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RESEARCH ARTICLE

Chemical Composition, *In Vitro* Fermentability, and Anti-Methanogenic Potential of Oak (*Quercus* spp.) Leaves with PEG Supplementation

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Abstract

This study evaluated the chemical composition, in vitro fermentability, and anti-methanogenic potential of oak (*Quercus* spp.) (*Quercus brantii*, *Quercus cerris*, *Quercus coccifera*, *Quercus ithaburensis* subsp. *macrolepis*, *Quercus infectoria*, *Quercus libani*, and *Quercus suber*) leaves, focusing on condensed tannins and the effect of polyethylene glycol (PEG) treatment. Leaves from seven species were collected, dried, and analyzed for dry matter, crude protein, ether extract, crude ash, neutral detergent fiber, acid detergent fiber, and condensed tannins. Fermentation characteristics, methane production, metabolizable energy, and organic matter digestibility were assessed *in vitro*, with and without PEG. Chemical composition and tannin content varied significantly among species ($P<0.001$), with crude protein ranging from 10.25% to 17.41% and condensed tannins from 1.18% to 9.54%. PEG treatment significantly increased total gas production, methane production, metabolizable energy, and organic matter digestibility across all species ($P<0.001$), although methane percentage remained largely unaffected, suggesting tannins indirectly reduce methane rather than acting directly on methanogens. Among species, *Q. cerris* exhibited the highest gas production and digestibility after PEG, whereas *Q. brantii* showed the lowest fermentation efficiency. Species with higher tannin content displayed lower *in vitro* fermentability but greater methane suppression. Findings suggest oak leaves are a valuable feed resource that can enhance ruminant nutrition while helping control methane emissions. Utilizing tannin-rich leaves with PEG provides a dual benefit: improving feed efficiency and contributing to sustainable livestock management.

Keywords: *Quercus* spp., Oak leaves, Condensed tannins, Polyethylene glycol (PEG), methane, *In vitro* gas production

Introduction

Livestock production in ruminant animals is challenged by two main issues: maximizing animal productivity and reducing environmental impacts, including emissions of enteric methane, which are responsible for large amounts of greenhouse gases in the atmosphere [1,2]. In this regard, tree leaves are seen as alternative or complementary feeds to traditional forages for ruminant animals because of their nutritional and bioactive properties. Of these, oak leaves are common and accessible in most parts of the world and are traditionally used as animal feed for ruminants because they provide them with protein, fiber, and mineral requirements for growth and maintenance [3,4].

Although oak leaves are good for ruminant animals, their use is limited by secondary metabolites such as condensed tannins and phenolic compounds, which are anti-nutritional in nature and are responsible for reducing nutrient availability and feed efficiency in ruminants [5,6]. Polyethylene glycol (PEG) is commonly used to reduce tannins and phenolic compounds in oak leaves. PEG binds tannins and phenolic compounds, thus eliminating their anti-nutritional activities and improving nutrient availability and feed efficiency in ruminants without affecting nutrient value [7,8].

Besides their effect on feed utilization, oak leaves also possess anti-methanogenic activity. Oak leaves are tannin-rich and are capable of reducing methane emissions in ruminants by inhibiting methanogenic archaea and reducing hydrogen availability, which is critical for methanogenesis [6,9,10]. As oak leaves possess both activities, they are therefore promising for sustainable ruminant production systems.

There are significant differences in chemical composition and tannin and fermentation potential in oak leaves, depending on oak tree species, which affect their nutritional value and methanogenic activity [3]. The interaction between oak leaves, PEG supplementation, and

microbial activity in ruminants is critical in order to fully understand and optimize oak leaves for ruminant feeding. This study aims to assess the chemical composition, *in vitro* fermentability, and anti-methanogenic activity of *Quercus* species *(Quercus brantii, Quercus cerris, Quercus coccifera, Quercus ithaburensis* subsp. *Macrolepis, Quercus infectoria, Quercus libani,* and *Quercus suber),* including PEG supplementation, in order to understand and optimize ruminant feeding for sustainable ruminant production.

