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Effect of Soapwort root **on rumen fluid fermentability characteristics and gas production**

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Abstract

The study involved randomly assigning rumen fluid from 10 Polish Holstein-Friesian cows, fed a standard balanced diet, to a four-replicated treatment: i. control (CTRL): 25% alfalfa, 25% green grass, and 50% corn grain; ii. SR1: CTRL with soapwort root in a 1:1 ratio; iii. SR2: CTRL with soapwort root in a 1:3 ratio; and iv. powdered soapwort root. Four 24-hour *in vitro* fermentations were conducted, analysing 40 samples. After fermentation, total gas production, pH, methane (CH_4) concentration, volatile fatty acids (VFA) production and profiles, hydrogen recovery, and hydrogen consumption ratio were measured. The study indicated that the gas production rate starts to decrease after 8 hours for all treatments concerning CTRL. Furthermore, it revealed that soapwort root reduced CH_4 production in all experimental feeding models, metabolic hydrogen recovery, and the CH_4 to VFA ratio. In addition to this, we observed an increase in the propionic acid content in all groups supplemented with the soapwort root. The low values of the VFA utilisation index (NGR) and the acetic–propionic ratio, as well as the high values of the propionic– butyric ratio, indicate a reduced level of methanogenesis without loss of metabolic energy. In our opinion, the root is characterised by a high level of saponins, which slightly affects the nutritional value of the feed, making it a good additive for reducing CH_4 emissions.

Key words: *Saponaria officinalis*, rumen fermentation, volatile fatty acids, methane

In recent years, there has been increasing interest in the greenhouse effect, which is linked to rising global temperatures. This heightened attention has underscored the serious consequences for humans, animals, and the environment (Shivanna, 2022; Króliczewska et al., 2023).

Methane, the second most significant greenhouse gas after CO_2, is 28 times more potent than CO_2 in causing global warming over 100 years (United States Environmental Protection Agency, 2013). The increase in atmospheric CH_4 concentrations is primarily driven by human population growth, food production, and anthropogenic sources (Qi and Feng, 2025). Although CH_4 remains in the atmosphere for a shorter period, with a half-life of 8.6 years, in contrast to CO_2 it presents a valuable target for short-term climate mitigation (Muller and Muller, 2017; Frank et al., 2024). This characteristic makes it a desirable target to mitigate global warming in the short term, as reducing its emissions can lead to immediate benefits. Nevertheless, global CH_4 emissions increased by 9% between 2000 and 2017, according to published data from the United Nations Environment Programme (UNEP) (Saunois et al., 2020).

A significant portion of agriculture-related CH_4 emissions (approximately 42%) results from enteric fermentation, paddy rice cultivation, manure management, and biomass burning (Gerber et al., 2013; Crippa et al., 2024; Qi and Feng, 2025). Domesticated animals, such as cattle, sheep, and goats, naturally produce CH_4 as part of their digestion processes. In 2020, cattle and dairy cows were among the primary contributors to CH_4 emissions, accounting for 72% of total agricultural sector emissions (Asselstine et al., 2021; FAO, 2022; Crippa et al., 2024). Despite some reduction in CH_4 emissions through improved production efficiency, the annual decline is minimal (less than 1%) and insufficient to counterbalance the rising emissions driven by increasing demand for animal protein (Beauchemin et al., 2020). Therefore, a global effort is required to effectively reduce CH_4 emissions from ruminants. Various methods are being employed to reduce CH_4 emissions in ruminants, including farming practices, dietary changes, feed additives, chemical inhibitors of methanogenesis, probiotics, vaccination targeting the rumen microbiome, selective breeding, and genetic techniques (Króliczewska et al., 2023). The use of feed additives to directly and indirectly suppress methanogens is a key component of these strategies (Honan et al., 2022; Króliczewska et al., 2023; Durmic et al., 2025). The rumen environment can be altered using feed additives to restrict methanogen growth and reduce CH_4 emissions without focussing on the specific methanogenesis pathway. Among the factors influencing CH_4 production are those related to the metabolism of carbohydrates and hydrogen (H_2) (Morgavi et al., 2010). Feed additives are specifically implemented to modify the rumen environment to reduce CH_4 production without affecting health or productivity. The most commonly used phytogenic additives include saponins, tannins, and flavonoids (Karásková et al., 2015; Zhou et al., 2020) as previously reviewed comprehensively (Hassan et al., 2020). However, the understanding of the metabolic processes influenced by secondary plant metabolites, such as saponins, remains incomplete and underexplored. This knowledge gap presents an opportunity for further research.

Plants containing saponins can serve as effective feed additives that alter rumen fermentation. Saponins are surface-active highly amphipathic glycosides composed of aglycone sapogenin and glycon-saccharide. The hydrophobic aglycone backbone and hydrophilic sugar molecules give them the ability to foam and emulsify (Moses et al., 2014; Rai et al., 2021).

The potential effects of saponins are mainly associated with nitrogen (N_2) metabolism, primarily due to their lethal impact on protozoa, which predominantly facilitate proteolytic activity in the rumen (Hassan et al., 2020). By reducing protozoal populations, saponins lower hydrogen (H_2) availability and subsequently reduce CH_4 production without negatively impacting rumen functionality. Saponins also inhibit certain bacteria and fungi in the rumen (Haque, 2018; Honan et al., 2022). Furthermore, the use of saponins can increase the efficiency of microbial protein synthesis and protein flow into the duodenum (Unnawong et al., 2021).

Saponins are found in various parts of the plant, including the leaves, seeds, roots, tubers, and bark. They are widely distributed across different plant species, resulting in a range of biological activities (Hassan et al., 2010). Major sources of saponins in ruminant diets include *Camellia sinensis*, *Quillaja saponaria*, *Yucca schidigera*, and *Medicago sativa* (Jayanegara et al., 2014; Yi et al., 2023), though saponins are widely present in many plants species.

