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Effects of plantago species herbage and silage on *in vitro* ruminal fermentation and microbiome

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ABSTRACT

The present study aimed to compare the nutrient composition, *in vitro* ruminal fermentation values and microbiome in the ruminal fermentation of herbage and silage of the *Plantago media, P. major* and *P. lanceolata* species. The lactic acid (LA) content of *P. lanceolata* silage was higher than those of other plantago silages ($p < 0.05$). The α-linolenic, w-3, polyunsaturated (PUFA), medium chain (MCFA) and long-chain fatty acids (LCFA) of plantago silages were lower than those of plantago herbages ($p < 0.05$). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents, total gas and methane production, metabolic energy (ME) and organic matter digestion (OMD) values and ammonia-nitrogen concentration in the *in vitro* fermentation fluid of *P. major* silage were lower than those of other plantago silages ($p < 0.05$). The *in vitro* ruminal methane production and community of archaea *Methanobrevibacter* in the microbiome of *P. major* herbage were higher than that of *P. media* and *P. lanceolata* herbages. The ensiling process significantly increased the *in vitro* total gas production, acetic acid concentration and *Prevotellaceae* bacteria of *P. media* and *P. lanceolate* compared their herbages. As a result, *P. lanceolata* and *P. media* silages provided high-quality silage fermentation; the nutrient composition was not lost to a great extent in the silage environment and the ruminal fibrolytic bacterial composition was positively affected. *Plantago major* silage could not provide a good silage quality and the *in vitro* ruminal fermentation and ruminal fibrolytic bacteria community value were negatively affected.

HIGHLIGHTS

- The crude protein (CP) and very long chain fatty acids contents of *P. major* herbage were higher than those of *P. media* and *P. lanceolata*. However, the *P. major* silage had high silage CP and ammonia-nitrogen concentration, low *in vitro* ruminal digestion (total gas production, ME and OMD), and plant cell wall substances.
- The ensiling of *P. lanceolata* and *P. media* positively affected the ruminal fibrolytic bacterial composition (*Rikenellaceae_RC9_gut_group*, *Oscillospiraceae_UCG-002* and *Prevotellaceae*).
- The *in vitro* ruminal methane production and community of *Methanobrevibacter* in the ruminal microbiome of *P. major* herbage were higher than those of *P. media* and *P. lanceolata* herbages.

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Forage; microbiome; organic acid; plantago; rumen fluid

Introduction

The quality of forage, which is general expression of the levels of CP, structural carbohydrates, non-structural carbohydrates, inorganic matter and fatty acids and the fermentation capacity in gastro-intestinal cannula, changes according to the preservation form (herbage, hay, silage), climatic conditions (annual rainfall, temperature etc.) and soil characteristics (irrigation, pH, salty etc.) (Kara et al. 2018). Nowadays, the driest period of the last 900 years is experienced in the countries of the south of Europe and the Mediterranean region, where the effect of global drought is more pronounced (Ramirez-Restrepo and Barry 2005; IPCC 2014). Semi-arid areas are considered arid, while arid regions become desert (Altın et al. 2012). The green pastures in the arid and semi-arid areas present at a certain level in the spring and early summer months; after early summer, most plants turn yellow and dry out. In addition, global warming now encountered in the producing of high-quality forages,

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especially in arid and semi-arid areas (IPCC 2014). Researchers focus their studies on forage crops resistant to drought and stay green throughout the year (Pommerrenig et al. 2007; Rozentsvet et al. 2015; Kara et al. 2018; Minnée et al. 2020). Mudrik et al. (2003) stated that the wide distribution of the plantago species demonstrated that these species effectively adapt to various environmental conditions, especially soil drought stress. These plants are also resistant to cold weather conditions and can continue to grow in winter. Plantago (250–300 species) belongs to the Plantaginaceae family and grows naturally in different parts of the world (Guil-Guerrero 2001). The plant height varies according to the species, up to 60 cm and the leaf length (lancet, oval, elliptical or sliced according to the species) can reach up to approximately 25 cm. They are perennial and cosmopolitan plants that can grow in salty soils used for feeding (Samuelsen 2000). In the leaf parts of plantago species, biologically active glycosides (aucubin and catalpol), flavonoids, flavone glucosides, caffeic acid derivatives, some effective mucilage polysaccharides, monoterpenoids, vitamins and tannins have been found (Samuelsen 2000; Oprica et al. 2015). Plantago species' resistance to plant diseases shows their superiority over other forage crops (Kara et al. 2016). Although it is a perennial plant, it can bloom in the first year. It usually blooms from April to August (Fons et al. 1998). Among these plants, the *P. lanceolata* (lanceolate plantain), *P. major* (broad-leaved plantain), and *P. media* species have the widest distribution (Bıçakçı et al. 2011). It has been reported that the leaves of plantago species were consumed voraciously by animals grazing in the pasture, as their flavour increases due to sorbitol, which accumulates at a high rate, especially in those grown in salty and arid soil conditions (Pommerrenig et al. 2007). Plantago species differs from each other in terms of their nutritional content. The leaf of *P. lanceolata* included about 15% crude protein (CP), 28%, neutral detergent fibre (NDF) and 50 mg/100g of total carotenoid (Guil-Guerrero 2001). A study reported that *P. lanceolata*, harvested in spring, summer, and winter, varied between 71 and 80% *in vitro* DM digestibility, between 37 and 52% NDF value and between 11 and 19% CP values (Sanderson et al. 2003). The lanceot and tonic plantago species cultured from plantago species were on average 19–20% of CP values, 28–39% of NDF values and 84–86% of *in vitro* DM digestion (Labreveux 2002). Hay, vital important source of feedstuffs used in the ruminant ration, contains 1–3% fat and its fatty acid profile affects health and animal product quality (NRC 2001). It has been determined that the linolenic acid fatty acids content (C18:3), which has important biological activity, differ significantly between plantago species (45% for *P. lanceolata,* and 40% for *P. major* in the total fatty acid profile) (Guil-Guerrero 2001). One of the nutritional ingredients of the plantago species that may affect its biological activity may be its fatty acid composition.

It is understood that it is important to determine the plantago species with the best feed quality, silage quality and effect on the rumen microflora when the effect of drought is evident as globally. The present study aims to compare the nutrient composition, *in vitro* ruminal fermentation values, and rumen microbiome of herbage and silage of *P. media*, *P. major*, and *P. lanceolata* species. The study hypothesises that the effects of plantago herbage and silage on nutrient composition, silage fermentation, *in vitro* ruminal fermentation, and microbiome will be variable between species, and the superior species will be determined.

Material and methods

Plantago herbages and silages

The herbage samples of *P. lanceolata, P. major,* and *P. media* were collected from Kayseri province, Türkiye (38°56 N, 34° 24 E). The samples of plantago herbage were collected from the same field (approximately 3000 m^2) in June 2021 (at the beginning of the summer season). The samples of plantago herbage were collected from six different points of the same field using the random sampling method (Kara et al. 2018). The samples (total of 30 kg) were collected from over 1 cm on top of the land. The chopped forage was ensiled within one hour of harvesting time. The plantago species were harvested at the early flowering stage, and included all aerial parts (leaf, stem, or bud flower). For each plantago species, the silage process was done in eight replicates. The *P. lanceolata*, *P. major*, and *P. media*, were ensilaged in a polyethylene (25 cm × 35 cm sizes) silage bag. Each silage bag contained approximately 600 g of silage material. The cutting length of plantago herbage was approximately 2–2.5 cm length. These polyethylene bags were vacuumed using a vacuum machine (Caso VC100, Germany). The silage bags were stored in laboratory conditions in a sun-free environment for 60 days, and, then, over time, all bags opened. All silage bags were analysed in triplicate for chemical composition values.

