Journal of Animal and Feed Sciences, 35, 4, 2026, XX, https://doi.org/10.22358/jafs/220272/2026
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

ORIGINAL PAPER

ARTICLE IN PRESS
(final version of manuscript before type-setted PDF)

Comparison of nutritional and anti-nutritional substances and *in vitro* fermentation values of ribwort plantain, Italian ryegrass and sainfoin herbages

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Received: 22 January 2026
Revised: 19 March 2026
Accepted: 1 April 2026

ABSTRACT. This study compared the nutritional composition, bioactive compounds, and *in vitro* ruminal fermentation characteristics of ribwort plantain (*Plantago lanceolata*; PLA) with Italian ryegrass (*Lolium multiflorum*; LOM) and sainfoin (*Onobrychis viciifolia*; OVI) cultivated under arid conditions. Herbage samples were analysed for nutrient composition, amino acids, fatty acids, minerals, carotenoids, and secondary metabolites. *In vitro* ruminal fermentation was evaluated using rumen fluid incubation, and differences between forages were assessed statistically. PLA showed significantly higher concentrations of non-fibrous carbohydrates (NFC), α-linolenic acid, and several amino acids, including aspartic acid, glutamic acid, glycine, isoleucine, leucine, and phenylalanine, compared to LOM and OVI ($P < 0.05$). It also contained higher levels of minerals (Ca, Fe, and S) and carotenoids (lutein, zeaxanthin, β-carotene, γ-carotene, lycopene, and β-cryptoxanthin), resulting in increased total carotenoid and provitamin-A contents. The concentrations of serine, histidine, arginine, threonine, and tyrosine in PLA were similar to those of LOM but higher than in OVI ($P < 0.05$).

Saponin content in PLA was comparable to OVI and higher than in LOM ($P < 0.05$). Although PLA produced lower herbage yield under arid conditions, it demonstrated superior nutritional quality and a richer bioactive profile. These findings suggest that PLA can be considered a valuable alternative forage source in arid environments due to its high content of essential nutrients, omega-3 fatty acids, carotenoids, and secondary metabolites.

KEY WORDS: amino acid, condensed tannins, fatty acid, *Plantago lanceolata*, saponin

Introduction

Meadow and pasture areas contain many species beyond the grass and legume families. Many of these plants possess natural forage value and are utilised by animals. A major limitation in such systems is the insufficient plant diversity, and under Mediterranean climatic conditions, meadows and pastures typically dry out after June, resulting in a marked decline in nutritional value. Therefore, research on alternative forage species capable of remaining green for most of the year while maintaining nutrient content and digestibility is justified (Kara et al., 2016; 2018). *Plantago* species, which grow naturally in the Mediterranean region and many other parts of the world, show potential as forage crops due to their drought tolerance, adaptability to different soil types, and suitability for cutting (Stewart, 1996; Küchenmeister et al., 2014). Belonging to the family *Plantaginaceae*, these perennial and cosmopolitan plants reach heights up to 60 cm, with leaves (lanceolate, oval, elliptical, or segmented, depending on the species) reaching approximately 25 cm. They can also grow in saline soils and are suitable for animal feeding. Historically, *Plantago* species were used by ancient Chinese, Ottoman, and Egyptian civilisations for wound healing due to their therapeutic properties. Flowering generally occurs between April and August. These plants are relatively tolerant of soil acidity and can grow naturally across a wide pH range (4.2–7.8). Although *Plantago* can establish in a variety of soil types without requiring high fertility, sandy and moist soils are considered optimal for growth (Stewart, 1996). Leaves of *Plantago lanceolata* (PLA) are characterised by

a favourable nutritional composition, as Guil-Guerrero et al. (2001) reported that leaf tissues contained 15.8% crude protein (CP), 27.7% neutral detergent fibre (NDF), and approximately 50 mg/100 g total carotenoids on a dry matter (DM) basis. Fresh leaves of PLA were shown to contain relatively high mineral contents, including 60 mg/100 g Ca, 49 mg/100 g Mg, 1.54 mg/100 g Fe, and 0.14 mg/100 g Cu (Guil-Guerrero et al., 2001). Supporting these findings, Sanderson et al. (2003) reported that cultivated PLA reached *in vitro* DM digestibility (DMD) values ranging from 71% to 80%, with CP contents of 11–19% and NDF levels of 37–52% depending on the season. Similarly, Labreveux (2002) demonstrated that *Plantago* species, particularly PLA, maintained relatively high CP concentrations (approximately 19–20%) and low NDF contents (28–39%) during individual growth stages, accompanied by consistently high *in vitro* DMD values (84–86%). In addition to its protein and fibre characteristics, *Plantago* species are notable for their fatty acid composition. Guil-Guerrero et al. (2001) showed that α-linolenic acid accounted for approximately 45% of the total fatty acid profile in PLA and 40% in *P. major*, while the remaining fatty acid fractions differed significantly between the two species. Biologically active iridoid glycosides, flavonoids, flavone glucosides and caffeic acid derivatives have also been identified in the leaves of *Plantago* species (Samuelsen, 2000). It is emphasised that biologically active substances present in *Plantago* species may represent a potential source of natural antioxidants due to their antioxidant activity (Beara et al., 2009; Oprica et al., 2015). Determining the nutritional properties of forages provides essential guidance for researchers and breeders regarding their appropriate inclusion levels in animal rations and their potential effects on animal health and product quality (NRC, 2001; Kara et al., 2018; Kara, 2021). The present study hypothesised that PLA differs from grass and legume forage crops in terms of hay yield, hay quality, and detailed nutritional characteristics when grown under identical climatic conditions. Therefore, accurate determination of chemical composition, energy content, and digestible nutrients is

crucial for evaluating feed quality (Kaplan, 2011; Kara, 2016). In addition to these parameters, assessing the methane mitigation potential of feeds is of particular importance due to their role in global warming (Ersahince and Kara, 2017). Accordingly, the aim of this study was to compare the detailed chemical composition and *in vitro* ruminal fermentation characteristics of herbages obtained from cultivated ribwort plantain (*Plantago lanceolata*, PLA) with those of cultivated Italian ryegrass (*Lolium multiflorum*, LOM) and sainfoin (*Onobrychis viciifolia*, OVI).

Material and methods

This study was approved by the Local Ethics Committee for Animal Experiments of Erciyes University (Kayseri, Türkiye; Approval No: 21/01, Date: 06.01.2021), covering both the plant cultivation trials and the *in vitro* ruminal fermentation phase in lamb nutrition.

