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Effects of fresh olive cake inclusion in ensiled total mixed rations on *in vitro* rumen fermentation kinetics and methane production

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Abstract: This study evaluated the effects of replacing maize silage with increasing levels of olive cake (OC) in ensiled total mixed rations (ETMR) on microbial quality, *in vitro* rumen fermentation kinetics, methane production, and estimated energy parameters. Three experimental diets were formulated to contain 0% (Control), 10% (OC10), and 20% (OC20) olive cake on a dry matter basis and were ensiled for 60 days. On day 60, ETMRs were opened, microbial composition was assessed, and *in vitro* ruminal fermentation was conducted for 24 h using the gas production technique. No significant differences were observed among treatments in bacterial counts ($P > 0.05$), and coliforms and enterobacteria were below detectable limits in all groups. Increasing OC inclusion showed a trend toward reducing butyric acid concentration ($P = 0.055$) and significantly reduced propionic acid concentration ($P < 0.05$), whereas acetic acid concentration remained unaffected. Total short-chain fatty acids (TSCFA, mmol/l) concentration tended to decrease with increasing OC inclusion ($P = 0.060$). Furthermore, OC inclusion significantly reduced total gas production, metabolisable energy (ME), organic matter digestibility (OMD), and net energy for lactation (NE_L) ($P < 0.001$). Methane production (ml/0.2 g DM) decreased numerically by approximately 14%, although this reduction was not statistically significant. Methane as a proportion of total gas was also unaffected. Ammonia-N concentrations did not differ among treatments. The reduction in methane output appeared to be associated with a general suppression of fermentative activity rather than a shift toward propionate-driven hydrogen utilisation. The lipid-rich and phenolic composition of OC may have contributed to reduced microbial fermentation intensity and hydrogen availability. Although higher inclusion levels negatively affected digestibility and energy values, moderate inclusion may offer a balance between environmental benefits and nutritional efficiency.

Keywords: agro-industrial by-products; enteric methane mitigation; gas production technique; silage microbiology; ruminal fermentation profile

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Recent discussions regarding methane emissions have centred largely on ruminants, particularly dairy cattle. In ruminants, ingested nutrients are fermented by rumen microorganisms, producing H_2 and CO_2, which are subsequently converted to methane by methanogenic archaea (van Zijderveld et al. 2010). This biological mechanism results in a loss of approximately 10% of the energy derived from feed and contributes to roughly 15% of global greenhouse gas accumulation (Xie et al. 2025). Consequently, dietary strategies to mitigate ruminal methanogenesis are of increasing interest (Baceninaite et al. 2022).

Since ensiled feeds undergo partial fermentation, they are associated with lower methanogenesis and CH_4 emissions (Boadi et al. 2004). Another advantage of ensiling process is that the resulting fermented feed is rich in bacteriocins, organic acids, antifungal peptides, and hydrogen peroxide, which have been reported to lower CH_4 emissions (Afonso et al. 2026).

As is well-established, the organic acid composition of silage varies according to the microbial load active during the fermentation process. Moreover, lactic acid bacteria (LAB) in ensiled feeds mitigate rumen methanogenesis through several pathways: modifying silage chemical profiles, suppressing pathogenic microbes, shifting rumen fermentation patterns, and enhancing organic matter digestibility (Ellis et al. 2016). Consequently, analysing the composition of microorganisms in ensiled feeds can provide significant insights into ruminal methane production.

While fat supplementation is generally used to increase the energy density of diets for high-yielding dairy cows, replacing carbohydrates with fats also leads to lower CH_4 emissions due to reduced fermentation. The methane-suppressing mechanism of fats involves the inhibition of organic matter fermentation, a reduction in cellulose digestibility, and the direct inhibition of ruminal methanogens through the biohydrogenation of unsaturated fatty acids, such as oleic, linoleic, linolenic, and arachidonic acids (Johnson and Johnson 1995).

Ensiled total mixed rations (ETMR) applications facilitate the use of high-moisture food industry by-products, such as olive cake, in animal nutrition (Bueno et al. 2020), while the pre-fermentation process is expected to suppress ruminal methane production. Olive cake (OC), the residue remaining after oil extraction, consisting of oil, pits, skins, and pulp, typically contains 6–16% residual oil on a dry matter basis. Due to this oil and phenolic compound content, it is hypothesised that OC may exert a methane-reducing effect in ETMR (Vastolo et al. 2025). Despite the increasing interest in OC as a ruminant feed ingredient, most previous studies have primarily focused on its direct inclusion in fresh TMR or its effects on nutrient digestibility and animal performance (Foti et al. 2022). In our previous study using the same experimental rations, the fermentation characteristics, chemical composition, and aerobic stability of ETMR containing fresh OC were evaluated (Er et al. 2025). However, the potential consequences of these fermentation-induced changes on ruminal fermentation dynamics and methane production remain unknown. In particular, the interaction between lipid-rich OC, pre-fermentation during ensiling, and subsequent ruminal fermentation dynamics remains insufficiently understood. In this context, the present study was designed as a follow-up experiment focusing specifically on rumen fermentation characteristics and methane production, using the same ETMRs previously described (Er et al. 2025). Furthermore, most previous studies have focused on fresh TMR rather than ETMR, and the potential synergistic or antagonistic effects of ensiling on the antimethanogenic properties of OC have not been clearly elucidated. Therefore, the objective of this study was to evaluate the effects of replacing maize silage with increasing levels of fresh OC in ETMR on silage microbial composition, *in vitro* rumen fermentation characteristics, methane production, and estimated energy parameters, with particular emphasis on fermentation modulation and methane mitigation potential. We hypothesised that replacing maize silage with increasing levels of fresh OC in ETMR would reduce methane production due to its lipid and phenolic content, modulate rumen fermentation patterns, and alter microbial dynamics without impairing silage quality.

MATERIAL AND METHODS

Ensiling the total mixed rations. Three total mixed rations (TMR) were formulated based on the nutrient compositions and dry matter (DM) requirements typically used in dairy cattle nutrition (30 kg milk yield, 600 kg body weight, and midlactation). Diets contained 50–55% DM and a forage-to-concentrate ratio of 50 : 50 (DM basis).

