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The ruminal and faecal microbiota, digestion processes, and milk composition of dairy cows are modified by the botanical biodiversity of pastures

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ABSTRACT

Pasture botanical diversity is known to change milk composition and improve dairy product quality. However, the chemical and physiological mechanisms behind this effect are only partially known and the role of ruminant's microbiota is still unclear. To study the effects of pasture botanical biodiversity on rumen microbiota, fermentation parameters and milk composition of dairy cows, an *in vivo* experiment, including *in vitro* measurements, was carried out with two balanced groups of seven dairy cows each. After a 5-week pre-experimental period on a common permanent grassland plot, the two groups of cows grazed for 4 weeks (experimental period) on two plots characterised by contrasting levels of plant biodiversity: low diversity (**LD**; 19 species, mainly grasses) and high diversity (**HD**; 56 species, mostly dicots). Samples of simulated bites, rumen fluid, faeces, and milk were collected at the end of the pre-experimental and experimental periods. The species richness (α-diversity) of bacteria and fungi in the rumen and faeces of the cows did not differ between treatments, contrary to the composition and relative abundance (β-diversity) of bacterial and fungal communities. In addition, during *in vitro* rumen fermentation, total gas production of HD herbage was lower compared with LD, probably because of the different chemical characteristics of the substrates and the partial inhibition of bacterial activity by tannins. Furthermore, methane production *in vitro* was reduced in the HD group compared to the LD one, as indicated by the higher CO_2:CH_4 ratio. Thus, the differences in β-diversity may be explained both by herbage fibre and plant secondary metabolite contents. Plant tannins also protected dietary proteins from degradation, as indicated by the lower ammonia to CP ratio obtained *in vitro* in HD than in LD digesta. Comparable proportions of C18:3 n-3 were found in milk, despite the lower total fatty acid and C18:3 n-3 contents of the HD herbage. Plant secondary metabolites in the rumen could have partially inhibited the activity of ruminal bacteria responsible for the biohydrogenation of polyunsaturated fatty acids. This study explains how grazing dairy cows on permanent grasslands rich in plant biodiversity helps transferring polyunsaturated fatty acids from herbage to milk and likely reduces methane and ammonia emissions by influencing ruminal and faecal microbiota thanks to plant secondary metabolites.

Implications

The effects of two permanent grasslands characterised by contrasting levels of plant biodiversity on the bacterial and fungal communities of rumen and faeces, *in vitro* rumen fermentation parameters, and lipid metabolism were investigated in dairy cows.

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Feeding dairy cows with a highly biodiversified pasture modified the structure of ruminal and faecal microbiota, reducing enteric methane production and ruminal ammonia, while it improved nitrogen efficiency. The herbage tannins modified rumen lipid metabolism by preserving C18:3 n-3 from biohydrogenation. This research helps to understand the interactions between permanent grasslands' plant biodiversity, ruminal and faecal microbial populations, and digestive processes in dairy cows.

Introduction

Grassland-based farming systems increasingly appear to be a promising option for ruminant livestock, as they enable the balancing of production, farm income, and the provision of a wide range of ecosystem services, including food products of high nutritional and sensorial quality (Martin et al., 2020). In grassland farming systems, ruminants fed forages produce dairy and meat products with a higher nutritional quality (Prache et al., 2020; Cabiddu et al., 2022), especially if they graze pasture. Their dairy and meat products are rich in health–promoting fatty acids (**FA**) such as α-linolenic (C18:3 n-3, **ALA**), rumenic (C18:2 *cis*-9 *trans*-11, **RA**), and vaccenic (C18:1 *trans*-11, **VA**) acids (e.g., Elgersma, 2015; Ferlay et al., 2017; Fan et al., 2023), liposoluble vitamins, carotenoids, terpenes, and have typical sensory characteristics (Cabiddu et al., 2022; Manzocchi et al., 2022b).

However, changes in grazing management can alter pasture characteristics, and therefore, the ruminal digestive processes which, in turn, affect dairy product quality. For instance, at the end of each paddock utilisation when cows are frequently moved, available herbage decreases and the remained yet uneaten presents lower digestibility and CP content (Coppa et al., 2015a). Also, in extensively managed pastures, the advance of herbage phenological stage causes modifications of herbage chemical composition (e.g., an increase in fibre fractions and a reduction of protein and lipid contents), reducing organic matter digestibility (Coppa et al., 2015b; Renna et al., 2020), and potentially increasing enteric methane production of grazing dairy cows (Martin et al., 2010). In contrast, the plant secondary metabolites (and especially tannins), often abundant in botanically diversified pastures, are able to modify *in vitro* rumen fermentation and reduce methane production and ammonia concentration (Carreño et al., 2015; Menci et al., 2021). Tannins, indeed, protect dietary proteins against rumen microbial degradation, thanks to the formation of tannin-protein complexes which hinder the proliferation of proteolytic bacterial communities (Patra and Saxena, 2011).

Plant secondary metabolites can modify ruminant digestive processes and, consequently, could affect milk composition (Leiber et al., 2005). It has been shown that botanically-diversified pastures rich in secondary metabolites can have a strong influence on milk FA profile (Renna et al., 2020; Cabiddu et al., 2022). Indeed, diets rich in tannins protect dietary linoleic acid (C18:2 n-6, **LA**) and ALA from ruminal biohydrogenation (**BH**) and then increase their content in milk (Henke et al., 2017).

The reduction of enteric methane production and the modulation of the BH processes derive in some extent from the inhibitory action of plant secondary metabolites on rumen microbiota (Vasta et al., 2019). Even so, the effect of dietary tannins on ruminal microflora has been scarcely studied on dairy cows grazing on highly botanically diversified pastures. We hypothesised that the proximate composition, FA profile, and plant bioactive compounds, such as tannins, of herbages may change the structure of rumen microbiota and consequently affect the digestive processes in ruminants. The aim of this study was to investigate through an *in vivo* trial the interactions that occur among pasture chemical composition (as a function of botanical diversity level), ruminal and faecal microbiota, rumen digestive processes, and milk quality in dairy cows. *In vitro* rumen simulation was achieved in order to accurately measure parameters that would be complicated or expensive at pasture, such as enteric methane production.

Material and methods

Experimental design and animal management

The experiment was carried out at the INRAE Herbipôle research facility in Marcenat, France (https://doi.org/10.15454/1.5572318050509348E12; 45°15′N, 2°55′E; altitude 1 135–1 215 m; average annual rainfall 1 100 mm) in spring and summer 2018. During a 5-week pre-experimental period (**P1**), 14 Holstein and Montbéliarde cows grazed on a common permanent grassland plot characterised by a moderate botanical diversity (36 botanical species). Subsequently, the cows were randomly allocated to two groups of seven individuals each, balanced by lactation stage, breed, parity, and milk yield. At the end of P1, the cows produced 17.1 ± 2.37 kg milk/cow × day, had a BW of 610 ± 56.3 kg, and were 228 ± 14.7 days in milk. The average milk fat and protein contents were 38.2 ± 2.95 g/kg and 34.0 ± 2.13 g/kg, respectively. The two groups were conducted for a 4-week experimental period (**P2**) on two plots characterised by contrasting levels of botanical diversity: low diversity (**LD**) and high diversity (**HD**). All along the experiment, the cows were fed at pasture with free and guaranteed access to clean and fresh water and to mineral blocks. The cows were milked twice a day, at 0630 and 1600 h, in a herringbone milking parlour. Individual milk yield was recorded at each milking. Herbage intake at pasture was estimated through the cow's potential intake capacity according to INRAE (2018). Live weight and body condition score were recorded in the last week of each period as described in Manzocchi et al. (2022a). Herbage, rumen fluid, faeces, and milk samples were collected individually during the last week of P1 and P2.

