REVIEWS

Influence of diets supplemented with naturally protected or unprotected eucalyptus oil on methane production and lactating buffalo productivity

Fawzy Mohamed Abo-Donia · Mohamed Youssif Elaref · Abd El-Moniem Ali Sayed Mahgoub · Tarek Abd-Elwahab Ahmed Deraz · Usama Aboelez Nayel

Received: 31 July 2021 / Accepted: 3 December 2021 / Published online: 10 December 2021
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Abstract
This study was designed to investigate the influence of naturally protected eucalyptus oil supplementation in a form of leaves (EUL) or mature seed capsules (EUS) compared to crude eucalyptus oil (EUO). The control group (G1) received a diet containing concentrate feed mixture, fresh berseem, rice straw, and corn silage, whereas the G2, G3, and G4 animals have a diet supplemented with 200 g/head/day of EUL or EUS or 4 mL/head/day EUO, respectively. Supplementation of EUL or EUS increased NH3-N, short-chain fatty acids, and concentrations of acetic acid in vitro. Bacterial total count, protozoa, and cellulolytic bacteria increased (P < 0.05) with EUL and EUS supplementation. Methane production dropped (P < 0.05) with EUS, EUL, and EUO supplementation. Milk fat decreased (P < 0.05) with EUO supplementation, while an adverse trend was shown for lactose. No differences in feed conversion were found among EUS, EUL, and EUO. Blood total protein, albumin, and urea increased (P < 0.05) with supplementation of EUL or EUS compared to EUO. EUO supplementation yielded increased (P < 0.05) AST, ALT, glucose, and creatinine. Supplementation with EUL, EUS, or EUO decreased (P < 0.05) DM, OM, and CP digestibility, while digestibility of EE with supplementation by EUL, EUS, or EUO was higher (P < 0.05). The digestion coefficient of NDF and ADF decreased (P < 0.05) with supplemental EUL, EUS, or EUO compared to the G1 diet. Feeding EUS increased the values of TDN and DCP compared to EUL, which increased than EUO. Our results confirm that the naturally protected form of leaves or seeds mitigates the undesirable effects of directly supplementing crude eucalyptus oil.

Keywords Eucalyptus oil · Eucalyptus leaves · Eucalyptus seed capsules · Methane · Degradability · Digestibility · Milk production

Introduction
The rumen is an intricate system in which nutrients used up by microorganisms at a suitable pH provide the main products of fermentation, short-chain fatty acids (SCFAs), and microbial biomass, which is exhausted by the host ruminants (Cieslak et al. 2013; Vakili et al. 2013). There has been increased interest recently to reduce the rate of rumen methane production. Methane (CH4) production from enteric fermentation is a concern because of the increased
accretion of greenhouse gases in the atmosphere as well as the losses of nutritious energy (Sallam et al. 2010). There has been an interesting effort to reduce CH₄ release by inhibition of ruminal methanogens to increase the efficiency of feed energy utilization by ruminants; this would also rally economic efficiency and the environment (Benchaar and Greathead 2011; Onel et al. 2021). Many studies have been carried out to investigate the impact of supplementation with eucalyptus leaves and eucalyptus oil (EUO) on methane production (McIntosh et al. 2003; Castillejos et al. 2006); however, much is still unknown about using dried or ground mature seeds. Additionally, Sallam et al. (2010) hypothesized that EUO might be used as a feed supplement to alter rumen biohydrogenation to reduce CH₄ release and increase the flow of SCFAs to the duodenum. Moreover, Abo-Donia and Naggal (2015) reported that tannins have been shown to alter rumen biohydrogenation, while Sallam et al. (2010) stated that eucalyptus has an ionophore effect by affecting SCFAs formation in the rumen lead to inhibition of the final step in the biohydrogenation of SCFAs to stearic acid. Due to the volatile and reactive of essential oils (ESOs), it is possible that their effectiveness, when included in an animal’s diet, possibly altered by conditions during the production season as well as storage of ESOs and conditions in the digestive system of the animals (Onel et al. 2021).

