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Using eco-friendly feedstuffs in ruminants to achieve a cleaner environment and reduced carbon footprint

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Using eco-friendly feedstuffs in ruminants to achieve a cleaner environment and reduced carbon footprint

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Editorial: Using eco-friendly feedstuffs in ruminants to achieve a cleaner environment and reduced carbon footprint

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Editorial on the Research Topic
Using eco-friendly feedstuffs in ruminants to achieve a cleaner environment and reduced carbon footprint

Introduction

The environmental impact of modern farming and the relentless drive for various extraction and production, often at the expense of replenishment and recycling, demand that we rethink our approach to livestock systems and nutrition. This rethinking must involve using alternative resources responsibly, promoting recycling, and reducing our carbon footprint. Given the growing urgency of this issue and the rapid expansion of research in this field, this editorial aims to highlight and discuss the latest findings, alongside alternative feed resources and additives that can be sustainably used for farm animals (1, 2). This Research Topic brings together contributions from around the world that focus on a common goal: adopting eco-friendly feed resources and approaches that improve or maintain animal performance while reducing environmental impacts, particularly greenhouse gas emissions. Collectively, these works reflect a shared vision for a sustainable, circular bio-economy in animal agriculture. The nine articles published (out of 11 submitted) span a broad range of innovative strategies, from valorizing agricultural by-products and developing functional feed additives, to exploring novel proteins derived from algae and insects. These papers came from geographic diverse sources with contributions from Egypt, Italy, South Korea, India, Turkey, China-Italy, Tunisia-Palestine, and two from China alone. The research covered a variety of animals including camels, sheep, cattle, pigs, insects, and even companion animals like dogs. All the papers are original research articles and employ diverse approach, including in vivo trials, *in-vitro* fermentation studies, and meta-analyses.

Findings

Valorizing wastes and agro-industrial by-products

The studies here were evaluated using *in vitro* means. For example, Ghazzawy et al. investigated the use of biochar derived from date palm (*Phoenix dactylifera*) seeds as a feed supplement for camels. Supplementation significantly enhanced fermentation parameters and reduced methane production *in vitro*. This shows the emerging role of biochar in not only soil amendment but also by-product and waste recycling (3), enteric emission mitigation, especially in arid regions where both camels and date palms are abundant. Vastolo et al. evaluated eight polyphenol-rich agro-industrial by-products, including grape, tomato, olive pomace, and hazelnut skin using sheep rumen fluid. By-products, from citrus and hazelnut by-products showed the greatest anti-methanogenic potential. Similarly, Ghzayel et al. examined carob leaves collected from Tunisia and Palestine that were treated with NaOH, urea, or polyethylene glycol. While treatment effects were highly dependent on the geographical origin of the leaves, the study confirmed the promise of agroforestry residues as a viable source of feed for ruminants, particularly in dryland ecosystems. These results promote the integration of regional waste streams into livestock diets as part of circular agricultural systems.

Exploring novel protein sources: insects, algae, and fermented gases

The global race to identify viable and scalable alternative feed resources continues, especially in countries where governments recognize the importance of livestock farming, whether from a food security, economic, or environmental perspective. A comprehensive meta-analysis by Gao et al. explored the feasibility of using insect-derived meals, such as those from *Tenebrio molitor*, *Hermetia illucens*, and *Bombyx mori* in ruminant diets. The authors found that moderate inclusion ($\leq$30%) of these high-protein feeds support digestibility and rumen fermentation, while also boosting growth performance in some trials. The oriental hornet (*Vespa orientalis*) was particularly promising, as it may hold untapped potential among underexplored insect species. Palangi et al. investigated the algae-nanoparticles relationship to assess the anti-methanogenic effect of *Chlamydomonas reinhardtii* combined with magnesium oxide and magnesium sulfide nanoparticles. I*n vitro* data showed significant improvements in gas production, digestibility, and volatile fatty acid profiles, pointing to the dual role of algae as a methane inhibitor and nutritional enhancer. While dogs may seem like an unusual inclusion in this Research Topic, it is important to understand that they are considered part of the broader category of farm animals, not necessarily as food animals (though this occurs in some cultures), but more commonly as companion animals. In a novel extension of microbial protein sources, Babu et al. conducted a pilot study on dogs using a fermented protein derived from methane gas. These alternative feeds imply the broader viability of insect and single-cell proteins as sustainable feed ingredients across species.

Additives and dietary interventions for methane reduction

Methane mitigation via dietary supplements was also examined by Zhou et al., who evaluated the combined use of 3-nitrooxypropanol (NOP) and L-malate in dairy cows. The NOP supplementation alone reduced enteric methane by 54%, with no adverse effects on milk yield. When combined with L-malate, methane emissions were reduced by 51%, with added benefits to milk fat and protein composition. These results reinforce the importance of precise feed additives as tools for emission reduction without compromising productivity.

Replacing conventional ingredients without sacrificing performance

The feasibility of replacing high-demand ingredients like soybean meal was addressed by Zhao et al., who demonstrated that mixed plant proteins, including rapeseed meal, palm kernel meal, and dried distillers grains with solubles (DDGS), could effectively substitute soybean meal in pig diets without negatively affecting growth or carcass quality. While not directly targeting methane reduction, these substitutions could contribute to feed sustainability and reduce deforestation-linked inputs. Meanwhile, Malik et al. conducted a meta-analysis on the effects of DDGS on methane emissions in cattle. Contrary to some expectations, DDGS had no significant effect on methane production or dry matter intake. However, its neutrality indicates that it can be used without exacerbating emissions, offering flexibility in diet formulation.

Conclusion

These studies collectively illustrated the need to integrate diverse feed innovations including locally available by-products, novel proteins, targeted additives, and smart replacements to enable meaningful reductions in the carbon footprint of ruminant systems. This Research Topic also emphasizes the need for contextual evaluation as the effectiveness of feed interventions often depend on species, geography, processing methods, and dietary inclusion levels. For example, the performance of carob leaves or polyphenol-rich by-products was found to vary by origin and treatment method, and the efficacy of insect meals differed by species and inclusion rate. As global demand for animal protein rises, sustainable intensification must become a priority and feed innovations offer one of the most immediate levers for change.

Author contributions

VP: Writing – review & editing, Writing – original draft. MA: Writing – review & editing, Conceptualization, Writing – original draft. SS: Writing – original draft, Writing – review & editing.

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Cutting-edge exploration of insect utilization in ruminant nutrition—feature and future: a systematic review and meta-analysis

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There has been a growing interest in using insects as sustainable protein sources for ruminant feed, such as the adults of the two-spotted cricket (*Gryllus bimaculatus*), larvae of the mealworm beetle (*Tenebrio molitor*), black soldier fly (*Hermetia illucens*), and pupae of the silkworm (*Bombyx mori*). The advantages of these insects over other plant materials lie in their elevated levels of crude protein and fat. However, this interest lacks a comprehensive understanding of the impact of insects on the ruminal fermentation processes, including digestibility and gas production, as well as the impact on animal performance and related health aspects. This review offers a comprehensive analysis of ruminal fermentation indices across diverse insect species. Employing descriptive and meta-analysis methodologies, we examined the impact of incorporating insect-derived meals in ruminants' diets. Moreover, we evaluated the growth performance and biochemical parameters of blood in ruminants when species such as *Tenebrio molitor*, *Hermetia illucens*, Oriental Hornet (*Vespa Orientalis*), and *Bombyx mori* were incorporated into ruminants' diets. The meta-analysis was performed on a limited dataset of 14 *in vitro* and eight *in vivo* trials, investigating insect meal as a potential feed source. A comparison is drawn between these insect-based feeds and conventional dietary sources such as soybean meal, alfalfa hay, and commercial concentrate diets. Our meta-analysis revealed that incorporating *Gryllus bimaculatus* and *Hermetia illucens* to partially replace protein sources in ruminants' diet did not adversely affect digestibility, ruminal fermentation, and ruminant production, supporting the feasibility as a feed ingredient for ruminant animals. In addition, the oriental hornet showed an overall higher outcome on the final BW, ADG, digestibility, and volatile fatty acid (VFA) production, suggesting the promising effect of this insect for future use in ruminants. The data also indicates that dietary insect inclusion levels

should not exceed 30% (DM basis) to achieve an optimal ruminal fermentation profile. Furthermore, it offers comparative insights into the nutritional value of these insects, which warrant further investigation at the *in vivo* level. Ultimately, the existing understanding of the nutritional utilization potential of these insects by ruminants, particularly concerning macro- and micronutrients, is evaluated and revealed to be significantly constrained.

KEYWORDS

insects as feed, *in vitro* digestibility, *in vivo*, methane, total gas production

1 Introduction

By the year 2050, the global human population is projected to reach approximately 9.5 billion, necessitating a corresponding 70% increase in demand for animal-based food production, such as milk and meat (1). The primary livestock categories include pigs, with a production of 112.33 million metric tons (MT); poultry, with 109.02 million MT; and cattle (including beef and buffalo meat), with 67.99 million MT, collectively representing 91.80% of global meat production (2). As the most populous country globally, China has a significant demand for livestock products, particularly those derived from ruminant animals. Consequently, this surge in demand for animal-derived products may escalate the need for livestock feed (3). Providing sufficient feed for livestock is anticipated to encounter challenges as available land for cultivating feed resources diminishes. Intensive livestock production systems heavily depend on soybean meal (SBM) as a primary source of protein and essential amino acids (4). Nevertheless, its extensive use raises concerns regarding environmental sustainability and its competition with human nutrition (5).

Insects represent promising and innovative feed ingredients due to their valuable chemical composition. They are notably rich in proteins and contain significant amounts of lipids, making them suitable as protein and energy sources in animal dietary formulations (6). Insects have been incorporated as a feed ingredient in various animal species, including broiler chickens (7), laying hens (8), turkey (9), ducks (10), quail (11), rabbit (12), swine (13), companion animals (14), and aquatic species (15, 16). Despite their widespread use across these species, the utilization of insects in ruminant diets has been relatively limited. The limited adoption of insects in ruminant diets may be attributed to concerns regarding the potential risk of transmitting bovine spongiform encephalopathy (mad cow disease) despite the absence of evidence supporting such a linkage to date (17). While ruminants primarily consume grasses, legumes, and agricultural by-products, they frequently require protein supplements to enhance their production efficiency (18).

Moreover, feed expenses represent a significant constraint on advancing the livestock production industry. Feed costs typically constitute around 50–70% of the total budget, with protein requirements alone accounting for more than 15% of the overall feed expenditure (19). Hence, to address the rising demand for animal products in the coming years, there is an urgent need for innovative solutions and sustainable alternatives to traditional protein sources in animal diets, aiming to minimize environmental impact. The utilization of insects as animal feed offers substantial environmental benefits compared to conventional sources. Insects play a key role in bioconverting waste materials, require less water and land for cultivation, and contribute to lower greenhouse gas (GHG) emissions (5). Therefore, this development has prompted policymakers in the European Union to approve the use of insects as feed for pigs and poultry in April 2021, in addition to their existing approval for aquaculture since July 2017 (20). Currently, there is limited available data on the utilization and effects of insects as alternative feed for ruminants. Therefore, further scientific research is needed to raise awareness of this topic among policymakers in Europe, China, and globally and to assist in establishing a regulatory framework for licensing insects as ruminant feed (21).

The utilization of insects as feed for monogastric animals has been extensively reviewed (22). However, there remains a gap in applying insects as feed for ruminants. In Asia, Africa, Oceania, and South America, insects have historically served as traditional food sources within these regions. They have recently garnered interest as alternative protein sources in additional regions, including Europe and North America (23). In the United States, the black soldier fly (*Hermetia illucens*) is utilized exclusively in aquaculture. In Canada, the use of *Hermetia illucens larvae* is approved for both aquaculture and poultry production. Brazil currently lacks specific legislation addressing this matter, with insects permitted only for feeding non-ruminant animals (24). In countries like China and South Korea, this matter has no restrictions or limitations (25).

Presently, there is a lack of statistical data regarding the commercial rearing of insects. However, numerous countries have begun farming insects like crickets for the feed market. Annual insect meal production is anticipated to increase to 1.2 million tons by 2025 (26). Cricket presents promising potential as an alternative feed resource for animals. Figure 1 illustrates the detailed life cycle of selected insect species in this context. Typically, crickets are reared for approximately 5–6 reproductive cycles before being discarded due to diminished productivity. These discarded crickets can subsequently be utilized as animal feed (27). The black soldier fly (*Hermetia illucens*)

Abbreviations: ADF, Acid detergent fibre; ADFD, Acid detergent fibre digestibility; ALT, Alanine transaminase; ALP, Alkaline phosphatase; AST, Aspartate transaminase; BUN, Blood urea nitrogen; BW, Body weight; CH_4, Methane; CO_2, Carbon dioxide; CP, Crude protein; C_2, Acetate; C_3, Propionate; C_4, Butyrate; DDM, Digestible dry matter; DM, Dry matter; DMD, Dry matter digestibility; EAA, Essential amino acid; HCT, Hematocrit test; HGB, Hemoglobin; IVDMD, *In vitro* dry matter digestibility; IVOMD, *In vitro* organic matter digestibility; IVNDFD, *In vitro* neutral detergent fibre digestibility; IVADFD, *In vitro* acid detergent fibre digestibility; Max, Maximum; Min, Minimum; NA, Not applied; NDF, Neutral detergent fibre; NDFD, Neutral detergent fibre digestibility; ND, Not detected; NEAA, Non-essential amino acid; NH_3, Ammonia; OM, Organic matter; OMD, Organic matter digestibility; RBC, Red blood cell; RMSE, Root mean square error; SD, Standard deviation; TGP, Total gas production; TVFA, Total volatile fatty acids; WBC, White blood cells.

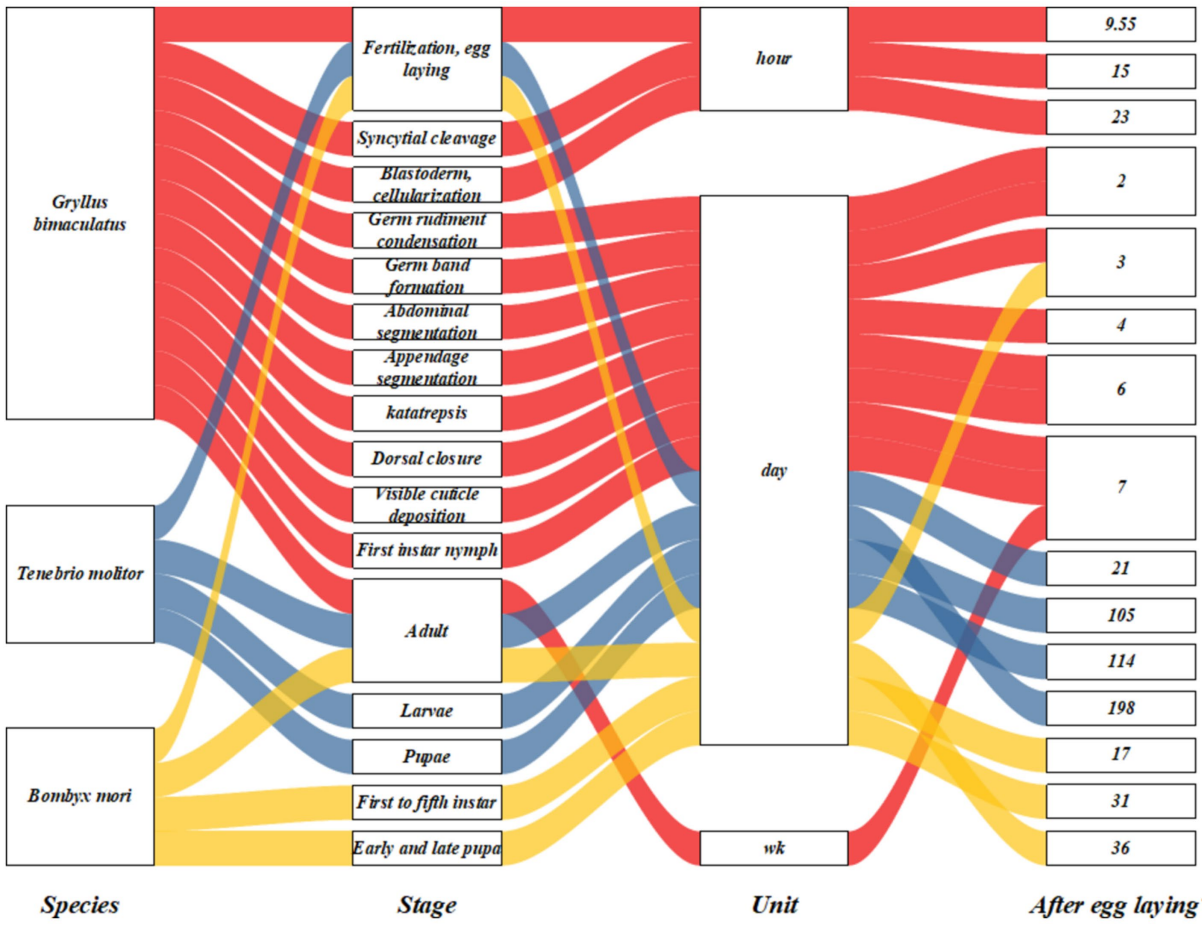

FIGURE 1
Schematic processes indicating a complete life cycle throughout major developmental *Gryllus bimaculatus*, *Tenebrio molitor*, *and Bombyx mori*; wk., week; *up to value, for example, in *Gryllus bimaculatus*, the egg-laying stage lasts from 0 to 9.55 h, etc.

exhibits a life cycle lasting approximately 40–43 days (28). Research interest in using edible insects as alternative feed has surged recently. As Hanönü et al. (29) reported, insect farming emerges as a more cost-effective option when evaluating land allocation for forage crop cultivation and their associated water requirements. Insects exhibit efficient feed conversion rates and rapid growth rates. It is estimated that approximately 2 kg of organic waste and 1 m^2 of space could yield 1 kg of insect protein. In particular, *Hermetia illucens* has gained increasing commercial utilization in animal feed due to its ease of rearing, high productivity, rich nutritional content, and efficient organic waste utilization. *Hermetia illucens larvae* have demonstrated the capacity to consume substrate ranging from 25 mg to 500 mg of fresh matter per larva per day, achieving a body length of approximately 27 mm, width of 6 mm, and weight of 220 mg by 14 days of age (30).

Moreover, Indonesia, characterized by its archipelagic geography and tropical climate, offers favorable conditions for *Hermetia illucens* production. Data from the Indonesian Ministry of Fisheries in 2021 indicate the presence of over 175 *Hermetia illucens* farmers spanning from the Sumatra Island (western) to the Papua Island (eastern) regions, with an average production rate of 100 kg per day (2). One challenge associated with small-scale production is the cost factor. Implementing good manufacturing practices could help mitigate the production costs of insects. Numerous insect species have undergone evaluation as potential components of ruminant diets, with notable candidates including the larvae of the mealworm beetle (*Tenebrio molitor*), pupae of the silkworm (*Bombyx mori*), larvae of the black soldier fly (*Hermetia illucens*), and adult two-spotted cricket (*Gryllus bimaculatus*) (Figure 2). However, research into consumer and stakeholder perspectives regarding the use of insects in farm animal diets remains limited. The chemical composition and average nutritional values of commonly studied insects used in ruminant nutrition research, such as *Gryllus bimaculatus* adults, *Tenebrio molitor* larvae, *Hermetia illucens* larvae, and *Bombyx mori* pupae are fully described in Table 1.

Consequently, this article is structured as follows: following the introduction, the second section discusses insects' effects on *in vitro* ruminal fermentation characteristics, mainly focusing on ruminal digestibility and gas production *in vitro*. The third section is centered on the evidence of *in vivo* studies investigating insects on ruminants, including the impacts on ruminal fermentation, productive performance, and health. The fourth section presents an economic evaluation of insect protein compared to alternative protein sources. The fifth section then thoroughly examines the legislative framework necessary for introducing a novel protein source into the specific sector of ruminant nutrition, specifically scrutinizing the

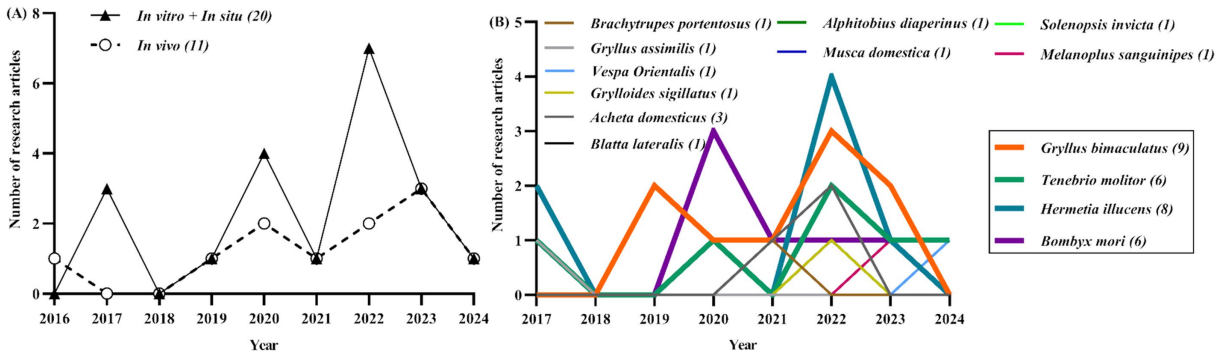

FIGURE 2
The number of original scientific research articles (excluding review articles) specifically investigating the relationship between insects and ruminant nutrition was determined using Google Scholar, Science Direct, PubMed, Web of Science, and Scopus databases. The search utilized the following keywords: **(A)** "insect, rumen" or **(B)** the names of insect species previously studied concerning ruminants by researchers. The highlighted insect species (bolded lines—*Gryllus bimaculatus*, *Tenebrio molitor*, *Hermetia illucens*, and *Bombyx mori*) were emphasized due to their frequent study in this context.

governmental regulations governing insect utilization in the European Union. The sixth section delves into ethical considerations surrounding the use of insects. The seventh section summarizes the current research gaps and outlines future directions for applying insects in ruminant nutrition. Subsequently, the eighth section (Supplementary material 1) offers insights into statistical modeling and prediction of the optimal inclusion level of insects in the ruminants' diet based on *in vitro* and *in vivo* studies, focusing on crucial aspects of ruminal fermentation driven by data availability. Finally, the conclusion will recapitulate the existing challenges and propose avenues for future research in this domain.

2 Ruminal digestibility and gas kinetics affected by various insects-based feeds

2.1 *Gryllus bimaculatus* adults

Supplementary Tables S1, S2 (Supplementary material 2) presents the *in vitro* experiments examined in this review regarding the impact of different insects on ruminal fermentations. The detailed ruminal *in vitro* fermentation profiles of specific insects, *Gryllus bimaculatus* and *Bombyx mori*, have been outlined in Supplementary Table S6 (Supplementary material 2). Figure 3 illustrates the ruminal fermentation metrics based on *in vitro* and *in vivo* studies examining the effects of various insect species and morphological stages. In a study conducted by Renna et al. (5), the *in vitro* ruminal fermentation characteristics were examined after 24 h of incubation using *Gryllus bimaculatus* adult meal as the incubation substrate, compared with control meals. The findings revealed significant reductions in total gas production with *Gryllus bimaculatus* adult meal treatments, showing 72.6, 70.6, and 57.3% for soybean, rapeseed, and sunflower meals, respectively. Similarly, methane (CH_4) production was notably lower with *Gryllus bimaculatus* adult meal treatments, with reductions of 79, 73.9, and 62.4% for meals of soybean, rapeseed, and sunflower, respectively. However, due to high fat and chitin content, the *in vitro* organic matter digestibility (IVOMD) in *Gryllus bimaculatus* adult meal treatments also decreased significantly by 45, 36, and 21% compared to soybean meal, rapeseed meal, and sunflower meal, respectively. Furthermore, the total saturated fatty acid (SFA) content was significantly lower by 6.79% compared to the soybean meal group. Nonetheless, the digestibility of *Gryllus bimaculatus* adult meal was relatively low, potentially limiting their utilization as feed ingredients.

To enhance the feeding value of *Gryllus bimaculatus* adult meals, specific treatments or processing methods targeting the removal of the exoskeleton fraction or chitin may be necessary. Comparative analysis of these findings with other studies demonstrates the impact of cricket exoskeleton removal through manual methods (removing the head, legs, and wings of oven-dried crickets) or chemical extraction (delipidation), resulting in a chitin reduction of 54.5 and 100%, respectively. When used as the sole substrate for *in vitro* ruminal fermentation, exoskeleton removal and chemical extraction of crickets were found to increase *in vitro* dry matter digestibility—IVDMD (and IVOMD) by 1.9% (2%) and 2% (1.7%) compared to whole cricket meal, respectively. Furthermore, in the same study, researchers reported that using crickets after exoskeleton removal and whole cricket meal to fully replace SBM at an inclusion level of 30% in the diet increased IVDMD by 1.9% and IVOMD by 2.7%, respectively. However, these increases were not statistically significant (27). In a study conducted by Ahmed et al. (19), it was observed that supplementing the diet with 10% *Gryllus bimaculatus* adult meal resulted in the replacement of 25% of SBM in the control group. This substitution significantly reduced total gas production by 16.5 and 12.1% per gram of DM and digestible dry matter (DDM), respectively. CH_4 production also decreased significantly by 26.7 and 22.5%, respectively. Interestingly, the digestibility of DM, OM, NDF, and ADF did not exhibit significant differences and yielded similar results between the experimental and control groups. These findings suggest that the investigated insects could be a sustainable alternative to replace 25% of the high-quality and expensive protein source, soybean meal, at a 10% inclusion level without inducing any adverse effects.

These findings highlight the potential of using *Gryllus bimaculatus* adult meal as a viable and environmentally friendly protein source in livestock feed formulations. Further research is warranted to explore long-term effects on animal performance and

TABLE 1 The nutritive value variability of the selected insect species[1].

Item	*Gryllus bimaculatus*		*Tenebrio molitor*		*Hermetia illucens*		*Bombyx mori*	
Commonly name	*Two-spotted cricket*		*Mealworm beetle*		*Black soldier fly*		*Silkworm*	
Family	*Gryllidae*		*Tenebrionidae*		*Stratiomyidae*		*Bombycidae*	
Order	*Orthoptera*		*Coleoptera*		*Diptera*		*Lepidoptera*	
Stage	*Adult*	(n)	*larvae*	(n)	*larvae*	(n)	*Pupae*	(n)
Dry matter, %	95.5 ± 1.16	4	95.4 ± 3.17	12	94.3 ± 4.24	10	93.5 ± 3.61	8
Organic matter, % DM	94.9 ± 2.25	7	95.5 ± 1.35	21	92.1 ± 3.68	16	93.7 ± 3.95	20
Crude ash, % DM	5.06 ± 2.24	7	4.47 ± 1.35	21	7.26 ± 2.78	15	6.33 ± 3.95	20
Crude protein, % DM	59.8 ± 4.95	8	51.7 ± 6.08	28	43.9 ± 8.51	17	59.7 ± 12.3	44
Crude fat, % DM	21.3 ± 11.3	8	29.7 ± 5.20	25	25.2 ± 9.98	15	23.2 ± 8.99	33
Crude fiber, % DM	8.75 ± 1.34	5	6.21 ± 1.06	8	6.00 ± 2.69	2	7.01 ± 4.20	16
Nitrogen-free extract, % DM	10.1 ± 5.13	3	14.5 ± 1.13	2	ND	-	5.44 ± 1.17	2
Neutral detergent fiber, % DM	35.4 ± 3.22	2	15.2 ± 5.90	6	22.7 ± 8.67	7	35.3 ± 7.01	2
Acid detergent fiber, % DM	18.5 ± 8.14	2	8.98 ± 1.80	9	10.5 ± 5.32	7	14.9 ± 8.10	2
Chitin, % DM	6.48 ± 0.95	2	6.59 ± 2.23	16	4.51 ± 1.29	8	8.61 ± 1.73	2
Gross Energy, kcal/100 g	456.3 ± 70.6	4	598.7 ± 66.2	9	499.3 ± 71.7	3	543.4 ± 77.9	3
Minerals, mg/100 g DM								
Macrominerals								
Calcium (Ca)	152.3 ± 77	4	29.2 ± 7.77	12	2,155 ± 879	15	97.6 ± 53.0	7
Phosphorus (P)	918 ± 235	3	926.7 ± 200	15	772.1 ± 202	14	689.4 ± 134	5
Potassium (K)	1,053 ± 38.7	2	911.5 ± 162.1	10	1,528 ± 519	16	618 ± 193	5
Sodium (Na)	334.2 ± 149.4	3	157.7 ± 69.4	8	344.2 ± 189	12	35.7 ± 8.67	2
Magnesium (Mg)	109 ± 35.3	3	227.7 ± 39.2	10	341.8 ± 135	17	211.5 ± 85.2	6
Microminerals								
Manganese (Mn)	6.97 ± 3.48	3	1.19 ± 0.41	9	18.2 ± 3.95	13	1.60 ± 0.44	6
Iron (Fe)	8.44 ± 3.32	4	5.52 ± 2.01	9	30.9 ± 8.11	12	3.55 ± 0.77	6
Copper (Cu)	3.42 ± 1.41	3	1.57 ± 0.35	10	0.84 ± 0.11	11	1.06 ± 0.31	5
Zinc (Zn)	18.6 ± 5.24	4	12.7 ± 3.04	10	10.6 ± 3.21	11	14.1 ± 6.90	5
Fatty acids, g/100g DM (unless otherwise stated)								
C18:1 *c*9 Oleic	6.16 ± 4.59	2	14.7 ± 1.17	2	12.4 ± 5.18	18	31.1 ± 8.99[a]	5
C18:2 *c*9*c*12 Linoleic	2.77 ± 1.95	2	7.38 ± 0.27	2	17.4 ± 6.49	18	6.80 ± 2.94[a]	18
C18:3 *c*9*c*12*c*15 α-linolenic	0.05 ± 0.04	2	0.12 ± 0.01	2	1.65 ± 0.59	18	30.9 ± 10.5[a]	18
∑ n-3 FA	0.08 ± 0.003	2	0.11 ± 0.00	1	2.06 ± 0.81	18	ND	-
∑ n-6 FA	2.90 ± 1.91	2	7.67 ± 0.00	1	18.6 ± 6.12	18	ND	-
∑ SFA	8.01 ± 6.73	2	6.70 ± 0.34	2	62.5 ± 10.6[a]	18	34.5 ± 0.00[a]	1
∑ MUFA	6.49 ± 4.75	2	16.6 ± 0.00	1	15.9 ± 5.09[a]	18	57.0 ± 0.00[a]	1
∑ PUFA	3.07 ± 1.79	2	7.78 ± 0.00	1	22.9 ± 3.79[a]	12	8.50 ± 0.00[a]	1
Total FA	17.6 ± 9.69	2	29.9 ± 2.05	2	23.4 ± 8.84	10	-	-
Amino acids, % DM (unless otherwise stated)								
EAA								

(Continued)

TABLE 1 (Continued)

Item	*Gryllus bimaculatus*		*Tenebrio molitor*		*Hermetia illucens*		*Bombyx mori*	
Commonly name	*Two-spotted cricket*		*Mealworm beetle*		*Black soldier fly*		*Silkworm*	
Histidine	2.04 ± 0.66	2	2.11 ± 0.67	14	1.19 ± 0.22	8	9.07 ± 6.32[b]	6
Lysine	2.66 ± 0.33	2	3.07 ± 0.84	14	2.22 ± 0.47	8	5.33 ± 2.23[b]	7
Threonine	1.84 ± 0.23	2	2.34 ± 0.56	14	1.60 ± 0.47	8	5.68 ± 2.43[b]	7
Isoleucine	2.26 ± 0.13	2	2.28 ± 0.42	14	1.68 ± 0.32	8	2.83 ± 0.95[b]	7
Leucine	3.93 ± 0.06	2	3.43 ± 0.55	14	2.51 ± 0.69	8	4.42 ± 2.31[b]	7
Methionine	0.57 ± 0.42	2	0.78 ± 0.24	13	0.79 ± 0.19	9	4.02 ± 1.74[b]	5
Phenylalanine	2.04 ± 0.29	2	2.27 ± 0.66	14	1.55 ± 0.27	8	4.08 ± 2.54[b]	7
Tryptophan	0.27 ± 0.00	1	0.36 ± 0.08	8	0.58 ± 0.05	3	1.58 ± 0.21[b]	5
Valine	3.35 ± 0.21	2	3.32 ± 0.71	14	2.32 ± 0.57	8	4.63 ± 1.04[b]	7
NEAA								
Arginine	3.54 ± 0.09	2	3.18 ± 0.71	14	1.77 ± 0.41	8	3.05 ± 1.44[b]	7
Tyrosine	2.75 ± 0.03	2	3.83 ± 0.76	13	2.63 ± 0.50	5	5.77 ± 1.27[b]	7
Cysteine	2.74 ± 0.34	2	1.04 ± 0.79	10	0.46 ± 0.38	10	0.92 ± 0.50[b]	5
Alanine	5.17 ± 0.67	2	4.27 ± 0.93	13	2.67 ± 0.81	8	5.72 ± 2.13[b]	7
Glycine	3.32 ± 0.01	2	2.64 ± 0.42	13	2.59 ± 1.09	10	6.95 ± 2.02[b]	7
Proline	2.40 ± 0.58	2	4.14 ± 1.24	13	2.43 ± 0.81	8	6.51 ± 2.68[b]	7
Glutamic	6.58 ± 0.27	2	6.45 ± 1.12	13	3.94 ± 1.56	8	16.2 ± 4.00[b]	7
Serine	2.03 ± 1.00	2	2.36 ± 0.42	13	1.69 ± 0.45	9	5.63 ± 2.97[b]	7
Aspartic	3.24 ± 0.52	2	4.47 ± 1.28	13	3.36 ± 0.93	8	8.23 ± 4.01[b]	5

[1]The presented values are based on the literature listed separately in Supplementary material 3.
[a]Values given as a percentage of the total fatty acid and their respective SD.
[b]Values are given as a percentage of the total AA and their respective SD.
EAA, Essential amino acid; NEAA, Non-essential amino acid; ND, Not detected. Data are presented as the mean ± SD.

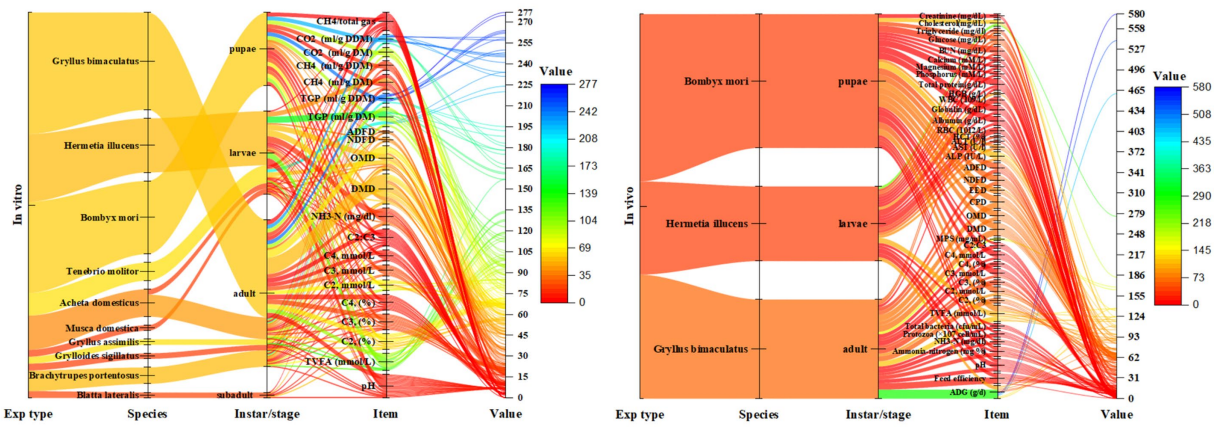

FIGURE 3
The comprehensive assessment of the impact of various insect species on ruminant nutrition, as examined through both *in vitro* and *in vivo* studies; NH_3, Ammonia; TVFA, Total volatile fatty acids; C_2, Acetate; C_3, Propionate; C_4, Butyrate; DMD, Dry matter digestibility; OMD, Organic matter digestibility; NDFD, Neutral detergent fiber digestibility; ADFD, Acid detergent fiber digestibility; TGP, Total gas production; DM, Dry matter; DDM, Degraded dry matter; CH_4, Methane; CO_2, Carbon dioxide. The value means of the corresponding measurements obtained from the original data gathered across all investigations encompassing insects on ruminants, as examined in this paper (both *in vitro* and *in vivo*), were depicted graphically.

health. The optimal inclusion levels of *Gryllus bimaculatus* adult's meal in ruminant diets have been demonstrated to play a pivotal role. In a recent study by Khonkhaeng et al. (31), the inclusion of *Gryllus bimaculatus* adult meal in ruminant diets was examined across a range from 65.1% to 70% by eight treatments, with a gradient increase of 0.7% for each treatment. The authors observed that when *Gryllus bimaculatus* adult meals were included at levels up to 67.9% in the diet, there was a significant linear decrease in IVOMD without

affecting total gas production. However, concerning IVDMD, it was suggested that *Gryllus bimaculatus* adult meal in ruminant diets should be maintained at levels below 65.1% to avoid compromising IVDMD. This outcome is contrary to that of Ahmed and Nishida (1), who observed a linear decrease in IVDMD with the inclusion of *Gryllus bimaculatus* adults at 30% of the diet compared to the control group; this study aligns with existing literature. Specifically, it observed reductions of 16.6% in total gas production and 12.5% in CH_4 production, consistent with prior research. Hence, the authors concluded that including *Gryllus bimaculatus* adult meal at up to 20% of the diet did not adversely affect nutrient digestibility.

To better understand the ideal inclusion levels, future trials could explore varying forage-to-concentrate (F:C) ratios, as investigated in studies such as Khonkhaeng et al. (31) (F:C 70:30) and Ahmed and Nishida (1) (F:C 60:40). These additional investigations would contribute valuable insights into optimizing *Gryllus bimaculatus* adult meal inclusion in ruminant diets. In a previous study emphasizing the significance of F:C ratios, researchers investigated the substitution of *Gryllus bimaculatus* adult meal for SBM at varying levels (25, 50, 75, and 100% replacement) that corresponded to inclusion levels of *Gryllus bimaculatus* adult meal in the diet at 4, 8, 12, and 16%, respectively. The F:C ratios were expressly set at 60:40 and 40:60. The study revealed that IVDMD was significantly higher with a F:C ratio of 40:60 compared to the corresponding ratio of 60:40. Similarly, a reduction in CH_4 production was observed when the F:C ratio was decreased while maintaining the same level of *Gryllus bimaculatus* adult meal in the diet (23). These findings underscore the importance of F:C ratios in optimizing nutrient utilization and CH_4 emissions in ruminant diets supplemented with *Gryllus bimaculatus* adult meal. In summary, these findings underscore the need for further investigation, including feeding trials *in vivo*, to better understand and optimize the utilization of *Gryllus bimaculatus* adult meal as a potential feed resource.

2.2 *Tenebrio molitor* larvae

Regarding the impact of *Tenebrio molitor larvae* meal on ruminal fermentation characteristics, this study revealed notable effects when used as the sole substrate. *Tenebrio molitor larvae* meal significantly reduced total gas production by 68.6, 66.2, and 51% compared to soybean, rapeseed, and sunflower meals, respectively. However, IVOMD decreased by 41, 32, and 17% compared to soybean, rapeseed, and sunflower meals, respectively. Additionally, the SFA content in the ruminal fluid was reduced by 53.2, 44, and 41.1% when compared to soybean (SBM), rapeseed, and sunflower meals, respectively. These outcomes are attributed to the higher fat content in *Tenebrio molitor larvae* meal (39.2%) compared to SBM (0.6%), rapeseed meal (2.8%), and sunflower meal (1.7%) (5). This discovery aligns with the findings of Jayanegara et al. (3), who reported that the higher fat content in *Tenebrio molitor* meal (20.3%) compared to SBM (2.7%) led to a significant reduction in IVDMD and IVOMD by 29.2 and 26.1%, respectively. Moreover, total gas and CH_4 production were significantly decreased by 46.7 and 55.1%, respectively, when *Tenebrio molitor* meal was used as the sole substrate during 24-h anaerobic *in vitro* fermentation. A noteworthy discovery emerged when comparing *Tenebrio molitor* with two other non-plant protein sources: grasshopper meal (*Melanoplus sanguinipes*) and ant egg meal (*Solenopsis invicta*). This study observed that *in vitro* ruminal fermentation decreased total gas and CH_4 production with these alternative protein sources while maintaining IVDMD (32). The research conducted by Hanönü et al. (29) demonstrated that supplementing alfalfa hay with *Tenebrio molitor larvae* meal at levels of 0.5, 1, and 1.5% led to a significant increase in IVOMD, both linearly and quadratically. A possible explanation for this effect could be attributed to the *in situ* ruminal dry matter (DM) digestibility of *Tenebrio molitor larvae* meal, which was determined to be 85.7% after 24 h, surpassing alfalfa. The *in vitro* degradable protein content was similar (around 60%) between SBM and *Tenebrio molitor larvae* meal (33).

2.3 *Hermetia illucens* larvae

The detailed ruminal *in vitro* fermentation profiles of specific insects, *Hermetia illucens* and *Acheta domesticus*, have been compiled in Supplementary Table S6 (Supplementary material 2). Regarding the impact of *Hermetia illucens larvae* meal on ruminant nutrition, studies have reported a significant decrease in both IVDMD and IVOMD by 35 and 34%, respectively, when used as the sole substrate compared to SBM after a 48-h incubation period. Additionally, total gas and CH_4 production were markedly reduced by 56.3 and 67.5%, respectively, in the *Hermetia illucens larvae* meal group compared to SBM (3). This finding broadly supports the work of other studies in this area linking *Hermetia illucens larvae* meal with IVOMD. According to Renna et al. (5), IVOMD was notably lower by 46, 37, and 22% in *Hermetia illucens larvae* meal compared to SBM, rapeseed meal, and sunflower meal, respectively. Consequently, there was a reduction in total gas production by 71.3, 69.2, and 55.3%, and CH_4 production by 77.5, 72.1, and 59.8% by *Hermetia illucens* larvae compared to SBM, rapeseed meal, and sunflower meal, respectively. This effect is attributed to the high fat (26.9%) and chitin content (5.2%) in *Hermetia illucens* larvae. Consistent with these findings, a prior study has shown that chemically defatted (using a hexane solution) and mechanically defatted (using an expeller) *Hermetia illucens* larvae led to significant increases in IVDMD and IVOMD by 26.7% (27.1%) and 14.9% (26.5%), respectively, compared to the intact *Hermetia illucens* larvae meal group. These effects were observed when these different insect inclusion levels were at 20% in the diet without influencing CH_4 production (34). Hence, extracting fat from *Hermetia illucens larvae* is essential for optimizing the insect's suitability as a feed ingredient for ruminant livestock. This finding aligns with recent research demonstrating that supplementing the diet with defatted *Hermetia illucens larvae* meal at 3.2%, representing a 20% substitution for SBM, led to notable increases in *in vitro* neutral detergent fiber digestibility (IVNDFD) over a 24-h incubation period, following a linear and quadratic trend compared to the control group. Moreover, IVDMD and IVNDFD exhibited enhancements of 6.31 and 4.64%, respectively, with defatted *Hermetia illucens larvae* meal at 3.2% in the diet during a 48-h incubation period. These outcomes may be attributed to the lower inclusion rate of *Hermetia illucens larvae* meal (3.2%) without significantly impacting the fat content (3.74%) when compared to the control group's fat content (3.19%) (24).

In summary, substituting SBM with *Hermetia illucens larvae* meal in ruminant diets often reduces nutritional quality *in vitro*. The main challenges associated with incorporating *Hermetia illucens larvae* meal include their significant chitin content, indicated by elevated levels of neutral detergent insoluble crude protein and acid detergent insoluble crude protein, as well as their high-fat content, which can adversely affect ruminal digestibility. Despite these challenges, a

distinct advantage of using *Hermetia illucens larvae* meal over SBM is their lower CH_4 emissions. Enhancing the nutritional value of *Hermetia illucens larvae* meal requires the application of specific treatments or processing methods.

2.4 *Bombyx mori* pupae

Ahmed et al. (19) reported that including 10% *Bombyx mori* pupae meal in the diet, replacing 25% of SBM, did not affect IVDMD, IVOMD, IVNDFD, or *in vitro* acid detergent fiber digestibility (IVADFD) compared to the control group, which included 40% SBM. However, the production of carbon dioxide (CO_2) and CH_4 per gram of DDM was significantly reduced by 13.7 and 19.4%, respectively, in the *Bombyx mori* pupae meal group compared to the control group. Notably, chitin is a component known to be poorly digested by animals and can contribute to lower IVDMD and IVOMD. The chitin content in the *Bombyx mori* pupae meal was measured at 9.83%. The inclusion of insects at a substitution level of 25% for SBM in this study did not negatively affect nutrient digestibility, likely due to the relatively low inclusion rate employed. Additional research is needed to explore the effects of higher inclusion levels of this insect, mainly when replacing soybean meal entirely. This investigation could assess their potential as effective options for reducing CH_4 production in ruminants.

Therefore, Ahmed and Nishida (1) conducted a study examining the inclusion of different levels (10, 20, 30, and 40%) of *Bombyx mori pupae* meal in the diet. The authors observed that including *Bombyx mori pupae* meal up to 30% in the diet resulted in a linear and quadratic decrease in IVDMD compared to the control group, which consisted of 300 mg of grass hay and 200 mg of concentrate mixture during a 24-h fermentation period. Furthermore, including 20% *Bombyx mori pupae* meal in the diet was deemed a safe threshold as it did not significantly impact IVDMD but led to a notable reduction in total gas and CH_4 production by 9.2 and 9.9%, respectively. It suggests that a 20% inclusion level of *Bombyx mori pupae* meal could be a suitable option for minimizing CH_4 emissions without affecting DM digestibility. Further trials were conducted using different F:C ratios to better understand the ideal inclusion levels. Based on the findings, it can be concluded that supplementing *Bombyx mori pupae* oil at a 2% level reduces CH_4 production by 12%–15% without negatively impacting feed fermentation. The reduction in CH_4 may be more notable when the oil supplement is added to a high-concentrate diet (F:C; 70:30) compared to a diet with a lower concentrate ratio (F:C; 40:60), resulting in reductions of 5.28 and 4.52%, respectively, compared to the control group (no oil supplement). Thirumalaisamy et al. (35) (Supplementary Table S7 in Supplementary material 2) presents the variations in ruminal fermentation parameters observed in response to different insects during the *in vitro* experiments. *Hermetia illucens* supplementation led to a notable decrease in acetate production by 34.5% compared to the control group ($p=0.03$). As a result, there was a pronounced reduction in the acetate:propionate (C_2:C_3) ratio ($p=0.03$; Supplementary Table S7 in Supplementary material 2).

3 Insects-based diet in ruminant feeding: *in vivo* trials overview

Supplementary Table S8 (Supplementary material 2) presents descriptive statistics for the variables used in assessing the impact of *Hermetia illucens*, *Tenebrio molitor*, *Bombyx mori*, and *Vespa orientalis* on blood biochemical parameters. Astuti et al. (36) documented that incorporating cricket meal at a concentration of 30% within the concentrate for post-weaning Etawah crossbred goats resulted in physiological responses (rectal temperature, heart rates, and respiration rate) that fell within normal ranges. However, the experimental group exhibited 182% significantly higher crude fat intake compared to the control group. Importantly, no adverse effects on ruminal fermentation profiles were observed, and the goats in the experimental group performed comparably to those on the control ration. These findings are consistent with earlier observations indicating that incorporating 15% cricket meal (replacing 100% soybean meal) as a protein source in lamb rations does not adversely affect palatability, performance, digestibility of DM and crude protein, feed efficiency, or blood metabolite profiles (including glucose, triglycerides, and total protein).

Furthermore, utilizing 7.5% cricket meal in lamb rations has been shown to reduce CH_4 production, as reported by the authors significantly. Therefore, replacing soybean meal with 7.5% cricket meal may be more advantageous, considering the positive impact on CH_4 reduction (37). Another example of this is the study carried out by Phesatcha et al. (4), which demonstrated that incorporating adult cricket meal (*Gryllus bimaculatus*) at 8% of the ration resulted in a significant linear increase of 25.6% in average daily gain and 7.46% in apparent digestibility of crude protein in Thai native male beef cattle. This increase was accompanied by linearly significant rises in rumen ammonia-nitrogen (26.5%) and blood urea nitrogen (6.4%). Furthermore, total volatile fatty acids (TVFA) were linearly increased by 26.5%, predominantly due to a 4.2% higher propionic acid level compared to the control group when cricket meal was included at 12% of the ration. Their study highlighted that cricket meal had a CP content of 62.4%, higher than soybean meal (SBM), influencing the alteration in TVFA.

Consequently, the C_2:C_3 ratio was significantly reduced. Moreover, estimated CH_4 emissions decreased by 20.9%, partially explained by a 35.9% decrease in protozoa when cricket meal completely replaced SBM in the ration. These findings suggest the potential benefits of cricket meal in improving cattle performance and reducing CH_4 emissions in feed formulations. Moreover, several research studies have recently investigated the use of defatted silkworm pupae meal in ruminant nutrition. Rashmi et al. (38) conducted a study that concluded that defatted silkworm pupae meal could be safely included at a level of 4.1% in cattle concentrate mixtures (substituting soybean meal up to 30%) without adverse effects on health or performance. This finding suggests that defatted silkworm pupae meal is a promising alternative to traditional protein sources for cattle, offering both nutritional benefits and cost advantages. A notable aspect of their study is the cost-effectiveness of defatted silkworm pupae meal compared to soybean meal. The price of defatted silkworm pupae meal was found to be 51.2% lower than soybean meal when calculated on per kilogram of crude protein basis. This cost advantage further enhances the appeal of defatted silkworm pupae meals as a viable protein source for cattle feed formulations. A notable finding from the earlier-reported results highlights the effective use of silkworm pupae oil to enhance ether extract digestibility by approximately 10% and reduce enteric CH_4 emissions by 17.5%–20.5%. These improvements were achieved without compromising nutrient intake or digestibility when oil

supplementation was administered continuously (daily) or intermittently (alternate week) at a consistent level of 2% of the diet.

