Comparison of chemical composition, antioxidant activity and nutrient degradability of Saffron (*Crocus sativus L.*) petals and alfalfa (*Medicago sativa L.*) hay

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Abstract

Introduction: Modern livestock production faces mounting environmental and sustainability challenges, including greenhouse gas emissions and competition for conventional feedstuffs. Regulatory restrictions on synthetic feed additives, particularly antibiotics in the world due to antimicrobial resistance concerns, have accelerated the need for alternative nutritional strategies. The strategic utilization of agro-industrial by-products offers a promising approach to address both environmental impacts and economic constraints. Saffron (*Crocus sativus* L.) cultivation generates substantial underutilized biomass; Iran, supplying 90% of global saffron production, produces approximately 53 kilograms of petals for every kilogram of harvested stigmas. Saffron petals (SP) are exceptionally rich in plant secondary metabolites including phenolic compounds, flavonoids, anthocyanins, and kaempferol derivatives, possessing documented antioxidant, antibacterial, and anti-inflammatory properties. However, a critical knowledge gap remains regarding mechanistic effects of SP on rumen fermentation dynamics, including gas production kinetics, volatile fatty acid production, methanogenesis, and nutrient degradability—information essential before recommending SP as a routine ruminant feed component.

Materials and Methods: Fresh SP and alfalfa hay were collected from Razavi Khorasan Province, Iran, shade-dried, and ground. Plant secondary metabolite profiles were quantified through spectrophotometric methods including total phenolic compounds (TPC), total flavonoids (TF), total tannins (TT), and anthocyanins (ANT). Antioxidant capacity was measured using FRAP and DPPH radical scavenging assays. Chemical composition analysis followed AOAC methods for DM, crude protein, ether extract, ash, and fiber fractions. In vitro gas production (IVGP) was performed using rumen fluid from two non-lactating fistulated cattle on a 50:50 concentrate-forage diet. Fermentation kinetics were monitored at intervals over 96 hours at 39 ± 0.5°C. Parameters including rapidly soluble fraction (a_1), slowly fermentable fraction (a_2), degradation rates (c_1, c_2), and lag phase (λ) were calculated using bi-compartmental logistic equations. Methane production was quantified after alkaline treatment. Ruminal fermentation characteristics such as pH, ammonia nitrogen (NH_3-N), and in vitro dry matter disappearance (IVDMD) were measured at 24 and 48 hours. In situ degradability kinetics were assessed using the nylon bag technique with polyester bags (5 × 10 cm, pore size 40-55 µm) incubated for 0-96 hours. Degradability parameters and effective degradability (ED) at passage rates of 0.04, 0.06, and 0.08 h^{-1} were calculated.

Results and Discussion: SP demonstrated substantially elevated plant secondary metabolites compared to alfalfa hay: total phenolic compounds (46.91 vs. 32.44 mg GAE/g DM), total tannins (7.88 vs. 2.74 mg/g DM), total flavonoids (29.93 vs. 12.85 mg QE/g DM), and anthocyanins (82.29 vs. 2.28 mg/100 g DM; all $P < 0.01$).

Antioxidant capacity was superior in SP, demonstrated by higher DPPH activity (69.14% vs. 44.30%) and FRAP values (277.7 vs. 182.1 µmol/g DM; $P < 0.01$). Chemically, SP contained lower crude protein (11.33 vs. 14.44% DM) but higher ether extract (3.39 vs. 1.22% DM) and substantially lower fiber (NDF: 23.04 vs. 40.72% DM; ADF: 15.10 vs. 32.39% DM), while non-fiber carbohydrates were markedly higher (56.36 vs. 33.73% DM; all $P < 0.01$). IVGP kinetics revealed important differences. The rapidly degradable fraction (a_1) was lower for SP (33.51 vs. 56.46 ml), while a_2 fraction was higher (42.66 vs. 35.35 ml). Notably, c_1 fraction was substantially faster for SP (0.101 vs. 0.065 ml/h), and λ was markedly shorter (0.94 vs. 1.95 h; all $P < 0.01$). However, organic matter digestibility (49.00 vs. 51.73%) and metabolizable energy (8.16 vs. 9.84 MJ/kg DM) were lower in SP ($P < 0.01$). Most significantly, methane production was dramatically reduced: SP generated only 3.34 ml methane compared to 6.76 ml for alfalfa hay, representing a 50.6% reduction ($P < 0.01$). This substantial mitigation involves condensed tannins inhibiting methanogenic archaea, selective suppression of fibrolytic bacteria, and hydrogen redirection toward alternative pathways. Ammonia nitrogen concentrations were substantially reduced in SP (6.50 vs. 11.40 mg/dL at 24 h; $P < 0.01$), indicating reduced protein degradation. This reflects tannins forming stable protein complexes at rumen pH. Short-chain fatty acid production was actually higher for SP (0.287 vs. 0.198 mmol/200 mg DM; $P < 0.01$). In situ degradability revealed lower effective degradability for SP at all passage rates ($P < 0.01$), indicating protective effects against ruminal degradation.

Conclusion: SP exhibited superior phenolic compounds and antioxidant profiles compared to alfalfa hay, with particularly elevated anthocyanins and flavonoids. These bioactive compounds substantially reduced methane production (50.6%), decreased ammonia concentrations, and lowered effective degradability—effects supporting nitrogen efficiency in ruminant diets. However, reduced dry matter digestibility and lower metabolizable energy content present nutritional constraints. Therefore, saffron petals should be incorporated as a strategically utilized functional feed ingredient at optimized levels (approximately 3% DM) rather than replacing conventional forages. This positions saffron petals as a specialized component for sustainability-focused production systems, simultaneously providing anti-methanogenic and antioxidant functions while moderating ruminal fermentation intensity.

Conflict of Interest: The authors declare no potential conflict of interest related to the work.

Keywords: Agro-industrial by-product, Flavonoid, Gas production kinetics, Methane mitigation, Phenolic compounds, Rumen degradability.