Soapwort (*Saponaria officinalis)*, a native to Europe, belongs to the *Caryophyllaceae* family. It has low cultivation requirements and often grows on roadsides, waste areas, and railroad tracks. This perennial plant typically reaches a height of 0.3 to 0.7 meters and grows at a moderate rate. It is sometimes regarded as invasive by certain individuals (Info Flora Plazi, 2021).

Among natural sources, soapwort is notable for its high saponin content, which can reach up to 21%. It contains a complex mixture of approximately 72 saponins (Kirshenbaum and Guegan, 2016; Jurado Gonzalez and Sörensen, 2020). The principal saponins present in soapwort are saponariosides A (SpA) and B (SpB). SpA is the predominant form in the root and is distinguished from SpB by the presence of an additional sugar (d-xylose) linked to the d-quinovose moiety (Jia et al., 1998; Jo et al., 2025). Furthermore, saponins extracted from soapwort exhibit high foam stability and capacity, remaining stable even in the presence of NaCl, sucrose, and low pH at food concentrations (Jurado Gonzalez and Sörensen, 2020).

Previous studies have shown that saponins possess antibacterial properties, significantly reducing the number of microorganisms such as bacteria, protozoa, and methanogens. This helps decrease CH_4 production in the intestines (Szczechowiak et al., 2013; Budan et al., 2014; Cieslak et al., 2014).

To our knowledge, evaluations of the effects of soapwort as well as other plants containing saponins on ruminal fermentation and gas production are still needed. The mechanisms of action of saponins remain unclear, and evidence regarding the beneficial effects of saponin extracts as health-enhancing agents and their efficacy in ruminants is still ambiguous (Guyader et al., 2015). A study found that administering approximately 25–50 g/d of *Yucca schidigera* to dairy calves reduced the concentration of VFA in their stomachs while administering saponins from *Terminalia chebula Retz.* to goats did not alter these levels (Gunun et al., 2022).

Furthermore, a meta-analysis by Yanza et al. (2024) revealed that the use of saponin extracts as dietary additives in ruminant nutrition significantly impacted ruminal fermentation parameters, specifically total VFA production and individual VFA composition. This effect was influenced by both the animal type ($p < 0.05$) and the source of saponin ($p < 0.001$), while pH and ammonia concentration did not show significant changes (Bharathidhasan et al., 2013). Ruminal protozoa exhibited a significant increase in response to higher levels of saponin extract, and there was a clear interaction effect between different sources of saponin. Additionally, the study indicates that different saponin sources may have varying effects on microbial populations. For example, extracts from *Sapindus rarak* and *Quilaja saponaria* successfully reduced the number of protozoa and bacteria, while certain amounts of *Yucca schidigera* and *Camellia sinensis* actually increased their numbers. These studies also show that different types and amounts of saponin extracts affect ruminal microbes in various ways and may influence levels of CH_4 reduction during fermentation (Lila et al., 2003; Wina et al., 2005; Belanche et al., 2016; Yanza et al., 2024).

These inconsistencies in the action of saponins could be attributed to factors such as the type and source of saponins, the dietary ration, the duration of supplementation, and the form of supplementation (liquid extract *vs.* powdered plant).

Saponin extract is harmful to ruminal protozoa when given directly to the rumen, especially in higher amounts and purities. However, the administration of saponins does not always show signs of toxicity, suggesting that additional factors within the oral environment of animals (such as mastication and salivary amylase) may play a role in detoxification. Alternatively, saponins might be shielded from detoxification or degradation by a matrix of feed particles (Patra and Saxena, 2009; Kholif, 2023). Moreover, the degradation of saponins in the rumen differs from that in other parts of the digestive system. Ruminal bacteria are capable of degrading saponins. In an *in vitro* study, the degradation rate initially increased slowly, followed by a rapid escalation after 6 to 8 hours of incubation. A rapid hydrolysis of saponins extracted from *Costus speciosus* rhizomes was observed in the sheep rumen after 1 hour of direct introduction (Meagher et al., 2001).

Therefore, the objective of this study was to evaluate the impact of the powdered soapwort root on the fermentation and gas production processes in the rumen, as well as its implications for animal nutrition using in vitro methods. We hypothesize that saponins present in powdered soapwort root reduce CH_4 emissions during fermentation in the rumen and extend their action time by protecting the saponins from degradation by microorganisms. Furthermore, this effect does not negatively impact other fermentation parameters, including the concentration of VFA.

Material and methods

Animals

The research was carried out at the Wrocław University of Environmental and Life Sciences, Research and Education Station, Wrocław, Poland. Rumen fluid was collected from 10 non-lactating Polish Holstein-Friesian cows with an average body weight of 650 ± 30 kg. The animals were kept in a locked system under welfare requirements and showed no signs of disease. They were fed a standard balanced diet according to the ruminant feeding recommendations: fodder chalk 0.174 g/100 g dry matter (DM). Fodder chalk is a raw mineral material that contains up to 98% $CaCO_3$, usually 91-93%, 0.11 to 0.64% Fe_2O_3 and trace amounts of macro and microelements, such as Mg, K, Na, P, Cu, Zn and Mn), premix 0.986 g/100 g DM, rapeseed meal 7.91 g DM, ground barley 9.53 g/100g DM, pasture hay 11.90 g/100 g DM, and grass silage 69.50 g/100 g DM, (IZ-INRA, 2016). All cows were fed the same diet before the experiment and received an appropriate amount of dry matter (DM) following the nutritional requirements for dry cows (IZ-INRA, 2016). Drinking water was available *ad libitum*.

Chemical analysis

The commercially available dry root of soapwort was purchased from Eko Herba Hajnówka, Poland. Representative samples of the plant material are stored for future reference at the Department of Animal Physiology and Biostructure, Wroclaw University of Environmental and Life Sciences, Norwida 31, 50-375 Wroclaw, Poland.