Experimental treatments were as follows:

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- Control group: 0% OC inclusion on a DM basis.
- OC10 group: 10% OC inclusion on a DM basis.
- OC20 group: 20% OC inclusion on a DM basis.

The forage component consisted of alfalfa hay, maize silage, and fresh OC depending on the treatment group, while the concentrate portion was composed of ground maize, ground barley, cottonseed meal, sunflower meal, soybean meal, mineral stone, sodium bicarbonate, salt, and a vitamin–mineral premix. In the control diet, the forage component consisted of 35.6% maize silage and 14.4% alfalfa hay, with no olive cake inclusion. In the OC10 treatment, fresh olive cake was incorporated at 10% of dietary dry matter by partially replacing maize silage (25.6%), while alfalfa hay remained constant at 14.4%. In the OC20 treatment, olive cake inclusion was increased to 20% of dry matter, with maize silage reduced to 15.6%, and alfalfa hay maintained at the same level. The concentrate portion of the rations remained identical across all treatments and consisted of 16.8% ground maize, 9.1% ground barley, 8.5% cottonseed meal, 12.2% sunflower meal, and 1.6% soybean meal, supplemented with 0.7% mineral stone, 0.5% sodium bicarbonate, 0.4% salt, and 0.2% vitamin–mineral premix. The detailed chemical composition of the fresh and ensiled TMRs is presented in Table 1. The formulation and ensiling procedure of the experimental diets (Control, OC10, and OC20) were previously described in detail (Er et al. 2025). Therefore, only a brief summary is provided here.

Before ensiling, the prepared TMRs were mixed manually for 6–8 min until homogeneous. Samples (300–350 g) were vacuum-sealed in polyethylene bags with a vacuum packaging machine to ensure anaerobic conditions. A total of 81 silage samples were ensiled, with 27 replicates per group scheduled to be opened on different days. For pH monitoring, three independent silos per treatment were opened on days 2, 4, 7, and 14. On days 21, 42, and 60, three additional independent silos per treatment were opened, and pH, DM loss, and aerobic stability were determined from each silo. At day 60, six further independent silos per treatment were opened for the determination of fermentation characteristics (organic acids and NH_3-N), chemical composition, feed value, and *in vitro* digestibility. Results were reported in the first publication of this study (Er et al. 2025). In the present study, six samples per group opened on day 60 were used to determine bacterial counts and to analyse total gas and methane production, short-chain fatty acids (SCFA), and ammonia nitrogen in the rumen fluid at the 24th h of *in vitro* rumen fermentation.

Microbial analysis. Samples (20 g) were homogenised with 180 ml of peptone water and incubated for 30 min at ambient temperature. Enumeration of total bacteria was by culture on plate count agar (all media from Oxoid,

Table 1. The chemical composition of fresh and ensiled total mixed rations

Chemical composition of fresh TMRs*	Control	OC10	OC20
DM (g/kg)	495	518	499
CP (g/kg DM)	150	148	147
EE (g/kg DM)	29.4	50.5	66.1
Ash (g/kg DM)	68.3	65.7	60.0
NDF (g/kg DM)	349	370	385
ADF (g/kg DM)	254	273	297
ADL (g/kg DM)	39.4	62.3	86.9
NFC (g/kg DM)	402	364	341
WSC (g/kg DM)	61.0	62.5	59.3
TP** (g/kg DM)	9.40	6.22	4.56
Chemical composition of ensiled TMRs*	**Control**	**OC10**	**OC20**
DM (g/kg)	470	501	495
CP (g/kg DM)	153	148	142
EE (g/kg DM)	22.0	50.1	77.3
Ash (g/kg DM)	68.5	66.1	59.8
NDF (g/kg DM)	378	398	407
ADF (g/kg DM)	263	283	305
ADL (g/kg DM)	44.1	68.7	93.1
NFC (g/kg DM)	377	336	313
WSC (g/kg DM)	26.3	22.9	23.4
TP** (g/kg DM)	9.80	5.97	6.17
pH	4.30	4.57	4.73

Chemical composition data were previously reported in Er et al. (2025) and are presented here for clarity

*The chemical composition was obtained from laboratory analyses (Er et al. 2025); **TP is presented as Gallic acid equivalent (GAE)

ADF = acid detergent fibre; ADL = acid detergent lignin; Control = 0% olive cake in TMR; CP = crude protein; DM = dry matter; EE = ether extract; NDF = neutral detergent fibre; NFC = non-fibrous carbohydrates; OC10 = 10% olive cake in TMR; OC20 = 20% olive cake in TMR; TMR = total mixed ration; TP = total phenolic compounds; WSC = water soluble carbohydrate

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Basingstoke, UK) for 1 day at 30 °C under aerobic conditions. The number of lactic acid bacteria was determined using de Man, Rogosa and Sharpe (MRS) agar (Merck KGaA, 64271; Darmstadt, Germany) after plates were incubated at 30 °C for 3 days. Yeasts and moulds were counted on yeast extract glucose chloramphenicol agar (Merck KGaA, Darmstadt, Germany) incubated at 30 °C for 24 hours. Enterobacteriaceae were enumerated using violet red bile glucose agar (VRBGA), while presumptive coliform bacteria were determined using Endo agar for 1 day at 30 °C under aerobic conditions. Results were expressed as $\log_{10}$ CFU/g.