Chemical compositions in herbage and silages

The silage materials and herbage were dried in a thermostatically controlled cabinet (Lovidond, Switzerland) for 48 hours at 60 °C, and dry matter (DM) content was calculated. Dried samples were mill in a grinder milled (IKA Werke, Germany) to a maximum of 1 mm particle size. The samples were analysed for the determination of DM, ash, crude protein (CP; nitrogen x 6.25), diethyl ether extract (EE) (AOAC 1995), neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Van Soest et al. 1991). The EE contents of herbage and silage were methylated with the three-stage modified procedure of Wang et al. (2015) in triplicate. A column of the fatty acid methyl esters (FAME's), used for the separation of fatty acid (cyanopropyl-phenyl-based phase, length: 60 m, internal diameter: 0.25 mm, film: 0.25 μm and maximum temperature 250–260 °C; Thermo Scientific TRACE^{TM}, TR-FAME GC Columns, catalogue number: 260M153P, USA). The FAME's in n-hexane were taken in a 1.5 ml screw neck ND-9 amber vial with 9 mm screw caps (silicone white/PTFE caps). The percentages of FAME's were detected in a gas chromatograph with flame-ionisation detection (GC-FID, Thermo Scientific, USA). The peak identification of each FAME was detected in a commercial FAME's mix standard solution in dichloromethane (Chem-Lab, catalogue number: CL.40.13093.0001, Zedelgem, Belgium). The FAME's identification was performed by comparing the retention times with the expected retention times of the standard mixture in chromatograms (Kara 2020).

Acidity values and ammonia-nitrogen concentration in silages

After the silage bags were opened, the pH values of the materials were immediately determined. Firstly, 25 g of wet silage sample was shredded for 15 seconds with 100 mL of distilled water in a laboratory-type blender (Waring Commercial, Torrington, CT, USA). The pH value of the filtered silage material was measured with a digital pH metre (Mettler Toledo, S220 pH/ion metre, Ohio, USA). The lactic acid (LA) content in silage fluid was determined in a spectrophotometrically (SI Analytics – Xylem Analytics Germany Sales GmbH & Co. KG, Mainz, Germany) method (Tekin and Kara 2020), modified from Barnett (1951). The analysis of straight short-chain fatty acids (SCFA) [acetic acid – AA (C 2:0), propionic acid - PA (C 3:0), butyric acid - BA (C 4:0), valeric acid - VA (C 5:0), and caproic acid - CA (C 6:0)] and branched SCFAs (BSCFA) [iso-butyric acid - IBA (C 4:0i), iso-valeric acid - IVA (C 5:0i) and iso-caproic acid – ICA (C 6:0i)] in silage fluid was measured by using a gas chromatograph- flame ionisation detector (GC-FID) (TRACE^{TM}1300, Thermo Fisher Scientific, Orlando, USA) with polyethylene glycol column (length: 60 m, i.d: 0.25 mm, film thickness: 0.25 μm; TG-WAXMS, Thermo Scientific, Orlando, FL, USA) device according to the method of Ersahince and Kara (2017). The ammonia-nitrogen concentration in silage fluid was determined using commercial ammonia assay procedure (Megazyme, K-AMIAR 02/20, Wicklow, Ireland).

The in vitro ruminal fermentation of forages

The *in vitro* ruminal fermentation values of plantago herbages (*P. lanceolata* herbage, *P. major* herbage, *P. media* herbage) and silages (*P. lanceolata* silage, *P. major* silage, *P. media* silage) were analysed using an *in vitro* gas production technique. Rumen fluid (approximately 1 L), used in the *in vitro* gas production technique, was taken from two Brown Swiss-Simmental mix breed cattle using a gastric tube. Rumen fluid was taken 3 hours after feeding. The cattle consumed the ration, which included maize silage (5 kg/day on as fed basis), wheat straw (1.7 kg/day on as fed basis), alfalfa herbage (1.5 kg/day on as fed basis), sugar beet pulp (4.5 kg/day on as fed basis) and concentrated mix feed (5 kg/day on as fed basis). Approximately one litter of rumen fluid was collected in a thermos, which included water, at 39 °C using CO_2 gas, and filtered with six layers of cheesecloth in the laboratory. The samples were incubated in rumen fluid and buffer mix in 100 ml capacity calibrated anaerobic glass fermenter (Fortuna®, Poulten & Graf Ltd., Wertheim, Germany) following the procedures of Menke et al. (1979). The reducing solution in the *in vitro* fermentation medium was added for providing the oxidation-reduction potential (ORP) and the anoxic conditions. The pH and ORP of the *in vitro* digestion fluids were measured using Mettler Toledo InLab® Expert Pro-ISM sensor probes in pH-Ion metre (Seven Compact^{TM} pH/Ion S220, Mettler-Toledo, Schwerzenbach, Switzerland). The 200 ± 10 mg of dried samples (substrates) were incubated with 20 ml of buffer mixture and 10 ml of filtered rumen fluid in an anaerobic glass fermenter at 39.0 ± 0.5 °C in an incubator for 24 h, as five repetitions (Menke et al. 1979). Besides, five blank glass fermenters (no samples) were incubated to provide correction values. The total gas volume and produced substrates were read from the volume lines on the glass fermenter at 24 h. The amount of methane gas in total gas produced at 24 h

was determined in an infra-red methane measurement device (Sensor, Europe GmbH, Erkrath, Germany) according to Kara et al. (2015), in all anaerobic glass fermenter. The metabolic energy (ME) and organic matter digestion (OMD) values of the herbage and silages were calculated with total gas production and nutrient matter composition using equations by Menke and Steingass (1988).

The SCFA and ammonia-nitrogen in the in vitro *fermentation fluid*

After *in vitro* incubation, SCFA and ammonia-nitrogen analyses of the *in vitro* fermentation fluid were carried out. The 2 mL of fermentation fluid was centrifuged at 15000 rpm x g for 15 min in a micro-centrifuge (Gyrozen 1524, Gyrozen Co. Ltd., Daejeon, Korea). The 1.25 mL of the supernatant and 0.25 mL of meta-phosphoric acid (25%, w/v) were mixed. Analysis of SCFA was determined by using a gas chromatograph (GC) device (Thermo Trace 1300, Thermo Scientific, USA) with an auto sampler (Thermo AI-1310, Thermo Scientific, USA). The GC device was equipped with a Flame Ionisation Detector (FID), with polyethylene glycol columns (length: 60 m, i.d: 0.25 mm × 0.25 μm, film thickness: 0.25 μm) (TG-WAXMS, Thermo Scientific, USA). The operation procedure of the GC-FID was according to the study of Ersahince and Kara (2017). The ammonia-nitrogen content in fermentation fluid was determined using a commercial ammonia assay procedure (Megazyme, K-AMIAR 02/20, Wicklow, Ireland) on a spectrophotometer (Xylem Analytics Germany Sales GmbH & Co. KG, Mainz, Germany).

Microbiome analyses of the in vitro *fermentation fluid*

After *in vitro* incubation, microbiome analyses of the *in vitro* fermentation fluid were carried out. Total DNA of *in vitro* rumen fluid contents was extracted using the commercially kit (GeneMATRIX Tissue & Bacterial DNA Purification Kit, EURx Moleculer Biology Products, Przyrodników, Gdańsk, Poland). The concentration and quality of DNA were evaluated using a fluorometer (The VICTOR3^{TM} Multilabel Plate Reader, Perkin Elmer, Shelton, CT, USA) using fluorescent-based double-chain Picogreen dye. The 16S rRNA (V3-V4) sequences were used to taxonomically identify bacterial and archaeal strains. This primer pair amplifies a region of approximately 460 bases. Before the next-generation sequencing readout, the sample's 16S V3- V4 regions are amplified by PCR during amplicon library preparation. The V3–V4 region was amplified using the 16S Amplicon PCR Forward Primer = 5′ (TCGTCG GCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGG C WGCAG) and the 16S Amplicon PCR Reverse Primer = 5′ (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGAC TACHVGGGTATCTAATCC) primers (Klindworth et al. 2013).