A topical study by Chouhan et al. (2017) found that the use of ESOs in a protected form has a high potential for antimicrobial resistance outstanding so that increasing chemical stability and solubility, reduced rapid evaporation, and reduced retardance of ESOs action. In addition, Lammari et al. (2020) established that the encapsulation of ESOs to make their release subject to continuous control enhances their bioavailability and effectiveness against microorganisms. In recent years, increasingly negative consumer perceptions encouraged increased interest in adding ESOs to ruminant feeds to increase milk production and improve the animals’ physiological performance (Thao et al. 2015). In the same line, Turek and Stintzing (2013) suggested that adding such oils in their natural form, whether in the form of leaves or grains, avoids the adverse effects of those crude oils and increases their effectiveness in ruminant nutrition. However, knowledge is scarce about the role of naturally protected as opposed to crude oil on animal performance (Maes et al. 2019). Therefore, we hypothesized that naturally protected eucalyptus oil supplementation may be better decrease CH₄ production, and the current study was designed to investigate the impact of supplementation of eucalyptus oil vs naturally protected in form (leaves or seeds capsules) on CH₄ production and productive performance.

Materials and methods

Ingredients and the trial diets

Eucalyptus leaves (EUL) and green mature seed capsules (EUS) collected from trees on the banks of the irrigation canals were dried under shade for a week, and then ground and stored at ambient room temperature until use. Eucalyptus oil (EUO) was obtained from El Hawag for Natural Oils, El Nasr City, Cairo, Egypt. Four experimental diets were formulated as total mixed ration (TMR) isonitrogenous and iso-caloric diets of lactating buffalo as recommended by Karel (1982). Animals G₁ received the basal diet consisting of a concentrate feed mixture (CFM), fresh berseem (FB), rice straw (RS), and corn silage (CS) at a 40:60 concentrate: roughage ratio. The second (G₂), third (G₃), and fourth (G₄) groups have received a control diet with a supplement of 200 g/head/day of EUL, EUS, or 4 mL EUO, respectively. Supplemental EUL, EUS, or EUO were dissolved daily in 1 L tap water, and then blended and mixed directly with the concentrated feed to ensure consistency. Weekly homogeneous samples of tested diets were dried and ground, and then held in glass bottles for analysis and in vitro studies. The chemical composition of ingredients and the tested diets are shown in Table 1.

Sixteen healthy lactating Egyptian buffalo (body weight: 457.4 ± 10.5 kg; in season: 2 to 4; after 14 days in lactation) were distributed into four similar groups and randomized according to their previous milk records using quadratic 4 × 4 Latin square experimental designs. Animals were individually fed the experimental diets twice daily (8 a.m. and 6 p.m.). The diet was offered for 28 d (21 days as a preliminary period + 7 days as a collection period), and it was adjusted every week based on changes in body weight and milk production. Multiminer salt blocks were supplied for the animals to lick freely, along with access to drinking water.

In vitro gas production and degradability

In vitro gas accumulation technique was conducted as confirmed by Theodorou et al. (1994) on obtained samples of the experimental diets. Rumen liquid was collected from two buffalo in each group before the morning meal using a stomach tube. About 600 mg of tested sample (1.0 mm) was incubated with 60 mL of previously prepared buffered rumen juice for each bottle (1:3 mL/mL) as proposed by Goering and Van Soest (1970) under continuous CO₂ reflux in a 100 mL calibrated glass bottle in a water bath at 39 °C. Samples were incubated in quadratic groups together with four bottles containing only an incubation medium (blank). Headspace gas pressure was measured at 2, 4, 8, 16, 24,
36, and 48 h. The kinetic parameters of GP(t) (mL/g DM) were fitted using the NLIN option as a model of France et al. (2000) as

$$G_{v(t)} = b \times \left(1 - e^{-c(t-L)}\right),$$

where $G_{v(t)}$ is the gas produced at time $t$, “$b$” is the asymptotic gas produced (mL/g DM) by the insoluble but slowly fermenting fraction, “$c$” is the constant gas production rate (mL/h), “$t$” is the time of fermentation, and “$L$” is lag time. In vitro CH$_4$ production was determined as the procedure by Pellikaan et al. (2011).

After termination of the incubation, the bottle contents were used for the determination of in vitro neutral detergent fiber degradability (IVNDFD). In vitro liquor from each bottle was collected after filtration to determine the pH using a portable pH meter (HANNA-pH meter, (model HI8424), Woonsocket, RI, USA), NH$_3$-N concentration measured as the procedure AOAC (2016), and the total SCFAs as mentioned by Eadie et al. (1967). Molar proportions of acetate, propionate, and butyrate concentrations were analyzed by gas–liquid chromatography (GC 2010; PerkinElmer, Inc., Shelton, CN, USA) capillary column (HPINNOWAX, 30m_0.250 mm_0.25 mm). The counting of rumen ciliate protozoa was performed under a light microscope according to Dehority (2003). Bacteria and cellulolytic bacteria were counting according to Wanapat et al. (2000).