The experimental material (substrates, SR) was chemically analysed for DM, ash (CA), ether extract content (EE) and crude fibre (CF) according to the method of AOAC (AOAC, 2012) using a Kjeltec 2300 Foss Tecator apparatus (Häganäs, Sweden) as previously described by Pikhtirova et al. (2024). Crude protein (CP) was calculated from nitrogen values (CP = N concentration × 6.25) using the Kjeldahl method, which gives a value for the protein content of the experimental substrates (AOAC, 2012). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) in substrates were determined according to Van Soest et al. (1991), adapted to an Ankom Fiber Analyzer A2000 (Ankom Technology Corp., Macedon, NY, USA). Acid detergent lignin (ADL) was evaluated by subjecting the ADF residue to 72% sulfuric acid for 3 h, filtering and recording the mass of the residue (Möller, 2019). The content of non-structural carbohydrate (NSC) and nitrogen-free extractives (NFE) (g/kg of DM) was calculated according to the National Research Council guidelines (NRC, 2001) as follows: NFC = 1000 – (CP + CA + EE + NDF), NFE = 1000 – (CP + CA + CF + EE). The contents of hemicellulose (HEM), holocellulose (HOL), and cellulose (CEL) were estimated as proposed by Javier-Astete et al. (2021). Briefly, the samples were subjected to 24 hours of extraction in 25% ethanol, then mixed with a solution of 96% sulfuric acid (VI): ice acetic acid 3:1 (V/V). The absorption of the final solution was measured at 450 nm using a BioTek spectrophotometer. The results were compared to the European Pharmacopoeia Reference Standard (Y0001537, Sigma-Aldrich).

Treatment

An *in vitro* study was conducted for gas production and other parameters analyses using a control substrate (no additive, CTRL) containing 25% alfalfa, 25% green grass, 50% corn grain, 2 doses of soapwort root mixed with the CTRL substrate (53.3 and 77.15 g/kg of substrate DM, SR1 and SR2 respectively) and powdered root of soapwort (SR3) in 24-h batch culture. The experimental group containing only powdered soapwort root (SR3) was included to specifically evaluate the direct effects of saponins on ruminal fermentation only in the presence of rumen fluid, a combination that, to the best of our knowledge, has not been previously investigated. Table 1 shows the amount of saponin in g/kg of substrate DM, in mg per ml of fresh rumen fluid (inoculum), and as a percentage in the buffered rumen fluids.

Fermentation analysis

The study involved the collection of rumen samples (biological replicates) from 10 cows using a probe, conducted 2 hours post-morning feeding (Pecka-Kiełb et al., 2025). We performed the experiments with 10 biological replicates for each experimental group (SR1, SR2, and SR3) and CTRL to measure the analysed parameters, ensuring robust and reliable data for statistical analysis. In total, 40 bottles were examined in this study. In addition, two blanks containing buffered rumen fluid were included to correct for gas production that was not associated with substrate digestion.

The fermentation process was carried out under anaerobic conditions at 39 °C using the Ankom RF Gas Production System equipped with glass bottles combined with temperature (5–60 °C) and pressure sensors (ranging from −69 to +3447 kPa ± 0.27 kPa, accuracy 0.1%) (ANKOM Technology, Macedon, NY, USA), as detailed in previous studies (Ahmed et al., 2021; Shaw et al., 2023; Tunkala et al., 2023; Pikhtirova et al., 2024).

Briefly, one litre of ruminal fluid was taken from the cows using sterile cannulas rinsed with 40 °C water two hours after morning feeding. It was then immediately and carefully transported to the laboratory in thermoses, maintaining an internal temperature of 39 °C, and sealed with parafilm to minimise air exposure. Subsequently, within 1 h post-collection, the rumen fluid was filtered through a 250 µm Nylon Monofilament Precision Woven Mesh (Fisher Scientific) to separate the liquid RF and the residual solids and mixed with prewarmed to 39 °C McDougall buffer solutions in a 1:3 volume ratio (Yáñez-Ruiz et al., 2016). The McDougall buffer was flushed with CO_2 for three hours before use until the pH was stable between 6.8 and 6.9.

Afterwards, 60 ml of buffered rumen fluid was added to four preheated to 39 °C glass bottles and supplemented with 1 g of freshly prepared substrates. The bottles were tightly closed with caps and then vented with CO_2 through a gas port to achieve anaerobic conditions until the internal pressure exceeded 8 psi. The continuous real-time gas production measurement was started immediately after the gases were released from the bottle.

The measurement parameters were recorded at a 10-minute interval for 24 h with a threshold of 1.5 psi for the automatic release of the accumulated gases to stop the transfer of CO_2 in the medium at a high gas pressure; a valve opening time was 250 ms, and a mixing interval was 50 cpm (Tagliapietra et al., 2010; Ankom, 2011; Suassuna et al., 2023; Pikhtirova et al., 2024). To ensure that the solution was at a stable temperature during measurements, the bottle was kept in shaking water baths at 39 °C. For kinetic analysis, gas production data collected at 4, 8, 12, 16, 20, 21, and 24 hours from the start of incubation were used. The 24-hour incubation time is enough time for *in vitro* studies to discriminate the differences between experimental treatments and allow for reliable interpretation of the results (Yáñez-Ruiz et al., 2016). Moreover, after 24 hours of incubation, cumulative gas and CH_4 production were measured and calculated as previously described by Pikhtirova et al. (2024) using the data recorded by the Ankom RF instrument and a gas chromatography analysis for CH_4. The measured gas pressure was converted into moles of gas produced using the ‘ideal’ gas law equation (see calculations and statistical analyses paragraph) and then converted to millilitres (mL) of gas produced by Avogadro’s law equation (gas in mL = n × 22.4 × 1000), where “n” is gas produced in moles, “p” is pressure in kPa, “V” is headspace volume in the bottle in L, “T” is temperature in Kelvin, and “R” is the gas constant (Suassuna et al., 2023).