Experimental design and field establishment

LOM and OVI seeds were obtained from a commercial forage seed supplier in Kayseri, Türkiye, and PLA seeds were sourced from the Afyonkarahisar Center for Medicinal and Aromatic Plants (Afyonkarahisar, Türkiye). Field trials for PLA, OVI, and LOM were conducted at the experimental plots of the Erciyes University Agricultural Application and Research Center during 2021–2022 to determine herbage yield and forage quality. Plots were established in a randomised complete block design with six replicates, and all species were harvested at the early flowering stage. While PLA and OVI (perennial species) were established once and monitored for two years, LOM (annual) was sown each year between September and October. PLA seeds were sown under rainfed conditions in six rows per plot with 20 cm row spacing at a rate of 400 g/da and a plot length of 4 m. At sowing, fertilisation consisted of

3 kg/daN and 7 kg/da P_2O_5, followed by an additional 3 kg/da N applied in spring. OVI and LOM were established using comparable experimental designs. OVI was sown in six-row plots with 50 cm row spacing at 10 kg/da, while LOM was sown annually in six-row plots with 30 cm row spacing at 4 kg/da (Çolak and Sancak, 2016). Fertilization included 3 kg/da N and 6 kg/da P_2O_5 for OVI and 4.5 kg/da N and 8 kg/da P_2O_5 for LOM at sowing, with an additional 4.5 kg/da N applied in spring. Border effects were minimised by excluding the two outer rows and the first 50 cm of each plot before harvesting the remaining area to determine fresh herbage yield.

Determinations of nutrient composition and forage quality

Forage samples were dried in a forced-air oven (Lovibond, Switzerland) at 55 °C for 48 h to determine DM content. Dried samples were subsequently ground to pass through a 1.0 mm screen using a laboratory mill (IKA A10, Germany). CP, ether extract (EE), and crude cellulose (CC) contents were determined according to AOAC (1995) methods. NDF, acid detergent fibre (ADF), and acid detergent lignin (ADL) contents were analysed following the method of Van Soest et al. (1991). Acid detergent insoluble nitrogen (ADIN) was determined by measuring CP in the ADF residue containing organic matter (OM), as described by AOAC (1995) and NRC (2001).

A 500 g subsample of fresh herbage from each experimental plot was dried at 70 °C until constant weight to determine DM content. DM and CP yields per decare were calculated based on DM yield and the DM and CP concentrations. All plants were harvested at the early flowering stage. Digestible dry matter (DDM), dry matter intake (DMI), relative feed value (RFV), and metabolisable energy (ME) were calculated using ADF, NDF, and CP values expressed on a DM basis, according to the equations described by Moore and Undersander (2002), and Belyea et al. (1993).

Determination of *in vitro* ruminal fermentation

The *in vitro* ruminal antimethanogenic effects of forage plant samples were evaluated for PLA, OVI, and LOM harvested at the early flowering stage (approximately 10% flowering). Rumen fluid was collected from 9 male Akkaraman lambs (approximately 3 months of age) using a rumen probe, 3 h after the morning feeding. The lambs were allocated into three dietary groups (n = 3 per group) and fed total mixed rations (TMR) containing PLA, LOM, or OVI as the main forage source. The ingredient composition and nutrient profiles of the experimental rations are presented in Table 1. Following a 1-month adaptation period to the experimental diets, rumen fluid was collected from each lamb. Approximately 200 ml of rumen fluid from each animal was pooled to obtain a composite inoculum for the *in vitro* gas production assays.

The pooled rumen fluid was transferred to the laboratory in screw-capped glass bottles placed in insulated containers and maintained at 39.0 ± 0.5 °C. Prior to use, the rumen fluid was filtered through four layers of cheesecloth under a continuous flow of CO_2. For the determination of ruminal methane production, forage samples (200 ± 10 mg, ground and passed through a 1.0 mm screen) were incubated in 100 ml calibrated glass syringes (Model Fortuna, Haberle Labortechnik, Germany) containing 20 ml of buffered medium (buffer, macrominerals, microminerals, reducing solution, and resazurin) and 10 ml of rumen fluid. Incubations were conducted in a water maintained at 39.0 ± 0.2 °C using the *in vitro* gas production technique described by Menke and Steingass (1988). Total gas production was recorded, and methane concentration in the gas produced after 24 and 48 h of incubation was determined using an infrared methane analyser (Sensor Europe GmbH, Erkrath, Germany) as described by Kara et al. (2015).

All analyses were performed in triplicate, and three blank syringes containing only inoculum and buffered medium were included to correct for endogenous gas production.

In vitro methane production was evaluated using a factorial arrangement consisting of three forage types (PLA, OVI, and LOM), three replicates, and two incubation times (24 and 48 h), resulting in a total of 18 experimental units.

In vitro DM, OM, NDF, and CP degradability (DMD, OMD, NDFD, and CPD, respectively) of PLA, OVI, and LOM hays at 24 and 48 h was determined based on the DM, OM, NDF, and CP contents of the undigested residues remaining in the incubation syringes after fermentation.

Determination of carotenoids

Carotenoid (α-carotene, β-carotene, γ-carotene, lutein, lycopene, and zeaxanthin) contents in forages were measured using a UV-spectrophotometer (SI Analytics UniLine 8100, Germany) according to the method of Ersahince and Kara (2017), modified from Biehler et al. (2010).

Determination of amino acids

Samples for amino acid analysis were hydrolysed with 6 N HCl at 150 °C for 4 h, and subsequently filtered through a 45 μm membrane filter. Free amino acid composition was determined using high-performance liquid chromatography (HPLC) according to the methods described by Aristoy and Toldrá (1991) and Antoine et al. (1999). Elution Buffer A consisted of Na_2HPO_4, $Na_2B_4O_7$, and NaN_3 (pH 8.2), while Elution Buffer B comprised a mixture of acetonitrile:methanol:water (45:45:10, v/v/v). Amino acid separation and quantification were performed using a Shimadzu LC-20AT Prominence liquid chromatograph equipped with a fluorescence detector and a Zorbax Eclipse-AAA column (4.6 × 150 mm, 3.5 μm). Orthophthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) were used as derivatisation reagents, and 0.4 N borate buffer (pH 10.2) was employed during derivatisation.

Determination of fatty acids

Fatty acid methylation of forage samples was performed using a three-step modified method described by Wang et al. (2015). Methylation was carried out using potassium hydroxide, methanol, and sulfuric acid (H_2SO_4, 10 M). The resulting fatty acid methyl esters (FAME) were extracted into n-hexane and centrifuged at 1600 *g* for 5 min. Subsequently, 1.5 ml of the supernatant was transferred into vials equipped with polytetrafluoroethylene (PTFE)/white silicone septa blue caps and analysed using a gas chromatograph (Thermo Scientific, USA) fitted with an automatic sampler (Thermo AI 1310, USA) Separation was performed on a FAME capillary column (60 m × 0.25 mm ID, 0.25 µm film thickness). The injector temperature was set at 255 °C, the initial oven temperature at 140 °C, and the maximum column temperature ranged from 250 to 260 °C. The carrier gas flow rate was 30 ml/min, and the total run time was 42 min. Fatty acids were identified by comparing chromatographic peaks in the chromatogram with their retention times (Kara, 2020).