Material and Methods

Collection and Preparation of Oak Leaves

Leaves of seven different oak species, namely, *Quercus brantii, Quercus cerris, Quercus coccifera, Quercus ithaburensis subsp. Macrolepis, Quercus infectoria, Quercus libani,* and *Quercus suber*, were collected from Kahramanmaraş province on May 12, 2025, and immediately transported to the lab. The leaves were dried at room temperature until complete desiccation was achieved. The dried leaves were then ground through a 1 mm sieve and finally stored in nylon bags. The leaves were reserved for further analysis of chemical compositions as well as *in vitro* gas production, ensuring standardized conditions for all experimental procedures.

Chemical Composition Analyses

The dry matter content was obtained by drying the sample at 105°C for 24 h. The crude ash content was obtained by incinerating the sample at 550°C for 6 h using a muffle furnace. The ether extract was obtained by using a Soxhlet extractor with petroleum ether. The crude protein was obtained using the Kjeldahl method as specified by AOAC [11]. The neutral detergent fiber and acid detergent fiber contents were obtained using the procedures specified by Van Soest et al. [12]. The condensed tannins contents were obtained using the butanol-HCl assay as specified by Makkar [13].

Determination of Gas and Methane Production of Oak Leaves

The *in vitro* gas production technique was employed to assess gas and methane production. According to Menke et al. [14]. fresh rumen liquor was collected from slaughtered animals (sheep) at a local abattoir. The fresh rumen liquor was filtered through four layers of cheesecloth under anaerobic conditions. Each sample, consisting of 0.2 g of oak leaf sample with or without PEG 6000 (1 g), was placed in individual 100 ml glass syringes. Thirty milliliters of filtered buffer solution containing rumen liquor was added to each syringe under anaerobic conditions. The glass syringes containing sample-free blanks were used to assess gas production due to buffer solution. Each sample was carried out in triplicate at 39°C for 24 h using a thermostatically controlled incubator. After 24 h of *in vitro* fermentation, gas produced was collected using a plastic syringe. Methane present in the collected gas was detected using an infrared methane analyzer (Sensor Europe GmbH, Erkrath, Germany) [15]. The concentration of methane was expressed as a percentage or milliliters. Net gas production and methane production for each sample were calculated by subtracting the respective blank values.

The volume of gas produced and methane concentration was employed to calculate metabolizable energy (ME) and organic matter digestibility (OMD) using equations developed by Menke & Steingass [16].

$$ME\ (MJ/kg\ DM) = 2.2 + (0.137*GP) + (0.057*CP) + (0.002859*EE^2)$$

$$OMD\ (\%) = 16.49 + (0.9042*GP) + (0.492*CP) + (0.387*CA)$$

GP: Gas production (ml) at 24 h incubation

CP: Crude protein (%)

EE: Ether Extract (%)

CA: Crude ash (%)

Statistical Analysis

Analysis of the data was done using SAS (2005). The effects of species, PEG supplementation, and their interaction on chemical composition and fermentation parameters were determined by two-way ANOVA. Tukey's multiple comparison tests were used to compare the means. Significance was considered at $P<0.05$.

Results

The effect of species on the chemical composition of oak leaves was provided in *Table 1*. The chemical composition of the leaves of seven *Quercus* species differed significantly ($P<0.001$). The range of dry matter (DM) composition was from 32.94% for *Q. suber* to 51.27% for *Q. brantii.* The highest crude protein (CP) composition was found in *Q. suber* (17.41%), while the lowest CP composition was found in *Q. coccifera* (10.25%). The fiber composition of the leaves of different oak species varied. *Q. brantii* contained the highest composition of both NDF (51.15%) and ADF (33.82%), while *Q. suber* contained the lowest composition of NDF (36.45%) and ADF (26.25%). Condensed tannin (CT) composition was highest in Q. cerris (9.54%), while the lowest in *Quercus ithaburensis subsp. macrolepis* (1.18%).