After 24 h of incubation, the gas was collected for the CH_4 analysis using a 10 mL gastight syringe (Agilent Technologies, Santa Clara, CA, USA) from a closed Ankom module bottle through a side port after the fermentation was complete. Four replicates per treatment were measured. During the sampling, the syringe was flushed a few times with the produced gas to ensure that a homogeneous sample was collected. Methane concentrations were determined by injecting 100 µl of the collected gas into a gas chromatograph Agilent Technologies 7890A GC System, Santa Clara, CA, USA, equipped with a thermal conductivity detector (TCD), a flame ionisation detector (FID) and two chromatography columns: Porapak

Q and HayeSep Q (Supelco, Bellefonte, PA, USA), with a Mol Sieve 5A PLOT Capillary GC Column was used to determine the concentration of CH_4 (Zhou et al., 2003; Pecka-Kiełb et al., 2025). Further details on the GC conditions have been reported previously (Pikhtirova et al., 2024). The experimental chromatograms were analysed by comparing the retention times of the CH_4 standards (Linde Group, Poland) using ChemStation software version B.03.02 from Agilent Technologies. The difference between total gas production (mmol/L) and CH_4 production over 24 hours was used to determine the level of non-methane gas (NMG) production.

To stop fermentation, formic acid was added at a ratio of 0.1 mL per 2 mL of solution. Following the completion of the fermentation process, the pH of the liquid fraction was measured. This was done using an inoLab® pH 7110 SET 2 (Karlsruhe, Germany), which was equipped with a SenTix 41 electrode and a temperature sensor.

Volatile fatty acids analysis

After stopping the fermentation, a 2 mL aliquot of ruminal fluid after 24 hours of fermentation was collected in a 2 mL microcentrifuge tube.. The samples were vortexed and centrifuged at 11.500 × g for 20 minutes. The supernatant was stored at −80 °C until VFA analysis. All analyses were conducted within two weeks after the fermentation was completed, and the analysis was repeated, generating three analytical replicates for each treatment. The liquid fractions were analysed using gas chromatography (7890A Gas Chromatograph, Agilent Technologies, Santa Clara, CA, USA) with a flame ionisation detector and an Agilent J&W DB-WAX-UI column, using helium as the carrier gas (flow: 25 mL/min). The analysis determined the total concentration of VFA expressed in mol%, as well as the content of acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid, and hexanoic acid. Furthermore, the acetate: propionate (A:P) and propionate: butyrate (P: B) ratios were calculated. Identification and concentration of VFA in the analysed samples were performed by comparing retention time and the peak area with the Sulpeco standard (Volatile Free Acid Mix Standards, CRM46975) using ChemStation software No. B.03.02 (Agilent Technologies, USA) (Zou, 2018).

Calculations and statistical analyses

CH_4 and GNM yields were calculated as follows (Dhakal et al., 2023):

$$\mathrm{CH_4\ mmol/L} = \frac{(\text{percentage concentration of}\ \mathrm{CH_4} \times \text{gas produced in mmol/L})}{100}$$

$$\mathrm{GNM} = \text{gas produced in mmol/L} - \mathrm{CH_4\ mmol/L}$$

The hydrogen recovery (H_2%) and the ratio of hydrogen consumption *via* CH_4/VFAs were calculated as described below:

$$\mathrm{H_2} = \frac{(4\mathrm{M} + 2\mathrm{P} + 2\mathrm{B}) \times 100}{(2\mathrm{A} + \mathrm{P} + 4\mathrm{B})}$$

$$\mathrm{CH_4/VFA} = \frac{(4\mathrm{M})}{(2\mathrm{P} + 2\mathrm{B})}$$

Where M represents CH_4, P- propionic acid, and B - butyric acid, all are expressed in mmol/L.

The fermentation efficiency (FE) coefficient is determined using the formula published by Baran and Žitňan (2002):

$$\mathrm{FE} = \frac{(0.62\ +\ 1.092\mathrm{P}\ + 1.56\mathrm{B})\ \times 100}{(\mathrm{A} + \mathrm{P} + 2\mathrm{B})}$$

Where A, P, and B represent the mol% of acetic acid, propionic acid, and butyric acid in the total VFA concentration, respectively.

The efficiency of fermented hexose energy to VFA energy (E1) and CH_4 energy (E2) was calculated according to the data published by Czerkawski (1987)

$$\mathrm{E1} = \frac{62\ +\ 0.47\ \times (\ \mathrm{P}\ + 2\ \mathrm{B}\ +\ 2\ \mathrm{V})\ \times 100\%}{(100 + \mathrm{B} + 2\mathrm{V})} = \frac{\text{VFA energy}}{\text{fermented hexose energy}}$$

$$\mathrm{E2} = \frac{28 -\ 0.47\ \times (\ \mathrm{P}\ +\ \mathrm{V})\ \times 100\%}{(100 + \mathrm{B} + \mathrm{V})} = \frac{\mathrm{CH_4}\text{ energy}}{\text{fermented hexose energy}}$$

Where A represents - acetic acid, P- propionic acid, B - butyric acid, and V - valeric acid, all are expressed in millimolar percentage [mol%].

The NGR index value was determined based on VFA levels and profiles. This index is expressed as the ratio of nonglucogenic to glucogenic VFAs (Orskov, 1975; Abrahamse et al., 2008).

$$\mathrm{NGR} = \frac{(\mathrm{A}\ + 2\mathrm{B}\ +\ \mathrm{Bc})}{(\mathrm{P} + \mathrm{Bc})}$$

Where A represents - acetic acid, P- propionic acid, B - butyric acid, and Bc – branched-chain fatty acids, all are expressed in mol%.

The cell yield index (CY) expressed in g/L of the mixed ruminal microorganisms was calculated using the equation developed by Chalupa (1977).

$$\mathrm{CY}\ =\ (\mathrm{A}\ +\ \mathrm{P}\ +\ \mathrm{B}\ +\ \mathrm{V})\ \times\ 0.03$$

Where: A, P, B, and V are the concentrations (mmol/L of ruminal fluid) of acetic, propionic, butyric, and valeric acid, respectively.

Before statistical analysis, the data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using Levene's test. If a normal distribution of residuals could not be achieved, the data were analysed using the Kruskal-Wallis test. We used the Statistica 13.3 software (STATISTICA for Windows (2001), Tulsa, OK: StatSoft, Inc.) to do a one-way analysis of variance (ANOVA) on all the data and then a multiple comparison Duncan test to see if there was a significant difference between the means. Differences between means were considered statistically significant at $P < 0.05$. The analysis data comprises 10 biological replicates ($n = 10$) accompanied by three analytical repeats for every substrate.