Furthermore, the observed reduction in CH_4 emissions is attributed to a decrease in protozoa population. Expressly, significant decreases were noted in total protozoa (39.8%–42%) and *Isotrichidae* (40.3%–41.8%) (39). These findings align with a meta-analysis by Dai et al. (40), which demonstrated that CH_4 emissions correlate positively with total rumen protozoa and *Isotrichidae* but not with *Ophyroscolecidae*. In summary, using silkworm pupae oil as a supplement in livestock diets shows promise for improving nutrient digestibility and reducing CH_4 emissions through targeted modulation of rumen microbial populations. Further research could contribute valuable insights into sustainable livestock production practices.

Only one study has explored the effects of *Oriental Hornet* meal on lamb nutrition (41). The findings from this study revealed significant improvements in the digestibility of DM, organic matter (OM), crude protein, and ether extract when *Oriental Hornet* meal was included at a level of 3.42% of the ration. Specifically, digestibility increased by 2.32, 2.99, 9.74, and 1.93%, respectively. Moreover, including *Oriental Hornet* meal at this level led to notable enhancements in average body weight gain (30.9%) and growth rate (30.7%) compared to the control group. This improvement can be attributed to the higher total digestible nutrients and digestible crude protein content in the experimental ration, which were 1.56 and 1.43% higher than the control group, respectively, due to the substitution of *Oriental Hornet* meal for SBM. An intriguing finding was the significantly increased economic efficiency of 19.1% observed in the experimental group compared to the control group. This higher economic efficiency suggests a higher net return from using *Oriental Hornet* meal, making it potentially well-suited for the Egyptian market. In summary, the limited study on *Oriental Hornet* meal in lamb nutrition demonstrated promising effects on digestibility, growth performance, and economic efficiency. Further research could provide valuable insights into the potential utilization of *Oriental Hornet* meals as a cost-effective and beneficial protein source for ruminants, particularly in specific regional markets like Egypt (41).

In addition, recent research comparing the supplementation of 4% *Hermetia illucens* oil to sheep ration vs. no supplementation has shown significant increases in both TVFA and total bacteria in the ruminal fluid, with increments of up to 44.8 and 77.1%, respectively (28). The variation in total bacterial population can be attributed to several factors, including differences in rations, types of feed, timing and methods of rumen fluid collection, and feeding frequency. Rations containing easily digestible protein and carbohydrates promote bacterial growth in the rumen. In the study by Ningsih et al. (28), the experimental diets exhibited a total digestible nutrients (TDN) content up to 5% higher than the control meal. This increase likely contributed to the observed rise in the TVFA in the rumen, presumably due to the higher bacterial population resulting from the addition of black soldier fly oil supplementation. Consistent with the findings of this study, previous research has shown that the addition of *Hermetia illucens* fat at a level of 0.2% in the ration of multiple-breeding black-motley cows resulted in a significant increase in TVFA production in the rumen (42).

Recent investigations have explored the impact of incorporating *Hermetia illucens* meal into sheep nutrition. Researchers observed that replacing soybean meal with black soldier fly larvae did not negatively affect the performance or hematological profile of the sheep. Notably, body weight gain tended to increase ($p = 0.082$), and feed conversion ratio tended to decrease ($p = 0.089$) when *Hermetia illucens larvae* meal was included at 2.5 and 5% of the ration, respectively. Furthermore, analysis of blood leukocyte differentiation, including lymphocytes, monocytes, neutrophils, eosinophils, and basophils, showed no significant differences, indicating that all animals maintained a healthy status (43). Because lymphocytes play a central role in adaptive immunity, recognizing and targeting specific pathogens. Monocytes can differentiate into macrophages upon entering tissues, where they play a vital role in engulfing and digesting pathogens. Neutrophils, the most abundant white blood cells, act as the body's primary defense against infections by engulfing and destroying bacteria through phagocytosis. Eosinophils combat parasitic infections and regulate allergic responses by releasing toxic proteins. Basophils release histamine and other chemicals involved in allergic reactions, contributing to the inflammatory response and defense against certain parasites (44).

Moreover, Supplementary Tables S1–S5 (Supplementary material 2) contains a comprehensive list of both *in vitro* and *in vivo* experiments discussed in the review, detailing experimental methodologies such as methods used, incubation times or experimental periods, information about animal donors including their status and feeding regimens, specifics of treatments applied, insect species studied, and ethical approvals obtained. Besides, depending on the species, form, and inclusion level of insects, substituting soybean meal can have varying degrees of impact on ruminal fermentation indices and performance, as detailed in Table 2. Moreover, descriptive statistics of the variables in the database used to evaluate the effect of *Gryllus bimaculatus, Hermetia illucens,* and *Bombyx mori* on ruminal fermentation parameters in ruminants (*in vivo*) have been shown in Supplementary Table S9 (Supplementary material 2). Supplementary Table S10 (Supplementary material 2) displays the impact of various insects on ruminal fermentation parameters. The *Gryllus bimaculatus* treatment yielded a significant increase ($p < 0.01$) in ruminal pH, rising by 3.76% compared to the control. *Vespa Orientalis* treatments enhanced the apparent digestibility of DM by 7% ($p = 0.003$) compared to the control.

Furthermore, the *Bombyx mori* treatment notably increased the apparent digestibility of acid detergent fiber (ADF) compared to the control treatment ($p = 0.007$; Supplementary Table S10 in Supplementary material 2). Supplementary Table S11 (Supplementary material 2) illustrates their influence on biochemical parameters in the context of *in vivo* experiments. None of the dietary insect interventions elicited discernible alterations in the blood biochemical profiles of ruminants compared to the control.

4 Economic evaluation of insect protein compared to alternative protein sources

Table 3 demonstrates the economic feasibility of selected insects relative to plant-based protein sources. The current prices of soybean meal, rapeseed meal, and sunflower meal feeds are approximately €0.486, €0.3, and €0.237 per kg, respectively. Meanwhile, the current prices of *Hermetia illucens* and *Tenebrio molitor* are approximately €7.25 and €14.5 per kg, respectively. Therefore, for a comprehensive assessment between insects and

TABLE 2 Effect of various invertebrate insects used as either protein or fat (energy) source carrier on the ruminant species response.

Source (*form*)	Species	Replaced meal	Inclusion level (*Substitution level*)	Results	Reference
Gryllus bimaculatus	Beef cattle[1]	Soybean meal	4%; 8%; 12%	Replacing SBM with cricket meal in the concentrated feed mixture at up to 100% improved nutrient digestibility and ruminal fermentation efficiency in Thai native beef cattle fed a diet primarily composed of rice straw. This substitution resulted in increased production of volatile fatty acids, particularly propionate, and enhanced microbial protein synthesis. Additionally, protozoal populations decreased, and CH_4 production in the rumen was mitigated.	(4)
(*Full-fat meal pellet*)			(*33%; 67%; 100%*)		
Hermetia illucens	Sheep[2]	Supplement oil	4%	The addition of calcium soap black soldier fly oil to the ration of Garut sheep has been shown to elevate total volatile fatty acid levels and bacterial population without affecting rumen pH, ammonia concentration, or protozoa population.	(28)
(*Full-fat*)			(*No specific substitution*)		
Gryllus bimaculatus	Goats[3]	Soybean meal	15%; 30%	Incorporating cricket meal at levels of up to 30% in the concentrate portion of diets for growing goats has demonstrated favorable palatability, with no discernible adverse impacts on ruminal fermentation profiles and comparable performance relative to control rations.	(36)
(*Full-fat meal*)			(*50%; 100%*)		
Gryllus bimaculatus	Lambs[4]	Soybean meal	7.5%; 15%	The study findings indicate that incorporating 15% cricket meal (as a complete replacement for soybean meal) in lamb rations is feasible without compromising palatability, performance, feed efficiency, or blood metabolite profiles. Additionally, offering lamb rations with 7.5% cricket meal leads to a notable reduction in CH_4 production. Considering these results, substituting soybean meal with 7.5% cricket meal may be more advantageous due to its CH_4-reducing effect.	(37)
(*Full-fat meal*)			(*50%; 100%*)		
Bombyx mori	Steers[5]	Soybean meal	1.4%; 2.7%; 4.1%	It was determined that dried silkworm meal could be incorporated into cattle concentrate mixtures at levels of up to 4.1% as a safe substitute for SBM without adverse effects on the health or performance of the animals. Therefore, silkworm meal presents itself as a promising alternative to traditional protein sources for cattle, offering benefits in terms of both nutritional quality and cost-effectiveness.	(38)
(*Defatted*)			(*10%; 20%; 30%*)		
Bombyx mori	Sheep[6]	Supplement oil	2%	Silkworm pupae oil, when included at 2% of the diet, has demonstrated the capability to achieve a significant reduction of approximately 15–20% in enteric CH_4 emissions while maintaining intake and nutrient digestibility. This reduction in CH_4 emissions results from a combination of reduced protozoa levels and alterations in the rumen methanogen community composition.	(35)
(*Full-fat*)			(*No specific substitution*)		
Vespa Orientalis	Lambs[7]	Soybean meal	1.14%%; 2.28%; 3.42%	Using Oriental Hornet meal, replacing soybean meal up to 30%, can enhance productive and reproductive performance, nutrient composition, physiological responses, and economic efficiency in Ossimi lambs without detrimentally affecting their performance.	(41)
(*Full-fat meal*)			(*10%; 20%; 30%*)		
Hermetia illucens	Beef cattle[8]	Supplement fat	0.02%; 0.2%	The data indicates that incorporating Black Soldier Fly Larvae fat can enhance cow productivity, immune defenses, and milk quality.	(42)
(*Full-fat*)			(*No specific substitution*)		
Hermetia illucens	Sheep[9]	Soybean meal	2.5%; 5%	Black soldier fly larvae have the potential to replace soybean meal in sheep diets without negatively impacting performance or hematological profiles.	(43)
(*Full-fat meal*)			(*50%; 100%*)		

[1]Thai native male beef cattle (2 years old; 230 ± 15 kg of BW).
[2]Garut sheep (No specific statement for BW).
[3]Post-weaning Etawah crossbred goat (2 months old; 12 ± 0.40 kg of BW).
[4]No specific species mentioned (2 months old; 11.24 ± 1.62 kg of BW).
[5]Crossbred steers (496.25 ± 5.39 kg of BW).
[6]Mandya sheep (16–18 months old; 24.1 ± 1.20 kg of BW).
[7]Ossimi lambs (20.58 ± 0.85 kg of BW).
[8]Black-motley cows (590 ± 4 kg of BW; BCS 3.15 ± 0.04).
[9]No specific species mentioned (6–8 months old; 20.42 ± 3.57 kg of BW).
BCS, Body condition score; BW, Body weight.

TABLE 3 The economic viability of insects in comparison to plant-based protein sources.

Potential source	*Hermetia illucens*[1]	*Tenebrio molitor*[1]	Soybean meal[2a]	Rapeseed meal[3a]	Sunflower meal[4a]
CP (%)	43.9	51.7	47.58	37.6	33.52
Lysine-L (%)	2.22	3.07	3.01	1.95	1.48
Methionine-M (%)	0.79	0.78	0.638	0.76	0.75
Sales prices (€/kg; SP)	7.25	14.5	0.486	0.3	0.237
Protein-prices (€/kg; PP)	16.5	28.0	1.021	0.798	0.707
Protein-L (€/kg; PL)	0.367	0.861	0.031	0.016	0.010
Protein-M (€/kg; PM)	0.130	0.219	0.007	0.006	0.005
PP to PP SBM*	16.2	35.2	1	1	1
PL to PL SBM*	11.9	55.3	1	1	1
PM to PM SBM*	20.0	36.1	1	1	1

[1]The sales prices for *Hermetia illucens* were obtained from the European Union market, particularly Germany. For Tenebrio molitor, the sales price data originated from the European Union market, specifically the Netherlands, as Niyonsaba et al. (46) indicated.
[2]The chemical composition data for SBM was adopted from Lagos and Stein (51). The average calculation was based on SBM chemical composition from five countries: China, Argentina, Brazil, the United States, and India.
[3]The chemical composition data for rapeseed meal was derived from the study by Cheng et al. (52).
[4]The chemical composition of sunflower meal was extracted from the research conducted by Liu et al. (53).
[a]The price data were obtained from the website (54) https://teseo.clal.it/en/?section=oilseeds-price-eu, accessed on April 24, 2024. The price of SBM was calculated as an average from markets in Germany, the Netherlands, Poland, Romania, and Spain. The price of rapeseed meal was calculated as an average from markets in Belgium, the Czech Republic, Denmark, Germany, Hungary, Lithuania, the Netherlands, Poland, and Romania. Similarly, sunflower meal prices were determined as an average from markets in Hungary, the Netherlands, Romania, and Spain.
*The analysis involved determining how much insect protein source would need to be allocated to match the cost of each euro of plant protein source.

traditional plant protein sources, it is essential to adjust the nutritional value based on parameters such as crude protein content or essential amino acids profile. This adjustment allows a more accurate comparison of their economic and nutritional merits. The findings indicate that replacing each euro of SBM with *Hermetia illucens* would cost 16.2 €/kg for protein, 11.9 €/kg for lysine (Lys), and 20 €/kg for methionine (Met) for farmers.

Similarly, replacing each euro of SBM with *Tenebrio molitor* would lead to costs of 35.2 €/kg for protein, 55.3 €/kg for Lys, and 36.1 €/kg for Met for farmers. The elevated cost of insect meal currently limits its application in ruminant diets. Nevertheless, to be competitive, expanding the scale of insect breeding operations within companies is expected to enhance efficiency and decrease the overall cost of insect protein production over time (45). Achieving mass production remains a distant prospect. While definitive conclusions on cost reduction or profit increase in insect production were not drawn, it has been proposed that greater mechanization could lead to reduced labor costs, and utilizing low-value feed substrates may decrease operational expenses. In terms of farm output sales, commercializing insect frass as fertilizer could offer an additional income stream for insect farmers (46). The potential of insects as a viable alternative feed component is attributed to their short life cycle.

Furthermore, projections from the International Platform of Insects for Food and Feed suggest a significant rise in the utilization of insects for food and feed within the European Union. The insect volume is expected to escalate from 500 tons in 2020 to surpass 1 million tons by 2025, reaching an estimated 3 million tons by 2030, encompassing both larvae and adult forms. This upward trajectory in market demand likely mirrors the lucrative opportunities available to stakeholders engaged in insect production. This growth is anticipated to contribute to heightened consumer awareness regarding the detrimental impacts of conventional animal feed production (45).

5 Review of regulations governing the use of insects as feed for ruminants

Insect meals are categorized as processed animal proteins and are subject to prohibitions on their utilization in numerous high-income nations (e.g., European countries). On the contrary, developing and emerging regions often lack specific legislation. For instance, in Asia, Thailand, a leading producer of crickets, is actively developing the first set of guidelines for insect breeding. In China, insects are widely used as feed and food components in various regions, yet they have not yet been officially recognized under food law (45). In the Americas, there is no specific prohibition or approval concerning the use of insect proteins in the processing, marketing, or incorporation into animal feed within this region. In the recent past, within the European Union, the approval for incorporating insects into farm animal feed was restricted to seven specific insect species, as outlined in Commission Regulation (EU) 2017/893 Commission Regulation-EU (47). These approved species encompassed two mealworm species (*Tenebrio molitor*, *Alphitobius diaperinus*), two fly species (*Hermetia illucens*, *Musca domestica*), and three cricket species (*Acheta domesticus*, *Gryllodes sigillatus*, *Gryllus assimilis*). Over time, there has been a growing expansion in the utilization of insect species for animal feed. Domestic silkworms, which exclusively consume mulberry leaves, pose no risk of contamination from animal-origin food sources that are not permitted for insect feed. Silkworms (*Bombyx mori*) have recently been added to the roster of authorized insect species for manufacturing processed animal protein utilized in animal feed, as delineated in Commission Regulation (EU) 2021/1925 (Commission Regulation-EU) (48).

Although legal regulations regarding the use of insects as feed vary regionally, researchers and feed manufacturers have a notable global interest in promoting innovation and research in this field. In the coming years, this interest may lead to legislative changes similar to those observed for monogastric animals, facilitating broader acceptance and

utilization of insects in ruminant feeding practices worldwide. In summary, regarding the current global legislative framework concerning the use of insects as feed for ruminants, both insect oil and meal are explicitly authorized in countries including Mexico, Colombia, Brazil, Morocco, Algeria, Niger, Nigeria, Sudan, South Africa, Namibia, Ethiopia, India, Australia, and New Zealand. Insect oils are authorized but not insect meals in countries such as Russia, Finland, Sweden, Norway, Iceland, the United Kingdom, Denmark, Belarus, Estonia, Ireland, France, Spain, Italy, Romania, Ukraine, and Poland. Some countries like Egypt, Ecuador, Chile, Canada, and Alaska (United States) lack specific insect regulatory frameworks. However, countries such as Argentina, Iran, Japan, North Korea, and Tunisia do not authorize insect oils or meals to be used as feed for ruminants (20). Moreover, a structured compilation of legislative documents from the European Parliament and the Council (EC) concerning insect production for food and feed is presented in Supplementary Table S12 (Supplementary material 2), arranged chronologically.

6 Ethical considerations for insects

Insects possess the potential to be incorporated into livestock production systems as a source of feed. However, insects must be cultivated on a large scale within a "mini-livestock" framework to be effective as feed. Because these large-scale rearing systems are relatively novel, formal industry standards and welfare regulations have not been fully established, resulting in unresolved questions related to insect welfare. Considering the significance of consumer attitudes in shaping the social acceptance of insect production, it is essential to analyze consumers' ethical perspectives on using insects as livestock feed. As per Fukuda et al. (49), sampling involved convenience sampling of 361 adult consumers in the United States. When queried about using insects as livestock feed, 34% of respondents expressed support, 52% remained neutral, and 15% voiced opposition. Among those opposed, 58% cited ethical concerns as their rationale for opposition. Among respondents who expressed support or neutrality regarding using insects as livestock feed, 29% identified concerns related to livestock welfare, while 26% identified concerns related to insect welfare as perceived risks. These observations suggest that insect producers have an incentive to implement best practices that are perceived as fostering high-welfare conditions for their "mini-livestock" when used for livestock feed. Moreover, the findings indicate that, although the existing research on consumer acceptance is limited, it is unlikely to impede the development of the insect protein industry for feed. Nonetheless, additional research is needed to investigate consumer willingness to pay for animal products derived from animals fed with insects and assess whether insects contribute to improved acceptability, both in terms of general perception and sensory appeal, compared to conventional products (50).

7 Current research gaps and future directions in applying insects to ruminant nutrition

The following issues warrant attention: (1) Nutrient requirements and digestibility—research gap: limited comprehensive studies on the specific nutrient requirements of ruminants when fed insect-based diets, especially the insects' CP conventional factor for proximate analysis not unified yet. Because a portion of the nitrogen is contained within chitin, it is also extracted during protein analysis using the traditional Kjeldahl method, resulting in overestimating the actual CP content. Future direction: to standardize the conventional factor for CP content in potential insect feeds for ruminants across various species and morphological stages of the insects. (2) Feed formulation optimization—research gap: insufficient knowledge about optimal feed formulations incorporating insect meals for different classes of ruminants (e.g., lactating cows, growing calves). Future direction: explore novel feed formulation strategies that maximize the nutritional value of insect-based feeds while ensuring balanced diets for ruminant health and performance. Investigate the synergistic effects of combining insects with other feed ingredients. (3) Long-term effects on animal health and performance—research gap: limited understanding of the long-term impact of insect-based diets on ruminant health, productivity, and reproductive performance. Future direction: conduct longitudinal studies to assess the effects of sustained insect feeding on rumen health, metabolic function, immunity, and overall animal performance over extended periods. Investigate potential benefits or challenges associated with prolonged insect-based feeding. More studies are required to understand the impact of insect-based diets on ruminal fermentation dynamics, microbial populations, and metabolite production. Investigating potential health risks or safety concerns associated with feeding insects to ruminants is essential. Studies should focus on assessing antinutritional factors, toxins, or allergens in insect-based feeds. (4) Environmental impact and sustainability—research gap: incomplete evaluation of the environmental sustainability aspects of using insects as feed in dairy production systems. Future direction: quantify greenhouse gas emissions, resource utilization, and ecological footprints associated with insect farming and incorporation into ruminant diets. Explore integrated systems that leverage insect farming for waste management and circular economy principles. (5) Consumer acceptance and market dynamics—research gap: limited understanding of consumer perceptions and acceptance of dairy products derived from ruminants-fed insect-based diets. Future direction: investigate consumer attitudes toward insect-fed dairy products, addressing concerns related to food safety, quality, and ethical considerations. Develop strategies to enhance market acceptance and promote the adoption of insect-derived feed in dairy production systems. (6) Regulatory framework and policy development—research gap: inadequate regulatory guidelines and policy frameworks governing the use of insects in ruminant nutrition. Future direction: collaborate with regulatory bodies to establish evidence-based standards for insect-derived feed safety and quality assurance. Advocate for policy changes that support the sustainable integration of insects into ruminant diets. (7) Innovative approaches and technology—research gap: limited exploration of innovative technologies and processing methods for optimizing insect-derived feed production and utilization in dairy systems. Future direction: explore novel approaches such as precision feeding, genetic selection for enhanced utilization of insect proteins, and advanced processing techniques to improve the efficiency and efficacy of insect-based ruminant nutrition. Research should explore different insect species and their processing methods to optimize nutrient bioavailability and ensure feed safety. Comparative studies between fresh, dried, and processed insects can provide valuable insights. Addressing these research gaps and advancing future directions will facilitate the broader adoption of insect-derived feed in ruminant nutrition, promoting sustainability, efficiency, and resilience in dairy production systems; especially for neonatal calves, particularly those with underdeveloped rumens, the abomasum assumes paramount importance. The abomasum comprises 60–70% of the calf's stomach capacity and secretes gastric juices rich in hydrochloric acid and digestive enzymes. These enzymes facilitate the breakdown of proteins, fats, and

carbohydrates in the ingested feed, whether insects or other nutrients, into simpler forms readily absorbed by the calf's body.

8 Conclusion

Recent data confirm the feasibility of integrating insects into ruminant diets, showing predominantly positive effects on growth performance, ruminal fermentation indices, and methane mitigation. However, the absence of global uniformity in insect products highlights the need for attention and standardization. To optimize the efficiency of insect and ruminant production, comprehensive assessments of economically viable insect species should be prioritized in future studies. Moreover, (1) environmental sustainability: using insects as feed aligns with sustainability goals by reducing reliance on conventional protein sources like soybean meal, which are resource-intensive and contribute to environmental degradation. Insects have a lower ecological footprint and can be produced using organic waste streams. (2) Improved feed efficiency: Insect-derived feeds offer opportunities to optimize feed efficiency in ruminants, potentially enhancing animal performance and productivity; nevertheless, the expenses associated with feed must be tackled. (3) Consumer acceptance and market trends: despite initial consumer reservations, there is growing interest in insect-fed dairy products due to their sustainability credentials and nutritional benefits. Dairy producers can leverage this trend to diversify product offerings and capture niche markets. (4) Research and development: continued research is needed to address knowledge gaps related to nutrient requirements, feed formulation, long-term health effects, and market dynamics surrounding insect-based ruminant nutrition. (5) Policy and regulatory considerations: policymakers and industry stakeholders should collaborate to establish clear guidelines and regulations governing the use of insects in ruminant diets, ensuring food safety and quality standards are met. Adopting insect-based feed strategies holds significant promise for enhancing ruminant nutrition and advancing environmental sustainability in dairy production. Dairy producers can benefit from diversifying feed sources, reducing reliance on traditional protein sources, and improving overall feed efficiency. Researchers should prioritize studies to optimize insect-derived feed formulations and assess their long-term impacts on ruminant health and performance. Policymakers and industry stakeholders play a crucial role in facilitating the adoption of insect-based feed by establishing supportive regulatory frameworks and promoting consumer acceptance. By embracing insect-based nutrition, the dairy industry can contribute to a more sustainable and resilient agricultural future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

MG: Conceptualization, Methodology, Project administration, Resources, Software, Visualization, Writing – original draft. ME: Data curation, Supervision, Writing – review & editing. MS-S: Data curation, Writing – review & editing. AC: Data curation, Writing – review & editing. YY: Methodology, Writing – review & editing. AI: Data curation, Methodology, Validation, Writing – review & editing. BX: Data curation, Validation, Writing – review & editing. Z-jC: Writing – review & editing. IF: Writing – review & editing. HJ: Writing – review & editing. AA: Data curation, Writing – review & editing. Y-bL: Data curation, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1484870/full#supplementary-material

SUPPLEMENTARY MATERIAL 1
Data processing and meta-analysis.

SUPPLEMENTARY MATERIAL 2
Datasheet.

SUPPLEMENTARY MATERIAL 3
References.

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Effects of distiller's dried grains with solubles on enteric methane emissions in dairy and beef cattle: a meta-analysis

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Introduction: Distiller's dried grains with solubles (DDGS), a by-product of grain fermentation for ethanol production, are extensively used in livestock feed. Given their nutrient composition, DDGS could potentially influence methane (CH_4) emissions, a significant greenhouse gas concern in ruminant production systems. This study utilized a multilevel random-effects meta-analysis to assess the impact of DDGS inclusion in cattle diets on CH_4 production and yield.

Methods: The literature search was conducted on 23 July 2024. Studies reporting CH_4 emissions and dry matter intake (DMI) in cattle fed DDGS-based diets were identified, and data extraction was performed. The meta-analysis calculated the mean difference (MD) for DMI and CH_4 yield and the relative mean difference (RMD) for CH_4 production across the selected studies.

Results: A total of $k = 25$ effect sizes from 10 studies were included in the DMI meta-analysis. DDGS had no significant effect on DMI in dairy or beef cattle ($p = 0.770$, MD = 0.070, 95% confidence interval [CI] from −0.420 to 0.561). For CH_4 production, $k = 24$ effect sizes from 10 studies were analyzed, revealing no significant effect ($p = 0.759$, RMD = −1.045, 95% CI: from −8.025 to 5.935). Similarly, the meta-regression model indicated that the diet's ether extract (EE) had no significant influence ($p = 0.815$, 95% CI from −1.121 to 1.409) on CH_4 production. For CH_4 yield, $k = 23$ effect sizes from 10 studies were included, with results showing no significant effect ($p = 0.475$, MD = −0.434 g/kg DMI, 95% CI: from −1.673 to 0.805). The regression model for the EE content of the diet also showed no significant impact on CH_4 yield ($p = 0.311$, 95% CI: from −0.366 to 0.122).

Discussion: The findings suggest that the inclusion of DDGS does not significantly affect DMI, enteric CH_4 production, or CH_4 yield in cattle. Moreover, the EE content in DDGS-containing diets does not significantly influence CH_4 outcomes. These results indicate that DDGS can be incorporated into cattle diets without exacerbating CH_4 emissions, contributing to sustainable livestock feeding practices.

KEYWORDS

distillers dried grains with solubles, methane, dairy cows, cattle, meta-analysis

1 Introduction

Distiller's dried grains with solubles (DDGS) are widely utilized as a feed ingredient in livestock systems due to their abundant availability and robust nutritional profile. As a by-product of ethanol production through grain fermentation, DDGS is produced when two-thirds of the corn starch is converted to ethanol, leaving behind nutrients concentrated in the stillage (1). These nutrients are then recovered and processed into DDGS, resulting in a product with significantly enhanced nutritional content compared to the original grain. Specifically, the fermentation process triples the concentrations of protein, fiber, fat, and phosphorus in DDGS relative to corn, with typical DDGS compositions including 10–30% crude protein (CP), 4–12% fat, 12–36% neutral detergent fiber (NDF), and 0.3–0.9% phosphorus on a dry matter (DM) basis (2). The growing demand for bioethanol has led to increased production of DDGS, making it an increasingly important component of livestock feed. For instance, in 2023 alone, the United States exported 10.8 million metric tons of DDGS (3). The widespread adoption of DDGS in feed not only reduces reliance on imported soybean meal and cereals but also contributes to lowering the carbon footprint and enhancing food security (4). Corn DDGS is particularly well-established in dairy cattle diets, with inclusion levels of up to 300 g/kg of diet DM reported without adverse effects on milk yield (5). Due to its high protein content, DDGS is primarily used as a protein source for ruminants (6). However, there is limited research exploring the impact of DDGS on enteric methane (CH_4) emissions in dairy and beef cattle.

Methane emissions are a critical issue in livestock production due to their significant contribution to greenhouse gases and their impact on climate change (7). Studies have shown mixed effects of DDGS inclusion on CH_4 emissions. In dairy cows, for instance, DDGS has been shown to reduce enteric CH_4 emissions without negatively impacting feed intake or milk production (8). However, DDGS inclusion has also been associated with increased manure CH_4 emissions by up to 15% (9). In beef cattle, high levels of DDGS supplementation (40% on a DM basis) can reduce CH_4 emissions but may simultaneously increase nitrous oxide emissions, highlighting a trade-off between different greenhouse gases (10).

Several studies have reported reductions in CH_4 emissions when feeding DDGS to beef (11, 12) and dairy cattle (8). Hünerberg et al. (10) also reviewed that DDGS consistently resulted in lower CH_4 emissions. The potential mechanism behind this reduction could be attributed to the higher fat content in DDGS (2), which can negatively affect ruminal fiber degradation, alter the acetate-to-propionate ratio, and reduce protozoa numbers, thereby decreasing CH_4 production (8).

Due to inconsistencies in the literature, with some studies indicating that CH_4 emissions are unaffected by varying levels of DDGS inclusion (13), animal nutritionists, policymakers, and farmers struggled to make informed decisions regarding the inclusion of DDGS as a CH_4-mitigating feed ingredient in dairy and beef ration. Therefore, this meta-analysis was conducted to quantify the effect of DDGS inclusion in the diet on CH_4 production and yield. Additionally, this study aimed to evaluate whether any reductions observed in CH_4 emissions in dairy or beef cattle-fed DDGS are associated with the fat content of the diet.

2 Materials and methods

2.1 Search strategy and data processing

The literature search was conducted on 23 July 2024, with no time restrictions applied. We selected two databases, PubMed[1] and Scopus,[2] along with Google Scholar, for our search. For PubMed and Scopus, we used the following keywords: DDGS OR dried distiller's grains with solubles AND methane OR CH_4 AND cattle OR cows OR beef OR steer OR cow OR heifer. For Google Scholar, the keywords were dried distiller's grains with solubles OR DDGS AND methane. The detailed information on the search strategy is presented in the PRISMA flowchart (Figure 1) (14).

Only English-language, peer-reviewed articles were included, and studies reporting enteric CH_4 emissions were selected. Articles that reported CH_4 emissions from *in vitro* studies were excluded. Eligible studies had to involve dairy cattle, heifers, or beef cattle (either steers or heifers) and provide CH_4 emission data. Data for CH_4 emissions (g/day) were considered as CH_4 production, and CH_4 yield was reported as grams per kilogram of dry matter intake (DMI). We extracted data for CH_4 production, CH_4 yield, and DMI, acetate, propionate, and butyrate, along with sample size, standard deviation (SD), or standard error of the mean (SEM). For studies providing variance as SED, we used the RevMan calculator (Version 5.4, 15) to compute the SEM. Study characteristics, such as experimental design, diet composition (including % of forage in the diet, % of concentrate in the diet, NDF, EE, CP, starch, % of DDGS in the diet, and types of DDGS: wheat or corn), and types of animals were extracted (Table 1). For the study by Bernier et al. (16), where EE of the diet was not reported, it was calculated using the nutritional dynamic system (NDS) Professional Software. Methane production and yield reported in liters were converted to g/day and g/kg DMI, respectively. Liters per day were converted to grams per day, assuming that a mole of CH_4, weighing 16.0 g, has a volume of 22.4 L (17).

2.2 Data analysis

The analysis utilized mean difference (MD) as the outcome measure for DMI and CH_4 yield (treatment mean – control mean). Methane production was calculated as relative mean difference (RMD) = [(treatment mean – control mean)/(control mean)] × 100. The RMD, a dimensionless variable, was used to account for large variations and is particularly useful for expressing percentage changes in methane production, which is of greater interest to readers (18). The standardized mean difference (SMD) for volatile fatty acids is a statistical technique commonly employed in meta-analyses to compare and synthesize findings from different studies that use varying measurement scales (19, 20). To calculate the SMD, the mean of the control group is subtracted from the mean of the treatment group, and the result is divided by the pooled standard deviation (19). A positive SMD indicates that the treatment group had a higher mean than the control group, while a negative SMD suggests the opposite.

1 https://pubmed.ncbi.nlm.nih.gov

2 https://www.scopus.com/home.uri

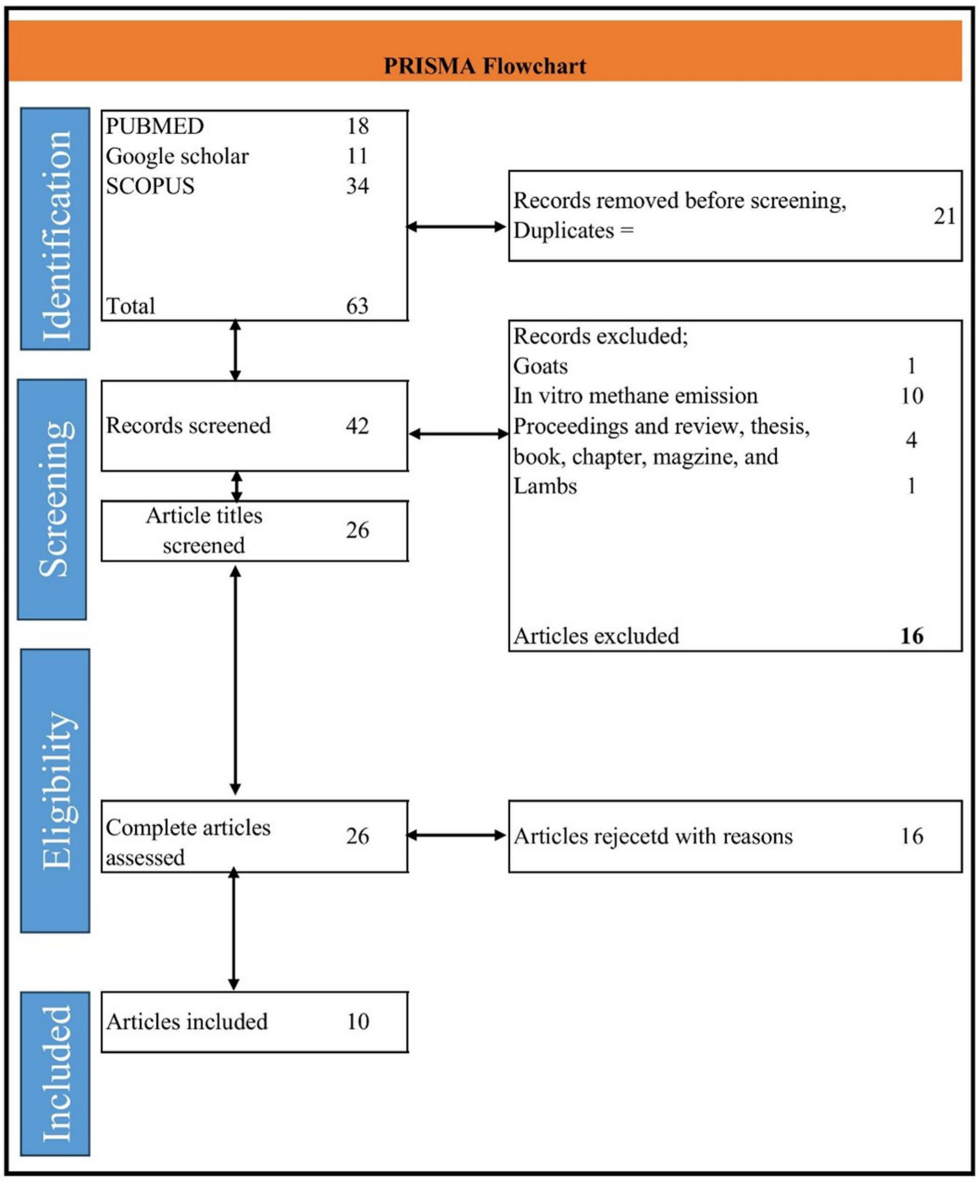

FIGURE 1
The preferred reporting items for systematic reviews and meta-analyses (PRISMA) flowchart for search strategy and details of study inclusion and exclusion.

We applied a multilevel random-effects model to address the dependency of effect sizes from the same study. This three-level meta-analytical model is appropriate for handling dependence and heterogeneity among studies. In this model, effect sizes extracted from the same study are considered nested within higher levels, making it suitable for scenarios with varying degrees of variation both within and between studies. The multilevel meta-analysis technique provides more precise effect sizes of treatment effects and helps identify sources of heterogeneity. The variance distribution in the model is as follows: level 1 = sampling variance, level 2 = effect sizes extracted from the same study, and level 3 = variance between studies. By accounting for the varying levels of variation within and between studies, the multilevel meta-analysis technique can provide more precise effect sizes of treatment effects and aid in identifying the sources of heterogeneity (21, 22). We applied an equal effect model for acetate, as the limited number of studies prevented the multilevel model from converging. Convergence refers to the optimizer's ability to identify the best-fitting parameters for the applied model. Successful convergence occurs when the algorithm effectively minimizes or maximizes the target function. Conversely, failure to converge can result from issues, such as poorly specified models, insufficient data, or constraints, that hinder the optimizer's ability to find an optimal solution. Additionally, a subgroup analysis was conducted based on the types of DDGS fed to the animals, with subgroups created for wheat and corn DDGS.

Heterogeneity (τ^2) was estimated using the DerSimonian-Laird estimator (23), and the I^2 statistic (24) was reported and calculated as follows:

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TABLE 1 Database characteristics of primary studies included in the meta-analysis.

Reference	Methane quantification method	DDGS %	Animal	DOE	DDGS	Forage %	Concentrate %	EE %	CP %	NDF %	Starch %
McGinn et al. (11)	SF_6	35.00	Beef	RCBD	Corn	60.00	40.00	5.10	17.40	42.50	
Bernier et al. (16)	SF_6	10.70	Beef	RCBD	Corn, wheat	87.50	12.50	2.48	8.70	63.40	
Bernier et al. (16)	SF_6	21.50	Beef	RCBD	Corn, wheat	76.50	23.50	3.04	11.40	58.60	
Benchaar et al. (8)	Respiratory chamber	10.00	Dairy cows	LSD	Corn	60.10	39.90	4.98	16.40	33.80	15.80
Benchaar et al. (8)	Respiratory chamber	20.00	Dairy cows	LSD	Corn	60.10	39.90	6.06	16.60	36.30	13.70
Benchaar et al. (8)	Respiratory chamber	30.00	Dairy cows	LSD	Corn	60.10	39.90	7.16	16.80	37.80	11.20
Hales et al. (32)	Respiratory chamber	15.00	Beef	LSD	Corn	10.00	90.00	4.80	14.30	16.80	58.70
Hales et al. (32)	Respiratory chamber	30.00	Beef	LSD	Corn	10.00	90.00	7.40	18.30	18.50	42.80
Hales et al. (32)	Respiratory chamber	45.00	Beef	LSD	Corn	10.00	90.00	8.30	20.20	18.70	39.10
Hales et al. (33)	Respiratory chamber	30.00	Beef	LSD	Corn	10.00	90.00	6.83	17.36	16.39	39.58
Hünerberg et al. (12)	Respiratory chamber	30.00	Beef	LSD	Corn	55.00	45.00	5.40	18.60	38.50	17.90
Hünerberg et al. (12)	Respiratory chamber	30.00	Beef	LSD	Wheat	55.00	45.00	3.70	23.50	33.90	16.80
Hünerberg et al. (46)	Respiratory chamber	40.00	Beef	LSD	Corn	8.00	92.00	5.40	19.60	27.90	34.70
Hünerberg et al. (46)	Respiratory chamber	40.00	Beef	LSD	Wheat	8.00	92.00	3.10	23.10	24.50	31.90
Castillo-Lopez et al. (47)	Indirect calorimetry	20.00	Dairy cows	LSD	Corn	50.70	49.30	3.90	17.10	38.10	21.40
Castillo-Lopez et al. (47)	Indirect calorimetry	20.00	Dairy cows	LSD	Corn	50.70	49.30	3.30	17.10	37.90	21.30
Castillo-Lopez et al. (47)	Indirect calorimetry	20.00	Dairy cows	LSD	Corn	50.70	49.30	3.60	17.10	38.00	21.30
Judy et al. (48)	Indirect calorimetry	20.00	Dairy cows	LSD	Corn	58.97	41.03	3.38	17.20	34.70	23.20
Judy et al. (48)	Indirect calorimetry	20.00	Dairy cows	LSD	Corn	58.97	41.03	4.76	16.90	35.10	21.90
Garnsworthy et al. (13)	Infrared analyzer	9.55	Dairy cows	LSD	Wheat	61.85	38.15	4.16	18.85	35.20	19.22
Garnsworthy et al. (13)	Infrared analyzer	19.15	Dairy cows	LSD	Wheat	62.25	37.75	4.15	18.97	37.65	17.70
Garnsworthy et al. (13)	Infrared analyzer	29.00	Dairy cows	LSD	Wheat	62.70	37.30	4.20	19.07	40.20	16.15
Garnsworthy et al. (13)	Infrared analyzer	6.80	Dairy cows	LSD	Wheat	61.10	38.90	4.19	19.00	34.80	27.20
Garnsworthy et al. (13)	Infrared analyzer	22.00	Dairy cows	LSD	Wheat	61.80	38.20	4.91	19.00	35.40	21.80
Garnsworthy et al. (13)	Infrared analyzer	27.10	Dairy cows	LSD	Wheat	62.00	38.00	5.51	19.00	35.70	20.00

DDGS, distiller's dried grains with solubles; DOE, design of experiment; EE, ether extract; NDF, neutral detergent fiber; CP, crude protein of the diet; LSD, Latin square design; RCBD, randomized control block design; SF_6, sulfur hexafluoride.

$$I^2 = \frac{Q-(k-1)}{Q} \times 100$$

where Q is the χ^2 statistic and k is the number of studies included in the meta-analysis.

A prediction interval for the true outcomes was also provided (25). The Knapp and Hartung adjustment method was used for the tests and confidence intervals (26). Potential outliers and influential studies were assessed using studentized residuals and Cook's distances (27). Meta-regression was performed to test the hypothesis

that CH_4 emissions decreased with increased EE contents in the diet, with EE included as a continuous variable in the multilevel random-effects meta-regression model. Studies with studentized residuals larger than the $100 \times [1–0.05/(2 \times k)]$ percentile of a standard normal distribution were considered potential outliers (Bonferroni correction with two-sided $\alpha = 0.05$ for k studies). Studies with Cook's distances larger than the median plus 6 times the interquartile range were deemed influential. Sensitivity analyses assessed the robustness of the results by removing statistical outliers with 95% confidence intervals lying outside the pooled effect size (27). Funnel plot asymmetry was checked using the rank correlation test (28) and the regression test by Sterne and Egger (29), with the standard error of observed outcomes as the predictor. Data analysis was performed using R (version 4.4.0) (30) and the metafor package (version 4.6.0) (31).

3 Results

3.1 Database characteristics

The data analysis included 6 studies on beef cattle and 4 studies on dairy cattle, yielding 11 effect sizes for beef and 14 effect sizes for dairy. The experimental design was a randomized complete block design (RCBD) in two studies and a Latin square design (LSD) in the remaining eight. Two types of DDGS were used: wheat-based DDGS in three studies and corn-based DDGS in seven. Methane quantification methods varied, with the sulfur hexafluoride (SF_6) trace gas technique used in one study, an infrared analyzer in another, indirect calorimetry in two, and a respiratory chamber in five (Table 1).

On average, the inclusion rate of DDGS was 29.74% for beef cattle and 19.54% for dairy cattle, with concentrate levels at 64.54 and 41.28%, respectively (Table 2). For dairy cattle, forage averaged 58.71% of the diet, with CP at 17.79%, NDF at 36.47%, and starch at 19.41%. In contrast, beef cattle diets had a higher DDGS content (29.74%) and more variable forage levels (35.45%), with CP averaging at 17.49%, NDF lower at 32.69%, and starch higher at 35.18% (Table 2).

A summary of the multilevel random-effects meta-analysis and meta-regression for DMI and methane production and yield is provided in Table 3, offering a concise overview of the statistical results.

3.2 Dry matter intake

A total of $k = 25$ effect sizes from 10 studies were included in the analysis. The observed mean differences ranged from −0.92 to 4.60, with 48% of the effect sizes being negative. The multilevel random-effects meta-analysis indicated that DDGS had no significant effect on DMI in dairy or beef cattle ($p = 0.770$, MD = 0.070, 95% CI: from −0.420 to 0.561). An orchard plot illustrating the observed outcomes and the effect size from the multilevel random-effects model is presented in Figure 2. The subgroup analysis for the different types of DDGS was also non-significant ($p > 0.05$) for corn, wheat, or a mixture of both. The effect sizes were as follows: corn DDGS ($p = 0.529$, MD = 0.146, 95% CI = from −0.328 to 0.612), wheat DDGS ($p = 0.135$, MD = −0.509, 95% CI = from −1.191 to 0.172), and a mixture of corn and wheat DDGS ($p = 0.189$, MD = 1.327, 95% CI = from −0.704 to 3.358). The Q-test revealed heterogeneity among the true outcomes (Q = 39.56, $p = 0.023$, $\tau^2 = 0.148$, $I^2 = 39.34\%$). Since the heterogeneity (I^2) was below 40% and the primary outcome was non-significant, meta-regression was not conducted, as adding covariates would be meaningless. An

TABLE 2 Descriptive statistics for the dietary characteristics of the studies included in the meta-analysis.

Variables	Mean	Minimum	Maximum	SD	Missing
Dairy cattle					
DDGS	19.54	6.80	30.0	6.887	0
Forage	58.71	50.70	62.70	4.496	0
Concentrate	41.28	37.30	49.30	4.496	0
EE	4.590	3.300	7.160	1.080	0
CP	17.79	16.40	19.07	1.090	0
NDF	36.47	33.80	40.20	1.806	0
Starch	19.41	11.20	27.20	4.161	0
Beef cattle					
DDGS	29.74	10.70	45.00	10.57	0
Forage	35.45	8.000	87.50	31.39	0
Concentrate	64.54	12.50	92.00	31.39	0
EE	5.050	2.480	8.300	1.893	0
CP	17.49	8.700	23.50	4.537	0
NDF	32.69	16.58	63.40	16.58	0
Starch	35.18	16.80	58.70	13.59	3

All units are in %, otherwise mentioned. SD, standard deviation; DDGS, distiller's dried grains with solubles; EE, ether extract; NDF, neutral detergent fiber; CP, crude protein.

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TABLE 3 Summary statistics of the multilevel random effects meta-analysis and meta-regression for dry matter intake and methane production and yield.

Variables	Effect size	SE	*T*-value	DF	*p*-value	95% CI	Q	I^2	Egger's test *p*-value
DMI	0.070	0.238	0.295	24	0.770	−0.420 to 0.561	39.56	39.34	0.001
Types of DDGS									
Corn	0.146	0.229	0.638	14	0.529	−0.328 to 0.612	–	–	–
Wheat	−0.509	0.328	−1.548	7	0.135	−1.191 to 0.172	–	–	–
Corn:Wheat	1.327	0.979	1.355	1	0.189	−0.704 to 3.358	–	–	–
CH_4 Production	−1.045	3.374	−0.309	23	0.759	−8.025 to 5.935	21.5	0	0.469
Types of DDGS									
Corn	−3.502	5.059	−0.692	13	0.496	−14.02 to 7.019	–	–	–
Wheat	−0.243	7.179	−0.034	7	0.973	−15.17 to 14.68	–	–	–
Corn:Wheat	4.347	12.26	0.354	1	0.726	−21.14 to 29.84	–	–	–
Ether extract	0.144	0.611	0.611	23	0.815	−1.121 to 1.409	–	–	–
CH_4 Yield	0.434	0.597	−0.726	22	0.475	−1.673 to 0.805	48.00	54.16	0.161
Types of DDGS									
Corn	−0.835	0.836	−0.998	12	0.330	−2.580 to 0.910	–	–	–
Wheat	0.903	1.308	0.690	7	0.498	−1.826 to 3.632	–	–	–
Corn:Wheat	−0.359	2.399	−0.149	1	0.882	−5.364 to 4.646	–	–	–
Ether extract	−0.122	0.117	−1.036	22	0.311	−0.366 to 0.122	–	–	–

SE, standard error; DF, degree of freedom (number of effect size); 95% CI, 95% confidence interval; DDGS, distiller's dried grains with solubles; DMI, dry matter intake.