The primers used for the gene-specific 16S rRNA V3-V4 region were taken from the Klindworth et al. (2013). Metagenomic sequencing was performed on an Illumina Novaseq 6000 platform (Illumina, San Diego, CA, USA). Illumina's 'Sequencing by Synthesis' technology detected each base as it was added to the DNA template during sequencing. Deoxyribonucleotide triphosphate (dNTP) mixes used in sequencing were special reversible, terminator-bound dNTPs. These dNTPs reduced the margin of error and the possibility of incorrect base addition due to their unique chemical structure in each sequencing cycle. The Illumina Novaseq instrument processes were read with the Novaseq Control Software program and bases were determined using the Real Time Analysis v1.18 program. Base reads were converted to FASTQ format with the bcl2fastq (v1.8.4) program. Scythe (v0.994 BETA) and Sickle programs extracted non-specific adapter sequences from the read results. With bioinformatics analysis, the taxonomic classification of the samples, alignment according to reference sequences, operational taxonomic unit determination, and grouping of species according to their similarities were performed.

Statistical analysis

Three different herbages and three silages were studied in five repetitions, and 30 anaerobic glass fermenters were used. For each plantago species, each silage bag was analysed as three replications (8 silage bags × 3 replications = a total of 24 replications). *In vitro* fermentation and end product analyses (total gas, methane, estimated fermentation values, SCFA, microbiome, etc.) were performed with five replications.

The experimental data were first subjected to Levene's test to detect the variance homogeneity. Statistical significance among plantago silages for silage acidity and ammonia-nitrogen were determined by one-way variance analysis. The multivariate analyses were implemented for homogeneous variances by General Linear Model procedures to test treatment differences for nutrient, fatty acids, and *in vitro* ruminal fermentation values of plantago herbage and silages. The two-way variance analysis was conducted on

silage chemical compositions and *in vitro* digestion values tested in different silages. Data (nutrient matter, fatty acids, and *in vitro* ruminal fermentation values) were analysed using the following statistical model: $Yijk = \mu + Ei + Dj + EDij + eijk$. Where, Y*ijk* – the overall mean for each parameter investigated; μ – over mean; E – effect of *i*: plantago species on the observed parameters; D – effect of *j* – forage type on the observed parameters; ED – interaction between the *i* – plantago species and *j* – forage type; eijk – the standard error term. Tukey post hoc test was used to determine the significant differences at $p < 0.05$.

Results

Chemical composition

The chemical compositions of plantago herbage and silages are shown in Table 1. The DM and ADF contents of plantago species' herbages were similar ($p > 0.05$). The DM and ash contents of *P. major* silage were higher than those of *P. media* silage and *P. lanceolata* silage ($p < 0.05$). The NDF and ADF contents of *P. major* silage were lower than those of *P. media* silage and *P. lanceolata* silage ($p < 0.05$). The average DM, ash and EE contents of plantago silages were higher than those of plantago herbages ($p < 0.05$). The CP, NDF and ADF contents of plantago silages were lower than those of plantago herbages ($p < 0.05$).

Silage ammonia-nitrogen and acidity values

The ammonia-nitrogen concentration and acidity values in silages of different plantago species are shown in Table 2. The ammonia-nitrogen concentration of *P. major* silage was higher than those of *P. media* and *P. lanceolata* silages ($p < 0.05$). The pH values in silage fluids of *P. media* and *P. major* were higher than that of *P. lanceolata* ($p < 0.05$). The LA content in silage of *P. lanceolata* was higher than those of *P. major* and *P. media* ($p < 0.05$). Besides, the BA, PA and IBA contents in silage of *P. media* were higher than those of *P. major* and *P. lanceolata* ($p < 0.05$).

Fatty acid composition

The compositions of fatty acid in total fatty acids of plantago herbage and silages are shown in Table 3. The percentage of palmitic acid of *P. media* herbage was lower than those of *P. major* and *P. lanceolata*

Table 1. The chemical composition of plantago herbage and silages.

Forage type	plantago species	DM	Ash	CP	EE	NDF	ADF	NFC
Herbages	*P. media*	20.26	12.79^{B}	12.64^{B}	1.85^{B}	43.07^{A}	32.20	29.64
	P. major	19.63	16.28^{A}	14.57^{A}	2.02^{A}	38.13^{B}	33.90	28.98
	P. lanceolata	21.64	12.64^{B}	11.88^{B}	1.85^{B}	46.27^{A}	31.02	27.35
	SEM	1.13	0.74	0.29	0.15	0.59	0.83	0.75
Silages	*P. media*	21.58^{B}	13.63^{B}	12.64^{A}	2.13	44.01^{A}	33.46^{A}	27.57^{B}
	P. major	27.05^{A}	14.56^{A}	9.62^{B}	2.23	23.02^{B}	22.88^{B}	50.55^{A}
	P. lanceolata	21.01^{B}	12.81^{B}	12.98^{A}	2.26	42.98^{A}	33.31^{A}	28.96^{B}
	SEM	0.98	0.06	0.25	0.01	0.51	0.72	0.65
Forage type	Herbages	20.68	13.42	12.72	1.89	43.36	32.07	28.59
	Silages	23.21	13.67	11.75	2.21	36.67	27.55	35.70
P values	Forage type	0.031	0.062	0.021	<0.001	0.001	0.003	0.001
	Plantago species	0.471	<0.001	0.573	0.008	<0.001	0.002	<0.001
	Interaction	0.029	<0.001	0.004	0.022	0.001	0.001	0.001

^{A,B}: The difference between the average values indicated by different letters of plantago silages or Plantago herbage for different Plantago species is important (one-way ANOVA-Tukey). ^{a,b}: The difference between the average values indicated by different letters for the silage type is important.
DM: Dry matter in wet basis, CP: crude protein in DM; EE: diethyl ether extract in DM; NDF: neutral detergent fibre in DM; ADF: acid detergent fibre in DM; NFC: non-fibre carbohydrate in DM (NFC, % = 100 – NDF, % - CP, % - EE, % - ash, %).

Table 2. The ammonia-nitrogen concentration and acidity values in silages of different plantago species.

			%, silage DM							
	Silage fluid			SCFA					BSCFA	
Silages	Ammonia-nitrogen, mg/L	pH	LA	AA	BA	PA	CA	VA	IBA	ICA
P. media	34.95^{b}	4.55^{b}	1.14^{c}	0.09	0.33^{a}	0.04^{a}	0.05	0.04	0.31^{a}	0.06
P. major	51.45^{a}	4.46^{b}	3.86^{b}	0.10	0.03^{b}	0.02^{b}	0.05	0.04	0.25^{b}	0.06
P. lanceolata	34.97^{b}	4.08^{a}	5.68^{a}	0.17	0.03^{b}	0.02^{b}	0.05	0.04	0.09^{b}	0.06
SD	11.87	0.23	2.15	0.07	0.17	0.01	0.01	0.0001	0.14	0.002
SEM	2.42	0.05	0.44	0.01	0.04	0.002	0.002	0.0001	0.03	0.001
P values	0.002	<0.001	<0.001	0.225	<0.001	<0.001	0.527	0.745	0.020	0.408

AA: acetic acid; BA: butyric acid; PA: propionic acid; CA: caproic acid; VA: valeric acid; IBA: iso-butyric acid; ICA: iso-caproic acid; SCFA: straight short-chain fatty acids; BSCFA: branched short-chain fatty acids; tSCFA: total short chain fatty acids.
^{a,b}The difference between the average values indicated by different letters for the silage type is important.