1. Introduction

Contemporary livestock production faces a convergence of environmental, regulatory, and ethical pressures. Global climate change and resource scarcity have intensified competition between human food systems and animal feed production, placing increasing demands on conventional feedstuffs such as cereals and high-quality forages (Vaghar Seyedin et al., 2024). Concurrently, regulatory restrictions on feed additives—particularly the European Union's prohibition of antibiotic feedstuffs due to bacterial resistance development—have necessitated alternative approaches (Ilias et al., 2023). In response, the strategic incorporation of agro-industrial by-products into ruminant diets has emerged as a solution addressing both environmental and economic concerns: mitigating environmental impacts while reducing production costs (Vaghar Seyedin et al., 2025; Vaghar Seyedin et al., 2023). Success requires identifying by-products that are nutritionally adequate, biologically functional, and available in sufficient quantities without competing with human food systems.

Saffron (*Crocus sativus* L.) cultivation generates substantial biomass of underutilized material. Iran, supplying approximately 90% of the world's saffron, produces this spice under arid and semi-arid conditions (Shokrpour, 2019). For over four millennia, the dried red stigmas have been valued as a culinary spice and component of traditional healing systems throughout Asia. However, each kilogram of harvested stigmas yields approximately 53 kilograms of petals, along with leaves, corms, and vegetative tissues—materials typically discarded as agricultural waste (Serrano-Diaz et al., 2012). From a nutritional perspective, saffron petals (SP) contain crude protein levels ranging from 10.20% to 11.86% and ether extract values between 1.58% and 5.3% (Ebrahimi et al., 2024a; Hosseini et al., 2018). Furthermore, SP are rich in secondary plant metabolites, including flavonoids, anthocyanins, kaempferol derivatives, crocin, and crocetin (Ebrahimi et al., 2024b; Termentzi & Kokkalou, 2008; Zeka et al., 2015). These compounds are well-documented for reducing oxidative stress and exerting antibacterial, anti-inflammatory, hepatoprotective, and hypolipidemic effects (Ebrahimi et al., 2024; Jafari-Sales & Pashazadeh, 2020). Recent phytochemical analyses have confirmed the presence of phenolic acids—gallic and chlorogenic acids—alongside quercetin and kaempferol, supporting their potential in managing digestive disorders and promoting rumen health (Feyzi & Reyhani, 2016).

Preliminary evidence in small ruminants supports SP's functional potential. When included at 3% (dry matter basis) in the diet of Afshar fattening lambs, SP improved blood antioxidant status and enhanced meat shelf-life durability without compromising growth performance (Ebrahimi et al., 2024a). Similarly, supplementation of up to 3% SP during lactation in Saanen goats significantly increased milk yield and improved both blood and milk antioxidant status, with no adverse effects on dry matter intake or nutrient digestibility (Ebrahimi et al., 2024b). Complementary in vivo studies in rodents have demonstrated that saffron and its bioactive constituents reduce oxidative stress, modulate lipid profiles, and improve systemic antioxidant status (Samarghandian et al., 2016). Despite these encouraging results, a critical knowledge gap remains: the specific effects of SP on rumen fermentation dynamics—including gas production kinetics, volatile fatty acid profiles, and in situ degradability—have not been systematically characterized. Such information is essential before recommending SP as a routine ruminant feed component, particularly given that the rumen environment is the primary site of fiber digestion and microbial metabolism.

To address this gap, validated in vitro and in situ techniques provide a robust methodological framework. The in vitro gas production (IVGP) system allows detailed characterization of fermentation kinetics and metabolite outputs, while the in-situ nylon bag technique provides direct measurement of ruminal degradability (Makkar, 2003; Ørskov & McDonald, 1979). Combined with antioxidant capacity assays and nutrient digestibility determinations, these methods enable comprehensive comparative assessment between SP and a standard forage reference. Therefore, the objective of this research was to compare the antioxidant capacity and nutrient digestibility of SP versus alfalfa hay (AH). Additionally, the study aimed to evaluate the effects of both feed materials on in vitro gas production, ruminal fermentation parameters, and in situ degradability. The overarching goal was to determine whether SP could serve as a functional and sustainable alternative feed component in ruminant diets.

2. Materials and Methods

2.1. Sample preparation and determination of bioactive compounds

Fresh SP and AH were collected from the Torbat-Heydarieh region of Razavi Khorasan Province, Iran. The samples were shade-dried for 72 hours, then ground into a fine powder using an electric grinder. To obtain an ethanolic extract, 50 g of dried SP was macerated in 1000 mL of 80% (v/v) ethanol for 72 hours at room temperature with continuous shaking at 150 rpm on an orbital shaker (GFL Orbital Shaker 3005, Burgwedel, Germany). After filtration and centrifugation (15 min, 3000×g), the extract was stored in the dark at 4°C for subsequent analysis of bioactive compounds, including total phenolic compounds (TPC), total flavonoids (TF), and total tannins (TT) (Makkar et al., 1993). TPC was quantified using the Folin–Ciocalteu method, with absorbance measured at 725 nm; results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter (DM) (Makkar et al., 1993). TF content was determined via the aluminum chloride colorimetric method, with absorbance read at 415 nm and results reported as milligrams of quercetin equivalents (QE) per gram of DM (Chang et al., 2002). Anthocyanin (ANT) content was measured using the differential pH spectrophotometric method: the extract was diluted 1:99 (v/v) in acidified methanol, and absorbance was recorded at 530 nm. Results were expressed as milligrams of cyanidin-3-glucoside per 100 g of DM (Coronado et al., 2002).

The difference between the pH values was calculated as follows equation (1):

$$A = [(A_{max} - A_{700nm})\,pH1.0] - [(A_{max} - A_{700nm})\,pH4.5] \qquad (1)$$

Then, ANT concentration (mg/L) was calculated using the following equation (2):

$$ANT = (\mathrm{A} \times \mathrm{MW} \times \mathrm{DF} \times 1000)/(\epsilon \times \mathrm{d}) \qquad (2)$$

In the equation (1) and (2), A is absorbance, A_{max} is absorbance at 510 nm, A_{700} is absorbance at 700 nm, ANT is anthocyanin concentration, MW is 433.39 g/mol (molecular weight of the pelargonidin 3-glucoside), DF is 10 (dilution factor), ϵ is 15600 (coefficient of molar absorptivity), and d is pathlength (cm) = 1.