***In vitro* total gas and methane production.** *In vitro* ruminal fermentation was conducted using the gas production technique described by Menke and Steingass (1988). The Erciyes University Animal Experiments Local Ethics Committee (24/189) approved the study as suitable for animal experiments. Rumen fluid used in the *in vitro* fermentation assay was obtained from two donor cattle and pooled prior to incubation to minimise individual animal variation and to provide a representative microbial inoculum. Each dietary treatment was incubated in quadruplicate within the same incubation run. These replicate bottles were considered analytical replicates representing fermentation responses of the same inoculum source. Donor animals were fed 9.8 kg DM/day, consisting of maize silage (5.0 kg/day), wet sugar beet pulp (4.5 kg/day), wheat straw (1.7 kg/day), and lucerne hay (1.5 kg/day) on a feed basis, in addition to a concentrate mixture. The total mixed ration contained 12.6% crude protein (CP) and 40.3% neutral detergent fibre (NDF) on a DM basis and provided 2 479 kcal/kg DM of metabolisable energy, in accordance with the recommendations of the National Research Council (2001). For each analysis, 0.20 g of ETMR sample (DM basis) was incubated in a 100-ml anaerobic glass fermenter (Model Fortuna; Haberle Labortechnik, Lonsee, Germany) containing 30 ml of a rumen fluid-buffer mixture (1 : 2, v/v). All samples were analysed in quadruplicate. After 24 h of incubation, total gas volume was measured using the calibrated scale of the anaerobic fermenters, as described by Menke et al. (1979).

At the end of the 24-h incubation period, the total gas volume (ml) produced in each syringe was determined by reading the graduated scales on the syringes. After recording the total *in vitro* gas production, approximately 50 ml of the headspace gas was collected into a plastic syringe. This gas was then injected into a computer-linked (Vestel, Türkiye) infrared methane analyser (Sensor; Europe GmbH, Erkrath, Germany) to determine the *in vitro* methane concentration as a percentage. The volumetric level of methane within the total gas (ml) was then calculated accordingly.

Metabolisable energy, net energy for lactation, and organic matter digestibility. The metabolisable energy (ME), net energy lactation (NE_L), and organic matter digestibility (OMD) were calculated based on the 24-h total gas production (ml/200 mg DM) nutrient composition (g/kg DM) using equations developed by Menke et al. (1979) and Menke and Steingass (1988):

$$\text{ME (MJ/kg DM)} = 2.20 + 0.136 \times \text{gas production} + 0.057 \times \text{crude protein} + 0.002\,9 \times \text{ether extract}^2 \quad (1)$$

$$NE_L \text{ (MJ/kg DM)} = 0.115 \times \text{gas production} + 0.005\,4 \times \text{crude protein} + 0.014 \times \text{ether extract} - 0.005\,4 \times \text{ash} - 0.36 \quad (2)$$

$$\text{OMD (\% DM)} = 14.88 + 0.889 \times \text{gas production} + 0.45 \times \text{crude protein} + 0.065\,1 \times \text{ash} \quad (3)$$

pH and SCFA composition and ammonia-nitrogen. Following 24 h of fermentation, the pH values of the fermentation fluids (15 ml) were measured using a digital pH meter (Mettler Toledo, USA). Subsequently, the short-chain fatty acid (SCFA) composition comprising acetic acid (AA, C2:0), propionic acid (PA, C3:0), butyric acid (BA, C4:0), and valeric acid (VA), isobutyric acid (IBA, C4:0i), and isovaleric acid (IVA, C5:0i), was determined using a gas chromatography system equipped with a flame ionisation detector (GC-FID) (TRACE™ 1300; Thermo Fisher Scientific, Orlando, USA). A polyethylene glycol column (length: 60 m, i.d.: 0.25 mm, film thickness: 0.25 µm, TG-WAXMS; Thermo Scientific, Orlando, FL, USA) was utilised for this analysis. The analytical procedure followed the methodology described by Ersahince and Kara (2017). Short-chain fatty acid (SCFA) concentrations were calculated as the sum of AA, PA, BA, and VA, while branched-chain fatty acid (BCFA) concentrations were calculated as the sum of IBA and IVA. Total short-chain fatty acid (TSCFA) concentrations were calculated as the sum of SCFA and BCFA. All analyses were performed in triplicate.

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Ammonia-nitrogen levels in the fermentation fluid were determined from 15 ml samples collected at the 24th h of *in vitro* ruminal fermentation. The analysis was conducted using a Megazyme commercial kit (Ireland) via the ELISA method. NH_3-N concentrations were expressed in mg/l, and analyses were performed in triplicate.

Statistical analysis. The experimental data were subjected to one-way analysis of variance (ANOVA) based on a completely randomised design, using SPSS software (v26.0; IBM Corp., Armonk, NY, USA). Differences among treatments were evaluated using Tukey's test. Significance was declared at $P < 0.05$.

RESULTS

Microbial composition. Table 2 presents the microbial evaluation (log_{10} CFU/g) of ETMRs. Total bacterial counts showed that the control group was 5.80 log CFU/g, while the group with 10% OC inclusion was 6.08 log CFU/g, and the 20% OC group was 6.28 log CFU/g. No statistically significant difference was detected among the groups in terms of total bacterial counts ($P = 0.880$).

Regarding lactic acid bacteria counts, the highest value was observed in the group containing 10% OC (5.87 log_{10} CFU/g), followed by the control group (5.38 log_{10} CFU/g) and the 20% OC group (5.11 log_{10} CFU/g). However, these differences were also not statistically significant ($P = 0.296$). Yeast and mold counts were measured at 2.66 log_{10} CFU/g in the control group, 2.30 log_{10} CFU/g in the OC10 group, and 2.37 log_{10} CFU/g in the OC20 group; no significant differences were observed between the groups. Coliforms and enterobacteria were not detected in any group.

pH and SCFA proportions and ammonia-nitrogen in *in vitro* fermentation fluid. Table 3 presents the pH and SCFA concentrations in the

Table 2. Effect of olive cake inclusion on the microbial composition of ETMRs

Variables (log_{10} CFU/g)	C	OC10	OC20	SEM	*P*-value
Total bacteria	5.80	6.08	6.28	0.668	0.880
Lactic acid bacteria	5.38	5.87	5.11	0.283	0.296
Mold and yeast	2.66	2.30	2.37	0.194	0.441
Coliform	nd	nd	nd	nd	–
Enterobacteriaceae	nd	nd	nd	nd	–

C = control, 0% olive cake in ETMR; nd = not detected; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR; SEM = standard error of mean

