Received: 5 December 2023 | Revised: 5 March 2024 | Accepted: 17 March 2024

DOI: 10.1111/gfs.12669

Grass and Forage Science BGS EGF WILEY

ORIGINAL ARTICLE

Mixed ensiling of drought-impaired grass with agro-industrial by-products and silage additives improves the nutritive value and shapes the microbial community of silages

Theresa Gruber[1] | Katerina Fliegerová[2] | Georg Terler[3] | Reinhard Resch[4] | Qendrim Zebeli[1] | Thomas Hartinger[1]

[1]Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Vienna, Austria

[2]Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Prague, Czech Republic

[3]Institute of Livestock Research, Agricultural Research and Education Centre Raumberg-Gumpenstein, Irdning-Donnersbachtal, Austria

[4]Institute of Plant Production and Cultural Landscape, Agricultural Research and Education Centre Raumberg-Gumpenstein, Irdning-Donnersbachtal, Austria

Correspondence
Thomas Hartinger, Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Vienna, Austria.
Email: thomas.hartinger@vetmeduni.ac.at

Funding information
Austrian Federal Ministry of Agriculture, Forestry, Regions and Water Management, Grant/Award Number: 101623

Abstract

Droughts lead to reduced biomass production and unfavourable nutrient composition in grassland. As an alleviation, yet unexploited strategy, mixed ensiling of grass with agro-industrial by-products may improve the ensilability and nutritive value of drought-impaired grassland. This study investigated first whether mixed ensiling of drought-impaired grass with either sugar beet pulp (SBP), wheat gluten feed (WGF) or brewers' grains (BG) has a beneficial impact on chemical composition, fermentation characteristics, in vitro gas production (GP) and physically effective neutral detergent fibre (peNDF) of silages. Secondly, it was tested whether the application of anaerobic fungi culture supernatant (AF), mixed ruminal fluid (RF) or lactic acid bacteria (LAB) provides further advantages. Additionally, the microbial community composition was evaluated in selected silages. All silages showed satisfying conservation characteristics with high lactic acid levels and low dry matter losses, and peNDF values typically found for conserved forages. Mixed ensiling with BG substantially increased the crude protein concentration, whereas SBP increased the total degradability and WGF enhanced both. The further addition of fresh AF resulted in the overall highest lactic acid levels, especially in SBP-based silages, but without changes in in vitro GP. The in vitro GP was higher with RF, particularly in mixed silages, suggesting an improved degradability. The LAB-treated silages showed lower pH compared to controls, but had no impact on in vitro GP kinetics. Concluding, mixed ensiling holds potential to produce high-quality silages from drought-impaired grassland. The further addition of silage additives can be useful for certain substrates, but appeared not mandatory.

KEYWORDS
anaerobic fungi, enzymes, forage, ruminant, silage additive

1 | INTRODUCTION

For both physiological and ecological reasons, forages represent the most important component in cattle feeding. This is especially true for grass silages that, in contrast to maize (*Zea mays* L.) silages, can be produced on cropland and permanent grassland and thus regionally and ecologically compatible in all temperate regions of Europe and worldwide. Grass silages are a valuable feed source that typically provide cattle with dietary energy and protein as well as physically effective fibre. However, in recent years, changes in the annual precipitation patterns have led to prolonged drought periods. This situation is further aggravated by the general stronger evapotranspiration due to rising air temperatures from global warming (Ionita & Nagavciuc, 2021; Kempf, 2023; Naumann et al., 2021), as a whole substantially affecting both the quantity and quality of grass silages. Besides reduced biomass production, drought-impaired grass has a compromised nutritive value, particularly a higher lignification that often goes along with lower concentrations of easily fermentable carbohydrates and crude protein (CP; Sheaffer et al., 1992; Habermann et al., 2019), altogether resulting in reduced ensilability, lowered degradability of organic matter, and eventually diminished animal performance. It is expected that drought periods will continue to be a rising challenge for forage production in long term in most areas of Europe (Forzieri et al., 2014). To prevent forage shortages and improve the nutritive value of grass silages, mixed ensiling of drought-impaired grass with regionally available agro-industrial by-products can be a promising alternative that has not yet been largely explored. Agro-industrial by-products typically have high nutrient concentrations, such as CP, starch or sugars, and are not in competition with human consumption, which also lowers their price on the market (Flachowsky et al., 2017). Such co-substrates could provide easily fermentable carbohydrates that also improve ensilability and/or further valorise the silages in terms of nutrient density. For instance, sugar beet pulp (SBP) contains high pectin and considerable sugar amounts to boost the energy content, while brewers' grains (BG) provide mostly CP and wheat gluten feed (WGF) delivers starch and CP content.

Apart from the impact of mixed ensiling itself, silage additives may further support the conservation success and improve the nutritive value of ensiled grass. Recent research from our team found the addition of anaerobic fungi culture supernatant (AF) or mixed ruminal fluid (RF) to be a tool to improve the silage fermentation quality in grass silages: Both additives resulted in lower silage pH and reduced dry matter (DM) losses. Furthermore, AF had significantly improved the in situ fibre degradability, which was attributed to a fibre-cleaving effect of fungal enzymes in the silage (Hartinger et al., 2022). Besides these two additives, other studies have suggested that lactic acid bacteria (LAB) could promote the silage fermentation in forages with unfavourable properties, including those with rather low water-soluble carbohydrate (WSC) concentrations (Guo et al., 2023), which may also apply to drought-impaired forages.

Based on these findings, the aim of our study was the comprehensive exploration of mixed silages prepared from drought-impaired grass and different by-products, that is, SBP, WGF, or BG, for producing high-quality forages. Additionally, we investigated whether the addition of AF, RF, or LAB could further improve the fermentation quality and nutritive value of those silages. Therefore, the chemical composition, silage fermentation characteristics, in vitro gas production (GP), which is indicative of ruminal digestibility, as well as the concentration of physically effective neutral detergent fibre (peNDF) of the silages were evaluated. We hypothesised that the co-ensiling of drought-impaired grass with by-products improves silage quality compared to silage from purely drought-impaired grass due to an increased availability of rapidly fermentable carbohydrates and, depending on the co-substrate, increases either the CP or energy content of the silages. Additionally, we expected a stronger lactic acid fermentation and consequently a lower pH with the addition of silage additives. Due to a fibre-cleaving effect during ensiling, we hypothesised a higher rumen degradability of silages treated with fresh AF that is expressed in an increased in vitro GP.

So far, there is no information on the impact of mixed ensiling or the addition of AF or RF on the microbial community in silages. As the bacteria present in silages are in fact decisive for the successful preservation (McDonald et al., 1991), such knowledge seems highly important to understand previously observed effects. Hence, in addition to the 'classical' assessment of the silages, we further analysed the microbial community composition in mixed silages, ensiled alone or with the addition of AF or RF using a culture-independent approach. Since comprehensive data about the impact of LAB inoculation on the microbial community in silages can be found in literature (e.g., Bai et al., 2021; Guo et al., 2018; Ridwan et al., 2023), those silages were omitted from this part of the analysis.

2 | MATERIALS AND METHODS

2.1 | Production of silage additives

Three different silage additives were used, that is, AF, RF, and LAB. The AF was obtained as described by Hartinger et al. (2024). Briefly, the AF was derived from the fungal strain *Feramyces* DF1 (GenBank accession number MW907584), which was isolated from deer rumen content. The strain was cultivated in batch culture for 4 days, and the resulting fungal mycelium was removed by centrifugation. The enzymatically active supernatant was collected and stored at −20°C until it was used for ensiling purposes. Before adding it to the silages, it was thawed overnight in the fridge. Heat-inactivated AF was used as control additive (InactAF) and therefore cooked for 30 min and subsequently cooled down to room temperature.

The RF was collected from two dry rumen-cannulated cows before the morning feeding at the University Clinic for Ruminants, University of Veterinary Medicine Vienna, Austria. The cows were kept according to the Austrian guidelines 114 of animal welfare (BGBl. II Nr. 485/2004 idF BGBl. II Nr. 151/2017) and fed hay ad libitum plus 1 kg of concentrate (KuhKorn PLUS Energie, Garant-Tiernahrung GmbH, Pölchlarn, Austria) per cow per day. The RF was

filtered through 4 layers of gauze (Wilhelm Weisweiler GmbH & Co. KG, Münster, Germany) and temporarily stored in flasks in a water bath at 39°C, maximum for 30 min. Heat-inactivated RF (InactRF) was used as a control by placing the RF in a forced-air oven at 103°C for 45 min. After cooling down to room temperature, the additive was used for ensiling.

The LAB additive was a commercial silage additive (Bonsilage Forte, H. Wilhelm Schaumann GmbH, Pinneberg, Austria) that contains three different bacterial species in a total concentration of at least 1.25×10^{11} LAB/g, namely *Pediococcus acidilactici*, *Lactobacillus paracasei* and *Lactococcus lactis*. The LAB additive was available as a powder and dissolved in untreated tap water before application. The final solution consisted of 200 mg of additive per litre, as recommended by the manufacturer.

2.2 | Production of silages

The main component of all silages was drought-impaired grass grown at the Agricultural Research and Education Centre Raumberg-Gumpenstein (Irdning-Donnersbachtal, Austria) in a drought experiment. The grass was a second cut and grown in 8 plots, that is, replicates that were randomized among treatments, under controlled drought conditions by protecting all grassland plots from precipitation (268 mm in total) using a roof construction with a plastic film (rainout-shelter). During the growth period from end of May 2021 until harvest at the end of July 2021, the average temperature was 18.6°C. The plots were composed of 93.4% grasses (predominantly *Arrenatherum elatius* (L.) P.Beauv. ex J.Presl & C.Presl, *Poa pratensis* L., *Lolium perenne* L. and *Dactylis glomerata* L.), 3.2% legumes (predominantly *Trifolium repens* L.) and 3.4% forbs (in weight percentages). Directly after harvest but before wilting, the fresh forage had a DM and CP concentration of 327 g/kg DM and 122 g/kg DM, respectively. The average yield was 1825 kg DM/ha. For logistical and organizational reasons, drought-impaired grass had to be air-dried on ground after harvest, so this wilted drought-impaired forage was used as main component in the present experiment.