### Digestibility and blood parameters

Composed feces were from the rectum directly of all animals in each group in the morning before feeding at the end of the collection period. Acid-insoluble ash (AIA) was used as an inner marker to estimate the digestion coefficient of nutrients (Van Keulen and Young 1977). For analysis of feeds, fecal samples were dried at 60 °C and grinding to pass through a 1-mm screen. Estimated dry matter (DM), crude protein (CP), ash, and ether extract (EE) were as procedures of AOAC (2016). Neutral detergent fiber (NDF) was estimated according to Van Soest et al. (1991). Nutrient digestibility coefficients and the nutritive value were counted as equations of Schneider and Flatt (1975).

Blood samples were obtained morning from the jugular vein of each experimental animal in each group before access to feed on the last day of the collection period. The samples were centrifuged at 4000 rpm/15 min to separate the serum and then stored at −18 °C until analysis. Total proteins, albumin, urea nitrogen (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, and glucose concentration were determined using commercial kits (Bio Mérieux SA, F-69280 Marcy l’Etoile, France).

### Milk production and composition

Lactating buffalo were milked twice daily (6:00 and 18:00), and milk production (MP) was recorded for each buffalo during the collection period. Daily milk samples were mixed in the morning and afternoon for each animal and stored at −20 °C for analysis of milk protein, fat, and lactose using infrared Milko-Scan (13BN Foss Electric, Denmark). Ash was estimated according to procedures of AOAC (2016), while total solids (TS) and solid not fat (SNF) were calculated as differences. Fat-corrected milk (FCM, 7%) was calculated according to Raafat and Saleh (1962) using the following equation:

$$\text{FCM} = \left[(0.265 \times \text{milk yield, kg}) + (10.5 \times \text{fat yield, kg})\right].$$

### Table 1

| Item         | Ingredients | Experimental diets |
|--------------|-------------|--------------------|
|              | CFM BF RS CS EUL EUS | $G_1$ $G_2$ $G_3$ $G_4$ |
| OM           | 91.06 86.97 83.18 92.11 95.07 94.93 | 88.09 88.76 88.76 88.09 |
| CP           | 16.35 15.07 2.69 6.82 6.63 6.63 | 12.28 12.37 12.34 12.28 |
| NDF          | 55.65 34.78 73.41 60.00 60.82 62.01 | 53.78 54.21 54.20 53.78 |
| ADF          | 38.26 23.91 48.64 45.64 45.01 49.8 | 58.64 37.22 37.21 36.86 |
| EE           | 3.62 2.34 1.19 2.58 2.59 2.85 | 2.60 2.89 2.86 2.90 |
| Ash          | 8.94 13.03 16.82 7.89 4.93 5.07 | 11.91 11.24 11.24 11.91 |

**CFM** concentrate feed mixture, **BF** fresh berseem, **RS** rice straw, **CS** corn silage, **EUL** eucalyptus leaves, **EUS** eucalyptus seeds, **EUO** eucalyptus oil, **OM** organic matter, **CP** crude protein, **NDF** neutral detergent fibre, **ADF** acid detergent fiber, **SEM** standard error of the mean, **EE** ether extract.
Energy-corrected milk (ECM) was calculated using fat and protein (adjusted to 3.5% fat and 3.2% protein) by Casasús et al. (2004) as the following formula:

\[
\text{ECM (kg)} = \frac{\text{milk production (kg)} \times (383 \times \text{fat }\% + 242 \times \text{protein }\% + 783.2)}{3140}.
\]

Statistical analysis

Kinetics of in vitro gas production was analyzed using Statistical Analytical System (version 9.2; SAS Institute, Cary, NC) according to the General Linear Model as follows:

\[
Y_{ij} = \mu + T_i + e_{ij}
\]  

where \( Y_{ij} \) is the observation, \( \mu \) is the overall mean, \( T_i \) is the fixed effect of the treatments, and \( e_{ij} \) is the random error term common for all observations.

All obtained data from the feeding experiments were subjected to analysis of variance according to a 4 × 4 Latin square design using the general linear model procedures of the Statistical Analysis System Institute (version 9.2; SAS Institute, Cary, NC). The model was

\[
Y_{ijkl} = \mu + P_i + C_j + T_k + e_{ijkl}
\]

where \( Y_{ijkl} \) is the dependent variable under examination, \( \mu \) is the overall mean, \( P_i \) is the fixed effect of the period \( (i = 4) \), \( C_j \) is the random effect of the cow \( (j = 4) \), \( T_k \) is the fixed effect of the dietary treatments \( (k = 4) \), and \( e_{ijkl} \) is the random error. The results are presented as mean values with the standard error of the means. Differences among means at \( P < 0.05 \) were accepted as representing statistical differences. Treatment means were compared by orthogonal polynomials using Duncan’s new multiple range test (MRT, 1955).