The statistical models and experimental design used for statistical analysis were as follows for ANOVA:

$$Z_{ij} = \kappa + \alpha_i + \nu_{ij}$$

Where Z_{ij} is the response variable, κ is the overall mean, α_i is the fixed effect of the treatment (different additives: CTRL, SR1, SR2, and SR3), and ν_{ij} is the residual error.

In the experiment, gas production data after 24 hours of fermentation were analysed as a factorial randomised fermentation run design with repeated measures on the same rumen fluid as a function of time to compare the means of the treatments.

$$y_{ijk} = a + x_i + z_j + xz_{ij} + \varepsilon_{ij},$$

where: y_{ijk} = observation, a = overall mean, x_i = substrate effect (i = 4), z_j = time (j = 6), xz_{ij} = substrate × time, and ε_{ij} = error.

Pearson's correlation coefficients were calculated using STATISTICA to examine correlations between the soapwort concentration and rumen fermentation parameters. The correlation was considered significant at $P < 0.05$.

Results

Chemical analysis

Table 2 presents a chemical analysis of the plant materials used in this study. Substrates containing soapwort root exhibited elevated saponin levels and reduced ether extract levels. The substrates SR1 to SR3 demonstrated low raw fibre and NDF content, resulting in higher levels of NSC and NFE. CP levels varied between 95.5 and 135 g/kg of DM. The highest levels were observed in CTRL, while the lowest levels were found in SR3. SR substrates showed higher amounts of crude ash and lignin but lower amounts of CEL, HEM, and HOL compared to CTRL.

Methane and gas production

Figure 1 illustrates the cumulative kinetics of gas production over a 24-hour incubation period. This analysis revealed significant differences ($P < 0.05$) in cumulative GP after accounting for the blank gas volume across various levels of the soapwort root. Following 24 hours of incubation, the CTRL treatment exhibited the highest gas production, followed by treatments SR1, SR3, and SR2. The gas production rate declines after 8 hours across all treatments compared to the CTRL group. After 20 hours of fermentation, the gas content in SR2 began to decrease relative to CTRL ($P < 0.05$).

The fermented SR2 and SR3 substrates showed a significant ($P < 0.01$) reduction in gas production after 24 hours of fermentation. A comparison of cumulative gas production from the CTRL substrate with other substrates indicates lower gas production up to 16 hours. Furthermore, when rumen fluid mixed with SR2 was fermented, it produced much less gas ($P < 0.05$) between 16 and 20 hours compared to SR1 and SR3.

Table 3 shows the alterations in CH_4 production, H_2 recovery, and the CH_4 to VFA ratio within 24 hours of the fermentation period. A notable reduction in NMG production was observed for both the SR2 substrate ($P < 0.01$) and the SR3 substrate ($P < 0.05$) when compared to the CTRL group. Furthermore, soapwort root demonstrated a significant decrease in CH_4 and H_2 levels. A comparable trend was noted for the CH_4/VFA ratio. The CH_4/VFA ratio in the CTRL group was significantly higher ($P < 0.01$) compared to the SR groups. The CH_4/VFA value on the SR3 substrate was significantly lower ($P < 0.05$) compared to the SR1 and SR2 substrates. The pH value exhibited a slight decrease ($P < 0.05$) in group SR3 relative to CTRL.

Volatile fatty acids analysis

When soapwort root was added to rumen fluid during *in vitro* fermentation, the amounts of isobutyric, isovaleric, valeric, and hexanoic acids went down, as shown in Table 4.

The CTRL group had much more propionic acid ($P < 0.05$) and less butyric acid ($P < 0.01$) and acetic acid ($P < 0.05$) in the rumen fluid, regardless of whether soapwort root was present. The amount of butyric acid was also significantly lower ($P < 0.05$) in the SR1 group compared to the SR2 and SR3 groups. The concentration of butyric acid was significantly reduced ($P < 0.05$) in the SR1 group relative to the SR2 and SR3 groups. Moreover, the use of SR3 led to a significant increase ($P < 0.05$) in total VFA generation in the rumen content, compared to the CTRL group.

Fermentation parameters

Furthermore, we examined the influence of the substrates on the parameters of the fermentation process (Table 5). The addition of substrate containing soapwort root (SR1, SR2, and SR3) to the incubated content resulted in a significant increase in the P:B ratio ($P < 0.01$). The FE and E1 parameters showed similarity; however, the E2, NGR, and A:P ratios demonstrated a reduction.

The CY value showed a significant increase in the SR2 group ($P < 0.01$) and the SR3 group ($P<0.05$) relative to the CTRL group. Statistical analysis indicated that root application affects the parameters of the fermentation process. The addition of soapwort root (SR1, SR2, and SR3) to the incubated content resulted in a significant increase in the P:B ratio ($P < 0.01$). The FE and E1 parameters exhibited similarity, whereas E2, NGR, and the A:P ratio showed a decline. The CY value was significantly higher in the SR2 group ($P < 0.01$) and the SR3 group ($P < 0.05$) relative to the CTRL group.

Correlation analysis

The correlation between the measured fermentation characteristic parameters is presented in Table 6. However, Table 7 illustrates a correlation between calculated fermentation parameters such as NGR, E1, E2, FE, CY A:P, and P:B, as well as their relationship to the CH_4/VFA ratio. Pearson’s correlation coefficient (r) was employed to assess the strength of associations between variables (ruminal fermentation parameters). The correlation coefficient (r) ranged from −1 to 1, with positive correlation indicated by $r > 0$ and negative correlation by $r < 0$. Pearson’s correlation analysis revealed significant associations between most VFA, except isocaproic acid. Also, correlation analysis for pH was conducted. A significant correlation was found solely between pH and butyric acid ($r = 0.379$, $P < 0.05$).