Table 3. Effect of olive cake inclusion in ETMRs on rumen pH and molar concentrations of SCFA and BCFA at 24 h of *in vitro* rumen fermentation

Variables	C	OC10	OC20	SEM	*P*-value
pH	6.44	6.29	6.33	0.169	0.572
Valeric acid (mmol/l)	0.915	0.894	0.874	0.014	0.153
Isobutyric acid (mmol/l)	0.876	0.850	0.843	0.013	0.168
Isovaleric acid (mmol/l)	0.971	0.959	0.932	0.023	0.475
Butyric acid (mmol/l)	9.20^{a}	8.97^{ab}	8.70^{b}	0.136	0.055
Propionic acid (mmol/l)	27.2^{a}	26.7^{ab}	26.3^{b}	0.243	0.046
Acetic acid (mmol/l)	43.6	42.5	42.1	0.452	0.067
BCFA (mmol/l)	1.85	1.81	1.78	0.034	0.339
SCFA (mmol/l)	80.9^{a}	79.1^{ab}	78.0^{b}	0.831	0.046
TSCFA (mmol/l)	82.8	80.9	79.7	0.863	0.060

^{a,b}Means within the same row with different superscripts differ significantly according to Tukey's post hoc test ($P < 0.05$)
BCFA = branched-chain fatty acids; C = control, 0% olive cake in ETMR; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR; SCFA = short-chain fatty acids; SEM = standard error of mean; TSCFA = total short-chain fatty acids

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fermentation fluid at the 24th h for ETMRs containing varying levels of OC. pH values were similar across treatments ($P > 0.572$). A statistically significant difference was observed in PA levels ($P < 0.05$), whereas BA levels showed a decreasing trend ($P = 0.055$). The highest BA level was found in the Control group (9.20 mmol/l), while the lowest was in the OC20 group (8.70 mmol/l). Similarly, PA levels decreased from 27.2 mmol/l in the C group to 26.3 mmol/l in the OC20 group.

No statistically significant differences were found for other SCFA and BCFA types, including VA, IBA, and IVA ($P > 0.05$). The SCFA concentration was significantly higher in the Control group compared to the OC20 group (80.9 vs 78.0 mmol/l, respectively; $P < 0.05$). No differences were observed between groups regarding BCFA concentrations ($P = 0.339$). Additionally, AA and total SCFA levels in the ETMRs exhibited a downward trend as the olive cake inclusion rate increased (respectively, $P = 0.067$ and $P = 0.060$).

The inclusion of OC did not lead to any significant changes in the proportions of SCFA and BCFA ($P > 0.05$; Table 4). However, the proportion of BA in the ETMR showed a downward trend as the OC inclusion rate increased ($P = 0.077$).

The effects of OC cake inclusion in ETMRs on the NH_3-N levels in the *in vitro* rumen fermentation fluid at the 24th h are presented in Figure 1. No statistically significant differences were observed among the groups for NH_3-N levels ($P = 0.356$).

Ruminal total gas and methane production, metabolisable energy, net energy for lactation, and organic matter digestibility. Figure 2 shows the effect of OC inclusion in ETMRs on total gas and methane production at 24 h of *in vitro* rumen incubation. The total gas production was significantly affected by the inclusion level of OC in the ETMRs ($P < 0.001$). The control treatment exhibited the highest total gas production, whereas the inclusion of OC at 10% (OC10) and 20% (OC20) of dietary dry matter resulted in a significant reduction in total gas output. Moreover, total gas production decreased progressively with increasing OC concentration, with OC20 showing the lowest value among the treatments. In contrast, methane production expressed on an absolute basis (ml) was not significantly influenced by OC inclusion levels at 24 h of *in vitro* rumen fermentation ($P = 0.182$). Although numerically lower methane production was observed in the OC10 and OC20 treatments compared with the control, these differences did not reach statistical significance. Similarly, methane production expressed as a percentage of total

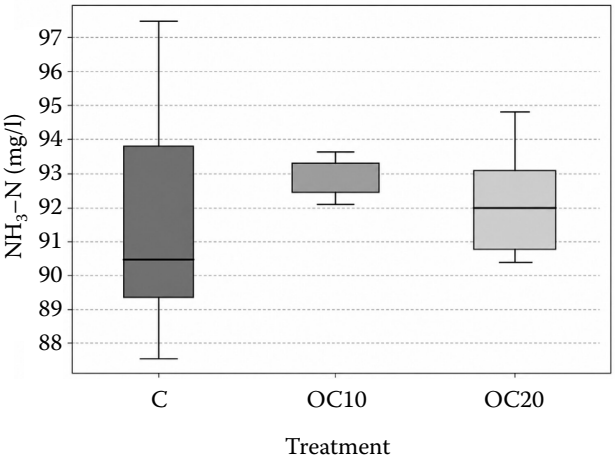

Figure 1. NH_3-N (mg/l) concentrations after 24 h of *in vitro* ruminal incubation of experimental diets containing increasing levels of olive cake

Box plots represent the median and interquartile range. Whiskers indicate the minimum and maximum values

C = control, 0% olive cake in ETMR; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR

Table 4. Effect of olive cake inclusion in ETMRs on proportions of SCFA and BCFA at 24 h of *in vitro* rumen fermentation

SCFA and BCFA proportions (% of TSCFA)	C	OC10	OC20	SEM	*P*-value
Valeric acid	1.098	1.106	1.098	0.007	0.722
Isobutyric acid	1.058	1.051	1.058	0.007	0.691
Isovaleric acid	1.172	1.185	1.169	0.017	0.786
Butyric acid	11.10	11.10	10.90	0.063	0.077
Propionic acid	32.90	33.00	33.00	0.079	0.456
Acetic acid	52.70	52.60	52.80	0.079	0.197

BCFA = branched-chain fatty acids; C = control, 0% olive cake in ETMR; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR; SCFA = short-chain fatty acids; SEM = standard error of mean; TSCFA = total short-chain fatty acids