The ensiling experiment comprised four different substrates and six different silage additives. The four substrates constituted wilted drought-impaired forage that was ensiled either solely (G) or with one of three different by-products. Therefore, the forage was mixed with (i) pelleted SBP (S; 370 g/kg DM), (ii) pelleted WGF (W; 630 g/kg DM), or (iii) fresh BG (B; 290 g/kg DM) that was obtained from nearby local producers. Those by-product inclusion levels were chosen to either produce a mixed silage moderate in energy (~6.0 MJ net energy for lactation (NEL)/kg DM) and high in CP (~180 g/kg in DM), or an energy-dense mixed silage, that is, ~6.3 MJ NEL/kg DM (Resch, 2021). The chemical composition of all substrates is presented in Table 1. These silages were then ensiled without a silage additive (CON) or with either fresh AF, InactAF, fresh RF, InactRF or LAB. The applied concentrations were 100 g/kg DM for RF and InactRF as well as 10 g/kg fresh matter (250,000 colony-forming units per g fresh matter) for the LAB, which corresponds to previous ensiling trials (Hartinger et al., 2022) or to the manufacturer's recommendation. For AF and InactAF, a dosage of 250 g/kg DM was applied, which is higher than in previous ensiling experiments (Hartinger et al., 2022) and was chosen to investigate whether the beneficial effects on silage quality and ruminal in situ degradability observed before (Hartinger et al., 2022) can be further enhanced – especially since AF constitutes a novel silage additive candidate for which no knowledge on optimal dosages exists.

Additionally, molasses (10 g/kg fresh matter) and tap water were added to all silages to achieve a final silage DM concentration of ~350 g/kg that is typically targeted in silages (McDonald et al., 1991).

TABLE 1 Chemical composition of the drought-impaired grass and by-products used as substrates for ensiling.

	Drought-impaired grass[a]	Sugar beet pulp	Wheat gluten feed	Brewer's grains[b]
Dry matter (g/kg)	928	934	939	281
Ash (g/kg DM)	80.9	74.3	60.8	41.8
Crude protein (g/kg DM)	98.6	147	246	255
Ether extract (g/kg DM)	23.0	7.50	47.4	101
aNDFom[c] (g/kg DM)	537	380	388	633
ADFom[d] (g/kg DM)	325	229	151	263
ADL[e] (g/kg DM)	75.6	11.6	65.8	72.1
Hemicelluloses[f] (g/kg DM)	212	151	237	370
Cellulose[g] (g/kg DM)	249	113	85.4	191
Water-soluble carbohydrates (g/kg DM)	179	120	89.5	13.9

[a]Air-dried forage.
[b]In fresh form.
[c]Neutral detergent fibre assayed with a heat stable α-amylase and expressed exclusive of residual ash.
[d]Acid detergent fibre expressed exclusive of residual ash.
[e]Acid detergent lignin.
[f]Calculated as aNDFom (g/kg DM) – ADFom (g/kg DM).
[g]Calculated as ADFom (g/kg DM) – ADL (g/kg DM).

Then, substrates were ensiled in vacuum bags (Plastar Pak, Concorezzo, Italy) with 900 g of substrate per bag and residual oxygen was removed using a vacuum sealer (HFE vacuum systems Henkovac, Netherlands). The bags were directly weighed after sealing and stored at ~20°C for 90 days. Each treatment was produced in quadruplicate, resulting in four independent replicates per treatment.

2.3 | Analysis of silage composition

After 90 days of storage, the bags were weighed to calculate the DM loss and subsequently opened. Representative samples of about 300 g were taken from each silage bag and dried at 65°C in a forced-air oven for 48 h. The dried samples were then ground through a 0.5 mm screen using an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany). All chemical analyses were conducted following the guidelines of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2012). The DM concentration was determined by drying the samples at 103°C for a minimum of 4 h (method 3.1). Then, DM concentration was corrected for the loss of volatile compounds from silages that occur during drying using the equation of Weißbach and Kuhla (1995). Ash concentration was determined by combustion in a muffle furnace at 580°C for at least 4 h (method 8.1). The CP was analysed using the Kjeldahl method (method 4.1.1) and ether extract (EE) was determined using the Soxhlet extraction system (Extraction System B-811, Büchi, Flawil, Switzerland; method 5.1.2). Proportions of neutral detergent fibre assayed with a heat-stable α-amylase and expressed exclusive of residual ash (aNDFom; method 6.5.1), acid detergent fibre expressed exclusive of residual ash (ADFom; method 6.5.2) and acid detergent lignin (ADL; method 6.5.3) were determined using the Fibre Therm FT 12 (Gerhardt GmbH & Co. KG, Germany). The analysis of WSC concentration was carried out following the procedure outlined in method 7.1.1. Afterwards, concentrations of hemicelluloses and cellulose were calculated as aNDFom – ADFom and ADFom – ADL, respectively.

2.4 | Analysis of silage fermentation characteristics

Immediately after opening the vacuum bags, a cold-water extract was prepared from each silage according to Hartinger et al. (2022). Briefly, a 50 g sample was taken and mixed with 100 mL of distilled water in a jar, sealed, and placed in the fridge at 4°C for 24 h. Then, the content was filtered through three layers of gauze (Wilhelm Weisweiler GmbH & Co. KG, Münster, Germany) and the pH was directly measured using a calibrated pH meter (S40-K SevenMulti™ pH meter, Mettler Toledo, Vienna, Austria). The remaining liquid was stored in aliquots at −20°C for further analyses.

Ammonia-N (NH_3-N) was analysed colorimetrically based on the Berthelot reaction (Hinds & Lowe, 1980) and lactic acid was analysed by high-performance liquid chromatography (UltiMate 3000 HPLC system, Thermo Fisher Scientific, Vienna, Austria) following the method of Weiß and Kaiser (1995). Volatile fatty acids and alcohols were determined by gas chromatography using a GC apparatus (Shimadzu GC Plus with FID detector, Shimadzu, Kyoto, Japan) that was equipped with a 30 m × 0.53 mm i.d. × 0.53 µm capillary column (Trace TR Wax, Thermo Fisher Scientific, Waltham, MA). Injector and detector had temperatures of 170°C and 220°C, respectively, and helium was used as carrier gas with a flow rate of 1 mL/min.

2.5 | In vitro gas production kinetics

The in vitro GP kinetics were determined using the Hohenheim gas test (Menke & Steingass, 1988). In brief, each dried sample (4 per treatment) was ground through a 1 mm screen using an ultra-centrifugal mill (ZM 200, Retsch, Haan, Germany) and incubated in duplicate (technical replicates) in two independent runs, meaning 8 true replicates per treatment (2 run means × 4 replicates). The ruminal fluid was collected before morning feeding from two rumen-cannulated dry Holstein cows at the University Clinic for Ruminants, University of Veterinary Medicine Vienna, which were also used for collection of RF applied as silage additive and kept as described in Section 2.1. Then, the syringes prepared with 200 mg DM of samples were filled with 30 mL of the inoculum and placed into the pre-warmed incubation chamber at 39°C. The GP was recorded at 0, 2, 4, 8, 12, 24, 32, 48, 56, and 72 h of incubation.

2.6 | Determination of particle size distribution

We applied a dry sieving method to measure the particle size distribution of all CON silages. A sieving cascade of three different screens was used, that is, 8 mm, 2 mm, and 1.18 mm, which were placed in a vibratory sieve shaker (AS 200 digit, Retsch, Germany). Approximately 35 g of fresh silage was placed on the top sieve before the start of the sieving. The sieving process lasted 5 min and the amplitude was set at 3 mm. To determine the different proportions, the screens were weighed before and after sieving. To obtain more accurate results, two aliquots of each silage sample were sieved. The calculation of peNDF >8 mm ($peNDF_{>8mm}$) was based on the aNDFom concentration of the silages multiplied by the percentage of particles retained on the screen larger than 8 mm (Mertens, 1997). The $peNDF_{>8mm}$ was calculated as particles >8 mm are most decisive for the fibre mat stratification in cattle rumen and therefore for promoting a sufficient rumination activity and the associated saliva-induced buffering (Zebeli et al., 2012).

2.7 | Microbial analysis of mixed silages

2.7.1 | DNA extraction and sequencing

To better understand the impact of both mixed ensiling as well as the novel silage additives AF and RF on the microbial community composition in the silage, we further performed a 16S rRNA gene sequencing

analysis on those silages. Therefore, ~10 g of fresh silage were taken directly after silo opening and ground using mortar and pestle with liquid nitrogen (Hartinger et al., 2020). Subsequently, DNA was extracted from an aliquot of the ground sample using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) in accordance with the manufacturer's protocol and the addition of mutanolysin, lysozyme and proteinase K. After the isolation, DNA quantity was determined using a fluorometer (Qubit Fluorometer 2.0, Thermo Fisher Scientific, Austria) and the respective kit (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific, Austria) by following the manufacturer's instructions. The hypervariable region V4 of the 16S rRNA gene (2 × 250 bp) was amplified using the primer pair 515F (5′- GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′- GGACTACHVG GGTWTCTAAT-3′). The gene sequencing was performed on the NovaSeq 6000 sequencing platform and both trimming of primers and stitching of paired-end reads was done by Novogene (Novogene Co., Ltd, Cambridge, United Kingdom).

2.7.2 | Bioinformatic analysis

The sequencing data set was processed using the Quantitative Insights into Microbial Ecology QIIME2 v2023.2 (Bolyen et al., 2019). The read quality was inspected using FASTQC with the PHRED score offset of 33. Before quality-filtering using 20 as a minimum acceptable PHRED score, sequence data were merged with VSEARCH (Rognes et al., 2016). Denoising into sub-operational taxonomic units (sOTU) was done by Deblur (Amir et al., 2017) and mitochondria or chloroplast sequences were then excluded from representative sequences and feature tables. The resulting sOTU were aligned with mafft (Katoh et al., 2002) and a phylogeny was constructed with FastTree2 (Price et al., 2010). Taxonomy was assigned to sOTU using a classify-sklearn naïve Bayes taxonomy classifier trained with the 515F/806R primer set against the SILVA Small Subunit rRNA database v138 (Quast et al., 2013). Afterwards, the filtered feature table, rooted tree and taxonomy were imported in RStudio v14.1717 for further analysis.

2.8 | Mathematical calculations and statistical analyses

The GP data was used to estimate the parameters of GP kinetics using the equation of Ørskov and McDonald (1979): $Y = a + b * (1-e^{(-c * t)})$, where Y is the GP at time t (mL/200 mg DM), a is the GP from the from the soluble, immediately available substrate (mL/200 mg DM), b is the GP from insoluble, fermentable substrate (mL/200 mg DM) and c is the GP rate per hour (/h). Therefore, technical replicates were averaged per each independent run.