Discussion

Soapwort is a perennial plant species belonging to the genus *Gypsophila* within the *Caryophyllaceae* family (Çam and Topuz, 2018). It is characterised by elevated saponin levels in the root varying from 25 to 80 mg/g DM (Wichtl and Anton, 2003). Cieslak et al. (2014) obtained 21.4 mg of saponins per gram of extract from 1 kg of soapwort root by using an aqueous methanol extraction method. Furthermore, it was also found that soapwort roots differ in terms of the qualitative and quantitative content of saponins depending on the origin of the plant. Saponin content ranged from 69.18 mg/g of DM for a plant originating from Zaklików (Poland) up to 30.63 mg/g for a plant originating from Opatkowice (Poland) (Moniuszko-Szajwaja, 2019). Our study found that the saponin concentration in the root was 55.62 mg/g DM in the root, aligning with existing literature data (Budan et al., 2014; Jo et al., 2025). Saponins, characterised by amphiphilic structures that link high-molecular-weight glycosides to a triterpene or steroid aglycone, are notable for their capacity to generate a metastable soap-like foam that exhibits high stability (Böttger et al., 2012; Kregiel et al., 2017; Rai et al., 2021). They can endure temperatures reaching 90 °C, low pH levels, and ionic presence (Çelik et al., 2007; McClements and Gumus, 2016; Jurado Gonzalez and Sörensen, 2020). Additionally, the root of soapwort exhibits a low NDF content of approximately 206 g/kg DM, along with fat at 5 g/kg DM and protein at 83 g/kg DM (Szczechowiak-Piglas et al., 2016). It also contains free carbohydrates including D-glucose, D-galactose, and D-sucrose (Slobodianiuk et al., 2021).

The experimental group (SR3), containing only powdered soapwort root, was included to specifically evaluate the direct effects of saponins on ruminal fermentation only in the presence of rumen fluid, a combination that, to the best of our knowledge, has not been previously investigated. Understanding the direct impact of saponins on ruminal fermentation is essential for evaluating their potential as a natural CH_4 mitigation strategy. Given the structural diversity and biological activity of plant-derived saponins, it is critical to determine their specific effects on microbial populations, fermentation parameters, total gas and CH_4 production. Investigating these effects in the presence of rumen fluid allows for a more accurate assessment of their mode of action, kinetics and stability under physiologically relevant conditions, contributing valuable data to the development of sustainable feed additives for ruminant nutrition.

This study showed that the soapwort root is distinguished by elevated saponin content and reduced levels of EE, CP, CF, and NDF. Only limited research exists on the fundamental composition of the soapwort root. The composition of soapwort root can be compared to that of the additive from group C, specifically, corn silage, which contains a protein content of 81.4 g/kg DM, an ether extract of 24.1 g/kg DM, and an NDF content of 481.6 g/kg DM (Pecka-Kiełb et al., 2021). The low nutritional value of the used additive suggests that saponins influenced the fermentation profile.

Previous studies reviewed by Kholif (2023) have shown that saponins significantly impact the rumen ecosystem by decreasing the population of protozoa and ciliates present (Kozłowska et al., 2020; Widyarini et al., 2021). Protozoa constitute approximately 25–50% of the rumen microbial biomass and play key roles in feed digestion and H_2 production. In rumen fermentation, H_2 is primarily produced by microbial hydrogenases. This hydrogen is then largely consumed by methanogenic archaea during methanogenesis, where they reduce CO_2 to CH_4. The result of this study indicates H_2 reduction with the increasing concentration of saponins, which may indicate a decrease in the amount of protozoa. Furthermore, this may decrease methanogenesis by diminishing the activity of methanogens in the rumen (Kholif, 2023; Króliczewska et al., 2023).

Saponins inhibit the activity of protozoa that produce acetic acid and butyric acid and stimulate those that produce propionic acid (Patra and Saxena, 2009). The results of our research may indicate such a mechanism. Furthermore, saponins facilitate the transfer of hydrogen generated during fermentation from the methanogenesis pathway to the propionic acid pathway (Patra et al., 2012; Budan et al., 2014). Previous research has shown that *S.*

officinalis root extract during *in vitro* fermentation decreases total gas and CH_4 production while modifying the propionic to butyric acid ratio (Budan et al., 2014; Cieslak et al., 2014).

This research demonstrated that soapwort root decreased CH_4 production, metabolic hydrogen recovery, and the CH_4-to-VFA ratio. Additionally, an increase in propionic acid content was observed in all groups supplemented with soapwort root. In contrast, a reduction in acetic acid levels was noted in the SR1 and SR2 groups. Holtshausen et al. (2009) find that *in vivo* supplementation with saponins from (*Y. schidigera* and *Q. saponaria*) resulted in reduced CH_4 production but was also associated with other undesirable effects on ruminal fermentation and feed digestibility, such as decreased feed digestibility, particularly NDF. Ammonia-N concentration, acetate proportion, and the A:P ratio were all significantly lower, regardless of the saponin concentration, and generally declined linearly ($P < 0.01$) with increasing saponin levels. Furthermore, *in vitro* studies show that total gas production, CH_4 concentration, DM and NDF digestibility also decreased, though some reductions were only numerical (Holtshausen et al., 2009). Several other studies have similarly reported decreased CH_4 production with saponin supplementation *in vitro* (Hess et al., 2003; Lila et al., 2003; Cieslak et al., 2014; Jayanegara et al., 2020). However, it was also found that the addition of saponins did not affect CH_4 production *in vitro* (Wang et al., 2000). Recently, Kim et al. (2023) reported that supplementation with *A. saponaria* (1–2% DM, containing 37.26–45.65 mg/g saponins) increased total gas production *in vitro* without altering CH_4 output, theoretical maximum gas production, or the fractional rate of gas production. Additionally, the abundance of ruminal archaea increased. Furthermore, tea saponin included in pelleted concentrates failed to decrease enteric CH_4 emissions in nonlactating dairy cows, moreover daily CH_4 production (g/d) was not affected, but CH_4 emissions (g/kg of dry matter intake) increased by 14% with tea saponins (Guyader et al., 2015; Guyader et al., 2017).