Table 3. The compositions of fatty acid in total fatty acids of plantago herbage and silages.

		% as in total fatty acids													
Forage type	Plantago species	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	MUFA	PUFA	n-3	n-6	n-9	MCFA	LCFA	VLCFA
Herbages	*P. media*	0.98	16.75^{B}	3.88^{A}	7.66^{A}	14.20^{B}	47.75	8.78^{A}	65.78	48.35	17.42	7.98	0.73^{A}	96.34^{B}	2.72^{B}
	P. major	1.06	18.28^{A}	2.13^{B}	4.11^{B}	15.87^{B}	50.40	6.90^{B}	69.48	50.80	18.67	6.46	0.22^{B}	96.12^{B}	3.47^{A}
	P. lanceolata	1.26	18.40^{A}	3.78^{A}	7.35^{A}	16.19^{A}	48.80	8.53^{A}	66.34	49.02	17.31	7.77	0.59^{A}	98.60^{A}	0.61^{B}
Silages	*P. media*	1.23	24.47	3.82^{B}	6.81^{B}	17.29^{A}	40.42	8.04^{B}	59.00	40.77	18.23	7.43^{B}	0.07^{B}	96.85^{A}	1.42^{B}
	P. major	1.26	23.55	4.13^{A}	8.72^{A}	16.43^{A}	37.40	11.97^{A}	56.32	37.85	18.46	11.33^{A}	0.16^{B}	95.33^{AB}	4.32^{A}
	P. lanceolata	0.98	23.08	3.28^{B}	4.73^{B}	15.06^{B}	40.60	8.47^{B}	60.32	41.43	18.89	7.95^{B}	0.45^{A}	93.54^{B}	5.72^{A}
Forage	Herbages	1.10	17.81	3.26	6.38	15.42	48.98	8.07	67.20	49.39	17.80	7.40	0.52	97.02	2.26
type	Silages	1.16	23.70	3.74	6.75	16.26	39.47	9.49	58.54	40.01	18.52	8.91	0.23	95.24	3.82
	SD	0.14	3.16	0.91	2.24	1.26	5.70	2.28	5.42	5.61	0.71	2.18	0.25	1.68	1.86
P values	Forage type	0.207	<0.001	0.189	0.658	0.069	<0.001	0.147	<0.001	<0.001	0.003	0.105	<0.001	<0.001	0.001
	Plantago species	0.488	0.750	0.264	0.511	0.575	0.897	0.610	0.871	0.868	0.029	0.488	<0.001	0.160	0.003
	Interaction	0.081	0.006	0.025	0.011	0.005	0.274	0.046	0.130	0.246	0.510	0.048	<0.001	<0.001	<0.001

C14:0 = Myristic acid, C16:0 = Palmitic acid, C18:0 = Stearic acid, C18:1 = Oleic acid, C18:2 = Linoleic acid, C18:3 = α-linolenic acid, MCFA: Medium chain fatty acids (fatty acids with chains containing from 6 to 12 atoms of C), MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, SFA: Saturated fatty acids, UFA: Unsaturated fatty acids, LCFA: Long chain fatty acids (fatty acids with chains containing from 14 to 20 atoms of C), VLCFA: Very long chain fatty acids (fatty acids with chains containing above 20 atoms of C), SD: Standard deviation of means
[A,B]The difference between the average values indicated by different letters of silages or herbage for different plantago species is important (one-way ANOVA-Tukey), [a,b]The difference between the average values indicated by different letters for the silage type is important.

Table 4. The *in vitro* ruminal fermentation values of plantago herbage and silages.

	Plantago species	*In vitro* total gas, mL	*In vitro* methane, mL	ME	OMD	Ammonia-nitrogen
Herbages	*P. media*	38.41	8.15^{B}	8.14	55.48	69.78
	P. major	38.44	9.57^{A}	8.34	57.26	70.98
	P. lanceolata	35.10	7.49^{B}	7.66	52.27	71.79
Silages	*P. media*	44.49^{A}	10.66^{A}	8.97^{A}	60.89^{A}	69.05
	P. major	38.15^{B}	7.03^{B}	7.93^{B}	54.07^{B}	70.54
	P. lanceolata	41.97^{A}	9.94^{A}	8.65^{A}	58.38^{A}	72.21
Forage type	Herbages	37.09	7.93	7.98	54.55	70.82
	Silages	41.54	9.20	8.52	57.78	70.60
	SD	4.21	1.60	0.59	2.87	1.95
P values	Forage types	0.029	0.131	0.046	0.036	0.862
	Plantago species	0.492	0.217	0.495	0.480	0.334
	Interaction	0.027	0.021	0.013	0.013	0.933

In vitro total gas: *in vitro* gas production as ml for 0.2 g DM at 24 h ruminal incubation; *in vitro* methane: *in vitro* methane production as ml for 0.2 g DM at 24 h ruminal incubation; ME: Metabolic energy calculated from *in vitro* total gas production, as MJ/kg DM; OMD: Organic matter digestion, calculated from in total gas production at 24 h ruminal incubation.
SD: Standard deviation of means, [a,b]: The difference between the average values indicated by different letters for the silage type is important.

herbage ($p < 0.05$). The percentages of stearic acid, oleic acid, monounsaturated fatty acids (MUFA) and medium chain fatty acids (MCFA) of *P. major* herbage were lower than those of *P. media* and P. lanceolata herbages ($p < 0.05$). The percentages of linoleic acid and long chain fatty acids (LCFA) of *P. lanceolata* herbage were higher than those of *P. media* and *P. major* herbages ($p < 0.05$). The percentages of oleic, linoleic and w-9 fatty acids of *P. lanceolata* silage were lower than those of *P. media* and *P. major* silages ($p < 0.05$). The percentages of very long chain fatty acids (VLCFA) in total fatty acids of *P. lanceolata* and *P. major* silages were higher than that of *P. media* silage ($p < 0.05$). The percentages of oleic, w-6 and VLCFA fatty acids in total fatty acids of plantago silages (average value) were higher than those of plantago herbages (average value) ($p < 0.05$). The percentages of α-linolenic, PUFA, w-3, MCFA and LCFA fatty acids in total fatty acids of plantago silages (average value) were lower than those of plantago herbages (average value) ($p < 0.05$).

The in vitro *ruminal fermentation and end-products*

The *in vitro* ruminal fermentation values of plantago herbage and silages are in Table 4. The *in vitro* ruminal fermentation values of different plantago species 'herbages did not same ($p < 0.05$). The *in vitro* total gas production, *in vitro* methane production, ME and OMD values and ammonia-nitrogen concentration in rumen fluid of *P. major* silage were lower than those of *P. media* and *P. lanceolata* silages. The *in vitro* total gas production, ME and OMD values of plantago silages were higher than those of plantago herbage ($p < 0.05$).

Short chain fatty acids (mmol/L) in the *in vitro* ruminal fermentation fluid of plantago herbage and silages are shown in Table 5. The concentrations of straight short-chain fatty acids (SCFA) and branched short-chain fatty acids (BSCFA) in the fermentation fluid of plantago herbages and silages did not differ ($p > 0.05$).

Table 5. Short-chain fatty acids (mmol/L) in the *in vitro* ruminal fermentation fluid of plantago herbage and silages.