Determination of mineral composition

In forage samples, macrominerals including calcium (Ca), magnesium (Mg), phosphorus (P), sodium (Na), and potassium (K), as well as microminerals such as iron (Fe), zinc (Zn), copper (Cu), selenium (Se), manganese (Mn), and cobalt (Co), were determined. For mineral analysis, a 0.20 g sample was weighed and digested with 2 ml HNO_3 and 3 ml H_2O_2 using a microwave digestion system under controlled temperature and pressure (wet digestion). The resulting clear digests were diluted to a final volume of 25 ml with ultrapure water. Prior to analysis, multi-element calibration standards containing the target elements were prepared at concentrations of 0, 1, 5, 10, 20, 30, 40, and 50 ppb. After instrument performance was verified using a tuning solution, calibration standards were analysed first, followed by the digested samples. Elemental concentrations were determined using an inductively coupled plasma mass spectrometer (ICP-MS; Agilent 7500a, USA).

Determination of phenolic compounds and saponins

Phenolic compound contents of the forages (proanthocyanidin, bound condensed tannin (BCT), extracted condensed tannin (ECT) and the BCT/proanthocyanidin ratio) were determined using the butanol-HCl method and UV-VIS-spectrophotometer (SI Analytics UniLine 8100, Germany) (Makkar et al.,1995; Kara 2016). Saponin content in dried forage samples was determined using a modified method of Vador et al. (2012), as described by Ersahince and Kara (2017).

Statistical analysis

The experimental data were first subjected to Levene's test to assess the homogeneity of variance. Normality of distribution was verified using the Shapiro-Wilk test. Statistical analysis were performed using SPSS software (version 17.0). The chemical composition and fermentation parameters of individual forages were evaluated using multivariate analyses under a General Linear Model (GLM) framework, assuming homogeneous variances. Linear and quadratic polynomial contrast analyses were applied to assess dose-response relationship.

The effect of chemical compositions and fermentation values of different forages on the variables were further evaluated using one-way analysis of variance (ANOVA). The following statistical model was applied:

$$Yi = \mu + Si + ei,$$

where: Y*i* – investigated parameter (e.g., herbage yield, forage quality, chemical composition), μ – general mean, S*i* – the effect of the *i*-th forage type and e*i* – the random error term.

Tukey's multiple range test was used to identify significant differences between means ($P < 0.05$).

Principal Component Analysis (PCA) was performed to evaluate multivariate relationships between nutrient composition, fatty acids, amino acids, digestibility parameters, mineral contents, and secondary metabolites of the forage samples. All variables were standardised using Z-transformation (mean = 0, SD = 1), and PCA was conducted based on the

covariance matrix. The first two principal components (PC1 and PC2), which explained most of the total variance, were visualised using a biplot to illustrate correlations and clustering patterns among forage samples (PLA, LOM, OVI) and the measured variables.

Results

Nutrient contents of forage samples harvested from trial plots

Under the arid climatic conditions of the present study, PLA had lower green herbage, DM and CP yields compared with LOM and OVI ($P < 0.001$). The DDM and metabolisable energy (ME) values of PLA were similar to those of LOM ($P > 0.05$) but higher than those of OVI ($P < 0.001$). The DMI of PLA was higher compared to other forages ($P < 0.001$). No significant differences were observed in RFV between the three forage species ($P > 0.05$) (Table 2). The DM content of LOM was higher than those of PLA and OVI ($P < 0.05$). OVI had the highest OM and ADF concentrations among the forage crops ($P < 0.05$). Based on the mean nutrient composition of the biennial PLA, the NFC and ash contents were higher than those of the other forages. The ADIN level of PLA was similar compared to LOM ($P > 0.05$) but higher than that of OVI ($P < 0.05$). EE, ADL and CC contents did not differ significantly between the forages ($P > 0.05$). The hemicellulose concentration of PLA was lower than those of OVI and LOM ($P < 0.05$) (Table 2).

In all three forages, the predominant fatty acids were α-linolenic acid (C18:3, n-3), linoleic acid (C18:2, n-6, cis), palmitic acid (C16:0), oleic acid (C18:1, n-9, cis), stearic acid (C18:0), and myristic acid (C14:0). The proportion of α-linolenic acid was highest in PLA (44.28%), followed by OVI (32.45%) and LOM (24.11%) ($P < 0.001$). In contrast, linoleic acid (n-6) content was lowest in OVI (17.44%) compared to PLA (20.24%) and LOM (21.24%) ($P < 0.001$). Regarding saturated fatty acids, PLA had lower proportions of palmitic acid (19.09%)

than OVI (22.16%) and LOM (26.51%) ($P < 0.001$) and also showed the lowest stearic acid (C18:0) concentration among the forages ($P < 0.01$). Similarly, the oleic acid (C18:1, n-9, cis) proportion was lower in PLA (6.22%) than in OVI (12.27%) and LOM (14.22%) ($P < 0.001$). Myristic acid (C14:0) content exceeded 2% in PLA and LOM and was significantly higher than in OVI (1.28%) ($P < 0.05$). The proportions of total unsaturated fatty acids (UFA), polyunsaturated fatty acids (PUFA), n-3 fatty acids, and the n-3/n-6 ratio were higher in PLA than in LOM and OVI ($P < 0.05$). The n-6 fatty acid proportion in PLA was similar to that of OVI ($P > 0.05$) but lower than in LOM ($P < 0.05$). In contrast, the percentage of very long-chain fatty acids (VLCFA) in PLA did not differ from LOM ($P > 0.05$) but was significantly lower than in OVI ($P < 0.05$) (Table 3).

The amino acid composition of the forages varied significantly (Table 4). PLA had higher concentrations of aspartic acid, glutamic acid, glycine, isoleucine, leucine, and phenylalanine compared to LOM and OVI ($P < 0.001$). Serine, histidine, arginine, threonine, and tyrosine concentrations in PLA were similar to those in LOM ($P > 0.05$) but higher than in OVI ($P < 0.01$). In contrast, lysine, proline, and histidine concentrations were lower in PLA compared to the other forages ($P < 0.001$). Methionine, valine, alanine, and total amino acid concentrations in PLA were intermediate between those of LOM and OVI ($P < 0.001$). The grouped amino acid profile indicated that PLA contained relatively higher proportions of acidic and polar uncharged amino acids, LOM was richer in nonpolar and basic amino acids, whereas OVI generally showed lower levels across most amino acid groups, with moderate proportions of aromatic amino acids (Figure 1).

The concentrations of Ca and S were markedly higher in PLA compared to LOM and OVI ($P < 0.05$). The Ca concentration in PLA was approximately three times higher compared to LOM and twice that of OVI. The S concentration was about fivefold higher than in LOM

and ninefold higher than in OVI ($P < 0.05$). While the concentrations of K, P, Mg, Na, Zn, and Cr were similar in all forages ($P > 0.05$), Fe and Ba levels in PLA were significantly higher than those in the other species ($P < 0.05$). Mn concentrations were comparable between PLA and OVI ($P > 0.05$) but lower in LOM ($P < 0.05$). The Fe concentration in PLA was approximately 50% higher than in LOM and nearly twofold higher that in OVI ($P < 0.05$) (Table 4).