Herbage characterisation, sampling, and analyses

The extension of the plots was planned to guarantee *ad libitum* herbage availability to the cows for the duration of the trial. The botanical composition of the pre-experimental and experimental plots was determined using the phyto-pastoral method (Daget and Poissonet, 1971) and is reported in Table 1. To collect herbage samples as much representative as possible of the herbage selected by the grazing cows, simulated bites were sampled on one day in the last week of P1 and P2, as described by Coppa et al. (2015a). Briefly, a sward patch alike to that grazed by the cow was collected, taking into consideration the botanical composition, the herbage height, and phenological stage. The simulated bites for each cow were sampled several times during the experimental period, both in the morning and in the afternoon, up to 10 simulated bites per cow. The different collected simulated bites were pooled per cow, divided into homogeneous sub-samples, and stored at −20 °C for the subsequent analyses. The botanical group composition of simulated bites was determined by hand separating the plants into groups of grasses, legumes, and non-legume dicots (Table 2). Each botanical group was weighed after oven drying at 60 °C for 72 h to calculate their proportion on a DM basis. Fibre content (NDF and ADF), CP, ash, and pepsin-cellulase DM digestibility were determined by near-IR reflectance spectroscopy, as detailed by Manzocchi et al. (2022a). The herbage phenolic compound content was assessed as described in Iussig et al. (2015). Briefly, total extractable phenols were extracted with aqueous acetone and non-tannin phenols were separated by total tannins using polyvinyl-polypyrrolidone. The butanol-HCl-iron method was used

Table 1
Botanical composition of the pre-experimental (PREXP), low–diversified (LD), and high–diversified (HD) plots offered to dairy cows.

PREXP		LD		HD	
Species	SC[1] (%)	Species	SC[1] (%)	Species	SC[1] (%)
Poa pratensis	22.6	*Bromus hordeaceus*	20.1	*Thymus pulegioides*	16.3
Dactylis glomerata	16.9	*Poa pratensis*	19.8	*Festuca nigrescens*	14.4
Trifolium repens	15.8	*Trifolium pratense*	16.3	*Agrostis tenuis*	10.3
Agrostis tenuis	14.6	*Dactylis glomerata*	12.5	*Hieracium pilosella*	6.7
Festuca nigrescens	11.2	*Trifolium repens*	9.0	*Carex sempervirens*	6.6
Taraxacum officinale	3.1	*Lolium multiflorum*	7.5	*Achillea millefolium*	4.3
Cirsium eriophorum	1.7	*Taraxacum officinale*	5.7	*Avenella flexuosa*	4.2
Holcus lanatus	1.4	*Cerastium holosteoides*	2.5	*Anthoxanthum odoratum*	3.9
Trifolium pratense	1.4	*Agrostis tenuis*	2.5	*Trifolium pratense*	2.5
Cerastium holosteoides	1.3			*Luzula campestris*	2.5
Plantago lanceolata	1.2			*Festuca ovina*	2.5
Phleum pratense	1.2			*Knautia arvensis*	2.1
Polygala vulgaris	1.1			*Nardus stricta*	1.8
Achillea millefolium	1.0			*Lathyrus montanus*	1.8
				Helianthemum nummularium	1.5
				Cerastium arvense	1.5
				Veronica arvensis	1.5
				Bromus erectus	1.4
				Galium verum	1.1
				Lotus corniculatus	1.1
				Trifolium repens	1.1
				Ranunculus bulbosus	1.1
				Plantago lanceolata	1.1
				Rumex acetosella	1.1
				Briza media	1.0
Grasses	70.0	Grasses	63.9	Grasses	41.1
Legumes	17.3	Legumes	25.3	Legumes	7.2
Non-legume dicots	13.2	Non-legume dicots	11.4	Non-legume dicots	53.5
Total species, n	36	Total species, n	19	Total species, n	56

Abbreviation: SC, specific contribution.
[1] Species with SC < 1% are not included.

Table 2
Botanical composition (%), proximate composition (g/kg DM, unless otherwise stated), phenolic compounds, and fatty acid profile (g/100 g total fatty acids) of simulated bites of dairy cows grazing a low diversified (LD) or a high diversified (HD) herbage plot.

Item	LD	HD	SEM	*P*-value
Botanical composition				
Grasses	67.6	57.1	2.97	0.010
Legumes	29.1	13.3	1.89	<0.001
Non-legume dicots	3.78	29.0	2.23	<0.001
Proximate composition				
DM (g/kg FM)	226	289	11.5	<0.001
Ash	86.8	71.7	2.14	<0.001
CP	199	142	2.85	<0.001
NDF	489	509	45.4	0.002
ADF	238	261	3.30	<0.001
Pepsin-cellulase DM digestibility (%)	77.6	64.2	0.59	<0.001
Phenolic compounds				
TEP[1]	10.7	24.6	1.21	<0.001
TT[1]	9.55	23.0	1.16	<0.001
CT[2]	0.32	3.40	0.23	<0.001
Fatty acid profile				
Total fatty acids (g/kg DM)	52.2	33.5	2.01	<0.001
C16:0	18.1	23.5	0.35	<0.001
C18:0	1.98	2.39	0.048	<0.001
C18:1 *c*9	2.50	3.87	0.121	<0.001
C18:2 n-6	12.7	11.4	0.62	<0.001
C18:3 n-3	37.4	27.3	3.50	0.037

Abbreviations: *c*, cis; CT, condensed tannins; FM, fresh matter; TEP, total extractable phenols; TT, total tannins.
[1] Content expressed in gallic acid equivalent.
[2] Content expressed in leucocyanidin equivalent.

to determine condensed tannins. Total tannins were calculated as the difference between total extractable phenols and non–tannin phenols. The total extractable phenols and total tannins were expressed in gallic acid equivalents, while condensed tannins were expressed in leucocyanidin equivalents. Finally, simulated bites were lyophilised and subsequently analysed for their FA composi-

tion. Briefly, methyl esters were separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil88) and determined by a gas-chromatograph equipped with a flame ionisation detector. The FA content (g/kg DM) was obtained using an internal standard (C23:0). The details of the applied method are described in Ferlay et al. (2010).

In vitro rumen fermentation

In vitro rumen fermentation was performed as detailed in Menci et al. (2021). Freeze-dried simulated bites of each cow in P1 and P2 were used as substrates for *in vitro* incubation. Rumen fluid was collected from three cannulated sheep (used as ruminant model) reared in the INRAE Herbipole experimental facilities immediately before the feeding. Sheep were daily fed 1.2 kg of total mixed ration composed of 80% hay from permanent grassland (in % DM, NDF: 50.6, ADF: 26.7, ADL: 3.0, CP: 14.2) and 20% concentrate (barley, pulp beet, soybean meal, molasses, minerals and vitamins) in two equal meals for 17 days prior the *in vitro* trial. Animals had free access to water and salt block supplement (Sel'pur, Salins, Paris, France). Donor animal characteristics and collection procedure are described by Niderkorn et al. (2020). Incubation started almost immediately after rumen fluid collection. The substrate was fermented at 39 °C and under anaerobic conditions for 24 h. Each sample was incubated in duplicate (technical repetitions) and the fermentations were repeated 3 times in 3 consecutive days (runs). For each fermenter, gas pressure was measured in the headspace, and gas samples were collected for gas composition analyses after 3.5 and 24 h. *In vitro* DM degradability was determined as the difference between the DM of the herbage substrate before fermentation and the DM of the residuals after fermentation. Ammonia in the supernatant was measured using the Berthelot reaction (Weatherburn, 1967). The gas composition (CH_4 and CO_2) was analysed by gas chromatography, using a Micro-GC 3000A (Agilent Technologies, France) equipped with two columns (Menci et al., 2021). The individual volatile FA in the supernatant fraction of the incubation medium was analysed by gas chromatography (Perkin Elmer Clarus 580CPG), according to Morgavi et al. (2013).

