Check for updates

*Grass and Forage Science*

WILEY

Grass and Forage Science

ORIGINAL ARTICLE OPEN ACCESS

# Screening of and Mechanistic Insights Into the Enteric Methane Mitigation Potential of European Native and Non-Native Forage Trees, Shrubs, and Herbs Using In Vitro Batch Culture

J. Van Mullem[1,2] | J. Jeyanathan[1] | N. Peiren[2] | P. Vermeir[3] | J. Valckx[4] | W. Govaerts[4] | J. Barbry[5] | K. Verheyen[6] | V. Fievez[1]

[1]Laboratory for Animal Nutrition and Animal Product Quality, Department of Animal Science and Aquatic Ecology, Ghent University, Ghent, Belgium | [2]Flanders Research Institute for Agriculture, Fisheries and Food, Merelbeke-Melle, Belgium | [3]Laboratory for Chemical Analysis (LCA), Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium | [4]Govaerts & Co Cvba, Veerle, Belgium | [5]Inagro, Rumbeke-Beitem, Belgium | [6]Forest & Nature Lab, Department of Environment, Ghent University, Ghent, Belgium

**Correspondence:** V. Fievez (veerle.fievez@ugent.be)

**Received:** 12 February 2025 | **Revised:** 23 May 2025 | **Accepted:** 10 June 2025

**Funding:** This work was supported by Flemish Government, Department of Agriculture and Fisheries; Fonds Wetenschappelijk Onderzoek, 1SC6324N.

**Keywords:** ensiling | enteric methane | fermentability | headspace gas composition | in vitro batch incubation | polyethylene glycol

## ABSTRACT

Enteric methane ($CH_4$) emissions from ruminant livestock must be mitigated to reduce their climate impact. Trees, shrubs, and herbs have gained attention for their nutritional value, climate resilience, and $CH_4$ reduction potential. This in vitro study evaluated 45 forage species harvested in Flanders, Belgium (July 2022), for their effects on enteric $CH_4$ production (μmol/g DM), total volatile fatty acid (VFA) production (μmol/g DM), and relative $CH_4$ production ($CH_4$:total VFA, mol/mol). Leaf traits from the TRY database were included. Twelve promising species were selected for a second experiment (July 2023) using fresh and ensiled substrate. Polyethylene glycol (PEG) was used to assess the activity of tannins in fresh substrates. Different headspace conditions (100% $CO_2$ vs. 50% $CO_2$/50% $H_2$) were used to assess the impact on methanogens. Reduced $CH_4$ production in the first experiment was mainly linked to reduced fermentability, and leaf traits are more closely related to fermentability than direct $CH_4$ mitigation. *Alnus glutinosa*, *Castanea sativa*, *Catalpa bignonioides*, *Populus nigra*, and *Hedera helix* emerged as the most effective $CH_4$ mitigators. Ensiling reduced the concentration of phenolic compounds. PEG assays confirmed the role of tannins in some species; however, PEG was ineffective against the hydrolysable tannins in *C. sativa*. *C. sativa*, *A. glutinosa*, *H. helix*, and *C. bignonioides* exhibited anti-methanogenic effects, likely due to plant secondary metabolites, some of which were only indirectly evaluated (tannins and total phenolic compounds). Further phytochemical and microbiological analyses, along with in vivo trials, are needed to confirm these forages' practical application in livestock diets.

## 1 | Introduction

Enteric methane ($CH_4$) is a major contributor to climate change, accounting for approximately 30% of global anthropogenic $CH_4$ emissions (FAO 2017). To limit the climate impact of ruminant livestock, feeding strategies can be implemented (Beauchemin et al. 2022). Forages play a central role in dairy cattle diets (Hassanat et al. 2013), providing essential nutrients such as carbohydrates and proteins (Vuolo et al. 2019). However, these forages also contribute to enteric $CH_4$ production, with their

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2025 The Author(s). *Grass and Forage Science* published by John Wiley & Sons Ltd.

impact influenced by factors such as chemical composition and fermentability (Russell and Hespell 1981). In response to the environmental challenges posed by dairy production, there is a growing interest in partially replacing traditional forages with alternative plant species such as herbs, shrubs, and trees. This approach is particularly relevant to organic farming, where many existing solutions (e.g., 3-NOP, nitrate) do not align with the principles or legal requirements. Fodder trees and shrubs are more resilient to climate change than grasses, maintaining foliage production in tough conditions (Balehegn 2017) and providing ecosystem services (Vandermeulen et al. 2018). Beyond providing essential nutrients, these alternative forages may also help to reduce enteric $CH_4$ production. Several studies performed in vitro screenings to identify the potential of alternative forage plants for their $CH_4$ mitigating potential (Jayanegara et al. 2011; Macheboeuf et al. 2014; Terranova et al. 2018). However, there is a high variability in their effects on ruminal fermentation variables between different plant species but also within a single species. The mitigating potential of these plants is often attributed to their chemical composition, and more specifically the presence of secondary plant metabolites (PSM) (Ku-Vera et al. 2020; Huang et al. 2023; Lambo et al. 2024). However, plants rich in PSM may also be less palatable or even toxic. Nevertheless, species with promising $CH_4$-mitigating properties may still be valuable as sources of bioactive compounds for extract-based applications. Additionally, some species may currently have limited availability, although this could potentially be overcome through cultivation.

Within the plant kingdom, leaf traits can be used to identify the resource use strategy. This concept is captured by the Leaf Economic Spectrum (LES), a universal gradient ranging from acquisitive (fast-growing) to conservative (slow-growing) strategies (Wright et al. 2004). Furthermore, several of these leaf traits (e.g., leaf N content, leaf dry matter content) are correlated with forage digestibility (Pontes et al. 2007; Gardarin et al. 2014). Additionally, plant defence strategies, including structural defences (e.g., lignin and cellulose) and chemical defences (e.g., phenolic content) are likely to depend on LES traits (Mohanbabu et al. 2023). If such correlations exist, leaf traits may serve as reliable indicators to predict the $CH_4$ mitigation potential of a plant species. PSM-like tannins and saponins are already studied extensively (Ku-Vera et al. 2020); however, understanding in the mode of action and potential to reduce enteric $CH_4$ production is rather limited. In vitro batch incubations can be a useful tool to evaluate the mitigating potential of different herbs, shrubs, and trees. Despite its limitations (Yáñez-Ruiz et al. 2016), small adjustments to this system can create artificial conditions that provide more insights into the $CH_4$ mitigation mechanism. For instance, polyethylene glycol (PEG) can be added to the in vitro system to assess the effect of tannins on fermentation variables (Makkar et al. 1995). The addition of PSM can inhibit $H_2$-producing ruminal microorganisms, indirectly reducing $CH_4$ production by limiting the availability of $H_2$ or directly by inhibiting methanogens (Ku-Vera et al. 2020; Lambo et al. 2024). Since $H_2$ uptake by methanogens is directly proportional to its concentration in the headspace, higher $H_2$ levels result in increased $CH_4$ production (Czerkawski et al. 1972). Thus, artificially increasing the $H_2$ concentration in the headspace may serve as an indicator of methanogen inhibition. While in vitro systems have limitations, they provide a controlled, simplified environment to examine impacts on ruminal fermentation.

This study aimed (1) to evaluate the $CH_4$-mitigating potential of a range of native and non-native European plant species, (2) to identify common leaf traits among those with effective mitigation properties, (3) to explore the effect on $CH_4$-mitigating properties under artificial in vitro conditions to provide insights into the underlying mechanisms of $CH_4$ reduction. To the best of our knowledge, our study is the first to combine screening with the investigation of potential mechanisms of action by making simple setup modifications to the in vitro system, such as adjusting the headspace composition.

## 2 | Materials and Methods

### 2.1 | Overview of the Experimental Setup

A schematic overview of the experimental setup is shown in Figure 1. Here an overview of the experimental setup is given, while details on collection, transport, storage and handling of the plant material as well as in vitro incubations, chemical analysis and statistical treatment are given below. In the first experiment, 45 plant species commonly found in Western Europe, including both native and non-native species, were selected for an initial screening of their $CH_4$ mitigation potential. Plant species were primarily selected based on their potential relevance for ruminant diets or their widespread occurrence in the landscape. However, not all selected plant species are intended for direct inclusion in ruminant feed. Data from the TRY Plant Trait Database (Kattge et al. 2020) was used to categorise the plant species into two groups: woody and herbaceous plant species. All plant species were collected within a single harvest window of 10 days in early July 2022. To introduce variation in plant material, each species was harvested from three different geographical locations in Flanders (Belgium). The specific locations, however, varied between plant species and were not fixed. Fresh plant material was incubated for 24 h with rumen inoculum, a bicarbonate/phosphate buffer under 100% $CO_2$ headspace. To assess their potential to reduce both absolute $CH_4$ production (mmol/g dry matter (DM)) and relative $CH_4$ production (expressed as mmol $CH_4$ per mmol volatile fatty acids (VFA)), a control mixture was included in the incubation. The control mixture was composed of 65% perennial ryegrass (*Lolium perenne*) and 35% white clover (*Trifolium repens*), harvested at the same time and from the three different locations, and represents the standard basal roughage used for organic ruminants in Flanders.

Based on the potential to reduce relative $CH_4$ production of the first experiment, 12 plant species were selected for a second experiment to explore the working mechanism underlying the $CH_4$ reduction (Tables 1–3). For this selection procedure, besides mitigating potential, practical relevance, effect on overall ruminal fermentation, and mitigation potential in literature were considered. The selected species were harvested within a 10-day window in July 2023, across the same three geographical locations sampled in 2022. Plant material was pooled over different locations and used as substrate in this experiment. Again, fresh material of the 12 selected plant