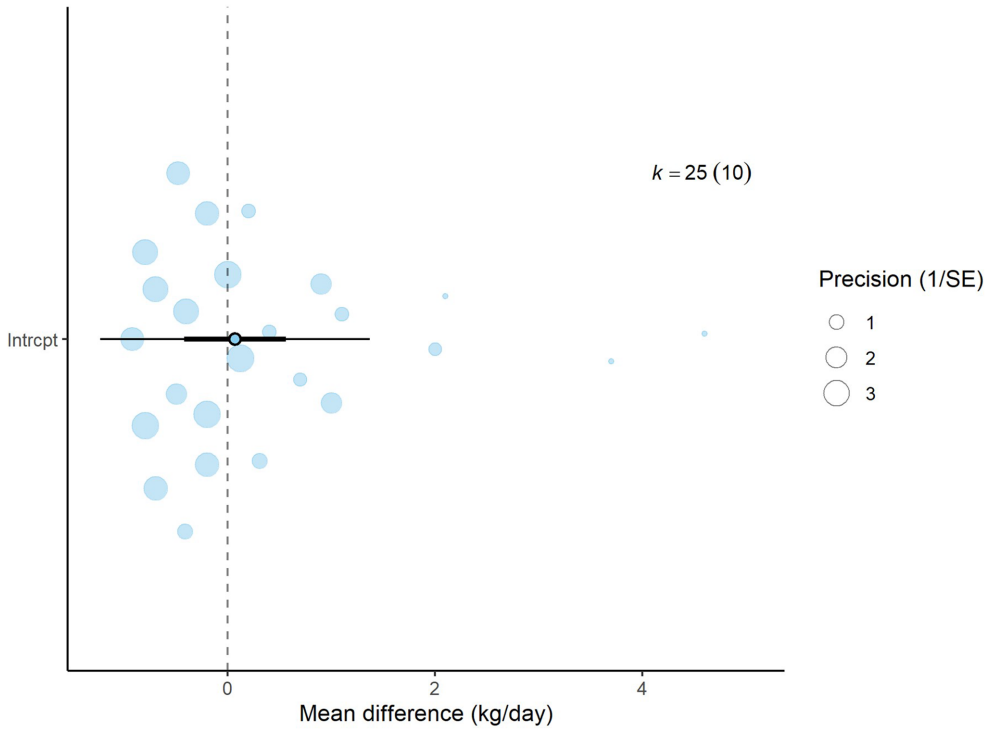

FIGURE 2
Orchard plot for dry matter intake (DMI): The overall effect size from a multilevel random-effects meta-analysis of 25 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line indicates the prediction interval, while the dotted vertical line marks the line of no effect.

examination of studentized residuals showed no outliers, with no values exceeding ±3.09. Additionally, Cook's distances indicated that none of the studies were overly influential. The funnel plot of the effect sizes, shown in Figure 3, indicated potential asymmetry, supported by the rank correlation and Egger's regression tests ($p = 0.009$ and $p = 0.001$, respectively).

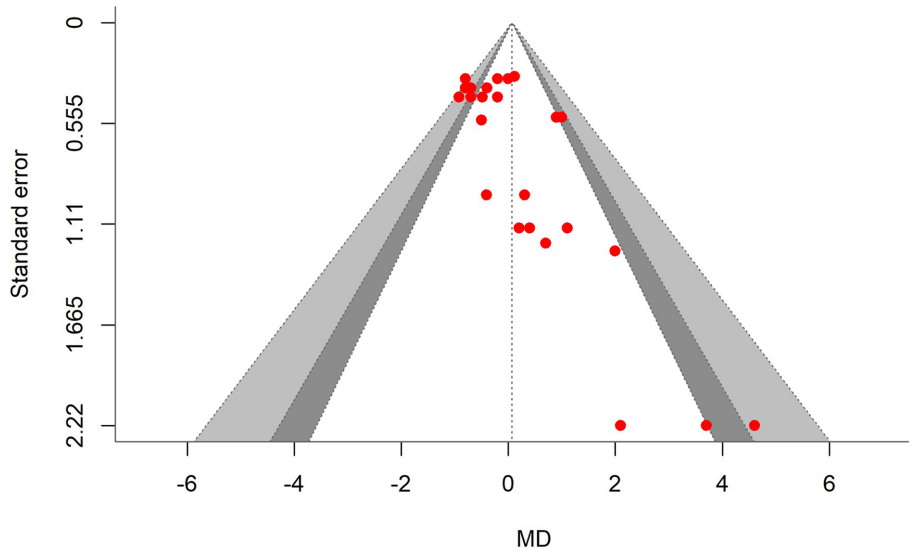

FIGURE 3
Contour-enhanced funnel plot showing asymmetrical distribution of effect sizes around the standard error, indicating bias in the dry matter intake (DMI) meta-analysis. MD, the mean difference.

3.3 Methane production

A total of $k = 24$ effect sizes from 10 studies were analyzed. Sensitivity analysis identified a treatment with 45% distiller grains as an outlier and overly influential, leading to its exclusion from the final analysis (32). The observed RMD was −1.045%, with 52% of the effect sizes being positive. Methane production was found to be non-significant ($p = 0.759$, RMD = −1.045, 95% CI: from −8.025 to 5.935). An orchard plot showing the observed outcomes and the prediction interval is presented in Figure 4. The subgroup analysis of different types of DDGS showed no significant impact on methane production. For corn-based DDGS, the effect size was non-significant ($p = 0.496$, RMD = −3.502, 95% CI = from −14.02 to 7.019). Similarly, wheat-based DDGS had no notable effect ($p = 0.937$, RMD = −0.243, 95% CI = from −15.17 to 14.68). The combination of corn and wheat DDGS also showed no significant influence ($p = 0.726$, RMD = 4.347, 95% CI = from −21.14 to 29.84). The regression model indicated that the EE of the diet had no significant effect on CH_4 production ($p = 0.815$, 95% CI: from −1.121 to 1.409), with an increase of 0.144% in CH_4 production per unit increase in EE. The Q-test suggested homogeneity among the true outcomes (Q = 21.5, $p = 0.550$, $\tau^2 = 0$, $I^2 = 0$), indicating no heterogeneity. The funnel plot in Figure 5 showed no asymmetry, as confirmed by the rank correlation and Egger's regression tests ($p = 0.549$ and $p = 0.469$, respectively).

3.4 Methane yield

A total of $k = 23$ effect sizes from 10 studies were included in the analysis. Sensitivity analysis identified two treatments with 30 and 45% distiller grains as outliers, which were subsequently removed from the final analysis (32). The observed mean differences for CH_4 yield ranged from −3.90 to 3.63, with 57% of the effect sizes being negative. Methane yield was found to be non-significant ($p = 0.475$, MD = −0.434 g/kg DMI, 95% CI: from −1.673 to 0.805). An orchard plot depicting the observed outcomes and the prediction interval is shown in Figure 6. The regression model for EE indicated no significant effect on CH_4 yield ($p = 0.311$, 95% CI: from −0.366 to 0.122), with a −0.122 g/kg DMI increase in CH_4 yield per unit increase in EE. The subgroup analysis for types of DDGS suggests that DDGS types have no significant effect on methane yield. The effect sizes were as follows: corn DDGS ($p = 0.330$, MD = −0.835, 95% CI = from −2.580 to 0.910), wheat DDGS ($p = 0.498$, MD = 0.903, 95% CI = from −1.826 to 3.632), and a mixture of corn and wheat DDGS ($p = 0.882$, MD = −0.359, 95% CI = from −5.364 to 4.646). The Q-test indicated heterogeneity among the true outcomes (Q = 48, $p = 0.001$, $\tau^2 = 0.55$, $I^2 = 54.16\%$). The funnel plot in Figure 7 showed no significant asymmetry, supported by the rank correlation and Egger's regression tests ($p = 0.183$ and $p = 0.161$, respectively) (Figure 8).

3.5 Acetate

A total of $k = 10$ effect sizes from four studies were included in the analysis. The observed SMD for acetate was found to be significant ($p = 0.005$, SMD = −0.463, 95% CI: from −0.749 to −0.176). Subgroup analysis by DDGS type indicated that corn DDGS significantly decreased rumen acetate production, with an effect size of ($p = 0.001$, SMD = −1.048, 95% CI: from−1.526 to −0.570) (Table 4). In contrast, wheat DDGS showed no significant difference ($p = 0.176$, SMD = −0.313, 95% CI: from −0.801 to 0.173). Due to the substantially reduced acetate production, a meta-regression was conducted to identify potential moderators influencing acetate levels. We found that increasing the inclusion level of DDGS in dairy cattle diets significantly reduced acetate ($p = 0.005$, SMD = −0.024, 95% CI: from −0.040 to −0.009). Similarly, the inclusion of EE had a significant effect on rumen acetate production ($p = 0.002$, SMD = −0.102, 95% CI: −0.159

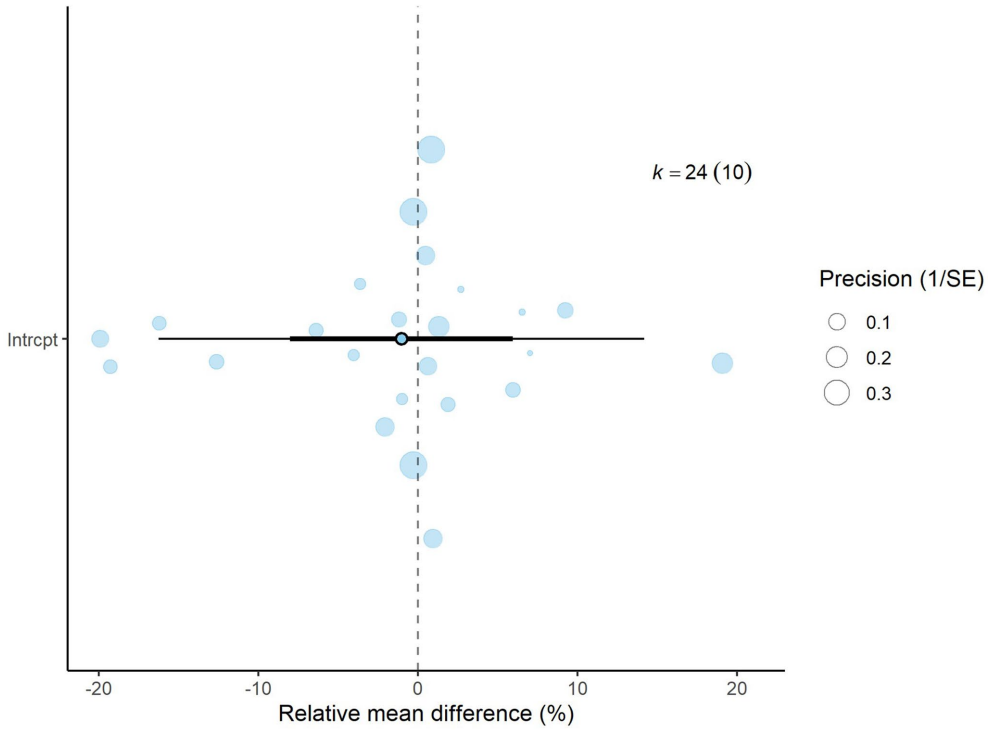

FIGURE 4
Orchard plot for methane production % (relative mean difference): The overall effect size from a multilevel random-effects meta-analysis of 24 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line represents the prediction interval, while the dotted vertical line marks the line of no effect.

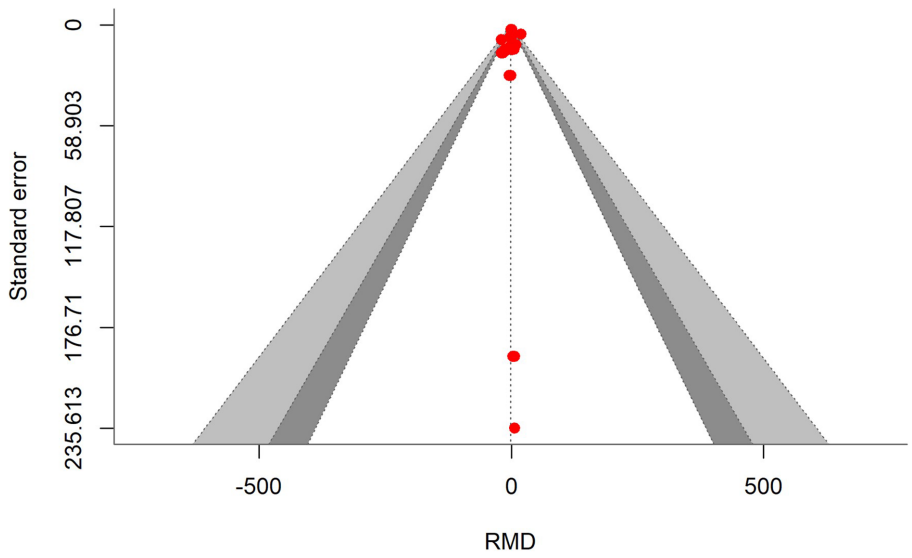

FIGURE 5
Contour-enhanced funnel plot showing symmetrical distribution of effect sizes around the standard error, indicating no bias in the methane production meta-analysis.

to 0.046). The Q-test indicated no significant heterogeneity among the true outcomes ($Q = 8.28$, $p = 0.506$, $\tau^2 = 0$, $I^2 = 0\%$). Funnel plot asymmetry was also non-significant, as supported by both the rank correlation and Egger's regression tests ($p = 0.216$ and $p = 0.461$, respectively).

3.6 Butyrate

A total of $k = 10$ effect sizes from four studies were analyzed. Rumen butyrate production was found to be non-significant ($p = 0.159$, SMD = 0.569, 95% CI: from −0.270 to 1.409) (Figure 9).

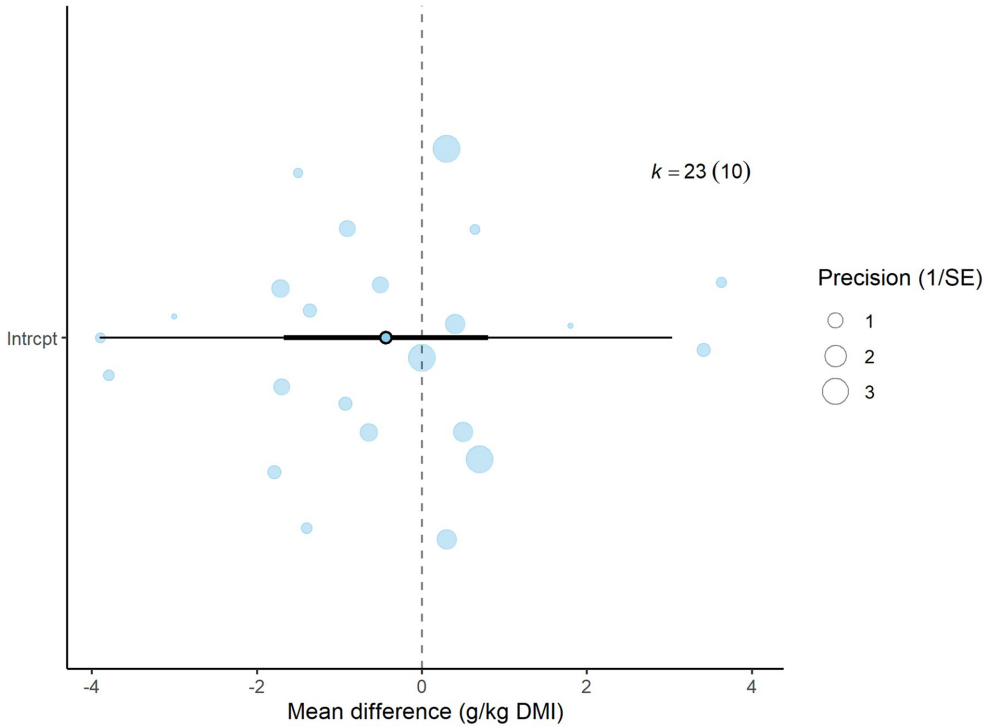

FIGURE 6
Orchard plot for methane yield: The overall effect size from a multilevel random-effects meta-analysis of 23 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line represents the prediction interval, while the dotted vertical line marks the line of no effect. SE, standard error.

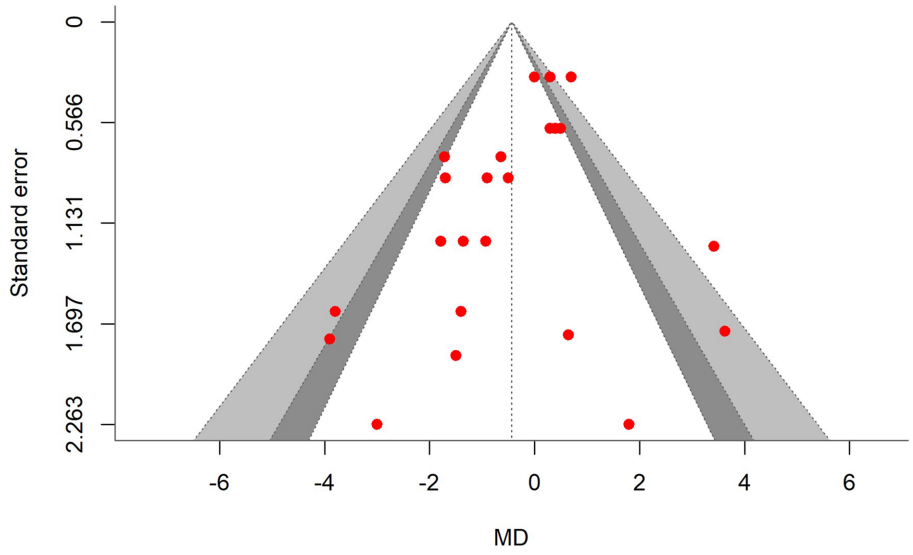

FIGURE 7
Contour-enhanced funnel plot for studies included in the methane yield meta-analysis: symmetrical distribution of effect sizes around the standard error indicates no bias.

Subgroup analysis by DDGS type showed no significant impact on butyrate production (Table 5). For corn-based DDGS, the effect size was non-significant ($p = 0.102$, SMD = 0.784, 95% CI: from −0.198 to 1.766), and wheat-based DDGS also showed no notable effect ($p = 0.384$, SMD = 0.389, 95% CI: from −0.586 to 1.365). The regression model indicated that the EE of the diet had no significant effect on CH_4 production ($p = 0.067$, SMD = 0.131, 95% CI: from −0.011 to 0.274). The Q-test suggested significant heterogeneity among the true outcomes ($Q = 32.33$, $p = 0.0002$, $\tau^2 = 0.563$, $I^2 = 76.58\%$). The funnel plot showed asymmetry; the rank correlation

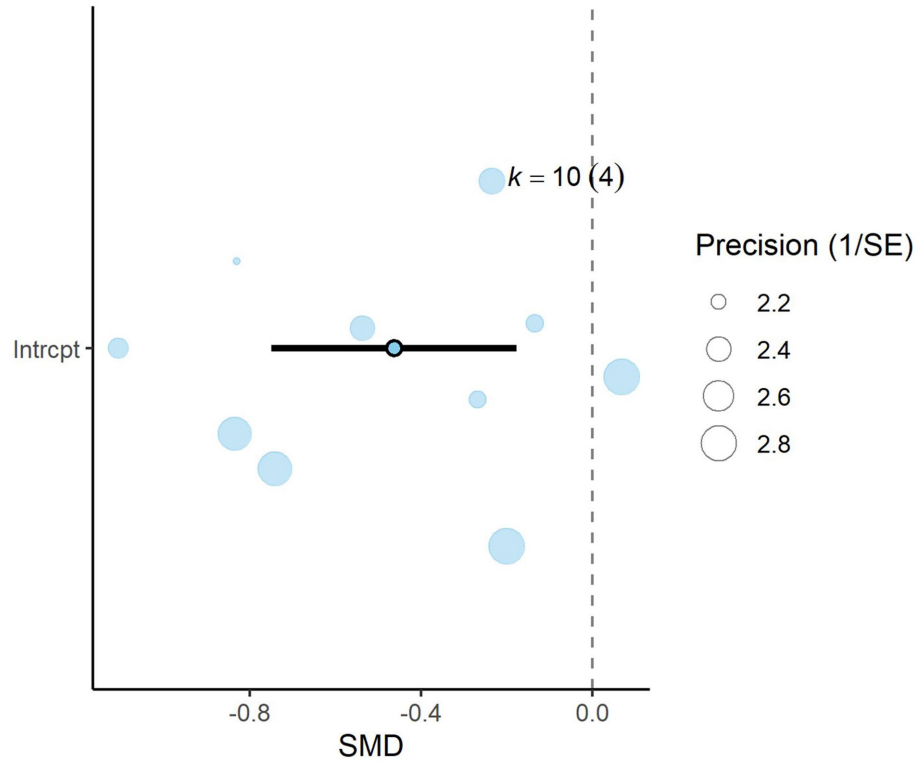

FIGURE 8
Orchard plot for acetate: The overall effect size from a multilevel random-effects meta-analysis of 10 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line represents the prediction interval, while the dotted vertical line marks the line of no effect. SE, standard error.

TABLE 4 Summary statistics for the equal effect meta-analysis and meta-regression for rumen acetate production.

Variables	Effect size	SE	*T*-value	DF	*p*-value	95% CI	Q	I^2	Egger's test *p*-value
Acetate	−0.463	0.126	−3.654	9	0.005	−0.749 to −0.176	8.28	0.0	0.461
Ether extract	−0.102	0.025	−4.099	9	0.002	−0.159 to 0.046	–	–	–
DDGS %	−0.024	0.006	−0.360	9	0.005	−0.040 to −0.009	–	–	–
Types of DDGS									
Corn	−1.048	0.207	−5.054	4	0.001	−1.526 to −0.570	–	–	–
Wheat	−0.313	0.211	−1.483	4	0.176	−0.801 to 0.173	–	–	–

SE, standard error; DF, degree of freedom (number of effect size); 95% CI, 95% confidence interval; DDGS, distiller's dried grains with solubles. All effect sizes are expressed as standardized mean difference (SMD).

test was non-significant ($p = 0.216$), while Egger's regression test was significant ($p = 0.006$).

3.7 Propionate

A total of $k = 10$ effect sizes from four studies were included in the analysis. The observed SMD for acetate was found to be non-significant ($p = 0.508$, SMD = −0.125, 95% CI: from −0.538 to −0.286) (Figure 10). Subgroup analysis by DDGS type indicated that corn DDGS had no effect on rumen propionate production ($p = 0.622$, SMD = 0.116, 95% CI: from −0.408 to 0.641). Similarly, wheat DDGS showed no significant difference ($p = 0.139$, SMD = −0.382, 95% CI: from −0.920 to 0.155) (Table 6). EE also had no significant effect on rumen propionate production ($p = 0.913$, SMD = −0.004, 95% CI: from −0.087 to 0.079). The Q-test indicated no significant heterogeneity among the true outcomes ($Q = 9.99$, $p = 0.350$, $\tau^2 = 0.029$, $I^2 = 30.73\%$). Funnel plot asymmetry was non-significant, as confirmed by the rank correlation and Egger's regression tests ($p = 1.0$ and $p = 0.417$, respectively).

4 Discussion

Methane emissions from livestock production are a significant contributor to climate change, posing a major challenge among

TABLE 5 Summary statistics for the multilevel random effects meta-analysis and meta-regression for rumen butyrate production.

Variables	Effect size	SE	*T*-value	DF	*p*-value	95% CI	Q	I^2	Egger's test *p*-value
Butyrate	0.569	0.371	1.532	9	0.159	−0.270 to 1.409	32.33	76.58	0.006
Ether extract	0.131	0.063	2.082	9	0.060	−0.011 to 0.274	–	–	–
DDGS %	0.022	0.012	1.729	9	0.117	−0.006 to 0.051	–	–	–
Types of DDGS									
Corn	0.784	0.426	1.840	4	0.102	−0.198 to 1.766	–	–	–
Wheat	0.389	0.432	0.919	4	0.384	−0.586 to 1.365	–	–	–

SE, standard error; DF, degree of freedom (number of effect size); 95% CI, 95% confidence interval; DDGS, distiller's dried grains with solubles. All effect sizes are expressed as standardized mean difference (SMD).

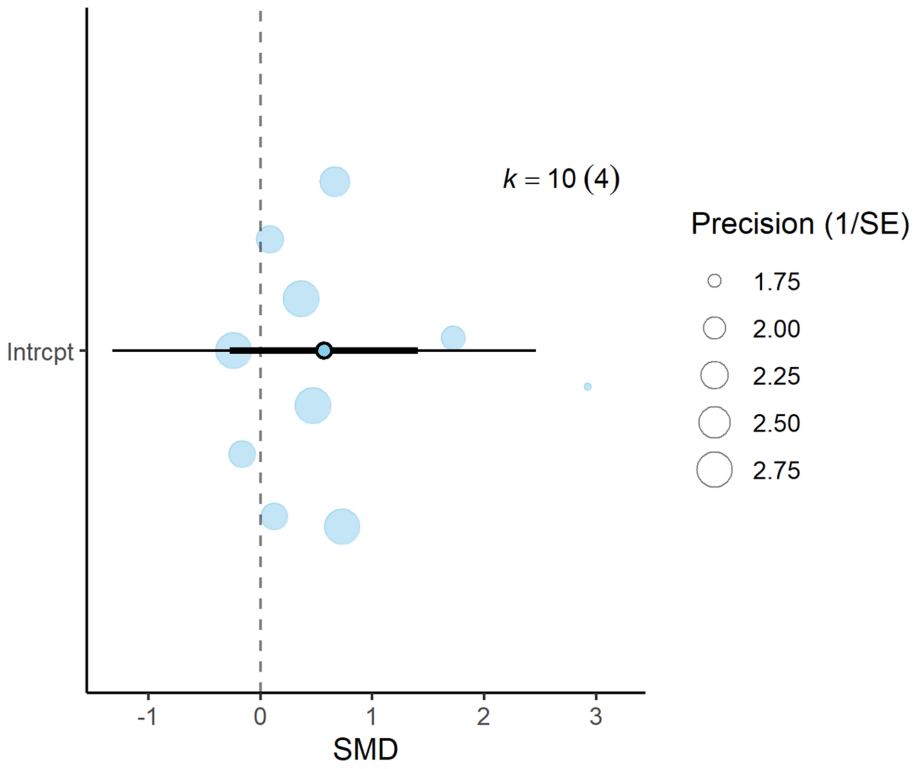

FIGURE 9
Orchard plot for butyrate: The overall effect size from a multilevel random-effects meta-analysis of 10 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line represents the prediction interval, while the dotted vertical line marks the line of no effect. SE, standard error.

TABLE 6 Summary statistics for the multilevel random effect meta-analysis and meta-regression for rumen propionate production.

Variables	Effect size	SE	*T*-value	DF	*p*-value	95% CI	Q	I^2	Egger's test *p*-value
Propionate	−0.125	0.182	−0.689	9	0.508	−0.538 to −0.286	9.99	15.5	0.417
Ether extract	−0.004	0.036	−0.111	9	0.913	−0.087 to 0.079	–	–	–
DDGS %	−0.006	0.005	−1.187	9	0.265	−0.018 to 0.005	–	–	–
Types of DDGS									
Corn	0.116	0.227	0.511	4	0.622	−0.408 to 0.641	–	–	–
Wheat	−0.382	0.233	−1.639	4	0.139	−0.920 to 0.155	–	–	–

SE, standard error; DF, degree of freedom (number of effect size); 95% CI, 95% confidence interval; DDGS, distiller's dried grains with solubles. All effect sizes are expressed as standardized mean difference (SMD).

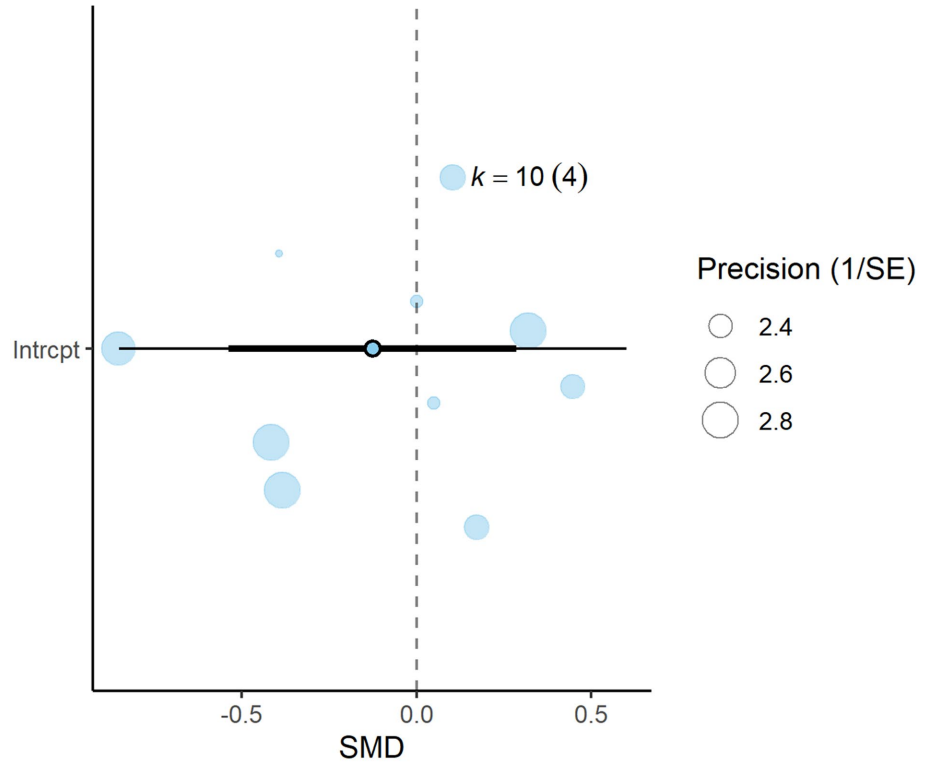

FIGURE 10
Orchard plot for propionate: The overall effect size from a multilevel random-effects meta-analysis of 10 effect sizes is centered on zero, with a 95% confidence interval (CI) spanning the line of no effect (dotted line). The thick black horizontal line represents the prediction interval, while the dotted vertical line marks the line of no effect. SE, standard error.

atmospheric pollutants. It is well established that CH_4 emissions are influenced by both the quantity and type of nutrients fermented in the rumen. Typically, CH_4 production increases with DMI, and the specific nutrients fermented play a crucial role in rumen methanogenesis.

Our findings suggest that DDGS have no significant effect on DMI in both dairy and beef cattle, consistent with previous studies in beef cattle (11, 33) and dairy cattle (16). However, this is in contrast to other studies that observed increased DMI when DDGS replaced soybean meal and corn in dairy cattle diets (34). The discrepancy may be attributed to our study's focus on literature that specifically evaluates enteric CH_4 emissions, potentially excluding studies that might have reported positive effects on DMI without examining CH_4 outcomes (8). This limitation highlights the need for a more comprehensive analysis that includes a broader range of studies.

Our meta-analysis found that both CH_4 yield and production were non-significant, indicating that DDGS does not influence CH_4 emissions in dairy or beef cattle. This finding contrasts with some studies that suggest DDGS can impact these emissions (11, 33). For instance, research has shown that feeding DDGS to dairy cows can mitigate enteric CH_4 emissions without negatively affecting intake and milk production (8). In beef cattle, DDGS inclusion has also been associated with reduced CH_4 emissions (11, 33), attributed to the high EE (EE) content of DDGS (12.7% of DM), which can range from 2.0 to 5.1% of DM (11).

The reduction in CH_4 production in these studies is often linked to increased EE supply from DDGS, which affects ruminal fiber degradation, the ratio of acetate to propionate, and protozoa numbers. These factors collectively contribute to lower CH_4 production. However, our meta-regression analysis did not find a significant influence of EE on CH_4 production or yield, contradicting the hypothesis that higher fat content from DDGS would reduce CH_4 emissions. This suggests that the relationship between dietary fat content in DDGS-supplemented cows and CH_4 emissions may be more complex than previously thought and warrants further investigation. Another potential mechanism could be related to sulfur concentration. Buckner et al. (35) analyzed 1,200 DDGS (corn =400 and wheat = 800) samples from six ethanol processing facilities over 10 months, reporting an average sulfur content of 0.78%. Higher sulfur content may reduce CH_4 emissions by redirecting ruminal H_2 from methanogenesis for CH_4 production (36) toward hydrogen sulfide (H_2S) production. Hydrogen sulfide has been shown to inhibit methanogenic archaea directly (37). The activity of sulfate-reducing bacteria (SRB) depends on the availability of H_2 and sulfate levels, as these bacteria use sulfate as a terminal electron acceptor in anaerobic respiration (38, 39). By increasing the sulfate level in the rumen, the capacity of SRB to outcompete methanogens as an H_2 sink is enhanced, which could further reduce CH_4 emissions (40). This suggests that sulfur and sulfate levels in DDGS may influence the microbial dynamics, favoring pathways that reduce CH_4 production. Our findings suggest that dietary fat is not responsible for CH_4 reduction

in DDGS-supplemented cows. The reduction in CH_4 emissions reported in some studies might be associated with higher sulfur contents, and variations in sulfur levels due to regional and processing differences could explain the differing results across studies.

The findings of the current meta-analysis suggest that acetate production decreases significantly in cows supplemented with DDGS, which aligns with previous studies in dairy cattle (8, 41, 42). This reduction in acetate may be linked to a decline in ruminal fiber digestion and a decrease in the ruminal degradability of hay as the proportion of DDGS in the diet increases (8). These results are further supported by the meta-regression model, which shows a linear decrease in acetate production with increasing DDGS inclusion. In contrast, butyrate and propionate production were not influenced by the percentage or type of DDGS. The literature shows inconsistencies regarding butyrate and propionate production. Leupp et al. (43) reported a decrease in acetate molar proportion alongside an increase in propionate, with no effect on butyrate in beef cattle. Meanwhile, Anderson et al. (44) observed a numerical decrease in acetate and increases in both propionate and butyrate molar proportions in dairy cows fed DDGS diets. There was evidence of publication bias in both DMI and butyrate. This bias may be linked to the unilaterally skewed effect sizes observed in the meta-analysis. Additionally, meta-analyses with a smaller number of studies are more susceptible to publication bias than those with a larger number of studies, which can affect the reliability and representativeness of the findings (45). The implications of our findings for livestock management and CH_4 mitigation are significant. Although DDGS may not consistently reduce CH_4 emissions, their diet inclusion offers other nutritional benefits, such as improved nitrogen utilization. However, it is essential to consider the environmental impact of increased nitrogen excretion when evaluating the overall sustainability of DDGS in cattle diets (8). Future research should focus on identifying the conditions under which DDGS can effectively reduce CH_4 emissions and exploring the underlying mechanisms in greater detail. Studies should also investigate the potential relationship between CH_4 reduction and sulfur content in cattle diets, particularly when supplemented with DDGS. Given that DDGS is rich in both fats and sulfur, it is important to distinguish the individual effects of these components on CH_4 emissions and overall cow health. Understanding how sulfur and fats interact within the rumen and their combined impact on CH_4 reduction will be crucial in developing more sustainable cattle diets that mitigate environmental impact while ensuring animal health. Additionally, addressing the significant variability observed in the literature could provide clearer insights into the role of DDGS in CH_4 mitigation.

5 Conclusion

Our meta-analysis indicates that the inclusion of DDGS has no significant impact on DMI in dairy or beef cattle in studies that evaluated enteric CH_4 emissions. Similarly, DDGS supplementation in cattle diets does not influence enteric CH_4 production or yield. Furthermore, the EE content of diets containing DDGS does not significantly affect CH_4 production or yield in these cattle.

These findings have important implications for livestock producers and policymakers seeking to balance the nutritional benefits of DDGS with the need for effective CH_4 mitigation strategies. Continued research is essential to refine our understanding of DDGS's role in CH_4 emissions and to explore alternative dietary strategies that can contribute to more sustainable livestock production systems.

A key limitation of this meta-analysis is the relatively small number of studies available on enteric CH_4 emissions and rumen volatile fatty acids in dairy and beef cattle supplemented with DDGS. This limited dataset may reduce the statistical power and generalizability of the results, as fewer studies can increase variability and the potential for bias. Future research involving a larger body of studies would be valuable in validating and expanding upon the conclusions drawn here.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Inquiries regarding the original data for the meta-analysis can be directed to Muhammad Irfan Malik, dr.irfan279@gmail.com.

Author contributions

MM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. JL: Funding acquisition, Writing – review & editing. MC: Writing – review & editing. TH: Writing – review & editing. XS: Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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Enteric methane emissions, rumen fermentation, and milk composition of dairy cows fed 3-nitrooxypropanol and *L*-malate supplements

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Twenty-four cows were used in a randomized complete block design. Cows were assigned to three groups: (1) Control, (2) 3-nitrooxypropanol (NOP) of 200 mg/kg feed dry matter (10% NOP), and (3) NOP × MAL (10% NOP at 200 mg/kg feed dry matter plus 99% *L*-malate at 10 g/kg feed dry matter). Cows were fed for 10-wk. NOP did not affect dry matter intake (DMI) or milk yield, whereas NOP × MAL decreased DMI but did not affect milk yield. Average methane production decreased by 54% in NOP and by 51% in NOP × MAL. Both NOP and NOP × MAL increased concentrations of milk fat and protein. In addition, concentrations of short-chain fatty acids and total saturated fatty acids increased in both NOP and NOP × MAL. However, total monounsaturated fatty acids and total polyunsaturated fatty acids only increased in NOP × MAL.

KEYWORDS

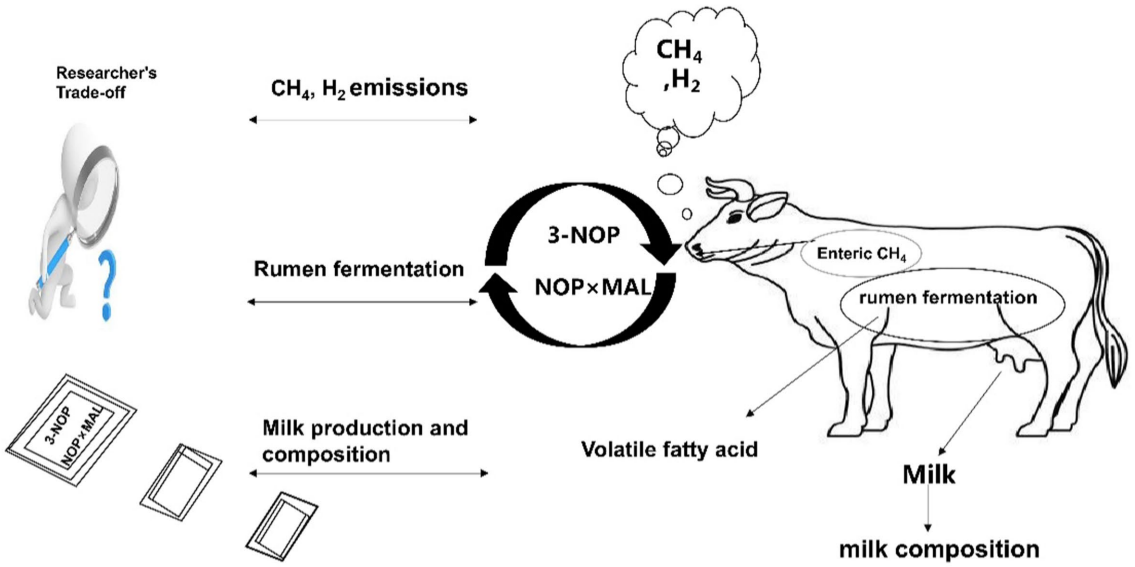
methane emissions, 3-nitrooxypropanol, 3-nitrooxypropanol plus *L*-malate, milk composition, milk fat

Highlights

- Average methane production decreased by 54% in NOP and by 51% in NOP × MAL.
- Both NOP and NOP × MAL decreased the molar ratio of acetate-to-propionate.
- Both NOP and NOP × MAL increased concentrations of milk fat and protein.

1 Introduction

Methane (CH_4) produced in ruminant intestines is a greenhouse gas with warming potential. Over 100 years, the global warming potential of CH_4 is 28–34 times that of carbon dioxide (1), but its greenhouse effect is 80 times that of carbon dioxide in the 10–20 years after its release (2). Methane in rumen is mainly produced by several methanogenic archaea that reduce carbon dioxide (CO_2) through hydrogen gas (H_2), and it is chemically very stable (3, 4). Methane is ultimately excreted in the form of rumen fermentation by-products (5). Globally, intestinal CH_4 emissions of ruminants account for approximately 3–5% of total greenhouse gas emissions (6) and 2–12% of total ruminant dietary energy intake (7). Reductions in CH_4 emissions from the intestines of ruminants can be a means to achieve the goal of the Paris Agreement, which aimed to stabilize the global climate– at 1.5°C to 2°C above



GRAPHICAL ABSTRACT

the preindustrial level (8). Therefore, it is urgent to reduce CH_4 emissions and to develop strategies to increase the energy utilization rate of ruminant diets. Meanwhile, to address climate change at the national level, the Chinese government has set the strategic goals of achieving peak carbon dioxide emissions in 2030 and carbon neutrality in 2060.

In exploring how to reduce intestinal CH_4 emissions, ruminant diets have been supplemented with tannins, saponins, monensin, bromochloromethane, and vegetable oil (9–11). Although those dietary additives reduced enteric CH_4 emissions to a certain extent, they also likely decreased the digestibility and production performance of animals and had some toxic effects on animals with unsustainable intestinal CH_4 emissions (10–14). Therefore, there are limitations with dietary supplements in animal production.

Feeding a supplement to ruminants with at least one organic molecule substituted by at least one nitrooxy group at any position has recently been shown to be very effective in reducing CH_4 production with no negative effects on rumen fermentation (15). However, when the nitrooxy group is replaced by other chemical groups with similar physical and chemical properties, the inhibitory effect on CH_4 production is lost. Thus, as reported by the patent inventors (15), the nitrooxy group is the key to reducing CH_4 emissions. Among several organic compounds listed by the inventors that are substituted by at least one nitrooxy group at any position, 3-nitrooxypropanol (NOP) has been the most studied. The nitrooxy group in NOP specifically binds to coenzyme M reductase (MCR), and as a result, the nickel ion in its nickel enzyme is oxidized from +1 to +2 to inactivate MCR, which further continuously inhibits CH_4 emissions (16). Although the results of NOP studies are slightly different, it consistently and continuously reduces CH_4 emissions and increases hydrogen gas (H_2) emissions (17–20). Hydrogen gas is a high-energy gas, and when hydrogen produced by ruminant fermentation in a diet cannot be effectively used by animals, it is an indirect waste of diets (21, 22). Therefore, a reasonable approach is urgently needed to promote H_2 use by ruminants.

In the process of biological oxidation in animals, *L*-malic acid is used as a hydrogen or an electron transporter to transfer hydrogen to mitochondria in rumen microbes and the mitochondria in the cow for oxidation to generate energy (23–25). Milk yields increase when *L*-malic acid is fed to dairy cows or dairy goats (26–28). The increases are likely because hydrogen is transferred to cell mitochondria by *L*-malic acid, and then, H^+ is oxidized into extra energy to improve animal production performance.

NOP is listed by Duval and Kindermann (15) and therefore, it was selected as the inhibitor of CH_4 emissions in one treatment group in the experiment in this study to determine whether the effect on reducing CH_4 emissions has a similar conclusion to that of previous researchers who studied NOP. NOP plus *L*-malic acid (NOP × MAL) was used in the other treatment group to determine whether H^+ was oxidized to adenosine triphosphate (ATP) by cell mitochondria to provide energy. Therefore, the purpose of this study was to determine the effects of NOP and NOP × MAL supplementation on CH_4 emissions, milk yield, rumen fermentation, and milk composition of dairy cows in the middle lactation period.

2 Materials and methods

All animals involved in the experiment were cared for according to the guidelines of the Animal Care and Use Committee of Henan Agricultural University (Zhengzhou, China). All experimental procedures were reviewed and approved by the committee. The NOP (10% NOP) compound was developed by DSM Nutritional Products Ltd. (Kaiseraugst, Switzerland) and was applied at 200 mg/kg feed dry matter. The organic acid MAL (99% MAL) was developed by Changmao Biochemical Engineering Institution (Changzhou, China) and was applied at 10 g/kg feed dry matter. The NOP dosage were supplemented in the diet of dairy cows in the middle lactation period according to Duval and Kindermann (15) and the report on malic acid used by previous researchers in dairy cows (29, 30). The milk of the

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cows in this experiment was abandoned for 7 days, and the cows were still used for production.

2.1 Experimental design, diet, and treatment

Twenty-four Holstein cows (parity 3) with similar age, weight (659 ± 20 kg), lactation stage (115 ± 10 d), and milk yield (24.6 ± 1.6 kg/d) were used in a 10-wk randomized complete block design and it consisted of 7-d sample collection. All cows were placed in a shaded open barn.

All cows were milked twice daily at 0600 and 1800 and were fed with a total mixed ration (TMR) (Table 1) diet twice daily at 0700 and 1900. During the whole experiment, cows could freely intake diet and drinking water. Control (CON) animals were not fed either supplement. The two treatment groups included animals fed 10% NOP at 200 mg/kg feed dry matter or 10% NOP at 200 mg/kg feed dry matter plus 99% MAL at 10 g/kg feed dry matter. The NOP and MAL were added as powders to the TMR and premixed, which allowed the dairy cows to consume the supplements all day by consuming diet.

TABLE 1 Ingredients and nutritional composition of the experimental diet.

Item, % dry matter (unless otherwise noted)	Total
Ingredient	
Dry ground corn	28.5
Soybean meal	8.4
Corn silage	19.3
Alfalfa haylage	17.8
Soyhull	8.1
Oat hay	7.5
Cottonseed, whole	5.0
$CaHPO_4$	0.5
Salt	0.5
$CaCO_3$	0.9
Molasses	3.0
Mineral and vitamin premix[1]	0.5
Nutrient composition	
DM[2], %	47.0
CP[3]	16.5
NDF[4]	29.9
ADF[5]	17.9
Ether extracts	3.2
Ash	9.0
Ca	1.0
P	0.4

[1]Premix per 1 kg of diet: vitamin A, 1,500 KIU/kg; vitamin D_3, 350 KIU/kg; vitamin E, 8,000 IU/kg; niacin, 5,000 mg/kg; biotin, 200 mg/kg; β-carotene, 600 mg/kg; Mn, 3,500 mg/kg; Cu, 2,500 mg/kg; Zn, 12,500 mg/kg; iodine, 200 mg/kg; Co, 60 mg/kg; Se, 65 mg/kg.
[2]DM, dry matter.
[3]CP, crude protein.
[4]ADF, acid detergent fiber.
[5]NDF, neutral detergent fiber.

2.2 Data and sample collection

When animals were fed, the ration provided to cows and the portion of diet rejected by all cows were recorded. Recording daily feeding and refusal allowed the amount of TMR fed to cows to be adjusted based on a daily refusal of 10%. Because the contents of NOP and MAL supplements were consumed by dairy cows after premixing with the TMR, they were not determined in the discarded diet. To determine the chemical composition of the diet, a TMR diet sample (approximately 600 g) was collected on days 69 and 70. Samples of TMR were dried in a forced-ventilation drying oven at 65°C for 72 h and then ground to pass through a 1-mm sieve. Samples were stored at 4°C until chemical analysis of feed components. The TMR diet was adjusted every 2 weeks to ensure that the concentrate-to-roughage ratio (5:5) fed to all animals was similar. The weights of cows were measured at the beginning and the end of this experiment. In addition, milk yield was continuously recorded from 10-wk. Milk samples collected in the morning and evening were mixed and divided into two 50-mL sterile test tubes. One milk sample was mixed with 2-bromo-2-nitropropane-1,3-diol and stored at 4°C, and the other sample was stored at −20°C until analysis of milk components and fatty acids (FAs). In addition, to determine enzyme activities in the serum of dairy cows, blood was collected from the cow tail vein with disposable venous blood collection needles and negative pressure blood collection tubes. Blood was collected before cows were fed at 0700 on days 69 and 70. Blood was centrifuged at 2,000 × *g* for 20 min at 4°C, and then, the serum was removed with plastic straws and stored at −30°C until analysis of enzyme activity.

Rumen fluid was collected before feeding at 0700 on days 69 and 70. Immediately after collection, and a 10-mL sterile syringe was used to inject rumen fluid into a 10-mL frozen tube, which was quickly stored at −80°C in liquid nitrogen until analysis of rumen microorganisms. In addition, the rumen fluids were filtered through two layers of filter gauze. Filtered samples were put into 50-mL centrifuge tubes and centrifuged at 4,000 × *g* for 20 min at 4°C, and then the samples were immediately stored at −20°C until analysis of volatile fatty acids (VFAs) and N-NH_3.

Methane emissions from the guts of all dairy cows were measured by using sulfur hexafluoride (SF_6) tracer gas technology (31) for five consecutive days (days 66–70). Halters and polyvinyl chloride neck yokes (internal capacity of approximately 2 L) modified by Johnson et al. were used as the devices to collect CH_4 gas. In addition, the design of the halters and yokes could be allowed to a half of 100% reduction in yoke vacuum pressure through the connected stainless steel capillary tube over 24 h. Pure SF_6 brass permeation tubes were made by members of our laboratory and were stored in an anaerobic environment at 39°C for 3 months before the experiment began to determine the permeation rates. For example, the average release rates (mean ± SD) of the groups 1, 2 and 3 were 5.16 ± 0.33, 4.91 ± 0.38, and 5.84 ± 0.36 mg/d, respectively. One week before gas collection, the SF_6 permeation tubes with known permeation rates were put into the rumen through a rumen catheter. In the first week of collection, the halters, yokes, and stainless steel capillary tubes were worn by cows to measure CH_4 emissions.