Rumen fluid and faeces sampling and analyses

For both P1 and P2 periods, rumen fluid and faeces samples were collected individually after the morning milking. The rumen fluid was sampled by stomach tubing and immediately filtered through a 250–µm nylon pore cloth. Then, a sub-sample was lyophilised, lipids in 100 mg of sample were methylated with 0.5 *M* NaOH in methanol and methanol acetyl chloride (10:1 v/v), and the FA profile was analysed (Zened et al., 2011). Another subsample was used for microbial analysis, as detailed by Morgavi et al. (2015). Briefly, rumen fluid samples were centrifuged (15 000 × g, 15 min at 4 °C) just after collection, 0.8 mL supernatant was discarded, and the pellet was stored at –80 °C until processing. Faeces were collected by intra-rectal sampling the day after rumen fluid sampling and immediately stored at –20 °C until preparation of the pellets. For a better representativeness of the faecal microbiota analyses, faeces suspensions were prepared by adapting a method detailed by McOrist et al. (2002). In brief, ten grams of thawed faeces were blended with 90 mL of sterile phosphate-buffered saline using a Stomacher® and centrifuged (8 000 × g, 20 min at 4 °C). After removing the supernatant, the obtained pellets were mixed with 1–mL sterile phosphate-buffered saline, centrifuged (13 000 × g for 5 min at 4 °C), the supernatant removed, and the pellets stored at –20 °C. Total DNA extraction was performed from the pellets of individual faeces and rumen fluid using a FastDNA Spin Kit for Soil (MP Biomedicals, Eschwege, Germany). The 16S rRNA genes of the bacterial population and the internal transcribed spacer 2 region of the fungal population were amplified, and the amplicons were sequenced as described by Verdier-Metz et al. (2022).

Milk sampling and analyses

Milk fat and protein contents and somatic cell counts were measured on samples collected on four consecutive milkings on the last week of both P1 and P2 periods. Milk samples were preserved with bronopol at 4 °C until analysis, which was conducted within 2 days after sampling with a spectrometric method (Fossomatic and MilkoScan FT6000, Foss, Hillerød, Denmark). On the last day of P1 and P2 periods, individual milk samples were obtained from the morning and evening milking for FA analyses. These samples were lyophilised and pooled based on milk yield, and the resulting daily sample was preserved at –20 °C before analyses. Milk FA were methylated by adding 2 mL of 0.5 *M* sodium methoxide and 1 mL methanolic HCl (5% HCl v/v in methanol) at 50 °C for 5 min. Milk FA were analysed by gas-chromatography as previously described for simulated bites (Ferlay et al., 2010).

Statistical analyses

Statistical analyses were performed using the SPSS software (version 27.0 for Windows; SPSS Inc., Chicago, IL, USA). The number of individuals was established *a priori* running a power analysis (power set at 80%) based on the variables that were already available before the experiment (milk production and fat and protein contents). All data of simulated bites (proximate composition, phenolic compounds content, FA profile, and *in vitro* fermentation parameters), rumen (FA profile), and milk (productivity, gross composition, and FA profile) were processed using the mixed linear model. For all parameters, homoscedasticity and normality were tested with Levene's and Shapiro-Wilk test, respectively. The somatic cell counts data were log-transformed to achieve normality prior to be statistically analysed. For all these parameters, except for *in vitro* trial data, fixed effect (treatment) and covariates (pre-experimental period and days in milk) were considered. For *in vitro* fermentation data, a mixed model was used including treatment as a fixed effect and fermentation run as a random effect. Differences were considered as significant when $P < 0.05$ and as a tendency towards significance when $0.05 \leq P < 0.10$.

The microbial communities were characterised using the workflow rANOMALY (Theil and Rifa, 2021) that uses amplicon sequence variants as taxonomic units. The 16S amplicon sequence variants were assigned with an environment–specific database (DAIRYdb, Meola et al., 2019) and a general database (SILVA r138) and the internal transcribed spacer amplicon sequences with UNITE v8.2 and UTOPIA v072019 (Theil and Rifa, 2021) databases. The α-diversity of the bacterial and fungal communities was measured using richness (Observed) and evenness (Shannon) indexes. The effects of treatment (LD, HD) and period (P1, P2) on the α-diversity indexes were assessed using a mixed linear model in R (4.3.1) software. The differences in microbial community composition between treatments (β-diversity) were estimated using Bray Curtis dissimilarity and metric multidimensional scale for visualisation and analysed by permutational multivariate analysis of variance (PERMANOVA Adonis) within R software. Differential analysis on the relative abundance of bacterial and fungal taxa according to treatment and period was performed with three methods in ExploreMetabar (Theil and Rifa, 2021): DeSeq2 (Love et al., 2014), MetaGenomeSeq (Paulson et al., 2017) and MetaCoder (Foster et al., 2017).

Results

Pasture botanical diversity and chemical composition and fatty acid profile of simulated bites

The P1 period pasture plot consisted of 36 botanical species, of which grasses were the majority (70.0%), followed by legumes and other dicotyledons (17.3 and 13.2%, respectively). Concerning P2, the HD plot had a higher number of botanical species compared to the LD plot (56 vs 19, Table 1). The HD plot was dominated by non-legume dicots (53.5%) and grasses (41.1%), whereas the LD plot was mainly composed of grasses (63.9%) and legumes (25.3%). The proportion of botanical functional groups, as well as the proximate composition and FA profile of the simulated bites, are reported in Table 2. The cows of LD group mainly fed grasses (67.6%) and legumes (29.1%), while those of HD fed grasses (57.1%) and non-legume dicots (29%). The NDF and ADF contents of the simulated bites were slightly lower in the LD compared to the HD group (−20 and −22 g/kg DM, respectively), while the CP content showed an opposite trend (+57 g/kg DM in LD than in HD). The total extractable phenols and the total tannin contents of the simulated bites were more than double in the HD than in the LD group (+2.3-fold and +2.4-fold, respectively). The condensed tannins were 10.5 times higher in the HD than in the LD simulated bites. Compared with HD, LD simulated bites had a higher total FA content (52.2 vs 33.5 g/kg DM, respectively). The proportions of LA and ALA were significantly higher in the LD than in the HD simulated bites (+1.3 and +10.1% points), whereas the HD simulated bites showed higher proportions of oleic (C18:1 *cis*-9; +1.3% points) acids compared to the LD ones.