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

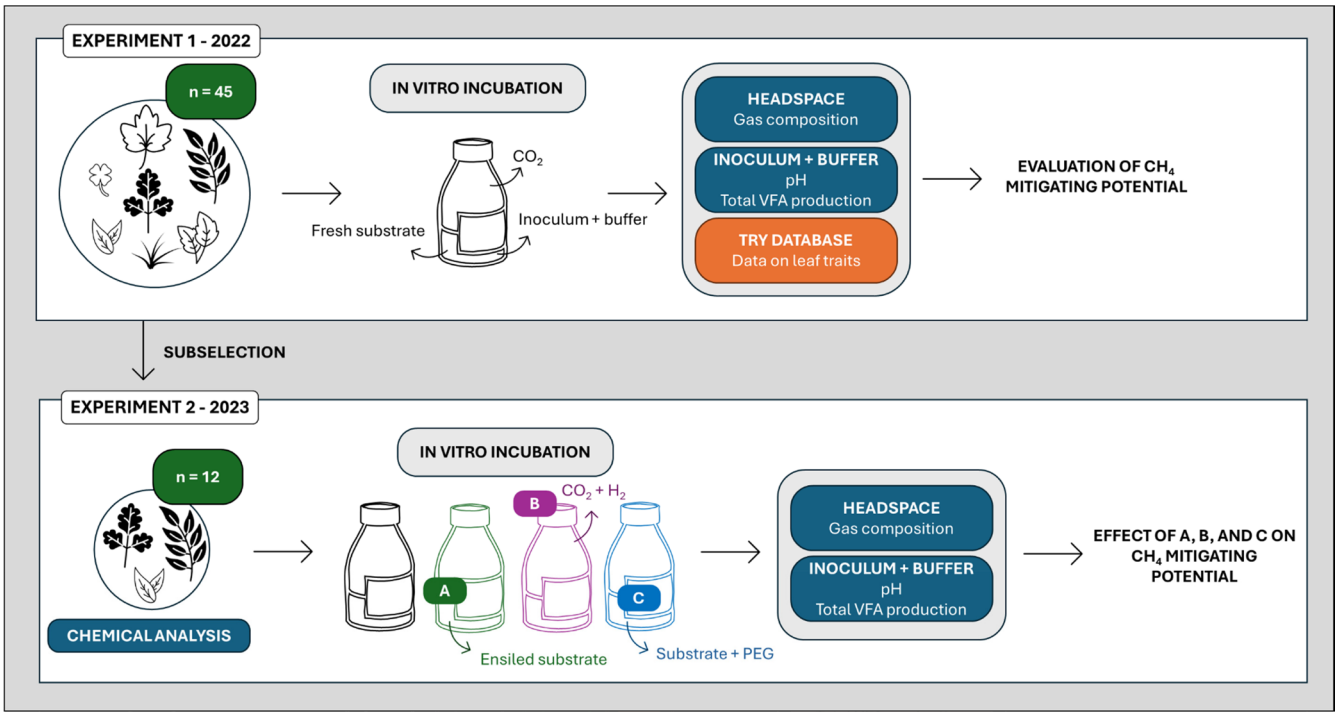



**FIGURE 1** | Schematic overview of the experimental set up.

**TABLE 1** | LS means of fermentation characteristics of fresh (F) and ensiled (S) plant material.

| | | | $CH_4$ (μmmol/g DM) | | Total VFA (mmol/g DM) | | Relative $CH_4$ (mol/mol) | |
|---|---|---|---|---|---|---|---|---|
| | No. | Group | F | S | F | S | F | S |
| Control mixture | 1 | A | 1.58 | 1.47 | 5.43$^{b}$ | 5.09$^{a}$ | 0.29 | 0.29 |
| *Alnus glutinosa* | 3 | B | 0.30* | 0.36* | 2.91*$^{a}$ | 3.17*$^{b}$ | 0.10* | 0.11* |
| *Castanea sativa* | 6 | C | 0.09*$^{a}$ | 0.93*$^{b}$ | 1.37*$^{a}$ | 3.63*$^{b}$ | 0.06*$^{a}$ | 0.26$^{b}$ |
| *Catalpa bignonioides* | 7 | C | 0.44*$^{a}$ | 1.16*$^{b}$ | 3.11*$^{a}$ | 4.15*$^{b}$ | 0.14*$^{a}$ | 0.28$^{b}$ |
| *Corylus avellana* | 9 | E | 0.62* | 0.67* | 2.59* | 2.66* | 0.24 | 0.25 |
| *Cydonia oblonga* | 10 | E | 0.55*$^{a}$ | 0.74*$^{b}$ | 2.61* | 2.74* | 0.21*$^{a}$ | 0.27$^{b}$ |
| *Hedera helix* | 13 | D | 0.93*$^{a}$ | 1.12*$^{b}$ | 5.22 | 5.06 | 0.18* | 0.22 |
| *Myrica gale* | 15 | E | 0.53*$^{a}$ | 0.73*$^{b}$ | 2.16* | 2.11* | 0.25$^{a}$ | 0.35$^{b}$ |
| *Populus nigra* | 17 | D | 0.82*$^{a}$ | 1.49$^{b}$ | 4.14*$^{a}$ | 4.49*$^{b}$ | 0.20*$^{a}$ | 0.34$^{b}$ |
| *Ribes × nidigrolaria* | 25 | E | 0.84*$^{a}$ | 1.03*$^{b}$ | 3.57*$^{a}$ | 3.84*$^{b}$ | 0.24 | 0.27 |
| *Sambucus nigra* | 29 | D | 1.06* | 1.17* | 4.89* | 4.99 | 0.22* | 0.24 |
| *Hypericum perforatum* | 36 | D | 0.85* | 0.94* | 3.96*$^{b}$ | 3.47*$^{a}$ | 0.21*$^{a}$ | 0.27$^{b}$ |
| *Rumex obtusifolius* | 41 | E | 0.65*$^{a}$ | 1.00*$^{b}$ | 2.65*$^{a}$ | 3.39*$^{b}$ | 0.24$^{a}$ | 0.30$^{b}$ |

*Note:* Different letters indicate a significant difference within a species between fresh and ensiled plant material based on Tukey post hoc test. Group (A, B, C, D, and E) indicate the plant species that cluster together in the PCA in Figure 8 and can be considered as one group.
*A significant difference with control mixture (65% *Lolium perenne*, 35% *Trifolium repens*) based on a Dunnet's post hoc test.

species and the control mixture was incubated under the same conditions as in the first experiment. Furthermore, all selected plant species and the control mixture were also subjected to three additional treatments, including (A) ensiled substrate, (B) 50% $CO_2$ + 50% $H_2$ headspace, and (C) fresh substrate + PEG. Throughout both experiments, in vitro batch incubation techniques were used.

## 2.2 | Harvesting and Storing Substrates

For the trees and shrubs, 300–400 g of fresh material was collected in a plastic bag that was closed airtight and stored in a cooling box. The aerial vegetative components were collected from multiple plants at the same site for each plant species. Only non-woody twigs with attached leaves or leaflets that



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

**TABLE 2** | LS means of absolute $CH_4$ production (mmol/g DM) for each of the selected plant species in different headspace gas compositions ($CO_2$ and $CO_2 + H_2$).

| | | | Absolute $CH_4$ production (mmol $CH_4$/g DM) | |
|---|---|---|---|---|
| | No. | Group | $CO_2$ | $H_2 + CO_2$ |
| Control mixture | 1 | A | 1.58[a] | 3.01[b] |
| *Alnus glutinosa* | 3 | B | 0.30* | 0.50* |
| *Castanea sativa* | 6 | C | 0.11* | 0.10* |
| *Catalpa bignonioides* | 7 | C | 0.44* | 0.51* |
| *Corylus avellana* | 9 | E | 0.62*[a] | 1.81*[b] |
| *Cydonia oblonga* | 10 | E | 0.55*[a] | 1.32*[b] |
| *Hedera helix* | 13 | D | 0.90* | 0.96* |
| *Myrica gale* | 15 | E | 0.53*[a] | 1.25*[b] |
| *Populus nigra* | 17 | D | 0.82*[a] | 2.30*[b] |
| *Ribes × nidigrolaria* | 25 | E | 0.84*[a] | 1.88*[b] |
| *Sambucus nigra* | 29 | D | 1.06*[a] | 2.16*[b] |
| *Hypericum perforatum* | 36 | D | 0.85*[a] | 1.45*[b] |
| *Rumex obtusifolius* | 41 | E | 0.65*[a] | 1.22*[b] |

*Note:* Different letters indicate a significant difference within a species between different conservation methods based on Tukey post hoc test. Group (A, B, C, D, and E) indicate the plant species that cluster together in the PCA in Figure 8 and can be considered as one group.
*A significant difference with the control mixture (65% *Lolium perenne*, 35% *Trifolium repens*) based on a Dunnet's post hoc test.

were grown during the growing season of 2022 or 2023 were collected at a plant height between 1 and 2.5 m as this represents the browsing height of cows. For the herbaceous plant species, 100 g of fresh material including leaves and stems from different plants was harvested and placed in an airtight plastic bag. The longest interval between harvesting and arrival at the laboratory was overnight, with all samples received the following morning. During transport, samples were stored in closed containers with ice packs, ensuring dark conditions and maintaining temperatures below 4°C. The fresh plant material (tree, shrubs, and herbs) was frozen at −20°C and subsequently freeze-dried, as this method best preserves the characteristics of fresh material (Fievez et al. 2004). Before using it as an incubation substrate, this plant material was ground and sieved (0.212 mm < substrate particles > 1 mm) and stored in a desiccator in the dark. Throughout the text, the freeze-dried substrate is referred to as "fresh" substrate, since freeze-drying. Lab-scale silages were made as previously described by Gadeyne et al. (2016) with small adaptations. Fresh material (100 g) of the harvest in July 2023 was cut (pieces of 1–2 cm), sprayed with formic acid solution (5%) and placed in a vacuum bag (250 × 300 mm, 90 μm, FPS-multiproducts NV). These bags were stored in a dark place at room temperature for up to 30 days. After the ensiling process was finished, substrates were frozen (−20°C), freeze-dried, ground, and sieved in the same way as the fresh samples.

## 2.3 | In Vitro Incubation

The in vitro incubation was performed as described in Vlaeminck et al. (2014) with small adaptations. In short, substrates were incubated for 24 h in an in vitro batch system using rumen fluid from Holstein Friesian rumen fistulated lactating dairy cows. The rumen fluid was collected just before morning feeding (8:00 a.m.) through the rumen cannula using a vacuum suction pump and a perforated sampling probe. Approximately 1 L of rumen fluid was collected from at least three different cows that were fed the same diet. The basal diet was composed of 50% maize silage and 50% wilted grass silage (on a DM basis) supplemented with some straw and urea. Additionally, soybean meal and balanced (based on net energy and protein digestible at the level of the small intestine, using the Belgian/Dutch VEM and DVE evaluation system) compound feed was administered to fulfil the animals' requirements for milk production. The collected rumen fluid was transferred into a pre-heated insulated flask and transported to the laboratory. After filtration through a sieve with a pore size of 1 mm and under continuous $CO_2$ flushing at 39°C, the rumen fluid of the different cows was combined and then mixed with a bicarbonate/phosphate buffer (containing 3.58 g $Na_2HPO_4 \cdot 12H_2O$, 1.55 g $KH_2PO_4$, 0.124 g $MgCl_2 \cdot 6H_2O$, 8.74 g $NaHCO_3$, and 1 g $NH_4HCO_3$ per litre of distilled water). Incubation was performed in triplicate in 120-mL flasks. The flasks were sealed and flushed with $CO_2$. Afterwards, $CO_2$-saturated bicarbonate/phosphate-buffered rumen fluid was added (buffer + water:rumen fluid 4:1, v:v) to reach a total liquid volume of 25 mL. The flasks were incubated at 39°C in a shaking incubator (Edmund Bühler GmbH, Hechingen, Germany) for 24 h. The amount of substrate used per flask was 250 mg. Blanks were not included as plant secondary metabolites can influence fermentation not only of the added substrate but also of organic matter present in the inoculum (Araujo et al. 2011). Although additive-specific blanks as suggested by Araujo et al. (2011) could help correct for this, such an approach was not possible in our study, as the substrate itself contained the secondary metabolites. For the second experiment on the working mechanism, the headspace was either filled with 100% $CO_2$ or 50% $H_2$ and 50% $CO_2$. To determine if tannins contribute to the reduction in $CH_4$ production, additional flasks containing 50 mg of Polyethylene glycol (PEG 6000) were included, as PEG is known to bind tannins and reduce their impact on ruminal fermentation variables (Makkar et al. 1995). After 24 h, the fermentation process was stopped by placing the incubation bottles in an ice bath to stop microbial activity.