At the beginning of measuring CH_4 emissions (days 66–70), the air in the yokes was pumped out at 0600 daily to induce negative pressure, followed by placing the yokes on the cows. Halters and cow yokes were replaced every 24 h. High-purity nitrogen (N_2) was used daily to check whether there was pipeline blockage in the halters. To obtain a representative sample, the yokes were pressurized with N_2 to induce positive pressure. Three 100-mL subsamples were collected from each yoke using syringes and then injected into three corresponding 100-mL gas sampling bags (Dalian, China), which were used to analyze background concentrations of CH_4, SF_6, and H_2. At the end of each sampling, the yokes were pressurized with N_2 three times and then decompressed. They were pressurized again and then remained under pressure until the next day to check whether there were any leaks. If there were no leakages, they were used again to collect samples. To calculate average daily CH_4 emissions, background yokes were treated in the same way as cow treatment yokes.

2.3 Sample analyses

Dried TMR samples were ground by a pulverizer and passed through a 1-mm mesh screen and then sent to the feed and detection analysis laboratory of Henan Agricultural University to determine the dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and ash by AOAC International official methods 930.15, 990.03, 973.18, and 942.05, respectively (32). Crude fat was determined by AOAC methods 2003.05 (33). The neutral detergent fiber (NDF) was determined by the method of Van Soest et al. (34). Concentrations of phosphorus (P) and calcium (Ca) were determined according to the guidelines for (35). Concentrations of milk fat, protein (CP), lactose, and milk urea nitrogen (MUN) were measured using infrared spectroscopy with a Milk Composition Somatic Cell Analyzer (CombiFossTM-7; Beijing, China) at the Henan Dairy Production Performance Testing Institution (Zhengzhou, China). Concentrations of FAs were measured using a gas chromatograph (GC-2010 Plus; Shanghai, China) at the Qingdao Yixin Testing Technology Service Institution (Qingdao, China). Residues and metabolites of NOP in milk were measured by using high-performance liquid chromatography (U 3000; Shanghai, China). After collection, the pH of rumen fluid was immediately measured with a pH meter (ST-20; Shanghai, China). To determine the concentration of VFAs in rumen fluid, rumen fluid samples were centrifuged at 10,000 × *g* for 20 min, and then, supernatants were analyzed by gas chromatography according to the method described by Schlau et al. (61). The concentration of N-NH_3 in rumen fluid was determined according to the guidelines described by Ivan et al. (62).

Rumen contents stored at −80°C were sent to Shanghai Meiji Biomedical Technology Institution (Shanghai, China) on dry ice. Frozen rumen content (approximately 2 g) was thawed on ice, and total DNA was extracted by a bead-beating method to determine the copy numbers of total bacteria, methanogenic archaea, and protozoa (36). Following DNA extraction, total populations of bacteria and methanogenic archaea were measured and analyzed by qPCR using the primer pairs U2 (37) and uniMet (38), respectively, and total protozoa copy numbers were measured and analyzed by qPCR and SYBR-green chemistry with the primer pair P-SSU-316F (39) and P-SSU-539R (40). Serum of the three treatment groups were sent to Nanjing Jiancheng Institute of Bioengineering (Nanjing, China), and activities of the enzymes for the malate dehydrogenase (MDH), phosphoenolpyruvate carboxykinase (PCK), pyruvate kinase (PK), and citrate synthase (CS) were determined spectrophotometrically by using enzyme-linked immunosorbent assay (ELISA) detection kits (Nanjing, China) (41).

In the gas detection center of Henan Agricultural University, gas chromatography was used to detect the background concentration of CH_4 in the gas obtained from cow yokes by hydrogen flame ionization (GC1120; Shanghai, China) and also by detecting the background concentration of SF_6 by electron capture detection (GC-4000A; Beijing, China). Methods were according to those described by Johnson et al. (42). The background concentration of H_2 was measured by a pump-type H_2 detector (SKY 2000; Beijing, China). In addition, the treatment of background yokes was same to that of cow yokes. However, the background concentration of SF_6 was usually very small compared with that of cow yokes, and thus, it was ignored. In the calculation of CH_4 emissions, the representative samples and data for 3 d in whole period were selected according to Hristov et al. (63). The background CH_4 level was only subtracted from the CH_4 concentration in the yokes of dairy cows, according to Johnson et al. (42). To facilitate statistical analysis, daily CH_4 emissions were averaged for all cows.

2.4 Calculations and statistical analyses

The methane emission rate (QCH_4) was calculated from the measured concentrations of CH_4 ($[CH_4]_y$) and SF_6 in the yokes, the CH_4 ($[CH_4]_b$ concentration in the background yokes, and the known release rate of SF_6 (QSF_6) (42) as follows:

$$QCH_4 = QSF_6 \times \left([CH_4]_y - [CH_4]_b\right) / [SF_6].$$

All data were analyzed as the analysis of variance model by using the one-way ANOVA program in SPSS 19.0 (2010; SPSS Inc., Chicago, IL, USA). The effects of NOP and the interaction of NOP × MAL on CH_4, DMI, milk yield, milk composition, VFAs, total bacteria, total methanogenic archaea, and total protozoa were analyzed. When there was a significant difference, multiple comparisons were made to determine the differences among the three treatments by using the Duncan. Differences were considered significant at $p \leq 0.05$, and significant trends were recognized at $0.05 < p \leq 0.10$.

3 Results and discussion

3.1 Effects of NOP and NOP × MAL on dry matter intake, methane emissions, milk yield, feed efficiency, milk composition, and serum enzyme activity of dairy cows

In this experiment, cows were fed NOP (10% NOP 200 mg/kg feed dry matter) or NOP × MAL (10% NOP at 200 mg/kg feed dry matter plus 99% *L*-malate at 10 g/kg feed dry matter) via the TMR. Dairy cow DMI and milk yield were not affected by NOP (Table 2), which are results generally consistent with those of previous studies with NOP (19, 43, 44). In NOP × MAL, compared with CON, DMI decreased ($p \leq 0.001$), but milk yield was not affected. Compared with CON, enteric CH_4 emissions decreased by 54% in NOP and by 51% in NOP × MAL (Figure 1). Simultaneously,

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TABLE 2 Effects of supplementing diets with 3-nitrooxypropanol (NOP) and 3-nitrooxypropanol plus *L*-malate (NOP × MAL) on dry matter intake (DMI), methane emissions, milk yield, body weight (BW), feed efficiency, milk composition, and serum enzyme activity of dairy cows.

Item	Treatment[1]			SEM	*p*-value[2]	
	CON	NOP	NOP × MAL		C vs. N	N vs. N × M
DMI, kg/d	24.5	23.6	22.5	0.27	0.10	0.05
Milk yield, kg/d	22.6	23.5	23.0	0.57	0.27	0.54
CH_4, g/d	484.3	223.3	236.8	16.38	<0.001	0.84
CH_4, g/kg of DMI	19.8	9.5	10.5	0.95	<0.001	0.58
H_2, ppm/d	0.0	7.53	5.20	0.30	<0.001	0.001
ECM,[3] kg/d	22.5	26.8	27.6	0.68	0.002	0.45
Milk NE_L,[4]Mcal/d	16.7	20.0	20.6	0.51	0.002	0.44
ECM feed efficiency,[5]kg/kg	0.92	1.14	1.23	0.03	0.001	0.11
Feed efficiency,[6]kg/kg	0.92	1.00	1.02	0.02	0.057	0.45
Milk fat, %	3.75	4.74	5.09	0.06	<0.001	0.01
Milk protein, %	3.67	3.99	4.08	0.09	0.03	0.48
Milk lactose, %	4.74	4.72	4.85	0.03	0.88	0.10
MUN, mg/dL	21.3	19.8	19.4	0.31	0.02	0.67
BW, kg	660	655	652	2.70	0.43	0.67
The activity of enzyme,[7]						
MDH (U/L)	53.8	59.0	66.0	2.65	0.21	0.10
PCK (U/L)	112	120	130	3.06	0.24	0.13
PK (m U/L)	329	353	379	5.45	0.07	0.05
CS (U/L)	16.37	18.45	21.13	0.56	0.07	0.02

[1]CON = Control.
[2]C vs. N, CON vs. NOP; N vs. N × M, NOP vs. NOP×MAL.
[3]ECM (kg/d) = kg of milk production × (383 × fat% + 242 × protein% + 165.4 × lactose% + 20.7) ÷ 3,140 (59).
[4]Milk NE_L (Mcal/d) = kg of milk production × (0.0929 × fat% + 0.0563 × protein% + 0.0395 × lactose%) (60).
[5]ECM yield ÷ dry matter intake (DMI).
[6]Milk yield ÷ DMI.
[7]MDH, malate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; CS, citrate synthase.

the H_2 concentration in the two treatments also increased ($p = 0.001$). The continuous CH_4 emission reduction effect of the two treatments is generally the same as that in previous studies with NOP (5, 14, 40). Treatment with NOP significantly increased milk fat concentration ($p \leq 0.001$) compared with that in CON, but the increase in NOP × MAL was greater ($p = 0.01$) than that in NOP. In dairy cow serum, the activities of PK ($p = 0.07$) and CS ($p = 0.07$) increased slightly in NOP compared with that in CON, whereas the activities of MDH ($p = 0.10$), PCK ($p = 0.13$), PK ($p = 0.05$), and CS ($p = 0.02$) were higher or tended to be higher in NOP × MAL than in NOP. To explain the results, coenzyme M reductase (MCR) might be inactivated by the nitrooxy group in NOP, which would result in continuous reductions in CH_4 emissions and simultaneous increases in the content of H_2 (16). Hydrogen gas is considered a high-energy gas, and a small part of the H^+ accumulated in the rumen can be converted into extra energy to increase milk fat concentration, which might be why milk fat concentration increased in NOP (35, 45). The compound *L*-malic acid is a hydrogen transporter, and as H_2 accumulates in the rumen, hydrogen ions can be carried by the hydrogen transporter and transferred to mitochondria in rumen microbes and the mitochondria in the cow to be oxidized into ATP, which provides additional energy for animals to use in production. Therefore, in a previous study, the addition of malic acid to the diet of dairy cows not only reduced CH_4 emissions but also increased

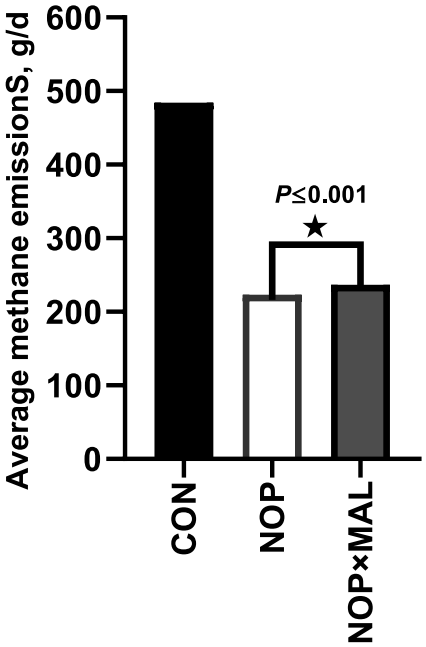

FIGURE 1
Methane emissions of three treatments in whole period.

milk production (46). Thus, the mechanism of malic acid might be explained. Similarly, activities of the enzymes MDH, PCK, PK, and CS in dairy cow blood serum were higher in NOP × MAL than in NOP. This result further indicated that NOP × MAL increased the processes of gluconeogenesis and glycolysis and activated the Krebs cycle and biological oxidation. As a result, NOP × MAL could provide more energy than NOP to increase milk fat concentration, which might explain why milk fat concentration in NOP × MAL was higher than that in NOP. In terms of CH_4 emissions, both *in vivo* and *in vitro* experiments lead to the conclusion that malic acid can reduce CH_4 emissions (29, 30). However, then reduction in CH_4 emissions in NOP × MAL was slightly lower than that in NOP, which could be explained by a slight competitive antagonism between NOP and MAL in reducing CH_4 emissions. In previous studies, malic acid increases the milk yield of dairy cows (27, 28, 47). However, contrary to the hypothesis and experimental conclusion in this study, NOP × MAL did not increase the milk yield of cows but instead reduced DMI and had no effect on milk yield. This result could be explained by the diet meeting the energy demand of dairy cows for production and not requiring further consumption (35).

3.2 Effects of NOP and NOP × MAL to dairy cows on the volatile fatty acid (VFA) profile in rumen fluid and rumen microbial profile counts

Compared with CON, NOP tended to decrease (Table 3) the molar ratio of acetate ($p = 0.08$) and increase the molar ratio of propionate ($p = 0.10$). Simultaneously, compared with NOP, NOP × MAL had the same tendency to decrease the molar ratio of acetate ($p = 0.07$) and increase the molar ratio of propionate ($p = 0.09$). As a result, the molar ratio of acetate-to-propionate decreased in cows fed the two treatments. In addition, compared with CON, molar proportions of butyrate and valerate increased in NOP, which are results generally consistent with those of previous studies on NOP (18, 44). Simultaneously, compared with NOP, the molar ratios of butyrate and valerate tended to increase in NOP × MAL. Compared with CON, NOP reduced the concentration of N-NH_3 ($p = 0.02$); however, there was no difference between NOP × MAL and NOP. Similarly, compared with CON, NOP tended to reduce copy numbers of total bacteria ($p = 0.15$) and methanogens ($p = 0.13$), but there was no distinction between NOP × MAL and NOP. This result could be explained by the fact that an increase of propionate in rumen fluid is considered to be a competitive alternative compared with an H_2 sink (44, 48). The concentration of H_2 in NOP × MAL and NOP was increased, but the H_2 discharged from the rumen was only a small part of the H_2 estimated by the two treatments to reduce CH_4 emissions. Because of possible adaptation of rumen ecosystems, an increase in dissolved H_2 concentration in the rumen is bound to replace H_2 sinks and the incomplete recovery of reduction equivalent in discharged H_2 (49, 50). Therefore, a decrease in the molar ratio of acetate and an increase in the molar ratio of propionate in this experiment were expected. In previous studies, malic acid decreased the molar ratio of acetate in rumen fluid and increased the molar ratio of propionate (29, 51). In this experiment, the acetate-to-propionate ratio in NOP × MAL was slightly lower than that in NOP, which might

TABLE 3 Effects of supplementing diets with 3-nitrooxypropanol (NOP) and 3-nitrooxypropanol plus *L*-malate (NOP × MAL) to dairy cows in the middle lactation period on the volatile fatty acid (VFA) profile in rumen fluid and rumen microbial profile counts.

Item	Treatment[1]			SEM	*p*-value[2]	
	CON	NOP	NOP × MAL		C vs. N	N vs. N × M
pH	5.48	5.40	5.63	0.05	0.43	0.11
Total VFA, mmol/L	108	107	105	1.20	0.71	0.52
VFA molar proportion (%)						
Acetate	65.80	62.70	59.30	1.13	0.08	0.07
Propionate	26.60	27.70	28.60	0.34	0.10	0.09
Butyrate	17.52	18.13	18.59	0.13	0.02	0.07
Isobutyrate	1.24	1.24	1.25	0.01	0.60	0.42
Valerate	2.28	2.34	2.38	0.01	0.04	0.10
Isovalerate	1.57	1.57	1.58	0.02	0.06	0.09
Acetate-to-propionate ratio	2.22	2.01	1.83	0.50	0.04	0.07
N-NH_3, mg/100 mL	13.11	10.01	8.80	0.60	0.02	0.36
Total bacteria copy numbers, × 10^7/g of rumen digesta	15.05	14.41	14.24	1.02	0.15	0.87
Methanogen copy numbers, ×10^6/g of rumen digesta	1.82	1.60	1.67	1.01	0.13	0.79
Protozoa copy numbers, ×10^5/g of rumen digesta	1.63	1.61	1.62	0.35	0.69	0.78

[1]CON = Control.
[2]C vs. N, CON vs. NOP; N vs. N × M, NOP vs. NOP×MAL.

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be explained by the synergistic effect of NOP and MAL in rumen fermentation. Melgar et al. (18) reported that valerate is produced by the condensation of acetate and propionate, which could explain why valerate increased in the two treatments. In addition, compared with NOP, the pH in NOP × MAL tended to increase, which indicated that malic acid increased the transport of hydrogen ions from H_2 sinks in the rumen to cell mitochondria to be oxidized to ATP. However, there are also endosymbiotic and ecto-endosymbiotic relations between protozoa and some methanogens, indicating there are also relations between CH_4 production and methanogens and protozoa (52). In studies on the effects of tea saponin and lipids, the diversity of methanogenic bacteria and CH_4 production often decreased with reductions in protozoa (53–55). Although NOP did not affect the total numbers of protozoa, it decreased copy numbers of total methanogenic archaea and bacteria, indicating that NOP had a highly specific effect on total methanogenic archaea in this experiment. Note that although MAL was not the focus of discussion, the effect of NOP × MAL on dairy cows was worth explaining in this experiment.

However, in terms of N-NH_3, compared with NOP (19, 44), NOP in this experiment reduced the concentration of N-NH_3. This result might be because NOP improved the utilization efficiency of N-NH_3 in dairy cows, which could also explain the increases in CP and decreases in MUN in milk.

3.3 Effects of NOP and NOP × MAL on fatty acids in milk of dairy cows (μg/mL of total fatty acids)

In terms of milk FAs (Table 4), NOP increased the short-chain FAs 4:0 and 10:0 ($p = 0.001$), 8:0 and 14:0 ($p = 0.02$), and the short-chain FAs 6:0 ($p = 0.015$) and 12:0 ($p = 0.01$). The NOP × MAL treatment resulted in a similar increase in milk FAs. Approximately 50% of the fat in milk comes from the absorption of FAs in the blood of dairy cows by mammary glands, with the other 50% from the *de novo* synthesis of FAs (56, 57). The main substrates for synthesizing FAs in dairy cows are acetate and butyrate, but butyrate only provides half of the four carbons (58). Therefore, the short-chain FAs in mammary glands are mainly synthesized from acetate. When CH_4 production in the rumen is inhibited, butyrate seems to be the main substrate for synthesis of short-chain FAs in mammary glands (18), which is also the result of a decrease in CH_4 emissions and an increase in the butyrate molar ratio. In addition, when CH_4 production in the rumen was inhibited in NOP, biooxidation of a small part of the H_2 sink might provide additional energy for the synthesis of FAs in milk. However, the NOP × MAL treatment could provide more additional energy for FAs synthesis, which might explain NOP × MAL had further the increase in FAs concentration in milk than NOP. In this experiment, the concentrations of monounsaturated fatty acids (MUFAs) 16:1 and 18:1 decreased in NOP, and thus, the

TABLE 4 Effects of supplementing diets with 3-nitrooxypropanol (NOP) and 3-nitrooxypropanol plus *L*-malate (NOP × MAL) on fatty acids in milk of dairy cows in the middle lactation period (μg/mL of total fatty acids).

	Treatment[1]				*p*-value[2]	
Item	CON	NOP	NOP × MAL	SEM	C vs. N	N vs. N × M
C4:0	44.0	60.1	84.8	1.90	0.001	<0.001
C6:0	31.8	40.3	76.5	1.96	0.015	<0.001
C8:0	26.5	32.6	48.6	1.40	0.02	<0.001
C10:0	45.4	56.5	78.9	1.40	0.001	<0.001
C12:0	67.6	73.9	86.2	1.11	0.01	<0.001
C14:0	225	267	398	10.25	0.02	<0.001
C14:1	25.36	22.48	32.1	0.92	0.13	<0.001
C15:0	31.1	28.3	40.1	1.97	0.59	0.01
C16:0	657	646	737	6.06	0.50	<0.001
C16:1	61.21	47.68	77.87	2.18	0.006	<0.001
C18:0	233	266	407	9.05	0.08	<0.001
C18:1	619	544	832	15.38	0.02	<0.001
C18:2	147	130	256	5.61	0.13	<0.001
C18:3n-3	33.0	35.9	39.4	0.79	0.07	0.04
C18:3n-6	22.0	23.8	35.5	1.12	0.52	<0.001
C20:0	29.0	31.2	40.8	0.48	0.03	<0.001
ΣSFAs[3]	1,440	1,553	2,048	17.29	0.004	<0.001
ΣMUFAs[4]	706	614	942	18.13	0.02	<0.001
ΣPUFAs[5]	202	190	331	5.62	0.30	<0.001

[1]CON = Control.
[2]C vs. N, CON vs. NOP; N vs. N × M, NOP vs. NOP×MAL.
[3]ΣSFAs = Total saturated fatty acids.
[4]ΣMUFAs = Total monounsaturated fatty acids.
[5]ΣPUFAs = Total polyunsaturated fatty acids.

concentration of total MUFAs also decreased. This result could be explained by the fact that biohydrogenation may provide a small absorption sink for H_2 when CH_4 production in the rumen is inhibited (43). Total concentrations of saturated fatty acids (SFAs), MUFAs, and polyunsaturated fatty acids (PUFAs) increased in NOP × MAL, which might be because when CH_4 production in the rumen was inhibited, additional H_2 sinks were transferred by MAL and eventually oxidized into extra energy to increase generation of SFAs, MUFAs, and PUFAs. In that situation, biohydrogenation seemed to be weakened compared with that in NOP. In addition, residues and metabolites of NOP in milk were not detected, which indicated that NOP can be completely metabolized by dairy cows and has no effect on animal health.

4 Conclusion

Supplementing with NOP (10% NOP 200 mg/kg feed dry matter) reduced CH_4 in the guts of dairy cows by 54%, and supplementing with NOP × MAL (10% NOP at 200 mg/kg feed dry matter plus 99% *L*-malate at 10 g/kg feed dry matter) decreased CH_4 in the guts of dairy cows by 51%. The NOP treatment did not affect DMI and milk yield, whereas the NOP × MAL treatment reduced DMI but did not affect milk yield. Both treatments reduced the ratio of acetate-to-propionate and tended to reduce copy numbers of methanogens, which could explain reductions in CH_4 emissions. In addition, NOP increased the concentrations of short-chain FAs and total SFAs but decreased those of total MUFAs because of the action of a small part of rumen biohydrogenation. Compared with NOP, NOP × MAL increased the concentrations of short-chain FAs, total SFAs, MUFAs, and PUFAs, which indicated that NOP × MAL increased the energy utilization rate of cows compared with that with NOP.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the Committee of Henan Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal studies were approved by the Committee of Henan Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XZ: Investigation, Methodology, Writing – original draft. SF: Investigation, Writing – review & editing. GL: Methodology, Writing – review & editing. ZY: Investigation, Writing – review & editing. XD: Investigation, Writing – review & editing. YZ: Investigation, Writing – review & editing. TG: Funding acquisition, Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sustainable, greenhouse gas derived fermented protein in canine diets—a pilot study

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Sustainability concerns have increased consumer demand for non-animal-derived proteins and the search for novel, alternative protein sources. The nutritional sustainability of the food system without compromising the nutrient quality, composition, digestibility and consumption is pivotal. As with farmed livestock, it is imperative to ensure the well-being and food security of companion animals and to develop sustainable and affordable pet foods. The current pilot study was conducted to determine the effect of greenhouse gas-derived novel, fermented protein ingredient in beagle dogs. The greenhouse gas-derived fermented protein is an alternative protein ingredient with optimal nutritional factors and provides traceability, significantly optimizes the use of land and water, and provides sustainability to the feed value chain of canine diets. Three experimental groups including control, 5 and 10% inclusion of high protein ingredients were included in the study and the results suggest that the fermented protein is palatable and acceptable at 5 and 10% inclusions in the diets of dogs. The present study shows no significant difference in general alertness, clinical symptoms, water consumption and social behavior of dogs between 5 and 10% fermented protein inclusion in canine diets. The diversity of the bacterial community did not change after supplementation with the tested protein source in dogs. Only a few bacterial genera differed significantly in relative abundance between the experimental groups. Feed consumption, faecal scoring and the microbiome data results of this pilot study on the use of novel, methane gas derived, bacterial SCP as a protein ingredient in the canine diets, would pave way for more and more inclusion of such novel alternative protein sources in the pet food industry.

KEYWORDS

fermented protein, greenhouse gas, microbiome, canine diet, palatable

Introduction

The global pet food market is projected to grow from 115.50 billion USD in 2022 to 163.70 billion USD by 2029 (1). The increasing trend for pet ownership, rising urbanization and pet humanization are factors for the pet owners to opt also for nutritious and quality food for their pets and act as major drivers in the petfood market. Proteins in pet diets sourced from animal origin are posing threats on the sustainability factor. Hence adoption of sustainable practices of developing feeds less reliant on non-renewable sources would significantly strike the right balance between nutritional, ecological, social and economic aspects.

Protein is the most expensive, indispensable macronutrient in pet foods. National Research Council (NRC) (73) provides a recommended allowance of 10 and 20% crude protein for adult dogs (2) whereas the recommendations made by AAFCO for adult dogs is 18% crude protein (3). The ideal amino acid profile for dog nutrition is provided by Baker and

Maulden (4). The advantages of high protein, low carbohydrate foods elicit lower glycemic index which can benefit dogs with insulin resistance and diabetes (5, 6). The protein content of the diet is positively associated with food selection in dogs (7). Studies have shown that pet foods with a higher protein content (103 g/1000 kcal) in addition to higher fiber content, decrease voluntary intake, increase the amount and rate of weight loss, and increase fat mass loss during weight loss in dogs (8, 9). Dog foods containing high protein and low energy maintain muscle mass during weight loss (10, 11). Additionally, high-protein diets can be beneficial for endurance exercise in dogs. Sled dogs fed a diet consisting of 35% of energy from protein had higher plasma volume than dogs fed a diet with 18% of energy from protein (12). The 18% protein diet also resulted in decreased maximal oxygen uptake (VO2 max) and greater rate of soft-tissue injuries.

Considering the critical role of protein in pet foods, and in response to consumer demand, sourcing constraints and sustainability concerns, research for novel protein sources have emerged as an important trend in the pet food industry. Dried whole-cell yeast (*S. cerevisiae*) is an alternative to conventional animal-derived protein sources that aligns with this trend and has been shown to have beneficial health effects in several animal species, including the modulation of the colonic microbiota in dogs (13–16). Insect based proteins are also tested in dogs wherein the diets were shown to alter the gut microbiota slightly (17). Bacterial based protein ingredients which are produced under controlled conditions, and which are scalable are considered viable alternative source to circumvent the problems of protein shortage. Existing pet foods are rich in ingredients of animal origin and are associated with drawbacks such as higher greenhouse gas emissions, land and water use. A recent study estimated that pet food, specifically dry diets from the U.S., could account for up to 2.9% of global CO2 equivalent emissions and up to 1.2% of agricultural land use. As greenhouse gas, methane contributes to the global warming potential (GWP_{20}) 84 times that of carbon di oxide (18, 19), methane removal technologies have gained significant attention (20, 21) and is also considered as a cost-efficient carbon and energy source from the biomanufacturing standpoint (22). Fermented proteins offer several advantages over animal and plant proteins such as low carbon footprint, low reliance on land, water, and seasonal variations coupled with a balanced amino acid and nutritional profile. The current study provides support for the acceptability and digestibility of dog diets containing such greenhouse gas derived microbial fermented protein as a sustainable alternative protein source with an ideal amino acid profile and is palatable.

Materials and methods

Animals, facilities, and experimental design

Clinically healthy, adult beagle dogs, of both sexes, between 12 and 20 kg of bodyweight were enrolled in the study. Beagles were utilized in the study due to their uniform sizes, excellent temperament and physiology suited to studies in controlled environments. The standard housing conditions required for canine studies such as provision of minimum 2–4 m^2 space for the dogs allowing for free movement, non-slip flooring, soft bedding materials (straw). The animals had constant human interaction in addition to additional props (chew toys) for environmental enrichment. Adequate environmental conditions (temperature, humidity, ventilation, lighting) were maintained as per standard.

Dry pet food preparation as kibbles

The dry feed (kibbles) for dogs used in this study were custom manufactured by a pet food manufacturer (Taiyo Group, Chennai, India) and consisted of three different formulations using fermented single cell proteins (SCP) of bacterial origin with three different inclusion levels (0, 5, and 10%) stored at room temperature in sealed packages. Each sample is derived from the same lot of production, using uniform production parameters. Based on the inclusion levels of fermented proteins, each formulation also comprises varying percentages of different cereals and grain byproducts and micronutrients as mentioned in Table 1 as control, test 1 (fermented protein 5% inclusion), test 2 (fermented protein 10% inclusion) respectively. The fermented protein was produced by the continuous aerobic fermentation process using a patented proprietary fermentation process of String Bio Pvt. Ltd., India within its String Integrated Methane Platform, SIMP™ technology, as described in Subbian et al. (23). The same extrusion technique was used for the production of all of the formulations, and processing was carried out under the same conditions. The protein content of the different formulations was maintained at around 24%.

Feeding trial

The feeding trial was conducted at Invetus, the largest Australasian veterinary contract research organization under the Institutional Animal Care and Use Committee protocol, authorised with the trial number RIU C 22179 W.

Seven healthy adult beagles with an average body weight of around 12 to 20 kg were individually housed in pens. The dogs received two feedings per day at time with water *ad libitum*.

Study design

A total of seven dogs were enrolled into this study of which 5 dogs were fed with two different trial diets (3 dogs were fed with 5% fermented protein and another 2 dogs were fed with 10% fermented protein formulation) and 2 dogs were fed a control diet that contains 0% fermented protein and served as negative control throughout the conduct of this study for a period of 21 days.

From day 1–4 the 5 trial dogs and 2 control dogs were fed with a transition diet that consists of 75% standard diet (Cobbers Working dog kibble routinely fed at WRC) and 25% new diet. On days 5–8, dogs were fed with a transition diet that consisted of 50% standard diet and 50% new diet and on days 9–12, dogs were fed with a transition diet that consisted of 25% standard diet and 75% new diet. Following these 12 days of transition feeding, the 5 trial dogs were fed with 100% of the trial diet between days 13–20. The individual faecal samples were collected on day 1 and on day 21 for comparative microbiome analyses. The 2 control dogs received 100% control diet. Feed consumption and faecal scoring were recorded daily for all dogs. The diet schedule and the details of the animals are mentioned in

TABLE 1 Ingredient composition (%) of dry dog feeds used in the study.

Ingredients	Control	Test 1 (fermented protein 5% inclusion)	Test 2 (fermented protein 10% inclusion)
Rice	26.97	26.87	27.08
Oats	15.88	15.37	12.88
Wheat	14.45	14.39	14.50
Corn Gluten	14.45	14.23	14.24
Wheat Gluten	10.12	5.92	3.15
Novel fermented protein	0.0	5.0	10.00
Beet pulp	1.93	1.92	1.93
Brewer's yeast	0.96	0.96	0.97
Calcium carbonate	1.45	1.44	1.45
Salt	0.96	0.96	0.97
Choline chloride 70%	0.16	0.12	0.15
Potassium chloride	0.63	0.62	0.59
DL-Methionine	0.14	0.14	0.15
L-Lysine	0.00	0.38	0.23
Naturox (Kemin)	0.1	0.10	0.1
Monocalcium phosphate	1.11	0.96	0.97
Vitamin & Mineral Premix	0.29	0.29	0.29
MCD	0.40	0.40	0.40
Potassium sorbate	0.20	0.20	0.20
Termox Dry (Kemin)	0.15	0.15	0.15
Toxin Binder	0.20	0.20	0.20
Sodium Benzoate	0.10	0.10	0.10
Termox Liquid (Kemin)	0.03	0.03	0.03
Fish oil	2.41	2.40	2.42
Sunflower Oil	5.95	5.88	5.90
Flaxseed Oil	0.96	0.96	0.97
Total	100	100	100

Tables 2, 3. Tash and Buk were fed with control diets whereas Annie, Queenie, Kale, Huxley, Jasmine were fed with the test diets by replacing the basal diet with the fermented protein as mentioned in the Table 2.

The nutrient composition of the fermented protein source and the experiment protein diets are presented in Tables 4, 5. The proximate composition of the fermented protein and the diets were analyzed by standard AOAC test methods.

Faecal scoring and testing

Faecal scoring was done on a daily basis from day 1 to 21 and the scoring was done as per the Waltham faeces scoring system (24). The Waltham scale utilizes a scale of 1–5 with half numerical increments,

TABLE 2 Diet schedule of the fermented protein (test) study in dogs.

Days	Diet schedule
Days 1–4	5 trial dogs (3 on 5% and 2 on 10% protein test diets) + 2 controls (0% protein diet); Housed dogs by group into three communal pens. Transition feeding with 75% (262.5 g) standard diet and 25% (87.5 g) new diet with fermented protein; Recorded food consumption and faecal scoring; Collected individual faecal samples into yellow-top jars and submerged in faecal storage solution. Jars were labelled with study number, dog ID and collection day and stored frozen at −20°C.
Days 5–8	5 trial dogs (3 on 5% and 2 on 10% protein test diets) + 2 controls (0% protein diet); Transition feeding with 50% (175 g) standard diet and 50% (175 g) new diet; Recorded food consumption and faecal scoring.
Days 9–12	5 trial dogs (3 on 5% and 2 on10% protein test diets) + 2 controls (0% protein diet); Transition feeding with 25% (87.5 g) standard diet and 75% (262.5 g) new diet; Recorded food consumption and faecal scoring.
Days 13–20	5 trial dogs (3 on 5% and 2 on10% protein test diets) + 2 controls (0% protein diet); New food 100% (350 g); Recorded food consumption and faecal scoring.
Day 21	Recorded food consumption and faecal scoring; Collected individual faecal samples into yellow-top jar and submerged in faecal storage solution. Jars labelled with Study number, Animal ID and collection date.

TABLE 3 Details of the test animals.

S. No.	Animal ID	Details
1	Tash 1347	Control
2	Buk 3850	Control
3	Annie 3610	Test 1 (5%fermented protein)
4	Queenie 4339	Test 1 (5% fermented protein)
5	Kale 5636	Test 1 (5% fermented protein)
6	Huxley 5632	Test 2 (10% fermented protein)
7	Jasmine 7812	Test 2 (10% fermented protein)

covering a range of very hard (score 1) and dry to entirely liquid faeces (score 5) (24, 25). The mean Waltham score was calculated over the trial period to determine the overall stool consistency. The cut-off score for diarrhea was set at a mean score of 3.5. This also entails that control animals whose mean Waltham score exceeded this value were labelled as diarrhea-positive and vice versa. The faecal consistency scoring system is the most common stool scoring manual which can reflect intestinal health of the animals (26). Faecal condition scores can provide insights into how a diet is being digested (otherwise utilized) by an animal. The colour is also helpful in understanding the digestibility of animals. Low scores (unformed, loose, diarrhea, etc) may indicate digestive upset, malabsorption, and/or possible hydration issues. On the other end of the spectrum, hard stools may indicate a lack of appropriate fiber, a water balance issue, etc. The routine use of faecal scoring systems with animals can provide an invaluable tool to veterinarians and animal managers when there are any changes with condition, consumption, and/or overall health.

TABLE 4 Nutrient composition of fermented protein.

S. No.	Description	Value	Test method
1.	Dry matter, %	94.4	By calculation; AOAC 930.15 (for moisture)
2.	Crude protein, %	72.2	AOAC 984.13
3.	Fat, %	5.3	AOAC 2003.06
4.	Crude Fiber, %	<1.0	AOAC 962.09
5.	Ash, %	7.7	IS 14827–2000
6.	Gross energy, MJ/kg	21.7	By calculation

TABLE 5 Nutrient composition of dry kibbles.

S. No.	Description	Control	Test 1 (fermented protein 5%)	Test 2 (fermented protein 10%)
1.	Moisture, %	7.5	7.5	7.5
2.	Crude protein, %	24.0	24.0	24.0
3.	Fat, %	10.0	10.0	10.0
4.	Crude Fiber, %	1.7	1.6	1.7
5.	Ash, %	5.2	5.7	5.8
6.	Gross energy, MJ/kg	14.6	14.6	14.5

Faecal DNA isolation and metagenome analysis/faecal DNA extraction and sequencing

DNA from the faecal samples was extracted using a Bioline Isolate Faecal DNA kit (Meridian, cat.no#BIO-52082). Primers were selected to amplify the V3–V4 region of 16S rRNA genes as these regions display the maximum discriminatory power demonstrating sufficient sequence diversity using the forward primer ACTCCTACGGGAGGCAGCAG and reverse primer GGACTACHVGGGTWTCTAAT. Sequencing was performed on an Illumina MiSeq platform using 2 × 300 bp paired-end sequencing. The microbiota of the dogs fed with 5 and 10% protein diets—namely Annie 3610, Queenie 4339, Kale 5636, Huxley 5632, and Jasmine 7812—was investigated on days 1 and 21. This analysis was conducted to assess changes in the microbiota of dogs fed diets containing the fermented bacterial Protein between day 1 (beginning of the study) versus day 21 (end of the study).

Data analysis

Sequence data were trimmed with Trimmomatic (v 0.39) and then fastq files were analyzed using DADA2 in QIIME2 v2020.6 to denoise and produce Amplicon Sequence Variants (ASVs). ASVs were clustered at 99% identity using the VSEARCH plugin. Taxonomy was assigned using the SILVA database (v138). Obtaining feature table was further filtered (features that were present in only a single sample and features with a total abundance of less than 10). A total of 172,249 reads remained for the analysis, with an average of 17,224 reads per sample. The number of features remains after the data filtering step is 557. The downstream statistical microbial data analyses and visualizations were done using Microbiome Analyst (27). The community profiling was conducted using the R Phyloseq and Vegan packages. Principal coordinates analysis (PCoA) employing the Bray-Curtis Index and PERMANOVA was utilized to visualize the clustering of samples based on their phylum and genus-level compositional profiles. The identification of significant features was performed using single-factor statistical comparisons with a p-value cutoff of 0.05 and the t-test/ANOVA statistical method. The sequence data used for analysis is available in NCBI under BioProject accession Number PRJNA1116051.

Results

Feed consumption and faecal score

All the trial dogs were fed with 350 g/day as per calorific requirements. The feed consumption of the dogs fed with the fermented protein at 5 and 10% inclusions appear to be comparable to the control, demonstrating that the product is palatable and accepted by the dogs. There was a slight discrepancy in the feed consumption observed in one dog (Jasmine 7,812), which was fed with 10% fermented protein on days 12 and 14, however it became comparable to the control at the end of the study on day 21 (Figure 1). As part of this pilot study, we did not consider weight gain as the prime metric. However, we do not see a drastic change between the weights before and after the study (Table 6) with an exception in one of the animals, Jasmine 7,812 fed with 10% of the fermented protein did show lesser feed intake in the transition period between day 12 and day 14 which is also observed in the faecal consistency record (Figure 2).

Faecal scores

Faeces obtained from 0% protein control dog typically had a yellowish colouring to some parts of most faeces. 5% test protein faeces typically had an orangish colouring to some parts of some faeces. 10% test protein faeces had no major noticable differences to the colouring of faeces during the study. The aroma of faeces was different between the start of the study and the completion of the study for all groups. Clinical observations suggest that the diet containing fermented protein is acceptable by dogs throughout the study and hence reflects no palatability issues in the diets.

Microbiome analyses

The microbiota of faecal samples from dogs fed with test protein diet and control diets was examined on days 1 and 21, respectively. The relative abundance, alpha and beta diversity were analyzed from the microbiome data.

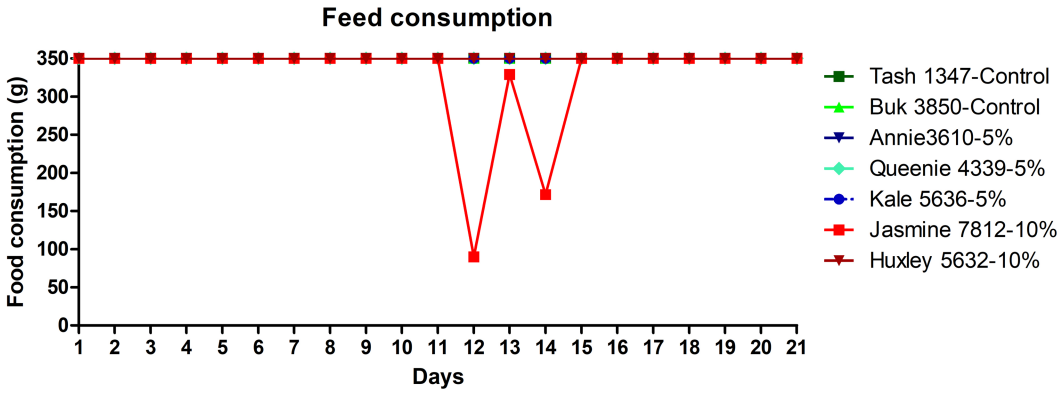

FIGURE 1
Feed consumption data of the dogs fed with the microbial fermented protein along with the control dogs. The line profiles demonstrate the feed consumption (in grams) of dogs over a period of 21 days. The feed consumption data points of the control dogs (Tash1347 and Buk3850) are marked in dark and light green colour respectively, 5% test protein fed dogs (Annie3610, Queenie4339 and Kale5636) are marked in dark blue, cyan and navy blue colours respectively, 10% test protein fed dogs (Jasmine7812 and Huxley5632) are marked in bright red and brown colour, respectively. The feed consumption of Jasmine7812 shows a dip on both 12th and 14th day.

Abundance profiling

The relative abundance at the phylum level is presented in Figure 3. Members of the Actinobacteriota phylum were reduced in the test protein group, while the Bacteroidota phylum showed an increased relative abundance (presented in yellow and purple, respectively, in Figure 3). Further statistical comparisons between the two groups demonstrated that these differences are significant (Figure 4). Firmicutes was the most abundant phylum detected in both the groups. The terms non-protein and protein corresponds to day 1 and day 21 diets fed to the animals.

The relative abundance of the top 10 genera, visualized in a stacked bar chart, shows that members of the *Bifidobacterium* genus (presented in light purple in Figure 5) has a reduced relative abundance in the protein diet group compared to the non-protein diet group, while members of the *Bacteroides* and *Fusobacterium* genera (presented in dark purple and dark green, respectively, in Figure 5) show an increase. However, statistical comparison between the two groups reveals that these differences are not significant (with a p-value cutoff of 0.05).

Single-factor statistical comparison between the test group studies

Single-factor statistical comparisons were used to determine if there were significant differences in the abundance of specific features between these groups. The results showed a significant reduction in the phylum Actinobacteriota ($p < 0.05$), but an increase in the phylum Bacteroidota in the protein diet group (Figure 4). No significant features were identified at the genus level with a p-value cutoff of 0.05.

Alpha and beta diversity profiling

No significant difference was found in the Shannon and Simpson alpha diversity values, along with the Chao1 richness index, between the two groups, at the genus and phylum levels ($p > 0.05$).

TABLE 6 Weight of the animals before and after the study.

Dog ID and group	Initial weight (Kg)	Final weight (Kg)
	Day 1	Day 21
Tash 1347 (Control −0% test protein)	20.0	20.0
Buk 3850 (Control – 0% test protein)	14.7	15.0
Annie 3610 (5% test protein)	13.1	14.5
Queenie 4339 (5% test protein)	15.2	14.5
Kale 5636 (5% test protein)	15.0	15.0
Huxley 5632 (10% test protein)	13.7	14.0
Jasmine 7812 (10% test protein)	15.8	15.9

Principal Coordinate Analysis (PCoA) was employed to explore and visualize similarities and dissimilarities in the overall microbiota compositions of the two groups. A statistically significant difference in beta diversity between two groups suggests distinctions in the composition of the communities within them ($p = 0.015$ at both phylum and genus level) (Figure 6). One of the leading indicators of a healthy gut microbiome is the increased richness and diversity of microorganisms (28). Dogs with gastro intestinal disorders have been reported to have lower diversity when compared to healthy dogs (29–32). Studies with corn fermented protein demonstrated good preservation of alpha and beta diversity (33). Hence, difference in beta diversity observed in the current study supports good intestinal health in the dogs fed with the fermented protein diets.

As canine health is influenced by diet and the gastrointestinal microbiome in terms of nutrient digestion and absorption (34), leveraging alternative, novel ingredients in the dietary supplementation of the pet foods without impacting their gut health is a significant factor. The gut microbiome harboring diverse bacteria is a cardinal immune and metabolic organ and evaluation of the faecal microbiome is the most accessible sample type for testing. Several lines of evidence

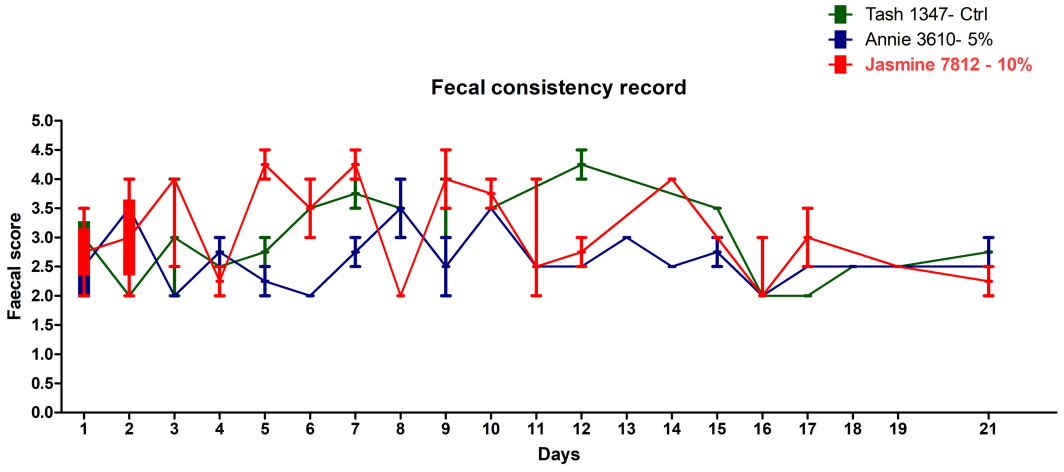

FIGURE 2
Representative faecal score of the dogs fed with the control (Tash1347, marked in green line) and fermented protein with 5% inclusion (Annie3610 marked in blue line) and 10% inclusion (Jasmine 7,812 marked in red line).

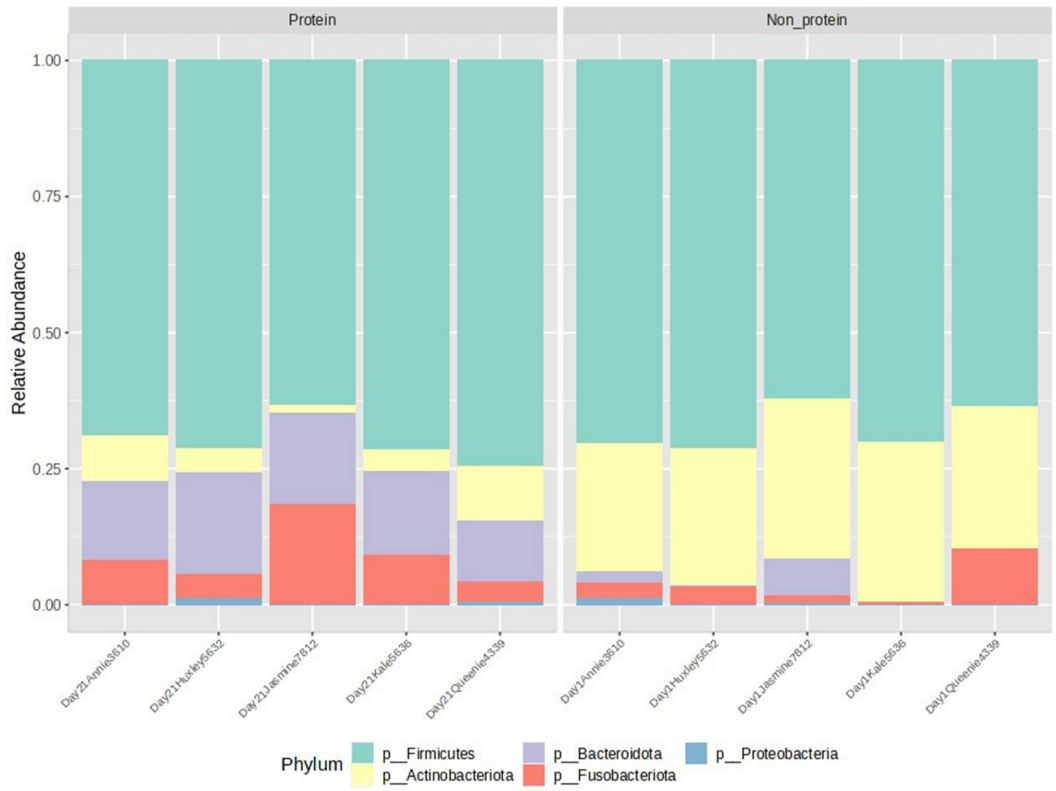

FIGURE 3
Relative abundance of the two groups. Members of the phyla Firmicutes, Actinobacteriota, Bacterioidota, Fusobacteriota, and Proteobacteria were observed. Protein group indicates animals fed with fermented protein (Day 21) and the non-protein group indicates animals fed with control diets (Day 1).

has demonstrated imbalances in the intestinal microflora is related to diseases like inflammatory bowel disease, irritable bowel syndrome, obesity and diabetes in humans and animal models (35–39) and are corroborated with the diagnosis and determining dysbiosis indices (40–43). Our current study demonstrates acceptance of the novel diet in the pet foods albeit the small sample size.