In vitro rumen fermentation

The pasture type significantly affected most of the studied *in vitro* rumen fermentation parameters (Table 3). The gas production, the *in vitro* DM degradability, and the ammonia concentration were lower when HD rather than LD simulated bites were incubated (−0.50 mmol/g DM, −5.85% points, and −0.64 mmol/g DM, respectively). The ratio between ammonia and CP of the simulated bites was lower for the HD treatment (0.0623) compared to the LD treatment (0.0750). The CO_2:CH_4 ratio was higher for the HD than for the LD simulated bites. The incubation of the HD simulated bites determined a lower content of total volatile FA than the incubation of the LD simulated bites (6.24 vs 7.00 mmol/g DM). The acetate proportion was higher when the HD rather than the LD simulated bites were incubated (+3%), while the proportions of propionate, butyrate, iso-volatile FA, and that of other minor volatile FAs showed an opposite trend. Caproate proportion was not affected by treatment.

Ruminal biohydrogenation of polyunsaturated fatty acids

As shown in Table 4, pasture type modified the FA profile of ruminal digesta. The rumen fluid of the HD cows had a higher proportion of polyunsaturated FA compared to that of the LD cows (+2.1% points). In particular, the proportions of ALA and LA were 0.6 and 1.6% higher in the rumen fluid of the HD cows, but the proportions of RA and C18:2 *trans*-11 *cis*-15 did not show any differences between treatments. The total monounsaturated FA proportion, as well as the proportion of VA, were lower in the rumen fluid of the HD cows when compared to that of the LD cows (15.2 vs 13.0%, 10.8 vs 8.2%, respectively), while an opposite trend was observed for the oleic acid proportion (1.1 vs 2.3%). The rumen fluid of the cows grazing on the HD plot had lower proportions of total saturated FA (57.0 vs 60.9%) and stearic acid (31.3 vs 39.9%) than that of the cows grazing on the LD plot. The total odd- and branched-chain FA proportion was 0.9% points higher in the rumen fluid of the HD cows when compared with the rumen fluid of the LD cows.

Ruminal and faecal microbiota

The main effects of the period and the pasture type are mainly related to the bacterial communities in rumen and the fungal communities in faeces. The pasture type had no major effect on the richness or evenness of neither bacterial nor fungal communities, whether in faeces or in rumen (Supplementary Table S1). However, it influenced the microbiota composition and the relative abundance of several microbial genera in rumen (Fig. 1 and Supplementary Fig. S1) as well as in faeces (Fig. 2 and Supplementary Fig. S1). Metric multidimensional scaling plotting based on Bray-Curtis dissimilarity showed that the two groups of cows presented a similar microbiota composition during the P1 period, both in rumen and in faeces. After 4 weeks on LD or HD pastures (end of P2 period), the microbial profiles in the rumen of HD cows were significantly different than those of LD cows, especially for bacteria (Fig. 1). In contrast, significant shifts in the fungal profiles were mainly observed in faeces (Fig. 2).

Twelve bacterial genera with an abundance greater than 0.5% were differentially abundant between HD and LD in rumen and 8

Table 3
In vitro rumen fermentation characteristics (%, unless otherwise stated) of simulated bites of dairy cows grazing a low diversified (LD) or a high diversified (HD) herbage plot.

Item	LD	HD	SEM	*P*-value
GP (mmol/g DM)	7.19	6.69	0.049	<0.001
IVDMD	63.5	57.6	0.721	<0.001
pH	6.21	6.16	0.030	<0.001
Ammonia (mmol/g DM)	1.51	0.87	0.068	<0.001
Ammonia:CP ratio	0.0750	0.0623	0.0015	<0.001
CO_2:CH_4 ratio	4.48	4.89	0.170	<0.001
Acetate	64.4	66.4	0.008	<0.001
Propionate	21.3	20.8	0.009	<0.001
Butyrate	8.80	8.50	0.003	0.024
Isobutyrate	1.40	1.10	<0.001	<0.001
Valerate	1.50	1.30	0.001	<0.001
Isovalerate	2.50	1.90	<0.001	<0.001
Caproate	0.114	0.096	<0.001	0.051
Total iso-VFA (mmol/g DM)	0.268	0.186	0.010	<0.001
Total VFA (mmol/g DM)	7.00	6.24	0.168	<0.001
Acetate:propionate ratio	3.03	3.22	0.165	<0.001

Abbreviations: GP, gas production; IVDMD, *in vitro* DM degradability; VFA, volatile fatty acid.

Table 4
Main fatty acid profile of rumen fluid of dairy cows grazing a low–diversified (LD) or a high–diversified (HD) herbage plot.

Fatty acid (g/100 g total FA)	LD	HD	SEM	*P*-value
Fatty acid groups				
SFA	60.9	57.0	0.66	<0.001
OBCFA	5.37	6.28	0.140	<0.001
Odd-chain FA	1.91	2.02	0.042	0.068
Iso branched	1.41	1.60	0.033	<0.001
Anteiso branched	2.04	2.66	0.091	<0.001
MUFA	15.2	13.0	0.47	<0.001
PUFA	5.41	7.51	0.396	<0.001
Individual fatty acids				
C6:0	2.05	2.00	0.303	0.916
C7:0	0.11	0.09	0.016	0.499
C12:0	0.51	0.67	0.049	0.025
C13:0	0.08	0.06	0.005	0.013
C13:0 *iso*	0.18	0.16	0.006	0.032
C14:0	1.19	1.73	0.053	<0.001
C14:0 *iso*	0.35	0.39	0.018	0.087
C15:0	1.16	1.21	0.031	0.275
C15:0 *anteiso*	1.77	2.20	0.085	<0.001
C15:0 *iso*	0.63	0.70	0.020	0.016
C16:0	11.9	14.8	0.24	<0.001
C17:0	0.56	0.66	0.009	<0.001
C17:0 *anteiso*	0.27	0.47	0.019	<0.001
C17:0 *iso*	0.26	0.36	0.014	<0.001
C18:0	39.9	31.3	1.04	<0.001
C18:1 *c*9	1.11	2.25	0.293	0.005
C18:1 *c*11	0.41	0.59	0.362	<0.001
C18:1 *c*12	0.16	0.16	0.016	0.816
C18:1 *t*6+*t*7+*t*8	0.33	0.37	0.010	0.002
C18:1 *t*9	0.21	0.29	0.011	<0.001
C18:1 *t*10	0.40	0.43	0.016	0.218
C18:1 *t*11	10.8	8.24	0.570	<0.001
C18:1 *t*12	0.46	0.50	0.016	0.111
C18:1 *t*13+*t*14	1.26	1.23	0.055	0.687
C18:1 *t*15	0.49	0.69	0.029	<0.001
C18:1 *t*16	0.72	0.64	0.028	0.029
C18:2 n-6	1.79	3.37	0.301	<0.001
C18:2 *c*9*t*11	0.04	0.14	0.042	0.114
C18:2 *t*11*c*15	0.80	0.75	0.047	0.433
C18:3 n-3	1.74	2.35	0.107	<0.001

Abbreviations: *c*, cis; FA, fatty acid; OBCFA, odd- and branched-chain fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; *t*, trans.

additional ones in faeces (Supplementary Table S2). *Prevotella* (Fig. 1b) was the dominant differentially abundant genus in rumen (33.5% in LD vs 24.7% in HD), followed by the genera *Ruminococcus* (4.6% in LD vs 2.1% in HD) and *Lachnospiraceae*_genus (2.6% in LD vs 0.7% in HD). Conversely, *Paludibacter* was more abundant in HD (5.8%) than in LD rumen (1.8%). In faeces, the genera *Intestinibacter* and *Anaerovorax* were more abundant in HD than in LD group (5.4 vs 3.5% and 1.0 vs 0.8%, respectively). The genus *Rikenellaceae*_RC9_gut_group was the only bacterial genus differentially abundant between HD and LD groups both in rumen and in faeces.