2.2. FRAP and DPPH assay

The FRAP reagent was prepared by combining 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl, 20 mM $FeCl_3$ solution, and 0.3 M acetate buffer (pH 3.6) in a 1:1:10 (v/v) ratio. 50 µl of each diluted ethanolic extract were then mixed with 3 mL of the freshly prepared FRAP reagent, and the resulting reaction mixtures were incubated at 37°C for 30 minutes. Absorbance at 593 nm was measured spectrophotometrically against a distilled water blank. FRAP values were reported as mmol of Fe^{2+} per g of DM weight of SPs and AH powder, following the methodology of Liu et al. (2009). Furthermore, a volume of 1 mL of varying concentrations of the SPs and AH extract was combined with 1 mL of a 0.004% methanol solution containing 2,2-diphenyl-1-picrylhydrazyl (DPPH). The mixture was thoroughly agitated and allowed to react in the dark at room temperature for 30 minutes (Molyneux, 2004). Absorbance was subsequently measured at 517 nm relative to a methanol blank. Radical scavenging activity (RSA) was calculated using the equation (3):

$$RSA = \big[(A_{blank} - A_{sample}\big)/A_{blank} \times 100 \qquad (3)$$

The extract concentration exhibiting 50% inhibition (IC50) was determined from the resulting dose-response curve plotting RSA (%) against extract concentration. Butylated hydroxytoluene served as a positive control, with an IC50 value of 0.174 ± 0.014 mg/mL.

2.3. Chemical composition analysis

Dry matter (DM), crude protein (CP), ether extract (EE), ash, neutral detergent fiber (NDF), and acid detergent fiber (ADF) content were determined using standard methods (AOAC, 2015) in the Animal Nutrition Laboratory of University of Birjand, Iran. Also, non-fiber carbohydrate (NFC) was calculated using the equation (4):

$$NFC = 100 - (CP + EE + NDF + Ash) \qquad (4)$$

In this equation NFC, CP, EE, NDF, and ash are non-fiber carbohydrates, crude protein, ether extract, neutral detergent fiber, and ash, respectively.

2.4. In vitro gas production

Gas production assays were performed on processed rumen samples according to the method of Blümel et al. (1997). Rumen fluid was collected from two non-lactating, rumen-fistulated cattle using a vacuum pump. The animals were fed twice daily with a ration consisting of 50% concentrate and 50% forage to maintain consistent feed intake. The rumen fluid was filtered through four layers of sterile gauze and immediately transported to the laboratory under anaerobic conditions. The fluid was mixed with McDougall's solution at a 2:1 ratio, and 50 mL of the mixture was added to 120 mL vials containing 500 mg of pre-weighed test samples. The vials were incubated in a water bath at 39 ± 0.5 °C, and gas production was measured at 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 hours using a pressure transducer (Mensor CPG 2400, Germany). Gas pressure readings were converted to volume using the standard gas volume–pressure equation at standard conditions (1 atm, 0°C). At 24 hours post-incubation, 4 mL of 10 M NaOH was added to three vials, which were then shaken thoroughly for 30 seconds, and gas volume was measured again (Fievez et al., 2005). The experiment was conducted in three separate runs, each consisting of 15 vials (4 for IVGP analysis, 3 for methane measurement, and 6 for fermentation parameter analysis). Blank vials (without sample) were used to correct for background gas production. The parameters a_1, a_2, c_1, c_2, and λ were calculated using the bi-compartmental logistic equation (5):

$$GP(t) = a_1/(1 + exp^{[2-4(c_1(t-\lambda)]})) + a_2/(1 + exp^{[2-4(c_2(t-\lambda)]})) \qquad (5)$$

GP(t) is cumulative gas production (ml); a_1 is gas volume produced from rapid digestion soluble fraction (ml); a_2 is gas volume produced from slow digestion insoluble fraction (ml); c_1 is rate of gas production due to the degradation of the soluble fraction (ml/h); c_2 is rate of gas production due to the degradation of the insoluble fraction (ml/h); t= incubation time (h); λ = delay time (h).

2.5. Computational parameters of IVGP and methane production

Next, the digestibility of organic matter degradability (OMD), short chain fatty acids (SCFA), and metabolizable energy (ME) were calculated using the method recommended by Menke et al. (1979) and Makkar (2005) (equations 6, 7 and 8). Microbial biomass production (MBP) was estimated using equation 9 (Makkar, 2005), and microbial biomass production efficiency (EMBP) was calculated according to Blümmel (1997) and equation 10. Also, equation 9 was used to calculate the microbial biomass production efficiency (Blümmel, 1997). Finally, the separation coefficient was calculated by dividing the degraded dry matter by the amount of gas produced after 24 hours of incubation (Blümmel et al., 1997).

$$OMD = 14.88 + 0.8893 \times GP24 + 0.0448 \times CP + 0.0651 \times Ash \qquad (6)$$

$$ME = 1.242 + 0.146 \times GP24 + 0.007 \times CP + 0.0224 \times EE \qquad (7)$$

$$SCFA = 0.0222GP24 - 0.00425 \qquad (8)$$

$$MBP = OMD - (GP24 \times 2.2) \qquad (9)$$

$$EMBP = \frac{OMD-(GP24\times2.2)}{OMD} \qquad (10)$$

$$PF = OMD/GP24 \qquad (11)$$

In these equations, the variables OMD, GP24, CP, Ash, ME, EE, SCFA, MBP, EMBP, 2.2 and PF represent, respectively, organic matter degradability (%), gas production in 24h incubation (ml/200 mg DM), content of crude protein of samples (g/kg DM), content of ash samples (g/kg DM), metabolizable energy (MJ/kg DM), content of ether extract of samples (g/kg DM), short-chain fatty acids (mmol/200 mg DM), microbial biomass production (mg/g DM), efficiency of microbial biomass production, stoichiometric constant coefficient, and partitioning factor (mg/ml).