Effect of species and PEG supplementation on *in vitro* gas, methane, metabolizable energy (ME), and organic matter digestibility (OMD) of different oak leaves is shown in *Table 2*. *In vitro* fermentation of the seven *Quercus* species was

Table 1. *The effect of species on the chemical composition (%) of oak leaves*

Species	DM	CA	EE	CP	NDF	ADF	CT
Q. branti	51.27^{a}	4.11^{cd}	2.34^{a}	11.69^{cd}	51.15^{a}	33.82^{a}	1.52^{e}
Q. cerris	50.11^{ab}	2.99^{e}	2.21^{ab}	11.35^{cd}	47.97^{ab}	32.20^{ab}	9.54^{a}
Q. coccifera	43.04^{c}	5.01^{b}	1.97^{ab}	10.25^{d}	43.40^{cd}	29.81^{c}	5.53^{b}
Q. ithaburensis subsp. *macrolepis*	48.68^{b}	3.42^{de}	1.74^{ab}	11.31^{cd}	46.60^{bc}	30.47^{bc}	1.18^{e}
Q. infectoria	49.25^{b}	6.33^{a}	1.87^{ab}	12.81^{c}	42.77^{d}	26.86^{de}	1.70^{e}
Q. libani	44.29^{c}	4.48^{bc}	1.51^{ab}	15.29^{b}	41.69^{d}	28.68^{cd}	3.84^{c}
Q. suber	32.94^{d}	4.79^{bc}	1.38^{b}	17.41^{a}	36.45^{e}	26.25^{e}	2.96^{d}
SEM	0.576	0.201	0.249	0.492	0.964	0.682	0.243
P	***	***	***	***	***	***	***

^{a,b,c,d,e} Column means with common superscript do not differ at P<0.05, ***DM:*** *Dry matter,* ***CA:*** *Crude ash,* ***EE:*** *Ether extract,* ***CP:*** *Crude protein,* ***NDF:*** *Neutral detergent fiber,* ***ADF:*** *Acid detergent fiber,* ***CT:*** *Condensed tannin, *** : p<0.001*

Table 2. *Effect of PEG supplementation on in vitro gas, methane, metabolizable energy (ME), and organic matter digestibility (OMD) of different Quercus species*

Examples	GAS (mL)		Methane (mL)		Methane (%)		ME (MJ/kg DM)		OMD (%)	
	PEG		PEG		PEG		PEG		PEG	
	-	+	-	+	-	+	-	+	-	+
Q. brantii	19.56^{b}	29.46^{b}	2.30	3.80^{b}	11.60^{ab}	12.90^{ab}	5.53^{b}	6.90^{b}	40.73^{b}	49.63^{b}
Q. cerris	26.16^{a}	39.00^{a}	2.30	5.73^{a}	8.83^{b}	14.66^{a}	6.43^{a}	8.20^{a}	46.33^{a}	57.90^{a}
Q. coccifera	24.70^{ab}	29.10^{b}	2.70	3.5^{b}	10.93^{ab}	12.10^{ab}	6.16^{ab}	6.73^{b}	44.80^{ab}	48.80^{b}
Q. ithaburensis subsp. *macrolepis*	26.90^{a}	34.60^{ab}	3.23	4.13^{b}	11.93^{a}	11.83^{b}	6.53^{a}	7.56^{ab}	47.00^{a}	54.00^{ab}
Q. infectoria	23.23^{ab}	32.02^{ab}	2.46	3.46^{b}	10.60^{ab}	10.73^{b}	6.06^{ab}	7.26^{ab}	44.96^{ab}	52.90^{ab}
Q. libani	24.70^{ab}	30.93^{b}	2.86	3.80^{b}	11.40^{ab}	12.30^{ab}	6.43^{a}	7.30^{ab}	47.20^{a}	52.80^{ab}
Q. suber	26.16^{a}	32.76^{ab}	2.60	4.00^{b}	9.83^{ab}	12.13^{ab}	6.76^{a}	7.60^{ab}	49.60^{a}	55.56^{ab}
SEM	1.674	2.208	0.319	0.435	0.812	0.798	0.227	0.300	1.510	1.985
P	0.010	0.007	0.106	0.002	0.023	0.009	0.002	0.005	0.002	0.006
Species	***		***		NS		***		***	
PEG	***		***		***		***		***	
Species x PEG	NS		NS		***		NS		NS	