Nine fungal genera with an abundance greater than 0.5% were differentially abundant between HD and LD in rumen (Supplementary Table S2). Seven of these genera varied in the same way also in faeces: 3 assigned to *Ascomycota*_genus (Fig. 2b), *Panaeolus*, and *Pilidium* were more abundant in both HD faeces and rumen compared to LD and conversely 4 assigned to *Neoascochyta*, *Trichoderma*, *Sordariomycetes*_genus, and *Monoblepharidales*_genus were more abundant in both LD faeces and rumen compared to HD. In addition, the HD faeces (Fig. 2b) showed increased abundance of the genera *Ascomycota*_genus (10.8%), *Neocallimastigaceae*_genus (8.4%), and *Preussia* (6.5%), while *Didymellaceae*_genus (11.7%) and *Ustilago* (19.9%) were more abundant in LD ones.

Focusing on the class of anaerobic fungi *Neocallimastigomycetes*, frequently found in cattle rumen, it was observed a significant period effect with higher Observed and Shannon indexes in the P2 than in the P1 period (Supplementary Table S1). We identified two fungal amplicon sequence variants assigned to *Neocallimastigaceae*_sp and *Anaeromyces*_sp._FFEX4 significantly more abundant in HD compared to LD faeces (Fig. 3). The HD rumen was differentiated by a higher abundance of amplicon sequence variant 357e assigned to *Orpinomyces*_sp and LD rumen by a dominance of amplicon sequence variant 3af2 assigned to *Neocallimastix*_species.

Animal performance and milk fatty acid profile

The milk yield as well as the milk fat and protein contents did not differ significantly between groups (Table 5). The estimated DM intake was 0.2 kg/cow × day higher for the HD than for the LD cows. The body condition score and BW were comparable between the LD and HD groups. The milk FA profile from the cows grazing on the HD and LD plots is reported in Table 6. The milk fat of the LD cows was richer in total polyunsaturated FA (+1.6% points), total conjugated linoleic acids (+1.2% points), RA (+1.1% points), and VA (+1.5% points) compared to that of the HD cows. On the opposite, the milk fat of the HD cows was richer in LA (+0.2% points), total saturated FA (+3.6% points), and stearic acid (+2.5% points) compared to that of the LD cows. The proportions of ALA, C18:2 *trans*-11 *cis*-15, total monounsaturated FA, oleic acid,

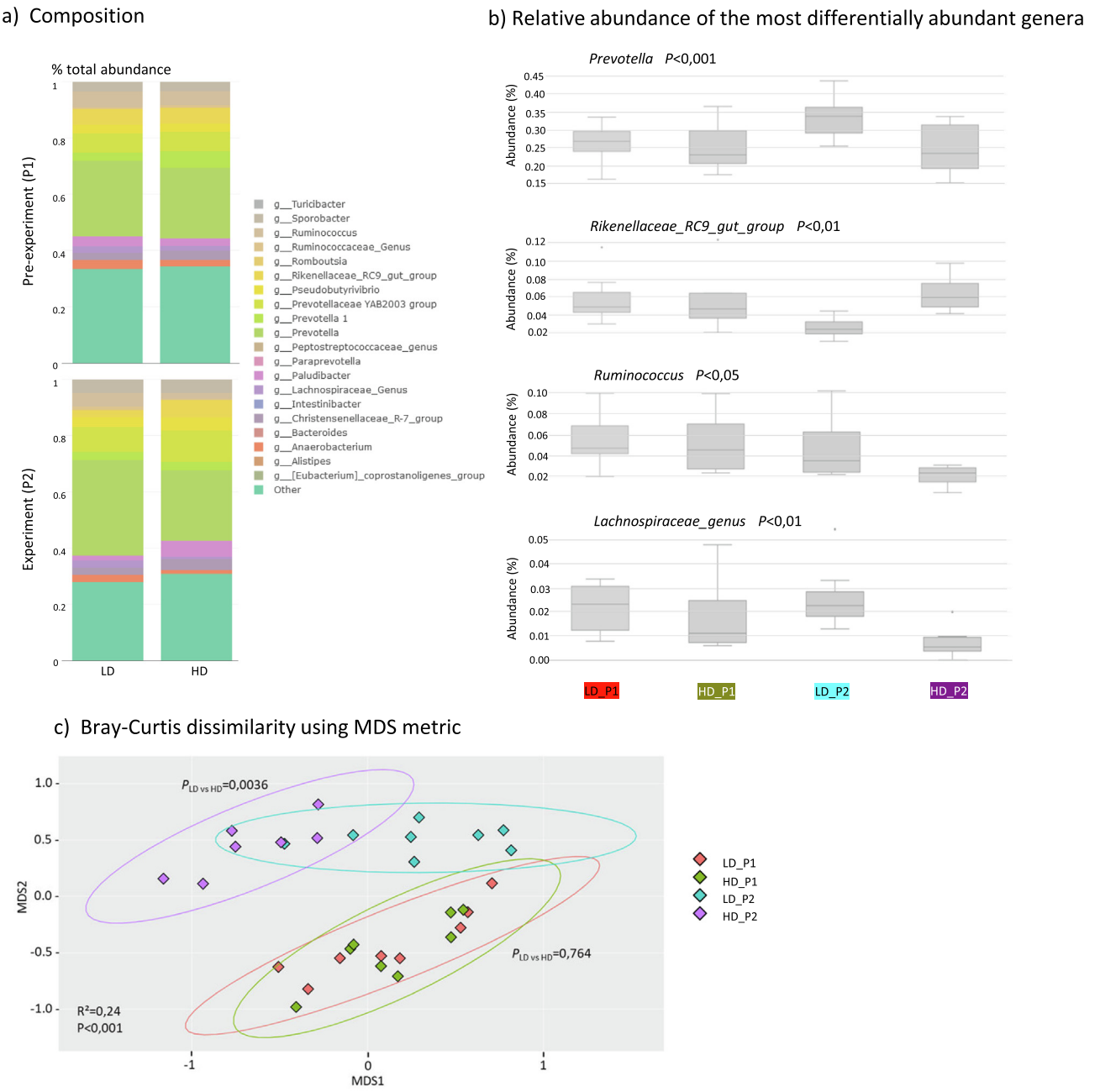

Fig. 1. Rumen bacterial microbiota (genus level) of dairy cows grazing the same herbage plot in pre-experimental period (P1), then a low–diversified (LD) or a high –diversified (HD) herbage plot in experimental period (P2). Abbreviation: MDS, multidimensional scale.

and total odd- and branched-chain FA were comparable between the treatments. Finally, the C14:1 *cis*-9/C14:0 ratio was lower in the HD than in the LD milk (0.073 vs 0.088, respectively).

Discussion

To the best of our knowledge, this is the first research that studied the effects of botanical diversity of permanent grasslands on rumen fermentation parameters, lipid metabolism, and rumen and faecal microbiota in dairy cows. Since a crossover design was not feasible under our mountainous conditions, we opted for a continuous experimental design with a pre-experimental period during which all animals grazed the same plot. Despite the quite low number of individuals chosen according to a power test ran on production variables, this experiment resulted in interesting findings relative to rumen and faecal microbiota, rumen fermentation parameters, and lipid metabolism.