Discussion

Our observations on feed acceptance, overall health and faecal microbiome profiling in dogs fed diets containing proteins derived from greenhouse gases show that these proteins are well tolerated, without any digestive problems and side effects. The feed consumption

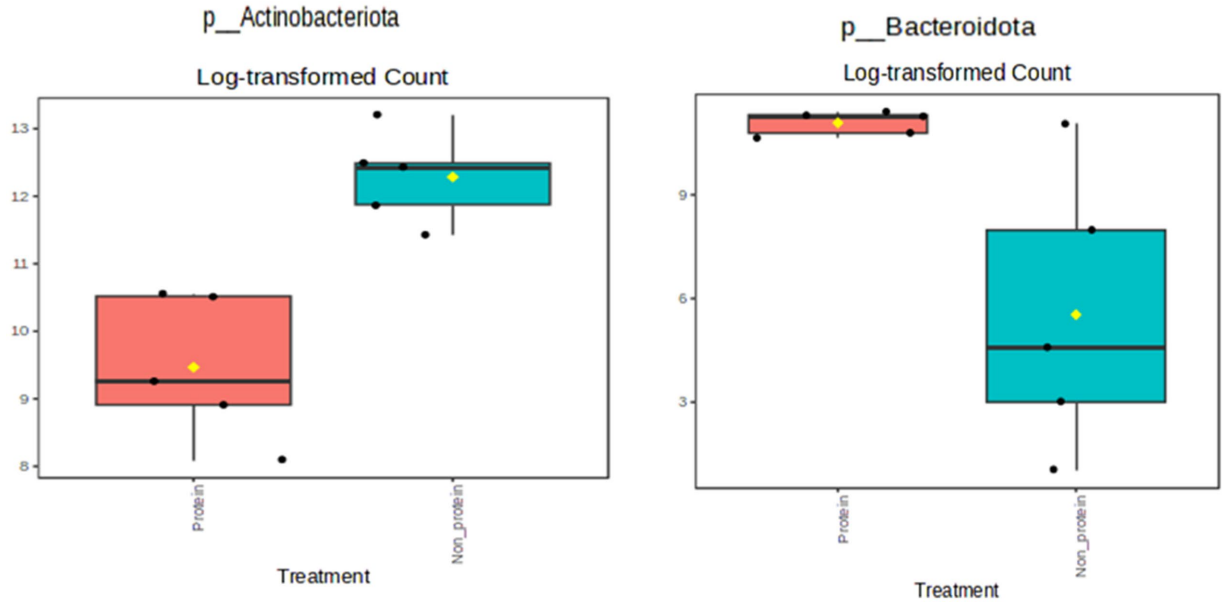

FIGURE 4
Single-factor statistical comparisons demonstrated a significant decrease in the phylum Actinobacteriota and an increase in the phylum Bacteroidota in the protein diet group ($p < 0.05$). Protein group indicates animals fed with fermented protein (Day 21) and the non-protein group indicates animals fed with control diets (Day 1).

and the faecal scores also support the acceptance of the fermented protein of bacterial origin in diets in dogs. Additionally, the microbiome profiling indicates no significant alpha diversity changes, indicating that the protein diet does not affect the overall richness and evenness of microbial community.

In terms of acceptance, throughout the study, both 5 and 10% protein mix were completely consumed by all the dogs indicating the smell and taste aspects of these sustainable protein fractions in the kibble mix were well accepted. This is in line with the earlier studies relating to acceptance of dry pet foods in dogs (44, 45). According to AAFCO recommendation, the minimum dietary protein requirement for a growing dog is 18% dry matter (DM) and 8% for an adult dog. Intake of fermented protein in the extruded products/kibbles can be considered as a good sign in the canine diets. Product appearance is one of the key characteristics in dry dog foods, as was found by Di Donfrancesco et al. (44) wherein the authors found that dry dog food kibble that is too light or too dark in appearance may receive lower overall liking scores from potential product purchasers. The colour of test diet kibbles used in the study were of light brown colour. In terms of weight changes, we observed that only a slight change in weight was observed in the dogs fed with both 5 and 10% protein diet. The weight changes were not expected for a short study duration kibble acceptance study (46).

The clinical observations indicated that both the diets containing 5 or 10% of the fermented protein did not cause any impact with respect to general alertness, water consumption and social behavior providing evidence that this alternative protein source is safe. Further, digestive health as measured through faecal colouring and consistency scoring also was supportive of a well-tolerated protein fraction in the diet, without any alterations to the stool formation. The elevated fiber levels in diets containing Torula Yeast and legume proteins reduced dry matter and organic matter digestibility and have shown lower apparent fat digestibility (47). Studies demonstrate that on comparing soybean meal to poultry byproduct meal in extruded dog diets, soybean meal tended to reduce the digestibility coefficients of dry matter, organic matter, acid hydrolysed fat, and gross energy (48) reported that the inclusion of a soluble yeast cell wall reduced the coefficient of fat digestibility in an extruded dog diet without affecting any other nutrient digestibility (49). In the current study, there were no issues observed in terms of digestibility with the fermented protein fed diets in dogs.

The potential impact of the protein diet on the faecal microbiota was investigated in dogs fed the protein diet, with day 1 representing the non-protein diet and day 21 representing the protein diet. Due to the limited number of dogs enrolled in the study, those fed both 5 and 10% protein diets were grouped together. There was no significant difference in alpha diversity between the two groups when investigating the Shannon, Simpson and Chao1 indexes. However, beta diversity analysis indicated a significant difference ($p = 0.015$) in community structure between the protein and non-protein diet groups, with clear separation of the clustered samples from each group. As described previously (50–52) the diversity of the bacterial community did not change after supplementation with the tested protein source in healthy dogs. Only a few bacterial genera differed significantly in relative abundance between the experimental groups. The results are similar to the study done with insect-based diets (house crickets and mulberry silkworm pupae) tested in dogs (17).

A significant reduction of the phylum Actinobacteriota in the dogs with protein diet group was observed. The most well-known Actinobacteriota are *Bifidobacterium*, which are homo—or heterolactic fermentative. Higher abundance of Actinobacteria has been observed in adult obese dogs, probably due to their role in the production of energetic SCFAs (53). In contrast to what was observed in the Actinobacteriota phylum, the Bacteroidota phylum showed an

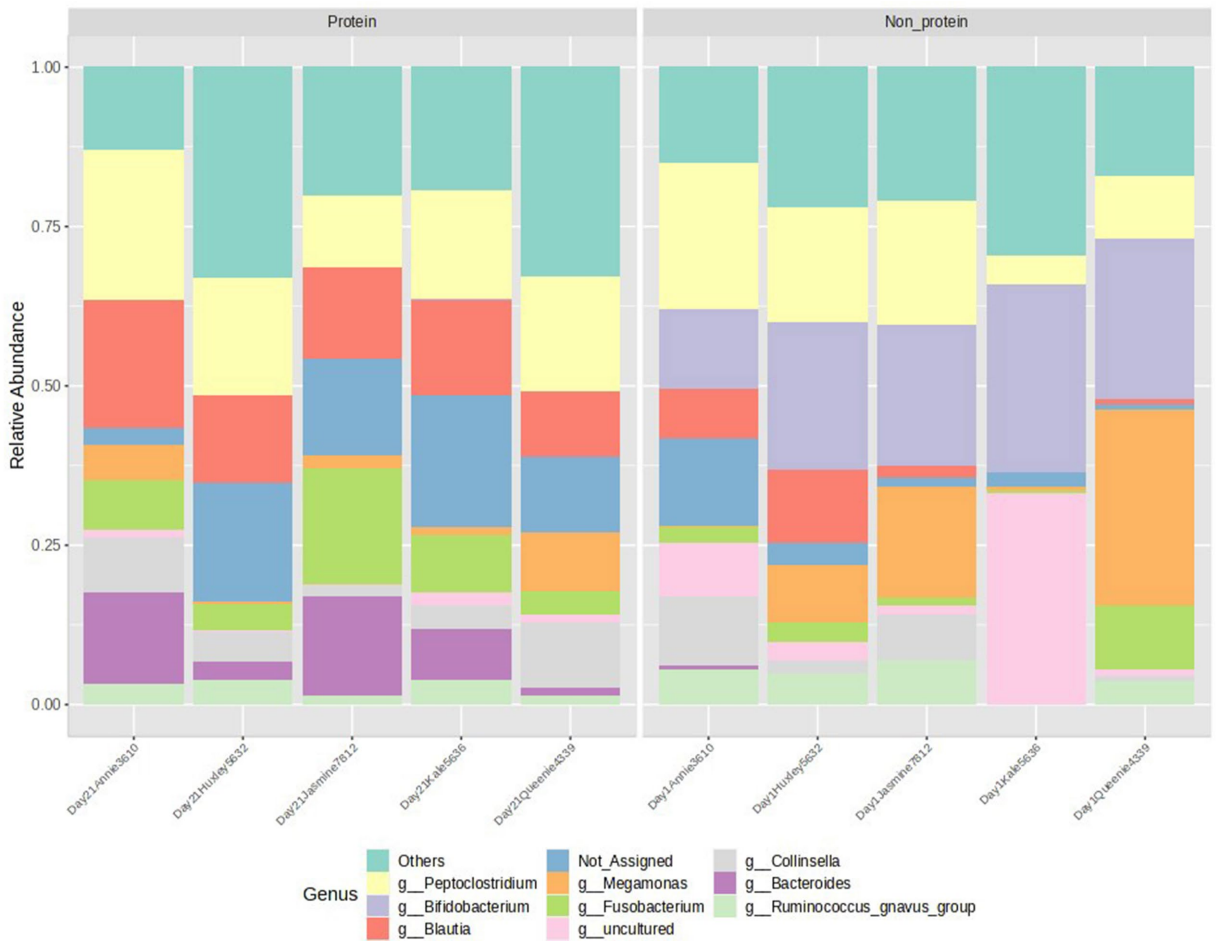

FIGURE 5
Relative abundance of top 10 genera detected in the two groups. Peptoclostridium, Bifidobacterium, Blautia, Megamonas, Fusobacterium, Collinsella, Bacteroides, Ruminococcus group of bacteria were observed. Protein group indicates animals fed with fermented protein (Day 21) and the non-protein group indicates animals fed with control diets (Day 1).

increased relative abundance in the test protein diet group. The most abundant genera of this phylum are *Bacteroides* and *Prevotella* (54), which play significant roles in human and animal gastrointestinal tracts and are known to reduce intestinal oxygen levels and promote the growth of strict anaerobic bacteria (55). Although not significant, members of the Bacteroides genus had an increased abundance in the protein diet group.

Similarly, abundance profiling showed an increase in the *Fusobacterium* genus in the protein diet group, although this was not statistically significant. It is worth noting that unlike in the humans, the *Fusobacterium* genus is one of the three predominant phyla composing the gut microbiota in adult dogs, representing around 20% of the total relative abundance (56). This phylum is commonly observed in healthy dogs (57) and is found in higher abundance in dogs and cats than in humans (58). Due to their ability to degrade proteins into amino acids and peptides (59), it is assumed that Fusobacteria are key bacteria in the gut metabolism of carnivorous animals (60).

Faecalibacterium and *Ruminococcus* belong to the Firmicutes phylum, which is one of the top three most abundant phyla of the gut microbiota, with a high diversity of species. *Faecalibacterium* uses metabolites to produce butyrate, serving as energy for enterocytes providing anti-inflammatory protection (61) or limiting the colonization of pathogens, such as *Salmonella* (62). Firmicutes was also the most abundant phylum in both the protein and non-protein diet groups in this study. The genus of Catenibacterium, that is part of the Erysipelotrichaceae family (phylum Firmicutes), was found to be higher in dogs fed with homemade diet, while it was decreased in faecal samples of dogs fed a dry kibble diet. Dogs fed a raw meat diet had the lowest abundances (63). A similar pattern was observed in this study, where Catenibacterium had low abundance in the faecal samples of both groups and was not among the top 10 genera detected which corroborates with the earlier research studies demonstrating their lower prevalence in the kibble diets. Blautia spp. is shown to have potential probiotic properties and are found to be involved in gut health (64, 65). Our studies also demonstrate the presence of these bacterial species which indicates the role of fermented protein in providing good gut health. Earlier reports wherein the canine diets fed with corn-fermented protein demonstrated that the overall richness and diversity of the faecal microbiota were maintained when compared to the traditional ingredients such as brewer's dried yeast and distiller's dried grains with solubles (33). The microbiome diversity in the current pilot study indicates a similar trend with good acceptance of the gas derived fermented protein diet and hence good gut health.

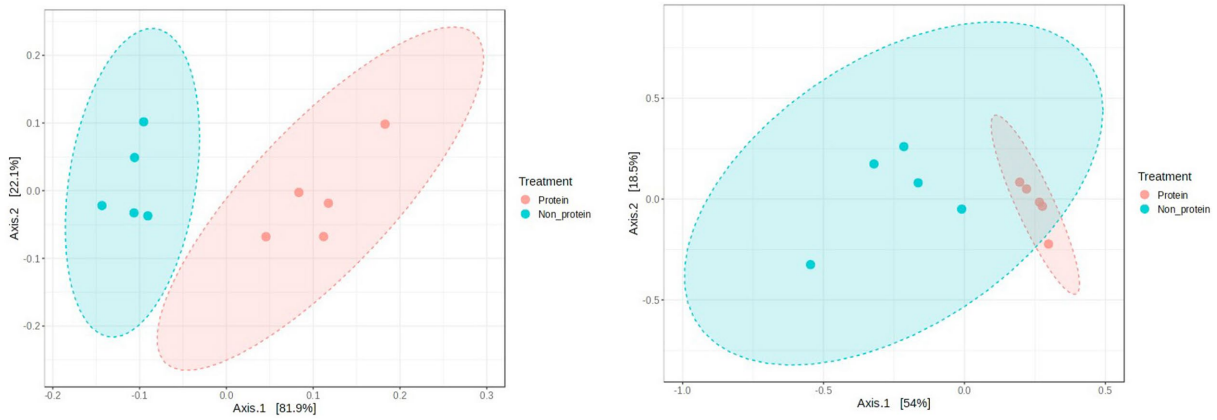

FIGURE 6
Principal coordinates analysis (PCoA) of gut microbiota composition in the two groups (left panel: at phylum level, right panel: at genus level). Distance method: Bray-Curtis index, Statistical method PERMANOVA'. *p*-value = 0.015. Protein group indicates animals fed with fermented protein (Day 21) and the non-protein group indicates animals fed with control diets (Day 1).

Beneficial gut bacteria play a crucial part in the regulation of the canine immune system, which is important for the growth of the gastrointestinal physiological structure (66). Thus, it is important that canine diets provide important and balanced nutrients for both the host and the gut microbime (34). Pet owners play a significant role in determining the canine diets. Plant-based diets are popular choices for vegetarian dog owners and for those with special health concerns such as GI diseases and food allergies (67). On the other hand, some owners prefer meat-based which consists of organs, meat, and bones (50, 68, 69). Meat-based diet seems to be the preferred diet by dog owners because of the stereotype that plant-based foods are indigestible fillers with lower concentrations of nutritional compounds (70). However, several health concerns have been raised against strictly meat-based diets that are nutritionally imbalanced, contaminated with heavy metal and excessive chronic intake has been related to toxicities across many species, including dogs (71). Hence, commercial pet foods are provided as alternative diets but might not be of high quality raw material (2). Considering the above factors, augmenting the canine diets with green house derived fermented protein seems to be a sustainable, cost-effective, alternative without compromising the gut health and palatability of the animals. Hence, the current study would pave way for a functional, alternative protein alternative in the canine diets paving new dimensions into the dietary adaptations in pet foods in the future.

Conclusion and future directions

Dietary protein sources in pet diets are largely sourced from animal byproducts and hence sustainability and societal changes become critical factors. The current study demonstrates that the microbial fermented protein source included in the canine diets is palatable and did not show any adverse effects on growth or welfare of the animals. Microbial fermented protein sources could be viable, sustainable solutions bringing lesser carbon footprint solution in the pet food sector without compromising on the quality of the nutritional profile. The microbiome changes with no major clinical symptoms is another positive sign in terms of overall digestive health of the animals.

Possible limitations of this study could be small sample size, and short study duration. It would have been beneficial to collect and analyze multiple faecal samples throughout the study for comparison. Also, an increase in the number of dogs enrolled in the study would provide more evidence of the potential impact of the protein diet on the faecal microbiota. Multicentered, large cohort studies coupled with biochemical, molecular and clinical parameters in future would help in validating and delineating the mechanisms of the fermented protein in the canine diets. For any study evaluating the impact of dietary intervention, there is concern regarding the study's duration and if it is long enough for adaptation. However, Lin et al. (72) reported that the microbiome of dogs stabilized 6 days after dietary intervention, suggesting that the 21-day adaptation in the current study should have been sufficient. Of note, the methods in the current study are similar to those previously utilized to evaluate the impact of dietary intervention on the faecal microbiota of dogs. Extended, long term studies would help in understanding the mechanism of the fermented protein in canine diets and warrants further research to delineate the long-term potential health implications of this novel protein source in pet foods. Considering the sustainability factors such as land and water use, the fermented protein is perennial source which is unaffected by any of the climatic factors. Leveraging fermented protein used in this study helps in achieving carbon negative and climate positive outcomes which in deed would help in overall reduction of the carbon footprint which is the critical need of the hour. With ongoing advancements in biology, fermentation technology, and process engineering, the commercial viability and scalability of fermented proteins are steadily improving, paving the way for their integration into commercial pet diets.

Data availability statement

The sequence data used for analysis is available in NCBI under BioProject accession Number PRJNA1116051.

Ethics statement

The animal study was approved by Invetus, the largest Australasian veterinary contract research organization (RIU C 22179 W). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RB: Methodology, Writing – review & editing. SP: Writing – original draft. RG: Formal analysis, Writing – review & editing. ES: Writing – review & editing. TV: Writing – original draft. RE: Writing – review & editing.

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Conflict of interest

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Partial replacement of soybean meal with mixed plant proteins yields comparable growth and carcass quality in growing-finishing pigs

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Objective: This study evaluated the impacts of partial replacement of soybean meal with different concentrations of mixed plant protein products (rapeseed meal (RSM) - palm kernel meal (PKM) -distillers dried grains with soluble (DDGS)) on growth performance and carcass quality of growing-finishing pigs.

Methods: A total of 180 crossbred [Yorkshire x Landrace] pigs with average initial weight of 29.72 + 1.65 Kg were randomly assigned to one of five dietary treatment groups on the basis of weight and sex, and the experimental duration was 105 days. The basal diet (C23ON) of growing and finishing pigs were partially replaced with increasing level of RSM-PKM-DDGS (1 to 5% for growing pigs, and 2 to 6% for finishers). Each treatment group had 9 replicate pens, each containing 2 barrows and 2 gilts. During the 15-week trial, body weight (BW), average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) were calculated for the periods of weeks 0–5, weeks 5–10, week 10–15, and for the entire experimental period.

Results: The partial replacement of soybean meal with mixed plant protein products (RSM, PKM-DDGS) showed no significant effect on the growth performance of pigs during the entire experimental period ($p > 0.05$). However, a decreasing ADG ($p = 0.0837$) and ADFI ($p = 0.0779$) were observed during weeks 0–5, while an increasing FCR was noted during weeks 10–15 ($p = 0.0835$) and the overall period. Furthermore, the replacement of soybean meal with mixed plant protein products (RSM-PKM-DDGS) showed no linear or quadratic effects on the digestibility of dry matter (DM), nitrogen (N), energy (E), fecal scores, or meat quality.

Conclusion: This suggests that mixed plant protein products (RSM, PKM, and DDGS) can effectively replace soybean meal as the primary protein source, providing comparable outcomes while potentially reducing feed costs.

KEYWORDS

plant protein products, growth performance, nutrient digestibility, fecal scores, meat quality

1 Introduction

Exploring feed in diets for pigs has garnered significant attention in the livestock industry, for example, soybean meal (SBM) is a major protein source for livestock, but it is also a relatively expensive feed ingredient, and feed costs account for approximately 50% of the total cost in pig production systems (1). Therefore, the partial substitution of feed ingredients to reduce feed costs has become increasingly important, with the aim of

achieving higher growth performance at lower production costs (2). Additionally, soybean meal (SBM), another primary feed ingredient, is a byproduct of soybean oil production, Due to its high protein content, well-balanced amino acid profile, and excellent digestibility, SBM is widely used in diets of pigs, serving as a critical protein source in the livestock industry that effectively promotes pig growth and improves feed utilization (3). Moreover, the sharp increase in soybean prices has driven many researchers to seek alternative, cost-effective protein sources (4). Rapeseed meal (RSM) has emerged as a favored protein source in pig's feed due to its high protein content and lower cost, becoming increasingly popular in pig nutrition (5). However, the high fiber content (10–20%) and lower oligosaccharide levels in RSM result in reduced energy utilization, which limits its efficiency in diets to some extent, thereby potentially reducing overall growth performance (6). Furthermore, some reports indicated that the inclusion of up to 4% RSM in the diets of growing pigs had no adverse effects on growth performance, nutrient digestibility, or meat quality (7). Also important is Palm kernel meal (PKM), a byproduct of palm kernel oil extraction, primarily produced in Southeast Asia, with Malaysia and Indonesia being the largest producers, due to its price advantage, low risk of mycotoxins, and relatively stable quality, PKM has been widely adopted as a substitute for SBM in animal feed (8). Studies have shown that the inclusion of PKM in pig diets can partially replace SBM while promoting growth, nutrient digestibility, and meat quality (9). With the rapid rise of the fuel ethanol industry, distillers dried grains with solubles (DDGS), a primary byproduct of ethanol production, has seen a significant increase in production capacity. During the dry-grind ethanol production process, the starch in grains is converted into ethanol and carbon dioxide, resulting in a concentration of nutrients in DDGS. Compared to original corn, DDGS contains approximately three times the protein, oil, fiber, and mineral content, making it a highly promising protein source for animal feed (10). The use of DDGS, particularly in the early stages of pig feeding, can help substantially reduce feed costs, thereby enhancing profitability while maintaining growth performance (11). Despite its notable nutritional advantages, research has shown that excessive inclusion of DDGS in the diet can negatively affect feed efficiency and lean meat percentage, significantly reducing carcass yield and impacting overall meat quality (12). Therefore, using DDGS as a partial replacement for SBM or combining it with other plant byproducts could improve the nutritional profile of DDGS, enhance growth performance while, allowing for the maintenance of growth performance while minimizing the negative effects in pigs. The objective of this study is to evaluate the effects of partial replacement of soybean meal with different concentrations of a blend of plant protein products (RSM, PKM, and DDGS) on the growth performance and carcass quality of growing-finishing pigs.

2 Materials and methods

2.1 Ethical statement

The Animal Care and Use Committee of Dankook University approved all experimental protocols used in the DK-1-2305.

2.2 Experimental animals, designs, diets and housing

A total of 180 crossbred pigs [(Yorkshire × Landrace) × Duroc], with an average body weight (BW) of 29.72 ± 1.65 kg, were used in a 15-week trial. The pigs were randomly assigned to one of five dietary treatments in a completely randomized block design comprising of nine replicate pens per treatment. Four pigs per pen (2 barrows and 2 gilts) were arranged according to their initial body weight and sex. The dietary treatments were as follows: The basal diets (CON) of growing and finishing pigs were partially replaced with increasing levels of RSM-PKM-DDGS (1 to 5% for growing pigs, and 2 to 6% for finishers). All diets were formulated to meet or exceed the nutritional requirements specified by the National Research Council (13) guidelines (Table 1). Pigs in both experiments were housed in controlled environments with plastic slatted flooring. Each pen was equipped with a self-feeder and a nipple drinker, allowing pigs to have ad libitum access to feed and water throughout the experiment.

2.3 Growth performance

During the 15-week duration of the experiment, the individual body weights (BW) of the pigs were recorded at the start of the trial, and then weighed at weeks 5, 10 and 15 of the experimental trial to estimate the body weight gain and average daily weight gain (ADG) on treatment basis. At the same time, the feed intake and feed leftovers were measured to estimate the average daily feed intake (ADFI) while feed conversion ratio (FCR) was evaluated.

2.4 Nutrient digestibility

To estimate the apparent total tract digestibility (ATTD), 0.20% chromium oxide (Cr_2O_3) was added to the diets 7 days prior to fecal collection in weeks 5, 10, and 15. Fecal samples were randomly collected from two pigs (one boar and one sow) per pen and pooled on a pen basis. The fecal samples were stored at −20°C in the laboratory before determining the ATTD for dry matter (DM), crude protein (CP), and gross energy (GE). Before chemical analyses, fecal samples were dehydrated at 70°C for 72 h. Feed and fecal samples were then ground and sieved through a 1 mm sieve to obtain a homogeneous sample. All samples were analyzed for DM, CP, and DE according to the Association of Official Analytical Chemists (14). Chromium concentration in the samples was measured using a UV spectrophotometer (Optizen POP, South Korea) according to (15). Total energy was measured by an oxygen bomb calorimeter (Parr instrument, United States). Nitrogen (N) was analyzed with a Kjeltec 2,300 nitrogen analyzer (Foss Tecator AB, Denmark). The ATTD was estimated using the following formula:

$$\mathrm{ATTD}(\%) = [1 - \{(N_f \times C_d) / (N_d \times C_f\}] \times 100$$

where: N_f indicated concentration in feces (% DM), N_d indicated nutrient concentration in diets (% DM), C_f indicated chromium concentration in feces (% DM), and C_d indicated chromium concentration in diets (% DM).

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TABLE 1 Composition of growing-finishing pig diets.

Item	(Growing phase)					(Finishing phase)				
	1%	2%	3%	4%	5%	2%	3%	4%	5%	6%
Ingredients (%)										
Corn	73.87	71.84	69.79	67.76	65.72	73.75	71.69	69.68	67.67	65.62
Soybean meal	17.50	16.22	14.95	13.66	12.40	15.02	13.76	12.48	11.20	9.92
Rapeseed meal	1.00	2.00	3.00	4.00	5.00	2.00	3.00	4.00	5.00	6.00
Palm kernel meal	1.00	2.00	3.00	4.00	5.00	2.00	3.00	4.00	5.00	6.00
DDGS	1.00	2.00	3.00	4.00	5.00	2.00	3.00	4.00	5.00	6.00
Tallow	2.35	2.69	3.03	3.37	3.71	2.42	2.77	3.10	3.43	3.78
MDCP	1.45	1.40	1.35	1.30	1.25	1.15	1.10	1.00	0.95	0.90
Limestone	0.75	0.76	0.78	0.80	0.80	0.65	0.67	0.72	0.72	0.74
Salt	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Methionine (99%)	0.05	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.05
Lysine (78%)	0.50	0.51	0.52	0.53	0.54	0.42	0.43	0.44	0.45	0.46
Mineral mix[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin mix[2]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline (25%)	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Calculated value										
CP, %	15.50	15.50	15.50	15.50	15.50	15.00	15.00	15.00	15.00	15.00
ME (kcal/kg)	3,300	3,300	3,300	3,300	3,300	3,300	3,300	3,300	3,300	3,300
Ca, %	0.70	0.70	0.70	0.70	0.70	0.60	0.60	0.60	0.60	0.60
P, %	0.60	0.60	0.60	0.60	0.60	0.55	0.55	0.55	0.55	0.55
Lys, %	1.10	1.10	1.10	1.10	1.10	1.00	1.00	1.00	1.00	1.00
Met, %	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
FAT, %	5.25	5.61	5.97	6.33	6.69	5.40	5.76	6.11	6.47	6.83

DDGS, distillers dried grains solubles; MDCP, mono dicalcium phosphate; CP, crude protein; ME, metabolizable energy; LYS, lysine; MET, methionine Provided per kg of complete diet: 16,800 IU vitamin A; 2,400 IU vitamin D_3; 108 mg vitamin E; 7.2 mg vitamin K; 18 mg Riboflavin; 80.4 mg Niacin; 2.64 mg Thiamine; 45.6 mg D-Pantothenic; 0.06 mg. Cobalamine; 12 mg Cu (as $CuSO_4$); 60 mg Zn (as $ZnSO_4$); 24 mg Mn (as $MnSO_4$); 0.6 mg I (as Ca $(IO_3)_2$); 0.36 mg Se (as Na_2SeO_3).

2.5 Fecal score

At the start of the experiment, as well as during the 5th, 10th, and 15th weeks, the fecal score was determined by averaging the scores of four pigs in each pen using a 5-grade scoring system. The standard of this system is as follows: 1 = hard, dry pellets in a small, hard mass; 2 = hard, formed stool that remains firm and soft; 3 = soft, formed and moist stool that retains its shape; 4 = soft, unformed stool that assumes the shape of the container; 5 = watery, liquid stool that can be poured. Scores were recorded on a pen basis following observations of individual pigs and signs of stool consistency in the pen, all pigs had mash form of feed.

2.6 Back fat thickness and lean meat percent of finishing pigs fed experimental diets

At the start of the second phase, and at the end of weeks 5 and 10, the backfat thickness and Lean Meat Percentage (LMP) of all pigs were measured. A real-time ultrasound instrument (Piglot 105; SFK Technology, Herlev, Denmark) was used to measure the carcass backfat thickness and LMP. LMP was calculated for all pigs (40 per treatment) from three different sites (shoulder, mid-back, and loin, just above the elbow, the last rib and the last lumbar vertebrae, respectively) 5 cm to the right of the midline according to the procedure described by Upadhaya et al. (16).

2.7 Meat quality

Carcasses were chilled at 2°C for 24 h and a piece of the right loin was taken through a perpendicular cut between the 10th and 11th ribs. Before evaluating meat quality, meat samples were thawed at ambient temperature. The color measurement of lightness (L*), redness (a*), and yellowness (b*) values were determined with a Minolta CR410 chromameter (Konica Minolta Sensing, Inc., Osaka, Japan). Sensory evaluation (color, marbling, and firmness scores) was carried out according to the National Pork Producers Council standards (17). At the same time, duplicate pH values of each sample were measured with a pH meter (Fisher Scientific, Pittsburgh, PA, United States). The water-holding capacity (WHC) was measured based on the procedure described in a previous report (18). Briefly, a 0.3 g sample was pressed at 3000 psi for 3 min on a 125-mm-diameter filter paper. The areas of the pressed sample and expressed moisture

were then determined with a digitizing area-line sensor (MT-10S, M.T. Precision Co. Ltd., Tokyo, Japan). The ratios of water to meat areas were calculated as a measure of WHC (a smaller ratio indicates higher WHC). The longissimus muscle area (LMA) was measured by tracing the longissimus muscle surface at the 10th rib, which also used the above-mentioned digitizing area-line sensor. Then, a 4 g of meat sample was stored in a plastic bag and treated in a water bath (100°C) for 5 min for measuring cooking loss. Then samples were cooled at room temperature. Cooking loss was calculated as:

$$\text{Cooking loss} = \frac{\text{sample weight before cooking} - \text{sample weight after cooking}}{\text{sample weight before cooking}} \times 100.$$

Drip loss was measured using approximately 4.5 g of meat sample according to the plastic bag method. On days 1, 3, 5, and 7, the meat samples were removed and dried on paper towels, then their weight was checked. Differences between sample weights were used to calculate the drip loss.

2.8 Carcass grade

Backfat thickness (BFT) (mm), carcass weight, and carcass grade were assessed. The quality of pork carcasses was graded into "Grade 1+," "Quality Grade 1," or "Grade 2," based on characteristics such as marbling, lean color, and conditions of belly streaks.

2.9 Statistical analysis

All data in this experiment were analyzed according to a completely randomized block design using GLM SAS (Statistical Analysis System, Version 9.2); each pen was treated as an experimental unit, except for meat quality, where individual pigs were considered an experimental unit. Duncan's multiple range test was performed to determine group differences. Orthogonal polynomials were used to evaluate the linear and quadratic effect of increasing (RSM-PKM-DDGS) supplementation to the diet. The initial body weight was utilized as a covariate for ADG and ADFI. Data variability was expressed as SEM, with a p-value less than 0.05 considered statistically significant, and a p-value from 0.05 to 0.10 considered a trend.

3 Results

The effects of partially replacing soybean meal in diets with different concentrations of a mixed plant protein product (RSM-PKM-DDGS) on the growth performance of growing-finishing pigs are shown in Table 2. Throughout the entire trial period, there were no significant differences ($p > 0.05$) in body

TABLE 2 Performance of growing-finishing pigs fed different inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	p- value	
Body weight, kg							Linear	Quadratic
Initial	29.72	29.72	29.72	29.72	29.72	0.003	0.9986	0.9985
Week 5	55.42	54.41	54.09	54.32	53.85	0.45	0.3514	0.4762
Week 10	83.66	83.31	83.35	82.85	83.12	0.56	0.6114	0.9436
Week 15	115.25	114.09	114.20	112.97	113.57	1.25	0.3334	0.9819
Week 0–5								
ADG, g	734[a]	706[ab]	696[ab]	703[ab]	689[b]	13	0.0837	0.1872
ADFI, g	1919[a]	1864[ab]	1845[ab]	1859[ab]	1830[b]	25	0.0779	0.1672
FCR	2.617	2.644	2.652	2.647	2.656	0.014	0.1397	0.6053
Week 5–10								
ADG, g	800	789	790	775	783	17	0.2939	0.8981
ADFI, g	2,201	2,192	2,194	2,178	2,186	27	0.6095	0.9005
FCR	2.753	2.783	2.780	2.815	2.796	0.030	0.1655	0.9171
Week 10–15								
ADG, g	903	879	881	861	870	21	0.1805	0.9545
ADFI, g	2,754	2,712	2,723	2,710	2,713	35	0.4300	0.6709
FCR	3.058	3.095	3.095	3.151	3.126	0.036	0.0835	0.7864
Overall								
ADG, g	847	830	832	815	823	18	0.2161	0.9793
ADFI, g	2,461	2,435	2,443	2,428	2,433	29	0.4785	0.8464
FCR	2.912	2.939	2.940	2.982	2.961	0.300	0.0903	0.7962

ADFI, average daily feed intake; ADG, average daily gain; FCR: feed conversion ratio; SEM: pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs. [a,b] means in the row with superscripts denotes statistically significant.

weight, ADG (average daily gain), ADFI (average daily feed intake), and FCR (feed conversion ratio) among the experimental groups except for 0 to 5 weeks. During 0 to 5 weeks, The ADG and ADFI of growing-finishing pigs fed group 4 diet were lower compared to those fed the CON group. Additionally, during weeks 0 to 5, the ADG ($p = 0.0837$) and ADFI ($p = 0.0779$) showed a decreasing trend, and during weeks 10–15, FCR ($p = 0.0835$) showed an increasing trend throughout the entire period. The effects of partially replacing soybean meal in diets with different concentrations of a mixed plant protein product (RSM-PKM-DDGS) on the fecal scores of growing-finishing pigs are shown in Table 3. Throughout the entire trial period, there was no significant difference ($p > 0.05$) in fecal scores among the experimental groups. The effects of partially replacing soybean meal in diets with different concentrations of a mixed plant protein product (RSM-PKM-DDGS) on the nutrient digestibility of growing-finishing pigs are shown in Table 4. Throughout the entire trial period, there was no significant difference ($p > 0.05$) in nutrient digestibility among the experimental groups. Throughout the entire trial period, there was no significant difference ($p > 0.05$) in backfat thickness and LMP, and meat quality among the experimental groups (Tables 5, 6). Furthermore, there was no significant difference ($p > 0.05$) in carcass grade among the experimental groups until week 15 (Table 7).

4 Discussion

In agricultural livestock production, various plant protein products can partially replace soybean meal, such as RSM. While RSM has a high protein content, making it a viable alternative to soybean meal, its high fiber content can impact nutrient digestibility. Therefore, many researchers have aimed to enhance the nutritional digestibility of RSM by improving processing techniques and adding enzymes, such as carbohydrase enzymes, to reduce fiber and anti-nutritional factors in RSM (7). Studies have reported that supplementing with RSM, in contrast to SBM, does not negatively affect body weight, ADG, ADFI, or G/F (19). Which aligns closely with our experimental results, albeit with a slight downward trend in ADG and ADFI during weeks 0 to 5. Evidence from previous research revealed that gradually increasing RSM levels in the diet (2, 4, and 6%) leads to a linear decline in ADG (2). In our study, the decreased ADG was caused by decreased ADFI. Transition from weaning diet (corn-SBM based) to unconventional diet containing RSM-PKM-DDGS can cause initial reduction of feed intake, we believe that the possible reason for this situation could be that RSM, PKM, and DDGS contain higher fiber levels compared to soybean meal, which reduces palatability and increases the gastrointestinal burden, making pigs less willing to consume the feed, thereby reducing feed intake. It is also possible that growing pigs need time to adapt to the texture, taste,

TABLE 3 Fecal scores of growing-finishing pigs fed varied inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	p-value	
Fecal score[1]							Linear	Quadratic
Initial	3.34	3.33	3.22	3.26	3.29	0.06	0.2221	0.7109
Week 5	3.13	3.15	3.17	3.16	3.20	0.07	0.6720	0.7477
Week 10	3.15	3.17	3.16	3.21	3.19	0.034	0.2386	0.5856
Week 15	3.07	3.10	3.09	3.12	3.11	0.030	0.3993	0.9113

SEM, pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs; [1]Fecal score = 1 hard, dry pellet; 2 firm, formed stool; 3 soft, moist stool that retains shape; 4 soft, unformed stool that assumes shape of container; 5 watery liquid that can be poured.

TABLE 4 Nutrient digestibility of growing-finishing pigs fed varied inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	*p*-value	
Week 5							Linear	Quadratic
Dry matter	80.68	80.54	80.36	80.42	80.08	0.83	0.7987	0.9006
Crude protein	77.15	76.97	76.71	76.80	76.52	0.89	0.7259	0.8776
Energy	80.99	80.87	80.67	80.75	80.46	0.52	0.7448	0.8760
Week 10								
Dry matter	75.23	74.96	74.99	74.82	74.90	0.91	0.7559	0.9525
Crude protein	71.58	71.41	71.43	71.20	71.28	0.83	0.7549	0.9702
Energy	74.26	74.07	74.10	73.90	73.99	0.81	0.7688	0.9950
Week 10–15								
Dry matter	72.23	71.95	71.97	71.77	71.86	1.01	0.7431	0.9686
Crude protein	69.24	68.97	69.02	69.76	68.85	0.54	0.5583	0.4202
Energy	71.18	70.98	71.01	70.76	70.86	0.71	0.6960	0.9730

SEM, pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs.

TABLE 5 BFT and LMP of growing-finishing pigs fed varied inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	*p*- value	
Initial							Linear	Quadratic
Backfat thickness, mm	12.40	12.38	12.36	12.35	12.36	0.02	0.9750	0.9938
LMP, %	62.17	62.18	62.16	62.17	62.18	0.01	0.9985	0.9985
Week 10								
Backfat thickness, mm	15.71	15.42	15.60	15.32	15.51	0.30	0.6861	0.9919
LMP, %	56.74	56.29	56.56	56.18	56.40	0.23	0.1785	0.8890
Week 15								
Backfat thickness, mm	18.96	18.68	18.82	18.57	18.71	0.28	0.6768	0.9835
LMP, %	52.33	51.85	52.10	51.77	51.97	0.23	0.1677	0.7629

LMP, Lean meat percentage; SEM: pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs.

TABLE 6 Meat quality of growing-finishing pigs fed varied inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	*p*- value	
Finish							Linear	Quadratic
pH	5.62	5.67	5.66	5.68	5.59	0.05	0.5399	0.7578
Water holding capacity, %	46.32	38.88	40.62	45.98	37.10	3.16	0.9653	0.0958
Longissimus muscle area, cm^2	8020.89	8081.20	8214.81	8443.65	7944.28	242.45	0.2098	0.7296
Meat color								
L*	54.75	53.59	55.93	52.95	54.89	1.00	0.4681	0.3348
a*	15.17	14.96	14.73	15.35	14.80	0.29	0.8232	0.1569
b*	7.3a	6.17b	7ab	6.32ab	6.77ab	0.36	0.1677	0.5021
Cooking loss, %	27.71	31.34	28.32	29.29	24.87	2.1	0.8566	0.5341
Drip loss, %								
d1	0.74	0.78	0.69	0.82	0.78	0.16	0.8560	0.7952
d3	2.07	2.40	1.84	2.03	2.66	0.31	0.6990	0.8584
d5	3.78	4.08	3.84	3.89	4.11	0.10	0.8473	0.2517
d7	5.87	6.17	5.99	6.01	6.20	0.11	0.5840	0.1904
Sensory evaluation								
Color	3.00	3.00	3.00	3.25	3.00	0.11	0.1544	0.2811
Marbling	3.00	3.17	4.00	2.83	3.08	0.39	0.8365	0.0820
Firmness	3.08	2.75	3.17	2.50	3.42	0.32	0.3431	0.5943

SEM, pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs.

and composition of the new diet, as well as to the new environment. During the initial transition phase, the reduction in feed intake is a normal phenomenon as pigs gradually adjust to the new diet and surroundings. It has also been reported that entirely replacing SBM with RSM can result in darker pork, with a significant reduction in meat lightness (L*) and yellowness (b*) values compared to pigs fed SBM. The lower L* values in pork might correlate with reduced fat content (5). These findings differ from our results, possibly due to the relatively low concentration of RSM used in our study. It remains unclear the possible effects of origin and storage temperature of RSM on growth performance of experimental animals, which may account for the absence of significant differences observed in our trial. Thus, further research and detailed analysis are essential. Soybean meal is a more balanced source of essential amino acids, while palm kernel meal (PKM) contains higher levels of methionine but is deficient in some other amino acids, such as threonine, cysteine, and proline. Despite these differences, PKM can still serve as a substitute protein source for corn-soybean meal due to its high protein content and cost-effectiveness, making it a satisfactory alternative (20). Some studies indicated that adding 4% PKM to the diet does not impact carcass characteristics or pork quality in finishing pigs (9), this supports the findings of our study. However, earlier studies show that higher inclusion of PKM in the diet can lead to a linear decline in ADFI, crude protein, and crude fiber digestibility, Pigs fed a PKM-based diet exhibit slower growth rates, poorer feed conversion ratios, and reduced feed intake (21). Additionally, research has shown that including 40% PKM in the diet can lower feed costs for weaning piglets; however,

TABLE 7 Carcass grade of growing-finishing pigs fed varied inclusion levels of mixed plant protein products.

Items	CON	TRT1	TRT2	TRT3	TRT4	SEM	*p*- value	
Finish							Linear	Quadratic
Carcass weight, kg	89.89	89.22	89.25	88.97	89.08	0.92	0.5107	0.8335
Backfat thickness, mm	18.78	18.31	18.56	18.14	18.47	0.56	0.4864	0.9586
1+, %	36.11	30.56	33.33	27.78	30.56	–		
1, %	33.33	33.33	36.11	33.33	30.56	–		
2, %	30.56	36.11	30.56	38.89	38.89	–		

SEM, pooled standard error of the mean. Dietary treatments were as follows: (1) CON-basal diet, (RSM-PKM-DDGS) 1% for growing pigs and 2% for finishing pigs; (2) TRT1, (RSM-PKM-DDGS) 2% for growing pigs and 3% for finishing pigs; (3) TRT2, (RSM-PKM-DDGS) 3% for growing pigs and 4% for finishing pigs; (4) TRT3, (RSM-PKM-DDGS) 4% for growing pigs and 5% for finishing pigs; (5) TRT4, (RSM-PKM-DDGS) 5% for growing pigs and 6% for finishing pigs.

PKM should not exceed 35% in the diet of growing pigs as it may significantly impact body weight (22). This is in contrast with the results of our findings, because the PKM was mixed with RSM and DDGS at relatively low inclusion levels, which mitigated any adverse effects. DDGS, a by-product of the bioethanol industry after ethanol extraction, is highly regarded as livestock feed due to its rich nutritional profile and cost-effectiveness. DDGS is abundant in crude protein, fats, and dietary fiber, and contains substantial amino acids, vitamins, and minerals. As a non-conventional feed ingredient, because DDGS contains lower levels of antioxidants and anti-nutritional factors, along with higher nutritional value, it can serve as an alternative protein source to soybean meal. This versatility has led to its widespread application in feed for swine, poultry, and ruminants (23). Studies indicate that incorporating 4–15% DDGS into diets generally does not adversely affect intake, ADG, or G/F in growing pigs (10). Another study noted that supplementing up to 25% DDGS in weaned piglet diets does not impact their overall growth performance (24), which aligns well with our experimental findings. However, other studies have reported that adding 15 and 30% DDGS to a SBM-based diet significantly reduces the ADG and ADFI of nursery pigs throughout the trial, although there is an upward trend in G/F (25). This discrepancy with our results may be due to variability in the nutritional composition of DDGS, which can vary with raw material quality and processing methods. Thus, in practical applications, it is essential to consider the source, batch variations, and inclusion levels of DDGS.

5 Conclusion

Our study demonstrates that partial replacement of soybean meal with varying concentrations of mixed plant protein products (RSM-PKM-DDGS) does not negatively impact growth performance, nutrient digestibility, fecal scoring, or meat quality in growing-finishing pigs. At the same time RSM, PKM, and DDGS are nutrient-dense, cost-effective, and widely used potential alternatives. The incorporation of mixed plant protein products (RSM-PKM-DDGS) around 1 to 5% in growing and 2 to 6% in finishing would be suitable levels to improve the performance and to reduce feed cost for sustainable pig production.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Committee of Dankook University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. JH: Investigation, Software, Visualization, Writing – review & editing. SC: Writing – review & editing, Methodology, Project administration, Supervision. IK: Funding acquisition, Resources, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Assessment of the effect of agro-industrial by-products rich in polyphenols on *in vitro* fermentation and methane reduction in sheep

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Introduction: This study aimed to evaluate, using the *in vitro* gas production technique, the effect of including eight agro-industrial by-products (carob, grape, two types of olive pomace, citrus pulp, tomato, and hazelnut skin) on fermentation end-products, ruminal degradability, and methane production in sheep diets.

Methods: The by-products were included at 10% dry matter in the control (CTR) diet, commonly adopted for adult sheep (80% natural grassland and 20% concentrate), and incubated at 39°C under anaerobic conditions.

Result and discussion: After 24 h of the incubation, the organic matter degradability (OMD24h) and methane production were assessed. After 120 h of the incubation, the organic matter degradability (OMD120h), volume of gas produced (OMCV), fermentation kinetics, pH, volatile fatty acids (VFAs), and ammonia were evaluated. Dunnett's test was used to compare the differences between the control and experimental diets, and multivariate analysis was performed to highlight the differences among the diets based on their *in vitro* characteristics. The results indicated that the inclusion of the by-products decreased the degradability and increased gas production after 120 h of the incubation. The by-products from the hazelnuts, citrus, grapes, and tomatoes significantly ($p < 0.001$) reduced the methane production, whereas the pomegranate, grape, 3-phase olive cake, tomato, and hazelnut by-products significantly ($p < 0.001$) increased the acetate production. The multivariate analysis showed that the butyrate concentration was a determining factor in the differences between the diets. The concentration of polyphenols in the selected agro-industrial by-products could modify fermentation parameters and metabolic pathways, leading to reduced methane production.

KEYWORDS

environmental impact, *in vitro* fermentation, methane, polyphenols, tannins

1 Introduction

According to the European Commission (1), the term "by-product" refers to any substance or object that results from a production process and whose existence is not intended in the primary process target (2). The volume of by-products, mainly originating from industrial processes, is constantly growing globally every year. In this regard, the largest proportion of residues (approximately 40–50% of total discards) consists of fruit and vegetable by-products (3). A total of 88 million tons (±14Mt) of food waste are produced along the supply chain in the European Union (EU). On a global scale, food losses and waste account for approximately 1.3 billion tons per year, or 16% of the total food supply. In the case of fruits and vegetables, food losses are in the range of 20–40%, beginning in initial agricultural production and continuing throughout processing, up to the final consumer (3, 4). This waste results in the loss of resources along the supply chain, such as water, land, and energy, and has a significant environmental impact (5–7). Considering the volatility of feed raw material prices, it is necessary to find alternative feeding options (8–10). By-products, particularly fruit and vegetable wastes, could serve as a feed resource rich in high-value nutrients for livestock.

Fruit and vegetable by-products, rich in tannins and flavonoids, may exhibit antimicrobial, antiparasitic, and antioxidant activity and could decrease methane and ammonia emissions, thereby reducing environmental impact (11–13). Indeed, *in vitro* trials (14–16) have demonstrated that some by-products, such as grape pomace and olive cake, could affect fermentation parameters and decrease methane emissions because of the presence of valuable bioactive molecules (17–19). Although by-products have long been included in the diets of livestock, providing added value to animal health and production (19), several issues, such as storage, seasonality, and variability in chemical composition (20, 21), make their inclusion in animal diet challenging (22).

Further studies are needed to gain a better understanding and characterization of the nutritional qualities of by-products. Therefore, the objective of this study was to evaluate, using the *in vitro* gas production technique, the effect of including eight agro-industrial by-products (carob, grape, two types of olive pomace, citrus pulp, tomato, and hazelnut skin) on fermentation end-products, ruminal degradability, and methane production in sheep diets.

2 Materials and methods

2.1 Chemical composition and bioactive compounds

The eight agro-industrial by-products (Table 1) were selected for their local availability in France, Italy, and Greece and were derived from different food industrial processing methods. In this study, two different types of olives were tested because a two-phase olive cake (OC2) by-product has higher moisture and lower fat content compared to a three-phase olive cake (OC3) and is derived from a more resourceful and environmentally friendly centrifugation process (23). The grape extract was obtained after the mechanical pressing of grapes to concentrate the polyphenols. Since bioactive compounds are very sensitive to high temperatures, all by-products were dried at 40°C for 3–4 d. All samples were milled (1.1 mm) and analyzed for dry matter (DM), crude protein (CP), ether extract (EE), and sugar contents (24). According to Van Soest et al. (25), the structural carbohydrate content (neutral detergent fiber, NDF; acid detergent fiber, ADF; and acid detergent lignin, ADL) was also determined, excluding the ash content. The total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) were also reported. The TPC of all samples was estimated using the spectrophotometric method (26), the TFC was estimated by modifying the aluminum chloride method of Pekal and Pyrzynska (27), and the TTC of the methanolic extracts was determined using a modified version of the spectrophotometric method (Table 2) (26).