2.6. Ruminal fermentation characteristics

pH, ammonia nitrogen (NH_3-N) concentration, and the amount of in vitro dry matter disappearance (IVDMD) were measured at 24- and 48-hours post-incubation. NH_3-N concentration was determined using the phenolphthalein hypochlorite method at a wavelength of 630 nm using a spectrophotometer (Broderick & Kang, 1980). pH was measured using a digital pH meter (Metrohm 744, Switzerland). To determine IVDMD, samples were filtered through a 42 µm membrane filter and the residue was dried in an oven at 60°C for 48 h. The IVDMD was then calculated using the following equation (12):

$$IVDMD = [A - (B - C)/A] \times 100 \qquad (12)$$

In the above equation (12), IVDMD is the in vitro dry matter disappearance (%), A is the weight of the sample (g DM), B is the weight after incubation (g DM), and C is the weight of the empty container (g DM after incubation).

2.7. In situ degradability kinetics

Ruminal degradation kinetics were assessed using the nylon bag method described by Ørskov et al. (1980). Two non-lactating, rumen-fistulated cattle were used. Approximately 3 g of SP or AH sample was placed into numbered polyester bags (5 × 10 cm, pore size 40–55 µm). To prevent leakage during early incubation, the bags were sealed with plastic sutures. Bags were incubated for 0, 2, 4, 6, 12, 16, 24, 36, 48, 72, and 96 hours. For zero-time degradation (water-soluble fraction), three bags were washed with lukewarm water to remove all soluble material. Each incubation cycle was performed in two stages using two rams. After incubation, bags were rinsed with cold water until the effluent became completely clear. The bags were then dried in an oven (Behdad Oven, Iran) at 55°C for 48 hours. Following drying, chemical analyses (DM, ash, CP) were performed according to AOAC (2000).

In situ Degradability and effective degradation rates were calculated using following equations (13) and (14).

$$P = a + b\left(1 - e^{(-ct)}\right) \qquad (13)$$

$$ED = a + [(b \times c)/(c + k)] \qquad (14)$$

These equations describe a as immediately soluble fraction (g DM); b as insoluble but potentially degradable fraction (g DM), c as rate constant for the degradation of fraction (h^{-1}); pd as potential degradable fraction (g DM), ED as the effective rumen degradation, and k as passage rate of 4, 6 and 8 h^{-1}.

2.9. Statistical model and data analysis

The determination of IVGP and in situ degradability parameters was performed using the NLIN procedure of the SAS software (version 9.4 M8 TS1). The variance analysis of the obtained data was performed using the GLM procedure of SAS software (version 9.4 M8 TS1) and the comparison of the data means was performed using the LSD test at a significance level of 5%. The statistical model was as equation (15).

$$Y_{ij} = \mu + T_i + e_{ij} \qquad (15)$$

Where Y_{ij} is the dependent variable, µ is the overall mean, T_i is the group effect, and e_{ij} is the experimental error. Run was not included as a random effect due to low variance between runs (CV < 5%).

3. Results and Discussion

3.1. Plant secondary metabolites and antioxidant activity

The concentrations of PSM and the antioxidant activity of SP and AH are summarized in Table 1. All measured parameters differed significantly between the two treatments ($P < 0.01$). SP exhibited significantly higher ($P < 0.01$) levels of TPC (46.91 vs. 32.44 mg/g DM), TT (7.88 vs. 2.74 mg/g DM), and TF (29.93 vs. 12.85 mg QE/g DM) compared to AH. In contrast, TC were markedly lower in SP (6.66 mg/100 g DM) than in AH (69.78 mg/100 g DM) ($P < 0.01$). ANT content was substantially greater in SP (82.29 vs. 2.28 mg/100 g DM) ($P < 0.01$). Regarding antioxidant capacity, SP demonstrated significantly higher radical scavenging activity, as measured by the DPPH assay (69.14% vs. 44.30%; $P = 0.01$). Similarly, FRAP was markedly greater for SP (277.7 vs. 182.1 µmol/g DM) compared to AH ($P < 0.01$).

Table 1. Comparison of plant secondary metabolites and antioxidant activity of Saffron petals and alfalfa hay

Item	TPC	TT	TF	TC	ANT	DPPH	FRAP
Saffron petals	46.91^a	7.88^a	29.93^a	6.66^b	82.29^a	69.14^a	277.7^a
Alfalfa hay	32.44^b	2.74^b	12.85^b	69.78^a	2.28^b	44.30^b	182.1^b
SEM	0.4155	0.0453	0.0989	0.4938	1.9074	1.0477	3.3144
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

TPC, total phenolic compounds (mg GAE/g DM); TT, total tannins (mg/g DM); TF, total flavonoids (mg QE/g DM); TC, total carotenoids (mg/100 g DM); ANT, anthocyanin (mg/100 g DM); DPPH, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (%); FRAP, ferric reducing antioxidant power (μmol Fe^{2+}/g DM).
*Significant differences between values in a column with various superscripts ($P< 0.05$)

The present study revealed substantial differences in secondary metabolite profiles between SP and AH, with SP demonstrating markedly superior concentrations of TPC, TT, TF, and ANT. This disparity is notable given the established role of phenolic compounds in modulating ruminal fermentation dynamics and enhancing ruminant antioxidant status (Vasta et al., 2019). The exceptionally high ANT content in SP (82.29 vs. 2.28 mg/100 g DM in AH) is particularly remarkable. Anthocyanins are water-soluble vacuolar pigments belonging to the flavonoid family, possessing potent free radical-scavenging capabilities through their ability to donate hydrogen atoms from hydroxyl groups on the B-ring to stabilize reactive oxygen species. The concentration differential observed (approximately 36-fold higher in SP) suggests that SP could serve as an effective dietary strategy to mitigate oxidative stress in ruminants, particularly during periparturient periods or under heat stress conditions when endogenous antioxidant defenses are compromised (Kazemi, 2024).

The IC_{50} values reported by Nasseri et al. (2015) (5.9 mg/ml) differ from the present study's DPPH percentage, likely due to differences in extraction solvent and assay endpoint. Ebrahimi et al. (2024a) reported SP total phenolic compounds, anthocyanins, and antioxidant capacity as 1257 mg GAE/100 g, 97.79 mg/100 g, and 6.30 μmol/100 g, respectively. The present results confirm their findings. These findings align with Kazemi (2024), who reported that SP by-products contain substantial quantities of kaempferol, a flavonol compound with demonstrated antioxidant and anti-inflammatory properties. Similarly, Goli et al. (2012) documented the phenolic profile of SP, identifying major compounds including quercetin derivatives and gallic acid, which collectively contribute to the observed antioxidant capacity. The DPPH radical scavenging activity (69.14% for SP vs. 44.30% for AH) and FRAP values (277.7 vs. 182.1 μmol/g DM) provide functional confirmation of the chemical analyses, indicating that the TPC in SP are biologically active rather than merely present as inert constituents.