After each incubation, gas composition of the headspace was analysed by connecting the incubation flasks directly to the micro gas chromatograph (990 Micro GC System, Agilent). The system automatically withdrew a subsample of the headspace gas for injection. Ethane was used as the internal standard. After opening the bottle, pH of incubation contents was measured. A 1 mL aliquot of the incubation content was acidified with 100 μL formic acid, containing the internal standard (10 mg 2-ethyl butyric acid/mL formic acid) for VFA analysis using a gas chromatograph (HP 7890A, Agilent Technologies) (Gadeyne et al. 2016). The net production of



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

**TABLE 3** | LS means of the fermentation characteristics of the incubation (24 h) with inoculum without (CTRL) and with the addition of polyethylene glycol (PEG).

| | | | Absolute $CH_4$ (mmol/g DM) | | Total VFA production (mmol/g DM) | | Relative $CH_4$ (mmol/mmol) | |
|---|---|---|---|---|---|---|---|---|
| | No | Group | CTRL | PEG | CTRL | PEG | CTRL | PEG |
| Control mixture | 1 | A | 1.58 | 1.53 | 5.43 | 5.23 | 0.29 | 0.29 |
| *Alnus glutinosa* | 3 | B | 0.30[a]* | 0.44[b]* | 2.91[a]* | 3.46[b]* | 0.10* | 0.13* |
| *Castanea sativa* | 6 | C | 0.11* | 0.10* | 1.37[a]** | 2.25[b]* | 0.06* | 0.05* |
| *Catalpa bignonioides* | 7 | C | 0.44* | 0.46* | 3.11* | 3.18* | 0.14* | 0.14* |
| *Corylus avellana* | 9 | E | 0.62[a]* | 1.16[b] | 2.59[a]* | 3.98[b]* | 0.24[a] | 0.30[b] |
| *Cydonia oblonga* | 10 | E | 0.55[a]* | 1.18[b] | 2.61[a]* | 4.47[b]* | 0.21[a] | 0.26[b] |
| *Hedera helix* | 13 | D | 0.91* | 0.93* | 5.27 | 5.26 | 0.17 | 0.18 |
| *Myrica gale* | 15 | E | 0.53[a]* | 0.95[b]* | 2.16[a]* | 3.33[b]* | 0.25[a] | 0.29[b] |
| *Populus nigra* | 17 | D | 0.82* | 0.83* | 4.14* | 4.22* | 0.20 | 0.20 |
| *Ribes × nidigrolaria* | 25 | E | 0.84[a]* | 1.37[b] | 3.57[a]* | 4.78[b]* | 0.24[a] | 0.29[b] |
| *Sambucus nigra* | 29 | D | 1.06* | 1.08 | 4.89* | 5.08 | 0.22 | 0.21 |
| *Hypericum perforatum* | 36 | D | 0.85[a]* | 1.03[b] | 3.96[a]* | 4.67[b]* | 0.21 | 0.22 |
| *Rumex obtusifolius* | 41 | E | 0.65[a]* | 1.26[b] | 2.65[a]* | 4.21b* | 0.24[a] | 0.30[b] |

*Note:* Different letters indicate a significant difference within a species between with and without PEG based on Tukey post hoc test. Group (A, B, C, D, and E) indicate the plant species that cluster together in the PCA in Figure 8 and can be considered as one group.
*A significant difference with the control mixture (65% *Lolium perenne*, 35% *Trifolium repens*) based on a Dunnett's post hoc test.

VFA was calculated by subtracting the amounts in rumen fluid before the incubation from the amounts measured after incubation.

## 2.4 | Chemical Analysis

Upon arrival in the laboratory, fresh plant material was weighed before and after freeze-drying to calculate the dry matter (DM) content. Crude fibre was analysed with the Ankom fibre analyser (Ankom Technology, USA) after boiling the freeze-dried samples in sulphuric acid and sodium hydroxide (EC 1992). Crude protein ($N \times 6.25$) was analysed using the Kjeldahl method (ISO:5983-2 2005). Carbon and nitrogen concentrations were measured by high temperature combustion at 1150°C using an elemental analyser (Vario MACRO cube CNS, Elementar, Germany) and the C:N ratio was calculated. For the analysis of minerals and trace elements, approximately 1 g (to the nearest 0.1 mg) of ground sample was weighed into a crucible. This crucible was placed in a muffle furnace at 500°C for 4 h. The resulting ash was dissolved in 5 mL of 6N HCl (Supelco) and heated at 100°C for 30 min. Subsequently, 5 mL of 3N HCl (Supelco) was added, and the solution was reheated at 100°C for another 30 min. After cooling, the solution was quantitatively transferred to a 50 mL volumetric flask with ultrapure water. Before ICP analysis, the solution was filtered using a Whatman 5 filter. All elements were analysed using an ICP-OES (iCAP 7000, Thermo Scientific) except for selenium, which was determined using ICP-MS (iCAP Q, Thermo Scientific). Total phenolic concentration was quantified using the Folin–Ciocalteu colorimetric method as described by Marinova et al. (2005) with small adaptations. Briefly, 0.125 g of the freeze-dried substrate was mixed with 10 mL of 70% ethanol to extract. The mixture was shaken in an orbital shaker for 2 h at $509 \times g$ and then centrifuged at $7633 \times g$ at 21°C. Then, 25 μL of the substrate extract or a gallic acid standard solution (ranging from 0.10 to 1.25 mg gallic acid/mL) was added to 250 μL Folin–Ciocalteu reagent. After 6 min, the mixture was combined with 750 μL of 20% (w/v) $Na_2CO_3$ solution. The samples were then kept in the dark at room temperature for 2 h to develop colour. The absorbance was measured at 740 nm (Infinite M nano, Tecan) and the results were expressed as grams of gallic acid equivalent (GAE) per 100 g of freeze-dried substrate.

## 2.5 | Leaf Traits

Data on leaf traits from the LES were obtained from the TRY plant trait database (www.TRY-db.org) (Kattge et al. 2020). Given the relevance of LES traits to resource use strategies and plant defence mechanisms, leaf traits were selected that are commonly associated with this spectrum. The following traits were chosen based on data availability for the plant species used in these experiments and their relevance to LES: leaf area ($mm^2$), leaf C (mg/g DM), leaf N (mg/g DM), C:N ratio, leaf density ($mg/cm^3$), leaf P (mg/g DM), leaf thickness (mm), leaf DM (mg/g FM). Due to varying availability, not all traits were recorded for every plant species. The number of available datapoints for each trait is as follows: leaf area: $n = 26$, leaf C: $n = 37$, leaf N: $n = 36$, C:N ratio: $n = 32$, leaf density: $n = 27$, leaf P: $n = 31$, leaf thickness: $n = 31$, leaf DM: $n = 37$.

## 2.6 | Statistical Analysis

All statistical analyses were done using the statistical software program R (version 4.1.2, www.r-project.org). The first



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gcb.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

experiment was analysed using a linear mixed model to evaluate the effect of plant species on absolute $CH_4$ production, total VFA production, and relative $CH_4$ production:

$$Y_{ij} = P_i + R_j + \varepsilon_{ij},$$

where $Y$ is the dependent variable; $P_i$ is the fixed effect of the $i$th plant species, $R_j$ is the random effect of the incubation run, and $\varepsilon_{ij}$ is the residual error.

For the second experiment, three linear mixed models (A, B, or C) were used to evaluate the effect of the different plant species and the treatment: ((A) fresh vs. ensiled substrate, (B) $CO_2$ vs. $CO_2 + H_2$ headspace, or (C) without vs. with PEG) on the fermentation characteristics using the following formula:

$$Y_{ijk} = P_i + T_j + R_k + \varepsilon_{ijk},$$

where $Y$ is the dependent variable; $P_i$ is the fixed effect of the $i$th plant species, $T_j$ is the fixed effect of the $j$th treatment (A, B, or C), $R_k$ is the random effect of the incubation run, and $\varepsilon_{ijk}$ is the residual error. For each of these models, ANOVA was used to test the model effects. A Dunnett's post hoc test was performed to compare all plant species with the control mixture. Tukey post hoc test was used to compare all plant species with each other. Least square means (LS means) were used in further analysis. In the first experiment, LS means were used to cluster the dataset using K-means clustering based on absolute $CH_4$ production and total VFA production. Plant traits of the LES were compared between clusters using a Tukey post hoc test. Pearson's correlations were calculated between the fermentation variables and leaf traits of the LES. Principal component analysis (PCA) was performed to uncover underlying patterns in leaf traits and to evaluate the effects of different treatments on ruminal fermentation patterns in the second experiment. For the leaf traits, a reduced dataset including only plant species and traits with sufficient data points was used, applying a cutoff of no more than 5% missing values.