		SCFA					BSCFA		
	Plantago species	AA	PA	BA	VA	CA	IVA	IBA	tSCFA
Herbages	*P. media*	67.52	12.79	11.56	1.11	0.34	1.58^{B}	0.81^{B}	95.72
	P. major	69.92	12.91	12.24	1.22	0.36	1.92^{A}	0.97^{A}	99.55
	P. lanceolata	65.32	12.53	11.08	1.02	0.33	1.39^{B}	0.74^{B}	92.42
Silages	*P. media*	72.95	14.92	12.37	1.35^{A}	0.45	2.12^{A}	1.06^{A}	105.22
	P. major	73.73	14.42	11.04	1.09^{B}	0.43	1.47^{B}	0.81^{B}	103.00
	P. lanceolata	71.61	14.65	12.37	1.32^{A}	0.44	2.10^{A}	1.04^{A}	103.54
Forage type	Herbages	67.59	12.74	11.62	1.12	0.35	1.63	0.84	95.90
	Silages	72.57	14.71	12.10	1.28	0.44	1.97	1.01	104.10
	SD	3.14	1.05	0.71	0.04	0.05	0.09	0.05	5.17
P values	Forage type	0.001	<0.001	0.451	0.037	0.001	0.005	0.006	0.003
	Plantago species	0.135	0.215	0.765	0.641	0.565	0.749	0.783	0.251
	Interaction	0.449	0.161	0.097	0.021	0.352	0.024	0.014	0.206

AA: acetic acid; BA: butyric acid; PA: propionic acid; CA: caproic acid; VA: valeric acid; IBA: iso-butyric acid; IVA: iso-valeric acid; SCFA: straight short-chain fatty acids; BSCFA: branched short-chain fatty acids; tSCFA: total short chain fatty acids.

Ensiling of plantago species increased the concentrations of AA, PA, VA, CA, iso-acids (IVA and IBA) and total short-chain fatty acids (tSCFA) in the *in vitro* ruminal fermentation fluid ($p < 0.05$).

Microbiome analyses

The relative abundance of the bacteria kingdom of the *in vitro* fermentation fluid microbiome differed for different plantago forages and ranged from 89.40 to 93.25% ($p < 0.05$). The archaea community of *P. major* herbage (9.71%) and *P. major* silage (7.09%) in the microbiome were higher than those of other forages ($p < 0.05$).

The most relative abundant bacteria phylums in the *in vitro* rumen fermentation fluid' microbiome of plantago forages were *Bacteriodota* (38.39–32.21%), *Firmicutes* (41.69–28.11%), *Planctomycetota* (5.62–2.61%), *Fusobacteriota* (9.22–0.13%), *Spirochaetota* (4.36–1.92%), *Proteobacteriota* (4.13–1.71%), *Verrucomicrobiota* (4.77–1.20%), *Patescibacteria* (2.00–1.22%), *Desulfobacterota* (1.33–0.95%) and *Actinobacteriota* (2.41–0.30%), respectively (Figure 1).

Relative abundance of the dominant genus with an average relative abundance ≥0.5%

At genus level of bacteria, the *Rikenellaceae_RC9_gut_group* (18.50–15.51%) *Prevotell*a (9.82–4.06%), *Solibacillus* (8.36–0.5%), *Fusobacterium* (9.18–0.44%), the *Bacteroideales* F082 *group* (3.82–2.90%), *Oscillospiraceae_NK4A214_group* (3.89–1.98%), *Christensenellaceae R-7 group* (2.27–1.98%), *Pirellula* (2.53–1.21%), Treponema (2.66–0.70%), *Muribaculaceae* (2.64–1.03%), *Lachnospiraceae* (1.80–0.77%), the *Eubacterium_coprostanoligenes group* (2.08–1.01%), *Lysinibacillus* (4.22–0.15%), the *Lachnospiraceae NK3A20 group* (2.07–0.80%), *Oscillospiraceae UCG-002* (1.81–0.87%), *Butyrivibrio* (2.07–0.59%), *Prevotellaceae UCG-001* (1.54–0.99%), the *Sphaerochaeta* (1.48–0.78%), *Desulfovibrio* (1.18–0.87%) were the most abundant species in microbiome of the *in vitro* fermentation fluid of plantago forages (Figure 2).

The relative abundance of genus *Sphaerochaeta* and *Treponema* in the *Spirochaetaceae* family in the rumen fluid of plantago silages were higher than those of plantago herbages ($p < 0.05$). Relative abundance of *Lachnospiraceae_NK3A20_group* (cellulolytic bacteria) in rumen fluid of *P. major* silage and *P. major* herbage were higher than those of *P. media* and *P. lanceolata* forage ($p < 0.05$). The relative abundance of *Oscillospiraceae_UCG-002* in rumen fluid of plantago silages was higher than those of plantago herbages ($p < 0.05$). *Prevotellaceae UCG-001* were relatively more abundant in *P. media* silage than in other plantago forages ($p < 0.05$).

Discussion

Nutrient content

The DM content of the plantago species (*P. media, P. lanceolata* and *P. major*) used in the present study at the time of harvesting was higher than the stated results (14.2, 13.4, and 12.3%, respectively) for these species in the previous research (Guil-Guerrero 2001). Besides, the DM (29%) and ash (15%) values reported by another researcher (Alghamdi 2018) for *P. lanceolata* herbage, the arid zones with a short scattered rainy season and prolonged dry period, were higher than that of the present study. Labreveux (2002) stated that the cultured lanceot and tonic plantago species included an average of 19–20% of CP and 28–39% of NDF from spring to late summer. The ash, CP, NDF and ADF contents of *P. lanceolata* herbage in

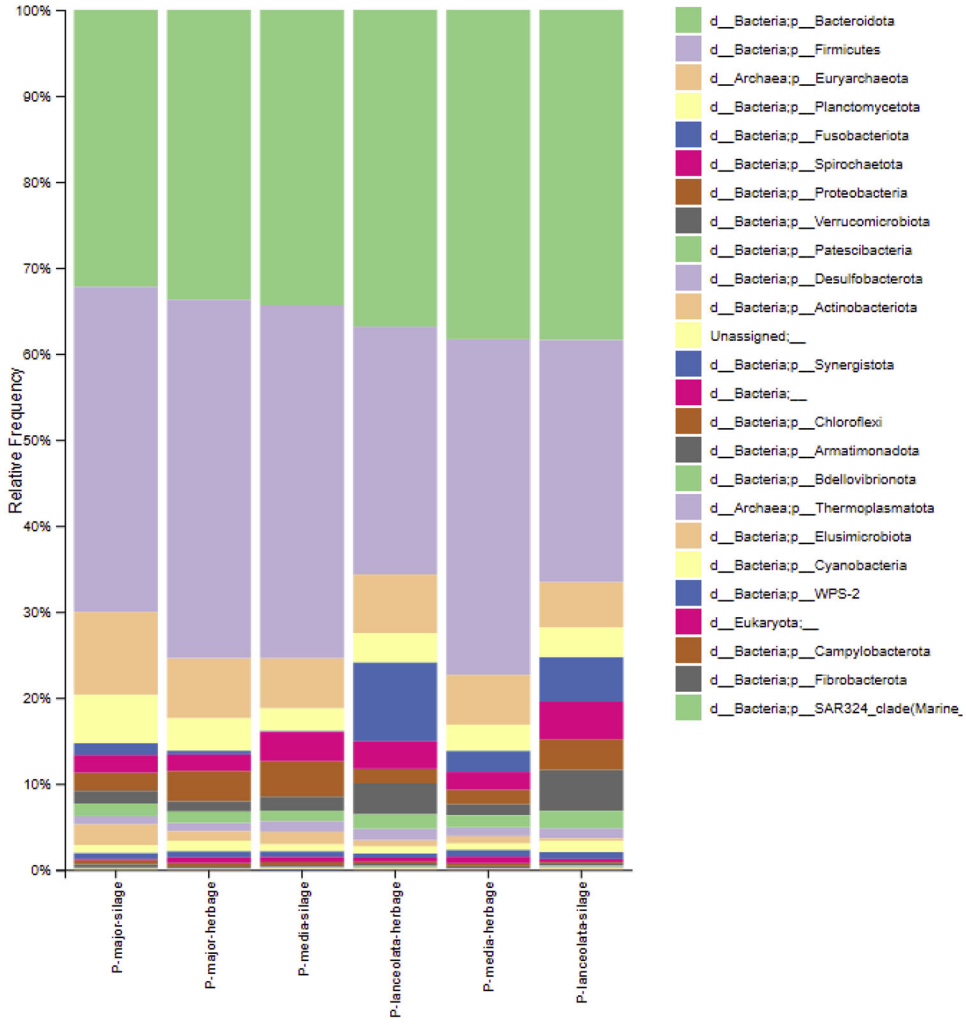

Figure 1. Bacteria and archaea phylum in microbiome of the *in vitro* ruminal fermentation fluid of plantago forages.