Phenolic compounds are secondary metabolites whose amount depends on the plant type and organ as well as stress severity. The most important positive effect of TPC is removal and prevention of free radical formation and deposition of oxidizing elements (Rao et al., 2025). Furthermore, TPC and TT in the rumen environment reduce protein solubility and its degradation by rumen bacteria by creating hydrogen bonds between tannin and protein segments, forming reversible indigestible complexes under the influence of pH. It has also been reported that rumen proteolytic enzymes are inhibited by tannins present in the diet, which leads to reduced CP degradation (Valcl and Lavrenčič, 2026). Reported results regarding tannin content effects on digestibility under in vitro and in vivo conditions are somewhat contradictory. For example, in an in vitro study, use of 6 to 9% TT (based on DM) in the diet did not have a negative effect on DM and CP digestibility (Jin et al., 2012).

3.2. Chemical Composition

The chemical composition of SP and AH is presented in Table 2. All measured parameters differed significantly between the two feedstuffs ($P < 0.01$). DM content was considerably lower ($P < 0.01$) in SP (13.37%) compared to AH (33.41%), reflecting the higher moisture content of fresh SP relative to preserved AH. Conversely, OM content was slightly but significantly higher ($P < 0.01$) in SP (94.11%) than in AH (90.14%). CP content was lower ($P < 0.01$) in SP (11.33% DM) than in AH (14.44% DM). EE content, was significantly higher ($P < 0.0001$) in SP (3.39% DM) compared to AH (1.22% DM). Also, content of NDF (23.04 vs. 40.72% DM) and ADF (15.10 vs. 32.39% DM) SP was lower than AH ($P < 0.01$). In contrast, NFC was substantially higher ($P < 0.01$) in SP (56.36% DM) compared to AH (33.73% DM).

Table 2. Comparison of chemical composition of Saffron petals and alfalfa hay

Item	DM	OM	CP	EE	NDF	ADF	NFC[1]
Saffron petals	13.37^b	94.11^a	11.33^b	3.39^a	23.04^b	15.10^b	56.36^a
Alfalfa hay	33.41^a	90.14^b	14.44^a	1.22^b	40.72^a	32.39^a	33.73^b

SEM	0.0947	0.0317	0.0302	0.1448	0.6864	0.4443	0.5694
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

DM, dry matter; OM, organic matter (% of DM); CP, crude protein (% of DM); EE, ether extract (% of DM); NDF, neutral detergent fiber (% of DM); ADF, acid detergent fiber (% of DM); NFC, non-fiber carbohydrates (% of DM).
[1]NFC = 100 – (CP+EE+NDF+Ash).
*Significant differences between values in a column with various superscripts ($P< 0.05$)

The chemical composition data reveal a distinct nutritional profile for SP that positions it as a complementary rather than replacement feedstuff relative to AH. The lower dry matter content of SP (13.37% vs. 33.41%) reflects its fresh, high-moisture nature, which has practical implications for storage, preservation, and inclusion rates in total mixed rations. However, the higher OM content (94.11% vs. 90.14% DM) and substantially lower NDF (23.04% vs. 40.72% DM) indicate that SP is rapidly fermentable and would likely promote higher passage rates when included in ruminant diets (Jadouali et al., 2022). The crude protein content of SP (11.33% DM) was lower than that of AH (14.44% DM), which is consistent with previous reports on saffron residues. Jadouali et al. (2022) documented similar CP values for saffron by-products harvested at various phenological stages, noting that protein content declines as the plant matures. This moderate protein level, combined with the high non-fiber carbohydrate content (NFC: 56.36% vs. 33.73% DM), suggests that SP would primarily supply readily fermentable energy rather than rumen-degradable protein. The EE content (3.39% vs. 1.22% DM) is notable, as the lipid fraction may contribute to delivery of fat-soluble bioactives, including crocetin derivatives, which have been implicated in the anti-inflammatory effects observed in some ruminant studies (Kazemi, 2024). In previous studies, the CP and EE ranges of SP have been reported as 6.7 to 11.86 and 1.58 to 8, respectively (Kardan Moghaddam et al., 2014; Ebrahimi et al., 2024), which is in agreement with the present findings. The composition of nutrients in forages, especially agricultural by-products, is highly variable due to their heterogeneity. Differences in forage chemical composition among different studies may be attributed to differences in species studied, regional climate, soil type, geographical location, and altitude (Wu et al., 2025; Gao et al., 2026).

3.3. Kinetic parameters of in vitro gas production

The kinetic parameters of IVGP derived from the fermentation of SP and AH over 96 h are presented in Table 3 and Figure 1. All estimated parameters differed significantly between the two substrates ($P < 0.01$). a_1 parameter was significantly lower ($P < 0.01$) for SP (33.51 ml) compared to AH (56.46 ml). Conversely, a_2 parameter was significantly higher ($P < 0.01$) for SP (42.66 ml) than for AH (35.35 ml). c_1 parameter was substantially higher ($P < 0.0001$) for SP (0.101 ml/h) than for AH (0.065 ml/h). In contrast, c_2 parameters was slightly but significantly lower ($P < 0.01$) for SP (0.010 ml/h) compared to AH (0.012 ml/h). λ parameters was markedly shorter ($P < 0.01$) for SP (0.94 h) than for AH (1.95 h), indicating that microbial fermentation of SP begins more rapidly upon exposure to rumen fluid.