Carotenoid concentrations differed significantly between the forages ($P < 0.001$). Overall, PLA contained the highest levels of lutein, zeaxanthin, β-carotene, γ-carotene, lycopene, β-cryptoxanthin, provitamin A, and total carotenoids. The concentrations of provitamin A, lutein, β-carotene, and total carotenoids in PLA were approximately twofold higher than in LOM and OVI. In addition, zeaxanthin levels were about sixfold higher than in LOM and threefold higher than in OVI, while lycopene and β-cryptoxanthin concentrations were fivefold higher than in LOM and three- to fourfold higher than in OVI. Similarly, γ-carotene concentration in PLA was three- to fourfold higher than in the other forages (Table 5).

The concentrations of antinutritional compounds varied between the forage species (Table 5). Saponin content was similar in PLA and OVI but higher than in LOM ($P < 0.01$). Proanthocyanidin and bound condensed tannin (BCT) levels in PLA were higher than in LOM but lower than in OVI ($P < 0.001$). The proanthocyanidin-to-BCT ratio was greater in PLA and LOM than in OVI ($P < 0.001$), whereas extractable condensed tannin (ECT) content did not differ among the forages ($P > 0.05$).

In vitro fermentation characteristics

In vitro ruminal fermentation was evaluated using rumen fluid collected from lambs (Table 6). Total gas production and *in vitro* DMD and OMD at 24 and 48 h were higher in PLA and OVI than in LOM ($P < 0.05$). Methane production from PLA at both incubation times was lower compared to OVI ($P < 0.01$ for 24 h; $P < 0.05$ for 48 h) and LOM ($P < 0.01$ for 24 h). At 24 h, NDFD was higher in PLA than in LOM and OVI ($P < 0.001$), whereas no differences were observed at 48 h ($P > 0.05$). CPD at 24 h and 48 h incubations in PLA was lower than in OVI ($P < 0.01$) but comparable to LOM ($P > 0.05$) (Table 6).

Multivariate relationships between nutrient and degradation variables

Principal component analysis (PCA) revealed clear differentiation among the forage species based on nutrient composition, fatty acid profiles, amino acid contents, and digestion parameters. The first two principal components (PC1 and PC2) explained a large proportion of the total variance, indicating that most variability in the dataset could be represented in a two-dimensional space (Figure 2). PLA was positioned on the negative side of PC1 and was mainly associated with higher levels of PUFA, particularly α-linolenic and linoleic acids. LOM was located in the upper region of the biplot and showed positive associations with several amino acids, including alanine, methionine, and leucine. In contrast, OVI was located on the positive side of PC1 and was characterised by higher digestibility parameters such as DMD, OMD, and CPD (Figure 2). Overall, the biplot indicated distinct biochemical and nutritional profiles for the three forage species.

A second PCA based on mineral elements and secondary metabolites also showed clear separation in individual forages (Figure 3). PLA forage was associated with higher concentrations of Ca, Fe, S, carotenoids, NDFD, saponins, and ECT. OVI was positioned in the

lower-left quadrant and was characterised by higher levels of CT, including BCT, together with higher DMD and CPD. LOM was located on the positive side of PC1 and was primarily associated with higher K and Zn concentrations. Methane production showed a negative loading on PC2 and was positioned closer to OVI on the biplot.

Discussion

Herbage yield, quality and nutrient composition of forage species

The nutrient composition of PLA has been reported to remain relatively stable under heat and drought stress, although herbage yield may decline (Küchenmeister et al., 2014). In the present study, the lower DM yield of PLA compared with the values reported by Dhamala et al. (2018) may be attributed to differences in climatic conditions, fertilisation rates, and irrigation practices. Under the dry conditions of the experimental area, green herbage, DM, and CP yields of PLA were lower than those of LOM and OVI. This variation may be related to the growth characteristics of these species, particularly the high tillering capacity of Italian ryegrass, which enables greater biomass production per unit area (Stewart, 1996). In addition, the wide distribution of *Plantago* species contributes to their adaptive growth responses under environmental stresses such as soil drought (Mudrik et al., 2002). The herbage yield of LOM observed in this study was within the range reported for Mediterranean climatic regions (Çolak and Sancak, 2016), although lower than the values reported by Lale and Kökten (2020), possibly due to differences in fertilisation and irrigation levels. OVI is an important leguminous species due to its high forage quality and beneficial effects on soil fertility and erosion control (Ertuş et al., 2012). The herbage yield and nutrient composition of OVI obtained in this study were similar to those reported by Koç and Akdeniz (2017), although ME and RFV values were lower than those recorded by Moharrery and Toghyani (2013). Previous studies have shown

that the DM yield of OVI varies widely depending on environmental conditions, ranging from 700 to 1500 kg/ha (Goplen et al., 1991) and from 510 to 650 kg/ha at different growth stages (Turk et al., 2011).

The present results indicated that PLA had higher DDM and ME values than OVI, as well as higher DMI compared with both LOM and OVI. According to RFV classification based on ADF and NDF contents (Richardson, 2001; Moore and Underander, 2002), all three forage species can be categorised as second-quality forage. The CP content of PLA was lower than values reported in Austria and New Zealand (Stewart, 1996; Sanderson et al., 2003; Roslon et al., 2015), likely due to differences in soil type and climatic conditions in Türkiye. However, the ash content of PLA was higher than that of the other species, indicating a relatively higher mineral content. The CP, NDF, and ADF values of LOM were consistent with meta-analysis results reported by Glasser et al. (2013), while the CP, ash, NDF, ADF, and total condensed tannin contents of OVI were comparable with outcomes of Bal et al. (2006). The NDF content of PLA was approximately 45%, which was lower compared to LOM and OVI harvested at similar phenological stages. In addition, PLA contained cell wall components typical of good-quality forage, including 36.7% ADF, 11.3% hemicellulose, and 8.84% ADL (NRC, 2001). NFC, including sugars, pectin, β-glucan, and starch, are mainly degraded by microbial and enzymatic digestion in the rumen and small intestine (Pu et al., 2020). The NFC/NDF ratio influences rumen fermentation and microbial populations. Here, PLA reached the highest NFC content (28%), suggesting potentially better digestibility compared with the other two species. ADIN, representing nitrogen bound to ADF and considered largely indigestible, was similar in PLA, LOM and OVI, indicating comparable effects on CPD in the small intestine (Nakamura et al., 1994).