2.2 *In vitro* gas production

The *in vitro* experimental design included a control (CTR) and seven experimental diets for adult sheep.

All diets consisted of 80% natural grassland and 20% concentrate (ingredients: soybean meal corn meal, wheat bran, and vitamin and minerals supplementation). Each by-product was included in an experimental diet at 10% on a concentrate DM basis. The dose was defined to exhibit the potential maximum effect of the by-products in the diet on ruminal fermentation. The diets were formulated to guarantee the following nutritional characteristics: NDF 42.8 ± 0.35% DM and CP 20.8 ± 0.38% DM.

All diets were incubated in serum flasks (one run, six replications per substrate, $n = 48$; mean weight: 1.0025 ± 0.00010 g) with pooled buffered sheep rumen liquor (10 mL) at 39°C under anaerobic

TABLE 1 Description and origin of the selected by-products.

Fruits	Family	Species	By-products	Origin
Citrus	*Rutaceae*	*Citrus senensis*	Pulp and peel	Italy
Olive	*Oleaceae*	*Olea europaea*	Cake (2-phase)	Italy
Hazelnuts	*Betulaceae*	*Corylysavellana*	Skin	Italy
Tomato	*Solanaceae*	*Solanum lycopersicum*	Skin	Italy
Carob	*Fabaceae*	*Ceratonia siliqua*	Pulp	Greece
Olive	*Oleaceae*	*Olea europaea*	Cake (3-phase)	Greece
Pomegranate	*Lythraceae*	*Punica granatum*	Peel and seeds	Greece
Grape	*Vitacea*	*Vitis vinifera*	Extract	France

TABLE 2 Proximate chemical composition and total content of the polyphenols, phenols, and tannins of the selected by-products.

By-products	DM	Ash	CP	EE	NDF	ADF	ADL	NSC	Sugar	TPC	TFC	TTC
	% as fed								% glucose	mg/ GAE/g	mg QE/g	mg CE/g
Citrus	44.8	4.50	8.37	1.55	16.6	11.0	0.80	69.0	7.43	109	10.2	23.2
Olive cake (3-phase)	66.5	5.39	9.18	19.7	53.2	36.6	17.2	12.5	1.36	81.3	11.9	12.1
Hazelnuts	95.9	3.22	10.8	20.9	51.8	45.4	32.1	13.3	4.06	768	31.0	692
Tomato	17.0	6.24	20.7	9.41	57.8	45.4	25.0	5.87	0.12	76.4	5.41	13.7
Carob	88.8	3.15	6.33	0.67	29.0	25.2	13.6	60.9	7.06	71.1	5.66	23.6
Olive cake (2-phase)	50.6	5.34	10.9	14.4	51.4	37.2	22.2	17.9	0.37	334	<LOD	24.2
Pomegranate	27.0	5.92	4.25	0.82	28.2	19.8	5.60	60.8	7.66	nd	nd	nd
Grape	33.5	1.05	3.67	0.57	8.00	4.00	2.00	86.7	6.96	732	17.36	713

DM, dry matter; CP, crude protein; EE, ether extract; GAE, Gallic Acid Equivalents; QE, Quercetin Equivalents; CE, Catechin Equivalents. NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; NSC, non-structural carbohydrates (=100 – CP– EE– NDF– Ash). TPC, total polyphenol content; TFC, total phenolic content; TTC, total tannin content; nd, not detected; LOD, limit of detectability.

conditions (28, 29). The rumen liquor was collected at the slaughterhouse from three healthy grazing adult sheep (age: 18–20 months; weight 45–50 kg). The rumen fluid was immediately stored in a pre-heated thermos and transported to the Feed Evaluation laboratory at the Department of Veterinary Medicine and Animal Production (University of Napoli Federico II) within 2 hours. In the laboratory, the rumen fluid was pooled to limit the donor effect, mixed, strained through four layers of cheesecloth, and diluted in a buffered medium (75 mL,1:7.5 rumen liquor:medium ratio). A reducing agent (4 mL) for oxidation was added to the flasks. In three bottles, the incubation lasted 120 h, and the produced gas was recorded 21 times (at intervals of 2 to 24 h) using a manual pressure transducer (Cole and Palmer Instrument Co, Vernon Hills, IL, United States). The cumulative volume of the gas produced was related to the incubated and degraded organic matter (OMCV and Yield, respectively, mL/g). After the incubation, the residue in each serum flask was filtered through crucibles (porosity #2) and burned in a muffle furnace at 550°C for 3 h to assess the organic matter degradability (OMD120h, %), determined by the weight difference between the empty crucible and the crucible after ashing.

2.3 Methane production assessment

The three flasks from the six replications of each diet were removed at 24 h for the methane (CH_4) and organic matter degradability (OMD24h) assessment. Three mL of the gas phase was sampled in duplicate from each serum flask using a gastight syringe and injected into a gas chromatograph (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy), equipped with a loop TC detector and a packed column (HaySepQ SUPELCO, 3/16-inch, 80/100 mesh) (30). The methane production was reported as a function of the incubated organic matter (CH_4iOM) and organic matter degradability (CH_4dOM).

2.4 *In vitro* fermentation end-products

At the end of the incubation period, the pH of the fermentation liquor was measured with a pH meter (ThermoOrion 720 A+, Fort Collins, CO, United States). The fermentation liquor (5 mL) of each serum flask was collected and centrifuged at 12,000 (x) g for 10 min at 4°C (Universal 32R centrifuge, Hettich FurnTech Division DIY, Melle-Neuenkirchen, Germany). Subsequently, 1 mL of the supernatant was mixed with 1 mL of oxalic acid (0.06 Mol). The volatile fatty acids (VFAs) were measured using gas chromatography (ThermoQuest 8000top Italia SpA, Rodano, Milan, Italy; fused silica capillary column 30 m, 0.25 mm ID, 0.25 µm film thickness). An external standard mixture consisting of acetic, propionic, butyric, iso-butyric, valeric, and isovaleric acids was used. The branched-chain fatty acids (BCFAs) proportion was calculated as follows: (Iso-Butyrate + Iso-Valerate)/total VFA. Ammonia was analyzed by spectrophotometric analysis (340 nm) using the Enzytec assay kit (art. n° E8390, R-Biopharm AG, Darmstadt, Germany).

2.5 Data processing and statistical analysis

For fermentation kinetics estimation, the gas production data were fitted to the sigmoidal model for each bottle (31):

$$G = A / \left(1 + \left(B / t\right)^{C}\right)$$

where G is the total gas produced (mL/g incubated OM) at time (t), A refers to the asymptotic gas production (mL/g), B is the time at which half of A is reached (h), and C is the curve switch.

The maximum fermentation rate (R_{max}, mL/h) and the time at which it occurred (T_{max}, h) were determined using model parameters (32):

$$R_{\max} = \frac{\left(A\, x\, B^{C}\right) x\, B\, x\, T_{\max}{}^{(B-1)}}{\left(1 + C^{B}\right) x \left(T_{\max} - B\right)^{2}}$$

$$T_{\max} = C\, x \left(B - 1\right) / \left(B + 1\right)^{1/B}$$

Statistical analyses for the *in vitro* fermentation parameters (OMD, OMCV, and Yield), kinetics (T_{max}, R_{max}), end-products (pH,

VFAs, and BCFAs), and OMD and CH_4 measured at 24 h were performed using one-way ANOVA (JMP®, Version 14 SW, SAS Institute Inc., Cary, NC, United States, 1989–2019) to evaluate the effect of the substrates as a fixed factor. The significance level was verified using Tukey's HSD test with p-values <0.01 and < 0.05. Dunnett's test was performed to observe the differences between the control and experimental diets. The Shapiro–Wilk test was performed for the normally distributed data. A stepwise discriminant analysis (STEPDISC, JMP software) was applied to the entire set of variables to select those that best discriminated between the diets. Afterward, the selected variables were used in canonical discriminant analysis (CANDISC procedure), a dimension reduction approach to derive canonical functions and summarize the variation among groups.

3 Results

3.1 *In vitro* parameters and fermentation kinetics

In Table 3, the *in vitro* parameters are presented. In all experimental diets, the addition of the by-products to the control diet significantly decreased ($p < 0.05$) the organic matter degradability (OMD), particularly when the olive cake from Italy (OC2) was included, followed by the pomegranate (PG). On the contrary, the inclusion of the by-products in the control diet significantly increased the gas production (OMCV and Yield) in all experimental diets during the first 6 h of the incubation (Figure 1). Regarding the fermentation kinetics (Figure 2), the pomegranate (PG), grape pomace (GR), olive cake from Greece (OC3), tomato (TO), and hazelnut (HZ) by-products significantly increased ($p < 0.001$) the time to the maximum fermentation rate (T_{max}) of the diet, while the citrus (CT) by-product supplementation to the control diet significantly decreased ($p < 0.001$) the T_{max} value. Apart from the PG diet, all other experimental diets, especially the one with the citrus (CT) by-products, showed a significant increase ($p < 0.01$) in the fermentation rate (R_{max}).

3.2 *In vitro* fermentation end-products

In Table 4, the end-products of the *in vitro* fermentation are reported. All experimental diets had significantly ($p < 0.001$) higher pH levels compared to the control diet. The addition of the OC3 and GR to a standard diet significantly decreased ($p < 0.05$) the ammonia production. The inclusion of the by-products to the control diet significantly decreased ($p < 0.001$) the production of the VFAs. All by-products, except for the OC2, significantly decreased ($p < 0.0001$) the BCFA production. Similarly, the inclusion of all by-products significantly decreased ($p < 0.001$) the propionate production, except for the CT and PG by-products. In contrast, the PG, GR, OC3, TO, and HZ diets significantly increased ($p < 0.0001$) the acetate production. The diets including the OC3, TO, and HZ demonstrated a lower percentage of the iso-butyrate compared to the CTR diet. Regarding the percentage of the butyrate, except for the CR and CT, the inclusion of all other by-products (i.e., PG, GR, OC2, TO, and HZ) in the control diet, significantly decreased ($p < 0.0001$) its production. Similarly, except for the OC2, the inclusion of the by-products in the control diet significantly

TABLE 3 *In vitro* organic matter degradability, gas production, and fermentation rate of the control and experimental diets.

Items	OMD120h	OMCV	Yield	T_{max}	R_{max}
	%	mL/g	mL/g	h	mL/h
CTR	81.1	257	314	3.77	9.37
CR	75.2 ***	285 ***	369 **	3.65 NS	11.3 ***
OC2	53.3 ***	294 ***	545 ***	4.60 NS	11.2 ***
PG	77.7 *	283 ***	364 ***	5.38 ***	9.33 NS
GR	73.4 ***	285 ***	387 ***	4.99 **	10.5 ***
OC3	72.2 ***	295 ***	419 ***	5.77 ***	10.2 ***
TO	72.2 ***	297 ***	411 ***	5.75 ***	10.6 ***
HZ	73.2 ***	294 ***	403 ***	6.58 ***	10.5 ***
CT	67.6 ***	300 ***	438 ***	2.03 ***	14.5 ***
MSE	1.11	8.34	12.7	0.233	0.05

CTR, control diet; CR, control diet + carob; OC2, control diet + Olive cake from Italy; PG, control diet + pomegranate; GR, control diet + grape; OC3, control diet + Olive cake from Greece; TO, control diet + tomato; HZ, control diet + hazelnuts; CT, control diet + citrus. OMD120h, degraded organic matter after 120 h of the incubation; OMCV, cumulative volume of gas related to incubated OM; Yield, cumulative volume of gas related to degraded OM; T_{max}, maximum time which occurs R_{max}, maximum fermentation rate. *, **, ***: $p < 0.05$, 0.01, 0.001, respectively; NS, not significant; MSE, mean square error.

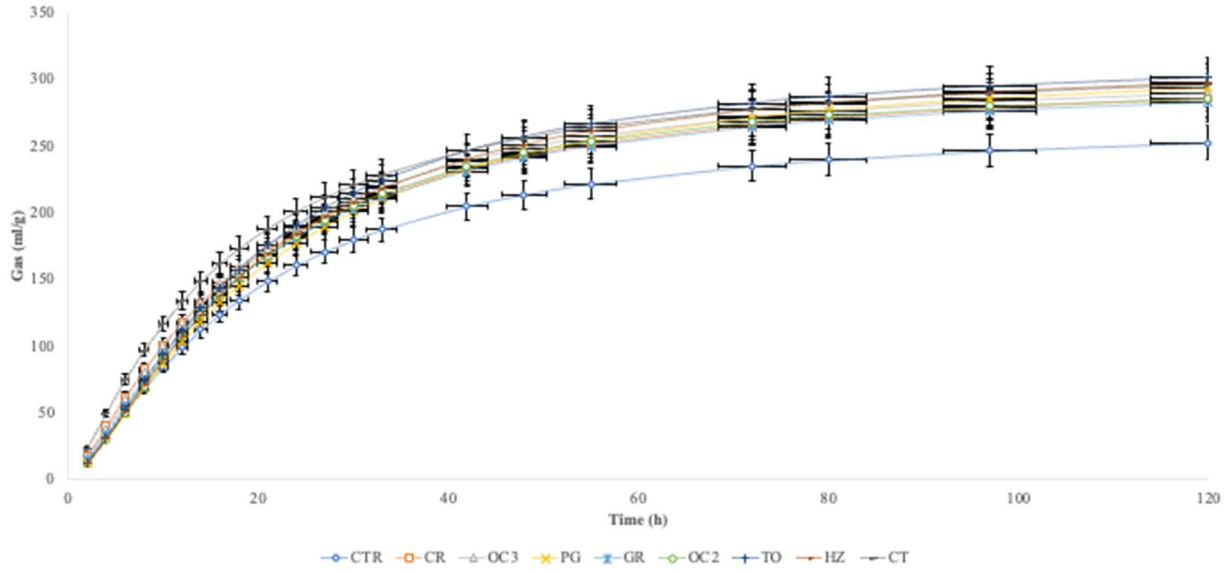


FIGURE 1
In vitro gas production over time.

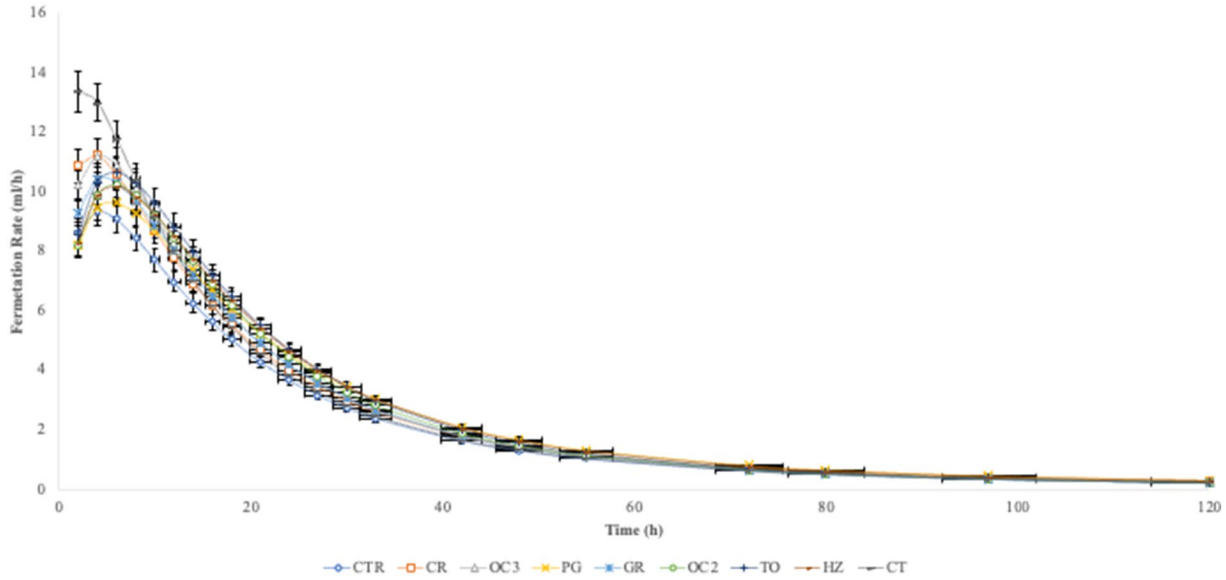


FIGURE 2
In vitro fermentation kinetic over time.

decreased ($p < 0.0001$) the iso-valerate percentage. The inclusion of the CR, OC2 from Italy, PG, GP, and TO by-products in the control diet significantly increased ($p < 0.0001$) the production of the valerate. The carob, GR, OC3, TO, and HZ diets significantly increased ($p < 0.0001$) the acetate/propionate ratio.

3.3 *In vitro* fermentation parameters

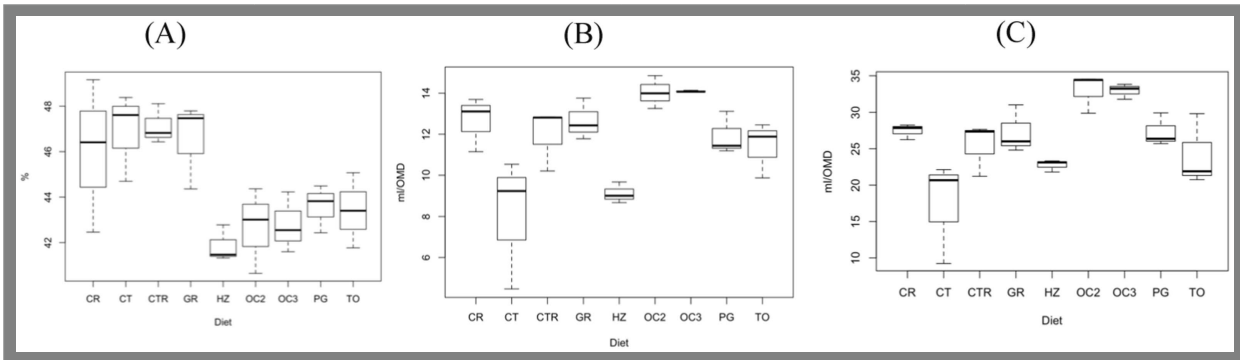
The *in vitro* parameters after 24 h of the incubation are presented in Table 5. Regarding the organic matter degradability (OMD24h), the inclusion of the olive cakes (OC2 and OC3), TO, and HZ decreased the values compared to the CTR diet. Few effects were observed on the methane production when expressed in ml/g iOM. Only the supplementation of the HZ and CT by-products to the control diet significantly decreased the methane production in terms of mL/giOM. The by-products of the HZ, CT, GR, and TO significantly decreased ($p < 0.01$) the methane production when related to the organic matter degraded (CH4dOM). On the contrary, the inclusion of the olive cakes in the control diet significantly increased ($p < 0.001$) the methane production when reported as mL/OMD.

TABLE 4 *In vitro* fermentation end-products of the control and experimental diets.

Diet	pH	NH_3	VFA	BCFA	Ace	Prop	Iso-but	But	Iso-val	Val	Ace/ Prop
		mmol/l		% VFA							
CTR	6.36	8.69	64.6	5.58	59.4	19.9	2.26	13.1	3.44	2.17	2.91
CR	6.41 ***	7.85 NS	54.3 ***	5.33 *	59.6 NS	18.2 ***	2.14 NS	14.2 ***	3.23 *	2.50 ***	3.37 **
OC2	6.47 ***	9.41 NS	56.8 ***	5.70 NS	59.7 NS	19.2 *	2.23 NS	13.0 NS	3.47 NS	2.35 ***	3.11 NS
PG	6.41 ***	7.19 NS	55.8 ***	4.98 ***	60.7 **	20.3 NS	1.99 NS	11.5 ***	3.01 ***	2.38 ***	3.01 NS
GR	6.42 ***	6.54 *	56.5 ***	5.15 ***	62.9 ***	17.4 ***	1.86 NS	12.1 ***	3.16 ***	2.37 ***	3.62 ***
OC3	6.46 ***	6.71 *	59.2 ***	4.46 ***	63.4 ***	18.6 ***	1.78 **	11.7 ***	2.67 ***	2.10 NS	3.47 ***
TO	6.45 ***	8.29 NS	60.3 ***	4.71 ***	64.2 ***	17.8 ***	1.83 *	10.8 ***	2.91 ***	2.47 ***	3.59 ***
HZ	6.41 ***	8.24 NS	57.6 ***	4.41 ***	62.7 ***	18.3 ***	1.81 *	12.4 ***	2.67 ***	2.21 NS	3.50 ***
CT	6.43 ***	8.00 NS	57.8 ***	5.14 ***	59.0 NS	19.3 NS	2.02 NS	14.1 ***	3.08 ***	2.24 NS	3.07 NS
MSE	42e-5	0.782	0.50	0.008	0.15	0.05	0.02	0.008	0.003	0.002	0.01

CTR, control diet; CR, control diet + carob; OC2, control diet + OC2 by-products; PG, control diet + pomegranate; GR, control diet + grape; OC3, control diet + OC3by-products; TO, control diet + tomato; HZ, control diet + hazelnuts; CT, control diet + citrus; NH_3, ammonia; VFA, volatile fatty acids; BCFA, branched-chain fatty acids; Ace, acetate; Prop, propionate; Iso-but, Iso-butyrate; But, butyrate; Iso-val, Iso-valerate; Val, valerate; Ace/Prop, acetate/propionate ratio. *, **, ***: $p < 0.05$, 0.01, 0.001, respectively; NS, not significant; MSE, means square error.

TABLE 5 *In vitro* organic matter degradability and methane production after 24 h of the incubation.



Diet	OMD24h	CH_4iOM	CH_4dOM
	%	mL/giOM	mL/OMD
CTR vs.			
CR	NS	NS	NS
OC2	*	NS	***
PG	*	NS	NS
GR	NS	NS	**
OC3	*	NS	***
TO	*	NS	***
HZ	**	***	***
CT	NS	**	***

In vitro organic matter degradability **(A)** and methane production by incubated **(B)** and degraded **(C)** organic matter after 24 h of incubation. CTR, control diet; CR, control diet + carob; OC2, control diet + OC2 by-products; PG, control diet + pomegranate; GR, control diet + grape; OC3, control diet + OC3 by-products; TO, control diet + tomato; HZ, control diet + hazelnuts; CT, control diet + citrus; OMD 24 h, degraded organic matter after 24 h of the incubation; CH_4iOM, methane related to incubated organic matter; CH_4dOM, methane related to degraded organic matter at 24 h of the incubation. *, **, ***: $p < 0.05$, 0.01, 0.001, respectively; NS, not significant; MSE, mean square error.

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3.4 Multivariate analysis

Table 6 shows the canonical structure; the first canonical variable explained more than 70% of the total variability, while the second explained less than 20%. As evidenced by the distribution of the diets in Figure 3, the first canonical variable was positively correlated with the OMD, Rmax, VFAs, BCFAs, and propionic, butyric, and iso-valerianic acids and was negatively correlated with the cellulose, OMCV, Tmax, methane production, and acetic and valerianic acids. The second canonical variable was positively correlated with the Rmax, methane production, BCFAs, propionate, butyrate, iso-valerate, and valerate and negatively

TABLE 6 Total canonical structure: correlations between the canonical variables and original variables.

Parameter	Can 1	Can 2
OMD120h	0.249	−0.419
OMCV	−0.445	−0.112
T_{max}	−0.609	−0.490
R_{max}	0.262	0.244
CH_4iOM	−0.310	0.338
CH_4dOM	−0.327	0.244
VFA	0.242	−0.319
BCFA	0.300	0.852
Ace	−0.631	−0.541
Prop	0.217	0.151
But	0.837	0.387
Iso-val	0.315	0.846
Val	−0.404	0.596
Variance explained (%)	71.7	19.1

Can 1, Canonical 1; Can 2, Canonical 2; OMD, degraded organic matter at 120 h of the incubation; OMCV, cumulative volume of gas related to incubated OM; T_{max}, time at which the maximum fermentation rate occurred; R_{max}, maximum fermentation rate; CH_4iOM, methane related to incubated organic matter; CH_4dOM, methane related to degraded organic matter at 24 h of the incubation; VFA, volatile fatty acids; BCFA, branched-chain fatty acids; Ace, acetate; Prop, propionate; Iso-but, Iso-butyrate; But, butyrate; Iso-val, Iso-valerate; Val, valerate.

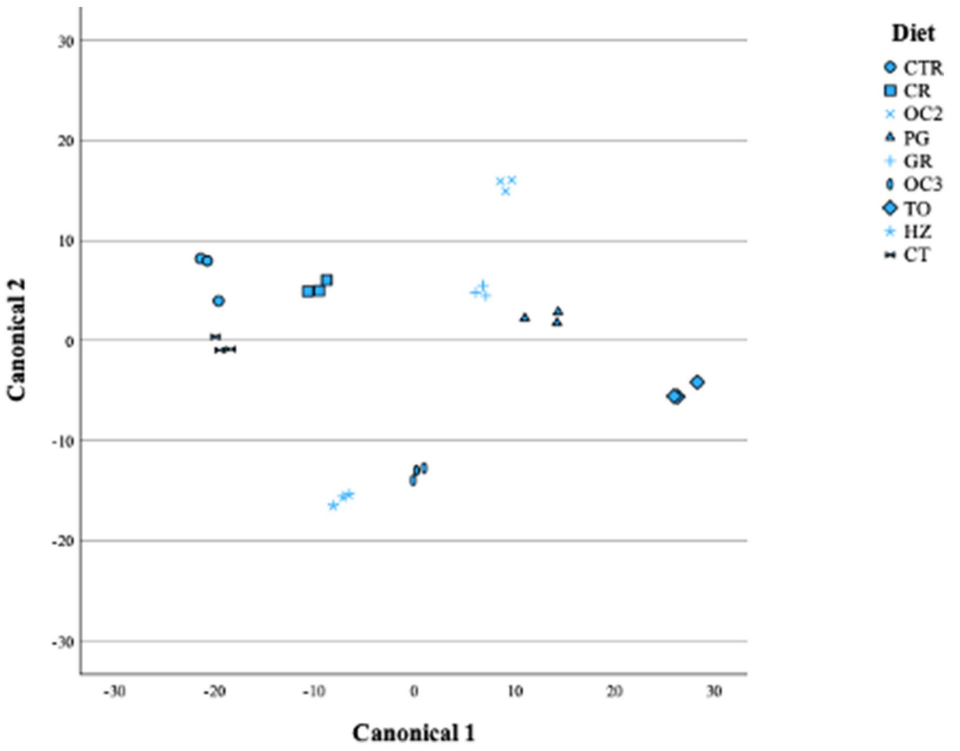

FIGURE 3
Plot of canonical 1 (Can 1) and canonical 2 (Can 2).

correlated with the OMD, OMCV, Tmax, volatile fatty acids, and acetate.

4 Discussion

The inclusion of the selected by-products in the diet, at a level of 10% DM, affected the fermentation parameters during the incubation (120 h). In particular, the experimental diets showed a reduction in the organic matter degradability and an increase in the gas production (OMCV and Yield). The chemical composition of the selected by-products likely contributed to these results. The high content of the lipids of some by-products, such as the olive cake and hazelnut skin, contributed to the reduced diet digestibility (33). Furthermore, the majority of the by-products reported high lignin content, which is a highly resistant compound that is only partially degraded by the microbial population in the rumen. However, lignin content is not directly responsible for diet digestibility; its association with other chemical components can influence the properties of fermentation, including the enzymatic degradation of structural carbohydrates (34). Indeed, by-products rich in phenolic compounds, such as hazelnut skin, grape pomace, and olive cake, could limit cellulolytic and fibrolytic microbial activity due to the formation of complexes with lignocellulose, which reduce fiber degradability (35). A previous *in vitro* study (36) showed that high content of condensed tannins bound proteins and reduced organic matter degradation. Moreover, tannins have a protein-binding property that leads to a reduction in dietary protein degradation by the proteolytic microbial population, limiting ammonia concentration (37). Notwithstanding the reduction in the digestibility, the cumulative gas production was higher in all samples compared to the control diet. The fermentation rate exhibited a similar trend, except for the PG diet. These results can be attributed to the presence of non-structural carbohydrates (38).

The variation in terms of the fermentation and gas production affected the pH level in the fermentation liquor at the end of the incubation, which was within normal values for the ruminants, ranging between 6.41 and 6.47 across all tested diets (39). The inclusion of the by-products in the diets did not affect the ammonia production, except for the GP and OC3 diets, in which it decreased the ammonia content and reduced the total VFA production. As previously reported, these results could be explained by the high content of polyphenols and tannins in these by-products, which could bind nutrients, such as protein and carbohydrates, leading to a reduction in fermentation products in the rumen (40).

The inclusion of agro-industrial by-products may lead to a shift in the metabolic pathways during the process of ruminal fermentation and the production of volatile fatty acids. Indeed, the GP, GR, OC3, TO, and HZ by-products increased the acetate levels in the diets compared to the propionate and butyrate. The decrease in the short-chain branched acids (iso-valerate, iso-butyrate, and BCFAs), which are end-products of protein metabolism, may be explained by the low protein content of the evaluated by-products and their high content of phenolic compounds (30).

Regarding the parameters obtained after 24 h of the incubation, the addition of the by-products to the control diet did not affect the organic matter degradability, except for the tomato, both olive cakes, and hazelnuts. The olive cakes demonstrated low *in vitro* degradability, which was also reported by several authors (23–41) and can be attributed to their chemical composition (high content of structural carbohydrates and lignin). Moreover, both OC2 and OC3 increased the methane production per gram of the OMD, with similar findings previously recorded by Marcos et al. (42), who observed an increasing trend in methane production when an exhausted olive cake was evaluated. On the contrary, most of the experimental diets showed lower methane production. When the methane production was related to the incubated organic matter (CH_4iOM), only the HZ and CT diets showed significant differences compared to the CTR diet. Tannins may exhibit a modulatory action on microbial populations, especially affecting archaea and protozoa, which have been correlated with methane production in the rumen (43–48).

Niderkorn et al. (49) evaluated *in vitro* rumen fermentation parameters in diets including sainfoin (*Onobrychis viciifolia* Scop.) pellets and/or hazelnut (*Corylus avellana* L.) pericarps using a batch culture system for 24 h. The authors concluded that the inclusion of the sainfoin pellets and hazelnut pericarps in a basal diet resulted in lower rumen fermentability and that condensed tannins decreased methane production and protein degradability. Atalay et al. (16) recorded a low methanogenic potential of grape pomace. In this regard, published data have reported different results regarding the potential of by-products for methane mitigation. These discrepancies could be explained by several factors, such as the industrial process (50).

The current results obtained through a stepwise multivariate discriminant analysis indicated that eight different canonical variables emerged, but only two completely explained the variance. Furthermore, most of the variance was explained by canonical 1 (Table 6), with the butyrate being the most discriminant parameter (showing the highest positive correlation). This result was also confirmed by the Mahalanobis distance (data not shown), with the CTR and TO diets showing the greatest distance (819, $p < 0.001$). In this regard, most of the experimental diets, particularly the TO diet, showed a decrease in the butyric acid production. This *in vitro* result could be promising for formulating a diet that prevents metabolic disorders. Indeed, increases in butyric and propionic acids could lead to metabolic disorders, such as subacute acidosis (SARA). Volatile fatty acids are the modulators of the inflammatory response as they can activate neutrophils, which are essential for host defense. Butyric acid decreases several neutrophil functions, such as phagocytosis (51). Moreover, β-hydroxybutyric acid (BHBA) is a metabolite of butyrate metabolism, normally used to monitor and prevent ketosis (52).

5 Conclusion

The obtained *in vitro* results demonstrated that the addition of the agro-industrial by-products at 10% DM affected the fermentation parameters (organic matter degradability and gas production). The addition of these by-products in a diet composed of natural grassland and concentrate promoted a reduction in the methane production during the first 24 h of the fermentation and increased the acetic acid production, which serves as a source of energy for ruminants. Further studies should be conducted to determine the appropriate inclusion dose of agro-industrial by-products in the basal diet of ruminants to avoid adverse effects on rumen fermentation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the Ethical Animal Care and Use Committee of the University of Napoli Federico II (Prot. 2019/0013729 of 08/02/2019). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AV: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. BM: Formal analysis, Writing – original draft. DK: Data curation, Writing – original draft. MN: Software, Writing – original draft. SH: Investigation, Writing – review & editing. RB: Project administration, Writing – review & editing. PL: Supervision, Writing – review & editing. MC: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. VN: Methodology, Writing – original draft, Writing – review & editing. SC: Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The authors declare that no Generative AI was used in the creation of this manuscript.

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The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1530419/full#supplementary-material

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Comparative anti-methanogenic ability of green algae (*C. reinhardtii*) with/without nanoparticles: *in vitro* gas and methane production

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Introduction: The purpose of this study was to investigate how in vitro gas production (GP) and ruminal fermentation characteristics were affected by increasing concentrations of green algae plant (*C. reinhardtii*) extracts in combination with nanoparticles MgO and MgS.

Methods: A solution containing 0.1 M $MgCl_2$ was prepared in 300 mL for the green production of MgCl nanoparticles. The mixture was refluxed for two hours at 85°C using a reflux condenser after 10 mL of pomegranate plant extract was added. The green algal plant (*C. reinhardtii*), which has many non-toxic antioxidants, was used as a carbon source to produce carbon quantum dots (CQD). Chemical analysis was conducted in accordance with AOAC (2005) recommendations. Rumen fluid from recently slaughtered calves is used to produce *in vitro* gas immediately following slaughter. Analysis of variance (ANOVA) was performed on the obtained data from the *in vitro* study in a completely randomized design using the mixed model of SAS (version 9.4; Inc., Cary NC, USA).

Results and Discussion: The variance analysis results and the average values of the chemical compositions were significantly influenced by the extracts (all $p < 0.0001$). In this line, the values of net gas, pH, OMD, ME, NEl, and ME were found to be the highest for Algae + 50 MgO and the lowest for Algae + 50 MgS, respectively (all $p < 0.0001$). These promising results imply that extracts from *C. Reinhardtii* may be able to mitigate the adverse consequences of rumen fermentation. To precisely ascertain the impact particular Rhodophyta on greenhouse gas emissions, additional investigation is needed.

KEYWORDS

gas production, nanoparticles, methane emission, *in vitro*, green algae

Introduction

In response to the increasing population and the need to provide animal protein, along with the lack of animal feed resources, humans and animals have competed for agricultural resources (1, 2). Thus, Sustainability of livestock production is currently a research priority due to the increasing demand for food by the growing world population. It has been predicted that green algae (*Chlamydomonas reinhardtii*) can provide biomass and animal feed in the future (3). Green algae are substantially more productive in terms of biomass than other photosynthetic organisms, and more crucially, growing microalgae does not compete with food crops on arable ground (4). It is possible to use the algae as a non-traditional alternative feed source owing to their efficacy in converting solar energy, independence from external environmental conditions, and high production rate compared to conventional crops (4).

Besides contributing to greenhouse gas emissions, methane loss is one of the greatest negative factors in ruminant production (5–7). Although causing energy loss in the rumen, CH_4 production reduces rumen acidity and keeps the rumen environment below normal via using H^+ ions by methanogenic bacteria (8). The concentration of dihydrogen in the rumen depends on factors such as methanogen growth and the rate of feed fermentation. Methane generation and volatile fatty acid production are determined by the equilibrium between pathways that create and combine metabolic hydrogen (9). A variety of methane inhibitors can prevent methane-related energy losses in ruminants and provide economic and ecological benefits (10).

Numerous resources have focused on the reduction of CH_4 generation, especially energy loss from methane production. In addition, studies on the transformation of fermentation products into chemicals useful for animals have been accompanied in the recent years. Accordingly, to reduce enteric methane production, unsaturated fatty acids (11, 12), lysozyme (13), organic acid salts (14), *S. cerevisiae* (15), enzymes (15), and ethyl acetate (16) are added to ruminant diets. Unlike specific CH_4 inhibitors, these compounds generally affect and suppress microorganism growth (17). Consequently, the feed value is reduced due to adverse effects on rumen fermentation. Many researchers suggest that, instead of adding additives that are thought to affect the rumen microbiome, the use of carbon quantum dots (CQD), magnesium sulfide (MgS) and magnesium oxide (MgO) nanoparticles, which are known as hydrogen receptors, is an appropriate alternative (18–21). However, there is a lack of information about the evaluating anti-methanogenic capabilities of nanoparticles of *C. reinhardtii* in *in vitro* system. Thus, this study assessed, using an *in vitro* gas and methane generation approach, the green algal (*C. reinhardtii*) anti-methanogenic capabilities with and without nanoparticles.

Materials and methods

Green synthesis and structural characterization of CQD, MgS and MgO NPs

Preparation of algae extract

For the green synthesis of MgCl NPs, 300 mL of a solution containing 0.1 M $MgCl_2$ was prepared. 10 mL of pomegranate algae extract was added to the solution and refluxed for 2 h at 85°C under a reflux condenser. It was then placed in a reactor via Teflon tube. Hydrothermal reactions were performed at 180–195°C for 4 h to reduce nano-particle (NP) size. The precipitated MgO NPs were washed first via pure water and ethyl alcohol. They were preserved in an atmosphere free of moisture after being dried for 48 h at 60°C in an oven. MgS NPs were synthesized using the same procedure. 1 mol of Na_2S was added to the synthesis medium and the same process was repeated to synthesize MgS NPs. In the synthesis of CQD, the green algae (*C. reinhardtii*), known for its high non-toxic antioxidant content, was used as a carbon source. For this purpose, the algae extract was placed in a reactor containing sodium citrate as a reducing agent. CQD was synthesized by incubating at 180–195°C for 8 h.

Characterization of CQD, MgS and MgO NPs

Green-synthesised CQD, MgS and MgO NPs were characterized at the High Technology Application and Research Center of Eastern Anatolia (DAYTAM) at Atatürk University. X-ray microscopy (XRD) and FTIR analyses were performed for the characterization of CQD, MgS, and MgO NPs. The synthesized CQD, MgS, and MgO nanoparticles were characterized, including their size and morphology.

Chemical analyses

AOAC (71) guidelines were followed for chemical analyses. Kjeldahl was used to determine N content (AOAC, 71, Method 984.13). For the determination of Acid Detergent Fiber (ADF) and Neutral Detergent Fiber (NDF), Van Soest et al. (22) were used.

In vitro gas production

In vitro gas production is performed by taking rumen fluid from newly slaughtered cattle (as soon as they are slaughtered), as mentioned by Palangi et al. (10). Using a method validated by Menke and Steingass (23), it was found that 0.2 g of treated (CQD, MgS, and MgO nanoparticles at levels of 0.50, 100 ppm) and ground (1 mm) green algae (*C. reinhardtii*) samples were incubated in rumen fluid via 100 mL standardized glass syringes to measure *in vitro* gas production. Methane and gas volumes of feed samples were measured 24 h after incubation.

Statistical analysis

The mixed model of SAS version 9.4 (SAS Institute, Inc., Cary, NC, United States) was used in a completely randomized design to examine the data gathered from the *in vitro* study. The following model was used to statistically analyze the experiment:

$$Y_{ij} = \mu + T_i + E_{ij}.$$

where μ is the overall mean for each parameter, T_i is the effect of treatment, and E_{ij} is residual error. Differences among sample means with $p < 0.05$ were accepted as statistically significant.

Results

Characterization of CQD, MgS, and MgO nanoparticles

XRD analysis

The fundamental method for examining crystal size, phase purity, and crystal structure is X-ray diffraction (XRD) examination. As

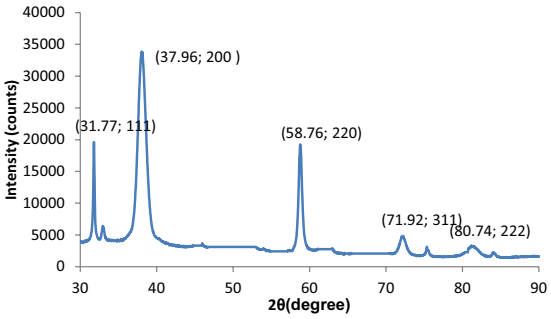

FIGURE 1
XRD patterns of MgO.

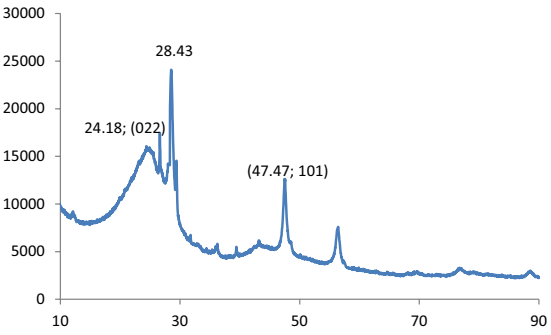

FIGURE 2
XRD patterns of CQD.

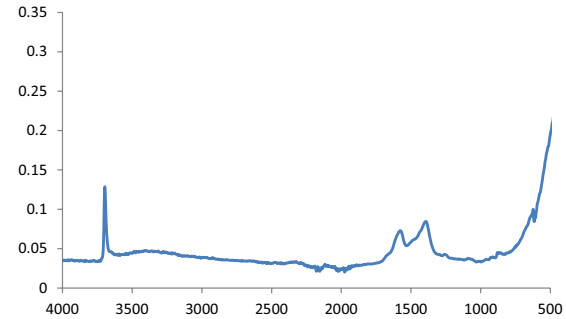

FIGURE 3
FTIR spectra of MgO NPs.

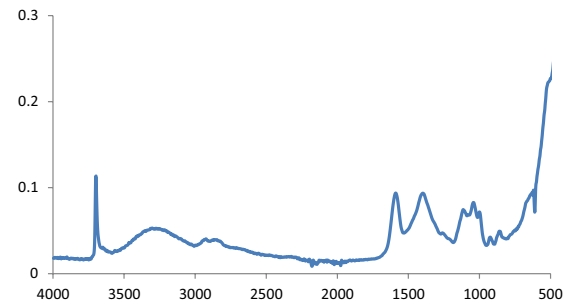

FIGURE 4
FTIR spectra of MgS NPs.

shown in Figure 1, the XRD pattern of the synthesized MgO exhibits various peaks corresponding to the (111), (200), (220), (311), and (222) reflection planes.

The particle size of the synthesized MgO NPs was determined from the Debye–Scherrer equation: D = K/cos (θ).

The MgO NPs' median dimension and d-spacing values have been determined to be 20 nm and 0.25 nm, correspondingly.

Figure 2 shows the XRD pattern of CNPs. The XRD pattern exhibited an intense peak at $2\theta = 22.90°$ and a weak peak at $2\theta = 41.60°$, corresponding to the (022) and (101) diffraction patterns of graphite carbon, respectively.

FTIR analysis

MgO NPs are characterized using the FTIR spectrum (Figure 3). In ambient settings, the spectra were captured at wavelengths ranging from 400 to 4,000 cm^{-1}. The peak at 651.94 cm^{-1} indicates the stretching peak vibration of Mg-O bond, confirming that the obtained product is magnesium oxide. Moreover, H_2O adsorption on the metal surface is indicated by the peaks at 1552.0 cm^{-1} and 3520.0 cm^{-1}.

The potential biomolecules in charge of the reduction of MgS NPs by green synthesis were found using Fourier transform infrared spectroscopy (FTIR) analysis. The FTIR spectra of MgS NPs made using Na_2S and pomegranate algae extract are displayed in Figure 4. In the spectrum, bands were observed at 3603.5, 1,725, 1,550, 1,232, 972 and 613 cm^{-1}. Particularly, the sharp band at 1,725 cm^{-1} represents the C=O vibrations specific to the structure of flavonoids that can be found in pomegranate extract.

TEM analysis

The characterisation of MgO NP production using pomegranate extract is depicted in Figure 5A, which is an image captured using a transmission electron microscope (TEM). Here, the scale bars are 500 and 200 nm. The images of TEM analysis of MgS NPs were taken and show the structures of MgS NPs (Figure 5B). The shape of these NPs was a small layer formation with a nearly spherical arrangement on a smooth surface. They had diameters ranging from 20–60 ± 1.6 nm and an average diameter of 55 ± 3.8 nm.

Chemical composition

The nutrient composition and relative feed value of algal at various concentrations are indicated in Table 1. The extracts impacted significantly the chemical compositions (all $p < 0.0001$). Regarding fiber fractions such as ADF and NDF, the highest values were recorded for Algae +100 MgS. In contrast, in related to the CP and EE fractions, Algae +50 Mgs had the highest values ($p < 0.0001$).

In vitro fermentation and gas production

The effects of algae extracts on *in vitro* rumen fermentation profiles are shown in Table 2. The parameters of gas production, pH, and OMD have influenced significantly by the different extracts (all $p < 0.0001$); the highest and lowest values regarding net gas, pH, OMD, ME, and NE_l were observed for Algae +50 MgO and Algae +50 MgS, respectively. The converse mentioned trend was observed for Algae +50 MgO and Algae +50 MgS in related to CH_4 production ($p < 0.0001$). Totally, not only measured total gas volume but also most of the measured parameters from the Algae-based rumen fluid

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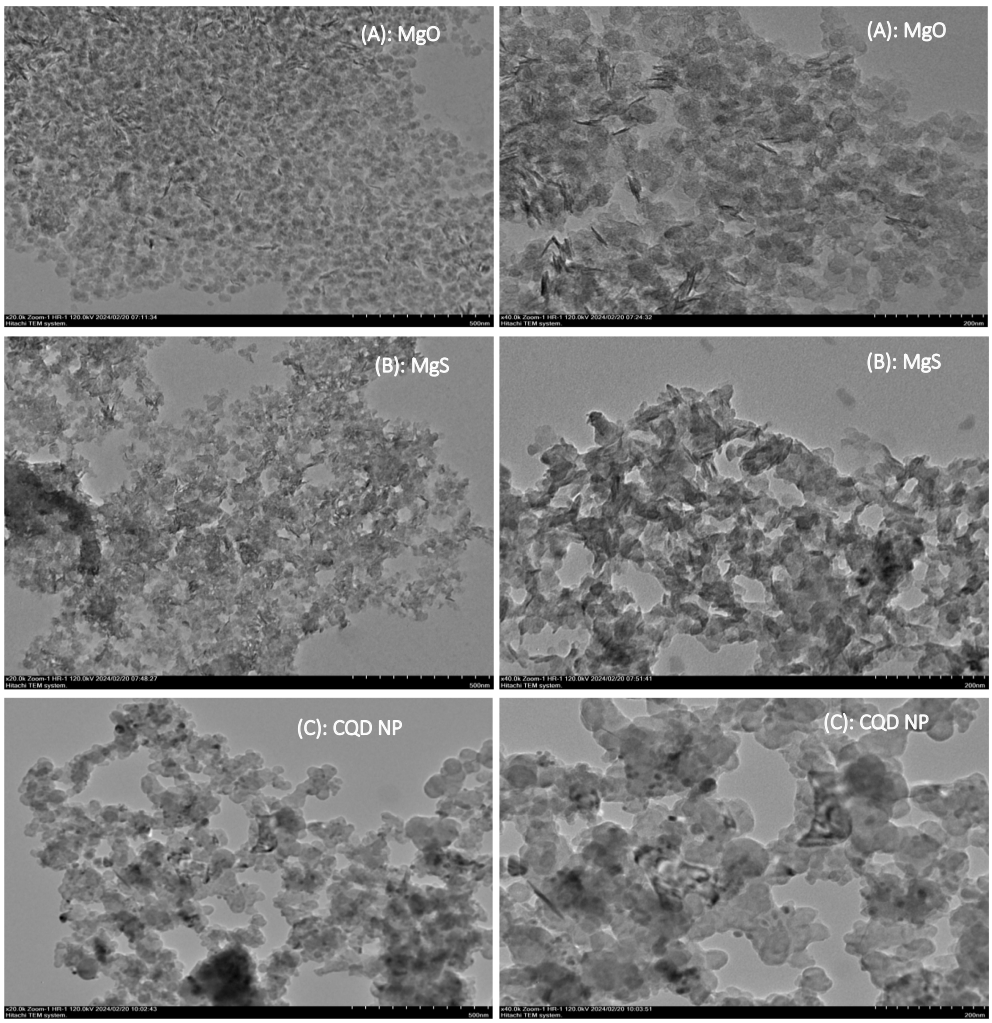

FIGURE 5
TEM images of **(A)**: MgO NP, **(B)**: MgS and **(C)**: CQD NPs.

TABLE 1 **Chemical nutrient composition and relative feed value of increasing doses of Algae at different levels of nanoparticles.**

Items	Treatment							SEM	*p*-value
	Algae Control	Algae +50 Carbon	Algae +100 Carbon	Algae +50 Mgo	Algae +100 Mgo	Algae +50 MgS	Algae +100 MgS		
CP, %	34.17^{ab}	34.68^{ab}	32.65^{b}	35.98^{ab}	32.25^{b}	37.23^{a}	37.08^{a}	1.49	<0.0001
DM, %	95.26^{ab}	95.86^{ab}	93.31^{b}	95.97^{ab}	96.80^{a}	95.22^{ab}	94.69^{ab}	0.86	<0.0001
Ash, %	28.40^{abc}	29.69^{a}	29.43^{ab}	27.87^{bc}	28.77^{abc}	27.46^{c}	28.51^{abc}	0.59	<0.0001
EE, %	1.25^{b}	1.78^{b}	1.42^{b}	1.17^{b}	1.48^{b}	2.82^{a}	1.68^{b}	0.31	<0.0001
NDF, %	31.98^{abc}	32.92^{ab}	32.67^{ab}	33.28^{ab}	28.94^{c}	30.09^{bc}	34.15^{a}	1.22	<0.0001
ADF, %	21.79^{a}	20.97^{ab}	21.45^{ab}	21.90^{ab}	20.96^{ab}	20.62^{b}	21.04^{ab}	0.38	<0.0001
ADL, %	11.54	11.69	11.88	11.96	10.57	10.40	12.14	0.73	0.069

CP, Crude protein; DM, Dry matter; Ash, ash; EE, Ether extract; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin. a-c, means within the column with unlike superscript differ significantly ($P < 0.01$).

were significantly influenced by the different nanoparticles ($p < 0.0001$).