The data sets of silage composition, fermentation parameters and GP kinetics were analysed with the GLM procedure of SAS v9.4 (SAS Institute Inc., Cary, USA) using the following model:

$$Y = \mu + c_i + s_j + (c \times s)_{ij} + e_{ij}$$

where μ is the mean, c_i is the main effect of the component ensiled together with drought-impaired grass, s_j is the main effect of silage additive, $(c \times s)_{ij}$ is the two-way interaction between the main effects and e_{ij} is the residual error. Thereby, the effect of silage additive was tested in three separate subsets, which were (i) CON, inactAF and fresh AF, (ii) CON, inactRF and fresh RF, plus (iii) CON and LAB. Differences between least square means were analysed by Tukey–Kramer post hoc test. The significance level was set at $\alpha = .05$ and a tendency was declared at $.05 < p < .10$ for all analyses.

The alpha diversity metrics were calculated in RStudio and then imported in SAS for statistical analysis using the GLM procedure and model as described above. Except for alpha diversity metrics, sequencing data was analysed in RStudio. The differences in beta diversity were calculated using the vegan package and the *adonis2* function (Anderson, 2001) and principal coordinates analysis (PCoA) plots were created using weighted UniFrac distance metrics. The differential abundances at genus level were calculated for the main factor silage additive using the package MaAsLin2 (Mallick et al., 2021). Thereby, changes in abundances were considered as relevant if coefficient was <−2.00 or >2.00 and Benjamini-Hochberg false discovery rate-adjusted *q*-values <.05. The Venn diagrams were created with the tool Venny v2.1 (Oliveros, 2007–2015) to determine microbial genera that were exclusively present in differently treated silages. Additionally, Spearman correlation coefficients between data sets of alpha diversity metrics, 10 most abundant microbial genera, differentially abundant microbial genera and silage quality characteristics were calculated and subsequently visualized in a heatmap.

3 | RESULTS

3.1 | Chemical composition, DM losses and peNDF$_{>8mm}$ concentration of silages prepared without additives

The chemical composition of the mixed silages without additives is presented in Table 2. The ash concentration was highest in G_CON, followed by S_CON and the lowest ash concentrations were observed in grass co-ensiled with WGF or BG (B; $p < .01$). Compared to G_CON and S_CON, the CP concentration was higher for the silages ensiled with the protein-rich by-products WGF and BG ($p < .01$). The EE concentration ranged between 20 g/kg DM and 50 g/kg DM and was higher in silages co-ensiled with protein-rich by-products than in G_CON or S_CON ($p < .01$). The highest aNDFom concentration was observed in B_CON followed by G_CON and S_CON and the lowest was found in W_CON ($p < .01$). The ADFom concentration was higher in G_CON than in B_CON, while W_CON and S_CON were intermediate and did not differ from any other feed ($p = .01$). The ADL concentration ranged from ~65 g/kg DM to 94 g/kg DM, with the lowest value in S_CON and the highest in W_CON, while the two other silages remained in between ($p < .01$). The concentration of hemicelluloses was higher in B_CON than in G_CON, S_CON or W_CON ($p < .01$). The cellulose concentration was 9.1 and 7.5 percentage

TABLE 2 Effect of mixed ensiling on chemical composition and silage fermentation characteristics of silages.

	Treatment[a]					
	Grass	SBP	WGF	BG	SEM[b]	*p*-value
Chemical composition						
DM[c] concentration (g/kg)	359	356	356	365	12.2	.97
Ash (g/kg DM)	86.8^{a}	82.2^{b}	74.8^{c}	76.8^{c}	0.62	<.01
Crude protein (g/kg DM)	119^{c}	109^{c}	216^{a}	165^{b}	7.38	<.01
Ether extract (g/kg DM)	23.5^{b}	19.8^{b}	43.9^{a}	50.8^{a}	3.22	<.01
aNDFom[d] (g/kg DM)	563^{b}	542^{b}	487^{c}	636^{a}	7.25	<.01
ADFom[e] (g/kg DM)	360^{a}	335^{ab}	331^{ab}	268^{b}	12.1	.01
ADL[f] (g/kg DM)	73.0^{b}	64.6^{c}	94.3^{a}	72.2^{b}	1.85	<.01
Hemicelluloses[g] (g/kg DM)	203^{b}	207^{b}	157^{b}	368^{a}	14.9	<.01
Cellulose[h] (g/kg DM)	287^{a}	271^{a}	237^{ab}	196^{b}	11.7	.01
WSC[i] (g/kg DM)	54.8^{bc}	80.5^{b}	128^{a}	36.3^{c}	5.13	<.01
Silage fermentation characteristics						
DM loss (%)	3.63	3.38	3.56	3.65	0.08	.53
pH	3.80^{ab}	3.73^{b}	3.80^{ab}	3.86^{a}	0.02	.03
Lactic acid (g/kg DM)	81.9	108	119	81.9	8.52	.05
Acetic acid (g/kg DM)	4.33	4.36	4.01	6.46	0.65	0.14
Propionic acid (g/kg DM)	n.d.[j]	n.d.	n.d.	n.d.	-	-
Butyric acid (g/kg DM)	n.d.	n.d.	n.d.	n.d.	-	-
Ethanol (g/kg DM)	1.44^{b}	1.97^{b}	8.93^{a}	1.56^{b}	0.34	<.01
Ammonia-N (g/kg total N)	29.3^{ab}	25.9^{ab}	20.4^{b}	31.1^{a}	1.57	0.03

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).
[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (Grass), with sugar beet pulp (SBP), wheat gluten feed (WGF), or brewer's grains (BG).
[b]Standard error of the mean.
[c]Dry matter.
[d]Neutral detergent fibre assayed with a heat stable α-amylase and expressed exclusive of residual ash.
[e]Acid detergent fibre expressed exclusive of residual ash.
[f]Acid detergent lignin.
[g]Calculated as aNDFom (g/kg DM) – ADFom (g/kg DM).
[h]Calculated as ADFom (g/kg DM) – ADL (g/kg DM).
[i]Water-soluble carbohydrates.
[j]Not detected.

points higher in G_CON and S_CON compared to B_CON with W_CON as intermediate ($p = .01$). The concentration of WSC was 5–7 percentage points higher in W_CON than in G_CON or S_CON ($p < .01$). Furthermore, WSC concentration was higher in S_CON than in B_CON with G_CON in between. The DM concentration was not different between silages ($p > .10$).

The results of fermentation characteristics analysis are also presented in Table 2. The pH was influenced by the mixed ensiling, B_CON had a 0.13 units higher value compared to S_CON with G_CON and W_CON as intermediate ($p = .03$). The concentration of ethanol was higher in W_CON compared to the others ($p < .01$). Moreover, the B_CON had a higher NH_3-N concentration compared to W_CON, whereby G_CON and S_CON were in between and not differing from other silages ($p = .03$). Lactic acid was the most dominant fermentation acid, with a concentration ranging from 82 g/kg DM to 119 g/kg DM but without differences between silages in post-hoc test ($p = .05$). Neither butyric acid nor propionic acid were detected in the silages.

Figure 1 displays the $peNDF_{>8mm}$ concentrations of silages, which was analysed only for CON silages. The $peNDF_{>8mm}$ ranged from 310 g/kg DM to 464 g/kg DM and was affected by mixed ensiling ($p < .01$) with G_CON and B_CON showing higher $peNDF_{>8mm}$ values than S_CON.

3.2 | Chemical composition and DM losses of silages prepared with AF

The chemical composition and silage fermentation characteristics of silages prepared with the AF additive are presented in Table 3. We observed an interaction of substrate and additive for the ash concentration, G_AF had a higher ash concentration compared to G_InactAF

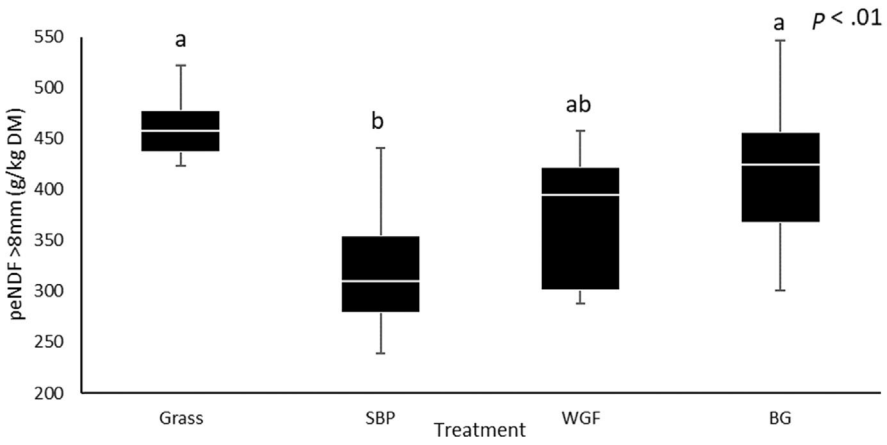

FIGURE 1 Physically effective neutral detergent fibre >8 mm ($peNDF_{>8mm}$) of pure drought-impaired grass silage (Grass), drought-impaired grass ensiled with sugar beet pulp (SBP), drought-impaired grass ensiled with wheat gluten feed (WGF), drought-impaired grass ensiled with fresh brewer's grains (BG).

($p = .03$), while supplementation of fresh or heat-inactivated AF had no effect on ash concentration in the mixed silages. Another interaction was present for aNDFom concentration ($p < .01$) with highest values for B_CON (636 g/kg DM) that differed from B_InactAF with B_AF in the middle ($p < .01$). In contrast, B_CON was lower in ADFom than B_InactAF and B_AF ($p < .01$). W_CON was higher in aNDFom and ADFom concentrations compared to W_AF ($p < .01$). The ADL concentration was increased by the addition of AF in pure grass silages and silages prepared with SBP and BG ($p < .01$), being approximately two times higher than for InactAF and CON. In contrast, the W_AF silages had a lower ADL concentraion than the other treatments ($p < .01$). Furthermore, hemicelluloses were higher in B_CON compared to B_InactAF and B_AF ($p < .01$). Additionally, we found an interaction for cellulose concentration with lower values in B_CON compared to the highest value in B_InactAF ($p < .01$). The WSC concentrations ranged from 35 g/kg DM to 128 g/kg DM and showed lower numbers for AF than CON in SBP and WGF silages ($p < .01$). For EE, a main effect of additive showed that InactAF-treated silages had lower values compared to AF and CON silages ($p = .01$).