Variability in the effects of saponins on CH_4 production across studies is likely due to differences in saponin source, dose level, and method of quantification (e.g., smilagenin equivalents vs. butanol-extracted solubles) or dose expression (g/kg DM vs. g/L). Possibly an increase in CH_4 emission was due to increased ruminal bacterial and fungal populations, which perhaps increase nutrient digestibility, especially fibres.

Gas production can be considered an important indicator of the nutritive value of feed. Ruminal microbes facilitate the digestion of feed, resulting in the production of gases, predominantly CH_4, CO_2, and H_2. Furthermore, gas production is anticipated to be influenced by the administration of saponins or plants containing saponins, owing to their impact on nutrient digestion (Kholif, 2023). On the other hand, the formation of propionate competes with CH_4 production for available H_2; thus, an increase in propionate production results in a decrease in CH_4 formation (Lipismita and Susanta Kumar, 2022). So, our studies indicate that soapwort may influence the fermentation profile, leading to a reduction in greenhouse gas emissions. Propionate, a primary energy source and byproduct of ruminal fermentation, significantly influences the food intake regulation in ruminants consuming cereal grains, subsequently affecting their satiety during meals (Allen, 2000).

Cardozo et al. (2005) demonstrated that saponins derived from *Y. schidigera* elevated propionate levels while reducing acetate levels at a pH of 5.5. At a pH of 7.0, they did not influence the concentrations of VFAs. Our findings indicate that acetate levels exhibited a slight yet significant decrease ($P < 0.05$) at a pH of approximately 6.5, with a notable reduction occurring solely at higher doses (SR3) of soapwort.

The pH of the medium in *in vitro* experiments in the rumen of animals significantly influences the sensitivity of ruminal bacteria to saponin administration (Patra and Saxena, 2009). Li et al. (2009) demonstrated that saponins exhibited *in vitro* antimicrobial activity at low pH levels, suggesting that ruminal pH may influence the efficacy of saponins based on dietary composition. The optimal pH range for rumen fermentation is typically 6.2 to 6.8 (Mao and Wang, 2025). The rumen microbial community is diverse and stable under normal pH conditions, including fibre-degrading bacteria like *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, starch-degrading bacteria such as *Streptococcus bovis* and *Ruminobacter amylophilus*, and methanogens like *Methanobrevibacter ruminantium*. The microbial community in the rumen experiences substantial alterations when pH decreases. Cellulolytic bacteria, which degrade fibre (e.g., *Fibrobacter succinogenes*), function best at pH ≥ 6.2. If pH drops below this, fiber digestion and VFA production from fibrous feeds decrease significantly (Mao and Wang, 2025). In our study, the supplementation of saponins resulted in a slight decrease in pH. This aligns with previously reported findings (Sliwiński et al., 2002; Liu et al., 2019; Darabighane et al., 2021). However, Mao et al. (2010) reported lower ruminal pH as a result of saponin supplementation, which was driven by an increased ruminal VFA concentration in sheep (Mao et al., 2010).

Additionally, propionic acid reduced feed intake in cows during the postpartum period and mid-lactation by diminishing both meal size and meal frequency (Choi and Allen, 1999; Maldini and Allen, 2018). The regulation of propionate feed intake in dairy cows has the potential to introduce innovative nutritional and pharmacological strategies to tackle the challenges associated with feeding cows during the transition period (Maldini and Allen, 2018).

Saponins also affect the concentration of VFA in the rumen, a primary fermentation product (Wang et al., 2019), However, the effects may vary, exhibiting no effect, positive, or negative outcomes, contingent upon diet, application level, and rumen pH (Lila et al., 2003; Lovett et al., 2006; Guo et al., 2008; Holtshausen et al., 2009; Patra and Saxena, 2009; Gunun et al., 2022; Kim et al., 2023). This study demonstrated that fermentation of soapwort root decreases the CH_4/VFA ratio in the rumen contents, consistent with the findings of Budan et al. (2014). This effect arises from the ability of saponins to enhance intestinal cell permeability and diminish the mechanisms of active nutrient transport, thereby promoting the absorption of substances that typically would not permeate the intestine (Kholif, 2023).

Inhibition of methanogenesis may lead to hydrogen accumulation, which does not result in the production of VFA. The observed reduction in hydrogen recovery (H_2%) in our study may suggest the transfer of H_2 to VFA, as was suggested by Lin et al. (2013). Consequently, the observed increase in VFA production within the SR3 group is deemed favourable in our studies. The increase in VFA production correlates with a minor reduction in the rumen content's pH level in the SR3 group; however, this outcome is not negative. Metabolic diseases in cows are characterised by rumen pH levels falling below 6.2-6.0 (Bannink et al., 2008; Fu et al., 2022).

Methanogenesis affects the ratio of non-glucogenic to glucogenic VFA (NGR), subsequently influencing energy balance and milk composition. The value of this parameter can vary between 1.3 to 5.9 (Morvay et al., 2011). Ruminants utilise propionic acid, a glucogenic fatty acid, for energy provision (Wilk et al., 2022). In fermentation processes, saponins significantly influence the rise of propionic acid and the reduction of butyric acid, resulting in a decrease in the A:P ratio and an increase in the P:B ratio (Lila et al., 2003). The observed low values of NGR and A:P, alongside high values of P:B, suggest a diminished level of methanogenesis while maintaining metabolic energy efficiency. Most ruminal cellulolytic microorganisms require branched-chain volatile fatty acids (isobutyric, isovaleric, valeric, and 2-methyl butyric acids) as a source of carbon skeleton for growth (Zhang et al., 2013). Ruminal branched-chain volatile fatty acids (BCVFA) mostly come from food protein or bacterial protein recycling. It does this by oxidatively deaminating and decarboxylating valine, leucine, and isoleucine in the rumen (Tedeschi et al., 2000). Previous studies have shown that BCVFA can improve rumen fermentation and enhance the digestion of cattle (Liu et al., 2008; Liu et al., 2009). Total gas, total VFA, and methane production, and digestibility are closely related. During fermentation, acetate and butyrate production are the main sources of gas production. The soapwort additive dose increase from SR1 to SR3 decreased the acetic acid proportion. A decrease in the proportion of acetic acid demands an increase in other VFAs. Propionate formation is an alternative to H_2 formation, since both pathways are electron-accepting. In propionate formation, pyruvate is reduced to propionate in one of two multi-step pathways, while in H_2 formation, protons (H^+) are reduced to H_2. Expectation, a strong inverse relationship between propionate and the A:P ratio, is confirmation that increasing the concentration of propionate will lower the ratio of acetate to propionate, thereby increasing energy efficiency. Therefore, increases in propionate formation are strongly associated with decreases in CH_4 production due to the stoichiometry of VFA production (Janssen, 2010; Dhakal et al., 2022).

