treatments

Vessel pH remained largely unaffected by the treatments, which suggests pH had no major influence on the reduction of CH_4 associated with the treatments (Table 3). Ammonia-N increased in both UHP (173.3 mg/L) and MgO_2 (160.5 mg/L) treatments ($p > 0.05$) relative to the control (100.3 mg/L) and was reduced in all other treatments. As UHP is a source of urea which is quickly hydrolysed by rumen microbiota to release NH_3, this likely accounted for a significant portion of this increase, as noted by O'Donnell et al. (2024). In contrast, O'Donnell et al. (2024), observed no difference in ammonia concentrations in MgO_2 treated vessels relative to untreated control. Hence, the elevated ammonia concentrations from MgO_2 treatment observed in this study are of interest and warrant further study at a range of doses. NH_3-N is a primary source of N for the rumen microbiome (Tedeschi et al., 2017) so its increase represents a potentially valuable means of enhancing microbial production, particularly in low quality forage diets (N limiting).

Large reductions in NH_3-N were seen with all other treatments ($p < 0.05$; Table 3). As protein degradability was similar in all treatments (excluding liquid H_2O_2), for both forage and concentrate (Table 2), perhaps this reduction in NH_3-N was related to the loss of certain microbial species. For example, Hackmann and Firkins (2015) hypothesised that the reduction in rumen NH_3-N frequently associated with removal of protozoa could be an indirect effect, where bacteria that efficiently assimilate NH_3-N proliferate in the absence of grazing from

Table 2
Effect of oxygen-releasing compounds on digestibility, expressed as % of nutrient disappeared.

		Treatment								
		Control	UHP	Liquid H_2O_2	Encap H_2O_2	SodP	CaO_2	MgO_2	*p* value	SEM
Concentrate	DM	55.74	53.39	51.20	54.83	53.09	60.19	59.28	0.765	1.346
	NDF	43.85	32.84	34.52	45.36	39.43	43.93	41.36	0.449	1.644
	OM	47.77	51.24	47.46	56.59	45.33	54.98	54.66	0.902	2.919
	Protein	49.10	46.04	41.79	49.66	45.96	54.15	52.99	0.551	1.945
	Starch	73.85	79.71	69.80	74.85	74.22	80.40	77.75	0.490	1.423
Forage	DM	77.90 ^{a}	61.77 ^{b}	54.94 ^{c}	73.66 ^{a}	60.34 ^{bc}	62.66 ^{b}	75.16 ^{a}	<0.0001	0.850
	NDF	61.02 ^{a}	31.06 ^{b}	18.78 ^{b}	54.38 ^{a}	28.23 ^{b}	32.55 ^{b}	55.12 ^{a}	0.0018	3.241
	OM	90.36 ^{a}	85.40 ^{cd}	81.96 ^{d}	88.66 ^{ab}	84.29 ^{cd}	85.43 ^{bc}	89.86 ^{a}	0.0025	0.647
	Protein	87.31 ^{a}	82.47 ^{b}	79.68 ^{b}	86.81 ^{a}	81.40 ^{b}	82.26 ^{b}	86.77 ^{a}	0.0044	0.676

a,b,c,d: for each variable, shared letters denote no difference in mean while unshared letters denote statistically significant differences in mean at the p value detailed for each row.
DM, dry matter; NDF, neutral detergent fibre; OM, organic matter.

Table 3
Effect of oxygen-releasing compounds on pH and fermentation products.