Regarding silage fermentation quality, results showed that InactAF addition increased DM losses compared to AF and CON in all silages ($p = .02$). Overall, the pH values ranged from 3.7 to 4.0, but in SBP and WGF silages, AF treatment increased silage pH compared to CON treatment ($p = .01$). In contrast, B_InactAF had a 0.12 and 0.18 units lower pH than B_CON and B_AF, respectively ($p = .01$). Also, lactic acid was 4.9 and 5.4 percentage points higher in G_AF and G_InactAF compared to G_CON ($p = .02$). Furthermore, InactAF supplementation also increased lactic acid concentration in SBP and BG silages. Acetic acid concentrations were generally low, ranging from 3 to 15 g/kg DM; yet higher in S_InactAF and S_AF than in S_CON ($p < .01$). Similarly, acetic acid in W_AF was higher than in W_InactAF and W_CON. Moreover, the acetic acid concentration in B_AF was higher and differed from both B_CON and B_InactAF. For ethanol, G_InactAF was higher than G_CON and G_AF, while W_CON had a higher concentration than W_AF and W_InactAF had the lowest value ($p < .01$). In BG silages, ethanol was lower in B_InactAF than in B_AF with B_CON as intermediate ($p < .01$). The NH_3-N concentration ranged between 20 and 55 g/kg total N and, apart from pure grass silages, CON treatments had lower concentrations than InactAF and AF ($p = .01$).

3.3 | Chemical composition and DM losses of silages prepared with RF

Table 4 presents the chemical composition and fermentation characteristics of silages treated with the RF additive. The aNDFom concentration of S_InactRF was lower compared to S_CON and S_RF ($p < .01$). Additionally, B_CON had more aNDFom compared to both B_InactRF and B_RF ($p < .01$). However, B_CON had lower ADFom concentrations compared to B_InactRF and B_RF ($p < .01$). Furthermore, an interaction for ADL showed that S_InactRF had lower values compared to S_CON ($p < .01$), The B_RF silages also had higher ADL levels compared to B_InactRF and B_CON. For hemicelluloses, B_CON had higher values than B_InactRF and B_RF ($p < .01$) and cellulose was higher for B_InactRF compared to B_CON with B_RF as intermediate ($p < .01$). Concerning DM losses, an interaction effect of additive showed that the addition of InactRF increased the losses compared to RF and CON in all silages ($p < .01$) with the greatest extent in WGF silages. Besides, a main effect of additive on EE concentration ($p = .04$) revealed no differences between treatments during the post-hoc test.

Regarding the fermentation characteristics, an interaction was found for acetic acid with S_RF showing higher values compared to S_CON ($p = .01$). For propionic acid, W_RF had higher numbers than W_CON, where this acid was not detected ($p < .01$). The ethanol concentration was higher in S_RF than in S_InactRF and S_CON, whereas W_CON had higher ethanol values than W_RF and W_InactRF ($p < .01$). Analysis of NH_3-N concentration showed a significant interaction ($p = .03$), but no differences were found in the post-hoc test. The main effect of additive, however, showed that CON was higher compared to InactRF ($p = .04$). Also, a main effect of additive was present for silage pH and RF treatment led to higher pH than InactRF, while CON was intermediate ($p = .03$). The lactic acid concentration was also higher for the InactRF treatment than for RF and CON ($p < .01$).

TABLE 3 Effect of anaerobic fungi supernatant on chemical composition and fermentation characteristics of silages prepared from drought-impaired grass solely or mixed with by-products.

	Treatment[a]													*p*-values	
	G_CON	G_InactAF	G_AF	S_CON	S_InactAF	S_AF	W_CON	W_InactAF	W_AF	B_CON	B_InactAF	B_AF	SEM[b]	Additive	Additive × silage
Chemical composition															
DM[c] concentration (g/kg)	359	342	351	356	343	355	356	367	351	365	356	360	12.2	.73	.92
Ash (g/kg DM)	86.8^{ab}	85.8^{bc}	91.0^{a}	82.2^{bcd}	81.6^{cde}	84.8^{bc}	74.8^{fg}	72.9^{fg}	77.7^{def}	76.8^{efg}	72.4^{g}	73.9^{fg}	1.01	<.01	.03
Crude protein (g/kg DM)	119	120	120	109	112	109	216	222	220	165	164	171	7.34	.76	.99
Ether extract (g/kg DM)	23.5	20.3	22.6	19.8	17.5	20.3	43.9	30.4	43.6	50.8	40.4	47.6	3.26	.01	.58
aNDFom[d] (g/kg DM)	563^{bc}	586^{bc}	552^{bcd}	542^{cde}	500^{efg}	510^{def}	487^{fg}	460^{gh}	440^{h}	636^{a}	579^{bc}	595^{ab}	7.28	<.01	<.01
ADFom[e] (g/kg DM)	360^{ab}	385^{ab}	411^{a}	335^{bc}	330^{bc}	366^{ab}	331^{bc}	283^{cd}	249^{d}	268^{d}	339^{bc}	383^{ab}	12.1	<.01	<.01
ADL[f] (g/kg DM)	73.0^{c}	76.7^{bc}	142^{a}	64.6^{c}	68.3^{c}	125^{a}	94.3^{b}	97.3^{b}	71.9^{c}	72.2^{c}	71.0^{c}	141^{a}	3.50	<.01	<.01
Hemicelluloses[g] (g/kg DM)	203^{bcde}	201^{bcde}	141^{e}	207^{bcd}	169^{cde}	144de	156^{cde}	178^{bcde}	191^{bcde}	368^{a}	241^{b}	212^{bc}	14.9	<.01	<.01
Cellulose[h] (g/kg DM)	287^{ab}	308^{a}	270^{ab}	271^{ab}	262^{ab}	241^{bcd}	237^{bcd}	185^{cd}	177^{d}	196^{cd}	268^{ab}	243^{bc}	11.7	.05	<.01
WSC[i] (g/kg DM)	54.8^{def}	45.6^{ef}	64.9^{cde}	80.5^{bcd}	59.4^{def}	34.8^{ef}	128^{a}	101^{ab}	94.1^{bc}	36.3^{ef}	43.6^{ef}	31.0^{f}	5.21	<.01	<.01
Silage fermentation characteristics															
DM loss (%)	3.63^{cd}	5.76^{ab}	3.66^{cd}	3.38^{d}	4.99^{bc}	3.35^{d}	3.56^{d}	7.13^{a}	3.51^{d}	3.65^{cd}	5.79^{ab}	3.91^{cd}	0.35	<.01	.02
pH	3.80^{bcdef}	3.79^{cdef}	3.85^{abcde}	3.73^{f}	3.75^{def}	3.86^{abcd}	3.80^{bcdef}	3.88^{abc}	3.96^{a}	3.86^{abcd}	3.74^{ef}	3.92^{ab}	0.02	<.01	.01
Lactic acid (g/kg DM)	81.9^{c}	136^{ab}	131^{ab}	108^{bc}	148^{a}	134^{ab}	119^{ab}	120^{ab}	117^{abc}	81.9^{c}	120^{ab}	102^{bc}	8.51	<.01	.02
Acetic acid (g/kg DM)	4.30^{e}	2.96^{e}	5.23^{de}	4.32^{e}	9.13^{cbd}	10.5^{bc}	4.08^{e}	10.3^{bc}	14.6^{a}	6.40^{cde}	3.41^{e}	10.5^{b}	1.03	<.01	<.01
Propionic acid (g/kg DM)	0.00	0.10	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.10	.58	.36
Butyric acid (g/kg DM)	n.d.[j]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	-	-
Ethanol (g/kg DM)	1.49^{cd}	7.67^{ab}	1.41^{cd}	1.93^{cd}	1.16^{cd}	1.73^{cd}	8.94^{a}	1.00^{cd}	6.73^{b}	1.51^{cd}	0.66^{d}	2.80^{c}	0.40	.02	<.01
Ammonia-N (g/kg total N)	29.3^{ef}	39.0^{cde}	41.3^{bcde}	25.9^{f}	42.3^{abcd}	44.5^{abc}	20.4^{f}	46.3^{abc}	54.8^{a}	31.1^{def}	48.0^{abc}	53.6^{ab}	2.31	<.01	.01

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).
[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (G), with sugar beet pulp (S), wheat gluten feed (W), or brewer's grains (B), and without an additive (CON), with heat-inactivated anaerobic fungi culture supernatant (InactAF), or with fresh anaerobic fungi culture supernatant (AF).
[b]Standard error of the mean.
[c]Dry matter.
[d]Neutral detergent fibre assayed with a heat stable α-amylase and expressed exclusive of residual ash.
[e]Acid detergent fibre expressed exclusive of residual ash.
[f]Acid detergent lignin.
[g]Calculated as aNDFom (g/kg DM) – ADFom (g/kg DM).
[h]Calculated as ADFom (g/kg DM) – ADL (g/kg DM).
[i]Water-soluble carbohydrates.
[j]Not detected.

TABLE 4 Effect of mixed ruminal fluid on chemical composition and fermentation characteristics of silages prepared from drought-impaired grass solely or mixed with by-products.