In our study, we also noticed a significant positive increase in the number of microorganisms expressed by the CY index (Chalupa, 1977) calculated based on VFA production, especially with reduced iso-acid (isobutyric, isovaleric) production levels (Pecka-Kiełb et al., 2021; Pikhtirova et al., 2024).

As was mentioned above, saponins exhibit toxicity toward ruminal protozoa when administered directly into the rumen, primarily due to their high concentration and purity (Patra and Saxena, 2009; Kholif, 2023). However, when delivered through dietary supplementation, no overt signs of toxicity are typically observed, suggesting that saponins may be either detoxified or physically protected from microbial degradation by their association with feed particle matrices (Patra and Saxena, 2009). The variability in the rate and extent of ruminal degradation across different saponin types suggests that certain saponins may retain bioactivity over extended periods within the rumen environment. Furthermore, in an *in vitro* study, the degradation rate was seen to start slowly and then increase very rapidly after 6 to 8 h of incubation. Meagher et al. (2001) observed rapid hydrolysis of saponins (ethanolic extraction of powdered Costus speciosus rhizomes followed by butanol–water partitioning) in the sheep rumen after 1 h of direct introduction. Our analysis did not indicate a reduction in microbial abundance with rising saponin levels, which may imply that, in contrast to extracts, saponins given in powdered form have a less potent toxic effect on microorganisms. Nonetheless, our studies indicated a reduction in total gas production following 20 hours of fermentation. This suggests the necessity for extended studies and the observation of CH_4 concentration kinetics during fermentation.

Factors such as animal species, breed, and environmental conditions may influence the rumen microbial community’s capacity to adapt and mitigate the antiprotozoal effects of saponins (Kholif, 2023). Microbial adaptation may involve the development of enhanced saponin-degrading capabilities, thereby reducing their effectiveness. Furthermore, it has been suggested that adding saponins increases the number of bacteria in the rumen, likely because they reduce the number of protozoa that engulf bacteria (Patra and Saxena, 2009). Rather than altering the surface tension of the extracellular environment, saponins primarily act by disrupting microbial cell membranes. Although the exact mechanism by which saponins promote the growth of specific bacterial species remains unclear, it has been suggested that low doses of saponins may enhance cell membrane permeability in a controlled manner, thereby facilitating greater nutrient uptake by bacterial cells (Kim et al., 2023). The effects of saponins on ruminal microbial populations appear to be highly species-specific and influenced by the source and concentration of saponins used. While some studies reviewed by Kholif (2023) have demonstrated stimulatory effects on beneficial bacterial species such as Selenomonas ruminantium and Prevotella ruminicola, others have reported inhibitory effects on cellulolytic and amylolytic bacteria, including *Butyrivibrio fibrisolvens, Streptococcus bovis, Ruminococcus albus,* and *R. Flavefaciens*. Additionally, several studies have shown that saponin supplementation can reduce protozoal populations and enhance overall bacterial abundance, potentially shifting rumen microbial dynamics in favour of more efficient fermentation pathways. These findings underscore the complex and variable nature of microbial responses to saponin supplementation, highlighting the need for targeted research to optimise saponin use in ruminant nutrition. Moreover, the relationship between enteric CH_4 emissions and the abundance of ciliate protozoa and bacteria remains poorly understood; therefore, microbiological investigations are essential to elucidate the shifts in microbial populations induced by soapwort (saponins) supplementation. Such studies are critical for understanding the underlying mechanisms through which saponins influence rumen microbial ecology and fermentation dynamics.

The FE index measures how fermentation in the rumen changes when feed additives are added and how they affect bacterial metabolism (Wilk et al., 2022). It was found that soapwort root increased the FE indices and the efficiency of fermented hexose energy to VFA energy (E1); however, it decreased the CH_4 energy (E2) value. The findings of Hundal et al. (2021) and Baran and Žitňan (2002) led us to conclude that the fermentation measurements indicate successful fermentation and that adding soapwort reduces CH_4 production.

Conclusion

In conclusion, soapwort root is characterised by a high saponin content, low fat content, and moderate protein levels, which only slightly affect the overall nutritional value of the feed, making it a promising additive for mitigating CH_4 emissions from ruminants. Regardless of its specific role in the fermentation process, supplementation with soapwort root increased propionic acid production, a key precursor for gluconeogenesis, and fermentation efficiency, and reduced CH_4 emissions. This study was limited by the use of a single feed substrate, which allowed for a focused investigation of temporal variations in fermentation parameters. Future research should evaluate the optimal inclusion rates of soapwort root to maximise its methane-reducing potential without adversely affecting animal performance. Furthermore, the CH_4 yield and concentration data indicate that sampling time points between 12 and 36 hours should be incorporated into future experimental designs to better understand the kinetics of fermentation and by-product formation in *in vitro* rumen systems supplemented with saponins.

Author contributions

EP-K and AP designed the research; EP-K, BK, JK, AP and AZ performed the research; EP-K, BK, JKr analysed data and performed statistical analysis; BK, EP-K and JKr wrote the paper and had primary responsibility for the final content. All authors critically reviewed the paper and approved the final manuscript and are accountable for all aspects of the work.

Conflict of interest disclosure

The authors have no conflicts of interest relevant to this article to disclose.

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Data sharing statement

Data will be made available on request.

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