Results

In vitro gas production kinetics and fermentation patterns

The pH value of the in vitro incubated diet in G4 was increased \( (P < 0.05) \) significantly compared to the other tested diets (Table 2). The NH3-N, SCFAs, and acetic acid concentrations were declined \( (P < 0.05) \) significantly in G4 as compared to G1, G2, and G3. The propionic acid concentrations of incubated rumen liquor in G2 and G3 were higher \( (P < 0.05) \) significantly compared to G4 and G1.

| Treatment | G1 | G2 | G3 | G4 | ± SEM | P-value |
|-----------|----|----|----|----|-------|---------|
| pH        | 5.99b | 6.05b | 6.07b | 6.25a | 0.037 | 0.0018  |
| NH3-N (mg/L) | 183.37a | 182.55a | 182.04a | 163.61b | 5.150 | 0.0522  |
| SCFAs (mM/L)  | 89.02a | 88.98a | 88.86a | 83.55b | 0.695 | 0.0002  |
| Acetic acid (mol/100 mol) | 59.99a | 59.95a | 59.93a | 57.22b | 0.728 | 0.0484  |
| Propionic acid (mol/100 mol) | 20.83b | 21.53a | 21.54a | 19.99c | 0.252 | 0.0027  |
| Butyric acid (mol/100 mol) | 11.77b | 11.68b | 12.23a | 11.26c | 0.080 | <.0001  |
| C2/C3 ratio | 2.88a | 2.78b | 2.78b | 2.86a | 0.016 | 0.0013  |

abc: Means within the same rows with differing superscripts are significantly different \( (P \leq 0.05) \)

SEM standard error of the mean, CB cellulolytic bacteria, A the exponential total gas mL, B the asymptotic gas produced (mL/g DM) by the insoluble but slowly fermenting fraction, C constant gas production rate (mL/h), L lag time, IVNDFD In vitro neutral detergent fiber degradability
The butyric acid concentrations of incubated rumen liquor in G3 were increased ($P < 0.05$) significantly compared to G1, G2, and G4. However, the C2/C3 ratio was reduced ($P < 0.05$) significantly in G2 and G3 compared to G4 and G1. The present study showed that EUO supplementation with buffalo diets led to a change in the end products of rumen fermentation with a drop in acetate. An in vitro incubated diet G4 reduced ($P < 0.05$) the total count of bacteria and cellulolytic bacteria compared to G2, G3, or G1. No significantly different found of the bacterial total count in G1, G2, and G3, but the cellulolytic bacteria count was lower ($P < 0.05$) in G3 than in G1. Conversely, the count of protozoa significantly increased ($P < 0.05$) with EUO supplementation (G4) compared to supplementation with EUL (G2), EUS (G3), or the control (G1).

As illustrated in Fig. 1, the cumulative gas volume (calculated as overall-means during incubation times) was lower ($P < 0.05$) significant for all treated diets (G2, G3, and G4) than for the control (G1). The lowest volume of gas accumulation was recorded with EUO (G4), followed by in vitro incubated diets with EUL (G2) and EUS (G3) (Table 2). The values of insoluble but slowly fermenting fraction (b) and constant gas released rate (c) were significantly ($P < 0.05$) higher with EUO supplementation (G4) compared to the other tested diets. Otherwise, the lag time was significantly ($P < 0.05$) lower with EUO supplementation (G4) compared to the other tested diets. Supplemental EUL, EUS, or EUO diets cause a significant ($P < 0.05$) lower CH$_4$ production and degradability of IVNDFD compared to the control diet. This study revealed that all supplemented forms of eucalyptus decreased ($P < 0.05$) CH$_4$ and total gas production.

**Feeding trials**

Eucalyptus supplementation of EUS, EUL, or EUO to buffalo diet caused a significant ($P < 0.05$) decrease in MP, 7% fat-corrected milk (FCM), and energy-corrected milk (ECM) compared to the control diet (Table 3). Buffalo in G4 had the lowest values of MP, 7% FCM, and ECM, followed by buffalo in G2 and G3. Milk fat was significantly ($P < 0.05$) decreased in G4 than the other groups. An adverse trend was obtained for the milk content of lactose, while no significant effect was noticed on the content of milk fat.