	Treatment[a]													*p*-values	
	G_CON	G_InactRF	G_RF	S_CON	S_InactRF	S_RF	W_CON	W_InactRF	W_RF	B_CON	B_InactRF	B_RF	SEM[b]	Additive	Additive × silage
Chemical composition															
DM[c] concentration (g/kg)	359	348	365	356	360	362	356	385	366	365	372	371	12.2	.72	.86
Ash (g/kg DM)	86.8	86.7	89.5	82.2	81.6	82.2	74.8	72.9	77.4	76.8	76.5	76.3	1.25	.06	.37
Crude protein (g/kg DM)	119	113	111	109	112	104	216	180	207	165	169	158	12.3	.36	.28
Ether extract (g/kg DM)	23.5	19.4	24.2	19.8	16.9	17.2	43.9	39.3	40.3	50.8	42.3	41.7	3.23	.04	.72
aNDFom[d] (g/kg DM)	563^{b}	555^{b}	551^{b}	542^{b}	475^{d}	528^{bc}	487^{cd}	458^{d}	458^{d}	636^{a}	572^{b}	560^{b}	7.99	<.01	<.01
ADFom[e] (g/kg DM)	360^{a}	356^{ab}	357^{a}	335^{abc}	301^{abcd}	332^{abc}	331^{abc}	294^{bcd}	275^{cd}	268^{d}	342^{ab}	336^{abc}	12.1	.98	<.01
ADL[f] (g/kg DM)	73.0^{bcd}	72.3^{bcd}	74.5^{bc}	64.6^{cd}	48.4^{e}	60.0^{de}	94.3^{a}	81.9^{ab}	82.6^{ab}	72.2^{bcd}	73.0^{bcd}	92.4^{a}	2.30	<.01	<.01
Hemicelluloses[g] (g/kg DM)	203^{bcd}	199^{bcd}	194^{bcd}	207^{bcd}	174^{bcd}	195^{bcd}	156^{d}	164^{cd}	183^{bcd}	368^{a}	229^{b}	224^{bc}	14.9	<.01	<.01
Cellulose[h] (g/kg DM)	287^{a}	283^{a}	282^{a}	271^{ab}	253^{abc}	272^{a}	237^{abcd}	213^{bcd}	193^{d}	196^{cd}	269^{ab}	244^{abcd}	11.7	.65	<.01
WSC[i] (g/kg DM)	63.7	57.9	54.8	73.9	74.6	80.5	121	145	128	26.0	40.4	36.3	8.12	.33	.48
Silage fermentation characteristics															
DM loss (%)	3.63^{c}	6.27^{ab}	3.33^{c}	3.38^{c}	5.06^{b}	3.53^{c}	3.56^{c}	6.90^{a}	3.36^{c}	3.65^{c}	5.04^{b}	3.60^{c}	0.20	<.01	<.01
pH	3.80	3.79	3.84	3.73	3.74	3.79	3.80	3.84	3.83	3.86	3.79	3.89	0.03	.03	.40
Lactic acid (g/kg DM)	81.9	124	102	108	146	121	119	120	117	81.9	120	93.1	8.56	<.01	.07
Acetic acid (g/kg DM)	4.31^{b}	3.53^{b}	4.52^{b}	4.33^{b}	7.62^{ab}	12.1^{a}	4.01^{b}	4.65^{b}	7.35^{ab}	6.43^{b}	4.68^{b}	7.53^{ab}	1.31	<.01	.01
Propionic acid (g/kg DM)	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}	0.20^{ab}	0.00^{b}	0.14^{ab}	0.31^{a}	0.00^{b}	0.20^{ab}	0.00^{b}	0.01	.01	<.01
Butyric acid (g/kg DM)	n.d.[j]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	-	-
Ethanol (g/kg DM)	1.43^{b}	1.96^{b}	3.01^{b}	1.96^{b}	1.21^{b}	7.40^{a}	8.94^{a}	1.03^{b}	1.84^{b}	1.55^{b}	1.39^{b}	1.60^{b}	0.63	<.01	<.01
Ammonia-N (g/kg total N)	29.3^{ab}	28.3^{abc}	22.3^{bcd}	25.9^{abcd}	23.1^{abcd}	27.9^{abc}	20.4^{cd}	17.9^{d}	20.7^{cd}	31.1^{a}	25.0^{abcd}	28.3^{abc}	1.55	.04	.03

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).

[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (G), with sugar beet pulp (S), wheat gluten feed (W), or brewer's grains (B), and without an additive (CON), with heat-inactivated mixed ruminal fluid (InactRF), or with fresh mixed ruminal fluid (RF).

[b]Standard error of the mean.

[c]Dry matter.

[d]Neutral detergent fibre assayed with a heat stable α-amylase and expressed exclusive of residual ash.

[e]Acid detergent fibre expressed exclusive of residual ash.

[f]Acid detergent lignin.

[g]Calculated as aNDFom (g/kg DM) − ADFom (g/kg DM).

[h]Calculated as ADFom (g/kg DM) − ADL (g/kg DM).

[i]Water-soluble carbohydrates.

[j]Not detected.

3.4 | Chemical composition and DM losses of silages with LAB

Table 5 presents the chemical composition and the fermentation characteristics of silages prepared with the LAB-based silage additive. The ADFom concentration was higher with the LAB than with the CON treatment for BG silages, but vice versa for WGF silages ($p < .01$). Regarding ADL, an interaction showed that W_LAB had lower concentrations compared to W_CON ($p < .01$). Higher cellulose concentrations were found for B_LAB than for B_CON, while the cellulose concentration was not affected by LAB in other silages ($p < .01$). Furthermore, aNDFom concentration was lower for all silages treated with LAB compared to CON ($p = .03$). In terms of hemicelluloses, B_CON had higher values than B_LAB ($p < .01$), while no differences were found between other silages. For WSC, the post-hoc test did not reveal differences although statistical analysis resulted in significant differences between additives.

Regarding the silage fermentation characteristics, pH values were 0.12 units lower in B_LAB than in B_CON ($p = .03$). Also, W_CON had higher ethanol concentrations compared to W_LAB ($p < .01$). Lactic acid was the most abundant fermentation acid and adding LAB to the silages resulted in higher concentrations compared to the CON treatment ($p < .01$), which was similarly observed for the acetic acid concentration ($p = .02$). Moreover, LAB treatment tended to cause lower NH_3-N values than CON ($p = .06$).

3.5 | Gas production kinetics of silages

The in vitro GP kinetics of silages are presented in Figure 2a–l. Among silages prepared without additives, S_CON had significantly lowest concentration of the soluble, immediately available fraction a ($p < .0.1$; Figure 2a), highest concentration of the insoluble but fermentable fraction b ($p < .01$; Figure 2b) and highest gas production rate c ($p < .01$; Figure 2c). Furthermore, GP rate was higher in W_CON than in B_CON with G_CON being intermediate.

Regarding silages treated with AF or InactAF, variable b was lower in W_CON than in W_InactAF, while W_AF was in between ($p < .01$; Figure 2e). Across all silages, variable a was higher for InactAF than for AF with CON as intermediate ($p = .05$; Figure 2d) and also the GP rate tended to be higher with InactAF treatment than for CON, while AF was intermediate ($p = .06$; Figure 2f). Within silages treated with RF or InactRF, variable a was higher for W_RF than for W_InactRF and W_CON ($p < .01$; Figure 2g), whereas no differences were observed in other silages. Supplementation of additive increased variable b in RF silages compared to CON silages with InactRF as intermediate ($p = .03$; Figure 2h). For variable c, no differences were observed in silages, despite a main effect of additive ($p = .05$; Figure 2i). For the LAB treatment, B_LAB had lower fraction a values than B_CON ($p < .01$; Figure 2j), whereas fraction b values were higher for W_LAB than for W_CON ($p < .01$; Figure 2k). The GP curves that were used to calculate all GP kinetic parameters are provided in Figures S1–S4.

3.6 | Microbial community composition

The data set comprised 2,705,360 sOTU after quality control and filtering out contaminants. The five most abundant genera were present in all analysed silages and accounted for around 94% of all present genera. These were *Lactobacillus* (67.5%), *Pediococcus* (15.0%), *Weissella* (6.18%), *Sphingomonas* (4.49%) and *Leuconostoc* (0.61%).

The mixed ensiling of grass with by-products strongly reduced all alpha diversity indices when compared to G_CON, while no differences were found for different by-products as co-substrates (each $p < .01$; Table 6). As illustrated in Table 7, the interaction of additive and silage substrate affected the number of observed sOTU and only showed differences between additives in pure grass silages ($p < .01$). Thereby, grass silages without an additive had most sOTU, whereas the addition of AF or RF reduced the number by around 80% and 40%, respectively. The same pattern was observed for the Fisher index ($p < .01$). For InvSimpson, index was again only affected in pure grass silages and lower for AF than CON ($p = .01$). Additionally, the Shannon index tended to be higher in G_CON than G_AF ($P = 0.06$). The main effect of additive revealed lowest Shannon index for AF, then RF and highest values in CON ($p < .01$).

The analysis of beta diversity is presented in Figure 3, showing that G_CON clustered separately from mixed silages along horizontal PCoA axis 1 ($p < .01$). Within the mixed silages, BG and SBP clustered apart along vertical PCoA axis 2 with WGF forming a slightly shifted cluster that still overlapped with SBP silages (Figures 3 and S5). The interaction of substrate and additive revealed that pure grass silages clustered separately from all other silages with no additive or RF, but AF treatment shifted the pure grass silages to the cluster of the other silages ($p < .01$). Additionally, it may be noted that one replicate of G_RF also shifted to the AF-treated silages.

The determination of differential abundances between control silages and AF-treated silages showed that 13 microbial genera were differently abundant (each $p \leq .05$; Figure 4) with two genera being higher abundant in AF-treated silages, that is, *Methanothermobacter* and *Pseudoclostridium*, while 11 genera were lower abundant in AF-treated silages, including the overall third most abundant genus *Weissella*. The addition of RF increased the abundance of *Enterococcus* when compared to CON ($p = .04$).

The Venn diagrams further showed that only 37 microbial genera (19.5%) were present in all silage types, while most genera (32.1%) were exclusive to pure grass silages (Figure S6). We found that nearly half of microbial genera were present with AF, RF and CON (95 genera or 45.2%). Further, majority of genera were shared between CON, AF and RF (on average 44 genera or 32.8%) and also many genera were shared between RF and CON, especially in pure grass silages, whereas the AF treatment caused a strong reduction in exclusive or shared microbial genera (Figure S7a–d). Additionally, the Spearman correlation coefficients-based heatmap showed that *Lactobacillus*, lactic acid, pH, NH_3-N and acetic acid were associated with reduced alpha diversity, whereas *Weissella* and all differently abundant genera were positively correlated with alpha diversity metrics (Figure S8).

TABLE 5 Effect of lactic acid bacteria on chemical composition and silage fermentation characteristics of silages prepared from drought-impaired grass solely or mixed with by-products.