VFA parameters

Table 2 shows the volatile fatty acid (VFA) composition of rumen fluid. The effects of extracts on total VFA (TVFA) were substantial ($p < 0.001$), with the highest value found in the "Algae +100 MgS" group (163.12 mM) and the lowest in the CON group (139.59 mM). For the individual VFA, extracts have resulted in fluctuated amounts between treatments, in which the treatments influenced the individual VFA significantly ($p < 0.001$).

TABLE 2 Effects of nanoparticles on *in vitro* gas, methane production quantities, and rumen fermentation variables of algae.

Item	Treatment								
	Algae Control	Algae +50 Carbon	Algae +100 Carbon	Algae +50 MgO	Algae +100 MgO	Algae +50 MgS	Algae +100 MgS	SEM	*p*-value
pH	6.77	6.79	6.76	6.78	6.77	6.75	6.79	0.02	0.074
CH_4, %	18.18^{bc}	17.14^{bc}	19.12^{bc}	16.11^{c}	20.77^{b}	28.98^{a}	25.93^{ab}	1.23	<0.0001
CH_4, mL	5.87	5.27	6.09	5.11	5.92	6.30	5.71	0.57	0.152
Gas, mL	32.32^{a}	30.64^{a}	31.67^{a}	32.04^{a}	28.59^{a}	21.81^{b}	21.99^{b}	1.94	<0.0001
TDMA, mg	364.7	389.17	355.83	370.2	395.4	390.41	365.88	13.52	2.672
ME, mj/kg KM	5.91^{ab}	5.85^{ab}	5.79^{ab}	5.99^{a}	5.59^{ab}	5.53^{b}	5.52^{b}	0.14	<0.0001
MPSE, mg	83.10^{c}	84.88^{ab}	83.69^{b}	83.36^{b}	86.45^{ab}	89.29^{a}	88.75^{a}	1.04	<0.0001
NEL, mj/kg KM	3.19	3.21	3.10	3.26	2.96	3.09	2.97	0.11	0.065
OMD, %	30.26^{a}	29.81^{a}	30.01^{a}	30.23^{a}	28.91^{a}	26.82^{b}	26.94^{b}	0.66	<0.0001
PF, mg/mL	364.7	389.17	355.83	370.2	395.4	390.41	365.89	13.52	1.281
TDD, %	70.25^{b}	75.00^{ab}	69.86^{b}	71.23^{b}	78.89^{a}	75.98^{ab}	71.48^{b}	2.43	<0.0001

a–c, means within the column with unlike superscript differ significantly ($p < 0.05$). TDMA, true digested matter amount; ME, metabolizable energy; MPSE, Microbial protein synthesis efficiency; NEL, net energy lactation; OMD, organic matter digestion; PF, Partition factor; TDD, true digestion degree; SEM, standard error of means.

Discussion

Characterization of CQD, MgS, and MgO nanoparticles

XRD analysis

The observed peaks demonstrate the cubic structure of MgO and assign it to the pure phase of periclase MgO. In the spectra of other phases, no additional peaks could be seen. It confirmed that the prepared MgO was crystallized and was free of impurities. In addition, the presented peaks exhibit higher intensity and narrower spectral widths, indicating the product is in good condition. The XRD graph obtained for the crystallographic analysis of synthesized MgS nanomaterials is given in Figure 6. The 2θ values for MgS NPs peak at 37.94° (200), 45.42 (220) and 58.71° (221) at 200, 210 and 222. The characteristic peaks of the XRD spectrum at $2\theta = 45.45°$ can be indexed at (220). Literature-based findings are consistent with the results obtained (24).

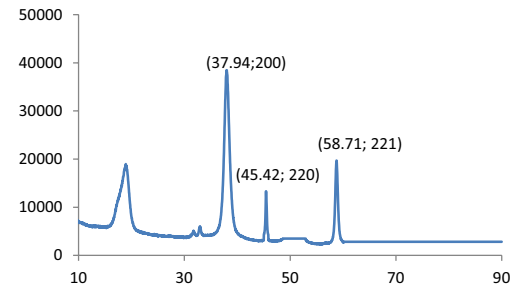

FIGURE 6
XRD patterns of MgS.

FTIR analysis

This is defined as OH stretching and bending, respectively. The metal-oxygen frequencies for the respective metal oxides published in the literature and observed frequencies coincide reasonably well. Using this method, MgO NPs can be analyzed for their chemical composition and surface properties (25). The -C-H bending vibrations in the aromatic amine groups of the flavonoid structure are linked to the absorption band at 550 cm^{-1}. Additionally, the peak at 972 cm^{-1} shows the existence of MgS NPs as well as the distinctive C-S bond structure peaks. Under the aliphatic chain structure, the observed 613.4 cm-1 peak is part of the –CH2 group. The pomegranate algae extract's bioactive components were verified using FTIR spectrum (26). Using this analysis, it is possible to determine the biomolecules involved in the synthesis of MgS NP. Figure 7 displays the carbon quantum dots of NP according to FTIR spectra. The band at around 3,242 cm^{-1} is indicative of OH stretching vibration, which may arise from either the hydroxyl groups found in C black NP or water absorption. The peak recorded at 1,652 cm^{-1} was exclusively found in pure CB and was ascribed to the material's C=C stretching vibration. Peaks at 2,040, 2,166, and 2,015 cm^{-1} are ascribed to the nanocarbon structure's carbonyl group and C–O stretching (27).

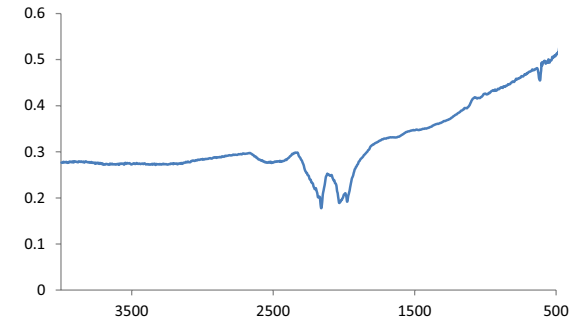

FIGURE 7
FTIR spectra of CQD NPs.

TEM analysis

TEM analysis is a very critical methodology to describe the particle size distribution, average particle size and shape of NPs. The produced MgO nanoparticles are less than 10 nm in size and spherical, as confirmed by TEM examination, despite being aggregated (28). The current green synthesis method approach has enabled the use of a simple and low-cost reducing agent for single-phase MgS NPs. This approach offers an effective method to synthesize MgS NPs in a non-toxic manner (24). Transmission electron microscopy (TEM) evaluated the morphology of pure carbon NP samples. These NPs are the samples showing the highest level of modification and are shown in Figure 5C. The TEM image shows semi-spherical primary particles with an average size ranging from 15 to 65 nm. These primary particles were formed and held together by agglomeration, resulting in agglomerates. The results corroborate other published studies in the literature, and the conclusions are consistent with current literature (29).

Chemical composition

The present results on the chemical composition of macroalgae were in line with previous reports (30, 31). In disagreement with our findings, a recent meta-analysis of 47 published papers containing a broad variety of macroalgae was conducted and demonstrated that the average content of CP, NDF, ADF, and organic matter (OM) was 734.2, 189.2, 321.3, and 208.5 g/kg DM, respectively (32). Additionally, to confirm our findings Min et al. (33) published the different levels of CP (7.8 to 38.1% DM), NDF (16.6 to 43.1% DM), ADF (6.6 to 13.1% DM), and EE (0.3 to 3.9% DM) across eight macroalgae species. It's important to note that the chemical composition and bioactive content of macroalgae are impacted by their taxonomic classification (brown, green, or red), and vary across genera and species. Seasonal fluctuations may also impact their composition during the growing and harvesting periods (31, 34). All algae and nanoparticles tested in our study had acceptable chemical compositions, particularly as a protein source; however, they should be included in a TMR ration to determine their potential advantages.

The current study's findings about NDF and ADF were congruent with those of Mahmood Ameen (35). Feeds typically comprise 100–120 g/kg DM of ash. The crude ash levels of the feeds were comparable to those reported by Kamalak et al. (36) and Karabulut et al. (37). Differences in nutrient composition of feeds between studies may be qualified to numerous elements, such as climate, fertilization, species and type, harvesting time, feed storage conditions, and vegetative phase (36, 38). Also, it has been stated that the *in vitro* gas production level is affected by the nutrient composition of feedstuff, the presence of compounds inhibiting (such as tannins) gas production, the microflora and microfauna content of the rumen fluid (donor animal's diet), and the quality of fermentation provided (36, 38).

Microalgae have mass balances ranging from 630 to 1,170 g kg^{-1}, although proximate analysis seldom provides 100% (39). Our investigation's findings regarding the mass balance deficit suggest that other soluble components such as B vitamins, nonprotein nitrogen, chlorophyll, and soluble carbohydrates may be responsible. Microalgae fiber is low in hemicellulose and lacks lignin, even though it has a high fiber content (50–55% of total carbohydrate) (40). This enhances the probability that the protein will be readily available due to its lack of lignin complexation. In addition, the cell wall fraction in microalgae is highly digestible (41). Drewery et al. (42) found that supplementing post-extraction algal residue (CP = 179 g kg^{-1} DM) increased OM digestibility in steers fed oat straw (CP; 45 g kg^{-1} DM). Similarly, Tetracystis sp., *N. bacillaris*, and *C. vulgaris* have a higher lipid content, which improves the calorie density of the diet. It has been widely shown that lipids frequently diminish enteric CH_4 emissions from ruminants (43, 44).

In vitro fermentation and gas production

The post-fermentation pH ranged from 6.75 to 7.79 among algal-extract treatments, demonstrating that algae supplementation promotes a more alkaline environment during microbial fermentation. Carbohydrates are the primary source of substrate for the creation of acetate and butyrate during ruminal fermentation, and as byproducts, CO_2 and hydrogen (H_2), are used by methanogenic archaea to produce CH4 (9). Furthermore, according to Kholif et al. (45), microalgae promote carbohydrate fermentation by rumen microbes, which is consistent with what was observed with the addition of microalgae and was attributed to the microalgae's fulvic acids, which can provide carbon to ruminal microorganisms (46) and thus favor microbial growth and increase DMD. In turn, the increased degradability resulted in higher production of SCFA and ME, ascribed to enhanced carbohydrate degradation (45).

Although not investigated in the current study, the increase in SFCA and ME with microalgae might be due to increased activity of the fibrolytic bacteria (47) and increased propionate production. In contrast, decrease of SFCA and ME are attributed to a reduction in other SCFAs, such as acetate (48). In the meantime, the effects on DMD and SCFA associated with the content and degradability of feed carbohydrates may be reflected in the computed variations in CH_4 per unit of SCFA, ME, and OM (49).

Biogas production (BG) is intimately related to feed degradability and, as a result, the availability of highly-fermented nutrients for rumen microbial activity and growth (15). Although their production is predominantly reliant on the fermentation of carbohydrates to SCFA and proteins, and BG is mostly made up of CO_2 and CH_4, their contribution to BG is negligible in comparison to that of carbohydrates (50). Furthermore, the production of acetate and butyrate during rumen fermentation produces more gas than the formation of propionate, accounting for the majority of the BG (51).

Natural compounds of microalgae have been proposed as potential methods for controlling rumen fermentation, contributing to CH_4 generation (52, 53). A previous *in vitro* investigation (54) demonstrated that Schizochytrium spp. inhibited CH_4. Furthermore, several research (55, 56) found an increase in CH_4-producing bacteria and protozoa, demonstrating that not all microalgae have CH_4-reducing properties.

The anti-methanogenic effect observed in this study has been reported in studies involving other microalgae (Spirulina platensis, *Chlorella vulgaris*, and Schizochytrium spp.). The studies attribute this effect to the presence of docosahexaenoic acid (C22:6 n − 3) and eicosapentaenoic acid (C20:5 n − 3), polyunsaturated acids that decrease the concentration of acetate and increase propionate, which results in reduction the abundance of methanogenic archaea, the primary microorganisms producing CH_4 (15, 51, 52). Likewise, Sheng et al. (57) found that humic compounds, including fulvic and humic acids, can lower CH_4 production in ruminants. They ascribed this to a decrease of the molar proportion of protozoa and acetate populations (58), which minimizes the amount of H_2 available for CH_4 production (59).

The addition of the microalgae reduced BG production in the current study, which is in line with Elghandour et al. (15), who observed that the BG decreased with the addition of the microalgae Schizochytrium spp. and associated it with the antimicrobial and cytotoxic effects of the compounds of the microalgae (60), as well as the long-chain fatty acid profile (48). Also, it is likely that the microalgae have modified the structure of the microbial community during fermentation which is caused variations in the final fermentation products, including the SCFA profile (61).

Among the treatments, the highest amount of gas produced was observed for the MgS nanoparticles group. Additionally, the MgO treatments demonstrated a notable decrease in the production of methane, which indicate the ability of MgO nanoparticles to meet the needs of rumen bacteria during the incubation period (62). The two main sources of *in vitro* gas generation are carbon dioxide and methane, which are derived directly from microbial fermentation, and carbon dioxide released from a bicarbonate buffer, obtained indirectly by buffering short-chain fatty acids. Menke and Steingass (23) affirm that the only variables influencing gas generation are the feed's physical and chemical composition. The fermentation rate, however, could be impacted by modifications in ruminal microbial activity.

VFA parameters

Volatile fatty acids (VFAs) have been considered one of the most significant factors in achieving anaerobic fermentation. According to Makkar (63), fluctuations in gas production might alter the amounts or ratios of VFA produced. VFAs' hydrophobic qualities enable them to penetrate the bilayer structure of the bacterial cell's plasma membrane (64). Therefore, by changing the membrane structure and increasing its flowability and permeability, they can lower the rate of bacterial growth (65).

Previous research has shown that adding red algae (*Asparagopsis taxiformis*) and lipid-extracted microalgae to forage diets dramatically boosted propionate and butyrate levels in the rumen (66). This is deemed advantageous since previous research demonstrated that the energy from propionate was used more efficiently than energy from acetate (67, 68). Lodge-Ivey et al. (68) found that adding lipid-extracted algae (Chlorella or Nannochloropsis) to the diet increased total rumen VFA content, which is in consistent with our findings. In contrast to our findings, it has been proposed that the high lipid content of Chlorella may suppress cellulolytic bacteria in the rumen and finally reduction of total VFA (53). Furthermore, adding algae to a corn silage-based diet raised ruminal pH and reduced total VFA by up to 18% after 19 days (69). Other *in vitro* investigations (53–55, 70) found that supplementation with DHA-rich microalgae or marine algae increased ruminal propionate while decreasing overall VFA and CH_4 synthesis. The differences could be attributed to variances in supplementation levels and oil extraction.

Conclusion

The results of our study indicate that the use of Algae+50 Mgo nano-particles, viable feed additive, highest in CP and EE, can reduce methane emission and gas production. Furthermore, all the treatments containing Algae decreased *in vitro* gas production. Also, addition of the Algae+50 Mgo nano-particles improved fermentation kinetics, VFAs, and nutrients' degradability compared to the other experimental treatments. These results are promising and suggest that the applied extracts could mitigate undesirable outcomes of rumen fermentation. Although more research is necessary to clarify the exact effects of the extracts on the aforementioned indices.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

VP: Conceptualization, Data curation, Project administration, Software, Writing – original draft, Writing – review & editing. AdK: Methodology, Data curation, Writing – original draft. MM: Supervision, Data curation, Writing – original draft. HN: Methodology, Data curation, Writing – original draft. HÜ: Methodology, Data curation, Writing – original draft. AlK: Methodology, Data curation, Writing – original draft. AF: Methodology, Data curation, Writing – original draft. AM: Methodology, Data curation, Writing – original draft. ML: Writing – review & editing, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chemical composition, *in vitro* gas production, and nutrient degradability of carob leaves as a sustainable feed for ruminants in Tunisia and Palestine

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Introduction: Carob leaves may be a potential roughage source for ruminants in arid areas. The nutritive value of this feedstuff may be considerably enhanced by the application of solid-phase chemical treatments. This study aimed to evaluate the nutritive value of carob leaves collected from Tunisia and Palestine untreated or treated with urea or sodium hydroxide (NaOH), or supplemented with polyethylene glycol (PEG) on chemical composition and *in vitro* ruminal fermentation.

Methods: Carob leaf samples were collected from either Palestine or Tunisia, and were used either untreated (control) or treated with urea, NaOH at 4% or PEG at 100 mg/g (dry matter (DM) basis), and analyzed for chemical composition. Carob leaves were incubated *in vitro* in diluted rumen fluid fermentation for 48 h, measuring fermentation gasses [methane (CH_4), and carbon dioxide (CO_2)], DM degradability and fermentation kinetics.

Results and discussion: Results showed a significant country × treatment interaction for most measured parameters, indicating that treatment effects are constrained by the origin of the leaves. Palestine untreated carob leaves had higher ($p < 0.001$) crude fat, crude protein (CP) and neutral detergent fiber (NDF), but less nonstructural carbohydrate (NSC), acid detergent fiber (ADF) and acid detergent lignin than Tunisia leaves. Tunisia carob leaves had higher concentration ($p < 0.01$) of flavonoids and tannins than leaves from Palestine. Of the three treatments tested, the addition of PEG increased ($p < 0.01$) the gas production during the incubation in diluted rumen fluid of carob leaves and this effect was greater with leaves from Palestine than with those from Tunisia. The other treatments had less noticeable effects, which were different when applied to the leaves from one or another country, given the significance of

the interaction country × treatment detected for most of the variables studied. PEG, NaOH and urea treatments of carob leaves can be applied to enhance the ruminal fermentation and energy value of this feedstuff. However, the effects of these treatments are highly dependent on the parent material, and seem to be more effective when applied to a low digestible material.

KEYWORDS

carob, degradability, *in vitro* rumen fermentation, methane, phytogenics

1 Introduction

The high cost of feed represents a significant constraint on global animal production. Recently, researchers have turned to alternative feed resources as a potential cost-effective source of animal fodder, aiming to reduce the production costs of animal products (1–3). There is an interest in bringing agroforestry into animal nutrition through the use of forestry resources in ruminant feed to gain competitive pricing compared to other products (1, 4). Among the promising plants for animal feed, the carob tree stands out. The carob tree (*Ceratonia siliqua*) is an agro-silvopastoral species of valuable socio-economic and ecological interest (5). It thrives in arid and semi-arid zones due to its adaptability to water constraints (6). The carob tree is gaining interest not only for the hardiness and quality of its wood, but also for most of its botanical parts, mainly for its fruits (pods and seeds) and leaves, which are utilized as animal feed (seeds also as a human food). Other parts of the tree are also exploited, such as the flower for carob honey production and the bark and roots for tanning due to their tannin content (2).

Carob fruits stand out for its high carbohydrate content (including primary soluble sugars like sucrose, fructose or glucose), dietary fiber, and polyphenols. It has lower levels of ash, lipids, and protein. Carob leaves, in particular, are highly appetizing and easily consumed by growing lambs (7). The *in vitro* gas production (GP) procedure is helpful for quickly screening feedstuffs and assessing their potential as energy sources for ruminants (8). Numerous studies have reported positive influences of various carob plant parts, such as seeds, pulps, and pods, on ruminants, yielding favorable results (9). Obeidat et al. (7) highlighted the positive effect of carob on milk yield, while digestibility was influenced by the proportion of carob pods in the diet. However, limited research has focused on carob leaves as potential ruminant feed. Additionally, many studies examining carob plants in ruminants have overlooked comparing the regional influence on carob effects in ruminants. Richane et al. (1) demonstrated that geographical location can significantly influence the chemical composition and bioactive components of feedstuffs. Furthermore, carob is noted for its tannin concentration, underscoring the need to investigate how different treatment strategies can effectively reduce tannin content in leaves before utilizing them to feed ruminants.

Several chemical treatments have been proposed to enhance the fermentation and degradability of agriculture byproducts for better valorization (10). Alkali and urea treatments pose no significant risk to animal health and can be used to improve the nutritional value of low-quality roughage (11, 12). Sodium hydroxide (NaOH) treatment breaks ester bonds between lignin and compounds such as acetic acid (C_2), phenolic acids, cellulose, and hemicellulose in the cell walls through saponification (12). This treatment also falls apart the plant cell wall making the polysaccharides (e.g., cellulose and hemicellulose) more accessible to hydrolytic enzymes for microbial digestion (11, 12). Urea treatment is another method for improving the nutritive value of low-quality forage. Urea treatment increases the nitrogen content of the forage and causes changes in the structure of the cell wall (13). Polyethylene glycol (PEG), a polyether, has a high ability to form stable complexes with tannins, thereby preventing the binding of tannins to proteins. It is widely used to mitigate the negative effects of condensed tannins in ruminant diets (12, 14). Brown and Ng'ambi (14) observed that supplementing *Acacia karroo* leaf meal with PEG at 23 or 30 g increased feed intake in goats without affecting the apparent digestibility of all nutrients or the final body weights of the goats. Recently, Zoabi et al. (12) demonstrated that NaOH treatment of almond hulls reduced crude protein (CP) and both structural and nonstructural carbohydrates (NSC), while urea treatment decreased the fiber fraction and increased CP content.

Consequently, the objectives of the present experiment were to evaluate the nutritive value of carob leaves from Tunisia and Palestine, both untreated and treated with urea or NaOH, or supplemented with PEG, focusing on *in vitro* GP, methane (CH_4) production, and *in vitro* ruminal fermentation. Our hypothesis was that the nutritive value of carob leaves would vary depending on the origin (country) and that treatment with NaOH or urea, or supplementation with PEG, would enhance the nutritive value of carob leaves as a ruminant feed.

2 Materials and methods

2.1 Sampling of carob leaves

Samples of leaves of *Ceratonia siliqua* L. (carob) were collected during September–October 2022 either (i) from a mountain area in Tunisia or (ii) from Jenin in Palestine. In Tunisia, samples were collected in the Parc National Djebel Zaghouan, a protected area of 1881 ha in the mountain of Zaghouan (latitude 36°24′10" N, longitude 10°8′35″ E and a peak altitude of 1,295 m above sea level) and covered mainly by Mediterranean forests and shrub lands. The average annual rainfall is 350 mm corresponding to a semi-arid Mediterranean climate. The soils are calcite characterized by a fragile structure. Sampling was stratified by altitude delimiting three separate areas at 250, 400 or 900 m altitude. In Palestine, samples were collected in the Um al-Tout Nature Reserve, a protected area of 36.3 ha in the southern part of Jenin, West Bank (latitude 32°27′33" N, longitude 35°18′03″ E and a peak altitude of 300 m above sea level). The average annual rainfall is 400–500 mm. Sampling was also stratified by altitude in three separate areas at around 90, 140 or 250 m altitude. At each country, leaves (without petioles) were harvested from four carob trees with scissors within each separate sampling area. Leaves were collected daily over the course of a week. Carob leaves collected from the trees of the same sampling area during the collection period (7 samples) were then mixed, and one composite sample was obtained per area, resulting in three independent samples (replicates, one per sampling area) per country. In the laboratory and after each collection, leaves were air-dried at room temperature (with

daily air temperatures ranging between 17 and 32°C during the day, similar in both countries at this season of the year) for 1 week and then stored in a dark room at room temperature. Then, three samples of carob leaves were collected from each country (one from each sample area) and all the samples from both countries were delivered to the National Research Centre (NRC) in Giza (Egypt) for further evaluation.

2.2 Treatments of carob leaves

In the NRC (Egypt), all received samples were ground using a blender mill (Grindomix GM 300, Normandie-Labo, Normandy, France) with a 1 mm sieve to achieve a uniform particle size. Air-dried carob leaves from Tunisia and Palestine were then treated with either urea or NaOH, while a portion remained untreated as a control. A 4% solution of urea or NaOH in water was applied at a rate of 1 L per kg of dried carob leaves. The treated material was packed into polythene bag silos and manually compressed to expel as much air as possible, minimizing the risk of fungal contamination. The treated biomass was stored in polyethylene bags for 40 days at room temperature (approximately 27°C). After the storage period, samples were oven-dried at 55°C for 48 h, ground, and stored in plastic bags for further analysis and *in vitro* fermentation. Proximate composition analysis and *in vitro* rumen fermentation were conducted at NRC (Giza, Egypt).

2.3 *In vitro* fermentation and biodegradation

The *in vitro* fermentation medium was prepared following the method of Goering and Van Soest (15). The detailed procedures for ruminal collection, the in vitro fermentation process, and gas collection for CH_4 and carbon dioxide (CO_2) production were previously described by Zoabi et al. (12), and Morsy et al. (16). Briefly, untreated or treated (with either urea or NaOH) leaves (three replicates per country and treatment) were evaluated in two incubation runs, with three bottles per replicate in each run. The untreated leaves were incubated either alone (control) or with the addition of PEG to the incubation medium at a rate of 100 mg PEG/g feed dry matter (DM). In each incubation run 2 blank bottles containing inoculum but no feed were included to establish baseline fermentation GP.

A sample of 1 g ± 10 mg carob leaves (untreated or treated with NaOH or urea) was weighed into filter bags (ANKOM F57; Ankom Technology, Macedon, NY, USA) and placed into 250 mL ANKOM bottles (Ankom^{RF} Gas Production System) equipped with an automatic wireless *in vitro* GP module (Ankom Technology, Macedon, NY, USA) with pressure sensors. Gas pressure was recorded every 10 min for 48 h, and the volume of gas produced by fermentation was calculated. After 48 h of incubation, CH_4 and CO_2 concentrations were measured in the gas contained in the bottle headspace using a Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK).

2.4 Sampling at the end of incubation and analysis of fermentation end-products

After 48 h of incubation, a sample of the liquid in the bottle was collected to measure pH, analyze total and individual short-chain fatty acid (SCFA) concentrations, and then all the contents were collected to determine the undigested residue to calculate degradability following the procedures previously described by Kholif et al. (17). Total gas and CH_4 production were expressed relative to degraded DM, neutral detergent fiber (NDF), and acid detergent fiber (ADF) after 48 h of incubation.

2.5 Chemical analysis

Samples of carob leaves (treated or untreated) were analyzed for the concentrations of proximate composition and of secondary metabolites as previously detailed by Kholif et al. (18). Methods described by AOAC (19) and Van Soest et al. (20) were used to determine the chemical composition. Plant extracts were obtained from finely ground dry carob leaves and used to determine plant secondary metabolites at the Ecochimie Laboratory, Department of Biological and Chemical Engineering, National Institute of Applied Sciences and Technology (INSAT), University of Carthage, Tunisia. The secondary metabolites analyzed were: total polyphenolics (Folin–Ciocalteu colorimetric method), antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical, condensed tannins [modified vanillin assay of Sun et al. (21)], total flavonoids [method of Dewanto et al. (22)], and anthocyanin [method of Gould et al. (23)].

2.6 Calculations and statistical analyses

The calculations of GP kinetic parameters including the asymptotic GP (A; mL/g DM); the fractional rate of GP (c; /h), and the discrete lag time (Lag; h) as well as the partitioning factor after 48 h of incubation (PF_{48}; mg degradable DM per mL gas), the metabolizable energy (ME) and microbial CP (MCP) production were previously described by Kholif et al. (24).

Data of chemical composition and fermentation were analyzed using the GLM procedure of SAS in a factorial experimental design with the model: $Y_{ijk} = \mu + T_i + R_j + (T \times R)_{ij} + \varepsilon_{ijk}$ where: Y_{ijk} is each individual observation, μ is the population mean, T_i is the treatment effect, R_j is the effect of country (Palestine or Tunisia), $(T \times R)_{ij}$ is the effect of treatment and country interaction, and ε_{ijk} is the residual error. The treatment effect had three levels (untreated, urea or NaOH) for chemical composition and four levels (untreated, urea, NaOH, PEG) for *in vitro* rumen fermentation studies (PEG was not used as a treatment for the leaves, but added to the medium when untreated leaves were incubated). The experimental unit was the composite sample of carob leaves collected from each sampling area in each country, resulting in three replicates per country.

3 Results

3.1 Chemical composition and secondary metabolites

Tables 1, 2 show chemical composition and secondary metabolite concentrations of carob leaves under different treatments. There was a significant ($p < 0.01$) country × treatment interaction for all chemical fractions (Table 1). The untreated carob leaves from Palestine had

significantly higher ($p < 0.001$) contents of EE, CP, and NDF compared to untreated carob leaves from Tunisia. Specifically, CP and EE in Palestinian untreated carob leaves were 1.89- and 2.79-fold higher than those in Tunisian untreated leaves, respectively. Conversely, untreated leaves from Tunisia showed higher ($p < 0.001$) OM, NSC, ADF, and acid detergent lignin (ADL) than Palestinian untreated leaves.

The CP content was lowest and ADL highest with NaOH treatment, and this trend was similar in leaves collected from both countries. In contrast, the treatment with urea increased by almost 2-fold the CP content of carob leaves from both countries. As for NDF, its content was reduced in leaves from Palestine treated with NaOH, but this effect was not observed with leaves from Tunisia, in which NaOH or urea treatment had no significant ($p > 0.05$) effects on NDF compared with the control. NaOH-treated leaves exhibit the highest ADF and ADL contents in leaves from both countries. For Tunisian carob leaves, untreated leaves had the highest ($p < 0.001$) values for EE, and NaOH-treated leaves the highest ($p < 0.006$) ADF and ADL.

Table 2 shows the secondary metabolite concentrations in carob leaves collected from both countries. No significant differences ($p > 0.05$) were observed in polyphenol and anthocyanin contents between carob leaves from Palestine and Tunisia. However, there were significant differences ($p < 0.005$) in flavonoids and tannins, with Tunisian carob leaves having higher values. Tannin content in Tunisian carob leaves was 2.5-fold higher ($p < 0.001$) than that in Palestinian leaves. Similarly, antioxidant activity [trolox equivalent antioxidant capacity (TEAC, Mmol/L and µmol/g)] and IC50 _DPPH (mg/mL) in carob leaves from Tunisia were higher ($p = 0.013$) than in those from Palestine.

3.2 Total fermentation gas, methane and carbon dioxide production

Figure 1 illustrates the *in vitro* ruminal GP (mL/g incubated DM) of carob leaves from Palestine or Tunisia untreated (Control) or treated with NaOH, or urea or supplemented with PEG for 48 h of incubation. The overall trend indicates a continuous increase in GP throughout the incubation time. The untreated, and urea and NaOH treated Palestine leaves produced less fermentation gas than Tunisian leaves at any incubation time. When carob leaves from Palestine were incubated with PEG, GP was increased compared with the untreated leaves. With leaves collected in Tunisia, the addition of PEG also increased GP compared with untreated leaves, but this effect was limited and non-significant.

Table 3 details the *in vitro* rumen GP kinetics of carob leaves from Palestine or Tunisia, untreated (control) or treated with PEG, NaOH, or urea. There were significant ($p < 0.05$) interactions between country and treatment for the asymptotic GP and total GP at 48 h of incubation. Gas production was higher when leaves from Tunisia were incubated in comparison with leaves from Palestine except for PEG treated leaves for which GP was similar regardless the origin. With leaves from Tunisia there were no treatment differences in *A* parameter, whereas total GP was increased with PEG addition compared with untreated leaves. With leaves from Palestine, both *A* parameter and total GP were increased with PEG compared with the other treatments. The fermentation rate (parameter *c*) was higher with NaOH than in untreated Palestinian leaves, whereas for Tunisian leaves the only significant difference was that rate was slower in urea-treated than in untreated leaves. The country by treatment interaction did not significantly ($p = 0.243$) affect the lag time.

Table 4 presents the CH_4 and CO_2 fermentation outputs of carob leaves from Palestine or Tunisia, either untreated (Control) or treated with PEG, NaOH, or urea after 48 h of incubation. Significant ($p < 0.05$) interactions between country and treatment were observed for CH_4 production and the proportions of CH_4 and CO_2 in total GP. The results indicate that there were not significant differences among treatments in CH_4 production (per g degradable substrate) or CH_4 concentration in total gas when carob leaves from Tunisia were incubated. However, with leaves from Palestine CH_4 was higher when PEG was added to untreated leaves. Overall, CO_2 concentration in fermentation gas from Tunisian carob leaves was not significantly affected by treatment ($p > 0.05$), whereas with leaves from Palestine treated with urea CO_2 percentage in total GP was higher than with the untreated leaves.

3.3 Degradability and fermentation

Table 5 provides insight into the *in vitro* rumen fermentation profile and degradability of carob leaves. Significant ($p < 0.05$) country

TABLE 1 Chemical composition of carob leaves untreated (Control) or treated with sodium hydroxide (NaOH), or urea (g/kg DM, unless otherwise stated).

Country	Palestine			Tunisia			SEM	*p* value		
Treatment	Control	NaOH	Urea	Control	NaOH	Urea		Country	Treatment	Country × Treatment
DM	930.2^{a}	927.8^{a}	931.1^{a}	827.0^{c}	902.9^{ab}	877.0^{b}	9.45	<0.001	0.006	0.005
OM	939.7^{b}	886.9^{e}	936.8^{c}	966.1^{a}	892.7^{d}	965.5^{a}	0.81	<0.001	<0.001	<0.001
EE	43.8^{c}	50.3^{a}	48.6^{b}	15.7^{d}	10.5^{f}	12.9^{e}	0.46	<0.001	0.125	<0.001
CP	98.4^{b}	77.2^{c}	171.7^{a}	52.2^{d}	45.8^{e}	102.6^{b}	1.67	<0.001	<0.001	<0.001
NSC	313.8^{f}	363.4^{d}	237.5^{e}	552.0^{a}	477.4^{c}	505.8^{b}	7.48	<0.001	<0.001	<0.001
NDF	483.8^{a}	396.0^{b}	479.1^{a}	346.2^{c}	359.0^{c}	344.1^{c}	4.95	<0.001	<0.001	<0.001
ADF	226.1^{e}	252.9^{b}	242.4^{cd}	237.4^{d}	282.5^{a}	248.7^{bc}	2.87	<0.001	<0.001	0.004
ADL	125.7^{e}	153.2^{c}	143.4^{d}	166.3^{b}	174.2^{a}	163.6^{b}	1.99	<0.001	<0.001	0.003

Means in the same row with uncommon letters are significantly ($p < 0.05$) different. ADF is acid detergent fiber; CP is crude protein; DM is dry matter; EE is ether extract; NDF is neutral detergent fiber; NSC is nonstructural carbohydrates; OM is organic matter; NSC is nonstructural carbohydrates; SEM is standard error of the mean. Multiple mean comparisons were presented only when the interaction was significant.

TABLE 2 Secondary metabolites of carob leaves.

Country	Polyphenol	Anthocyanin	Flavonoid	Tannins	Antioxidant activity[1]		IC_{50} _ DPPH
					TEAC, Mmol/L	TEAC, µmol/g	
Palestine	9.91	0.734	17.3	6,740	0.426	1.28	82.7
Tunisia	9.21	0.730	18.3	16,825	0.469	1.41	84.3
SEM	0.467	0.0150	0.12	88.6	0.0071	0.021	0.28
p value	0.348	0.863	0.004	<0.001	0.013	0.013	0.013

SEM, Standard error of the mean. IC_{50} _DPPH (mg/mL): the inhibitory concentration of the sample needed to inhibit 50% of the DPPH radicals.
Polyphenol contents in mg/g equivalent gallic acid (GAE)/g DM.
Anthocyanin contents: µg/mL.
Flavonoid contents in catechin equivalent per 100 g DM (CE/100 g).
Tannins contents: catechin equivalent/100 g (CE/100 g DM).
[1]Expressed as trolox equivalent antioxidant capacity (TEAC) in both Mmol/L and µmol/g.

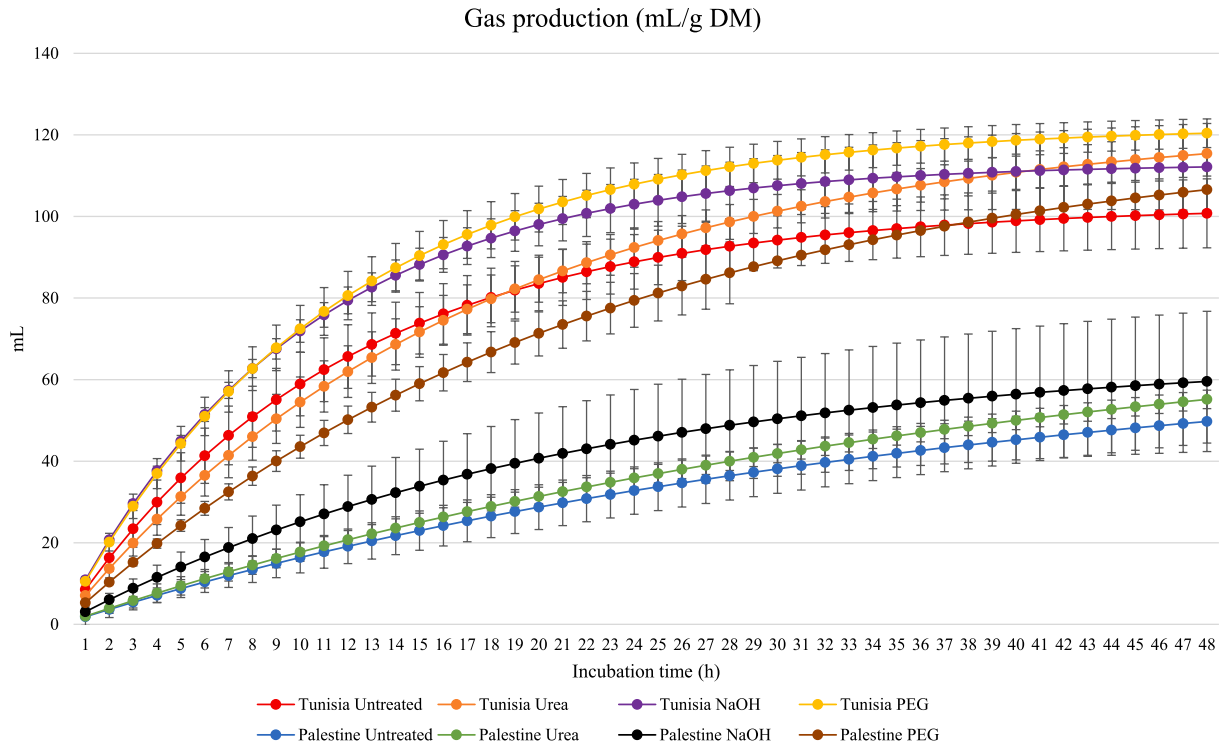

FIGURE 1
In vitro ruminal gas production (mL/g incubated DM) of carob leaves from Palestine or Tunisia untreated or treated with polyethylene glycol (PEG), sodium hydroxide (NaOH), or urea for 48 h of incubation. Error bars represent the variability between the three samples collected form each country and among incubation runs.

× treatment interactions were observed for DM degradation, C_2, C_3, C_2/C_3 ratio, pH, ME, and PF_{48}. DM degradability was lower in NaOH or urea treated- than in untreated leaves from Palestine, but no significant differences among treatments were observed with leaves from Tunisia. Fiber degradability was not significantly ($p > 0.05$) influenced by any treatment. SCFA increased with PEG addition when leaves from both countries were incubated. With leaves from Tunisia, urea treatment increased C_2 and C_2/C_3 ratio compared with the untreated leaves. In contrast, with leaves from Palestine, treatment of leaves with NaOH or the addition of PEG resulted in lower C_3 and in higher C_2 and C_2/C_3 ratio compared with the untreated or urea-treated leaves. Fermentation parameters showed that within each country untreated leaves showed the highest pH and the lowest ME. In Palestine-sourced leaves, PF_{48} was greatest for the untreated carob leaves, whereas no differences were observed among treatments in the Tunisian leaves.

4 Discussion

4.1 Chemical composition

The significant country × treatment interaction for nutrient concentrations indicates that the effect of the treatment was not the

TABLE 3 *In vitro* rumen gas production (GP) kinetics[1] of carob leaves from Palestine or Tunisia untreated (Control) or treated with polyethylene glycol (PEG), sodium hydroxide (NaOH), or urea after 48 h of incubation[2].

Country	Palestine				Tunisia				SEM	*p* value		
Treatment	Control	NaOH	PEG	Urea	Control	NaOH	PEG	Urea		Country	Treatment	Country × Treatment
A	70.0^{b}	66.5^{b}	121.0^{a}	78.1^{b}	103.0^{a}	113.1^{a}	122.1^{a}	124.0^{a}	7.26	<0.001	0.008	0.021
c	0.027	0.049	0.045	0.027	0.087	0.101	0.090	0.060	0.0065	<0.001	0.009	0.231
Lag	2.52	0.75	0.78	0.91	1.92	1.63	1.72	2.23	0.483	0.081	0.169	0.243
Total GP	49.7^{c}	59.6^{c}	106.6^{ab}	55.2^{c}	100.8^{b}	112.2^{ab}	120.4^{a}	115.4^{ab}	5.35	<0.001	<0.001	0.002

Means in the same row with uncommon superscripts are significantly ($p < 0.05$) different. *p*-value is the observed significance level of the *F*-test for treatment; SEM, standard error of the mean. Multiple mean comparisons were presented only when the interaction was significant.

[1]GP parameters: *A* is the asymptotic GP (mL/g DM), *c* is the rate of GP (/h), Lag is the initial delay before GP begin (h).

[2]Carob leaves untreated (Control treatment) or treated with sodium hydroxide (NaOH treatment) or urea (Urea treatment) or supplemented with polyethylene glycol (PEG treatment).

TABLE 4 Methane (CH_4) and carbon dioxide (CO_2) production[1] of carob leaves from Palestine or Tunisia untreated (Control) or treated with polyethylene glycol (PEG), sodium hydroxide (NaOH), or urea after 48 h of incubation[2].

Country	Palestine				Tunisia					*p* value		
Treatment	Control	NaOH	PEG	Urea	Control	NaOH	PEG	Urea	SEM	Country	Treatment	Country × Treatment
CH_4 mL/g DM	14.7^{e}	17.3^{de}	39.3^{a}	14.9^{e}	24.8^{cd}	32.9^{abc}	27.3^{bc}	34.4^{ab}	2.94	0.001	0.003	0.003
CH_4 mL/g degradable DM	27.8^{c}	37.1^{ab}	66.7^{a}	33.4^{c}	39.7^{ab}	50.5^{b}	42.1^{ab}	53.9^{ab}	5.13	0.163	0.009	0.002
CH_4 mL/g degradable NDF	29.4^{d}	38.4^{d}	71.3^{a}	34.7^{cd}	42.8^{bcd}	53.8^{abc}	46.2^{bcd}	58.5^{ab}	5.72	0.109	0.010	0.003
CH_4 mL/g degradable ADF	33.2^{c}	41.3^{bc}	88.2^{a}	32.9^{c}	45.8^{bc}	58.4^{b}	50.4^{bc}	63.2^{b}	6.68	0.256	0.003	0.006
CH_4% of total GP	29.3^{ab}	28.7^{b}	36.7^{a}	26.7^{b}	24.7^{b}	29.3^{ab}	22.7^{b}	30.0^{ab}	2.39	0.046	0.719	0.011
CO_2 mL/g DM	31.2	40.9	64.5	38.3	73.7	77.3	90.2	78.5	4.06	<0.001	<0.001	0.209
CO_2 mL/g degradable DM	58.2	88.4	109.8	85.5	117.8	120.2	139.1	123.0	8.43	<0.001	0.005	0.297
CO_2 mL/g degradable NDF	61.6	91.3	116.8	88.8	126.7	127.7	152.9	134.7	10.16	<0.001	0.009	0.466
CO_2 mL/g degradable ADF	69.1	97.5	144.7	84.4	135.7	138.8	166.9	144.7	10.58	<0.001	0.001	0.189
CO_2% of total GP	62.5^{bc}	68.9^{ab}	60.7^{c}	69.8^{a}	73.1^{a}	68.9^{ab}	74.9^{a}	67.9^{ab}	2.17	0.002	0.919	0.004

Means in the same row with uncommon letters are significantly ($p < 0.05$) different. *p*-value is the observed significance level of the *F*-test for treatment; SEM, standard error of the mean. Multiple mean comparisons were presented only when the interaction was significant.

[1]DM is dry matter, NDF is neutral detergent fiber, ADF is acid detergent fiber, GP is gas production.

[2]Carob leaves untreated (Control treatment) or treated with sodium hydroxide (NaOH treatment) or urea (Urea treatment) or supplemented with polyethylene glycol (PEG treatment).

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TABLE 5 *In vitro* rumen fermentation profile and degradability of carob leaves from Palestine or Tunisia untreated (Control) or treated with polyethylene glycol (PEG), sodium hydroxide (NaOH), or urea after 48 h of incubation[1].

Country	Palestine				Tunisia				SEM	*p* value		
Treatment	Control	NaOH	PEG	Urea	Control	NaOH	PEG	Urea	SEM	Country	Treatment	Country × Treatment
Degradability[2]												
DM	0.548^{b}	0.457^{c}	0.587^{ab}	0.457^{c}	0.625^{ab}	0.648^{a}	0.648^{a}	0.639^{a}	0.0251	<0.001	0.046	0.034
NDF	0.515	0.445	0.552	0.443	0.582	0.610	0.593	0.587	0.0254	<0.001	0.157	0.078
ADF	0.470	0.414	0.446	0.462	0.545	0.561	0.542	0.545	0.0267	<0.001	0.876	0.549
SCFA[3]												
Total	23.7	24.0	24.7	23.8	25.0	25.3	25.7	25.0	0.12	<0.001	<0.001	0.403
C_2	10.3^{e}	12.3^{bc}	12.1^{bcd}	10.6^{e}	11.7^{cd}	11.2^{de}	12.7^{b}	13.6^{a}	0.29	0.002	0.001	<0.001
C_3	9.71^{a}	7.99^{bc}	7.69^{c}	9.25^{abc}	9.49^{ab}	9.13^{abc}	9.68^{a}	8.45^{abc}	0.483	0.144	0.175	0.042
C_4	3.66	3.65	4.99	3.93	3.73	4.93	3.27	2.95	0.549	0.400	0.412	0.072
C_2/C_3	1.06^{b}	1.57^{a}	1.61^{a}	1.16^{b}	1.24^{b}	1.23^{b}	1.32^{ab}	1.61^{a}	0.094	1.000	0.022	0.002
Fermentation[4]												
pH	6.93^{a}	6.77^{bc}	6.50^{d}	6.77^{bc}	6.78^{b}	6.67^{c}	6.76^{c}	6.67^{bc}	0.034	0.343	<0.001	<0.001
ME	3.65^{d}	3.87^{cd}	4.92^{b}	4.14^{c}	4.92^{b}	5.26^{a}	5.40^{a}	5.29^{a}	0.107	<0.001	<0.001	0.003
PF_{48}	11.19^{a}	7.97^{bc}	5.53^{c}	8.63^{b}	6.22^{bc}	5.78^{c}	5.40^{c}	5.54^{c}	1.283	<0.001	0.004	0.023
MCP	475.9	358.0	412.6	378.0	429.5	421.4	411.9	435.8	25.83	0.326	0.137	0.150

Means in the same row with uncommon superscripts are significantly ($p < 0.05$) different. *p*-value is the observed significance level of the F-test for treatment; SEM, standard error of the mean. Multiple mean comparisons were presented only when the interaction was significant.

[1]Carob leaves untreated (Control treatment) or treated with sodium hydroxide (NaOH treatment) or urea (Urea treatment) or supplemented with polyethylene glycol (PEG treatment).

[2]DM is dry matter degradability (g/g incubated), NDF is neutral detergent fiber degradability (g/g incubated), ADF is acid detergent fiber degradability (g/g incubated).

[3]SCFA is short chain fatty acids (mmol/g DM), C_2 is acetate (mmol/g DM), C_3 is propionate (mmol/g DM), C_4 is butyrate (mmol/g DM).

[4]ME is metabolizable energy (MJ/kg DM), PF_{48} is the partitioning factor at 48 h of incubation (mg degradable DM/mL gas), MCP is microbial CP production (mg/g DM).

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same depending on the origin of the leaves, suggesting that regional factors such as climate, soil quality, forage composition, and management practices influence chemical composition and how this is affected by the chemical treatments. This significant interaction implies that the treatment does not have a uniform effect in leaves from different origins, requiring tailored recommendations. The statistical significance confirms that this variation is unlikely due to chance, highlighting the importance of considering regional differences when evaluating treatment effects on nutrient concentrations. As expected, the significant interaction of origin × treatment on chemical composition are also reflected on subsequent significant effects of this interaction on *in vitro* degradability and rumen fermentation. The differences between both countries (Palestine and Tunisia) in the chemical composition of untreated carob leaves can be attributed to various factors, including environmental parameters such as temperature, altitude, and rainfall, as well as considerations related to variety, cultivation practices, harvesting methods, storage conditions, and processing techniques (12). The results align closely with a study on carob pulp conducted by Richane et al. (1).

Notably, the CP contents observed in this study were considerably higher than those reported in a previous research, especially from Tunisia (1, 25). The elevated CP content in the urea-treated carob leaves can be attributed to the nitrogen supplied by the urea (12). Given that the CP of a feed substance is determined by its nitrogen (including the non-protein N), the increased nitrogen content must be due to the urea treatment. The variation in CP between carob leaves from Palestine and Tunisia may stem from genetic variety/cultivar or environmental multiple factors (12), such as genetic diversity, soil nutrient levels, water availability, etc. Another factor contributing to nutrient and plant metabolite variation could be the cultivars, as El Hajaji et al. (26) demonstrated variation in antioxidant and phenolic components among three varieties of carob tree leaves from Morocco. The leaf maturity (phenological) stage before harvesting in both countries could also play a role in the observed differences.