^{a,b} Column means with common superscript do not differ at P<0.05, ***ME:*** *Metabolisable energy (MJ/kg DM), OMD (%),* ***SEM:*** *Standard error mean,* ***PEG:*** *Polyethylene glycol,* ***NS:*** *Not significant,* ***+:*** *With PEG added,* ***-:****Without PEG added, *** : p<0.001*

significantly affected by PEG supplementation (P<0.001). The total gas production and methane production were significantly enhanced in all the tested species after PEG supplementation (P<0.01). Among the tested species, *Q. cerris* had the highest gas production (26.16 mL without PEG, 39.00 mL with PEG) and methane production (2.30 mL without PEG, 5.73 mL with PEG), while *Q. brantii* had the lowest gas production (19.56 mL without PEG, 29.46 mL with PEG) and OMD (40.73% without PEG, 49.63% with PEG). Methane proportion (%) was not significantly affected by PEG supplementation (P>0.05). The metabolizable energy (ME) and OMD were significantly enhanced by PEG supplementation in all the tested species (P<0.01). The highest ME and OMD were observed in *Q. cerris* and *Q. suber* after PEG supplementation. Significant differences were observed among the tested species in gas production, methane production (mL), ME, and OMD, while the interaction between species and PEG supplementation was significant only in methane proportion (%), suggesting that the relative response of methane production to PEG varies among species.

Discussion

The present study revealed a strong species-specific variation with respect to the chemical composition as well as the *in vitro* fermentability of the leaves of *Quercus*, which largely accounted for the variations observed

with respect to the ruminal fermentation characteristics. Species like **Q. suber** and **Q. libani**, which possessed a higher crude protein content as well as a lower fiber content, showed a higher digestibility as well as energy availability, as previously established with respect to the nutritive value of oak leaves [3]. On the contrary, species with a higher content of condensed tannins, like **Q. cerris**, revealed a reduced fermentability but a higher potential for the suppression of methane production. This result is consistent with the well-established trade-off between the nutritive value and anti-methanogenic properties of tannin-containing feeds, as previously established [5,6].

The inclusion of polyethylene glycol significantly increased gas production, methane yield, metabolizable energy, and organic matter digestibility of all the species, validating the biological activity of tannins present in oak leaves. PEG effectively complexes with tannins, thus neutralizing their inhibitory effect on the activity of microbe-derived enzymes and substrate degradation [5,7]. This consequently allows rumen microflora to have greater access to available substrates for degradation, hence increasing the rates of fermentation. These results are consistent with other studies that have shown an increase in gas production and digestibility of feedstuffs supplemented with PEG in tannin-containing diets *(Fig. 1)* [8,17,19,20].

Though there was an increase in the production of total methane (mL), the percentage of methane production (%) remained unaffected which indicates that tannins do not have a direct inhibitory effect on methanogenic microorganisms but have an indirect inhibitory effect by reducing the production of methane through the suppression of the overall process of microbial fermentation *(Fig. 2)*. This has been supported by earlier studies, which have suggested that the anti-methanogenic effects of tannins are related more to the reduced availability of hydrogen and the suppression of microorganisms as a whole, rather than the direct toxicity of tannins towards methanogens [5,9,18,21,22].