	Treatment								
	Control	UHP	Liquid H_2O_2	Encap H_2O_2	SodP	CaO_2	MgO_2	*p* value	SEM
NH_3-N (mg/L)	100.3 ^{abc}	173.3 ^{a}	35.5 ^{d}	68.7 ^{bcd}	45.9 ^{cd}	30.1^{d}	160.5 ^{ab}	<0.0001	0.917
pH	6.96	6.97	7.05	6.92	6.99	7.07	6.92	0.2568	0.016
Total VFA (mmol/d)	48.02 ^{ab}	38.78 ^{abc}	29.74 ^{c}	45.54 ^{ab}	38.59 ^{bc}	35.53 ^{bc}	49.57 ^{a}	0.0003	1.135
A:P ratio	2.10 ^{a}	1.49 ^{b}	1.34 ^{b}	2.01 ^{a}	1.58 ^{b}	1.52 ^{b}	1.95 ^{a}	<0.0001	0.025
VFA molar proportion (%)									
Acetate	52.99 ^{a}	50.93 ^{ab}	52.04 ^{ab}	50.20 ^{ab}	50.00 ^{b}	52.08 ^{ab}	50.43 ^{b}	0.0125	0.203
Propionate	25.04 ^{a}	34.05 ^{bc}	38.68 ^{c}	25.00 ^{a}	31.47 ^{b}	34.28 ^{bc}	26.19 ^{a}	0.0042	0.418
Butyrate	15.53 ^{a}	8.18 ^{cd}	5.43 ^{d}	13.10 ^{ab}	10.09 ^{bc}	7.54 ^{cd}	14.23 ^{a}	<0.0001	0.295
Valerate	5.80 ^{a}	5.78 ^{ac}	3.46 ^{c}	9.33 ^{d}	7.10 ^{b}	5.37 ^{ab}	7.62 ^{d}	<0.0001	0.110
Caproate	0.64 ^{a}	1.06 ^{ab}	0.37 ^{a}	2.44 ^{d}	1.34 ^{bcd}	0.73 ^{abc}	1.53 ^{cd}	<0.0001	0.083

a,b,c,d: for each variable, shared letters denote no difference in mean while unshared letters denote statistically significant differences in mean at the p value detailed for each row.

protozoa. Microbial community analysis would serve to answer such a question in the context of this research.

A decrease in total VFA concentrations (mmol/d) during the measurement week was observed in UHP, liquid H_2O_2, SodP and CaO_2 treatments to varying degrees (Fig. 2, Table 3), and is likely due to the reduction in digestibility that was observed in forage DM and NDF digestibility under these same treatments (Table 2). Total VFAs in MgO_2 (49.6 mM/d) and Encap H_2O_2 (45.5 mM/d) however remained stable, and similar to the control (48.0 mM/d), during the measurement period. An increase in total VFAs with MgO_2 was observed in the final days of the trial (Fig. 2). The most notable detrimental effect on total VFAs was from treatment with liquid H_2O_2, which reduced VFAs by around 38 % ($p < 0.05$) and demonstrates the release of oxygen species from this treatment was too rapid, significantly impeding the microbial community. Reductions in acetate lowered the acetate to propionate ratio (A:P) in all additive-treated samples, again with the exception of MgO_2 and Encap H_2O_2, which remained relatively unchanged (Table 3). The molar proportions of VFAs were again the least affected by the MgO_2 and Encap H_2O_2 treatments which were the same as the control for C2 – C4 fatty acids, but showed increased proportions of the longer chain fatty acid valerate ($p < 0.05$), as well as the medium chain fatty acid caproate ($p < 0.05$).

When viewed in light of the digestibility and VFA data, the additional reduction in biogas volume in other treatments, when compared to MgO_2 and Encap, is likely mostly attributable to the reduced NDF digestion. As noted by Vargas-Ortiz et al. (2022), this would decrease the amount of pyruvate which is subsequently converted to acetate and therefore liberate less hydrogen for reduction of CO_2 to CH_4, so indirectly reducing CH_4. This is corroborated by the lower amount of acetate seen in these treatments. MgO_2 and Encap H_2O_2 treatments, however, resulted in no significant differences in forage NDF destruction and thus reduced NDF destruction did not explain the lower biogas production seen with these slow-release treatments. MgO_2 and Encap H_2O_2 treatments did not either influence the total production of VFAs.