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**Table 3** Effect of leaves, seeds, and eucalyptus oil supplementation on milk production, its constituents, and feed conversion

| Item                      | Experimental diets | ± SEM | P-value |
|---------------------------|--------------------|------|---------|
| TDMI (kg/h/d)             | G1     | G2       | G3       | G4       | T×P |
|                           | 15.956  | 16.137   | 16.135   | 15.964   | 0.133 | 0.6265 | 0.2937 | 0.7791 |
| MP (kg/h/d)               | 7.00a   | 6.84b    | 6.80b    | 6.54c    | 0.047 | <0.0001 | 0.8900 | 0.6526 |
| FCM (kg/h/d)              | 6.38a   | 6.19b    | 6.10b    | 5.82c    | 0.059 | <0.0001 | 0.9926 | 0.9713 |
| ECM (kg/h/d)              | 9.04a   | 8.78b    | 8.66b    | 8.24c    | 0.068 | <0.0001 | 0.9969 | 0.8982 |
| Milk composition (%)      |         |         |         |         |      |         |         |         |
| Fat                       | 6.16a   | 6.08ab   | 6.02ab   | 5.95b    | 0.060 | 0.1013 | 0.7209 | 0.6964 |
| Protein                   | 3.78    | 3.78     | 3.77     | 3.69     | 0.031 | 0.2065 | 0.6721 | 0.8121 |
| Lactose                   | 4.54ab  | 4.57ab   | 4.66ab   | 4.72a    | 0.050 | 0.0612 | 0.3893 | 0.8416 |
| Ash                       | 1.39    | 1.38     | 1.38     | 1.38     | 0.018 | 0.9647 | 0.9634 | 0.1685 |
| SNF                       | 9.71    | 9.73     | 9.80     | 9.79     | 0.061 | 0.6439 | 0.2387 | 0.4375 |
| TS                        | 15.87   | 15.82    | 15.82    | 15.74    | 0.071 | 0.6798 | 0.5617 | 0.9866 |
| Feed conversion (kg intake/kg FCM 7% fat) |         |         |         |         |      |         |         |         |
| TDMI/FCM                  | 2.503c  | 2.616b   | 2.649b   | 2.746a   | 0.032 | <0.0001 | 0.6758 | 0.5986 |
| TDNI/FCM                  | 1.563b  | 1.642a   | 1.633a   | 1.609ab  | 0.019 | 0.0333 | 0.6652 | 0.5867 |
| NI/FCM                    | 0.049c  | 0.052b   | 0.053ab  | 0.054a   | 0.001 | <0.0001 | 0.6015 | 0.6106 |

Abc: Means within the same rows with differing superscripts are significantly different ($P \leq 0.05$)

SEM: standard error of the mean, TDMI: total dry matter intake, MP: milk production, FCM 7%: fat-corrected milk, ECM: energy-corrected milk, SNF: solid not fat, TS: total solids; NI: nitrogen intake.
of proteins, ash, SNF, and TS by supplementing EUS or EUL.

Feed conversion values identified as DMI/FCM, TDNI/FCM, and NI/FCM were increased \((P < 0.05)\) significantly by either supplemental form of eucalyptus in experimental diets compared to the control. The favorite values of feed conversion were noticed with either EUL or EUS supplementation in G2 and G3 compared to EUO supplementation. There is a discrepancy in the results of studies conducted in vitro gas production kinetics and fermentation patterns (Sallam et al. 2010; Thao et al. 2015; Giller et al. 2020; Onel et al. 2021).

Table 4  Effect of leaves, seeds, and eucalyptus oil supplementation on apparent digestibility coefficients and blood parameters of experimental diets

| Item                        | Experimental diets | ± SEM | \(P\)-value |
|-----------------------------|--------------------|------|-------------|
|                            | \(G_1\)            | \(G_2\) | \(G_3\) | \(G_4\) | \(T\) | \(P\) | \(T \times P\) |
| Nutrient digestibility and nutritive values (%) | | | | | | | |
| DM                          | 66.63a             | 65.86a | 63.88b | 61.73c | 0.358 | <.0001 | 0.3414 | 0.4809 |
| OM                          | 68.41a             | 67.60b | 66.41c | 63.51d | 0.107 | <.0001 | 0.0101 | <.0001 |
| CP                          | 70.62a             | 69.43b | 67.33c | 63.03d | 0.131 | <.0001 | 0.1097 | 0.1292 |
| EE                          | 67.15c             | 76.07a | 75.25a | 73.08b | 0.462 | <.0001 | 0.7607 | 0.8997 |
| NDF                         | 68.11a             | 67.28b | 64.24c | 60.01d | 0.209 | <.0001 | 0.0409 | <.0001 |
| ADF                         | 62.59a             | 62.05b | 59.36c | 57.40d | 0.127 | <.0001 | <.0001 | <.0001 |
| TDN                         | 62.45a             | 62.75a | 61.63b | 58.60c | 0.093 | <.0001 | 0.0109 | <.0001 |
| DCP                         | 8.67a              | 8.55b  | 8.31c  | 7.74d  | 0.016 | <.0001 | 0.1179 | 0.1125 |