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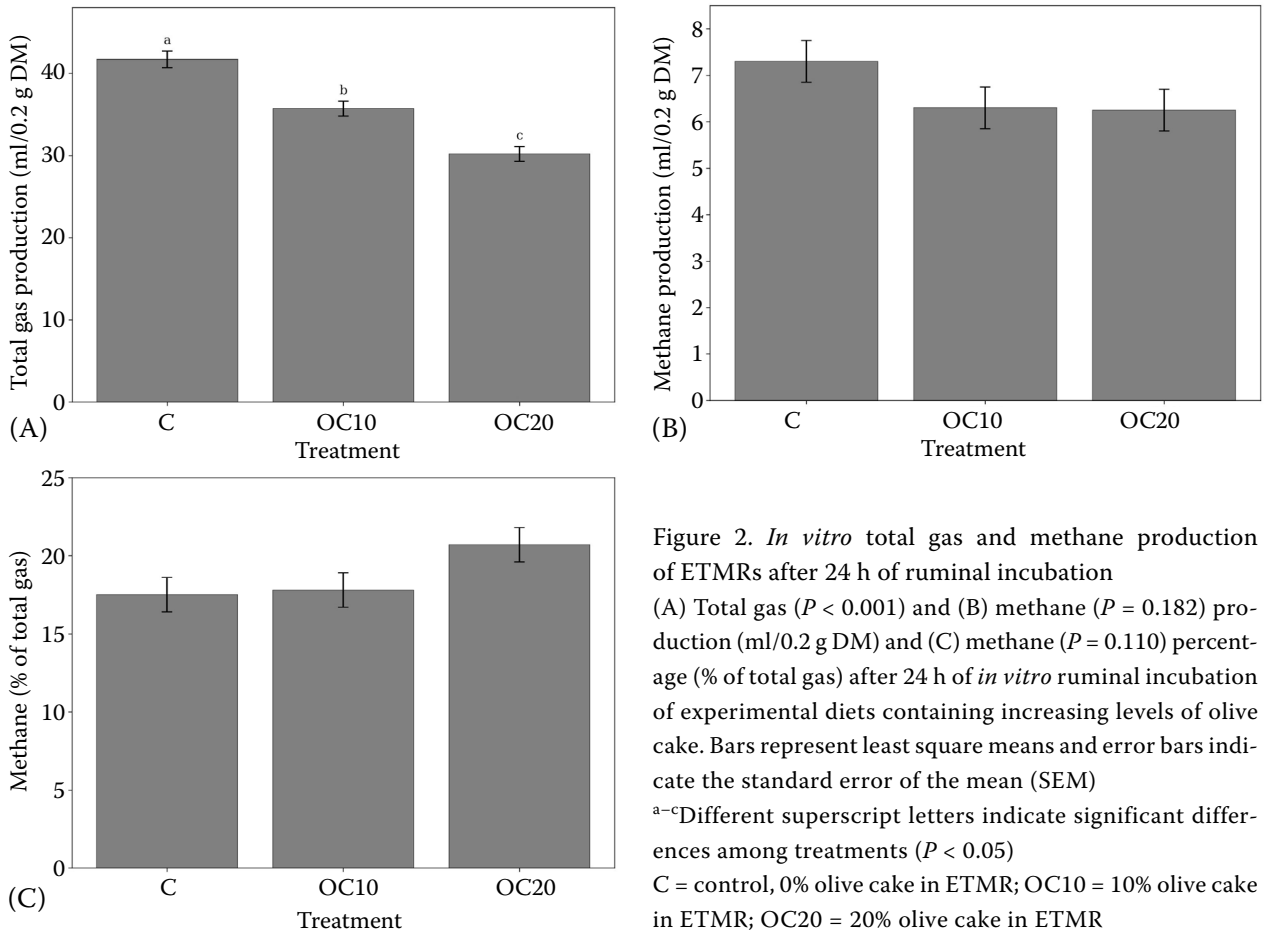

Figure 2. *In vitro* total gas and methane production of ETMRs after 24 h of ruminal incubation

(A) Total gas ($P < 0.001$) and (B) methane ($P = 0.182$) production (ml/0.2 g DM) and (C) methane ($P = 0.110$) percentage (% of total gas) after 24 h of *in vitro* ruminal incubation of experimental diets containing increasing levels of olive cake. Bars represent least square means and error bars indicate the standard error of the mean (SEM)

[a–c]Different superscript letters indicate significant differences among treatments ($P < 0.05$)

C = control, 0% olive cake in ETMR; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR

gas production was not significantly affected by dietary treatments ($P = 0.110$). However, a numerical increase in methane proportion was observed with increasing olive cake inclusion, with the highest value recorded in the OC20 group.

The inclusion of OC in ETMRs significantly affected estimated energy values and organic matter digestibility (Table 5). The ME content decreased progressively with increasing OC inclusion level ($P < 0.001$). The control diet exhibited the highest ME value, whereas diets containing 10% (OC10) and 20% (OC20) OC showed significantly lower ME concentrations, with the lowest value observed in OC20. Similarly, OMD was significantly reduced as the proportion of OC increased in the ration ($P < 0.001$). The control group showed the highest OMD, while OC10 and OC20 treatments resulted in a stepwise decline, indicating a negative effect of olive cake inclusion on digestibility. Net energy for lactation was also significantly influenced by dietary treatments ($P < 0.001$). Although both OC containing diets exhibited lower NE_L values compared with the control, no significant difference was observed between OC10 and OC20.