The increase in molar proportions of valerate and particularly caproate in MgO_2 and Encap H_2O_2 treatments may suggest a trend towards chain elongation, however, as only fatty acids of up to six carbons were measured herein, it would have been beneficial to know if longer chain fatty acids were also being produced, at the cost of acetate and butyrate. Chain elongation is a known alternative H_2 sink when methanogenesis is reduced (Baleeiro et al., 2021), whereby increased levels of H_2 within the rumen serve to potentially promote H_2 uptake pathways which benefit from excess H_2 levels within the environment (Greening et al., 2019). This sequestration of excess H_2 can also occur in response to the biohydrogenation, or alteration, of ruminal fatty acids profiles from unsaturated fatty acids to saturated fatty acids (Toprak, 2015).

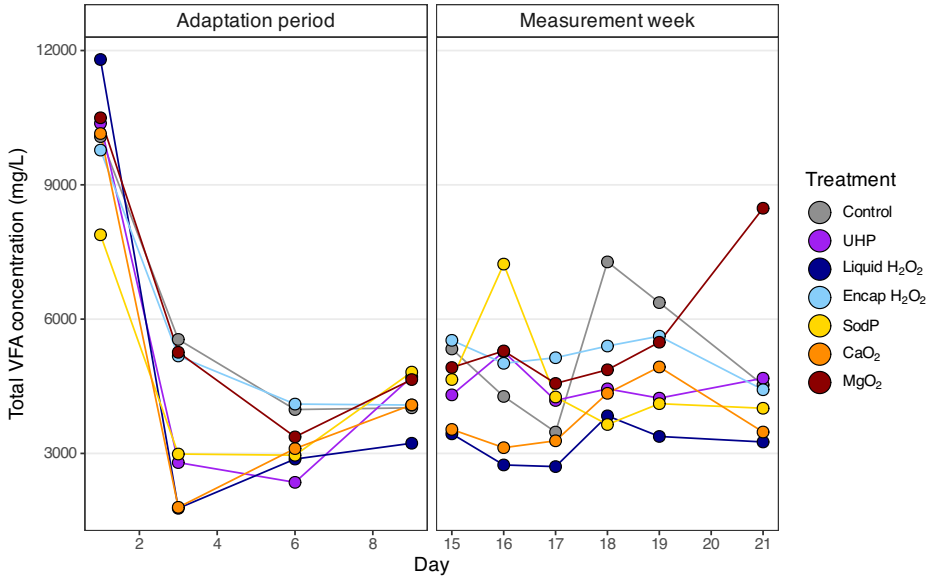

Fig. 2. Concentration of VFAs within the RUSITEC vessels during the 21-day trial, split into the adaptation period and the stable, measurement week. Each point represents the mean of replicate vessels per treatment ($n = 4$).

3.4. Changes in ORP from oxygen-releasing additives and their relationship with fermentation and CH_4 suppression

Ex-situ ORP, as a function of time post-feed, was recorded on day 13 of the trial (Fig. 3) where all samples, including the control samples saw an increase in ORP from below −300 mV to above −50 mV at two hours post addition of feed. Rumen ORP fluctuates naturally and has been shown to increase briefly after feeding (Richter et al., 2010), due to the influx of oxygen with feed. Facultative anaerobes rapidly consume this oxygen and revert the rumen to the highly reductive environment favourable for methanogenesis (Huang et al., 2018). Likewise in this study, this natural addition of oxygen *via* the forage and concentrate feed bags was demonstrated by a rising ORP observed in control vessels following addition of feed each day. Vessels were flushed with CO_2 for the period of feeding and treatment addition, but it is possible that trace amounts of oxygen entered the vessels and potentially contributed to this ORP rise.