the present study were similar to the result of the previous studies (Sanderson et al. 2003; Kara et al. 2018; Alghamdi 2018; Bariroh et al. 2021). Although it was determined that the CP content of *P. major* herbage was higher than those of *P. media* and *P. lanceolata* herbages in the present study, in the previous survey (Alghamdi 2018), it was reported that CP content of *P. media* was higher than those of *P. lanceolata* and *P major* herbage. In the present study, decreased CP and increased DM contents of *P. media* and *P. lanceolata* herbages with the ensilaging process were similar to the previous survey of Bariroh et al. (2021). Significant differences in nutrient compositions (particularly fatty acid profile) of different plantago species to the present study are similar in the previous studies (Sanderson et al. 2003; Kara et al. 2018; Alghamdi 2018; Bariroh et al. 2021).

In the present study, there are differences between the nutrient composition results of plantago species. The results of previous studies may depend on the cultivation status or collection from natural pasture, climatic conditions, vegetation period, and soil type. Anaerobic fermentation in the silage medium causes the bacterial breakdown of easily digestible carbohydrates, causing silage gas formation increased silage acidity. In the present study, the decrease in CP content with the ensilaging of *P. major* showed that protein compounds were broken down in the silage enviroment. Decreasing CP content in forage material was undesirable for ruminant feeding and forage quality. However, the fact that the CP value did not change in *P. lanceolata* and *P. media* silages stands out as an important advantage compared to *P. major*. The significant decrease in the NDF value of *P. major* herbage, which was already lower than the other herbage, with silage production indicates that this material had a high fibre digestibility in the silage medium. The structural carbohydrate components of *P. major* suffered a significant loss after ensilaging. Considering these results, silage of *P. major* was a

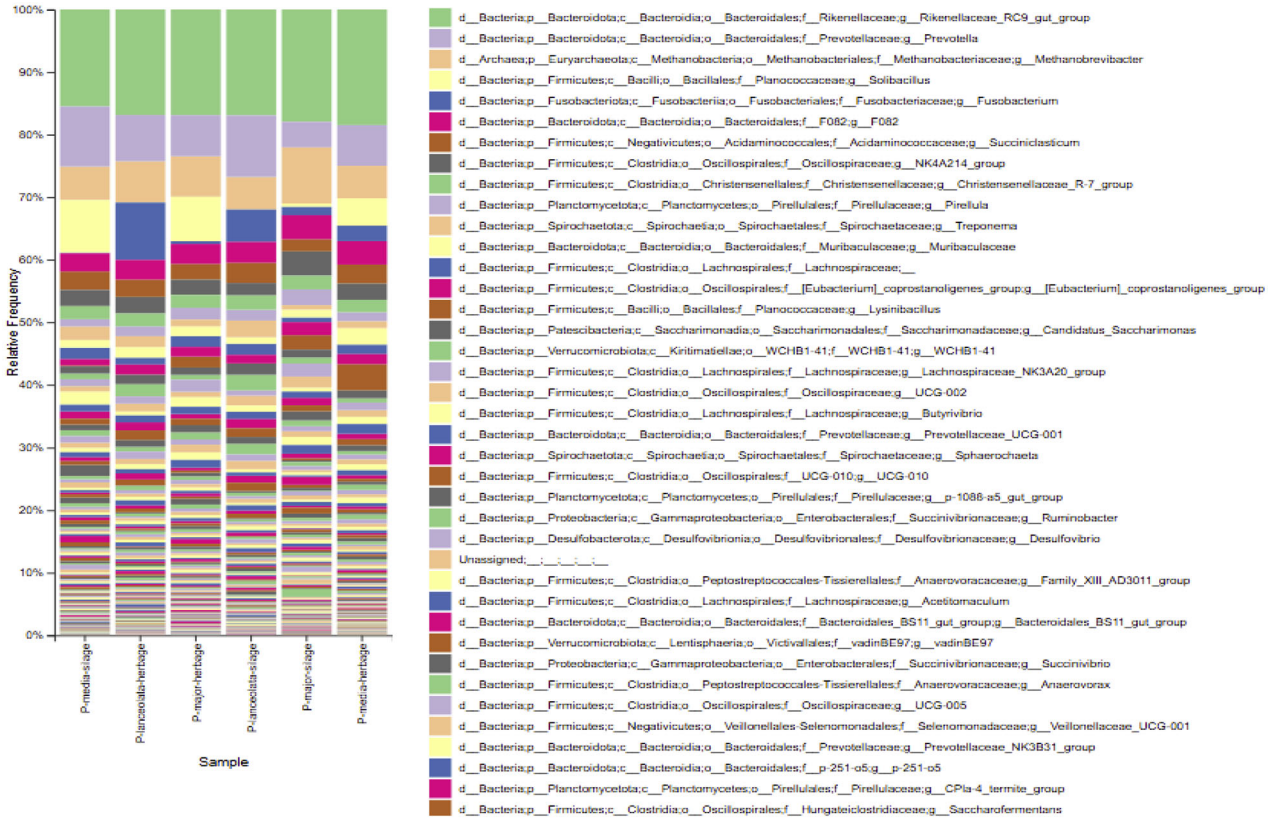

Figure 2. Bacteria and archaea genus in microbiome of the *in vitro* ruminal fermentation fluid of plantago forages.

disadvantage for *P. media* and *P. lanceolata* silages in terms of loss of CP and structural carbohydrates.

In the present study, the major fatty acids in total fatty acids of plantago herbages can be depicted in a series based on their content in the following order: C18:3>C16:0>C18:2>C18:1>C18:0 > C16:0. The palmitic, stearic, oleic and linoleic acids were fatty acids that differed between *P. media, P. major* and *P. lanceolata* herbages. Rozentsvet et al. (2015) demonstrated that the C16 plus C18 fatty acids content of *P. media* leaves comprised more than 90% of total fatty acids. The main fatty acid component of *P. media* herbage was fatty acids with chains containing from 14 to 20 carbon atoms. It included about 2% with hydrocarbon chains longer than 20 carbon atoms in total fatty acids (Rozentsvet et al. 2015). The main component of unsaturated fatty of plantago species in the present study was linolenic acid (C18:3; 47.8–50.4% for herbage and 37.4–40.6% for silages), and their predominant saturated fatty acid was palmitic acid (C16:0; 16.8–18.4% for herbage and 23.1–24.5% for silages). Generally, the percentages of unsaturated fatty acids in plantago forages were similar to those of *P. media* leaves (major unsaturated fatty acids; C18:3 was 33–58% of total fatty acids) by previous researchers (Rozentsvet et al. 2015). Although lower than the results of the present study, Guil-Guerrero (2001) determined that the main fatty acids in leaves for all plantago species was the α-linolenic acid, which was 38.1% for *P. media* leaves, 40.0% for *P. major* leaves and 45.1% *P. lanceolata* leaves. Presently, the differences in fatty acid composition for plantago species may be related to the leaf: stem ratio, leaf width and environmental conditions. Plantago's fermentation in the silage environment caused a change in the fatty acid concentrations (especially C16:0, C18:0, C18:1, C18:2, and C18:3); the effect also differed between species. The effect of oxidation and differentiation on fatty acids composition in a silage environment has been explored recently (Han and Zhou 2013; Kara 2021). Increased oxidation in the silage environment changes the fatty acid profile of forage materials (Kara 2021). In the present study, the α-linolenic acid % in total fatty acids of plantago species diminished (about 49 vs. 39% in total fatty acids) with the ensiling process was similar to results, for *Melilotus officinalis* silages (Kara 2021). The palmitic acid percentages in total fatty acid of plantago silages were higher than those of plantago herbages demonstrating that palmitic acid level in