The lipid content of forages commonly used in ruminant nutrition generally ranges between 1 and 3% DM. The fatty acid composition of these lipids contributes to the functional characteristics of forages and represents an important source of fatty acids for animals, apart from dietary fat supplements and concentrate feeds (NRC, 2001). The predominant fatty acids in forage species were α-linolenic acid (C18:3 ω-3), linoleic acid (C18:2 n-6), palmitic acid (C16:0), oleic acid (C18:1 n-9), and stearic acid (C18:0), which was consistent with previous studies (Mir et al., 2006; Dierking et al., 2010; Kara, 2021). In the current study, the proportion of linoleic acid in PLA was similar to that of LOM but higher compared to OVI, while the α-linolenic acid proportion was higher than in both other forages. In addition, palmitic and stearic acids were lower in PLA compared with LOM and OVI (Kara et al., 2022). Diets rich in linoleic and arachidonic acids stimulate PGF2α synthesis, whereas α-linolenic acid promotes progesterone production and plays a role in reproductive processes such as follicle development, ovulation, implantation, and pregnancy maintenance (Thatcher et al., 1994). Therefore, the favourable fatty acid profile of PLA may positively influence productivity and reproductive performance of dairy cattle.

Environmental stress conditions can induce oxidative processes in plants, leading to degradation of biomolecules such as lipids and proteins (Kumar et al., 2023). Secondary metabolites, particularly proanthocyanidins and other phenolic compounds, enhance antioxidant capacity and protect plant tissues against oxidative damage (Varela et al., 2016). In this context, the relatively higher proanthocyanidin content of PLA may contribute to the preservation of PUFA fractions such as α-linolenic and linoleic acids under drought conditions. Fatty acids also facilitate the intestinal absorption of fat-soluble compounds such as carotenoids (Schweiggert et al., 2012). The higher carotenoid and PUFA contents observed in PLA suggest that carotenoid absorption from this forage may be greater than from the other forages examined in this study.

Amino acids are essential nutrients for ruminant growth and metabolism. Most amino acids utilised by ruminant tissues originate from microbial protein synthesised in the rumen, although undegradable dietary protein (RUP) contributes to the amino acid supply through post-ruminal digestion (NRC, 2001; Gilbreath et al., 2021). In the present study, PLA contained higher levels of several amino acids, including aspartic acid, glutamic acid, glycine, isoleucine, leucine, and phenylalanine compared to LOM and OVI. Although its CP content was lower than that of OVI, the favourable amino acid profile suggests that PLA may represent a valuable protein source under arid or semi-arid conditions.

Mineral composition also differed among forage species. PLA contained higher concentrations of Ca and S compared to LOM and OVI, with Ca levels approximately three- to fourfold higher. The concentrations of Ca, P, and Zn were consistent with previous reports (Guil-Guerrero, 2001), whereas levels of some other minerals were higher than previously reported, possibly due to differences in plant cultivation conditions. Overall, PLA had relatively high macro- and micromineral contents, particularly Ca, Mg, Fe, and Mn, indicating its potential as a mineral-rich forage.

Carotenoids are important plant pigments with antioxidant properties and act as precursors of vitamin A in animals (Nozière et al., 2006). Here, the concentrations of lutein, zeaxanthin, β-carotene, γ-carotene, lycopene, β-cryptoxanthin, and total carotenoids in PLA were higher than those in LOM and OVI. These elevated carotenoid levels suggest that PLA may positively influence animal health, fertility, and the nutritional quality of animal products.

Condensed tannins, also referred to as proanthocyanidins, are polyphenolic compounds composed of oligomeric and polymeric chains of flavan-3-ol units linked by carbon-carbon bonds. At moderate concentrations, they can reduce excessive ruminal protein degradation and improve protein utilisation, whereas high levels may reduce digestibility (Schofield et al., 2001;

Min et al., 2003). In the present study, condensed tannin concentrations in PLA were higher than in LOM but lower than in OVI, suggesting a potentially beneficial level for ruminal protein utilisation. Saponins, which are triterpenoid glycosides, possess antimicrobial and anti-protozoal properties and may influence ruminal fermentation processes (Moses et al., 2014). In this study, saponin concentrations ranged between 3 and 6 mg/g DM, indicating that these forages are not saponin-rich plants but may still exert functional effects on rumen microbial activity.

In vitro ruminal fermentation provides useful information on feed digestibility and fermentation characteristics (Menke and Steingass, 1988). Total gas production for PLA was similar to OVI and exceeded that of LOM, which may be associated with its higher NFC content. Methane production of PLA was lower than that of OVI, potentially due to the presence of tannins and saponins, which are known to inhibit methanogenic microorganisms (Bhatta et al., 2009; Kara et al., 2015). Higher NDFD of PLA at 24 h incubation may be related to its lower ADF and ADL contents, whereas the higher CP degradation observed in OVI may be associated with its lower ADIN concentration. Gas production values indicate greater digestibility for PLA compared with LOM, while the similarity between PLA and OVI suggests comparable fermentation end-products. Although reducing methane emissions from the rumen is desirable, lower methane output in PLA relative to OVI indicates that reduction occurred without negatively affecting ruminal fermentation. In addition, the higher NDFD and OMD values of PLA compared to LOM further supported the view that inhibition of methanogenic microorganisms did not impair fibrolytic activity. The absence of negative effects on fibre digestion indicated that fibrolytic microbial populations were not adversely affected. Therefore, *Plantago lanceolata* can be considered a forage with rumen-modifying properties and anti-methanogenic effects.

PLA herbage grown under arid and semi-arid conditions demonstrated functional nutritional characteristics, together with moderate herbage yield and beneficial ruminal digestion properties. Although PLA herbage yield per decare was lower than that of LOM and OVI, nutritional quality was higher. PLA contained higher concentrations of certain minerals (Ca, Fe, and S), essential amino acids (aspartic acid, glutamic acid, glycine, isoleucine, leucine, and phenylalanine), and carotenoids compared to LOM and OVI. Moreover, higher levels of bioactive compounds such as β-carotene, γ-carotene, lycopene, β-cryptoxanthin, n-3 α-linolenic acid, and secondary metabolites such as saponins and proanthocyanidins further support its functional forage potential.

Plantago lanceolata showed favourable ruminal fermentation characteristics, with higher NDF and organic matter degradation compared to *Lolium multiflorum* and similar total gas production to *Onobrychis viciifolia*. Importantly, methane production was lower than in *O. viciifolia,* indicating a potential anti-methanogenic effect without negatively affecting fibre degradation or overall ruminal fermentation. These findings suggest improved rumen fermentation efficiency combined with reduced methane emissions.

Overall, *P. lanceolata* shows strong potential as a functional forage in ruminant feeding systems in arid and semi-arid regions, due to its favourable nutrient composition, efficient ruminal digestion, and capacity to reduce methane production.

Conflict of interest

The Authors declare that there is no conflict of interest.