Table 5. Effect of olive cake inclusion in ETMRs on ME, OMD and NE_L at 24 h of *in vitro* rumen fermentation

Parameters	C	OC10	OC20	SEM	*P*-value
ME (MJ/kg DM)	8.77[a]	7.89[b]	7.15[c]	0.127	<0.001
NE_L (MJ/kg DM)	5.28[a]	4.93[b]	4.65[b]	0.097	<0.001
OMD (% DM)	63.5[a]	57.6[b]	52.3[c]	0.851	<0.001

[a–c]Means within the same row with different superscripts differ significantly according to Tukey's post hoc test ($P < 0.05$)

C = control, 0% olive cake in ETMR; DM = dry matter; ME = metabolisable energy; NEL = net energy lactation; OC10 = 10% olive cake in ETMR; OC20 = 20% olive cake in ETMR; OMD = organic matter digestibility; SEM = standard error of mean

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DISCUSSION

Agro-industrial by-products significantly contribute to environmental pollution by degrading soil, water, and air quality. Accordingly, the utilisation of agro-industrial by-products, such as OC, in ETMR systems for ruminant nutrition represents a promising strategy. This approach not only enables the valorisation of waste materials but also holds the potential to influence ruminal fermentation and mitigate methane emissions through the beneficial effects of pre-fermentation. In this context, the present study evaluated the potential of fresh OC as a sustainable agro-industrial by-product in ETMR systems, with particular emphasis on rumen fermentation and methane mitigation.

Total bacteria and LAB counts were not affected by the OC inclusion (Table 2). It is widely established that LAB thrive by fermenting WSC found in fresh materials, thereby creating an optimal environment for microbial growth (Muck 2010). However, the use of varying levels of OC in ETMR had a limited impact on WSC concentrations (Table 1). Additionally, in the first paper of this experiment, Er et al. (2025) reported that no significant differences were observed in WSC contents across fresh TMRs. Consequently, no significant differences in bacterial counts were observed among the experimental groups.

In the present experiment, the counts of yeast and moulds (2.30–2.66 $\log_{10}$ CFU/g) remained considerably lower than the upper limit reported for good-quality corn-based silages (<5 $\log_{10}$ CFU/g; Kung et al. 2018). Likewise, coliforms and enterobacteriaceae counts remained below detectable limits in all treatment groups, consistent with the low microbial contamination typically observed in well-preserved, low-pH silage (Driehuis et al. 2018). Furthermore, it can be suggested that the organic acid load provided by the maize silage used in the ETMR formulations exerted a protective preservative effect.

In this study, OC inclusion in ETMRs did not affect pH levels of rumen fluid (Table 3). Although the ETMRs exhibited higher acidity (Table 1), observed pH levels were above the ideal rumen pH range (5.8–6.2) for optimal microbial activity and nutrient utilisation (Yilmaz et al. 2025). Rumen acid production primarily results from the microbial fermentation of WSC into SCFA and lactic acid (Dijkstra et al. 2020). However, the ensiling process markedly reduced the concentration of WSC in ETMRs (Table 1). Moreover, the increased dietary EE with OC inclusion may have suppressed ruminal microbial activity (Er et al. 2025). In addition, ruminal acid production generally far exceeds the amount of acid directly ingested with the diet (Aschenbach et al. 2011).

Increasing OC inclusion led to a decrease in BA and PA and SCFA, while AA remained unaffected. A numerical decrease (P = 0.060) in TSCFA concentration was observed as the density of OC in the ETMR increased. The literature contains controversial results regarding the stimulating or depressing effects of olive oil by products on rumen SCFA production. Pallara et al. (2014) reported that stoned OC inclusion increased ruminal propionic and butyric acid concentrations *in vitro*. Yanez Ruiz et al. (2004) obtained higher rumen AA concentration in goats and wethers fed diets based on two-stage olive cake. These differences are most likely strongly related to the olive variety, different oil extraction procedures used to obtain olive cakes and to the associative effects of the olive by-product with other dietary components. Furthermore, the decrease in PA and BA observed in current study may instead be attributed to the higher NDF and lignin content of fresh OC compared with the maize silage it replaced, which is known to limit fibrolytic and amylolytic fermentation. Zhang et al. (2025) reported that the forage-to-concentrate ratio does not always affect AA and TSCFA concentrations. The lack of change in AA concentration in this study may be due to the limited difference in NDF content between rations (378, 398, and 407 g/kg DM) and the fact that samples were taken at the 24th hour. Since the digestion of fibrous components takes longer, it is suggested that SCFA concentrations should also be evaluated at 48 and 72 h in similar future studies.

The inclusion of OC did not affect NH_3-N concentrations in the rumen fluid at the 24th h (Figure 1). Ruminal NH_3-N production is associated with protein digestibility and the CP content of the ration (Firkins et al. 2007). The similar CP content of the ETMRs (15.3%, 14.8%, and 14.2% DM) likely accounts for the similar NH_3-N concentrations. This also suggests that the ruminal degradable protein ratios of maize silage and the OC that replaced it are comparable.

The study demonstrated that increasing OC in ETMRs significantly reduced total gas production and led to a numerical decrease in methane production (approximately 14%; Figure 2). However,

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the proportion of methane within the total gas produced remained similar across ETMRs. *In vitro* gas production is generally used as a key indicator of ruminal digestibility potential. Hadhoud et al. (2020) also observed a decrease in total gas production as the amount of OC in fresh TMR increased.

Gas production typically decreases due to the high EE content of OC (Vastolo et al. 2025), potentially reflecting increased biohydrogenation of unsaturated fatty acids. These results may also be related to the high levels of tannins and lignin (anti-nutritional factors) found in OC, which can negatively affect microbial activity in the rumen (Patra and Saxena 2011). Additionally, ME, OMD, and NE_L values showed an inverse correlation with increasing OC concentration in ETMRs. This outcome is explained by the decrease in total gas production used to calculate these parameters.

Interestingly, despite the observed decrease in PA concentration, methane production showed a numerical reduction in the OC containing treatments. Under typical ruminal fermentation conditions, an increase in propionate is associated with reduced methane production due to its role as an alternative hydrogen sink (McAllister and Newbold 2008). However, the present findings suggest that other mechanisms may have contributed to the reduction in methane formation. One possible explanation is the high lipid content of fresh OC, particularly its unsaturated fatty acids, which have been reported to inhibit methanogenic archaea and reduce hydrogen availability through biohydrogenation processes (Yang et al. 2022). In addition, phenolic compounds and tannins present in OC may suppress methanogenic activity and protozoal populations independently of shifts in volatile fatty acid pathways (Dhanasekaran et al. 2020). Although tannin/phenolic content of OC was not directly determined in the present study, OC is generally known to contain considerable amounts of phenolic compounds (Er et al. 2025), and a similar mechanism may partly explain the methane-mitigating effect observed here. Furthermore, the decrease in total gas production observed in the present study suggests a general suppression of microbial activity, which may have limited substrate degradation and hydrogen generation. Therefore, methane reduction in this study may be related more to the inhibition of fermentation intensity rather than a shift toward propionate production. This hypothesis is supported by the observed decline in TSCFA concentration and organic matter digestibility with increasing OC inclusion.