silage fermentation did not oxidise or decompose. In the overall assessment, ensilaging of plantago species caused a decreasing of approximately 20% ratio in α-linolenic acid and an increasing of approximately 30% ratio in palmitic acid in total fatty acids according to those of their herbages.

Making silage of green-fresh forage plants provides forage for ruminant nutrition throughout the year. Acidity is important for ideal anaerobic silage fermentation (Tekin and Kara 2020). In the silage environment, lactic acid (pKa of 3.86), produced by lactic acid bacteria, is the organic acid, found in the highest concentration in the silage environment. It contributes the most to the decline in pH during anaerobic fermentation of ensiled material because it is about 10 to 12 times stronger than any of the other primary organic acids (acetic acid; pKa of 4.75 and propionic acid; pKa of 4.87) found in silage environment (Kung et al. 2018). The lowest pH value and the highest lactic acid level in the silage medium were determined in *P. lanceolata* silage. These acidity values were the targeted values for quality silage. The higher ammonia-nitrogen level in the silage fluid of *P. major* silage compared to other silages may have buffered the silage acidity with its alkaline feature. In parallel with the finding that ensiling of *P. major* herbage decreased the CP content in the forage, and increased the concentration of ammonia-nitrogen in the silage fluid. In the present study, concentrations of AA, the second major acid that inhibits yeasts in silage environments, in plantago silages were <0.2% in DM. Concentrations of BA, which should not be detectable in well-fermented silages, for plantago silages in the present study were at very low levels, especially for *P. major* and *P. lanceolata*.

Ruminal fermentation

In vitro gas production technique is a method that can be interpreted for the *in vitro* fermentation of feed material in the rumen and the energy and digestion level according to the amount of gas produced. In the study, *in vitro* total gas production and estimated digestion values (ME and OMD) of plantago species were within the expected values for quality forages (Kamalak et al. 2011; Kara et al. 2016) were found to be at a reasonable level. In the present study, it was observed that there was no difference between the *in vitro* gas productions values (35–38 ml/0.2 g DM) for herbages of plantago species. Previous researchers (Kara et al. 2016; Kara et al. 2018) have found that *P. lanceolata* herbages have higher *in vitro* gas production and estimated digestion values at 24 hours than those in the present study. Ensiling of plantago species increased *in vitro* gas production, ME and OMD values, and *in vitro* digestion values. The *in vitro* digestion values of *P. media* silage and *P. lanceolata* silage were higher than *P. major* silage. Low values of *P. major* silage compared to other plantago silages may be due to the failure to reach ideal silage acidity, the significant loss of CP value, and the fermentation of structural carbohydrates. The production amount for studied plantago forages of methane, an important vital gas produced by carbohydrate fermentation in the rumen, was within the values specified for forages (Kara et al. 2016; Kara 2021). The ensilaging increases the *in vitro* methane production in parallel with the increase in the *in vitro* total gas production for *P. media* and *P. lanceolata*. In the present study, ensilaging of plantago species increased the concentrations of SCFA (AA, PA, VA, and CA), BCFA (IVA and IBA) and tSCFA in the *in vitro* fermentation fluid was compatible with the increase in the *in vitro* gas production and estimated digestion values. Isoacids is the collective term for the branched-chain fatty acids: IBA and IVA and the straight-chain valeric acid, naturally produced in ruminant' digestive tracts (Andries et al. 1987). They are mainly built up from the degradation products of the amino acids valine, isoleucine, leucine and proline and should in turn be used for the biosynthesis of those amino acids and higher branched chain volatile fatty acids. Apajalahti et al. (2019) demonstrated that the 60% of valine, leucine, and isoleucine, untaken with ruminant feedstuffs, was recovered as IBA, 2-methylbutyric, and IVA (products of decarboxylation and deamination), respectively. The concentrations of iso-acids in the *in vitro* fermentation of *P. major* herbage were higher than those of *P. media* and *P. lanceolata* herbages may be related to the high CP content of *P. major* herbage. According to previously mentioned results, the low concentrations of iso-acids in *P. major* silage may be associated with the low CP content in *P. major* silage. In the present study, the differences in nutrient content with ensiling of plantago herbages changed the SCFA concentration in the *in vitro* fermentation fluid.

Ruminal microbiome

Ruminal microbiota plays a vital role in the feedstuffs fermentation, gas production (carbon dioxide, methane, etc), SCFA, BCFA and ammonia-nitrogen in the rumen (Patra and Yu 2014). Ruminants have various micro-organisms in their rumen, including

bacteria, protozoa, fungi and archaea (Kim et al. 2011). An 83.40–93.34% of the species belonged to the Bacteria kingdom, 5.44–9.71% to archaea, 0.73–1.30% to unclassified and 0.07–0.2% were Eukaryota. The bacteria community was mainly composed of *Bacteriodota* and Firmicutes. This was in agreement with previous studies, which presented the rumen microbiota composition in cattle, that reported that these phyla comprise around 90% of the 16S rRNA gene abundance (Delgado et al. 2019). The archaea community, rumen methanogens, in the *in vitro* fermentation fluid of plantago forages in the present study is composed of *Euryarchaeota* (mainly; *Methanobrevibacter* and *Methanosphaera* genus in *Methanobacteriales* order) and *Thermoplasmatota (Methanomassiliicoccales* order). These orders have all been characterised as hydrogenotrophic, utilising H_2/CO_2 or H_2/methanol produced by various fermentative bacteria in the anaerobic degradation of plant biomass (Poulsen et al. 2013).

The hydrogenotrophic pathway catalysing the conversion of CO_2 to methane is dominant in the rumen, and occurs in *Methanobrevibacter* (Danielsson et al. 2017). In the present study, it was observed that the *in vitro* methane production of *P. major* herbage (9.57 ml) was higher than that of *P. media* and *P. lanceolata* (8.15 and 7.49 ml) herbages, which can be in relation to the high community of *Methanobrevibacter* (9.68% in the total microbiome) in the *in vitro* fermentation fluid and AA concentration (an increasing trend) of *P. major* herbage. The NDF content of *P. major* herbage was lower than that of other plantago species and the NFC content of it was similar to other herbage. However, the *in vitro* total gas and methane production of *P. major* herbage was high, could be attributed to the fact that lignin plus cellulose complexes were not very prominent in this species and cellulose fermentation was higher than those in other herbages. On the other hand, the *in vitro* methane production of *P. major* silage (7.03 ml) was lower than those of *P. media* and *P. lanceolata* silages (10.66 and 9.94 ml), and the *Methanobrevibacter* in fermentation fluid of *P. major* silage was higher than those of *P. media* and *P. lanceolata* silages. The complex microbial ecosystem in the rumen produces the SCFA through the fermentation of dietary OM. In the present study, the relative abundance of *Prevotellaceae* bacteria in the *in vitro* fermentation fluid of *P. major* silage was found to be low in those of other plantago silages demonstrating parallel to the low fermentation of OM. Decreased methane production of *P. major* silage may be related to the decrease in the NDF content with good fermentation of *P. major* silage in the silage environment and the increase in the NFC content. The methane precursor is SCFA, and methanogen archaea are responsible for converting SCFA to methane. The AA and BA promote methane production, while PA production can be considered a competitive pathway for hydrogen use in the rumen (Moss et al. 2000). The BA and VA of ruminal acidifications in *P. major* silage being lower than those of other silages may be related to low methane production.