## 3 | Results

### 3.1 | Screening of Plant Species for Their Methane-Mitigating Potential

Absolute $CH_4$ production (mmol/g DM), total VFA production (mmol/g DM), and relative $CH_4$ production (mol $CH_4$/mol VFA) differed ($p < 0.05$, Table S1) between plant species. Initially, K-means clustering was used as an explorative method to cluster plant species based on absolute $CH_4$ and total VFA production. The clustering resulted in three clusters (Figure 2): (1) no significant or partial effect; (2) moderate effect; and (3) strong effect. In the 'no significant or partial effect' cluster, 7 of the 17 plant species had no significant effect on either $CH_4$ or VFA production, 2 species significantly affected $CH_4$ production only, and 6 species significantly influenced VFA production only. *Melilotus albus* (no. 39) and *Saponaria officinalis* (no. 42) even showed an increased total VFA production of 15% and 22% respectively. *Hedera helix* (no. 13) was allocated to the 'moderate effect' cluster, although it uniquely

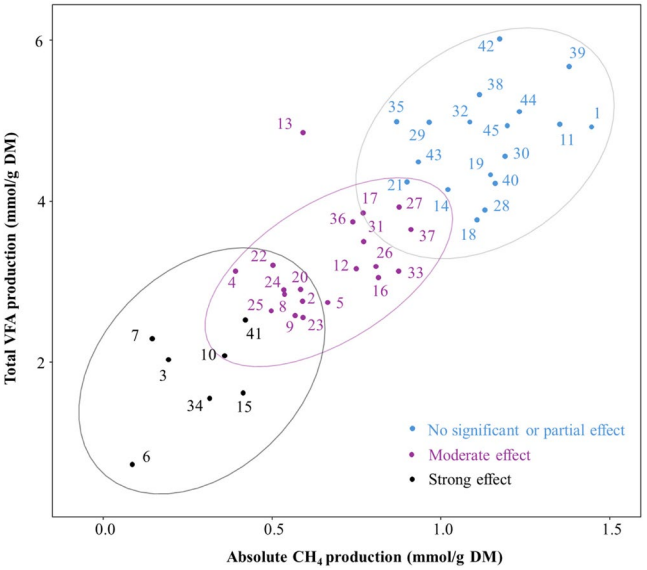



**FIGURE 2** | Clustering of plant species of the first experiment using K-means method based on absolute $CH_4$ production (mmol/g DM) and total VFA production (mmol/g DM). Three clusters could be identified: (●) no significant or partial effect ($n = 18$); (●) moderate effect ($n = 20$); and (●) strong effect ($n = 7$). Each point represents an individual plant species, identified by its corresponding number (Table S1). Ellipses indicate the 95% confidence interval for each cluster.

showed a 59% reduction ($p < 0.001$) in absolute $CH_4$ production without affecting total VFA production. *Castanea sativa* (no. 6) exhibited the lowest absolute $CH_4$ production and total VFA production, placing it at the edge of the 'strong effect' cluster. Figure 3 illustrates the absolute $CH_4$ production, total VFA production and relative $CH_4$ production of the different clusters. The correlation matrix of parameters of fermentation and leaf traits of the LES is shown in Figure 4. Absolute $CH_4$ and total VFA production were negatively correlated with leaf C ($r = -0.39$ and $r = -0.45$) and leaf DM ($r = -0.44$ and $r = -0.52$). However, these Pearson correlations disappeared in relative $CH_4$ production. The best multiple linear regression to predict absolute $CH_4$ production from leaf traits ($R^2 = 0.19$, $p < 0.01$) is: $y = 1.261 - 1.588x_1$ with $y$ = absolute $CH_4$ production (mmol/g DM), and $x_1$ = leaf DM (g/kg FM). To predict total VFA production, the best multiple linear regression ($R^2 = 0.33$, $p < 0.01$) is: $y = 10.636 - 4.976x_1 - 0.011x_2$ with $y$ = total VFA production (mmol/g DM), $x_1$ = leaf DM (g/kg FM), and $x_2$ = leaf C (g/kg DM). From all screened traits of the LES, leaf DM (mg/g FM) was the only leaf trait that showed a trend ($p = 0.061$) for a difference between the 'no effect' cluster (0.261 mg/g FM) and the 'moderate effect' cluster (0.329 mg/g FM) (Figure 5). The 'no effect' cluster is mainly composed of herbaceous plant species, with lower DM content. A PCA was performed to generate linear combinations of these leaf traits. The first two PCs accounted for 43.8% and 21.2% of the variance, respectively (Figure 6). Leaf N and Leaf P were correlated with PC1, while Leaf C, Leaf DM, and Leaf thickness were correlated with PC2. However, no clear relation between the leaf traits and the predefined clusters could be observed. An overview of the chemical composition of the plant species selected for the second experiment is shown in Table S2.



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

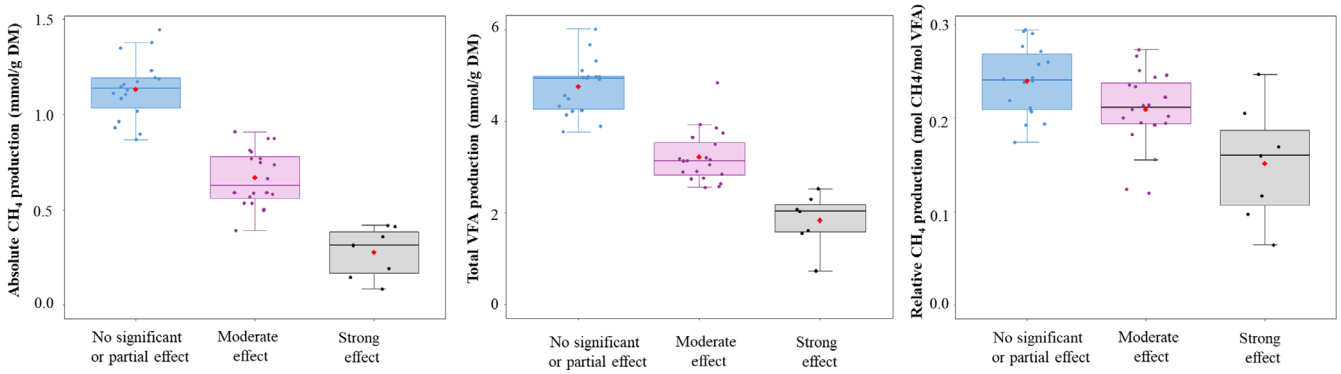



**FIGURE 3** | Boxplot showing the distribution of the LS means of absolute $CH_4$ production (mmol/g DM), total VFA production (mmol/g DM), and relative $CH_4$ production (mol $CH_4$/mol VFA) of the different clusters in the first experiment: (●) no significant or partial effect ($n = 18$); (●) moderate effect ($n = 20$); and (●) strong effect ($n = 7$) cluster. The box spans the interquartile range (IQR) from the first quartile (Q1) to the third quartile (Q3), with the horizontal line indicating the median (Q2). Whiskers extend to the smallest and largest values within 1.5 times the IQR. The mean is displayed as a red dot, and any dot beyond the whiskers is considered an outlier.

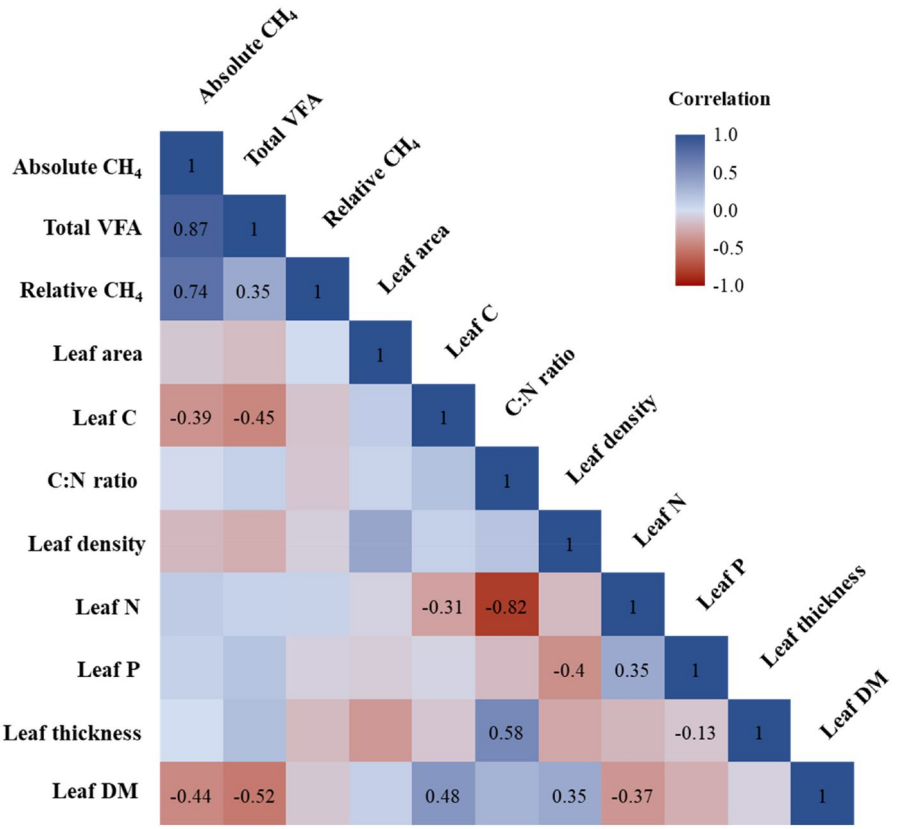



**FIGURE 4** | Correlation matrix of fermentation parameters (absolute $CH_4$ (mmol/g DM), total VFA (mmol/g DM), and relative $CH_4$ (mol $CH_4$/mol VFA)) of the first experiment and traits of the LES (leaf area ($mm^2$, $n = 26$), leaf C (mg/g DM, $n = 37$)), leaf N (mg/g DM, $n = 36$), C:N ratio ($n = 32$), leaf density ($mg/cm^3$, $n = 27$), leaf P (mg/g DM, $n = 31$), leaf thickness (mm, $n = 31$), leaf DM (mg/g FM, $n = 37$). Pearson correlation coefficients with $p$-value $< 0.05$ are displayed with their corresponding values.

## 3.2 | Effect of Ensiling on the Methane Mitigating Potential

Overall, absolute and relative $CH_4$ productions were not significantly affected by ensiling (Table 1). However, a significant interaction effect between plant species and substrate type was observed ($p < 0.001$), indicating that some plant species exhibited increased absolute and relative $CH_4$ production after ensiling, whereas others did not. Total VFA production was affected by plant species ($p < 0.001$), substrate type ($p < 0.01$), and the interaction between both ($p < 0.001$). In most plant species where a difference in absolute $CH_4$ production was observed between fresh and ensiled substrates, there was an associated increase in total VFA production. Only ensiled *Hypericum perforatum* (no.



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

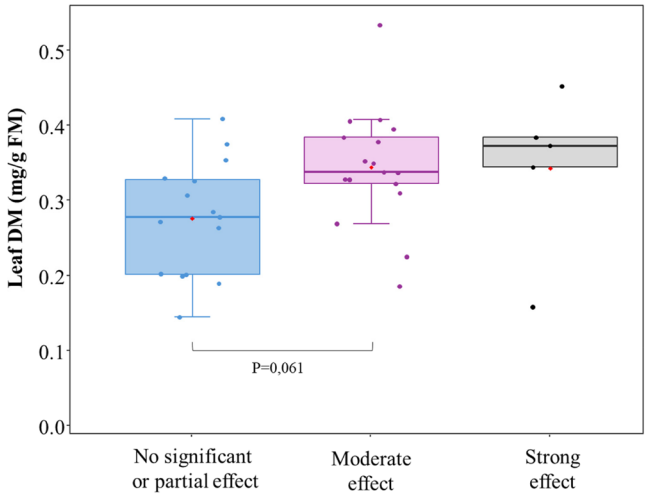



**FIGURE 5** | Boxplot showing the distribution the LS means of Leaf DM (mg/g FM) of the different clusters (● no significant or partial effect ($n=18$); ● moderate effect ($n=20$); and ● strong effect ($n=7$)). The box spans the interquartile range (IQR) from the first quartile (Q1) to the third quartile (Q3), with the horizontal line indicating the median (Q2). Whiskers extend to the smallest and largest values within 1.5 times the IQR. The mean is displayed as a red dot, and any dot beyond the whiskers is considered an outlier.