All treatments induced spikes in ORP following addition of treatment in excess of that observed in the untreated controls. In the Encap H_2O_2 treatment, an elevated ORP (mV) was observed at four hours post-addition of treatment, while an elevated ORP (mV) occurred at two hours post-addition of treatment for all other additives and the control (Fig. 3). This was a preliminary indication that a controlled- or slower-release was occurring in Encap H_2O_2. The treatments CaO_2, liquid H_2O_2, and SodP increased ORP values to the largest extent, with peaks of up to +140 mV, and these were the three treatments that had the most consistent effect on emissions as a function of the whole 21-day trial (Fig. 1). UHP treatment produced peaks of between +80 mV, while an elevated ORP peak in MgO_2 occurred just under +50 mV, which was similar to that seen in the Encap H_2O_2 treatment. Positive ORP values were not observed in the control, with an ORP peak of −20 mV observed at two hours post-addition of treatment.

When viewing continuous ORP data from a single *in-situ* probe that was available for part of this trial (Fig. 4a), ORP values prior to treatment addition were far below those from the *ex-situ* ORP measurements (Fig. 3). As the *ex-situ* ORP data were obtained from sub-samples removed from experimental units and then measured, the values are potentially higher than those that would be observed using *in-situ* probes, (however the trends relative to the untreated controls remain). The same variability in ORP values was observed by Huang et al. (2018), in a review which highlighted the inconsistency in rumen ORP values among methods of *in-vivo vs. in-vitro* measurement.

The *in-situ* probe was placed in one vessel treated with the Encap H_2O_2. The treatment Encap H_2O_2 was observed to successfully induce reproducible spikes in ORP (Fig. 4a). Although the profiles reached less elevated ORP values when compared with the static ORP profile for Encap H_2O_2 (Fig. 3), the ORP remains slightly elevated after 4 h, and returns to the baseline by 8 h post-addition of feed. Additionally, a trend of a "falling staircase" type visual was observed (Fig. 4b), whereby the ORP decreases slowly in the hours following addition of treatment. This phenomenon again likely indicates that small volumes of H_2O_2 are being released as the encapsulated sodium alginate bead dissolves, inducing staggered ORP values during the drop from positive to negative.

The extent of ORP modulation resulting from the different oxygen-generating additives is closely mirrored by their effect on digestibility, where those inducing higher peaks in ORP had more detrimental effects upon digestibility. This is likely a consequence of these more rapidly decomposing peroxide sources (UHP, liquid H_2O_2, SodP and CaO_2) releasing more oxygen (and potential reactive oxygen species; ROS) that the microbial community was either i) able to use, in the case of oxygen and facultative anaerobes or ii) detoxify in the case of ROS. Therefore, fibrolytic microbes were impeded and digestibility reduced. However, the release of oxygen from Encap H_2O_2 and MgO_2 did not have this detrimental effect on digestibility yet was able to reduce CH_4 production, potentially by altering the ORP above that which is favourable for methanogenesis but not too high to interfere with other fermentative processes. This was likely a consequence of the slower release of oxygen from these two treatments, where encapsulation with alginate slowed release from the encapsulated version, and the very slow reaction time of MgO_2 (Waite et al., 1999) accounted for its slow release.

While this proof of concept paper was based on transient increases of ORP over short periods of time, it revealed that an optimised