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Table 1. Feedstuffs and chemical composition of total mixed rations fed to lambs containing *Plantago lanceolata*, Italian ryegrass, or sainfoin

	TMR with *P. lanceolata*	TMR with Italian ryegrass	TMR with Sainfoin
		g/day	
P. lanceolata herbage	1000	–	–
Sainfoin herbage	–	1000	-
Italian ryegrass herbage	–	–	1000
Barley flake	250	250	250
Maize flake	250	250	250
Cotton seed meal	100	100	100
Marble dust	7	7	7
Dicalcium phosphate	10	10	10
Vitamin-mineral premix**	4	4	4
Salt	3	3	3
	Analyses values, % DM		
Starch	18.47	19.96	18.00
CP	13.88	14.31	14.46
Ash	7.50	7.35	7.24
EE	2.09	2.07	1.76
NDF	35.46	42.62	40.52
ADF	25.62	28.01	27.63
ADL	5.70	4.95	6.97
NFC	41.67	33.65	36.02
ME*	3215.25	3215.18	3215.19

CP – crude protein, EE – diethylether extract, NDF – neutral detergent fiber, ADF – acid detergent fiber, ADL – acid detergent lignin, DM – dry matter, NFC – non-fiber carbohydrates (100-NDF-EE-CP-ash), TMR – total mixture ration, ME – metabolic energy (kcal/kg DM) = 3216 + 0.013 %CP − 0.036 %ADF;** the vitamin-mineral premix was included at a level of 4 g per ration. Accordingly, each 4 g of premix supplied: IU: vit. A 4000, vit. D_3 800; mg: vit. E 80, niacin 400. In addition, the premix provided: mg: manganese 240, organic chelated zinc 160, iron 60, copper 4, cobalt 5, iodine 1, selenium 1. The premix also contained *Saccharomyces cerevisiae* at 1.6 × 10^3 CFU and 0.40 mg DL-methionine

Table 2. Herbage yields and nutrient contents of *Plantago lanceolata*, Italian ryegrass and sainfoin

	P. lanceolata	Italian ryegrass	Sainfoin	SD	SEM	*P*-value
Green herbage yield, kg/da	1458.93^{b}	2100.68^{a}	2192.52^{a}	342.66	89.81	<0.001
DM yield, kg/da	393.29^{c}	718.08^{a}	558.32^{b}	142.21	33.52	<0.001
CP yield, kg/da	61.62^{c}	125.14^{a}	100.88^{b}	27.96	6.59	<0.001
DDM, %	60.92^{a}	60.34^{a}	57.58^{b}	1.73	0.445	<0.001
DMI, %	2.59^{a}	2.06^{c}	2.35^{b}	0.26	0.06	<0.001
RFV	97.59	98.30	105.00	30.71	8.49	0.437
ME, MJ/kg	8.36^{a}	8.33^{a}	7.81^{b}	0.29	0.08	<0.001
DM (for green herbage), %	26.99^{b}	33.82^{a}	25.27^{b}	4.73	0.79	<0.001
			%, DM			
OM	87.89^{c}	89.87^{b}	92.95^{a}	2.48	0.39	<0.001
CP	14.56^{b}	17.01^{a}	17.94^{a}	2.46	0.39	<0.001
Ash	11.16^{a}	10.11^{b}	7.22^{c}	1.86	0.52	<0.001
EE	1.72	1.78	1.41	0.41	0.07	0.063
NDF	44.64^{c}	55.79^{a}	51.28^{b}	5.12	0.77	<0.001
ADF	34.70^{b}	35.25^{b}	38.63^{a}	2.62	0.43	<0.001
ADL	8.29	8.89	9.29	1.60	0.28	0.353
Hems	10.56^{c}	19.56^{a}	12.94^{b}	4.39	0.74	<0.001
NFC	26.83^{a}	16.31^{c}	22.23^{b}	5.06	0.89	<0.001
CC	25.98	26.37	29.09	3.40	0.62	0.079
ADIN*	9.53^{a}	8.20^{ab}	6.08^{b}	2.89	0.60	0.052

CP – crude protein, CC – crude cellulose, EE – diethyl ether extract, NDF – neutral detergent fiber, ADF – acid detergent fiber, ADL – acid detergent lignin, Hems – hemicellulose (NDF-ADF), DM – dry matter, NFC – non-fiber carbohydrates (100-NDF-EE-CP-ash), OM – organic matter, ADIN – nitrogen insoluble in ADF, DDM – digestible dry matter, DMI – dry matter intake, RFV – relative feed value, ME – metabolic energy, SEM – standard error of the means, SD – standard deviation of the means; * given as a % of total N; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

Table 3. Percentages of fatty acids in the total fatty acids of *Plantago lanceolata*, Italian ryegrass and sainfoin herbages

Fatty acids	*P. lanceolata*	Italian ryegrass	Sainfoin	SD	SEM	*P*-value
Myristic acid (C14:0)	2.46^{a}	2.05^{a}	1.28^{b}	0.53	0.14	<0.001
Palmitic acid (C16:0)	19.09^{c}	26.51^{a}	22.16^{b}	3.20	0.83	<0.001
Stearic acid (C18:0)	2.86^{b}	4.35^{a}	5.13^{a}	1.29	0.33	0.006
Oleic acid (C18:1 ω-9 cis)	6.22^{b}	14.22^{a}	12.27^{a}	3.86	1.00	<0.001
Linoleic acid (C18:2 ω-6 cis)	20.24^{a}	21.24^{a}	17.44^{b}	1.83	0.47	<0.001
α-Linolenic acid (C18:3 ω-3)	44.28^{a}	24.11^{c}	32.45^{b}	8.78	2.27	<0.001
Heneicosanoic acid (C21:0)	0.10^{b}	0.09^{b}	0.18^{a}	0.05	0.01	<0.001
cis-11,14,17-Eicosatrienoic acid (C20:3 ω-3)	0.74	1.01	1.21	0.53	0.14	0.401
cis-13,16-Docosadienoic acid (C22:2)	0.43^{b}	0.69^{a}	0.83^{a}	0.20	0.05	<0.001
Lignoseric acid (C24:0)	0.24^{b}	0.21^{b}	0.37^{a}	0.10	0.03	0.010
EPA (C20:5 ω-3)	0.02	0.06	0.04	0.04	0.01	0.288
DHA (C22:6 ω-3)	0.08^{b}	0.19^{a}	0.15^{ab}	0.07	0.02	0.023
SFA	26.03^{c}	34.95^{a}	31.90^{b}	3.93	1.01	<0.001
UFA	73.96^{a}	65.19^{c}	68.89^{b}	3.86	1.00	<0.001
MUFA	7.32^{b}	15.84^{a}	14.03^{a}	4.14	1.07	<0.001
PUFA	66.65^{a}	49.35^{c}	54.86^{b}	7.86	2.03	<0.001
ω-3	45.12^{a}	25.34^{c}	33.89^{b}	8.60	2.22	<0.001
ω-6	21.52^{b}	24.01^{a}	20.97^{b}	1.78	0.46	0.005
ω-9	6.41^{b}	14.67^{a}	12.76^{a}	3.97	1.02	<0.001
ω-3/ω-6	2.10^{a}	1.06^{c}	1.62^{b}	0.45	0.12	<0.001
MCFA	0.23^{c}	0.41^{b}	0.67^{a}	0.19	0.05	<0.001
LCFA	98.20	97.90	97.66	1.08	0.28	0.756
VLCFA	1.54^{b}	1.81^{ab}	2.44^{a}	0.57	0.15	0.021