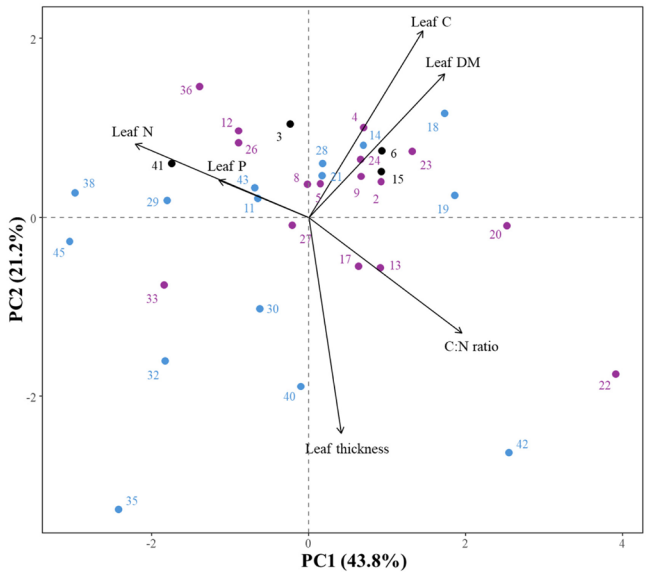



**FIGURE 6** | Biplot PCA of the reduced dataset with traits of the LES (Leaf C (mg/g DM), leaf N (mg/g DM), C:N ratio, leaf P (mg/g DM), leaf thickness (mm), leaf DM (mg/g FM)). Different colours indicate the different clusters shown in Figure 1: ● no significant or partial effect ($n=15$); ● moderate effect ($n=16$); and ● strong effect ($n=4$) on absolute $CH_4$ and total VFA production. Each point represents an individual plant species included in the PCA, identified by its corresponding number (Table S1), although some species are missing due to a lack of leaf trait data (i.e., *Catalpa bignonioides*, *Cydonia oblonga*, *Phyllostachys* spp., *Ribes* × *nidigrolaria*, *Vitis* spp., *Lotus pendunculatus*).

36) led to a 12% reduction in total VFA production. *Alnus glutinosa* (no. 3) was the only plant species that consistently reduced relative $CH_4$ production in both fresh and ensiled substrates. In contrast, all other species that reduced relative $CH_4$ production in fresh substrates, that is, *C. sativa* (no. 6), *Catalpa bignonioides* (no. 7), *Cydonia oblonga* (no. 10), *H. helix* (no. 13), *Populus nigra* (no. 17), *Sambucus nigra* (no. 29), and *H. perforatum* (no. 36) did not differ from the control when ensiled. Total phenolic concentration was measured in the ensiled substrates (Figure 7) and was affected ($p<0.05$) by substrate type, resulting in an overall reduced concentration in ensiled substrates compared to the fresh substrates.

### 3.3 | Effect of Headspace Composition on Absolute Methane Production

The effect of different headspace gas compositions ($CO_2$ or $CO_2+H_2$) on absolute $CH_4$ production is shown in Table 2. Absolute methane production was influenced ($p<0.001$) by the gas composition of the headspace. However, this effect differed over the plant species (head space composition × plant species, $p<0.001$). Based on the additional $CH_4$ production under $CO_2+H_2$ conditions compared to $CO_2$ conditions, three groups can be distinguished: (1) substrate incubations where the additional $CH_4$ production does not differ from that in the control medium under $CO_2+H_2$ conditions; (2) substrate incubations where additional $CH_4$ production is significantly higher under $CO_2+H_2$ conditions but remains lower than the additional $CH_4$ production in the control medium (e.g., *H. perforatum* (no. 36) and *Rumex obtusifolius* (no. 41)); and (3) substrate incubations where $CH_4$ production does not differ between $CO_2+H_2$ and $CO_2$ conditions (e.g., *A. glutinosa* (no. 3), *C. sativa* (no. 6), *C. bignonioides* (no. 7), and *H. helix* (no. 13)).

### 3.4 | Effect of PEG on Methane Production and Digestibility

The effect of the addition of PEG on the absolute $CH_4$ production of individual plant species differed (PEG × Plant species $p<0.001$, Table 3). In some of the plant species, the addition of PEG did not result in a significant effect on $CH_4$ and total VFA production (*C. bignonioides* (no. 7), *H. helix* (no. 13), *P. nigra* (no. 17), and *Sambucas nigra* (no. 29)). *C. sativa* (no. 6) showed no significant effect on absolute $CH_4$ production, but an effect on total VFA production. Additionally, the addition of PEG led to an effect on absolute $CH_4$ production and total VFA production in *A. glutinosa* (no. 3), *Corylus avellana* (no. 9), *C. oblonga* (no. 10), *H. perforatum* (no. 39), *Myrica gale* (no. 15), *Ribes* × *nidigrolaria* (no. 25), and *R. obtusifolius* (no. 41).

A PCA was performed to combine the effects of ensiling, addition of $H_2$ in the headspace, and supplementation of PEG to the substrate on the fermentation variables. The PC1 and PC2 explained 42.59% and 26.59% of the variance (Figure 8). Relative $CH_4$ production was positively correlated to PC1. Plant species on the negative side of the PC1 axis (*A. glutinosa* (no. 3), *C. sativa* (no. 6), *C. bignonioides* (no. 7), *H. helix* (no. 13), and *P. nigra* (no. 17)) showed the highest potential to reduce relative $CH_4$ production. Absolute $CH_4$ and total VFA production had a strong contribution to PC2, with plant species on the positive side of this axis showing lower absolute $CH_4$ and total VFA production. The effect of ensiling on fermentation variables and on total phenolic



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

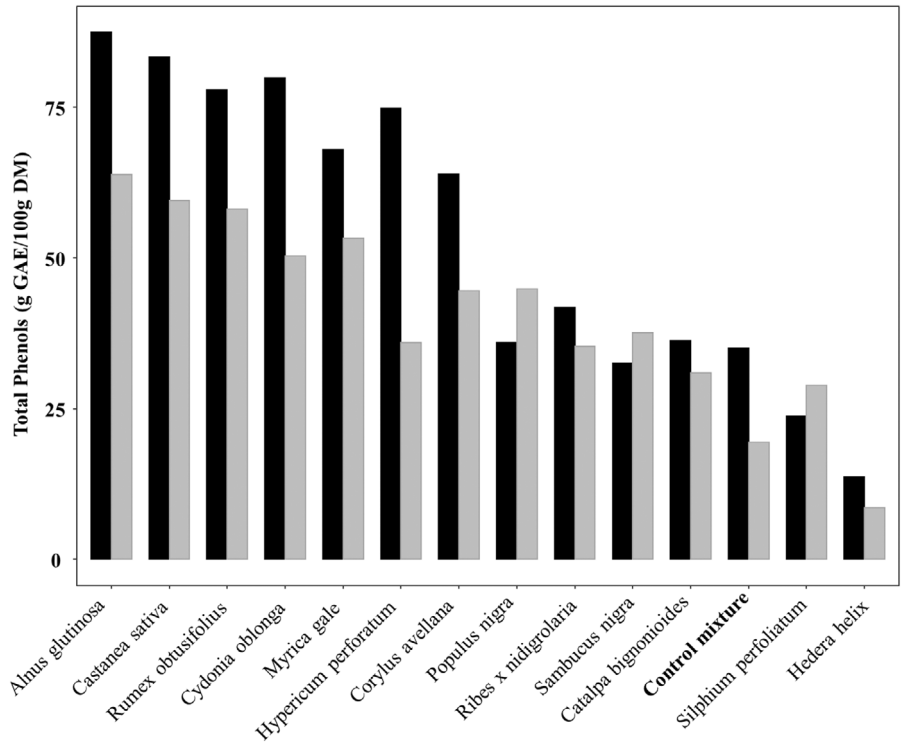



**FIGURE 7** | Total phenol content (g GAE/100 g DM) of fresh (black) and ensiled (grey) substrates.

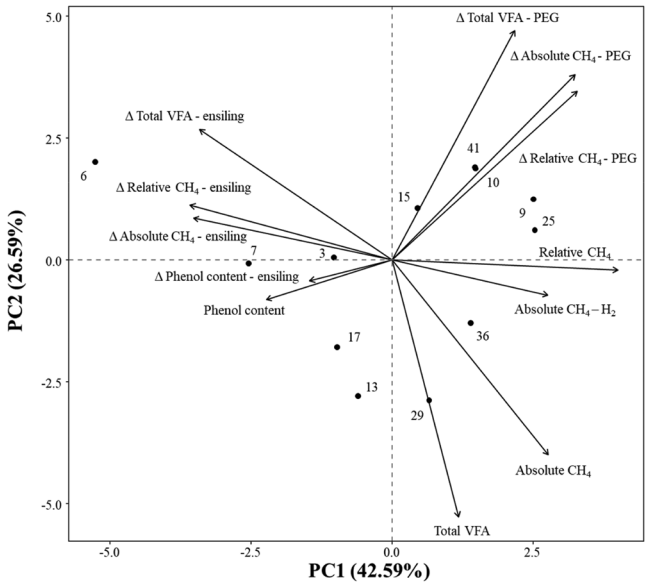



**FIGURE 8** | PCA based on the fermentation parameters and difference (Δ) in fermentation parameters as a result of ensiling, addition $H_2$ in the headspace, and addition of PEG. Each point represents an individual plant species included in the PCA, identified by its corresponding number (Table S1).

concentration was negatively correlated with PC1. The addition of PEG influenced both absolute and relative $CH_4$ production, contributing to both PC1 and PC2, while its effect on total VFA production primarily contributed to PC2. Additionally, the effect of $H_2$ in the headspace on absolute $CH_4$ production was positively correlated with PC1.