Impact of pasture biodiversity on rumen and faecal microbiota

The grassland botanical diversity did not affect ruminal and faecal bacterial α-diversity measures despite a difference in fibre content between LD and HD pastures and simulated bites. However, α-diversity refers to diversity on a local (single sample) scale, describing species richness (total number of species and how evenly these species are distributed). These metrics provide no information about changes in community composition (Yuan et al., 2016; Buckland et al., 2017); for instance, communities

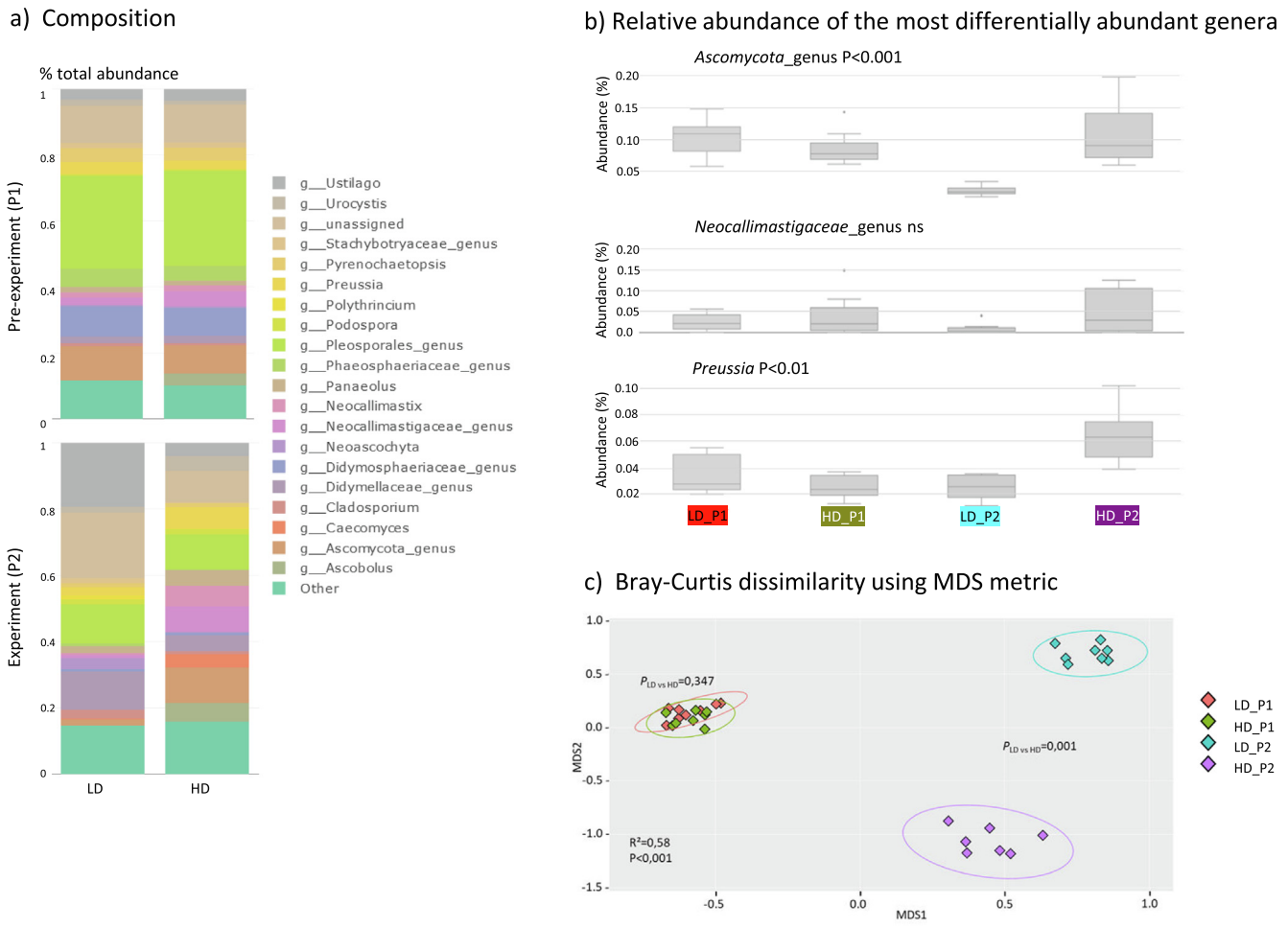

Fig. 2. Faeces fungal microbiota (genus level) of dairy cows grazing the same herbage plot in pre-experimental period (P1), then a low–diversified (LD) or a high–diversified (HD) herbage plot in experimental period (P2). Abbreviation: MDS, multidimensional scale.

may have similar α-diversity metrics even when their composition has shifted entirely. In contrast, β-diversity analysis allows changes in community composition to be taken into account. Calculating β-diversity indices for all combinations of sample pairs produces a distance matrix, which is often used for ordination (metric multidimensional scale in this paper) and data exploration in microbiota data analysis. Using this approach, we confirmed that the rumen microbial community of cows within the two groups was similar in the pre-experimental period (P1). Therefore, the major community shifts we observed during the experimental period (P2) could be directly related to the type of organic matter entering the rumen.

Diet is a major driver of rumen bacterial diversity (Huws et al., 2018). The NDF and ADF contents of the HD simulated bites were higher than that of the LD pasture. The higher fibre content should have stimulated the growth of fibrolytic rumen microbes. However, the relative abundance of key rumen fibrolytics, such as *Ruminococcaceae* and *Lachnospiraceae*, decreased in the rumen contents of cows grazing the high botanical diversity pasture. On the other hand, the HD simulated bites also had higher levels of plant bioactive compounds, namely the total extractable phenols and the total and condensed tannins measured in the study. Tannins may complex with lignocellulose and thus reduce substrate availability; tannins may also have a direct toxic effect on cellulolytic microorganisms (Vasta et al., 2019). Therefore, interactions between dietary chemical compounds should be considered when assessing the effects on the rumen microbial community.

Impact of botanical composition on digestive processes and enteric methane production

In vitro measurements may be subject to limitations because they are not accurately representative of *in vivo* measurements (Danielsson et al., 2017). In the present study, the rumen microbiota of the cows was adapted to the pasture diet. In contrast, the donor sheep were fed an 80:20 forage:concentrate total mixed ration diet, which did not strictly resemble the diet of the cows in the *in vivo* experiment. However, *in vitro* measurements can be a useful tool when *in vivo* conditions do not allow precise measurements, such as in the case of enteric methane detection in mountain pasture (Zhao et al., 2020). For this reason, we decided to use the *in vitro* study to compare the treatments while avoiding a direct extrapolation to *in vivo* conditions.