The main disparities in secondary metabolite composition are associated with environmental and natural factors, such as region, and variety (27, 28). Factors like the maturity stage, genetic diversities, or the cultivation environment could contribute to changes in the concentration of plant secondary metabolites. Phenolic compounds in carob leaves, known for their antioxidant properties in scavenging free radicals and preventing oxidative damage to cells, have been identified as effective antioxidants in plant foods, including fruits and vegetables (2). Utilizing this by-product as an energy supply for livestock may thus mitigate the risk of diseases related to oxidative stress (29). Polyphenolic compounds, including tannins, are recognized for forming complex linkages with metal ions and macromolecules such as proteins and polysaccharides (30). In the present study, polyphenol content and antioxidant capacity (IC_{50}) of carob leaves were consistent with the literature. The low IC_{50} value and the flavonoid content suggest robust antioxidant activity (31). Furthermore, these compounds are known to have beneficial effects on protein metabolism in ruminants, promoting reduced breakdown of dietary proteins in the rumen and enhancing by-pass protein and the uptake of amino acids in the small intestine (32).

4.2 Ruminal fermentation and degradability

The *in vitro* GP method is a reliable tool for animal feed assessment, as GP correlates well with MCP synthesis and *in vivo* and *in vitro* digestibility (33, 34). Pastorelli et al. (2) suggest a relationship between rumen fermentation of organic matter and GP, which aligns with the results of the present study. In this regard, it should be noted that greater GP indicates greater fermentative activity and nutrient degradation by rumen microorganisms. The range in chemical composition led to variability in rumen fermentation kinetics and degradability (35). The GP kinetics for carob leaves collected from Palestine or Tunisia revealed a rapid and significant degradation of carob leaves, which may be associated with their richness in carbohydrates easily bioavailable to the ruminal microbiota (36). The untreated carob leaves from Tunisia were fermented at a faster rate releasing more fermentation gas than those from Palestine, what may be attributed to the higher nonstructural carbohydrates (NSC) content that are more rapidly available to the rumen microbes, leading to increased GP from the substrate (37). The higher *in vitro* digestibility coefficients confirm that untreated carob leaves from Tunisia were fermented to a greater extent than those from Palestine, and the increased nutrient digestibility resulted in higher volatile fatty acid (SCFA) concentration in the medium after in vitro incubation. Carbohydrate fractions affect fermentation kinetics differently. Non fiber carbohydrates favor a more intense and faster fermentation, while on the contrary, structural carbohydrates are degraded to a lesser extent and at a slower rate, limiting the access of microorganisms to cell contents, reducing nutrient degradability (8). Gioxari et al. (38) reported that carob fatty acid composition contains proportions of C12: 0 and C14:0 which can have some antimicrobial activity that could have affected the fermentation from Palestine carob leaves. PEG seemed to be the most effective treatment among those tested to enhance ruminal fermentation of carob leaves, as GP increased when PEG was added to untreated leaves collected from both countries. PEG has been applied to animal diet by various methods such as spraying of tannin rich green leaves, treatment of harvested leaves, infusion into the rumen and drenching of the animal (39). Furthermore, PEG added to tannin containing plant samples increased *in vitro* digestibility of DM and CP (40). Priolo et al. (41) reported that carob pulp supplemented with PEG improved lamb performance, even similar to maize-based diet level by eliminating the effects of condensed tannin. The ability of PEG to bind tannin reduces the formation of protein-tannin complexes (42) thus preventing the anti-nutritional effects of tannins. Polyethylene glycol is a polymer containing many oxygen atoms capable of forming hydrogen bonds with hydroxyl groups of tannins (43). The extent of the effect on ruminal fermentation was greater when PEG was added to leaves from Palestine than to those from Tunisia, indicating that PEG was more effective with low degradable substrates. Both CO_2 and CH_4 are important enteric gasses from ruminants. They are generally reported to contribute to the greenhouse gasses (GHG) emissions from livestock. Therefore, studies are consciously looking for options to reduce their emission and perhaps shift emissions from CH_4 to CO_2 since CO_2 GHG warming potential is lower than CH_4. In this study, the CH_4 production was higher when total GP was higher, with subtle differences in CH_4 concentration in the fermentation gas. Some treatment effects on CH_4 to CO_2 *in vitro* were observed when leaved from Palestine were incubated, whereas there were no significant differences among treatments with the leaves from Tunisia. The only consistent effect was a higher CH4 production when PEG was added to Palestine-sourced carob leaves, confirming that PEG effects may become more noticeable with low degradable feedstuffs.

A decreased PF_{48} reflects a lower conversion of degraded substrate into microbial biomass (44). The addition of PEG to carob leaves from both countries increased the estimated ME content of these feedstuffs, suggesting that PEG may enhance energy utilization. Silanikove et al. (42) reported that PEG supports enhanced ME and CP availability to ruminants. With leaved from both countries, all the treatments applied increased the calculated ME concentration compared with the untreated leaves. The increase in energy value was greater with the carob leaves collected from Palestine, supporting that these treatments are more effective with less digestible roughages.

5 Conclusion

The current study revealed that the chemical composition and potential nutritional value of carob leaves were substantially influenced by geographical origin. Carob (*Ceratonia siliqua* L.) is widely used as a source of fiber for small ruminants in the Mediterranean region. Carob leaves have a potentially great nutritional value for ruminants. The results of this study suggest the possibility of using carob leaves in the diets of small ruminants, with the advantage that, being local natural resources, they are better adapted to the climate and agronomic conditions and limit the impact on the environment. PEG, NaOH and urea treatments of carob leaves can be applied to enhance the ruminal fermentation and energy value of this feedstuff. The effects of these treatments are highly dependent on the parent material, and seem to be more effective when applied to a low digestible material. Further research will be required to establish the most appropriate level of inclusion of this feedstuff in ruminants diets according to their production responses and, therefore, their economic impact.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Ethical review and approval for this study were exempted from IACUC (New Valley University, Egypt) because the experiment was conducted *in vitro*, and ruminal fluid was collected from local slaughterhouse facilities. All the experimental work was done in vitro, and no animal was involved further. Slaughtering of the animal was done by following animal welfare regulations, and was not exposed to pain, suffering, discomfort, or any animal abuse. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SG: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Project administration. HA: Validation, Writing – original draft, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Visualization, Writing – review & editing. HZ: Conceptualization, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. BA: Validation, Writing – original draft, Writing – review & editing. AEK: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing, Project administration. MA: Validation, Writing – original draft, Data curation, Software, Visualization. RB: Writing – review & editing, Formal analysis, Methodology, Writing – original draft. MH-M: Software, Validation, Visualization, Writing – original draft, Writing – review & editing. SL: Writing – original draft, Writing – review & editing, Investigation, Software, Visualization. MC: Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Phoenix dactylifera seed-derived biochar as a sustainable and environmentally feed supplement in camel: impacts gas production, methane emissions, nutrient degradability and fermentation parameters, performance predictions

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Introduction: Climate change poses a significant environmental challenge to all living organisms. Camels exhibit notable resilience to these changes. Concurrently, the date palm (*Phoenix dactylifera*), a widely cultivated plant in tropical and subtropical regions, generates substantial seed waste. Valorizing *Phoenix dactylifera* seed-derived biochar (PSB) to enhance feed supplements and mitigate environmental impacts presents a potentially sustainable and eco-friendly solution. This study investigated the potential of date palm seed-derived biochar as a sustainable feed additive for dromedary camels to reduce methane (CH_4) emissions and improve gas production, nutrient degradability, fermentation parameters, and performance predictions using *in vitro* models.

Methods: The PSB was synthesized and stored at 4°C until use. Ruminal fluids were collected from growing camels (24-36 months old) at the nutrition laboratory and subsequently incubated at 37°C. The basal diet was supplemented with PSB at 0, 1, 2, and 4%, and the resulting data were analyzed using polynomial analysis. Gas production, methane emissions, nutrient degradability, fermentation parameters, and performance predictions were assessed.

Results: At 6, 12, and 36 hours of incubation, all levels of PSB biochar supplementation resulted in a significant linear increase in gas production ($p < 0.05$). The inclusion of PSB significantly reduced CH_4 emissions in a quadratic manner ($p < 0.001$). The lowest reduction in CH_4 production was observed at the 1% and 2% PSB inclusion levels, with a greater reduction at the 4% level (quadratic effect; $p < 0.001$). A significant quadratic increase in TVFA production was observed with increasing PSB inclusion levels during the *in vitro* fermentation of camel diets

(quadratic effect; $p < 0.01$). Furthermore, pH values significantly decreased with biochar supplementation, exhibiting a linear trend with the lowest values at the 4% level, followed by 2% and 1% (linear effect; $p < 0.01$). Short-chain fatty acid (SCFA) production was improved by the addition of PSB compared to the control diet in camels (quadratic effect; $p < 0.01$). The inclusion of 1% or 2% PSB quadratically improved organic matter digestibility (%), metabolizable energy (DM), and net energy for lactation (NEL) in camels. Microbial crude protein (MCP) and purine derivatives (PD) were not significantly affected by PSB supplementation ($p > 0.05$).

Conclusion: In summary, the addition of PSB enhanced gas production, nutrient degradability, fermentation parameters, and performance predictions, while concurrently mitigating methane emissions *in vitro*. This study underscores the potential of utilizing PSB as a valuable feed supplement and a sustainable feed additive for dromedary camels in extensive production systems.

KEYWORDS

***Phoenix dactylifera* seed-derived biochar, gas production, methane emissions, degradability, fermentation parameters, predicted camel's performance**

1 Introduction

Developing cost-effective strategies to convert agricultural residues into valuable products is a global approach that directly addresses waste accumulation, a key contributor to environmental pollution. This aligns with the United Nations Sustainable Development Goals and offers considerable advantages across various sectors, particularly the livestock industry (1, 2). The date palm (*Phoenix dactylifera* L.), belonging to the Arecaceae family, is among the oldest cultivated fruit trees globally, a history in the Middle East and North Africa spanning over 5,000 years (1). Originating in the Persian Gulf region (3), these long-lived trees can exceed a century in lifespan. Major global date production is concentrated in countries such as Egypt, Saudi Arabia, Iraq, Algeria, and Iran (4). Consequently, date palm cultivation generates substantial residual biomass, particularly abundant in the Arabian region (1). This residual biomass is frequently disposed of through open-field burning, a practice that significantly contributes to environmental pollution in date-producing countries. Although some nations have incorporated this biomass into livestock feed, there remains a critical need to develop more sustainable and widely adopted applications for these currently underutilized resources (5, 6).

Given the escalating challenges of global climate change and rising temperatures, exacerbated by growing concerns regarding water scarcity, the date palm has emerged as a strategically significant crop. This is primarily due to its exceptional resilience to adverse climatic conditions and minimal water requirements (6). Furthermore, beyond its nutritious fruit, the date palm provides substantial environmental and economic advantages.

Global date palm cultivation, comprising over 120 million trees, produces substantial quantities of dates and significant secondary biomass, including midribs, fronds, stems, leaves, and coir (4). Over 84 million of these trees are concentrated in Egypt, Saudi Arabia, Algeria, Iraq, Iran, Morocco, and Tunisia (4). These plantations, which occupy approximately 3% of the world's cultivated land, generate an estimated 12 million metric tons of biomass waste annually (7). Despite this considerable volume, date palm seed residues remain largely underexploited, primarily due to a lack of cost-efficient processing methods (5, 6). More effective utilization of this biomass could not only enhance its economic value but also promote environmental sustainability by mitigating ecological burdens (5).

Several studies have investigated the effects of date palm seeds supplementation in the diets of both terrestrial (8–10), and aquatic (11) animals. The findings suggest potential benefits for animal health and productivity, while also addressing environmental concerns associated with date palm seed waste accumulation (4, 10). However, livestock systems are a significant contributor to global methane emissions, accounting for approximately 18% of the total and thereby exacerbating climate change. To mitigate this impact, various strategies have been introduced, including the incorporation of biochar into animal feed (12, 13).

Biochar, an economical soil enhancer with widespread agricultural applications, is typically synthesized via the thermochemical conversion of agricultural residuals byproducts (14). Biochar's effectiveness is largely determined by its characteristics, including its surface area, porosity, and the functional groups present on its surface (15). These properties are significantly influenced by the pyrolysis conditions and the original biomass feedstock. A recent area of investigation involves using biochar made from date palm seeds that has been magnetized with Fe_3O_4. This modified biochar shows promise for efficiently removing copper ions (Cu^{2+}) from contaminated water solutions (16). Biochar derived from date palm seeds are rich in minerals, carbon, and various fibers (17). Lignin, a prominent fiber in date palm seeds, makes up 21.2 to 24.06% of its composition (18). Research indicates that probiotic-inoculated biochar (at an inclusion rate of 50 g/kg dry matter) used as a dietary supplement for livestock can lead to several improvements. These include enhanced dry matter digestibility, increased microbial protein synthesis, and a higher milk fat concentration, all while maintaining total milk yield (19). Similarly, the inclusion of *Phoenix dactylifera* seed-derived biochar (PSB) in sheep diets has been linked to reduced gas emissions and improved growth performance (13).

Research indicates that biochar derived from waste materials can reduce methane production when added to cattle feed. For instance, A study by Leng et al. (20) reported a 22% reduction in methane production. Furthermore, Winders et al. (21) observed that supplementing cattle diets with 0.8% biochar led to a 9.5 and 18.4% reduction in enteric methane generation (g kg dry matter intake) during the growth and finishing stages, respectively. Similarly, a 0.5% biochar addition to an *in vitro* rumen experiment resulted in a 25% reduction in methane production (22). Adding date palm seed to sheep diets (up to 20%) improved the digestibility, milk yield, and composition in sheep (23) and other ruminant (24). Supplementing ruminant diets with

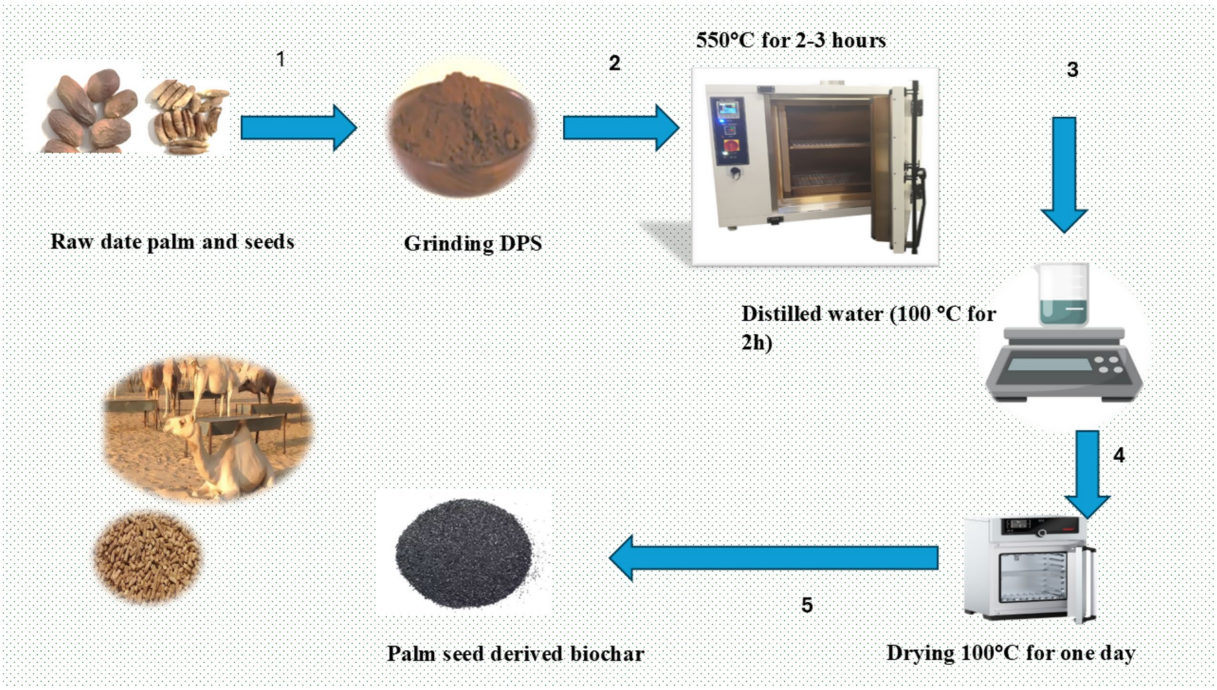

FIGURE 1
The steps of synthesized date palm seed-derived biochar.

biochar can beneficially alter rumen fermentation, leading to increased propionic acid and decreased methane emissions (25). Nevertheless, other studies suggest that non-inoculated biochar may not significantly impact milk yield, physiological indicators, or methane emissions in dairy cows (12). Given these inconsistent findings, the current *in vitro* experiment aims to assess the impact of a novel *Phoenix dactylifera* seed-derived biochar (PSB) on gas production, methane emission, degradability, fermentation dynamics, and predicted camel performance.

2 Materials and methods

2.1 Ethical statement

All experimental procedures and animal handling were reviewed and approved by the Animal Use in Research Committee (IACUC) at Zagazig University, Egypt, under approval number ZU-IACUC/2/F/25/2023. Throughout this experiment, all efforts were made to minimize animal suffering in accordance with the ARRIVE guidelines. The present study was carried out in the Laboratory of Animal Nutrition, Animal Production Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

2.2 Biochar synthesis

Date palm seeds, sourced from Linah Farm, Monufia Governorate, Egypt,[1] were chopped and used as feedstock for biochar production. Pyrolysis was performed at 550°C for 2–3 h under oxygen-depleted conditions to produce the biochar (Figure 1). After pyrolysis, the resulting biochar was ground and sieved to obtain a particle size range of 1–2 mm. The pyrolysis process involved two distinct steps, each employing a controlled heating rate of 10°C per minute (16). In the first step, the temperature gradually increased to 300°C and maintained for 1 h. Following this, the system was allowed to cool to room temperature over a 12-h period, facilitating a gradual transition to the subsequent phase. The second step involved raising the temperature to 600°C, which was sustained for 1 h to further enhance the biochar's characteristics. After this final holding period, the system was again cooled down to room temperature. The two-step pyrolysis process was selected over a single-step approach due to its enhanced control and efficiency in converting biomass to biochar, resulting in the formation of larger and more uniform pores (16). The resulting biochar was then ground and sieved to a particle size of 2 mm for uniformity. The processed biochar was stored in a dry environment until its use in this experiment (26). The yield was stored in a dry environment until used in this experiment.

2.3 Diet, treatment and chemical analysis

Four experimental diets were formulated, supplemented with *Phoenix dactylifera* seed-derived biochar (PSB) at levels of 0% (control), 1% (PSB1), 2% (PSB2), and 4% (PSB4). The basal substrate for the *in vitro* fermentation consisted of 30% berseem hay (*Trifolium alexandrinum*) and 70% concentrate mixture. The concentrate and berseem hay were finely ground (1 mm) and mixed at a ratio of 30:70, respectively. This dried substrate was used for both chemical analysis and *in vitro* gas production studies; the chemical

1 https://linahfarms.com/?srsltid=AfmBOorVRJBNBIV8wWvFmNe-c4BVDIK9ZdW12kzNs5_uSg_GvxS_T2te

composition of the substrate is detailed in Table 1. Biochar was added to the diet at 1, 2, and 4%, replacing an equivalent percentage of berseem hay. The substrate was analyzed for dry matter (DM), ash, organic matter (OM), ether extract (EE), and crude protein (CP) according to the methods of the Association of Official Analytical Chemists (27). Neutral detergent fiber (NDF) content was determined using the method described by Van Soest et al. (28). In brief, feed samples are ground to 0.1 mm and boiled in a neutral detergent solution containing sodium lauryl sulfate, EDTA, sodium borate, disodium hydrogen phosphate, 2-ethoxyethanol, heat-stable amylase, and sodium sulfite. This process solubilizes cell contents like sugars, starches, proteins, and lipids. The remaining insoluble residue, primarily hemicellulose, cellulose, and lignin, represents the NDF. This residue is then filtered, washed with hot water and acetone, dried, and weighed. The NDF content is calculated from the initial sample weight and the final residue weight.

2.4 *In vitro* incubations

Ruminal fluid was collected from a slaughtered camel at a slaughterhouse located in Zagazig, Sharkia Governorate, Egypt according to the method of Lutakome et al. (29). The rumen fluid was rapidly transported to the laboratory in a pre-warmed (39°C) insulated flask and maintained under anaerobic conditions until use.

TABLE 1 Formulation and chemical composition of the concentrate mixture, berseem hay, and basal diet.

Ingredients	Kg/Ton
Yellow corn	385
Soybean meal	154
Wheat barn	126
Common salt	10.5
Limestone	17.5
Sodium bicarbonate	3.5
Mineral and vitamin mixture[a]	3.5
Berseem hay	300

Chemical composition (%, on DM basis)	
Nutrient	basal diet[b]
Organic matter	94.81
Crude protein	15.96
Ether extract	2.98
Neutral detergent fiber	29.21
Acid detergent fiber	16.66
Ash	4.84
Crude fiber	12.70
Nitrogen free extract	63.18

[a]Minerals and vitamins mixture contained: Copper 30,000 mg, Iodine 800 mg, Selenium 300 mg, Iron 10,000 mg, MgO 80,000 mg, Zinc 100,000 mg, Cobalt 400 mg, Vit. A 10000000 IU, Vit. D_3 2,500,000 IU, Vit. E 35000 IU, and $CaCO_3$ to 3 Kg.
[b]The basal diet was a total mixed ration containing 50% Berseem hay (Trifolium alexandrinum) and 50% concentrate mixture. Biochar was added to the diet at 1, 2, and 4%, replacing an equivalent percentage of berseem hay.

Upon arrival, the rumen fluid was filtered through four layers of cheesecloth, then incubated in a water bath at 39°C and saturated with CO_2 until inoculation.

The buffered incubation medium (MB9) consisted of NaCl (2.8 g/L), $CaCl_2$ (0.1 g/L), $MgSO_4{\cdot}7H_2O$ (0.1 g/L), Na_2HPO_4 (6 g/L), and $KH_2PO_4{\cdot}H_2O$ (2 g/L). The pH of the MB9 medium was adjusted to 6.8, and anaerobic conditions were maintained by flushing with CO_2 for 30 min (30). The MB9 medium was mixed with the filtered rumen fluid at a 2:1 ratio (v/v). For incubation, glass tubes were used, each loaded with 200 mg of the experimental diet amended with date palm seed biochar (PSB) at various concentrations.

Each tube was injected with 30 mL of the mixed ruminal fluid, immediately sealed with a gas-release rubber stopper connected to a three-way valve and a calibrated plastic syringe for gas volume measurement. Gas production was recorded at 3, 6, 12, 24, 36, and 48 h of incubation. Blank tubes (without substrate) were included to correct for gas production from the inoculum. Each experimental run included four blank bottles and six replicate bottles for each treatment.

At the end of the incubation period, after the final gas volume was recorded, methane emission was estimated by NaOH (10 M) absorption according to Fievez et al. (31). Methane intensity was then calculated and expressed as mL CH_4/g TDDM, mL CH_4/g TDOM, and as a percentage of total gas produced.

2.5 Estimation of pH, ammonia-N, volatile fatty acids concentration, partitioning factor, and true nutrient degradation

At the end of the *in vitro* incubation, ruminal pH was measured immediately using a digital pH meter (model 6,010 N, Jenco Instruments Inc., San Diego, CA, USA). Following 48 h of incubation, 30 mL of neutral detergent solution was added to the contents of three replicate tubes per treatment, and the tubes were placed at 105°C for 3 h to determine truly degraded dry matter (TDDM). The residual dry matter weight was then estimated after filtering each sample through pre-weighed Gooch crucibles and drying at 105°C for 3 h (32). This value was subsequently used to estimate crude fiber degradability (CFD) according to (27). The contents of another three replicate tubes per treatment were used to determine the concentrations of ammonia-nitrogen (NH_3-N) and total volatile fatty acids (TVFA). TVFA concentration was determined using the steam distillation method as described by Warner (33). Briefly, to prepare for VFA concentration analysis by steam distillation, 10 mL of rumen fluid was combined with 2 mL of 25% (wt./vol) metaphosphoric acid and then frozen at −20°C to preserve the sample until analysis.

Ruminal NH_3-N concentration was measured using the method proposed by Conway (34). The partitioning factor (PF) was calculated as the ratio of organic matter (mg) degradability to gas production volume (mL at 24 h) (32).

2.6 Estimation of nutrients digestibility calculations

The equation of Menke and Steingass (35) was used to calculate The net energy of lactation (NEL, MJ/kg DM) and metabolizable energy (ME, MJ/kg DM).

$$ME\left(MJ/kg\ DM\right)=\left(0.157\times GP\right)+\left(0.0084\times CP\right)+\left(0.022\times EE\right)-\left(0.0081\times CA\right)+1.06.$$

$$NEL\left(MJ/kg\ DM\right)=\left(0.115\times GP\right)+\left(0.0054\times CP\right)+\left(0.014\times EE\right)-\left(0.0054\times CA\right)-0.36.$$

Where,

GP = net gas production (mL/0.2 g DM) at 24 h of incubation; EE = ether extract; CP = crude protein; CA = crude ash.

Short-chain fatty acid concentrations (SCFA) were calculated according to Getachew et al. (36) as:

$$SCFA\left(mmoL/200mg\ DM\right)=\left(0.0222\times GP\right)-0.00425.$$

Where GP is the 24-h net gas production (ml/200 mg DM).

Microbial CP biomass production was estimated, according to Blümmel et al. (32) as follows:

$$MCP\left(mg/g\ DM\right)=mg\ DMD-\left(ml\ gas\times 2.2mg/mL\right).$$

Where: 2.2 mg/mL is a stoichiometric factor that expresses mg of C, H, and O required to produce SCFA gas associated with production of 1 mL of gas.

Menke et al. (37) equation was used to calculate the *in vitro* organic matter digestibility (OMD %) as OMD (%) = 14.88 + (0.889 × GP) + (0.45 × CP) + (0.0651 × XA),

Where XA = Ash (%).

2.7 Data analysis

Shapiro–Wilk and Levene's tests were used to confirm data normality and homogeneity of variance. Statistical analyses were conducted with SPSS 25.0 (SPSS Inc., Chicago, IL, USA) using a mixed-effects model (PROC MIXED). To assess the dose–response relationships (linear and quadratic) of biochar (0, 1, 2, and 4% g/kg diet) on each dependent variable, orthogonal contrasts were applied. Statistical significance was set at $p < 0.05$, and Duncan's multiple range test was used for post-hoc comparisons.

3 Results

3.1 Effects of biochar derived from date palm seeds on gas production

At the 3-h time point, the inclusion of *phoenix dactylifera* seed-derived biochar (PSB) in the *in vitro* fermentation of camel diets resulted in a significant linear increase in gas production ($p < 0.001$, Table 2). At 6, 12, and 36 h, all levels of PSB biochar supplementation led to a significant linear increase in gas production ($p < 0.05$). At 24 h, while the 1 and 2% PSB biochar inclusion levels exhibited higher gas production with a quadratic effect, the 4% level did not differ significantly from the other groups ($p > 0.05$). After 48 h, PSB biochar supplementation significantly increased gas production, particularly at the 2 and 4% inclusion levels, whereas the 1% level showed similar gas production to the control group ($p > 0.05$).

3.2 Effects of biochar derived from date palm seeds on methane emissions

The inclusion of PSB significantly reduced methane (CH_4) emissions in a quadratic manner ($p < 0.001$, Table 3). The lowest reduction in CH_4 production (mL/g DMD or mL/g OMD) in response to date palm seed-derived biochar was observed at the 1 and 2% inclusion levels, followed by a greater reduction at the 4% level (quadratic effect; $p < 0.001$). No statistically significant difference was found between the 2 and 4% inclusion levels for CH_4 production expressed per gram of OMD or as a percentage of total gas produced ($p > 0.05$). Furthermore, the incorporation of PSB-derived biochar into camel diets reduced the CH_4 proportion of total gas production (GP) in a quadratic manner ($p < 0.001$).

3.3 Effects of biochar derived from date palm seeds on degradability and fermentation parameters

The effects of date palm seed-derived biochar inclusion on nutrient degradability are presented in Table 4. The incorporation of various levels of PSB-derived biochar did not significantly affect dry matter degradability (DMD; $p = 0.522$, Table 4) or crude fiber

TABLE 2 Dose–response effects of date palm seed biochar on *in vitro* gas production in camel rumen fluid.

Item	Biochar supplementation				SEM	*p*-value		
	Control	Biochar 1%	Biochar 2%	Biochar 4%		*ANOVA*	*Lin.*	*Quad.*
Gas production, mL/g DM								
3 h	21.67^{d}	26.25^{c}	36.67^{a}	35.00^{ab}	1.924	0.007	0.001	0.320
6 h	47.92^{b}	57.92^{a}	60.42^{a}	57.92^{a}	1.728	0.039	0.026	0.052
12 h	76.67^{b}	85.83^{a}	85.42^{a}	85.00^{a}	1.391	0.045	0.037	0.065
24 h	110.00^{b}	122.50^{a}	124.58^{a}	117.92^{ab}	1.867	0.017	0.079	0.006
36 h	136.25^{b}	148.33^{a}	152.08^{a}	152.08^{a}	2.124	0.014	0.004	0.101
48 h	149.17^{b}	160.42^{ab}	166.67^{a}	162.92^{a}	2.262	0.027	0.013	0.067

Polynomial contrasts to determine the p-values for the ANOVA (ANOVA) and the linear (Lin.) and quadratic (Quad.) effects of different biochar addition levels (0, 1, 2, and 4%) were used in this study. Means within the same row with different letters (^{a,b}) are significantly different ($p < 0.05$). The pooled standard error of means (SEM).

TABLE 3 Dose–response effects of date palm seed biochar on *in vitro* methane emission in camel rumen fluid.

Item	Biochar supplementation				SEM	*p* values		
	Control	Biochar 1%	Biochar 2%	Biochar 4%		*ANOVA*	*Lin.*	*Quad.*
CH_4 mL/ 1 g	53.33^{a}	32.08^{c}	36.00b^{c}	40.83^{b}	1.971	<0.0001	0.003	<0.0001
CH_4 mL / g DMD	84.89^{a}	50.46^{c}	56.77^{bc}	64.91^{b}	3.172	<0.0001	0.003	<0.0001
CH_4 mL / g OMD	127.12^{a}	72.56^{c}	80.91^{bc}	94.12^{b}	4.866	<0.0001	<0.0001	<0.0001
CH_4, % of GP	35.74^{a}	19.98^{c}	21.58^{bc}	25.08^{b}	1.399	<0.0001	<0.0001	<0.0001

Polynomial contrasts to determine the p-values for the ANOVA (ANOVA) and the linear (Lin.) and quadratic (Quad.) effects of different biochar addition levels (0, 1, 2, and 4%) were used in this study. Means within the same row with different letters ($^{a, b, c}$) are significantly different ($p < 0.05$). The pooled standard error of means (SEM). Total dry matter degradability (TDDM).

TABLE 4 Dose–response effects of date palm seed biochar on *in vitro* degradability and fermentation parameters in camel rumen fluid.

Item	Biochar supplementation				SEM	P value		
	Control	Biochar 1%	Biochar 2%	Biochar 4%		*ANOVA*	*Lin.*	*Quad.*
Degradability								
DMD	62.83	63.58	63.41	62.91	0.410	0.92	0.99	0.522
CFD	21.30	24.86	24.23	28.69	1.265	0.24	0.07	0.851
Fermentation parameter								
Ammonia (mg/100 mL)	12.29^{b}	12.39^{b}	10.08^{c}	13.04^{a}	0.483	0.013	0.99	0.117
TVFA (mL/L)	174.33^{b}	206.00^{a}	205.33^{a}	201.67^{a}	4.422	<0.001	<0.001	0.005
pH	6.31^{a}	6.06^{ab}	6.01^{ab}	5.90^{b}	0.055	0.055	0.010	0.486

Polynomial contrasts to determine the p-values for the ANOVA (ANOVA) and the linear (Lin.) and quadratic (Quad.) effects of different biochar addition levels (0, 1, 2, and 4%) were used in this study. Means within the same row with different letters ($^{a, b}$) are significantly different ($p < 0.05$). The pooled standard error of means (SEM). Total volatile fatty acids (TVFA), dry matter degradability (DMD), and organic matter degradability (OMD).

degradability (CFD; $p = 0.07$) in the *in vitro* fermentation of camel diets. In contrast, ammonia-nitrogen (NH_3-N) concentrations were influenced by biochar supplementation, with the highest values observed at the 4% inclusion level, while the lowest values were noted at the 2% level. No significant differences in NH_3-N concentrations were detected between the 1% biochar level and the control diet; however, the 4% level exhibited significantly higher NH_3-N concentrations ($p < 0.05$). Notably, a significant quadratic increase in total volatile fatty acid (TVFA) production was observed in response to increasing inclusion levels of PSB-derived biochar in the *in vitro* fermentation of camel diets (quadratic effect; $p < 0.01$). Furthermore, the pH values significantly decreased with biochar supplementation, showing a linear trend with the lowest values at the 4% level, followed by 2 and 1% (linear effect; $p < 0.01$).

3.4 Effects on predictive value

Short-chain fatty acid (SCFA) production was improved by the addition of PSB-derived biochar compared to the control diet in camels (quadratic effect; $p < 0.01$) (see Table 5). The inclusion of 1% or 2% PSB-derived biochar quadratically improved organic matter digestibility (OMD, %), metabolizable energy (ME, MJ/kg DM), and net energy for lactation (NEL, MJ/kg DM) in camels. In contrast, the high inclusion level (4%) did not result in significant differences in OMD (%), ME (MJ/kg DM), and NEL (MJ/kg DM) in camels compared to the other treatments ($p > 0.05$). Microbial crude protein (MCP, mg/g DM) and purine derivatives (PF, mg TDOM/mL gas) were not significantly affected by the addition of PSB-derived biochar to camel diets ($p > 0.05$).

4 Discussion

Climate change poses a significant environmental threat to all living things. In this context, camels demonstrate a degree of resilience. Interestingly, the extensive cultivation of date palms (*Phoenix dactylifera*) across tropical and subtropical regions produces a substantial amount of seed waste. A promising sustainable and eco-friendly strategy involves transforming this waste into biochar from PSB (38). This PSB can then be used to enhance animal feed supplements, simultaneously reducing ecological impact. For some time, various strategies have been explored to mitigate the effects of environmental stressors on livestock, including the use of phytochemicals, organic acids, and probiotics. Coinciding with these efforts, date palm cultivation has continued to generate substantial byproducts, particularly date palm seeds. These seeds have already shown success when incorporated into livestock feed, maintaining animal performance and productivity. Transforming the date palm seed waste into biochar is a game-changer (38). It tackles a major waste problem head-on and offers a powerful one-two punch: it helps make livestock, like camels, more resilient by boosting their feed, and it contributes to a much more sustainable agricultural system overall.

This *in vitro* study explored the use of date palm seed-derived biochar (PSB) as a feed additive for dromedary camels. Researchers found that PSB supplementation increased gas production and improved nutrient digestion and fermentation parameters. Notably, PSB quadratically reduced methane emissions, with the highest reduction at a 4% inclusion level. Supplementation with PSB led to increased TVFA and SCFA production, alongside a reduction in ruminal pH. Optimal enhancements in feed digestibility and energy

TABLE 5 Dose–response effects of date palm seed biochar on *in vitro* predictive value in camel rumen fluid.

Item	Biochar supplementation				SEM	P values		
	Control	Biochar 1%	Biochar 2%	Biochar 4%		*ANOVA*	*Lin.*	*Quad.*
SCFA (mmol)	0.48^{b}	0.54^{a}	0.55^{a}	0.52^{a}	0.008	0.016	0.064	0.006
ME (MJ/Kg DM)	4.67^{b}	5.07^{a}	5.13^{a}	4.92^{ab}	0.059	0.017	0.081	0.006
NE_L (MJ/Kg DM)	2.27^{b}	2.56^{a}	2.61^{a}	2.45^{ab}	0.043	0.017	0.081	0.006
OMD (%)	41.94^{b}	44.16^{a}	44.53^{a}	43.34^{ab}	0.332	0.018	0.080	0.006
MCP (mg/g DM)	581.36	583.04	579.81	576.31	4.672	0.97	0.71	0.816
PF (mg/TDOM/ mL gas)	1.95	1.82	1.80	1.82	0.027	0.17	0.09	0.142

Polynomial contrasts to determine the p-values for the ANOVA (ANOVA) and the linear (Lin.) and quadratic (Quad.) effects of different biochar addition levels (0, 1, 2, and 4%) were used in this study. Means within the same row with different letters (a, b) are significantly different ($p < 0.05$). The pooled standard error of means (SEM). Short-chain fatty acids (SCFA), metabolizable energy (ME), net energy lactation (NEL), microbial crude protein production (MCP), organic matter degradability (OMD), partitioning factor (PF) at 72 h of incubation.

values were evident at 1 and 2% PSB inclusion levels. While MCP and PD values were not significantly altered, these findings suggest that PSB holds potential as a sustainable feed additive for camels. Its application could contribute to improved animal productivity and a reduction in their environmental footprint, primarily through attenuated methane emissions.

Biochar consists of many minerals, such as potassium (K), phosphorus (P), sulfur (S), and calcium (Ca) (39). With the global population continuously expanding and anticipated to reach around 10 billion by 2,100, ensuring future food and water security remains a formidable challenge. Overcoming this challenge requires the promotion of sustainable agriculture and the intensification and expansion of both livestock and crop production to meet the growing demand for food (40, 41).

Dromedary camels are well-adapted livestock for harsh environments, capable of producing milk and meat and reproducing efficiently. Moreover, camels are considered non-conventional ruminants with distinctive features in their digestive physiology, particularly in the composition and activity of the rumen microbiome, making them of ecological and economic importance in arid and semi-arid regions (42). Addition, camels are playing an important role in utilizing low-quality forages efficiently due to their unique rumen microbial composition (43). The present study's findings demonstrate that the inclusion of biochar derived from PSB in camel diets led to substantial improvements in gas production dynamics, a pronounced decrease in methane emissions, and advancements in fermentation parameters and nutrient utilization efficiency. Our findings align with previous research suggesting that biochar plays a dual role in reducing methane emissions. Firstly, biochar provides a habitat that promotes the growth of methanotrophs (15, 20). These crucial microbes oxidize methane within the gut, directly leading to a reduction in released methane. Secondly, biochar's inherent ability to adsorb and absorb gases (15) is another significant factor in lowering enteric methane production.

The data showed that the addition of PSB to growing camel diets significantly increased gas production at most points of time. A linear increase ($p < 0.05$) was observed at 3, 6, 12, and 36 h of incubation, while at 24 h, a quadratic response ($p > 0.05$) showed at 1 and 2% levels with no significant difference at 4%. At 48 h, gas production significantly ($p > 0.05$) increased at 2 and 4% levels, but 1% showed no difference from the control. Date palm seed (PSB) biochar supplementation significantly enhanced gas production in growing camel diets over time, especially at 2 and 4% inclusion levels, this delayed increase in gas production can be attributed to the porous structure and high adsorption capacity of biochar, which contribute to the stabilization of microbial communities and the enhancement of enzymatic digestion (44). Similar patterns have been previously reported in cattle and sheep fed various types of biochar (21, 45).

Our study found that adding palm seed-derived biochar to the diets of growing camels significantly reduces methane emissions. Specifically, methane emissions were reduced by 39.85, 32.50, and 23.44% when biochar was supplemented at 1, 2, and 3% levels, respectively. The most substantial reduction occurred with just 1% biochar inclusion. This aligns with previous research suggesting that biochar works by altering microbial hydrogen pathways, thereby redirecting electrons away from methanogenesis (44, 46). Addition, camel rumen fluid harbors a uniquely structured microbial population, characterized by a higher abundance of highly efficient methanogens; the inhibitory effect of biochar on these communities may be more pronounced in camels than in cattle or sheep (47). These findings are consistent with previous studies involving biochar supplementation in sheep diets, which reported methane reductions between 65.58 and 78.39% (13). Also, incorporating biochar into dairy manure has methane reductions close to 58% (48), while other investigator fed sheep inoculated biochar registered a 9% reduction in methane emissions than controls (41).

Meta-analyses and controlled studies show that biochar can reduce methane emissions by an average of 21% in ruminants, but results vary widely depending on the type, dose, and delivery method of biochar used (49–51). Some controlled pen trials in cattle have shown modest reductions in methane emissions (8.8–12.9%) without negative effects on feed intake or fermentation, but these effects were not observed in grazing conditions (50).

Beyond simple pyrolysis, treating biochar with mineral salts or weak acids significantly boosts its properties. This process, called chemical activation, increases the biochar's pore size and surface area. It also adds various functional groups, like organic acids and phosphate groups, which in turn enhance the biochar's adsorption capabilities and chemical characteristics (15, 52). Additionally, acidic biochars have been found to enhance interspecies hydrogen transfer among microbial populations. This is especially advantageous in environments like the rumen, as it can significantly boost microbial activity and fermentation (53, 54). The facts support biochar's argument of lowering methane emissions; however, confirming this with other studies remains inconsistent. Some author's reported that no conclusions from the use of pine derived biochar and its effects on

methane emissions and milk production of cattle (8, 55, 56). In contrast, another study showed a 40% reduction of methane emissions with the addition of 0.6% of biochar added to the diet of cattle (20). A study determined that date seeds are successful in producing porous biochar due to their properties, such as low ash (1.14%), high volatile matter (65%), and high bulk density (0.5 g/mL) (17). It's a high-value material that can be used as a soil amendment and for energy generation, which helps mitigate climate change (57).

Although specific studies on date palm seed biochar are sparse, one study noted that it did improve growth, nutrient digestibility, and health in sheep, which was observed (13). In contrast, other studies highlight the need for more extensive *in vivo* studies. For instance, Winders et al. did not observe a reduction in methane yield for steers on finishing diets (21). Leng et al. illustrated a 24% reduction in methane production in Laos yellow cattle on biochar supplemented diets at 0.6% and a 40% when bound with potassium nitrate at 6% (20).

Biochar supplementation had varying effects on rumen fermentation parameters. Date palm seed-derived biochar had no significant effect on DMD ($p = 0.522$) or ($p = 0.07$) CFD degradability, but it significantly influenced fermentation parameters, including increased NH_3-N ($p < 0.05$) and TVFA ($p < 0.01$) levels, particularly at higher ($p < 0.01$) inclusion rates (4% level). Additionally, dietary biochar led to a significant linear decrease in ruminal pH, with the lowest values observed at the 4% level. Biochar can act as an electron shuttle in redox reactions within the rumen. This can influence the metabolic pathways of certain microbial populations, potentially diverting hydrogen away from methanogenesis (methane production) towards other pathways, such as propionate production (58). Propionate is a more energetically efficient volatile fatty acid (VFA) for the host animal. The highly porous nature of biochar provides extensive surface area for microbial colonization and adsorption (52). As previously mentioned, biochar contains lignin, a hydrophobic, amorphous polymer with a very high molecular weight. Lignin's structure includes an aromatic substructure and various functional groups (59).

Some theories suggest biochar might directly capture or bind gases like methane and CO2 to some extent; however, this needs further confirmation. Recent findings indicate that dietary biochar in ruminant diets has shown variable effects, with some *in vivo* studies reporting no significant impact on animal performance, rumen fermentation, or methane emissions in lactating Holstein dairy cows (12). These inconsistencies are likely due to differences in biochar characteristics (such as dosage, source material, and composition), the type of basal diet, and the physiological status of the animals studied. However, in the current study, the inclusion of PSB-derived biochar in camels diet at 1, 2, and 4% levels significantly ($p < 0.01$) increased concentrations of short-chain fatty acids (SCFAs), metabolizable energy (ME), net energy for lactation (NEL), and organic matter degradability (OMD), while microbial crude protein (MCP, mg/g DM) and purine derivatives (PF, mg TDOM/mL gas) were not significantly affected by the addition of PSB to camel diets ($p > 0.05$). These improvements, particularly at the 1 and 2% inclusion levels, suggest that PSB in camels' diet can enhance animal productivity. The observed increase in SCFAs during *in vitro* fermentation reflects a more efficient rumen fermentation process, as SCFAs are key energy substrates that support optimal growth, productive performance, and reproductive efficiency in ruminants (60). Moreover, the observed increase in total volatile fatty acids and short-chain fatty acids suggests enhanced fermentative activity, likely driven by improved microbial stabilization and nutrient utilization facilitated by biochar's porous structure and surface chemistry (61).

The relationship between diet and SCFAs plays a crucial role in maintaining a healthy gut microbiota. SCFAs support microbial diversity and intestinal barrier integrity in healthy animals, while also enhancing gut resilience under acidic conditions (62). Supplementation of date palm seed-derived biochar in sheep diets has been shown to significantly improve growth rates, likely due to enhanced nutrient digestibility and improved rumen fermentation dynamics (13). These benefits may result from biochar's ability to influence the passage of digesta through the gastrointestinal tract, thereby promoting more efficient digestion and potentially suppressing harmful bacterial populations (63). Bacteroidetes play a crucial role in methane generation by producing ample VFAs, thus aiding in anaerobic hydrolysis (64). Furthermore, the addition of biochar was observed to stimulate denitrification (the conversion of nitrate to dinitrogen) (65). This was evidenced by the presence of terminal electron acceptors and facilitate nitrate reduction.

Despite these promising findings, biochar supplementation did not significantly affect daily dry matter intake, milk yield, or feed conversion ratio (FCR) in sheep (19). Nonetheless, the current *in vitro* study provides valuable insights into the potential of date palm seed-derived biochar in camel diets as a sustainable feed additive for reducing methane emissions while enhancing nutrient utilization and animal performance. However, certain limitations must be acknowledged. Primarily, since the study focused only on camels, the generalizability of these results to other ruminant species may be limited. A limitation of this study was the need for further research on biochar inclusion in other animals to confirm its beneficial effects on the rumen ecosystem. Future *in vivo* studies involving multiple species are warranted to elucidate the underlying mechanisms of biochar's effects and to establish optimal inclusion rates for effective methane mitigation, ultimately contributing to more climate-resilient livestock systems.

5 Conclusion

In the era of climate change, camels are gaining significant attention as a promising livestock species due to their remarkable adaptability to harsh environmental conditions. As camel farming expands, it's crucial to find ways to sustain production while reducing greenhouse gas emissions from these animals in extensive systems. The results of this *in vitro* study found that PSB significantly increased total gas production and short-chain fatty acid concentrations, with the most notable improvements at 2% PSB inclusion. Importantly, methane emissions were markedly reduced, with the most substantial decrease (approximately 40%) observed at 1% PSB inclusion. Fermentation profiles were improved, as indicated by elevated volatile fatty acid levels and moderate shifts in rumen pH and ammonia concentrations. Digestibility and energy utilization metrics (ME and NEL) were also enhanced, without negative effects on microbial protein synthesis. These findings highlight PSB's potential as an eco-friendly strategy to mitigate GHG emissions from camels while simultaneously boosting rumen fermentation and

feed efficiency. Further research is needed to clarify the mode of action of biochar in *in vivo* experiments, specifically examining its effects on various physiological pathways and other reproductive and productive traits.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by animal use in Research Committee (IACUC) at Zagazig University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HG: Conceptualization, Writing – original draft, Writing – review & editing. NA: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. AS: Data curation, Methodology, Writing – original draft, Writing – review & editing. ME: Data curation, Methodology, Writing – original draft, Writing – review & editing. RM: Data curation, Methodology, Writing – original draft, Writing – review & editing. HA-D: Methodology, Writing – original draft, Writing – review & editing. EE-H: Methodology, Supervision, Writing – original draft, Writing – review & editing. MA-E: Investigation, Project administration, Validation, Writing – original draft, Writing – review & editing. SA: Investigation, Project administration, Validation, Writing – original draft, Writing – review & editing. ASS: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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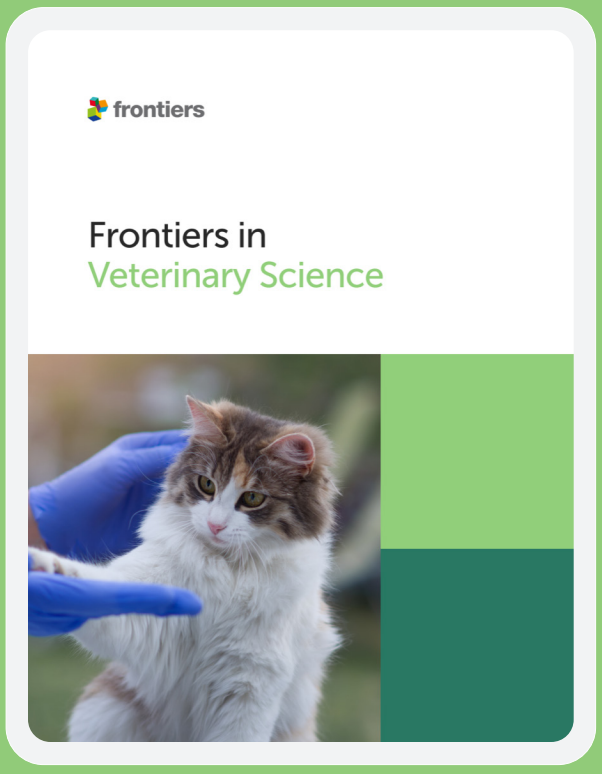

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