## 4 | Discussion

Results indicated a strong relationship between absolute $CH_4$ production and total VFA production. Leaf C content in this study (47.3% for woody plants, 44.5% for herbaceous plants) aligns closely with Ma et al. (2018), who reported 47.8% and 44.7%, respectively. The C-content in plants is positively correlated ($r^2 = 0.29$) with lignin content in plants (Ma et al. 2018), known to have a negative influence on forage digestibility (Grabber et al. 2009). Furthermore, structural defences, such as lignin, were positively correlated with resource-conservative plant strategies (Mohanbabu et al. 2023). Wright et al. (2004) demonstrated that leaf traits associated with resource-use strategies can be represented along a single axis. Our results align with this framework, with *Urtica dioica* (no. 45) reflecting resource-acquisitive traits, and *Pseudotsuga menziesii* (no. 22), reflecting resource-conservative traits. These findings indicate that leaf traits are more related to fermentability than to a specific $CH_4$ mitigating potential. However, within the current dataset, the relationship between DM and C with absolute $CH_4$ and total VFA production is biased by the woodiness of the plant species. *R. obtusifolius* (no. 41) was the only herbaceous species in the 'strong effect' cluster, while other species in this cluster were woody plants. Most herbaceous species were in the 'no significant or partial effect' cluster. Among the tested plant species, *H. helix* (no. 13) is an outlier within the 'moderate effect' group as it reduced absolute $CH_4$ production, without affecting total VFA production. This is in line with findings of Ramos-Morales et al. (2017), who found no influence of *H. helix* on total VFA concentration during an in vitro experiment.

Based on their reduction in relative $CH_4$ production observed in the first experiment or, in cases where this was not observed,



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

evidence of mitigation potential from the literature, 12 plant species were selected for further investigation. This second experiment indirectly explored the $CH_4$ reduction mechanisms and involved lab-scale experiments with fresh versus ensiled material, variable in vitro headspace gas-phase $H_2$ concentrations, and PEG addition. Ensiling resulted in an overall decrease in total phenolic content. This reduction may be related to cell rupture during chopping and ensiling (Huang et al. 2016). Besides decreased concentrations, PSM are known to undergo modifications and degradation during ensiling (Rodríguez et al. 2009; Muklada et al. 2021). For example, in willow, ensiling has been shown to result in complete metabolization of catechin, gallocatechin, and epicatechin (Muklada et al. 2021), a finding supported by De Bellis et al. (2022). This metabolization may be provoked by lactic acid bacteria involved in the ensiling process, which possess enzymatic activities such as tannase (Rodríguez et al. 2008) and glycosidase (Landete et al. 2014). As a result, deglycosylation of PSM is promoted during ensiling (Muklada et al. 2021). Based on these findings, it is plausible that the ensiled substrate in this study may exhibit reduced antimicrobial activity as glycosylated forms of these compounds have generally been reported to exhibit higher antibacterial and anti-virulence properties compared to their aglycones (Barros et al. 2024). The ensiled substrate of *C. sativa* (no. 6) strongly affected all fermentation variables, resulting in the loss of its reduction potential in relative $CH_4$ production compared to the fresh control mixture. A reduction in the total phenolic concentration can be observed. Based on the tannase activity of lactic acid bacteria as mentioned above, hydrolysable tannins present in *C. sativa* are possibly degraded. On the other hand, *P. nigra* (no. 17), *H. helix* (no. 13), and *S. nigra* (no. 29) showed no or only minor loss of methane-mitigating potential after ensiling, which coincided with their total phenolic content remaining largely unaffected. This suggests that their effect may be linked to phenolic (or other) compounds that remain stable during ensiling, possibly due to their presence in the aglycone form. Quercetin, a stable aglycone flavonoid found in all three species, may be one such compound contributing to this effect (Napoli et al. 2018; Gavrila et al. 2023). *C. avellana*, *C. oblonga*, *M. gale*, and *Ribes* × *nidigrolaria* exhibited reduced total phenolic concentrations, yet ensiling had little to no effect on fermentation variables. These findings suggest that the compound(s) responsible for the effects on fermentation variables were not affected during ensiling. Notably, all these plant species showed tannin activity (Makkar et al. 1995), as demonstrated by the positive effect of PEG on fermentation variables. Although tannase activity from lactic acid bacteria has been suggested to reduce the effects of hydrolysable tannins during ensiling, tannases specifically target hydrolysable tannins (Belmares et al. 2003). Therefore, it is possible that the observed effects were, at least partly, due to condensed tannins, which may have remained active in both fresh and ensiled substrate. Although the phenolic content of *C. sativa* (92 and 131 g/kg DM in 2009 and 2010; Jayanegara et al. 2011) has been previously reported to be largely composed of (hydrolysable) tannins (78 and 119 g/kg DM in 2009 and 2010), and chestnut tannin extracts have been shown to reduce $CH_4$ production in vitro (Hassanat et al. 2013; Battelli et al. 2023), the addition of PEG to *C. sativa* (no. 6) in the current study did not influence either absolute or relative $CH_4$ production. However, total VFA production increased. It is suggested that PEG influences hydrolysable tannins less compared to condensed tannins (Bhatta et al. 2009; Pellikaan et al. 2011). The increase in total VFA production suggests some effect of tannins on substrate fermentability, likely driven by the presence of condensed tannins in *C. sativa* alongside hydrolysable tannins (Terranova et al. 2020).

*A. glutinosa* (no. 3), *C. sativa* (no. 6), *C. bignonioides* (no. 7), *H. helix* (no. 13), and *P. nigra* (no. 17) showed little or no effect of $H_2$ addition on absolute $CH_4$ production, suggesting inhibitory effects on methanogens. *C. sativa* is known to mainly contain hydrolysable tannins, which have a more direct effect on the methanogens compared to condensed tannins (Patra and Saxena 2011). However, contrasting to what this study suggests, Battelli et al. (2023) found no effect on archaea as a result of the addition of hydrolysable tannins from *C. sativa*. Additionally, $H_2 + CO_2$- versus $CO_2$-headspace incubations with *A. glutinosa* (no. 3) suggest a direct anti-methanogenic effect. Its bark is known to be rich in oligomeric proanthocyanidins (a type of condensed tannin; Skrypnik et al. 2019), while *A. glutinosa* acorns predominantly contain ellagitannins (a type of hydrolysable tannin) as their main phenolic compound (Nawirska-Olszańska et al. 2022). However, neither the bark nor acorns have been addressed in the current study and no literature data were found on oligomeric proanthocyanidins and ellagitannins in leaves and twigs of *A. glutinosa*, which were the subject of the current study. Nevertheless, the observed effect of PEG on fermentation variables suggests the presence of tannins in the *A. glutinosa* leaves and twigs. Furthermore, another group of phenolic compounds, the diarylheptanoids, predominantly oregonin, have been identified as major phenolic compounds in *A. glutinosa* (Smeriglio et al. 2022). Both oregonin and its glycosylated form have been associated with antimicrobial properties (Altınyay et al. 2015; Abedini et al. 2016). In the present study, *H. helix* (no. 13) and *C. bignonioides* (no. 7) showed anti-methanogenic effects. *H. helix* is known to contain saponins such as hederacoside C, α-hederin, and hederagenin, which have been reported to possess antimicrobial properties and may inhibit methanogens (Uddin et al. 2011; Tatia et al. 2023), in line with our results of the $H_2 + CO_2$- vs. $CO_2$-headspace incubations. Similarly, *C. bignonioides* has shown antimicrobial effects in some studies (Torres et al. 2015), with saponins likely playing a role (Muñoz-Mingarro et al. 2003). Saponins are known to interact with the sterols present in the membrane of ciliate protozoa, resulting in reduced numbers or activity of protozoa (Patra and Saxena 2009). Methanogens are often found in endo- and ectosymbiotic associations with protozoa, contributing to approximately 37% of total methane production (Finlay et al. 1994). *H. perforatum* (no. 36) and *R. obtusifolius* (no. 41) both exhibit partial inhibition of methanogens, as $CH_4$ production increased, but less than in the control mixture. *H. perforatum* has been reported to contain the aglycone quercetin (Napoli et al. 2018), a compound that has demonstrated a strong anti-methanogenic activity with both methanogens and protozoa being inhibited (Nørskov et al. 2023). Other species in this study, such as *S. nigra*, *H. helix*, and *P. nigra*, also contain quercetin (Senica et al. 2017; Tebbi and Debbache-Benaida 2022; Gavrila et al. 2023) which may contribute to the observed effects. On the other hand, *R. obtusifolius* is known to contain condensed tannins, mainly procyanidin polymers (Spencer et al. 2007; Rochfort et al. 2008). Different n-hexane, chloroform, and aqueous fractions of *R. obtusifolius* showed antimicrobial properties



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

(Orbán-Gyapai et al. 2017), but methanol extracts showed particular efficacy (Harshaw et al. 2010). However, to fully understand the impact on ruminal microbiota, microbiological assessments are essential.

This study highlights the methane-mitigating potential of various plant species in modifying rumen metabolism. However, practical challenges must be considered when integrating these species into ruminant feed. Many investigated alternative forage species have lower competitiveness and limited tolerance to frequent cutting, making them less suitable as primary forage crops (Hamacher et al. 2021; Shackleton et al. 2024). Cultivating these species in monoculture on agricultural land may reduce the area available for high-yielding crops, potentially leading to production losses. However, incorporating trees, shrubs, and herbs into forage systems could enhance landscape diversification and biodiversity, contributing to more resilient agroecosystems with improved ecosystem services, such as erosion control, nutrient cycling, and weed suppression (Cappelli et al. 2022). Furthermore, although this study concentrated on ruminal fermentation, PSM may also affect feed palatability (Häring et al. 2008). Additionally, some species could present potential toxicity risks when consumed in large quantities (Acamovic and Brooker 2005).

## 5 | Conclusion

This study highlights the mitigating potential of plant species and the important role of their PSM in modifying rumen metabolism. However, the observed mitigation was often associated with a reduced fermentability, as supported by an analysis of leaf traits and lower total VFA production. An in-depth analysis of a subselection of 12 plant species identified *C. sativa*, *C. bignonioides*, *A. glutinosa*, *P. nigra*, and *H. helix* as most promising $CH_4$ mitigators. It is crucial to note that the effects of PSM on rumen metabolism are highly compound-specific, species-specific and influenced by abiotic and biotic factors with high impact on the $CH_4$ mitigating potential of plant species. On the other hand, this study showed that controlled adjustments to the in vitro setup can aid in identifying potential underlying mechanisms. While this vitro setup provided valuable insights, identifying the specific phytochemical profiles responsible for $CH_4$ mitigation and microbial analysis will help further elucidate the mechanisms behind these effects. To fully validate their $CH_4$-mitigating potential, future research should explore synergistic effects and the inclusion of plants in mixed diets and conducting in vivo trials.


### Acknowledgements

We would like to acknowledge the INSTALAB core facility at Ghent University for providing us with their analytical service and granting us access to their instrumental equipment. Special thanks to Charlotte Melis for the laboratory work.


### Ethics Statement

The ethical commission of the Institute of Agriculture, Fisheries and Food, Belgium (EC2015-257), approved all experimental procedures, including the fistulation of dairy cows, required for this study.

### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### References


Abedini, A., S. Chollet, A. Angelis, et al. 2016. "Bioactivity-Guided Identification of Antimicrobial Metabolites in *Alnus Glutinosa* Bark and Optimization of Oregonin Purification by Centrifugal Partition Chromatography." *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences* 1029–1030: 121–127. https://doi.org/10.1016/j.jchromb.2016.07.021.