In the present study, the high *in vitro* methane production of *P. major* herbage was higher than those of other herbages. The low the *in vitro* methane production of *P. major* silage was higher those of other silages related to the percentages of palmitic acid, oleic acid and MUFA fatty acid of forages samples (O'Brien et al. 2014). A previous study stated that oleic acid supplementations at different percentages in the *in vitro* fermentation of forage dose-dependent manner decreased *in vitro* methane production in the rumen (Wu et al. 2016). The relative abundance of *Prevotellaceae* bacteria, which can ferment the structural carbohydrate components (xylan/xyloglucan and pectin) and proteins in the rumen (Seshadri et al. 2018), in the *in vitro* fermentation fluid of *P. major* silage, high MUFA contents, found to be low in those of other plantago silages can be connected to the decreasing methane production. The *P. major* silage with high C18:0 and C18:1 (according to other plantago silages) decreased the methane production and relative abundances of archaea and fibrolytic bacteria in the present study in the rumen, parallel to the results of Ding et al. (2012). However, Judy et al. (2019) indicated that increasing C18:3 in the ruminant diet might not affect methane production or the digestibility of the diet.

In the present study, at the genus level of bacteria, *Rikenellaceae_RC9_gut_group* (18.50–15.51%) and *Prevotell*a (9.82–4.06%) were in relative abundance in the *in vitro* ruminal fermentation fluid. Huang et al. (2021) stated that at the genus level, the ruminal microbiome of the grazing group showed a higher abundance of *Rikenellaceae RC9 gut group* and *Prevotellaceae UCG-003*. Rabee et al. (2022) demonstrated that the dominant genera were *RC9_gut_group, Ruminococcus, Saccharofermentans, Butyrivibrio, Succiniclasticum, Selenomonas,* and *Streptococcus*, indicating the critical role of these genera in lignocellulose fermentation in the rumen. In the present study, the high relative abundance of

Rikenellaceae_RC9_gut_group in the *in vitro* ruminal fermentation fluid liquid of plantago herbage and silages with high structural carbohydrate (NDF, ADF) were consistent with the results of the studies (Zened et al. 2013; Zhang 2017) that feedstuffs materials containing high fibre or high structural carbohydrates increased the relative abundance of ruminal *Rikenellaceae_RC9_gut_group* bacteria. *Prevotellaceae* is involved in plant cell wall polysaccharides degradation, and can utilise branched-SCFA and participate in glucose metabolism (Liu et al. 2019). The relative abundance of *Prevotellaceae UCG-001* in rumen fluid in the present study was similar to the results of Qiu et al. (2022). In the present study, the relative abundance of *Prevotellaceae* bacteria in the *in vitro* fermentation fluid of *P. major* silage was lower than those of other plantago silages. It is thought that the decrease in BCFA (iso-acids; IVA and IBA) in the *in vitro* fermentation fluid of *P. major* silage may be related to the decreased relative abundance of *Provetella* bacteria in the *in vitro* fermentation fluid. The *Prevotellaceae* bacteria can also produce acetate and propionic acid by fermentation of structural carbohydrate components (xylan/xyloglucan and pectin) in the rumen (Seshadri et al. 2018). The increase in the *in vitro* total gas production and the AA, PA, VA, and CA concentrations with the ensilaging of plantago species can be associated with the relative abundance *of Prevotellaceae* bacteria in the rumen fluid. The ensiling process increased the *in vitro* total gas, acetic acid concentration and relative abundance of *Prevotellaceae* bacteria in the rumen fluid of *P. media* and *P. lanceolata*. The *Treponema*, positively correlated with dietary fibre (Billenkamp et al. 2021), in the *in vitro* fermentation fluid increased by about 40–50% with the ensilaging of *P. media* and *P. lanceolata* in the present study. Liu et al. (2014) showed that *Treponema saccharophilum* was a pectinolytic bacterium isolated from the bovine rumen. Ensilaging of herbages in the present study increased the PA concentration in the *in vitro* rumen fluid. *Muribaculaceae* are predicted to produce PA as a fermentation end product and are abundant and diverse in the guts of mice, although few isolates are available (Smith et al. 2021). Besides, the relative abundance of cellulolytic bacteria, such as *Ruminococcus, Lachnospiraceae_NK3A20_group, Ruminiclostridium* and *Lachnospiraceae_UCG-008* was compatible with the results of Wang et al. (2021). In the present study, the relative abundance of genus *Sphaerochaeta* and *Treponema* in the *Spirochaetaceae* family in rumen fluid of plantago silages were higher than those of plantago herbages. Some species within *Treponema* and *Sphaerochaeta* in the rumen show pectinolytic activities instead of being pathogens (Xie et al. 2018) and also produce AA, which is a lower energy source for the animal in comparison with BA (Abt et al. 2012). The production of H_2S in the rumen depends on the availability of sulphate reduction by ruminal sulphate-reducing bacteria (Howard and Hungate 1976). In the rumen, so many bacteria, fungi and protozoa are present, some bacteria are sulphate-reducing bacteria and these bacteria are anaerobic (Howard and Hungate 1976). *Desulfovibrio* is important sulphate-reducing strain found in the rumen by Howard and Hungate (1976). In the present study, *Desulfovibrio* ranged from 0.87% to 1.18% in the microbiome of rumen fluid for plantago forages. These bacteria can reduce sulphate into hydrogen sulphide in the animals' rumen, as *Desulfovibrio* reduces sulphate to sulphide and methanogens reduce CO_2 to produce CH_4. *Desulfovibrio* can also competitively attach to hydrogen ions. In the rumen, *Desulfovibrio*'s compatibility with methanogens depends mainly on the sulphate levels (Jiang et al. 2010).

Conclusion

The *P. major* herbage was more advantageous than *P. media* and *P. lanceolata* in terms of the CP and VLCFA contents of *P. major* herbage. Compared with other plantago silages, the ensiled *P. major* silage could not provide a good silage quality (nutrient composition - such as CP and NDF, and silage acidity). The ensiling of plantago herbage positively affected the ruminal fibrolytic bacterial composition (*Rikenellaceae_ RC9_gut_group*, *Oscillospiraceae_UCG-002,* and *Prevotellaceae*) of *in vitro* fermentation fluid. Besides, the *in vitro* ruminal methane production and community of *Methanobrevibacter* in the microbiome of *P. major* herbage were higher than those of *P. media* and *P. lanceolata* herbages. In general, the increase in the *in vitro* total gas production and the acetic, propionic, valeric, and caproic acid concentrations with the ensilaging of plantago species were associated with the increase in the relative abundance of *Prevotellaceae* bacteria in the rumen fluid.

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Ethical approval

This study was approved (number: 22/198) by the Local Ethics Committee for Animal Experiments of Erciyes University, Kayseri, Türkiye.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data supporting this study's findings are available on request from the corresponding author, [initials]. The data are not publicly available due to restrictions [e.g. information that could compromise the privacy of research participants].

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