The *in vitro* incubation of HD simulated bites showed a general depression of fermentation products, probably caused by the intrinsic characteristics of the herbage and the content of plant secondary metabolites. Indeed, the reduction of *in vitro* DM degradability and total volatile FA production may be caused by the high concentration of tannins in the HD pasture that partially inhibited the activity of rumen bacteria and fungi (Carreño et al., 2015; Niderkorn et al., 2020). Although tannins are the plant metabolites that most interact with ruminal metabolism (Frutos et al., 2020), a complex of other plant bioactive compounds – including essential oils – may have also contributed to inhibit the fermentative processes of the HD cows (Vasta and Luciano,

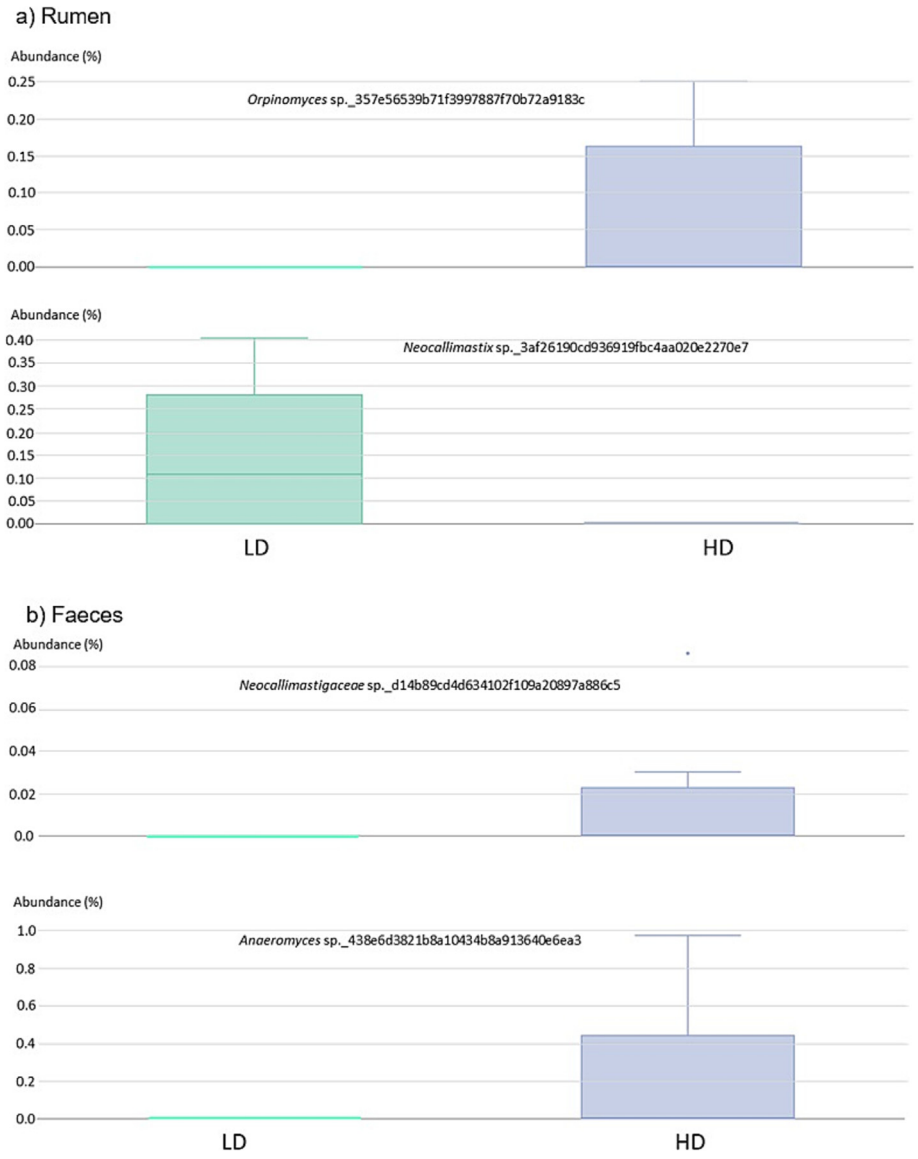

Fig. 3. Relative abundance of a) rumen and b) faeces fungal amplicon sequence variants (ASV) of the *Neocallimastigomycetes* class of cows grazing a low–diversified (LD) or a high–diversified (HD) herbage plot at the end of experimental period (P2).

Table 5
Milk production, milk composition, and performance of dairy cows grazing a low–diversified (LD) or a high–diversified (HD) herbage plot.

Item	LD	HD	SEM	*P*-value
Milk yield (kg/cow/day)	14.0	12.9	0.52	0.111
Fat (g/kg)	39.8	41.4	0.89	0.184
Protein (g/kg)	33.2	32.2	0.48	0.118
BCS (0–5)	1.92	1.86	0.04	0.274
BW (kg)	619	629	4.5	0.084
Estimated DMI (kg DM/head/day)[1]	18.8	19.0	0.07	0.004

Abbreviations: BCS, body condition score; DMI, DM intake.
[1] Calculated as reported by INRAE (2018).

2011). For instance, essential oils of *Thymus* species, notable for their content in plant metabolites including flavonoids and terpenes (Tohidi et al., 2018) and abundant in the HD pasture, have been shown to reduce *in vitro* DM degradability, fibre degradation, and volatile FA production (Martínez et al., 2006), and to inhibit the activity of *Streptococcus bovis* and *Selenomonas ruminantium* (Evans and Martin, 2000). Similarly, an extract from *Achillea millefolium*, a botanical species present in the HD pasture and rich in bioactive compounds, was shown to reduce microbial biomass and directly inhibit rumen methanogenesis (Kahvand and Malecky, 2017). The presence of *Knautia arvensis* in the HD pasture, known for its high content of condensed tannins, may also have impacted the rumen fermentation (Macheboeuf et al., 2014). The results found by Evans and Martin (2000), Martínez et al. (2006), Kahvand and Malecky (2017), and Macheboeuf et al. (2014) on *Thymus*, *A. millefolium* and *K. arvensis* are in agreement with the

Table 6
Fatty acid profile of milk from dairy cows grazing a low–diversified (LD) or a high–diversified (HD) herbage plot.

Fatty acid (g/100 g total FA)	LD	HD	SEM	*P*-value
Fatty acid groups				
SFA	58.6	62.2	1.14	0.031
OBCFA	5.40	5.47	0.114	0.664
Odd-chain fatty acids	2.31	2.26	0.032	0.344
Iso branched	1.82	1.89	0.065	0.482
Anteiso branched	1.27	1.33	0.027	0.115
MUFA	33.2	31.5	0.87	0.175
C18:1 *cis*	23.1	23.1	0.63	0.972
C18:1 *trans*	6.85	5.19	0.452	0.009
PUFA	7.30	5.67	0.343	<0.001
PUFA n-3	2.45	2.13	0.122	0.056
PUFA n-6	1.94	1.97	0.067	0.728
CLA	3.06	1.89	0.232	<0.001
Individual fatty acids				
C4:0	0.99	1.04	0.051	0.512
C6:0	1.13	1.14	0.052	0.837
C8:0	0.886	0.837	0.045	0.449
C10:0	2.20	1.90	0.114	0.069
C10:1 *c*9	0.251	0.219	0.017	0.175
C13:0	0.089	0.072	0.003	<0.001
C14:0 *iso*	10.50	9.61	0.378	0.116
C14:1 *c*9	0.878	0.748	0.060	0.113
C15:0 *anteiso*	0.786	0.813	0.018	0.275
C15:0 *iso*	0.387	0.423	0.008	0.002
C15:0	1.38	1.29	0.023	0.005
C16:0 *iso*	0.398	0.427	0.013	0.092
C16:0	24.9	25.9	0.73	0.357
C16:1 *c*7	0.280	0.272	0.004	0.192
C16:1 *c*9	1.31	1.23	0.103	0.597
C17:0 *iso*[1]	0.732	0.679	0.037	0.302
C17:0 *anteiso*	0.463	0.494	0.013	0.085
C17:0	0.698	0.766	0.015	0.001
C17:1 *c*9	0.254	0.277	0.011	0.181
C18:0	10.1	12.7	0.61	0.003
C18:1 *c*9[2]	22.0	22.1	0.60	0.980
C18:1 *c*11	0.587	0.582	0.031	0.901
C18:1 *c*12	0.142	0.119	0.007	0.031
C18:1 *c*15[3]	0.226	0.206	0.006	0.028
C18:1 *t*6+*t*7+*t*8	0.028	0.252	0.011	0.073
C18:1 *t*9	0.223	0.210	0.011	0.410
C18:1 *t*10	0.036	0.049	0.011	0.381
C18:1 *t*11	5.53	4.00	0.441	0.014
C18:1 *t*12[4]	0.383	0.295	0.007	<0.001
C18:1 *t*16[5]	0.382	0.365	0.015	0.388
C18:2 n-6	1.00	1.23	0.045	<0.001
C18:2 *c*9*t*13[6]	0.980	0.221	0.015	<0.001
C18:2 *c*9*t*11[7]	2.80	1.67	0.224	<0.001
C18:2 *t*11*c*15	0.568	0.496	0.080	0.520
C18:3 n-3	0.971	0.923	0.046	0.446
C20:0	0.124	0.222	0.008	<0.001
C20:1 *c*9	0.115	0.163	0.004	<0.001
C20:1 *c*11	0.018	0.015	0.003	0.494
C20:4 n-6	0.078	0.089	0.004	0.097
C22:5 n-3	0.149	0.148	0.005	0.913
C14:1 *c*9/C14:0 ratio	0.088	0.073	0.004	0.009