Acamovic, T., and J. D. Brooker. 2005. "Biochemistry of Plant Secondary Metabolites and Their Effects in Animals." *Proceedings of the Nutrition Society* 64: 403–412. https://doi.org/10.1079/PNS2005449.

Altınyay, Ç., M. Eryılmaz, A. N. Yazgan, B. Sever Yılmaz, and M. L. Altun. 2015. "Antimicrobial Activity of Some Alnus Species." *European Review for Medical and Pharmacological Sciences* 19: 4671–4674.

Araujo, R. C., A. V. Pires, G. B. Mourão, A. L. Abdalla, and S. M. A. Sallam. 2011. "Use of Blanks to Determine in Vitro Net Gas and Methane Production When Using Rumen Fermentation Modifiers." *Animal Feed Science and Technology* 166: 155–162. https://doi.org/10.1016/j.anifeedsci.2011.04.009.

Balehegn, M. 2017. *Silvopasture Using Indigenous Fodder Trees and Shrubs: The Underexploited Synergy Between Climate Change Adaptation and Mitigation in the Livestock Sector*, edited by W. Leal Filho, S. Belay, J. Kalangu, W. Menas, P. Munishi, and K. Musiyiwa. Springer International Publishing.

Barros, T. F., J. S. Borges, D. B. Silva, and D. S. Trentin. 2024. "Glycosylated Flavonoids Have Fewer Antibacterial Activity Than Corresponding Aglycone: Is It True for Antivirulence Activity?" *Revista Brasileira de Farmacognosia* 34: 910–926. https://doi.org/10.1007/s43450-024-00557-0.

Battelli, M., S. Colombini, P. Parma, et al. 2023. "In Vitro Effects of Different Levels of Quebracho and Chestnut Tannins on Rumen Methane Production, Fermentation Parameters, and Microbiota." *Frontiers in Veterinary Science* 10: 1178288. https://doi.org/10.3389/fvets.2023.1178288.

Beauchemin, K. A., E. M. Ungerfeld, A. L. Abdalla, et al. 2022. "Invited Review: Current Enteric Methane Mitigation Options." *Journal of Dairy Science* 105: 9297–9326. https://doi.org/10.3168/jds.2022-22091.

Belmares, R., J. Contreras-Esquivel, R. Rodríguez-Herrera, A. Coronel, and C. Aguilar. 2003. "Microbial Production of Tannase: An Enzyme With Potential Use in Food Industry." *LWT - Food Science and Technology* 37: 857–864. https://doi.org/10.1016/j.lwt.2004.04.002.

Bhatta, R., Y. Uyeno, K. Tajima, et al. 2009. "Difference in the Nature of Tannins on in Vitro Ruminal Methane and Volatile Fatty Acid Production and on Methanogenic Archaea and Protozoal Populations." *Journal of Dairy Science* 92: 5512–5522. https://doi.org/10.3168/jds.2008-1441.

Cappelli, S. L., L. A. Domeignoz-Horta, V. Loaiza, and A.-L. Laine. 2022. "Plant Biodiversity Promotes Sustainable Agriculture Directly and via Belowground Effects." *Trends in Plant Science* 27: 674–687. https://doi.org/10.1016/j.tplants.2022.02.003.

Czerkawski, J. W., C. G. Harfoot, and G. Breckenridge. 1972. "The Relationship Between Methane Production and Concentrations of Hydrogen in the Aqueous and Gaseous Phases During Rumen Fermentation in Vitro." *Journal of Applied Bacteriology* 35: 537–551. https://doi.org/10.1111/j.1365-2672.1972.tb03735.x.




13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

De Bellis, P., A. Maggiolino, C. Albano, P. De Palo, and F. Blando. 2022. "Ensiling Grape Pomace With and Without Addition of a Lactiplantibacillus Plantarum Strain: Effect on Polyphenols and Microbiological Characteristics, in Vitro Nutrient Apparent Digestibility, and Gas Emission." *Frontiers in Veterinary Science* 9: 808293. https://doi.org/10.3389/fvets.2022.808293.

EC. 1992. "Determination of Crude Fibre." *Official Journal of the European Communities* L344: 35–37.

FAO. 2017. Livestock solutions for climate change.

Fievez, V., M. Ensberg, T. T. Chow, and D. Demeyer. 2004. "Effects of Freezing and Drying Grass Products Prior to Fatty Acid Extraction on Grass Fatty Acid and Lipid Class Composition—A Technical Note." *Communications in Agricultural and Applied Biological Sciences* 69, no. 1: 93–102.

Finlay, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley, and R. P. Hirt. 1994. "Some Rumen Ciliates Have Endosymbiotic Methanogens." *FEMS Microbiology Letters* 117: 157–161. https://doi.org/10.1111/j.1574-6968.1994.tb06758.x.

Gadeyne, F., K. De Ruyck, G. Van Ranst, N. De Neve, B. Vlaeminck, and V. Fievez. 2016. "Effect of Changes in Lipid Classes During Wilting and Ensiling of Red Clover Using Two Silage Additives on *in Vitro* Ruminal Biohydrogenation." *Journal of Agricultural Science* 154: 553–566. https://doi.org/10.1017/S0021859615001203.

Gardarin, A., É. Garnier, P. Carrère, et al. 2014. "Plant Trait–Digestibility Relationships Across Management and Climate Gradients in Permanent Grasslands." *Journal of Applied Ecology* 51: 1207–1217. https://doi.org/10.1111/1365-2664.12293.

Gavrila, A. I., C. M. Zalaru, R. Tatia, et al. 2023. "Green Extraction Techniques of Phytochemicals From *Hedera helix* L. and In Vitro Characterization of the Extracts." *Plants (Basel)* 12, no. 22: 3908. https://doi.org/10.3390/plants12223908.

Grabber, J. H., D. R. Mertens, H. Kim, C. Funk, F. Lu, and J. Ralph. 2009. "Cell Wall Fermentation Kinetics Are Impacted More by Lignin Content and Ferulate Cross-Linking Than by Lignin Composition." *Journal of the Science of Food and Agriculture* 89: 122–129. https://doi.org/10.1002/jsfa.3418.

Hamacher, M., C. S. Malisch, T. Reinsch, F. Taube, and R. Loges. 2021. "Evaluation of Yield Formation and Nutritive Value of Forage Legumes and Herbs With Potential for Diverse Grasslands due to Their Concentration in Plant Specialized Metabolites." *European Journal of Agronomy* 128: 126307. https://doi.org/10.1016/j.eja.2021.126307.

Häring, D. A., A. Scharenberg, F. Heckendorn, et al. 2008. "Tanniferous Forage Plants: Agronomic Performance, Palatability and Efficacy Against Parasitic Nematodes in Sheep." *Renewable Agriculture and Food Systems* 23: 19–29. https://doi.org/10.1017/S1742170507002049.

Harshaw, D., L. Nahar, B. Vadla, G. M. Saif-E-Naser, and S. D. Sarker. 2010. "Bioactivity of *Rumex obtusifolius* (Polygonaceae)." *Archives of Biological Sciences* 62: 387–392. https://doi.org/10.2298/ABS1002387H.

Hassanat, F., R. Gervais, C. Julien, et al. 2013. "Replacing Alfalfa Silage With Corn Silage in Dairy Cow Diets: Effects on Enteric Methane Production, Ruminal Fermentation, Digestion, N Balance, and Milk Production." *Journal of Dairy Science* 96: 4553–4567. https://doi.org/10.3168/jds.2012-6480.

Huang, Q. Q., L. Jin, Z. Xu, et al. 2016. "Effects of Conservation Method on Condensed Tannin Content, Ruminal Degradation, and In Vitro Intestinal Digestion of Purple Prairie Clover (Dalea Purpurea Vent.)." *Canadian Journal of Animal Science* 96, no. 4: 524–531. https://doi.org/10.1139/cjas-2016-0006.

Huang, R., P. Romero, A. Belanche, et al. 2023. "Evaluating the Effect of Phenolic Compounds as Hydrogen Acceptors When Ruminal Methanogenesis Is Inhibited *In Vitro* – Part 1. Dairy Cows." *Animal* 17: 100788. https://doi.org/10.1016/j.animal.2023.100788.

ISO:5983-2. 2005. Animal Feeding Stuffs – Determination of Nitrogen Content and Calculation of Crude Protein Content – Part 2: Block Digestion/Steam Distillation Method, Standard 5983-2.

Jayanegara, A., S. Marquardt, M. Kreuzer, and F. Leiber. 2011. "Nutrient and Energy Content, In Vitro Ruminal Fermentation Characteristics and Methanogenic Potential of Alpine Forage Plant Species During Early Summer." *Journal of the Science of Food and Agriculture* 91: 1863–1870. https://doi.org/10.1002/jsfa.4398.

Kattge, J., G. Boenisch, S. Diaz, et al. 2020. "TRY Plant Trait Database—Enhanced Coverage and Open Access." *Global Change Biology* 26, no. 1: 119–188. https://doi.org/10.1111/gcb.14904.

Ku-Vera, J. C., R. Jiménez-Ocampo, S. S. Valencia-Salazar, et al. 2020. "Role of Secondary Plant Metabolites on Enteric Methane Mitigation in Ruminants." *Frontiers in Veterinary Science* 7: 584.

Lambo, M. T., H. Ma, R. Liu, B. Dai, Y. Zhang, and Y. Li. 2024. "Review: Mechanism, Effectiveness, and the Prospects of Medicinal Plants and Their Bioactive Compounds in Lowering Ruminants' Enteric Methane Emission." *Animal* 18: 101134. https://doi.org/10.1016/j.animal.2024.101134.

Landete, J. M., J. A. Curiel, H. Rodríguez, B. de las Rivas, and R. Muñoz. 2014. "Aryl Glycosidases From *Lactobacillus plantarum* Increase Antioxidant Activity of Phenolic Compounds." *Journal of Functional Foods* 7: 322–329. https://doi.org/10.1016/j.jff.2014.01.028.

Ma, S., F. He, D. Tian, et al. 2018. "Variations and Determinants of Carbon Content in Plants: A Global Synthesis." *Biogeosciences* 15: 693–702. https://doi.org/10.5194/bg-15-693-2018.

Macheboeuf, D., L. Coudert, R. Bergeault, G. Lalière, and V. Niderkorn. 2014. "Screening of Plants From Diversified Natural Grasslands for Their Potential to Combine High Digestibility, and Low Methane and Ammonia Production." *Animal* 8: 1797–1806. https://doi.org/10.1017/S1751731114001785.

Makkar, H. P. S., M. Blümmel, and K. Becker. 1995. "Formation of Complexes Between Polyvinyl Pyrrolidones or Polyethylene Glycols and Tannins, and Their Implication in Gas Production and True Digestibility in In Vitro Techniques." *British Journal of Nutrition* 73: 897–913. https://doi.org/10.1079/BJN19950095.