	Treatment[a]									p-values	
	G_CON	G_LAB	S_CON	S_LAB	W_CON	W_LAB	B_CON	B_LAB	SEM[b]	Additive	Additive × silage
Chemical composition											
DM[c] concentration (g/kg)	359	357	356	350	356	354	365	360	12.2	.71	1.00
Ash (g/kg DM)	86.8	85.1	82.2	82.5	74.8	76.0	76.8	72.6	0.94	.20	.14
Crude protein (g/kg DM)	119	115	109	103	216	231	165	159	7.30	.97	.48
Ether extract (g/kg DM)	23.5	25.3	19.8	18.7	43.9	42.4	50.8	43.1	3.22	.31	.44
aNDFom[d] (g/kg DM)	563	574	542	507	487	477	636	579	14.4	.03	.12
ADFom[e] (g/kg DM)	360^{a}	372^{a}	335^{a}	343^{a}	331^{a}	266^{b}	268^{b}	347^{a}	12.1	.38	<.01
ADL[f] (g/kg DM)	73.0^{bc}	69.4^{bc}	64.6^{c}	61.6^{c}	94.3^{a}	80.1^{b}	72.2^{bc}	80.0^{b}	2.09	.12	<.01
Hemicelluloses[g] (g/kg DM)	203^{b}	202^{b}	207^{b}	164^{b}	156^{b}	211^{b}	368^{a}	232^{b}	14.9	.02	<.01
Cellulose[h] (g/kg DM)	287^{ab}	303^{a}	271^{ab}	282^{ab}	237^{bc}	186^{c}	196^{c}	267^{ab}	11.7	.20	<.01
WSC[i] (g/kg DM)	54.8	54.3	80.5	49.7	128	131	36.3	23.8	5.24	.05	.09
Silage fermentation characteristics											
DM loss (%)	3.63	3.35	3.38	3.30	3.56	3.22	3.65	3.45	0.17	.12	.92
pH	3.80^{ab}	3.78^{ab}	3.73^{b}	3.72^{b}	3.80^{ab}	3.81^{ab}	3.86^{a}	3.74^{b}	0.02	.03	.03
Lactic acid (g/kg DM)	81.9	108	108	123	119	124	81.9	101	8.53	<.01	.64
Acetic acid (g/kg DM)	4.32	4.71	4.31	12.8	4.08	4.02	6.45	13.8	2.94	.02	.15
Propionic acid (g/kg DM)	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.01	.33	.41
Butyric acid (g/kg DM)	n.d.[j]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-	-	-
Ethanol (g/kg DM)	1.47^{d}	0.49^{d}	1.98^{bcd}	3.95^{bc}	8.93^{a}	4.05^{b}	1.56^{cd}	0.37^{d}	0.42	<.01	<.01
Ammonia-N (g/kg total N)	29.3	27.1	25.9	24.1	20.4	19.9	31.1	25.3	1.50	.06	.53

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).

[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (G), with sugar beet pulp (S), wheat gluten feed (W), or brewer's grains (B), and without an additive (CON) or with lactic acid bacteria (LAB).

[b]Standard error of the mean.

[c]Dry matter.

[d]Neutral detergent fibre assayed with a heat stable α-amylase and expressed exclusive of residual ash.

[e]Acid detergent fibre expressed exclusive of residual ash.

[f]Acid detergent lignin.

[g]Calculated as aNDFom (g/kg DM) – ADFom (g/kg DM).

[h]Calculated as ADFom (g/kg DM) – ADL (g/kg DM).

[i]Water-soluble carbohydrates.

[j]Not detected.

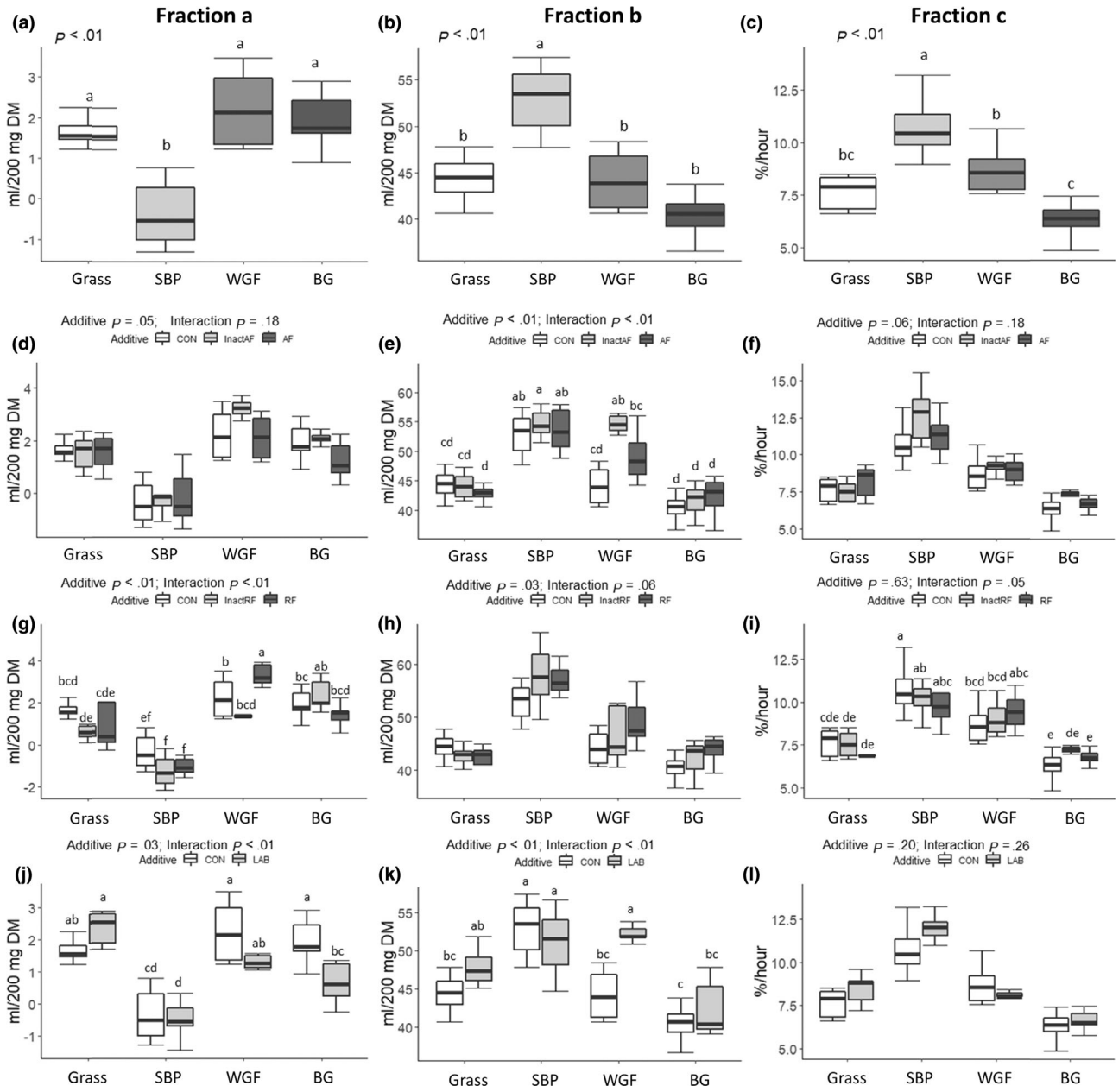

FIGURE 2 Boxplots illustrating the in vitro gas production (GP) kinetics of all silages. Thereby, (a–c) illustrate the data of silages prepared from drought-impaired grass solely (G_CON) or with sugar beet pulp (S_CON), wheat gluten feed (W_CON), or brewer's grains (B_CON). (d–f) illustrate the data of silages prepared with no additive (CON), fresh (AF) or heat-inactivated (InactAF) anaerobic fungi culture supernatant. (g–i) illustrate the data of silages prepared with no additive (CON), fresh (RF) or heat-inactivated (InactRF) mixed ruminal fluid. (j–l) illustrate the data of silages prepared with no additive (CON) or lactic acid bacteria (LAB). Boxplots on the left represent the initial GP from the soluble, immediately available substrate (fraction a), in the middle the GP from insoluble, fermentable substrate (fraction b), as well as the GP rate on the right (fraction c). Different superscript letters within the same figure indicate significant difference between least square means ($p \leq .05$).

4 | DISCUSSION

4.1 | Mixed silages of drought-impaired grass and by-products

We observed an overall satisfying silage fermentation with low pH, high lactic acid concentrations and no butyric acid in all silages. Consequently, this overall good silage quality was also reflected in low DM losses and seemed to refute our hypothesis that co-ensiling of drought-impaired grass with by-products would improve silage quality compared to ensiling solely drought-impaired grass. The silage pH ranged from 3.7 to 3.9 and was therefore indeed lower than typically found for grass silages. Likewise, lactic acid varied from 82 to 119 g/kg DM and thus was in the upper range for grass silages (Kung et al., 2018; Resch, 2021), which was presumably promoted by the high WSC concentrations in the initial substrates including molasses,

TABLE 6 Effect of mixed ensiling on alpha diversity metrics in silages prepared from drought-impaired grass solely or mixed with by-products.

	Treatment[a]					
	Grass	SBP	WGF	BG	SEM[b]	*p*-value
Observed sOTU[c]	297[a]	98.3[b]	77.7[b]	86.2[b]	26.3	<.01
Shannon	2.60[a]	1.36[b]	1.01[b]	1.24[b]	0.18	<.01
InvSimpson	7.41[a]	2.61[b]	2.06[b]	2.36[b]	0.64	<.01
Fisher	44.0[a]	11.75[b]	8.89[b]	9.91[b]	4.24	<.01

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).

[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (Grass), with sugar beet pulp (SBP), wheat gluten feed (WGF), or brewer's grains (BG).

[b]Standard error of the mean.

[c]Sub-operational taxonomic unit.

except for BG. Still, we observed differences in fermentation intensity as the BG silages had lower levels of lactic acid that, together with the buffering from BG-derived CP, may explain the higher pH values compared to other silages. The low acetic acid concentrations (<10 g/kg DM) indicated a predominantly homolactic fermentation, which has the greatest power for lowering silage pH and effectively inhibits other microorganisms in the silage, such as enterobacteria and clostridia (Pahlow et al., 2003). However, such low acetic acid concentrations could translate into a reduced aerobic stability and so be a disadvantage during the feed out phase, which may be followed up in future research.