Blood parameters

| Item                        | Experimental diets | ± SEM | \(P\)-value |
|-----------------------------|--------------------|------|-------------|
| Total protein (TP), g/dL    | 6.04b              | 6.12a | 6.13a | 5.98c | 0.009 | <.0001 | 0.6034 | 0.3950 |
| Albumin (A), g/dL           | 3.04b              | 3.15a | 3.15a | 3.00b | 0.027 | 0.0003 | 0.7482 | 0.0150 |
| AST, \(\mu/L\)              | 36.38b             | 36.47b | 36.57b | 37.36a | 0.314 | 0.0394 | 0.7197 | 0.0019 |
| ALT, \(\mu/L\)              | 16.06b             | 16.10b | 16.16b | 16.59a | 0.145 | 0.0508 | 0.6927 | 0.0003 |
| Urea (BUN), mg/dL           | 15.39a             | 15.22a | 15.23a | 14.10b | 0.073 | <.0001 | 0.1348 | 0.1178 |
| Glucose, mg/dL              | 58.50d             | 61.80c | 63.73b | 65.23a | 0.120 | <.0001 | 0.2217 | 0.1760 |
| Creatinine, mg/dL           | 1.52b              | 1.50b  | 1.57a  | 1.58a  | 0.014 | 0.0003 | 0.0334 | 0.1999 |

abc: Means within the same rows with differing superscripts are significantly different \((P \leq 0.05)\)

\(SEM\) standard error of the mean, \(DM\) dry matter, \(OM\) organic matter, \(CP\) crude protein, \(EE\) ether extract, \(NDF\) neutral detergent fibre, \(ADF\) acid detergent fibre, \(TDN\) total digestible nutrient, \(DCP\) digestible crude protein, \(AST\) aspartate aminotransferase, \(ALT\) alanine aminotransferase

In contrast, AST and ALT concentrations in the serum of buffalo fed a diet containing EUO \((G_4)\) were significantly \((P < 0.05)\) increased compared to the other groups. The urea concentrations significantly \((P < 0.05)\) decreased in \(G_4\) serum compared to the other groups, while no significant differences were shown among \(G_2\), \(G_3\), and \(G_1\). The concentration of serum glucose was significantly \((P < 0.05)\) affected by supplementation form. Buffalo in \(G_4\) had the highest serum glucose concentration, followed by buffalo in \(G_3\) and \(G_2\), respectively, and the lowest serum glucose concentration was estimated in buffalo in \(G_1\). Creatinine in buffalo fed a diet containing EUS or EUO were significantly \((P < 0.05)\) elevated compared to those fed a control diet \((G_1)\) or a diet containing EUL \((G_2)\).

Discussion

In vitro gas production kinetics and fermentation patterns

There is a discrepancy in the results of studies conducted on ESOs supplements, especially eucalyptus oil, in vitro rumen fermentation, and the extent of their various effects on gas accumulation and CH\(_4\) production (Sallam et al. 2010; Thao et al. 2015; Giller et al. 2020; Onel et al. 2021).
Nevertheless, the current study indicates that natural protection of eucalyptus oil (EUL and EUS) reduces \( \text{CH}_4 \) release with the same ability as EUO, but the protected form reduces also the negative effects of in vitro fiber degradability and fiber and in vivo protein digestibility compared to using EUO, as shown in Table 4. Several results also showed a variation in the effect of these oils on the rate of degradability of nutrient components (Sallam et al. 2010; Castillejos et al. 2006). The reduction in pH values during in vitro incubation of supplemental EUO in the diet was in line with the low values observed in several previous studies (Wang et al. 2009; Thao et al. 2014). Meanwhile, no observed effect of pH values with incubating of EUL or EUS supplementation, which was in line with the results obtained via Manh et al. (2012); Thao et al. (2015). These results suggest that the use of natural protection for supplemental eucalyptus oil as the leaves or seed form reduces the undesirable effects of supplemental eucalyptus crude oil.