Table 3. Comparison of *in vitro* gas production parameters of Saffron (*Crocus sativus L.*) petals and alfalfa (*Medicago sativa L.*) hay in 96h incubation

Item	**a_1**	**a_2**	**c_1**	**c_2**	**λ**
Saffron petals	33.51^{b}	42.66^{a}	0.101^{a}	0.010^{b}	0.94^{b}
Alfalfa hay	56.46^{a}	35.35^{b}	0.065^{b}	0.012^{a}	1.95^{a}
SEM	0.5358	0.1164	0.0006	0.0001	0.0451
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

a_1, gas volume derived from the degradation of the rapid digestion soluble fraction; a_2, gas volume derived from the degradation of the slow digestion insoluble fraction when; c_1, rate of gas production due to the degradation of the soluble fraction (ml/h); c_2, rate of gas production due to the degradation of the insoluble fraction (ml/h); λ, the initial delay before gas production begins (h).
*Significant differences between values in a column with various superscripts ($P< 0.05$)

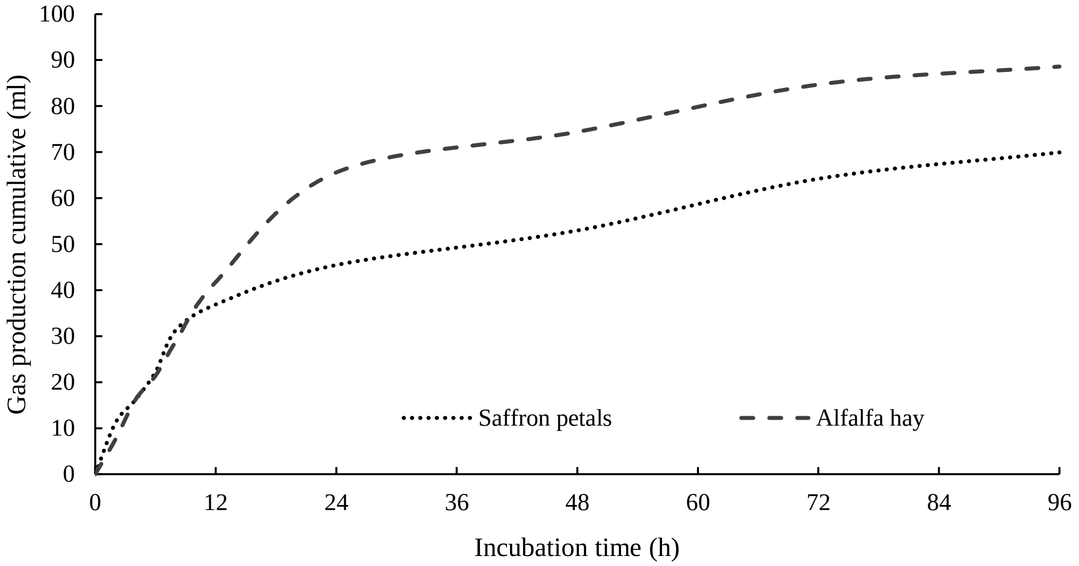

Figure 1. in vitro gas production cumulative Saffron (*Crocus sativus L.*) petals and alfalfa (*Medicago sativa L.*) hay in 96 h incubation

The IVGP parameters offer critical insight into how SP alters ruminal fermentation dynamics compared to AH. A biphasic gas production model revealed that SP exhibits a significantly lower immediately degradable fraction (a_1) but a larger colonizable fraction (a_2) than AH. This indicates that SP has a smaller pool of rapidly available nutrients and a greater proportion that requires microbial attachment and enzymatic breakdown before fermentation can begin. Notably, the degradation rate constant for the soluble fraction (c_1) is considerably higher for SP, meaning that once microbes gain access, fermentation progresses faster than with AH (Vasta et al., 2019). The shorter lag phase (λ) for SP is especially significant, as this parameter reflects the time needed for microbial colonization, enzyme synthesis, and fermentation onset. The reduced lag suggests that SP's structural traits—lower fiber content and potentially greater surface area—promote quicker microbial attachment. Alternatively, phenolic compounds in SP may exert selective pressure on the rumen microbiota, possibly favoring fibrolytic populations that rapidly utilize substrates (Kelln et al., 2020). Furthermore, Akbari Shooshood et al. (2024) found that adding 3% SP to a control diet significantly increased in vitro gas production after 24 and 72 hours of incubation.

Phenolic compounds exert a selective inhibitory effect within the rumen, disproportionately suppressing slower-growing fibrolytic bacteria—such as Fibrobacter succinogenes and Ruminococcus flavefaciens—while having minimal or no negative impact on, or even stimulating, rapidly fermenting amylolytic and saccharolytic populations (e.g., *Streptococcus bovis, Selenomonas ruminantium*). The consequence of this selective pressure is a biphasic fermentation pattern: rapid initial fermentation of soluble substrates followed by a reduced overall extent of fermentation, owing to impaired breakdown of structural carbohydrates (Díaz Carrasco et al., 2017; Bhatta et al., 2015; Guo et al., 2025; Yunilas et al., 2023). A second contributing mechanism involves the soluble, readily fermentable components present in SP—including simple sugars, anthocyanin glycosides, and other low-molecular-weight phenolics. Although these compounds are highly accessible to rumen microbes, they are available only in limited total quantity (Yunilas et al., 2023). Their rapid fermentation generates a steep initial gas production curve; however, once these substrates are exhausted, the remaining phenolic compounds suppress further fermentation of less accessible substrates (Palza et al., 2026).

3.4. In vitro gas production calculation parameters

Table 4 presents a comparison of the calculated gas production parameters. Significant differences were observed between the two substrates across all measured parameters ($P < 0.01$). OMD was marginally lower for SP petals (49.00%) than for AH (51.73%), a difference that was statistically significant ($P < 0.01$).

Similarly, ME content was lower in SP (8.16 MJ/kg DM) compared to AH (9.74 MJ/kg DM; $P < 0.01$). In contrast, SCFA production was significantly lower for AH (0.198 mmol/200 mg DM) than for SP (0.287 mmol/200 mg DM; $P < 0.01$). Microbial biomass production (MBP) was numerically higher for AH than for SP, and this difference reached statistical significance ($P < 0.01$). EMBP followed a similar trend, with SP showing slightly lower values than AH ($P < 0.0001$). PF was also lower for SP (11.45 mg/ml) relative to AH (12.37 mg/ml; $P < 0.0001$). In contrast, short-chain fatty acid (SCFA) production was significantly lower for AH (0.198 mmol/200 mg DM) than for SP (0.287 mmol/200 mg DM; $P < 0.01$). MBP was numerically higher for AH than for SP, and this difference reached statistical significance ($P < 0.01$).