Abbreviations: *c*, *cis*; CLA, conjugated linoleic acid; FA, fatty acid; OBCFA, odd- and branched-chain fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; *t*, *trans*.
[1] Coeluted with C16:1 *t*9.
[2] Coeluted with C18:1 *c*10 and C18:1 *t*15.
[3] Coeluted with C19:0.
[4] Coeluted with C18:1 *c*6.
[5] Coeluted with C18:1 *c*14.
[6] Coeluted with C18:2 *c*10*t*14.
[7] Coeluted with C18:2 *t*7*c*9 and C18:2 *t*8*c*10.

lower *in vitro* methane production of the HD than LD simulated bites, as indicated by a higher CO_2:CH_4 ratio in HD compared to LD. Probably, a high tannin content in the HD herbage was responsible for the depression of methanogenic bacteria (Jayanegara et al., 2012; Aboagye and Beauchemin, 2019). This result is also relevant because the high fibre content of permanent grasslands was expected to increase the enteric methane production (Martin et al., 2010). This *in vitro* result suggests that grazing on tannin-rich

botanically diversified pastures may be useful to compensate for or even reduce methane emission by ruminants but in future, it should be validated in *in vivo* conditions.

Impact of grassland biodiversity on protein degradation

Noteworthy, the lower ammonia content (when expressed in reference to the CP herbage content) in the liquid fraction of HD compared to LD indicates a better exploitation of dietary proteins (Jayanegara et al., 2020). The complex characteristics of HD herbage and the content of bioactive compounds have the potential to preserve the HD protein from the degradation operated by bacteria and fungi. Among the plant bioactive molecules, tannins are able to bind to dietary proteins, protecting them from ruminal degradation (Getachew et al., 2008). Herremans et al. (2020) observed that tannin-rich diets improved nitrogen metabolism in dairy cows by reducing nitrogen degradation in the rumen. Also, these authors suggested that tannin-rich diets may improve the general utilisation of nitrogen, reducing nitrogen losses in urine and faeces. This protective action could be even more useful in low protein diets; in fact, the HD simulated bites had a lower CP content than the LD ones.

Impact of pasture type on lipid metabolism

Remarkable was the comparable ALA proportion of the LD and HD milk fat. This result was not related to ALA supply by the simulated bites, because the HD herbage had a considerably lower ALA content than the LD one. Many authors reported that cows grazing highly diversified pastures may preserve milk ALA despite a low concentration of ALA in herbage compared with low botanically diversified pasture (e.g., Leiber et al., 2005; Lourenço et al., 2008; Coppa et al., 2011). We can suppose that bioactive secondary metabolites contained mainly in dicots of highly biodiverse pastures, including tannins, depressed the activity of ruminal bacteria involved in lipid metabolism, resulting in a decreased BH process for the HD cows. In particular, the condensed tannins present in high proportion in the HD pasture appear to have an inhibitory action against ruminal bacteria (Vasta et al., 2019), as they are known to be more resistant to degradation by hydrolytic enzymes (Hill, 2003).

Concerning other milk FA derived from dietary polyunsaturated FA BH, the higher proportion of RA in the LD milk was probably related to the higher LA content in the LD herbage. The ruminal BH of polyunsaturated FA has generated high amounts of intermediate products, including RA and VA (Shingfield et al., 2010). Contrary to what was observed in ruminal fluid, the proportion of stearic acid, final product of BH, was higher in the HD milk. Our results are in agreement with other studies, showing an increase in milk stearic acid in the presence of highly diversified pastures (e.g., Leiber et al., 2005; Lourenço et al., 2005; Coppa et al., 2011). We hypothesise that the HD tannins inhibited the initial stages of BH, preserving dietary polyunsaturated FA (including ALA and LA), while the intermediate and final stages of BH were less affected by the HD plant secondary metabolites. Indeed, the LD milk was rich in BH intermediate products, such as RA and VA, thanks to the higher quantity of FA precursors in the LD herbage and to an incomplete BH process. However, milk stearic acid is the balance between polyunsaturated FA BH and the activity of the Δ^9-desaturase enzyme in the udder. Therefore, the activity of Δ^9-desaturase enzyme in the udder might have reduced the stearic acid proportion, transforming it into oleic acid (Bernard et al., 2008). Indeed, the activity of Δ^9-desaturase enzyme, estimated by the C14:1 *cis*-9/C14:0 ratio, appeared to be higher in the LD than the HD cows.

Conclusion

Dairy cows grazing a highly botanically diversified pasture, rich in fibre and plant secondary metabolites, showed a different share of ruminal and faecal microbiota genera compared to cows grazing a low botanically diversified pasture, less fibrous and lower in bioactive compounds. The high fibre content of the HD pasture increased the relative abundance of fibrolytic bacteria and fungi. The high content of plant secondary metabolites in the HD herbage modified the microbial population activity, reducing enteric methane and ammonia production *in vitro*. Tannins of the HD herbage protected dietary proteins from ruminal degradation and improved protein availability for cows. Furthermore, although the HD herbage had a lower content of ALA, the milk produced by the cows grazing on both pastures had a comparable ALA proportion, thanks to the protective action of tannins that inhibited the activity of the ruminal bacteria responsible for the first BH step. The differences observed between the HD and the LD milk FA profiles seemed to support this hypothesis. This research contributes to explain why grazing botanically diversified permanent grasslands by dairy cows promotes the transfer of polyunsaturated FA from herbage to milk and probably limits enteric methane and ammonia emissions through the effect of plant secondary metabolites on ruminal and faecal microbial populations.

Supplementary material

Supplementary Material for this article (https://doi.org/10.1016/j.animal.2025.101537) can be found at the foot of the online page, in the Appendix section.

Ethics approval

All animal-related procedures were conducted in accordance with the French guidelines for animal welfare and were approved by the local ethics committee (approval number 2015043014541577).

Data and model availability statement

The data/models were not deposited in an official repository but are available from the authors upon reasonable request to the corresponding author.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

None.

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