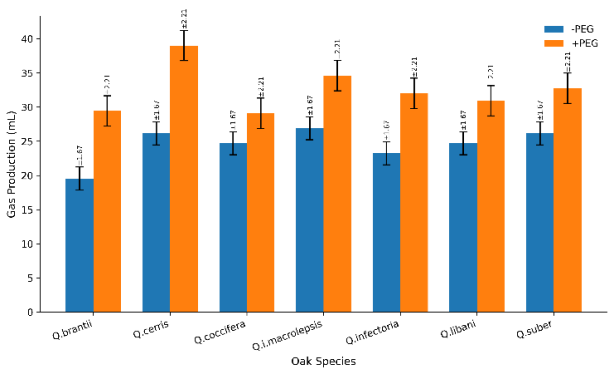

Fig 1. Effect of PEG addition on gas production

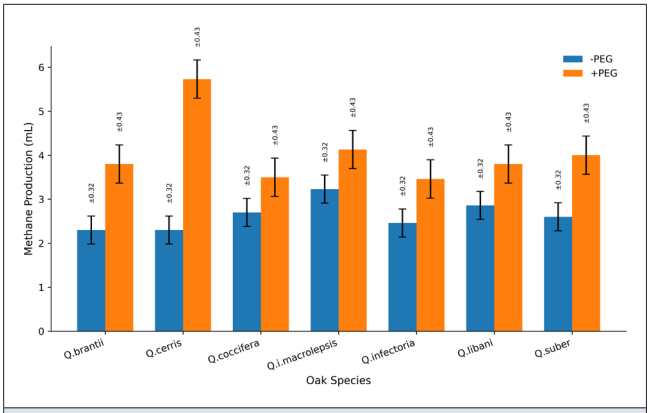

Fig 2. Effect of PEG addition on methane production (mL)

Most importantly, the failure to establish a clear relationship between CT concentration and fermentation parameters suggests that the effects of tannins cannot be accounted for by their concentration. The biological activity of tannins depends on their chemical structure, such as molecular weight, degree of polymerization, and protein binding affinity [5,6]. Consequently, it can be suggested that tannins at similar concentrations may have significantly different effects on rumen microbe fermentation. This may account for the variation observed between species.

Other compositional factors, aside from tannins, may have played a role in the observed variability, which include fiber composition and the potential involvement of secondary metabolites beyond tannins. The variability in NDF and ADF composition may affect degradation, while the structural composition, which may include lignin and other phenolic compounds, may have additional effects on microbial processes [9,18]. The above studies indicate that the responses to oak leaves in ruminal fermentation processes are likely the result of complex interactions among nutrient composition, tannin characteristics, and other plant secondary metabolites.

The positive effects of ME and OMD after PEG supplementation have again emphasized the negative effect of tannins on the nutritive values of feed *(Fig. 3, Fig. 4)* [23-25]. Tannins are known to have negative effects on digestibility by binding proteins and inhibiting fibrolytic activity [26-28]. This negative effect on digestibility is closely related to the positive effect of tannins on mitigating methane emission. From a practical point of view, the species that have greater effects on inhibiting fermentation may have greater mitigation effects on methane emission, while the species that have lower effects on tannins may have greater nutritive values.

Overall, the findings support the notion that tannins in oak leaves have a twofold impact on ruminant animals, reducing methane production while simultaneously reducing animal feed utilization. The twofold impact