Table 4. Computational parameters and CH_4 production of saffron petals and dried alfalfa in 24 hours of incubation

Item	OMD	ME	SCFA	MBP	EMBP	PF	CH_4
Saffron petals	49.00^b	8.16^b	0.287^a	372.9^b	0.944	11.45^b	3.34^b
Alfalfa hay	51.73^a	9.84^a	0.198^b	390.0^a	0.959	12.37^a	6.76^a
SEM	0.2444	0.0698	0.0080	1.5293	0.0017	0.0987	0.0477
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

OMD, organic matter degradability (%); ME, metabolizable energy (MJ/kg DM); SCFA, short-chain fatty acids (mmol/200 mg DM); MBP, microbial biomass production (mg/g DM); EMBP, efficiency of microbial biomass production; PF, partitioning factor (mg/ml); CH_4, methane (ml/200 mg DM).

*Significant differences between values in a column with various superscripts ($P< 0.05$)

Also, the estimated values of ME, NEL, SCFA and DOM of SP were 10.79 (MJ/kg DM), 3.62 (MJ/kg dry matter), 0.75 (mmol/g DM) and 43.74 (%), respectively. The estimated values of ME and DOM for saffron forage were reported to be 9.7 MJ/kg DM and 65.3%, respectively (Kardan Moghaddam et al., 2012), indicating lower OM digestibility of SP compared to saffron forage. In another study, the amount of ME, SCFA, and OMD was mentioned as 8 (MJ/kg DM), 0.89 (mmol), and 53.9 (%), respectively (Kardan Moghaddam et al., 2012). Akbari Shooshood et al. (2024) reported the metabolizable energy content of diets containing 0, 1, 2 and 3% SP as 9.08, 9.38, 9.50 and 9.71 (KJ/ kg DM) respectively. Also, in their study, the amount of OMD and the PF in the diet containing 3% SP were reported as 68.8 and 2.84 respectively. The methane production data are among the most compelling findings of this study. SP generated only 3.34 ml methane compared to 6.76 ml for AH, representing a 50.6% reduction. This substantial mitigation effect has significant environmental implications given that enteric methane accounts for approximately 17-37% of anthropogenic methane emissions globally (Bao et al., 2025). The mechanism underlying this reduction likely involves multiple pathways. Condensed tannins and other polyphenols in SP may directly inhibit methanogenic archaea, as documented by Vasta et al. (2019), who reported that tannins reduce methane production through both fiber digestion inhibition and direct interaction with methanogen populations.

The efficiency of microbial protein synthesis relative to substrate fermentation—expressed as PF—was lower for SP (11.45 vs. 12.37 mg/ml). Although this 7.4% difference is statistically significant, it should be viewed alongside the much more pronounced reductions in methane production and ammonia concentrations. The modest decline in microbial biomass production (MBP: 372.9 vs. 390.0 mg/g DM) may represent a trade-off: the phenolic compounds in SP exert antimicrobial effects that not only reduce overall microbial population density but also shift community composition toward more efficient, less methanogenic populations. This interpretation is supported by the observation that SCFA production was actually higher for SP (0.287 vs. 0.196 mmol/200 mg DM), suggesting that despite lower biomass, the resident microbial community remains highly fermentative (Vasta et al., 2019). Additionally, the redirection of hydrogen ions toward alternative electron sinks, such as propionate production or reductive acetogenesis, may contribute to the observed methane suppression. Recent research on alfalfa hay saponins by Bao et al. (2025) demonstrated that enzymatically transformed saponins reduced methane proportions by 20-45% while simultaneously enhancing volatile fatty acid production. The present study extends these findings by showing that SP, without enzymatic pretreatment, achieves comparable or superior methane mitigation, likely due to its diverse polyphenolic profile including flavonoids, tannins, and anthocyanins that act synergistically.

3.5. Ruminal fermentation parameters

The rumen fermentation parameters showed significant modulation due to SP inclusion (Table 5). 24- and 48 h after incubation, rumen pH was significantly higher in AH treatment ($P < 0.01$), indicating improved buffering conditions. NH_3-N concentration was significantly reduced in SP ($P < 0.01$), suggesting decreased protein deamination. IVDMD in SP at 24 and 48 h after incubation was 32.05 and 54.07%, respectively, which was significantly lower than AH ($P < 0.01$).

Table 5. Comparison of ruminal fermentation parameters of Saffron petals and alfalfa hay in 24- and 48-hours incubation

Item	pH		NH_3-N		IVDMD	
	24	48	24	48	24	48
Saffron petals	7.10^b	6.98^b	6.50^b	5.75^b	32.05^b	54.07^b
Alfalfa hay	7.27^a	7.11^a	11.40^a	8.71^a	48.48^a	62.65^a
SEM	0.0100	0.0152	0.1113	0.0042	0.1271	0.2137
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

NH_3-N, ammonia nitrogen concentration (mg/dL); IVDMD, in vitro dry matter disappearance (% of DM)
*Significant differences between values in a column with various superscripts ($P< 0.05$)

The significantly lower ammonia nitrogen concentrations in SP fermentations (6.50 vs. 11.40 mg/dl at 24 h; 5.75 vs. 8.71 mg/dl at 48 h) indicate reduced proteolysis and deamination of nitrogenous compounds. This finding is consistent with the well-established protein-binding capacity of condensed tannins, which form stable complexes with both dietary protein and microbial enzymes at rumen pH (typically 6.0-7.0) (Kelln et al., 2020). These complexes dissociate at the lower pH of the abomasum (approximately 2.5-3.5), allowing for intestinal digestion and absorption of amino acids, thereby increasing the supply of rumen-undegradable protein (RUP) to the small intestine.