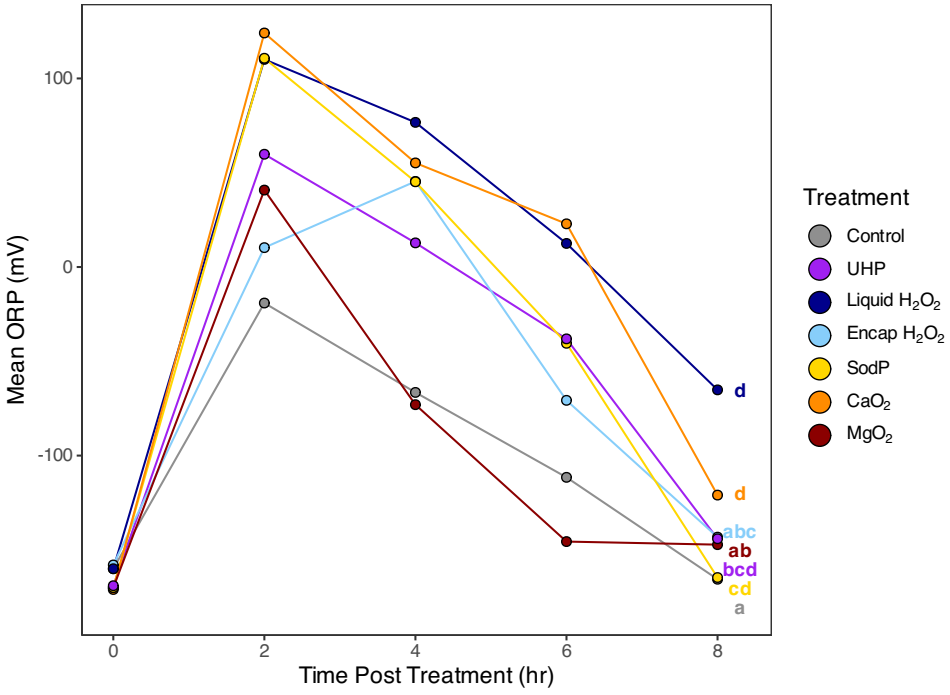

Fig. 3. Effect of oxygen-releasing compounds on the ORP (mV) during an 8 h period post feeding and treatment on day 13 of the trial. Shared letters denote no difference in mean over the 8 h, while unshared letters denote statistically significant differences in mean ($p < 0.05$) as a function of treatment where letters are coloured according to the treatment they represent.

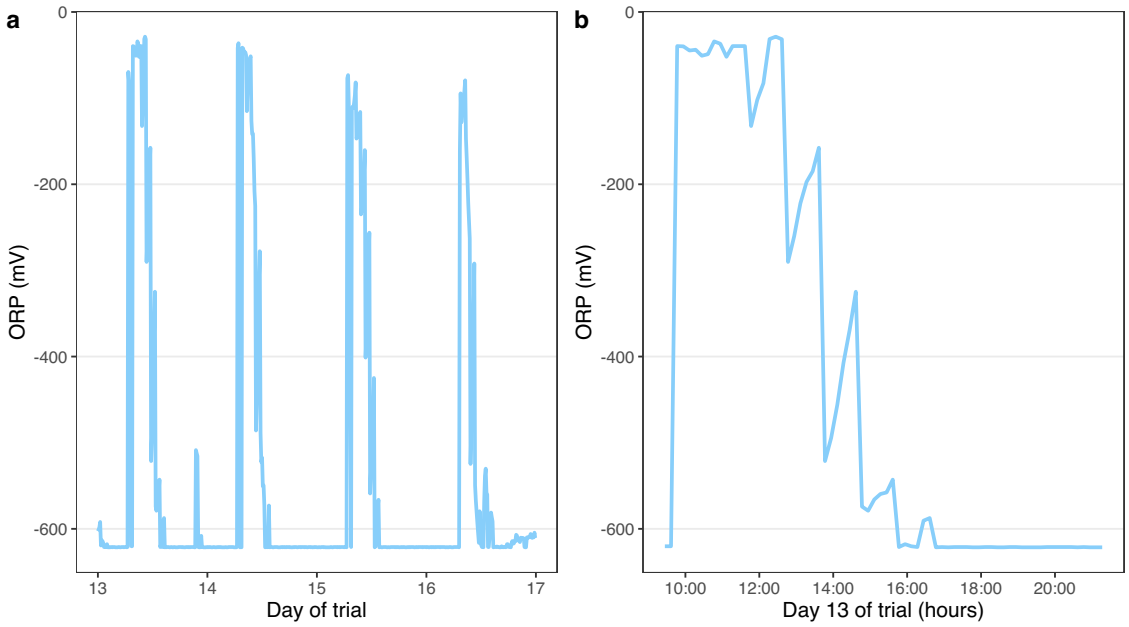

Fig. 4. Effect of Encapsulated liquid H_2O_2 on ORP (mV) during (a) 96 h period post treatment on days 13 to 16 of the trial and (b) 24 h period post treatment on day 13 of the trial.