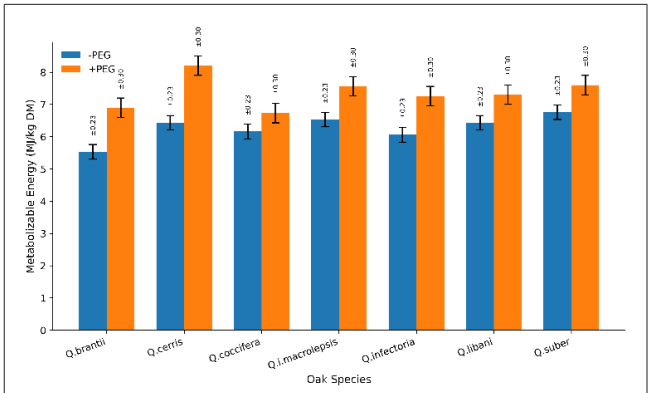

Fig 3. Effect of PEG supplementation on metabolic energy content

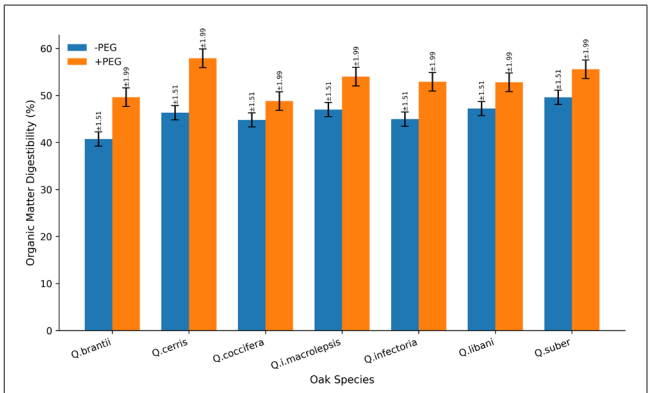

Fig 4. Effect of PEG supplementation on the degree of organic matter digestibility

highlights the need to develop strategic feeding models in which tannin-containing forages or tannin-binding agents like PEG have the potential to be optimized in terms of animal production and environmental sustainability [1,2,23-26].

The present findings are consistent with previous reports indicating that condensed tannins exert complex and dose-dependent effects on rumen fermentation dynamics. Tannins are known to suppress methanogenesis primarily through indirect inhibition of methanogenic archaea and protozoal populations, as well as by decreasing fiber degradation and hydrogen availability in the rumen. This mechanism may explain the observed reduction in methane production associated with oak leaf inclusion. Similar reductions in enteric methane emissions have been reported in studies evaluating tannin-rich tree leaves and shrub species, suggesting that tannin-containing feed resources could contribute to environmentally sustainable ruminant production systems. However, the beneficial environmental effects of tannins are often accompanied by negative nutritional consequences. The reduced feed utilization observed in the present study may be attributed to the strong binding affinity of tannins to dietary proteins, carbohydrates, and digestive enzymes, resulting in lower nutrient digestibility and microbial activity. Excessive tannin concentrations can particularly impair cellulolytic bacteria, thereby reducing fiber degradation and overall ruminal fermentation efficiency. Consequently, although methane mitigation represents an important advantage, decreases in nutrient utilization and animal performance may limit the practical applicability of high-tannin forages when used without dietary balancing strategies [9,18,29,30].

Nonetheless, as the present findings were conducted under *in vitro* conditions, caution is required when extrapolating the findings to *in vivo* systems. The response of animals may vary depending on certain parameters such as feed intake, adaptation of rumen microbiota, and the composition of the diet. Further *in vivo* research is required to determine the optimal inclusion levels, to assess the performance of the animals, and to confirm the methane mitigating potential under practical feeding conditions.

In conclusion the oak leaves are indicative of a promising natural feed source with co-benefits in providing protein to ruminants with moderate protein content while offering potential benefits in mitigating enteric methane production with its biologically active condensed tannins. The *in vitro* PEG assay confirmed that tannins in oak leaves are active in inhibiting rumen microbial fermentation because they increased gas and methane production in PEG-treated samples. This confirmed that the potential benefits of oak leaves in mitigating methane production are more dependent on the biological activity and chemical structure of tannins rather than their concentration. Oak leaves are promising as a sustainable and locally available feed source for mitigating enteric methane production in ruminants, but its inclusion in ruminant diets needs to be carefully optimized to meet protein protection, digestibility, palatability, and fermentation efficiency. Therefore, oak leaves are promising in providing substantial potential benefits as a feed source in mitigating enteric methane production in ruminants while promoting sustainable productivity.

Declarations

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Declaration of Generative Artificial Intelligence (AI): The article and/or tables and figures were not written/created by AI and AI assisted technologies.

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