EPA – cis-5,8, 11,14,17-Eicosapentaenoic acid, DHA – cis-4,7,10,13,16,19-Docosahexaenoix acid, SFA – saturated fatty acids, UFA – unsaturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, w-3 – omega 3 fatty acids, w-6 – omega 6 fatty acids, w-9 – omega 9 fat acids, MCFA – medium-chain fatty acids (6–12 C), LCFA – long-chain fatty acids (14–20 C), VLCFA – very long chain fatty acids (>20 C), SEM – standard error of the means, SD – standard deviation of the means; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

Table 4. Amino acid and mineral contents of *Plantago lanceolata*, Italian ryegrass and sainfoin herbages

	P. lanceolata	Italian ryegrass	Sainfoin	SD	SEM	*P*-value
Amino acids, % DM						
Aspartic acid	0.363[a]	0.255[b]	0.233[c]	0.144	0.024	<0.001
Glutamic acid	0.970[a]	0.817[b]	0.270[c]	0.306	0.051	<0.001
Serine	0.368[a]	0.370[a]	0.282[b]	0.044	0.007	<0.001
Glycine	0.747[a]	0.684[b]	0.596[c]	0.065	0.011	<0.001
Histidine	0.074[c]	0.217[a]	0.123[b]	0.061	0.010	<0.001
Arginine	0.590[a]	0.541[a]	0.260[b]	0.172	0.029	<0.001
Threonine	0.460[a]	0.489[a]	0.307[b]	0.078	0.013	<0.001
Alanine	0.876[b]	0.922[a]	0.658[c]	0.119	0.020	<0.001
Proline	0.983[c]	1.866[a]	1.334[b]	0.380	0.063	<0.001
Tyrosine	0.345[a]	0.310[b]	0.278[c]	0.051	0.009	0.003
Valine	0.777[b]	0.847[a]	0.622[c]	0.098	0.016	<0.001
Methionine	0.120[b]	0.161[a]	0.033[c]	0.056	0.009	<0.001
Isoleucine	0.699[a]	0.671[b]	0.530[c]	0.078	0.013	<0.001
Leucine	1.216[a]	1.029[b]	0.902[c]	0.136	0.023	<0.001
Phenylalanine	0.747[a]	0.706[b]	0.565[c]	0.084	0.014	<0.001
Lysine	0.453[c]	1.055[a]	0.918[b]	0.311	0.052	<0.001
Total amino acids	9.787[b]	10.920[a]	7.701[c]	1.379	0.230	<0.001
Macro minerals, g/kg DM						
Ca	18.28[a]	6.71[b]	9.85[b]	7.36	1.34	<0.001
K	18.10	19.57	16.73	5.32	0.97	0.505
P	2.27	2.54	2.06	0.61	0.11	0.207
Mg	1.47	1.59	1.26	0.50	0.09	0.330
Na	2.67	2.54	2.34	2.11	0.39	0.943
S	1.47[a]	0.32[b]	0.16[b]	0.49	0.22	0.021
Micro minerals, mg/kg DM						
Zn	17.97	20.56	19.23	9.88	1.80	0.852
Fe	1084.57[a]	646.25[b]	534.15[b]	400.11	73.05	0.002
Mn	116.38[a]	82.52[b]	157.51[a]	59.91	19.19	0.022
Cr	50.28	80.11	58.46	61.83	11.29	0.553
Ba	115.59[a]	55.84[b]	50.20[b]	44.52	8.13	<0.001

DM – dry matter, SEM – standard error of the means, SD – standard deviation of the means; [abc] – means within a row with different superscripts are significantly different at $P < 0.05$

Table 5. Carotenoid, saponins, and phenolic compounds concentrations of *Plantago lanceolata*, Italian ryegrass, and sainfoin herbages

	P. lanceolata	Italian ryegrass	Sainfoin	SD	SEM	*P*-value
Carotenoid concentration, mg/kg DM						
Provitamin-A carotenoids	56.61[a]	29.70[b]	29.51[b]	13.39	2.40	<0.001
α-carotene	11.65	9.83	9.27	2.64	0.42	0.088
Lutein	29.01[a]	13.60[b]	14.03[b]	7.43	1.24	<0.001
Zeaxantine	17.59[a]	3.32[b]	5.88[b]	6.61	1.19	<0.001
β-carotene	27.25[a]	14.58[b]	13.56[b]	6.37	1.06	<0.001
γ-carotene	12.91[a]	2.97[b]	3.39[b]	4.86	0.87	<0.001
Lycopene	10.64[a]	2.23[b]	3.50[b]	3.98	0.71	<0.001
β-cryptoxanthin	17.70[a]	3.56[b]	5.80[b]	6.65	1.20	<0.001
Total caroteinoids	126.75[a]	52.95[b]	56.48[b]	35.74	6.42	<0.001
Saponin and phenolic compounds						
Saponin, mg/g DM	6.05[a]	3.31[b]	4.92[ab]	2.72	0.36	0.006
Proanthocyanidin, g/100 g DM	0.99[b]	0.68[c]	1.57[a]	0.44	0.06	<0.001
BCT, g/100 g DM	0.31[b]	0.17[c]	0.94[a]	0.34	0.05	<0.001
ECT, g/100 g DM	0.68	0.51	0.62	0.27	0.04	0.150
BCT ratio, %	33.13[b]	25.36[b]	61.80[a]	17.56	2.37	<0.001

Provitamin-A carotenoids – α-carotene + β-carotene + β-cryptoxanthin, BCT – bound condensed tannin, ECT – extractable condensed tannin, DM – dry matter, SEM – standard error of the means, SD – standard deviation of the means, Proanthocyanidin – total condensed tannin; BCT ratio, % = (BCT/Proanthocyanidin) × 100; [ab] – means within a row with different superscripts are significantly different at $P < 0.05$

Table 6. *In vitro* total gas and methane productions and in vitro nutrient degradation rates of forages

	Plantago lanceolata	Italian ryegrass	Sainfoin	SD	SEM	*P*-values
In vitro total gas production, ml/0.2 g DM						
24 h	49.68^{a}	42.56^{b}	49.87^{a}	4.42	1.27	0.009
48 h	56.37^{a}	49.25^{b}	59.25^{a}	6.42	1.85	0.059
In vitro methane production, ml/0.2 g DM						
24 h	6.79^{b}	8.06^{b}	9.40^{a}	1.25	0.36	0.001
48 h	8.73^{b}	9.64^{ab}	11.62^{a}	1.64	0.47	0.018
DMD, %						
24 h	60.71^{a}	52.82^{b}	65.63^{a}	1.36	0.70	0.047
48 h	61.52^{a}	58.36^{b}	72.00^{a}	1.40	0.53	0.015
OMD, %						
24 h	65.33^{a}	55.42^{b}	66.01^{a}	5.20	1.73	<0.001
48 h	86.56^{a}	64.46^{b}	86.56^{a}	11.07	3.69	<0.001
NDFD, %						
24 h	74.44^{a}	67.80^{b}	64.68^{c}	4.41	1.47	<0.001
48 h	77.01	73.26	73.45	3.01	1.23	0.474
CPD, %						
24 h	74.72^{b}	74.95^{b}	77.43^{a}	1.37	0.45	0.001
48 h	79.92^{b}	78.84^{b}	83.41^{a}	2.10	0.70	<0.001