The review by Kelln et al. (2020) emphasized that moderate concentrations of condensed tannins (5-10 g/kg DM) can reduce bloat risk, increase RUP flow, and improve nitrogen utilization efficiency without compromising fiber digestion. The TT concentration in SP (7.88 mg/g DM) falls precisely within this optimal range, suggesting that SP is ideally positioned to confer these benefits. The lower ammonia levels observed are particularly advantageous for lactating dairy cows and growing lambs, where excessive ruminal ammonia production represents both a nitrogen loss to the animal and an environmental pollutant when excreted as urinary urea (Akbari Shooshood et al., 2024). In contrast, the inclusion of 50–100 g/kg TT has been shown to cause a slight reduction in CP degradability of soybean meal (Valcl & Lavrenčič, 2026). It has been reported that medicinal plants prevent amino acid deamination, thereby reducing ammonia NH_3-N concentration and CH_4, and increasing concentrations of propionate and butyrate in the rumen (Guo et al., 2025).

3.6. In situ nylon bag test

The degradability characteristics obtained from the nylon bags trial showed significant differences between SP and AH (Table 6). The fraction of a was significantly higher in SP ($P < 0.01$), Conversely, the fraction of b and pd fraction were significantly higher in AH ($P < 0.01$). Also, the degradation rate constant (c) was significantly lower in SP compare AH ($P < 0.01$). Furthermore, the ED of AH was significantly higher at passage rates of 0.04, 0.06, and 0.08 h^{-1} compared to SP ($P < 0.05$), indicating a protective effect of SP against rumen degradation.

The in-situ nylon bag degradability data provide complementary insights into the ruminal disappearance kinetics of SP and AH. The fraction of a was significantly higher for SP, consistent with the lower fiber content and greater water-soluble carbohydrate fraction (Jadouali et al., 2022). Conversely, the fraction of b was lower, and the total potentially degradable fraction was reduced accordingly. The constant of c was also lower for SP (0.114 vs. 0.128 h^{-1}), suggesting that once the rapidly fermentable fraction is depleted, the remaining substrate is degraded more slowly than alfalfa (Kelln et al., 2020). Kardan Moghaddam et al. (2014) reported parts A, B and C as 0.32, 0.39 and 0.043 respectively. Similarly, the values of ed4 and ed6 were reported as 52.1 and 48.2 respectively (Kardan Moghaddam et al., 2014). ED values, calculated at passage rates of 0.04, 0.06, and 0.08 h^{-1}, were consistently lower for SP, indicating greater ruminal escape.

This characteristic is advantageous for protein-rich fractions but requires consideration for energy-yielding components. For high-producing dairy cows with rapid passage rates (approximately 0.08 h^{-1}), the lower ED of SP suggests that a substantial portion of its organic matter would escape ruminal fermentation and be digested in the small and large intestines. The partitioning of digestion between the rumen and hindgut has significant implications for the site and extent of nutrient absorption, with post-ruminal digestion potentially providing more efficient energy utilization due to reduced methane losses (Akbari Shooshood et al., 2024). Valcl and Lavrenčič (2026) reported that hydrolyzable chestnut tannins at 100 g/kg combined with high water content increased the bypass protein content of soybean meal from 138 to 563 g/kg, representing an approximate 308% increase in the bypass protein fraction. This shift enhances the supply of RUP, which is then digested and absorbed as amino acids and CP in the small intestine. CP and amino acid utilization in the small intestine is more efficient for milk and meat production than reliance on microbial protein alone (López-Herrera et al., 2026). Regarding energy-yielding components, when energy sources (primarily starch) bypass rumen fermentation, they become available for small intestinal digestion. However, ruminants have an inherently limited capacity for intestinal starch hydrolysis due to relatively low pancreatic α-amylase activity and limited brush border enzyme expression compared to non-ruminants (Harmon et al., 2020). Additionally, ruminal fermentation of starch yields VFAs, which are the primary energy substrate for ruminants and are absorbed efficiently across the rumen wall. Diverting substantial starch to post-ruminal digestion may reduce VFA production while exceeding the animal's limited capacity for intestinal glucose absorption and utilization (López-Herrera et al., 2026).

Table 6. Comparison of *in situ* degradability of Saffron petals and alfalfa hay in 96h incubation

Item	**Parameters**				**Effective degradability (ED)**		
	a	b	c	pd (a+b)	ED4	ED6	ED8
Saffron petals	0.170^{a}	0.300^{b}	0.114^{b}	0.469^{b}	0.391^{b}	0.366^{b}	0.346^{b}
Alfalfa hay	0.114^{b}	0.483^{a}	0.128^{a}	0.598^{a}	0.483^{a}	0.444^{a}	0.412^{a}
SEM	0.0003	0.0004	0.0001	0.0002	0.0002	0.0002	0.0001
P-Value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

a, immediately soluble fraction; b, insoluble but potentially degradable fraction, c, rate constant for the degradation of fraction; pd, potential degradable fraction. ED4, ED6, ED8 = effective degradability at passage rates of 0.04, 0.06, and 0.08 h^{-1}, respectively

*Significant differences between values in a column with various superscripts ($P< 0.05$)

4. Conclusions

SP has a distinctly superior profile of phenolic compounds, including TPC, TT, TF, and ANT, along with markedly higher antioxidant activity (DPPH and FRAP) compared to AH. Regarding in vitro gas production kinetics, SP shows a shorter lag phase and a faster fermentation rate of the non-structural fraction; however, its overall OMD and ME are lower than those of AH. Most notably, SP reduces in vitro methane production by approximately 50%, likely due to its TT and TF content, and lowers ruminal NH_3-N concentrations and in situ effective degradability, indicating a protein-sparing, rumen-protective effect. Nevertheless, these environmental benefits must be explicitly weighed against a key biological limitation: reduced DM digestibility and lower energy availability, which may constrain animal performance and feed efficiency. However, these benefits come with reduced DM digestibility and energy availability, which may limit animal performance and feed efficiency. Thus, while SP holds promise as a methane-mitigating feed additive, it should not replace high-quality forages like AH. Instead, SP should be used strategically at optimized inclusion levels to balance methanogenesis suppression against nutrient utilization. Future research should focus on decoupling anti-methanogenic effects from digestive efficiency losses. Overall, SP is best positioned as a functional feed ingredient, not a conventional forage substitute.

5. Conflict of Interest

No conflicts of interest have been declared by the authors.

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