Although the present findings provide valuable preliminary insights into the effects of fresh OC inclusion in ETMR on rumen fermentation characteristics, several limitations inherent to the *in vitro* gas production technique should be acknowledged. *In vitro* systems do not fully replicate the dynamic and adaptive nature of the rumen ecosystem. Factors such as feed intake regulation, passage rate, microbial adaptation to dietary lipids and phenolic compounds, host–microbe interactions, and continuous absorption of fermentation end products cannot be accurately simulated under laboratory conditions (Yanez Ruiz et al. 2016). Moreover, rumen microorganisms *in vivo* may gradually adapt to lipid-rich or phenolic-rich substrates, potentially altering fermentation pathways and methane production over time. The 24-hour batch culture incubation used in this study represents a short-term fermentation response and may not reflect long-term microbial adaptation or shifts in methanogenic archaeal populations. Additionally, methane production *in vitro* is influenced by the closed-system accumulation of fermentation gases, which may modify hydrogen dynamics differently from *in vivo* conditions, where eructation continuously removes gases. Therefore, the numerical reduction in methane observed in this study should be interpreted cautiously. Further *in vivo* studies, including direct measurements of enteric methane emissions and rumen microbial profiling (e.g. archaeal abundance and protozoal populations), are required to confirm the practical methane mitigation potential of fresh olive cake–based ETMR.

Another limitation of the present study is the absence of detailed rumen microbial profiling, particularly methanogenic archaea and protozoal populations, which play a central role in methane production (Kara et al. 2015). Although the microbial evaluation performed in this study focused primarily on silage quality indicators such as lactic acid bacteria, total bacteria, and spoilage microorganisms, these parameters do not directly reflect the rumen microbial ecosystem. Therefore, the mechanisms underlying the observed numerical reduction in methane production could not be fully elucidated. However, the primary aim of this study was to investigate the fermentation-modulating potential of fresh OC within an ETMR system rather than to characterise rumen microbial shifts. The

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microbial stability and hygienic quality of ETMR are critical for ensuring consistent fermentation and feed safety, which justified the focus on silage microbiology (Driehuis 2013). Future studies should incorporate high-throughput sequencing approaches, such as 16S rRNA-based rumen microbiota profiling, archaeal abundance, and protozoal enumeration, to better understand the antimethanogenic mechanisms associated with olive cake inclusion.

Although methane production was not statistically reduced, a consistent numerical decrease (approximately 14%) was observed with increasing OC inclusion. From a biological perspective, even moderate reductions in methane may be meaningful, particularly when achieved through dietary strategies utilising agro-industrial by-products. It is important to emphasise that methane production should be interpreted not only on an absolute basis but also in relation to fermentation intensity. In the present study, OC inclusion markedly reduced total gas production, organic matter digestibility, and estimated energy values, indicating a suppression of overall fermentative activity. Therefore, the numerical reduction in methane appears to be associated with decreased substrate degradation rather than a specific redirection of hydrogen toward alternative sinks. This distinction is important, as methane mitigation strategies may operate through two different pathways: (*i*) shifting hydrogen utilisation toward propionate production, or (*ii*) directly inhibiting microbial fermentation and methanogenic activity (Ungerfeld 2018). The present findings suggest that fresh OC in ETMR primarily acted through direct inhibition of fermentation and methanogenesis, rather than propionate-mediated hydrogen redirection. This may be attributed to its lipid fraction and phenolic compounds, which are known to impair cellulolytic bacteria, protozoa, and methanogens. Biohydrogenation of unsaturated fatty acids could also have contributed as an additional hydrogen sink (Yang et al. 2022), though this could not be confirmed without fatty acid or hydrogen measurements. Overall, fresh OC appears to hold potential as a strategic ingredient for improving the sustainability of ruminant production systems.

CONCLUSION

Fresh OC inclusion in ETMR reduced total gas production, SCFA concentration, OMD, and estimated energy values. Methane production declined numerically as well, though not significantly likely a sign of suppressed fermentation. These findings suggest fresh OC as a fermentation-modulating ingredient with potential methane mitigation value. Around 10% DM inclusion appears to strike the best balance between environmental and nutritional outcomes. Confirming this, however, will require *in vivo* trials with direct methane measurements and rumen microbial profiling.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Afonso RB, Adivi LN, Linehan K, O'Donovan M, Hill C, Ross RP, Stanton C. Potential of silage inoculants to mitigate methane production from the rumen: A systematic review. J Dairy Sci. 2026 Feb;109(2):2053-65.

Aschenbach JR, Penner GB, Stumpff F, Gabel G. Ruminant nutrition symposium: Role of fermentation acid absorption in the regulation of ruminal pH. J Anim Sci. 2011 Apr;89(4):1092-107.

Baceninaite D, Dzermeikaite K, Antanaitis R. Global warming and dairy cattle: How to control and reduce methane emission. Animals (Basel). 2022 Oct 6;12(19):2687.

Boadi D, Benchaar C, Chiquette J, Masse D. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can J Anim Sci. 2004 Apr;84(3):319-35.

Bueno AVI, Lazzari G, Jobim CC, Daniel JLP. Ensiling total mixed ration for ruminants: A review. Agronomy. 2020 Jun;10(6):879.

Dhanasekaran DK, Dias-Silva TP, Filho ALA, Sakita GZ, Abdalla AL, Louvandini H, Elghandour MM. Plants extract and bioactive compounds on rumen methanogenesis. Agrofor Syst. 2020 Aug;94(4):1541-53.