Regarding ammonia concentration, the obtained result agreed with those of Vakili et al. (2013); Thao et al. (2015). A study by Castillejos et al. (2006) showed that long-term EUO supplementation caused a reduction of rumen \( \text{NH}_3\text{-N} \) than the control diet. Moreover, Onel et al. (2021) suggested that ESOs may inhibit bacterial production of excess ammonia in the rumen, resulting in reduced deamination of amino acid consequently lowering rumen \( \text{NH}_3\text{-N} \). These findings are consistent with the hypothesis of the current study, which presented the addition of eucalyptus oil in a protected form. Similarly, McIntosh et al. (2003) demonstrated that EUO inhibited the growth of some hyper-ammonia-producing bacterial species (i.e., \textit{Clostridium sticklandii} and \textit{Peptostreptococcus anaerobius}), but other bacterial species such as \textit{Clostridium aminophilum} were less sensitive. Hyper-ammonia-producing bacteria are extant in few numbers in the rumen (\( P < 0.01 \)) population, but they have very high deamination activity (Castillejos et al. 2006). In the same line, Vakili et al. (2013) reported that a higher level of EUO supplementation drove a slight reduction of SCFAs concentrations in the rumen. Similar findings were observed by Wang et al. (2009) when EUO supplementation was used in the sheep diet. Additionally, McIntosh et al. (2003) concluded that the effect of EUO supplementation in the rumen could be ascribed to chemical structures and bioactive components. In the same context, Castillejos et al. (2006) and Giannenas et al. (2011) emphasized that EUO supplementation leads to modification in the end products of rumen fermentation with a drop in acetate. These results may explain the decreased fiber digestibility with the addition of EUO compared to EUL and EUS. The studies by Cobellis et al. (2015) found similar results to those in this study, which indicated a decrease in feed degradability in the rumen. This could be attributed to the non-selective antimicrobial activities of supplemental ESOs, which affect a wide range of microbial subgroups such as cellulolytic bacteria. Furthermore, Morsy et al. (2012) and Onel et al. (2021) showed that adding all the tested ESOs of clove, eucalyptus, garlic, oregano, and peppermint reduced the abundance of rumen archaea and protozoa, especially cellulolytic bacteria.

Along the same lines, Cieslak et al. (2013) stated that ESOs supplemented with ruminant diets could alter digestion and fermentation, and methanogenesis of diets in the rumen by microbial populations. Besides, Sallam et al. (2010) proposed that the potential effect of supplementation with fresh and residual eucalyptus leaves on mitigation of \( \text{CH}_4 \) production be ascribed to a decrease in fermentable substrate rather than to a direct effect on methanogenesis. Analogous results were observed by Manh et al. (2012) in cows that received 100 g/day of eucalyptus leaf meal, which led to the mitigation of rumen \( \text{CH}_4 \) emissions. The study by Thao et al. (2014) reported a drop in \( \text{CH}_4 \) production by less than 15% when using eucalyptus extract.

**Animal feeding and performance**

Several studies have implied supplementation of eucalyptus leaves or eucalyptus oil on feed intake and palatability, but their results were variable and inconsistent (Ahmed et al. 2005). No differences were found in DMI with EUS, EUL, and EUO supplemented with buffalo diets. Similar findings were recorded by Benchaar et al. (2007) and Vakili et al. (2013), while Giannenas et al. (2011) and Abd El Tawab et al. (2020) stated that the quantity of feed intake relies on the dose of ESOs supplemented. On the other hand, Cardozo et al. (2006) revealed that EUO supplementation decreases DMI. The effects of eucalyptus supplementation on DMI may fluctuate with the eucalyptus source, diet type, diet interactions, or adaptation of rumen microbial groups (Yang et al. 2010b). In another study, Sallam et al. (2010b) mentioned that the major component of eucalyptus is 1,8-cineole, followed by \( \alpha \)-pinene could be responsible for the degradation of the chemical constituent and also lead to the acceleration of oxidation. Increased feed conversion efficiency was observed when dairy cows were supplemented with eucalyptus leaf material (Thao et al. 2015) and also with eucalyptus oil (Giller et al. 2020; Al-Suwaiegh et al. 2020). Despite MP and ECM decreased with supplanting eucalyptus, the reverse result was found by Giannenas et al. (2011) and Abd El Tawab et al. (2020) who confirmed that MP was increased with ESOs supplementation into the diets of dairy ewes. Milk contents of protein, fat, and lactose were very contradictory with ESOs supplementation. Some studies have reported an increase in protein content in milk (Wall et al. 2014), while others showed raising in milk fat (Santos et al. 2010). Nevertheless, other studies have found an increase in milk lactose (Benchaar et al. 2007) when dairy cows and ewes diets are supplemented with ESOs.
The digestibility of DM, OM, CP, NDF, and ADF differed ($P > 0.05$) among treatments in studies by Thao et al. (2014; 2015). Similarly, Sallam et al. (2010) concluded that supplementation with EUO influences the degradability of DM and OM in vitro. Furthermore, Santos et al. (2010) found that feed digestibility was affected when using ESOs as a supplement to the diet of lactating dairy cows. The current results are supported by those found by Benchaar et al. (2007) who recognized that apparent total tract digestibilities of DM, CP, and NDF were affected in lactating cows supplemented with 2 g/day of ESOs.