The proximate nutrient analysis showed a steep increase in CP content by including BG and especially WGF during ensiling of the drought-impaired grass. Notably, these high CP concentrations of up to 215 g/kg DM were accompanied by low NH_3-N concentrations of ≤31 g/kg total N, meaning an excellent protein conservation in the silages and the provision of high amounts of true protein from both BG and WGF silages. This indeed confirmed our hypothesis that mixed ensiling with BG and WGF can increase the nutritive value of drought-impaired grass. Prior studies suggested that CP is not a nutrient that is strongly affected or may even increase by droughts (Dumont et al., 2015; Küsters et al., 2021; Sheaffer et al., 1992), but when considering the deficient CP concentration in the present drought-impaired grass (98.6 g/kg DM), it became obvious that drought-impaired grassland may by far not provide sufficient amounts of CP to lactating dairy cows or growing cattle. Taking into account the high ADL levels, in fact almost as high as in straw, an accelerated maturation of the crops seemed causative and unlike sugars, CP was the actual scarce nutrient in the present drought-impaired grass. Indeed, the G_CON silages had on average around five percentage points less CP compared to grass silages examined throughout Austria over the last 20 years (Resch, 2021), and also increments of lignin in response to drought conditions have been found in other grassland research, as well (Habermann et al., 2019). Therefore, the co-ensiling with BG and WGF is a promising approach to provide the ruminant's need for nitrogenous compounds from silages. The high CP concentrations in BG and WGF silages are also remarkable as such high levels are usually only realized in legume silages, which, however, bear a considerable risk for butyric acid malfermentation and intensive protein breakdown during storage, even when supplying rapidly fermentable carbohydrates (Hartinger et al., 2019).

Regarding the practical implementation, mixed ensiling may be especially applicable for the very moist by-product BG that inherently needs prompt conservation and is generally very prone to spoilage and mycotoxin contaminations if ensiled alone (Marston et al., 2009; Penagos-Tabares et al., 2022), while WGF is commonly available in dry form. Likewise, SBP could be obtained in fresh form to be used as a suitable co-substrate, which would omit the energy-intense and so costly drying process of SBP. In this context, we also emphasize that drought-impaired grass should not be air-dried before ensiling, but may indeed be directly ensiled with co-substrates to not foil applicability of mixed ensiling by overstraining labour resources and time. In the present study, the grass was intermediately air-dried to reach well-defined and controlled ensiling conditions to allow comparability between treatments in the present experiment. Consequently, while this reservation is acknowledged, our research still reliably proofs the successful feasibility of the concept of mixed ensiling to valorise drought-impaired grass. Further experiments should now evaluate ensiling of the treatments tested herein in larger silos and under on-farm conditions.

The comparably high EE concentration observed in WGF and BG silages should derive from the high EE concentrations in WGF and BG, especially BG showed a EE concentration of 101 g/kg DM that is about 20 g higher than usually found (Universität Hohenheim, 1997). However, it must be acknowledged that the present BG batches were obtained from only one local brewery and comparably high in EE, so variation between breweries may be taken into account.

The in vitro GP data, which served as an indicator for rumen degradability, showed that the GP kinetics of WGF silages did not differ from those of pure grass silages, and BG silages showed the lowest GP of all silages, which was also true for the GP rate. Thus, CP-rich mixed silages did not perform as good as expected during the in vitro incubations. Presumably, the high CP concentration itself may be part of the explanation since protein fermentation only yields low gas amounts (Getachew et al., 1998), which may have negated the actually high WSC levels in WGF silages. Furthermore, the relatively high EE levels in BG silages should have also contributed to this, as its fermentation results in marginal GP (Getachew et al., 1998) and, in the

TABLE 7 Main effect of silage additive and interaction on alpha diversity metrics in silages prepared from drought-impaired grass solely or mixed with by-products.

	Treatment[a]											p-values		
	G_CON	G_AF	G_RF	S_CON	S_AF	S_RF	W_CON	W_AF	W_RF	B_CON	B_AF	B_RF	SEM[b]	Additive	Additive × silage
Observed sOTU[c]	484^{a}	102^{c}	305^{b}	119^{c}	73.0^{c}	103^{c}	88.8^{c}	47.0^{c}	97.3^{c}	74.5^{c}	86.8^{c}	97.3^{c}	35.5	<.01	<.01
Shannon	3.39^{a}	1.78^{bc}	2.64^{ab}	1.67^{c}	0.93^{dc}	1.47^{c}	1.51^{c}	0.47^{d}	1.07^{cd}	1.33^{cd}	1.12^{cd}	1.27^{cd}	0.21	<.01	.06
InvSimpson	11.3^{a}	3.82^{bc}	7.15^{ab}	3.25^{bc}	1.72^{c}	2.87^{bc}	2.91^{bc}	1.27^{c}	2.00^{c}	2.41^{c}	2.29^{c}	2.37^{c}	1.01	<.01	.01
Fisher	75.6^{a}	12.4^{c}	44.2^{b}	14.5^{c}	8.16^{c}	12.6^{c}	10.2^{c}	5.00^{c}	11.4^{c}	8.38^{c}	9.98^{c}	11.4^{c}	5.75	<.01	<.01

Note: In each row, different superscript letters indicate significant difference between least square means ($p \leq .05$).
[a]Silages prepared with different substrates, that is, drought-impaired grass ensiled either alone (G), with sugar beet pulp (S), wheat gluten feed (W), or brewer's grains (B), and without an additive (CON), with fresh anaerobic fungi culture supernatant (AF), or with fresh mixed ruminal fluid (RF).
[b]Standard error of the mean.
[c]Sub-operational taxonomic unit.

present concentrations, can be harmful for the rumen microbiota and impair rumen fermentation processes (Getachew et al., 1998; Maccarana et al., 2016). On the other hand, SBP inclusion boosted the in vitro GP, showing highest potential gas production, that is, the sum of GP from the soluble, immediately available substrate and from insoluble, fermentable substrate, plus highest GP rate of all silages and this may have several reasons. First, SBP is known for its high concentration of NFC, especially pectin, which is very rapidly fermented (Villalba et al., 2021). Second, the addition of SBP in dairy diets can enhance the fibre digestibility of all components (Münnich et al., 2018) and third, the lignin concentration was lowest for SBP silages. Consequently, our hypothesis of an elevated energy density with SBP as co-substrate was confirmed. In terms of further practical implementation, SBP may not be needed in dried form, as was the case in the present study, but fresh SBP could be used as a co-substrate and thus, the energy for the drying process can be saved.

In addition to providing dietary energy and nutrients, grass silages are an essential source of structure in the diet, needed to maintain a healthy forestomach system. As physically effective fibre is a combination of chemical and physical properties, peNDF$_{>8mm}$ is commonly analysed (Zebeli et al., 2008) and recommended (GfE, 2023). In our study, the peNDF$_{>8mm}$ concentrations ranged from 310 g/kg DM in SBP silages to 464 g/kg DM in BG silages. The highest value found for BG silages was presumably associated with the high aNDFom concentrationof BG, that is, 633 g/kg DM. Therefore, the physical effectiveness of such apparently high peNDF$_{>8mm}$ values would need backup by future feeding trials. Nevertheless, all silages provided sufficient physically effective fibre to be used without restriction in a balanced ruminant diet and the recommended minimum peNDF$_{>8mm}$ concentration for typical dairy cow diets of 185 g/kg DM (GfE, 2023; Zebeli et al., 2012) is feasible with all of the present silages.

4.2 | Additional application of silage additives

Regarding the impact of the AF treatment, one of the most important findings was the highest lactic acid concentration in all silages with AF addition that confirmed our hypothesis. It is conceivable that additional sugars released during fungal fibre degradation in the silos were metabolized into lactic acid. Surprisingly, the effect was also present for the InactAF treatment, so clarification is needed regarding the mode of action as the active components were not present in the heat-inactivated additive and such effects have not been observed in our previous studies (Hartinger et al., 2022; Hartinger et al., 2024). In contrast to these findings, the pH was not as low as in CON silages, which is unexpected because lactic acid predominantly contributes to the pH decline in silages (Kung et al., 2018) and also disagrees with previous findings on AF-treated grass silages (Hartinger et al., 2022). In terms of CP preservation, protein degradation was significantly higher in WGF silages with AF that may come from fungal proteases, which are introduced via the AF additive (Hartinger et al., 2018). However, this should not be over interpreted as the highest NH_3-N concentration (55 g/kg total N) was still

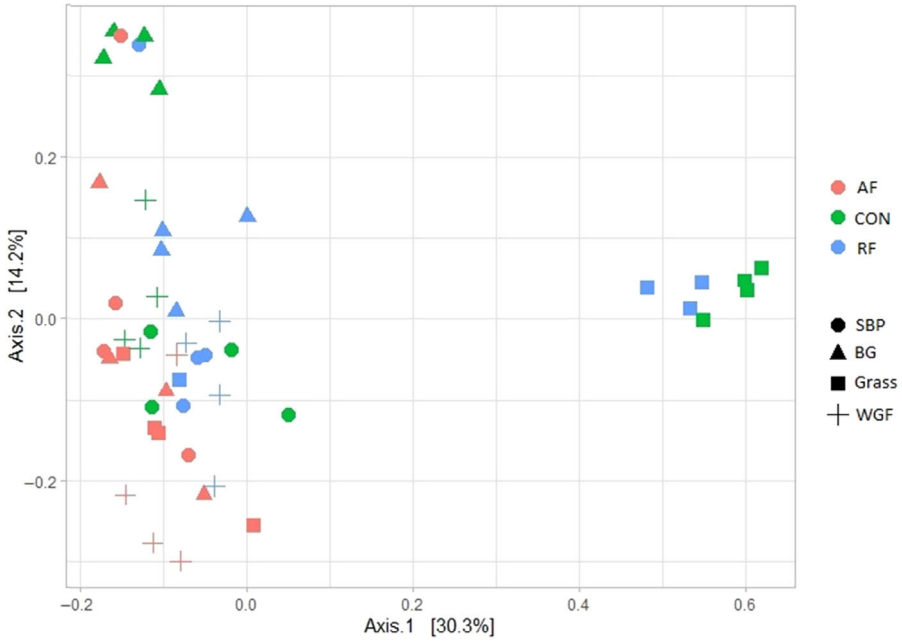

FIGURE 3 Changes in microbial community composition associated with different silage substrates and additives visualiszed as a principal co-ordinate analysis using weighted UniFrac distance metrics. The percentage of variation explained is indicated on the respective axes.