Dijkstra J, van Gastelen S, Dieho K, Nichols K, Bannink A. Review: Rumen sensors: Data and interpretation for key rumen metabolic processes. Animal. 2020 Mar;14(S1): s176-86.

Driehuis F, Wilkinson JM, Jiang Y, Ogunade I, Adesogan AT. Silage review: Animal and human health risks from silage. J Dairy Sci. 2018 May;101(5):4093-110.

Driehuis F. Silage and the safety and quality of dairy foods: A review. Agric Food Sci. 2013;22(1):16-34.

Ellis JL, Hindrichsen IK, Klop G, Kinley RD, Milora N, Bannink A, Dijkstra J. Effects of lactic acid bacteria silage

https://doi.org/10.17221/48/2026-CJAS

inoculation on methane emission and productivity of Holstein Friesian dairy cattle. J Dairy Sci. 2016 Sep;99(9): 7159-74.

Er M, Ahsan U, Kuter E, Ramay HAMS, Karagoz DM, Takka MO. Fermentation quality and in vitro digestibility of total mixed ration ensiled with fresh olive cake. J Anim Plant Sci. 2025 Sep;35(5):1335-44.

Ersahince AC, Kara K. Nutrient composition and in vitro digestion parameters of Jerusalem artichoke (Helianthus tuberosus L.) herbage at different maturity stages in horse and ruminant. J Anim Feed Sci. 2017 Sep;26(3):213-25.

Firkins JL, Yu Z, Morrison M. Ruminal nitrogen metabolism: Perspectives for integration of microbiology and nutrition for dairy. J Dairy Sci. 2007 Jun;90(Suppl_1):E1-16.

Foti P, Pino A, Romeo FV, Vaccalluzzo A, Caggia C, Randazzo CL. Olive pomace and pate olive cake as suitable ingredients for food and feed. Microorganisms. 2022 Jan 21; 10(2):237.

Hadhoud FI, Shaaban MM, Abd El Tawab AM, Khattab MSA, Ebeid HM, Gouda GA, Abdo MM. Olive cake silage as alternative roughage for ruminant: Effect on rumen degradability and in vitro gas production. Egypt J Nutr Feeds. 2020 Jun;23(2):265-72.

Johnson KA, Johnson DE. Methane emissions from cattle. J Anim Sci. 1995 Aug;73(8):2483-92.

Kara K, Guclu BK, Baytok E. Comparison of nutrient composition and antimethanogenic properties of different Rosaceae species. J Anim Feed Sci. 2015 Nov;24(4):308-14.

Kung L Jr, Shaver RD, Grant RJ, Schmidt RJ. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J Dairy Sci. 2018;101(5):4020-33.

McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. Aust J Exp Agric. 2008; 48(2):7-13.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D, Schneider W. The estimation of the digestibility and metabolizable energy content of ruminant feedstuffs from the gas production when incubated with rumen liquor. J Agric Sci. 1979 Aug;93(1):217-22.

Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim Res Dev. 1988;28:7-55.

Muck RE. Silage microbiology and its control through additives. Rev Bras Zootec. 2010 Jul;39:183-91.

National Research Council. Nutrient requirements of dairy cattle. 7th revised ed. Washington (DC): National Academies Press; 2001. 408 p.

Pallara G, Buccioni A, Pastorelli R, Minieri S, Mele M, Rapaccini S, Messini A, Pauselli M, Servili M, Giovannetti L, Viti C. Effect of stoned olive pomace on rumen microbial communities and polyunsaturated fatty acid biohydrogenation: An in vitro study. BMC Vet Res. 2014 Nov 26; 10:271.

Patra AK, Saxena J. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J Sci Food Agric. 2011 Jan 15;91(1):24-37.

Ungerfeld EM. Inhibition of rumen methanogenesis and ruminant productivity: A meta-analysis. Front Vet Sci. 2018 Jun 19;5:113.

van Zijderveld SM, Gerrits WJ, Apajalahti JA, Newbold JR, Dijkstra J, Leng RA, Perdok HB. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. J Dairy Sci. 2010 Dec;93(12):5856-66.

Vastolo A, Mora B, Kiatti DD, Nocerino M, HaroutounianS, Baka RD, Ligda P, Cutrignelli MI, Niderkorn V, Calabro S. Assessment of the effect of agro-industrial by-products rich in polyphenols on in vitro fermentation and methane reduction in sheep. Front Vet Sci. 2025 Jan 30;12:1530419.

Xie X, Cao Y, Li Q, Li Q, Yang X, Wang R, Zhang X, Tan Z, Lin B, Wang M. Mitigating enteric methane emissions: An overview of methanogenesis, inhibitors and future prospects. Anim Nutr. 2025 Feb 8;21:84-96.

Yanez Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi DP, O'Kiely P, Hristov AN. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants – A review. Anim Feed Sci Technol. 2016 Jun;216:1-18.

Yanez Ruiz DR, Moumen A, Martin Garcia AI, Molina Alcaide E. Ruminal fermentation and degradation patterns, protozoa population, and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: Effect of PEG supply. J Anim Sci. 2004 Jul;82(7): 2023-32.

Yang Z, Liu S, Xie T, Wang Q, Wang Z, Yang H, Li S, Wang W. Effect of unsaturated fatty acid ratio in vitro on rumen fermentation, methane concentration, and microbial profile. Fermentation. 2022 Oct;8(10):540.

Yilmaz K, Kara K, Oztas SY, Pirci G, Aslan O, Soylu Y. Effect of polyol supplementation in forage-based total mixed rations on in vitro rumen gas production and fermentation in beef cattle. J Anim Feed Sci. 2025 Apr;34(4): 644-50.

Zhang G, Li J, Liu S, Hou P, Tao X, Gao W, Sun L, Lv L, Liang J. Recent advances in bioconversion of lignocellulosic biomass for volatile fatty acid production with rumen microorganisms. Biotechnol Adv. 2025 Oct;83: 108654.

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