In the present study, eucalyptus supplementation in buffalo diets led to changes in blood components (Table 4). Meanwhile, Morsy et al. (2012) found that dietary supplementation with different ESOs (anise, clove, and juniper) or their combination significantly elevated total protein, albumin, and globulin. In addition, Malekkhahi et al. (2015) and Abd El Tawab et al. (2020) demonstrated that sheep fed garlic ESOs or lambs fed a combined (thymol, carvacrol, eugenol, limonene, and cinnamaldehyde) supplemental diet did not affect plasma total protein and albumin. According to a review by Huang and Lee (2018), the improvement of serum protein of animals fed an ESOs blend could be because of the content of phytochemicals, which have immune stimulation and anti-inflammatory and antioxidative activities. Moreover, Yang et al. (2010b) reported that concentrations of some blood metabolites such as total protein and albumin could be influenced by the type of ESOs by changing the feed intake, and the lack of change in glucose and creatinine concentration may be attributable to lack of DMI alteration by the ESOs.

Therefore, the synthesis of urea in the liver is performed from ammonia absorbed from the rumen; as a result, urea N concentration in the serum of buffalo is highly associated with the rumen NH$_3$-N concentration (Davidson et al. 2003). This interpretation is consistent with the results obtained, as the concentrations of rumen NH$_3$-N (Table 2) were not affected by EUS and EUL supplementation compared with the EUO supplement, which was reflected in BUN. These results disagree with those obtained from Yang et al. (2010a), who investigated different doses of ESOs in beef cattle but were consistent with some of those gotten by Davidson et al. (2003). Moreover, supplementation of EUO in the finishing diet of calves was expected to have pharmacological activity; however, these compounds did not affect the liver enzymes. Many previous studies have revealed that supplementation with ESOs did not affect significantly blood glucose concentration (Vakili et al. 2013; Yang et al. 2010b). These findings agreed with Malekkhahi et al. (2015) who reported that glucose levels showed an alteration in the blood of growing lambs when supplementation of different ESOs or blends of them. However, Yang et al. (2010b) found an increase in creatinine concentration in the blood with the addition of eucalyptus leaves, eucalyptus oil, or ESOs blend to the diet compared to G1 (Al-Suwaiegh et al. 2020). In contrast, Castillo et al. (2012) reported that the ESO blend (carvacrol, cinnamaldehyde, and capsaicin) supplementation decreased the serum creatinine level in calves.

**Conclusions**

Although crude eucalyptus oil can lower in vitro methane release, however, its negative effects on in vitro fermentation parameters (SCFAs and ruminal microorganisms), total tract fiber, and protein digestibility, and animal performance may limit its use. Under the conditions of the present study, the results emphasized that supplementing naturally protected eucalyptus oil either in leaves form or capsules with mature seeds reduced the release of in vitro methane with the same efficiency as using the (unprotected) crude oil and also mitigated its negative effects. The obtained results also indicated that the leaves followed by the seed capsules could be a good alternative option as evidenced by its results compared to the choice of crude oil supplements, especially in its effect on digestion, the production of milk, and its components as well as the blood.

**Author contribution** Fawzy Abo-Donia: Conceptualization, Software, Writing—Original Draft, Writing — Review and Editing, Visualization, Supervision. Usama Naye: Data curation, Methodology, Writing draft preparation. Mohamed Elaref: Methodology, Writing-Original draft preparation, Investigation. Abd El-Moniem Mahgoub: Conceptualization, Resources, Formal analysis, Visualization. Tarek Deraz: Software, Validation Investigation, Formal analysis.

**Funding** Joint support between all of the following: Animal Production Research Institute; Faculty of Agriculture, Sohag University, and Faculty of Agriculture, Menoufia University, Egypt.

**Data availability** All data generated or analyzed during this study are included in this published article.

**Code availability** Not applicable.

**Declarations**

**Ethics approval** All procedures and experimental protocols were carried out according to the guidelines for the care and use of animals in research and teaching per the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

**Consent to participate** All authors agree on their participation in the work herein reported.

**Conflict of interest** The authors declare no competing interests.
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