Marinova, D., F. Ribarova, and M. Atanassova. 2005. "Total Phenolics and Flavonoids in Bulgarian Fruits and Vegetables." *Journal of the University of Chemical Technology and Metallurgy* 40: 255–260.

Mohanbabu, N., M. Veldhuis, D. Jung, and M. Ritchie. 2023. "Integrating Defense and Leaf Economic Spectrum Traits in a Tropical Savanna Plant." *Frontiers in Plant Science* 14: 1185616. https://doi.org/10.3389/fpls.2023.1185616.

Muklada, H., R. Davidovich-Rikanati, S. Awabdeh, et al. 2021. "Ensiling Willow (*Salix acmophylla*) Fodder Modifies the Contents of Plant Specialized Metabolites, but Not Nutritional Attributes." *Animal Feed Science and Technology* 278: 115019. https://doi.org/10.1016/j.anifeedsci.2021.115019.

Muñoz-Mingarro, D., N. Acero, F. Llinares, et al. 2003. "Biological Activity of Extracts From *Catalpa bignonioides* Walt. (*Bignoniaceae*)." *Journal of Ethnopharmacology* 87: 163–167. https://doi.org/10.1016/S0378-8741(03)00111-9.

Napoli, E., L. Siracusa, G. Ruberto, et al. 2018. "Phytochemical Profiles, Phototoxic and Antioxidant Properties of Eleven *Hypericum* Species – A Comparative Study." *Phytochemistry* 152: 162–173. https://doi.org/10.1016/j.phytochem.2018.05.003.

Nawirska-Olszańska, A., E. Zaczyńska, A. Czarny, and J. Kolniak-Ostek. 2022. "Chemical Characteristics of Ethanol and Water Extracts of Black Alder (*Alnus glutinosa* L.) Acorns and Their Antibacterial,



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Anti-Fungal and Antitumor Properties." *Molecules* 27: 2804. https://doi.org/10.3390/molecules27092804.

Nørskov, N. P., M. Battelli, M. V. Curtasu, D. W. Olijhoek, É. Chassé, and M. O. Nielsen. 2023. "Methane Reduction by Quercetin, Tannic and Salicylic Acids: Influence of Molecular Structures on Methane Formation and Fermentation in Vitro." *Scientific Reports* 13: 16023. https://doi.org/10.1038/s41598-023-43041-w.

Orbán-Gyapai, O., E. Liktor-Busa, N. Kúsz, et al. 2017. "Antibacterial Screening of *Rumex* Species Native to the Carpathian Basin and Bioactivity-Guided Isolation of Compounds From *Rumex aquaticus*." *Fitoterapia* 118: 101–106. https://doi.org/10.1016/j.fitote.2017.03.009.

Patra, A. K., and J. Saxena. 2009. "The Effect and Mode of Action of Saponins on the Microbial Populations and Fermentation in the Rumen and Ruminant Production." *Nutrition Research Reviews* 22: 204–219. https://doi.org/10.1017/S0954422409990163.

Patra, A. K., and J. Saxena. 2011. "Exploitation of Dietary Tannins to Improve Rumen Metabolism and Ruminant Nutrition." *Journal of the Science of Food and Agriculture* 91: 24–37. https://doi.org/10.1002/jsfa.4152.

Pellikaan, W. F., E. Stringano, J. Leenaars, et al. 2011. "Evaluating Effects of Tannins on Extent and Rate of *In Vitro* Gas and CH4 Production Using an Automated Pressure Evaluation System (APES)." *Animal Feed Science and Technology* 166: 377–390. https://doi.org/10.1016/j.anifeedsci.2011.04.072.

Pontes, L. D. S., J.-F. Soussana, F. Louault, D. Andueza, and P. Carrère. 2007. "Leaf Traits Affect the Above-Ground Productivity and Quality of Pasture Grasses." *Functional Ecology* 21: 844–853. https://doi.org/10.1111/j.1365-2435.2007.01316.x.

Ramos-Morales, E., G. de la Fuente, S. Duval, et al. 2017. "Antiprotozoal Effect of Saponins in the Rumen can be Enhanced by Chemical Modifications in Their Structure." *Frontiers in Microbiology* 8: 399. https://doi.org/10.3389/fmicb.2017.00399.

Rochfort, S., A. J. Parker, and F. R. Dunshea. 2008. "Plant Bioactives for Ruminant Health and Productivity." *Phytochemistry* 69: 299–322. https://doi.org/10.1016/j.phytochem.2007.08.017.

Rodríguez, H., B. de las Rivas, C. Gómez-Cordovés, and R. Muñoz. 2008. "Degradation of Tannic Acid by Cell-Free Extracts of *Lactobacillus plantarum*." *Food Chemistry* 107: 664–670. https://doi.org/10.1016/j.foodchem.2007.08.063.

Rodríguez, H., J. A. Curiel, J. M. Landete, et al. 2009. "Food Phenolics and Lactic Acid Bacteria." *International Journal of Food Microbiology* 132: 79–90. https://doi.org/10.1016/j.ijfoodmicro.2009.03.025.

Russell, J. B., and R. B. Hespell. 1981. "Microbial Rumen Fermentation." *Journal of Dairy Science* 64: 1153–1169. https://doi.org/10.3168/jds.S0022-0302(81)82694-X.

Senica, M., F. Stampar, R. Veberic, and M. Mikulic-Petkovsek. 2017. "The Higher the Better? Differences in Phenolics and Cyanogenic Glycosides in *Sambucus nigra* Leaves, Flowers and Berries From Different Altitudes." *Journal of the Science of Food and Agriculture* 97: 2623–2632. https://doi.org/10.1002/jsfa.8085.

Shackleton, J., T. M. Boland, J. Kennedy, et al. 2024. "Annual and Seasonal Dry Matter Production, Botanical Species Composition, and Nutritive Value of Multispecies, Permanent Pasture, and Perennial Ryegrass Swards Managed Under Grazing." *Grass and Forage Science* 79: 630–650. https://doi.org/10.1111/gfs.12691.

Skrypnik, L., N. Grigorev, D. Michailov, M. Antipina, M. Danilova, and A. Pungin. 2019. "Comparative Study on Radical Scavenging Activity and Phenolic Compounds Content in Water Bark Extracts of Alder (*Alnus Glutinosa* (L.) Gaertn.), Oak (*Quercus Robur* L.) and Pine (*Pinus sylvestris* L.)." *European Journal of Wood and Wood Products* 77: 879–890. https://doi.org/10.1007/s00107-019-01446-3.

Smeriglio, A., V. D'Angelo, A. Cacciola, et al. 2022. "New Insights on Phytochemical Features and Biological Properties of *Alnus glutinosa* Stem Bark." *Plants* 11, no. 19: 2499. https://doi.org/10.3390/plants11192499.

Spencer, P., S. Sivakumaran, K. Fraser, et al. 2007. "Isolation and Characterisation of Procyanidins From *Rumex obtusifolius*." *Phytochemical Analysis: PCA* 18: 193–203. https://doi.org/10.1002/pca.967.

Tatia, R., I. Tarcomnicu, Z. Moldovan, A. D. Raiciu, L. Moldovan, and C. M. Zalaru. 2023. "In Vitro Antiproliferative Activity of Triterpenoid Saponins From Leaves of *Hedera helix* L. Grown in Romania." *South African Journal of Botany* 158: 1–7.

Tebbi, S. O., and N. Debbache-Benaida. 2022. "Phytochemistry, Chemical Composition and Therapeutic Uses of *Populus nigra* L. Aerial Parts From 1991–2021 Onwards: An Overview." *Sustainable Chemistry and Pharmacy* 30: 100880. https://doi.org/10.1016/j.scp.2022.100880.

Terranova, M., M. Kreuzer, U. Braun, and A. Schwarm. 2018. "In Vitro Screening of Temperate Climate Forages From a Variety of Woody Plants for Their Potential to Mitigate Ruminal Methane and Ammonia Formation." *Journal of Agricultural Science* 156: 929–941. https://doi.org/10.1017/S0021859618000989.

Terranova, M., S. Wang, L. Eggerschwiler, U. Braun, M. Kreuzer, and A. Schwarm. 2020. "Dose-Response Effects of Woody and Herbaceous Forage Plants on In Vitro Ruminal Methane and Ammonia Formation, and Their Short-Term Palatability in Lactating Cows." *Animal* 14, no. 3: 538–548. https://doi.org/10.1017/s1751731119002076.

Torres, C. A., M. B. Nuñez, M. I. Isla, M. P. Castro, A. M. González, and I. C. Zampini. 2015. "Antibacterial Activity of Tinctures From Tree Leaves Belonging to the Bignoniaceae Family and Their Synergistic Effect With Antibiotics." *Pharmacognosy Journal* 7, no. 6: 400–405.

Uddin, G., A. Rauf, M. Qaisar, T. U. Rehman, A. Latif, and M. Ali. 2011. "Preliminary Phytochemical Screening and Antimicrobial Activity of *Hedera helix* L." *Middle-East Journal of Scientific Research* 8, no. 1: 198–202.

Vandermeulen, S., C. A. Ramírez-Restrepo, C. Marche, V. Decruyenaere, Y. Beckers, and J. Bindelle. 2018. "Behaviour and Browse Species Selectivity of Heifers Grazing in a Temperate Silvopastoral System." *Agroforestry Systems* 92: 705–716. https://doi.org/10.1007/s10457-016-0041-x.

Vlaeminck, B., T. Braeckman, and V. Fievez. 2014. "Rumen Metabolism of 22:6n-3 In Vitro Is Dependent on Its Concentration and Inoculum Size, but Less Dependent on Substrate Carbohydrate Composition." *Lipids* 49: 517–525. https://doi.org/10.1007/s11745-014-3905-8.

Vuolo, M. M., V. S. Lima, and M. R. Maróstica Junior. 2019. *Chapter 2 – Phenolic Compounds: Structure, Classification, and Antioxidant Power*, edited by M. R. S. Campos. Woodhead Publishing.

Wright, I. J., P. B. Reich, M. Westoby, et al. 2004. "The Worldwide Leaf Economics Spectrum." *Nature* 428: 821–827. https://doi.org/10.1038/nature02403.

Yáñez-Ruiz, D. R., A. Bannink, J. Dijkstra, et al. 2016. "Design, Implementation and Interpretation of In Vitro Batch Culture Experiments to Assess Enteric Methane Mitigation in Ruminants—A Review." *Animal Feed Science and Technology* 216: 1–18. https://doi.org/10.1016/j.anifeedsci.2016.03.016.

## Supporting Information

Additional supporting information can be found online in the Supporting Information section.



13652494, 2025, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/gfs.70001 by Universiteitsbibliotheek Gent, Wiley Online Library on [26/08/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License