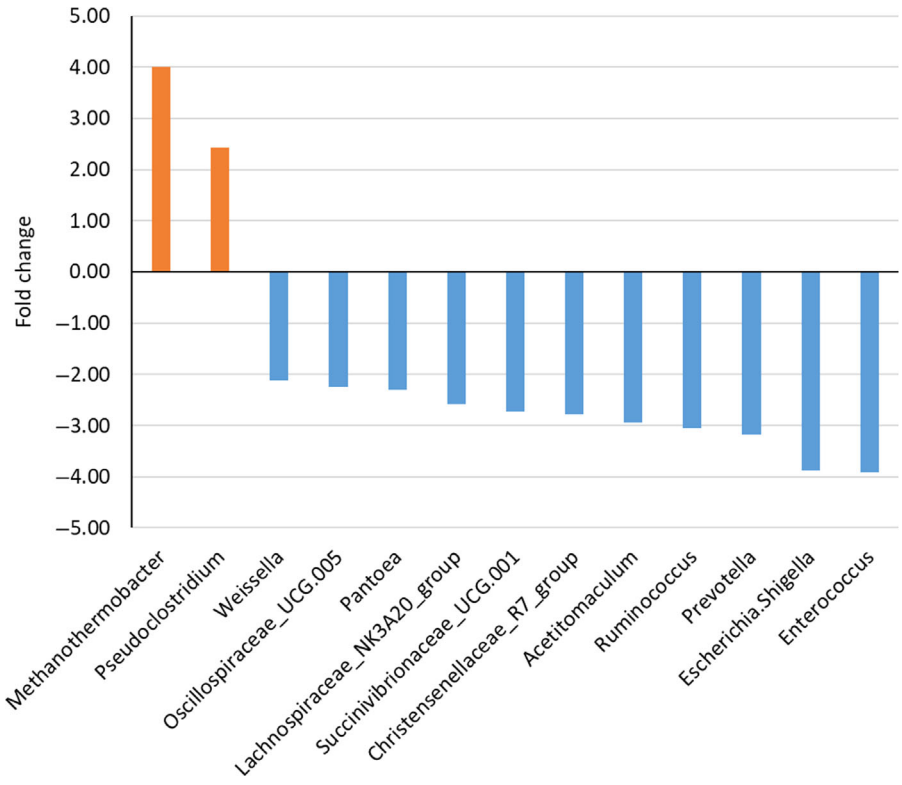

FIGURE 4 Differential abundances of microbial genera that were significantly affected by treatment with fresh anaerobic fungi culture supernatant with control silages serving as benchmark.

considerably below the threshold for sufficient silage protein quality of 100 g/kg total N (Kung et al., 2018). Another interesting finding was related to the fibre composition, in which the AF treatment indeed showed remarkable reductions of hemicelluloses in BG silages and of cellulose and lignin in WGF silages, so confirming our hypothesis of a fibre-cleaving effect mainly for WGF silages. Especially the cellulose but also lignin degradation could derive directly from fungal enzymes (Hagen et al., 2021; Lankiewicz et al., 2023), while the reduction in hemicelluloses could also be indirect from a stronger acid hydrolysis (Dewar et al., 1963) due to more lactic acid with the AF treatment. As similar reduction effects on fibre composition have also been observed when medium-quality grass was ensiled with AF in lower concentrations, thst is, 100 g/kg DM (Hartinger et al., 2022), it can be concluded that the higher dosages used in our study (250 g/kg DM) may not be necessarily needed to exert fibre-cleaving effects during ensiling, which eventually also means lower application costs.

Notably, these alterations in the fibre structure were not strongly reflected in in vitro GP, meaning only a numerically improved rumen degradability of W_AF compared to W_CON silages, whereas W_InactRF actually showed a higher in vitro GP than W_CON.

Similarly to AF, RF can also provide a large number of fibre-cleaving enzymes and metabolically active microbes that together can efficiently degrade plant cell wall structures (Li et al., 2020). However, a consistent pattern regarding the influence of RF on structural carbohydrates was not found. Nevertheless, we observed a significant increase in in vitro GP in response to RF addition when compared to CON silages, which was particularly pronounced in silages with by-product inclusion. In terms of silage fermentation characteristics, the InactRF treatment enhanced the lactic acid fermentation, whereas the addition of fresh RF led to more acetic acid. The latter can be interpreted as beneficial since overall, the silage pH was sufficiently low and more acetic acid would thus provide more protection from yeast metabolism during the feed out phase (Danner et al., 2003). However, treatment with InactRF substantially increased the DM losses by about three percentage points compared to fresh RF and CON silages, thus being an explicit disadvantage of this treatment. This observation was not expected since the InactRF treatment led to the highest lactic acid concentrations and a coherent explanation is consequently lacking. It may be speculated that dead microbial cells and their debris present in the InactRF additive may have been inefficiently metabolized by other microbes or simply corroded in the silage, consequently causing higher DM losses instead of contributing to substrate preservation like metabolically active microbes would have done. It should be noted that the composition and activity of the rumen microbiome can hardly be standardized, as well as it can harbour potential pathogens (Khafipour et al., 2011), which together may exclude the possibility of RF to directly become a silage additive in practice. Still, our research provides first orientation on how specific constituents of RF, such as enzymes, could act as silage additives and thus encourages further investigations.

The addition of LAB as well improved the silage fermentation characteristics as evidenced by significantly lower pH in LAB-treated silages, especially in B_LAB, along with less ethanol and higher lactic acid concentrations. The latter may be mainly associated with the homofermentative LAB present in the commercial additive. Therefore, our hypothesis of a stronger lactic acid fermentation and pH drop upon addition of LAB was confirmed. Interestingly, we also observed a higher acetic acid concentration in S_LAB and B_LAB silages that could be attributed to *Lactobacillus paracasei*, which can switch between homo- and heterofermentative metabolism (Makras et al., 2005). The B_LAB silages had lower hemicelluloses but more cellulose than B_CON silages, which may be associated with an acid hydrolysis due to more lactic acid in silages (Dewar et al., 1963) and consequently a relative increase in cellulose. Nevertheless, these changes had no impact on in vitro GP of BG silages. In contrast, W_LAB silages had a higher in vitro GP that could be related to the reductions in cellulose and lignin.

4.3 | Microbial community in selected mixed silages

The predominant genera of the microbial communities in our silages were typical genera found in silages (Ávila & Carvalho, 2020; Eikmeyer et al., 2013). Indeed, the three most abundant genera were *Lactobacillus*, *Pediococcus* and *Weissella*, together accounting for nearly 90% of sequences. These are all lactate producers and therefore represent desirable microorganisms, essential for a rapid acidification and stable conservation (Pahlow et al., 2003). As the dominance of these three genera was independent of the treatments, it is consistent with our findings of overall high lactic acid and low silage pH, although undetected changes at species level are possible (Hartinger et al., 2020).

Still, differences in the microbial community structure between silages were present. Hereby, all co-substrates reduced microbial diversity without differences between by-products and pure grass silages harboured a microbial community with the highest diversity and richness. Similarly, silage additives showed an analogous effect when comparing CON silages to silages treated with AF or RF. Hereby, the AF treatment led to a significant reduction in diversity and richness, significant changes in beta diversity structure, and reductions of several high and low abundant genera. It therefore seems that this additive could possess to some extent bactericidal properties that has not been yet described and deserves deeper investigation in the future. Interestingly, these substantial differences in the microbial community structure between silages were marginally reflected in the concentrations of microbial metabolites, such as fermentation acids, which may be explained by the functional redundancy found in microbial communities of silages (Langer et al., 2015). The RF addition, however, only caused minor changes in the microbial community strcuture, which was surprising as we expected significant shifts due to the introduction of various new microbes from the complex rumen ecosystem.

5 | CONCLUSIONS

Our study demonstrated that mixed ensiling of drought-impaired grass with agro-industrial by-products substantially enhances the nutritive value of the silages by increasing the otherwise low CP concentration and lowering concentrations of fibrous fractions, but without compromising the silage fermentation quality. The additional treatment with the silage additives AF, RF and LAB all intensified the lactic acid fermentation in the silages, while improvements in rumen degradability were only sparsely detected for certain treatments. Although co-substrates as well as the addition of silage additives, especially fresh AF, shifted the microbial community structure, this did not adversely affect the silage quality and lactate producers remained dominant in all silages. Thus, mixed ensiling can enhance the resilience of ruminant livestock production, helping farmers to adapt to drought conditions by optimizing the use of local feed resources, especially when using moist by-products as co-substrates.

Considering the overall satisfying conservation of the mixed silages, the further application of silage additives can in parts develop beneficial effects but seems not compelling for successful substrate preservation and high ruminal degradability.

ACKNOWLEDGEMENTS

The authors thank AGRANA Beteiligungs-AG, Vienna, Austria, for providing the sugar beet pulp and wheat gluten feed, as well as Schwechater Brauerei, Brau Union Österreich AG, Linz Austria, for providing the brewer's grains and H. Wilhelm Schaumann GmbH, Pinneberg, Germany for the provision of the lactic acid bacteria-based silage additive. The authors further acknowledge the work of A. Dockner, S. Sharma, S. Leiner, A. Sener and M. Hollmann from the Institute of Animal Nutrition and Functional Plant Compounds at the University of Veterinary Medicine Vienna during the chemical analyses of samples, as well as L. Štrosová from the Institute of Animal Physiology and Genetics, Czech Academy of Sciences, for the AF culture supernatant preparation. Furthermore, we would like to thank Dr. Andreas Schaumberger from the Institute of Plant Production and Cultural Landscape, Agricultural Research and Education Centre Raumberg-Gumpenstein, Irdning-Donnersbachtal, Austria for providing the drought-impaired forage.

FUNDING INFORMATION

This study was funded by the Austrian Federal Ministry of Agriculture, Forestry, Regions and Water Management, Grant Number 101623.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The sequences are deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the accession number PRJNA1026084. The R codes as well as the data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Theresa Gruber https://orcid.org/0009-0009-6813-0550
Katerina Fliegerová https://orcid.org/0000-0001-9439-8168
Georg Terler https://orcid.org/0000-0001-8881-6407
Qendrim Zebeli https://orcid.org/0000-0001-5188-9004
Thomas Hartinger https://orcid.org/0000-0001-5709-0500

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SUPPORTING INFORMATION

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How to cite this article: Gruber, T., Fliegerová, K., Terler, G., Resch, R., Zebeli, Q., & Hartinger, T. (2024). Mixed ensiling of drought-impaired grass with agro-industrial by-products and silage additives improves the nutritive value and shapes the microbial community of silages. *Grass and Forage Science*, 1–19. https://doi.org/10.1111/gfs.12669