encapsulated peroxide version would allow for more controlled, lower ORP elevations within the rumen, and therefore maintain slightly elevated levels of rumen ORP for extended durations. To fully understand the alternative electron processes that are taking place a result of this change in ORP, further analyses such as metabolomics and/or metatranscriptomics would need to be performed.

When employed as a process control, ORP can serve as an immensely valuable tool in establishing the capacity of diverse oxygen-releasing compounds to inhibit methanogenesis. As described by Liu et al. (2013), ORP can control the metabolite profile of microbial consortia, thus manipulating it is a valuable tool for re-directing to more favourable pathways, as demonstrated by Grimalt-Alemany et al. (2021) to enhance ethanol production and by Nghiem et al. (2014) to reduce unwanted gaseous end-products like hydrogen sulphide (H_2S) in anaerobic digestion systems.

4. Conclusions

This proof-of-concept paper, a continuation of work by O'Donnell et al. (2024), aimed to screen a number of slow oxygen-releasing compounds to identify potential CH_4-mitigating feed additives. Certain treatments, at the doses tested, reduced digestibility, particularly of forage NDF, which suggests future *in-vitro* work at lower doses. Encapsulated H_2O_2 and MgO_2 demonstrated the best performance in terms of reducing CH_4 without negatively impacting formation of end products, despite small, statistically insignificant reductions in NDF in forage bags incubated for 48 h. By altering the formulation of these additives to deliver a longer lasting, yet smaller increase in ORP, it could be possible to maintain mitigative effects upon CH_4 without impacting forage digestibility. This research demonstrates that controlled ORP alteration can potentially be harnessed as a tool for rumen modulation to achieve benign CH_4 suppression.

The successful screening of six oxygen releasing compounds has identified two additives that could move forward to dose-dependent *in-vitro* tests, with repeated runs for added statistical value. To gain a deeper understanding of the mode of action of these oxygen-releasing compounds within the rumen, ongoing and future work will also examine ruminal microbial interactions, dynamics and responses to the potential CH_4 inhibitors assessed in this research, to provide much needed clarity on what might be occurring when H_2 or CO_2 are diverted away from ruminant CH_4 production with the final goal of safely reducing ruminant CH_4 production *in-vivo*.

CRediT authorship contribution statement

Alison Graham: Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **Camilla Thorn:** Formal analysis, Conceptualization, Writing – review & editing, Writing – original draft. **Michael McDonagh:** Investigation, Data curation. **Caroline O'Donnell:** Investigation, Data curation. **Stuart F. Kirwan:** Methodology, Investigation, Conceptualization, Writing – review & editing. **Sandra O'Connor:** Investigation, Data curation. **Corine O. Nzeteu:** Formal analysis. **Alejandra C.V. Montoya:** Formal analysis. **Andrew Bartle:** Formal analysis. **Alison Hall:** Formal analysis. **Cathy Abberton:** Methodology, Conceptualization. **Ruairi Friel:** Conceptualization. **Sinead M. Waters:** Funding acquisition, Conceptualization, Writing – review & editing. **Vincent O'Flaherty:** Funding acquisition, Conceptualization, Writing – review & editing. **Stephen Nolan:** Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vincent O'Flaherty has patent pending to GlasPort Bio Ltd. Ruairi Friel has patent pending to GlasPort Bio Ltd. GlasPort Bio Ltd. has applied for patent protection of aspects of the treatments described in this paper. Vincent O'Flaherty (VOF) and Ruairi Friel (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. The other authors declare no real or perceived conflicts of interest. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

The raw data supporting the conclusions of this article can be made available upon request.

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