DM – dry matter, OM – organic matter, NDF – neutral detergent fiber, CP – crude protein, DMD – DM degradation, OMD – OM degradation, NDFD – NDF degradation, CPD – CP degradation, SEM – standard error of the means, SD – standard deviation of the means; ^{abc} – means within a row with different superscripts are significantly different at $P < 0.05$

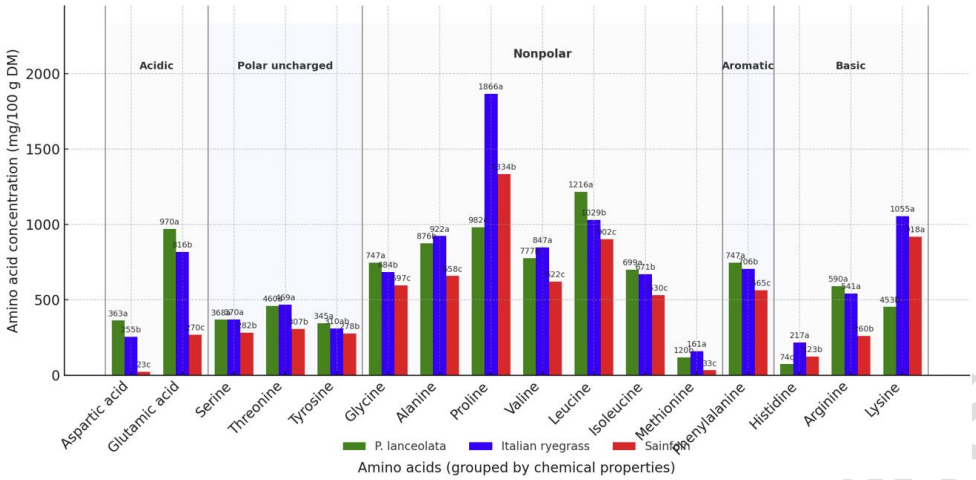

Figure 1. Comparison of amino acid profiles of *Plantago lanceolata*, Italian ryegrass, and sainfoin. Different superscript letters (^{abc}) indicate significant differences ($P < 0.05$) among forage species within each amino acid (One-way ANOVA).

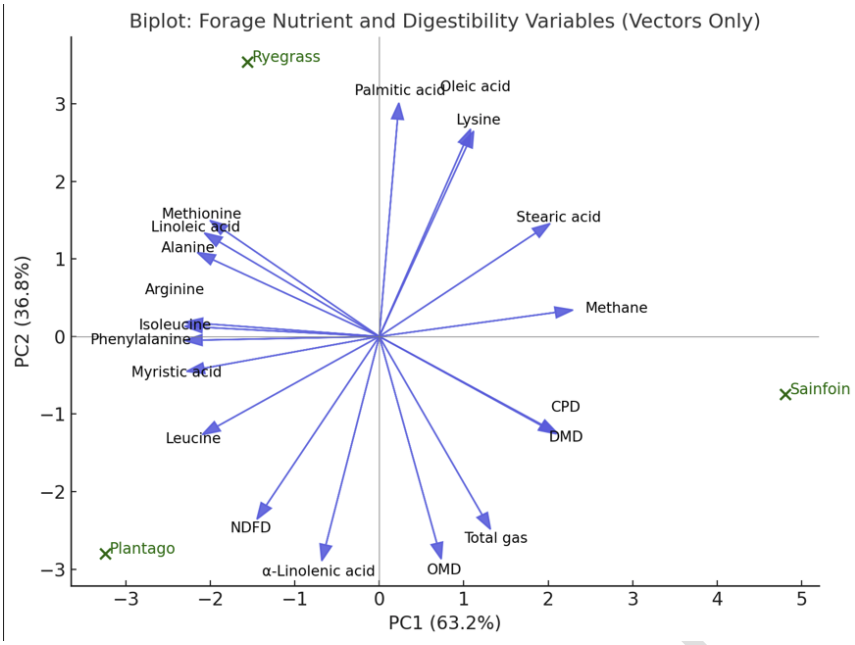

Figure 2. PCA biplot showing relationships among fatty acids, amino acids, and digestibility parameters in three forage species. DMD – dry matter degradation, OMD – organic matter degradation, NDFD – neutral detergent fiber degradation, CPD – crude protein degradation, ME – metabolizable energy, NEL – net energy for lactation, α-Linolenic acid – alpha-linolenic acid, PC – principal component

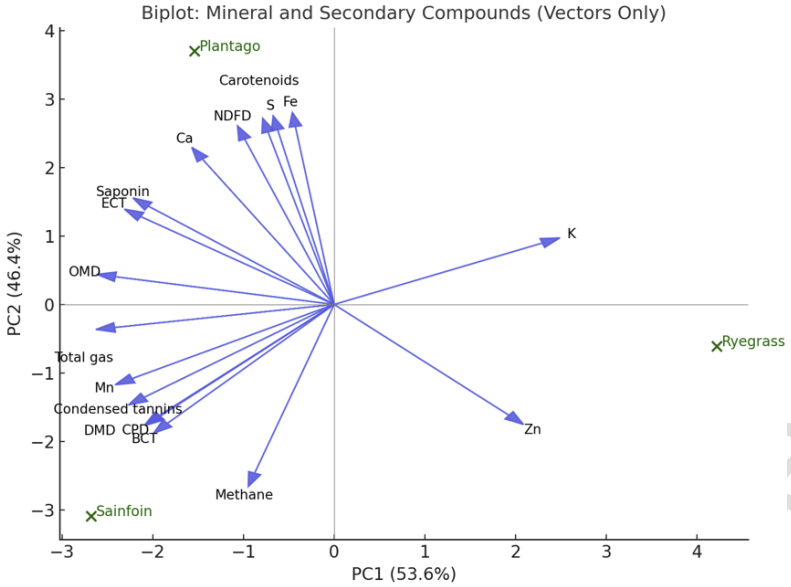

Figure 3. PCA biplot of minerals and secondary compounds in three forage species.

Ca – calcium, K – potassium, S – sulfur, Zn – zinc, Fe – iron, Mn – manganese, BCT –bound condensed tannins, ECT – extractable condensed tannins, DMD – dry matter degradation, OMD – organic matter degradation, NDFD – neutral detergent fiber degradation, CPD – crude protein degradation, PC – principal component