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Effect of multispecies fungal extract supplementation on growth performance, nutrient digestibility, ruminal fermentation, and the rumen microbiome composition of beef cattle fed forage-based diets

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Abstract

Our objective was to evaluate the effect of a multispecies fungal extract (**MFE**) on growth performance, apparent total tract digestibility (**ATTD**), fermentation characteristics, and rumen microbiome composition of beef cattle fed forage-based diets. For experiment 1, ruminally cannulated Angus × SimAngus cows ($n = 4$; body weight [**BW**] = 569 ± 21 kg) were used in a randomized crossover design with two 21-d study periods and a 23-d washout period to evaluate the effect of dietary inclusion of an MFE on in situ digestion, ruminal fermentation, and the composition of the rumen microbiome. Treatments consisted of a forage-based diet with or without the inclusion of a MFE. Rumen samples were collected on days 5, 10, and 20. Experiment 2 evaluated different inclusion rates of the MFE in a randomized complete block design using Angus × SimAngus-crossbred steers ($n = 80$; BW = 370 ± 44 kg). Steers were blocked by BW and randomly assigned to one of four treatments (2 pens/treatment): diet with no MFE, 0.02%, 0.04%, and 0.08% of the MFE (dry matter [**DM**] basis). Steers were fed a forage-based diet for 122 d. Subsets of 10 steers/treatment were randomly selected for the determination of ATTD on d 20, 40, and 60. All data were analyzed using the MIXED procedure of SAS. In exp 1, adding the MFE to the diet tended to increase the ruminal disappearance rate of the DM on day 10 ($P = 0.06$). No interactions or treatment effects were observed for the short-chain fatty acid profile of the rumen fluid ($P \geq 0.13$). Metagenomic analysis of the rumen microbiome showed an MFE × d interaction for the *Fibrobacter* genus ($P = 0.01$), which on day 20 was less abundant in the rumen of cows fed the MFE. In exp 2, steers supplemented with 0.04% of MFE had a lower average daily gain and were lighter at the end of the experiment (cubic, $P \leq 0.04$) compared to steers supplemented with 0.02% MFE. Steers fed the diet with 0.02% of MFE had the greatest gain-to-feed ratio among the MFE-supplemented groups (cubic, $P < 0.01$). Dietary inclusion of the MFE increased neutral detergent fiber digestibility (linear, $P = 0.05$). Steers supplemented with 0.04% of MFE had the greatest acid detergent fiber digestibility among treatments (quadratic, $P = 0.03$). Collectively, results showed that ruminal disappearance rate and digestibility of forage-based diets increased due to MFE supplementation, but did not translate into growth performance improvements or beneficially alter rumen fermentation.

Lay Summary

Enhancing the digestibility of fibrous feeds from cattle diets will benefit the productivity, efficiency, and sustainability of beef cattle operations. These experiments aimed to evaluate the effect of a multispecies fungal extract (**MFE**) on fiber digestibility and ruminal fermentation; and how these might be associated with growth performance in beef cattle. Diets (forage-based) were offered for ad libitum intake with or without the inclusion of an MFE. Growth performance, nutrient digestion, rumen metabolites, and changes in the rumen microbiome composition were measured over time. Overall, adding an MFE to forage-based diets offered to beef cattle transiently increased the disappearance rate of the diet dry matter from the rumen without modifying the rumen microbiome composition or fermentation characteristics. Total tract digestibility of the fibrous fraction of the diet was improved by the MFE but did not translate into growth performance improvements.

Key words: apparent total tract digestion, beef cattle, multispecies fungal feed additive, rumen microbiome

Abbreviations: ADF, acid detergent fiber; ADG, average daily gain; ATTD, apparent total tract digestibility; ASV, amplicon sequence variants; BW, body weight; CP, crude protein; G:F, gain to feed ratio; DM, dry matter; DMI, dry matter intake; MFE, multispecies fungal additive; NDF, neutral detergent fiber; SCFA, short-chain fatty acids

Introduction

Strategies that effectively enhance the digestibility of fibrous feeds are not only necessary for improving the productivity and efficiency of the beef industry but have also become a relevant approach for methane emission mitigation (Vijn et al., 2020). From the numerous dietary interventions proposed to increase fiber digestibility, fungal-derived fibrolytic enzymes (**FFE**) have received significant attention. Nevertheless, the inconsistent growth performance results observed in previous trials that

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evaluated the effect of including FFE in cattle diets have discouraged this technology from being extensively adopted at a farm level (Beauchemin et al., 1999; Meale et al., 2014). For example, including FFE to diets with varying forage sources improved growth performance of beef cattle (Beauchemin et al., 1995; McAllister et al., 1999). However, other researchers working under similar dietary scenarios were not able to achieve positive responses when supplementing FFE to beef cattle (ZoBell et al., 2000; Eun et al., 2009). Although unclear why discrepancies are present in the scientific literature, it has been primarily attributed to the substrate-specific nature of the fibrolytic enzymes as well as the heterogeneity of experimental conditions (Beauchemin et al., 2004). Alternatively, fungal extracts containing cells and fermentation products in addition to fibrolytic enzymes have not been adequately investigated. Furthermore, most studies that assessed the impact of supplementing beef cattle with fungal extracts fed an *Aspergillus oryzae*-derived extract (Kreikemeier et al., 1997; Podversich et al., 2023). Recently, we evaluated the inclusion of a multispecies fungal extract (**MFE**; *Aspergillus oryzae*, *Aspergillus terreus*, *Trichoderma reesei*, and *Trichoderma viride*) to a forage-based diet at 0.04% of the diet dry matter (**DM**) and observed growth performance improvements of beef cattle (Pittaluga and Relling, 2023). Despite rumen degradation kinetics and apparent total tract digestibility (**ATTD**) not being previously measured, based on the literature (Newbold 1997; Beauchemin et al., 2004), adding the MFE to the diet might increase the digestion rate of feed particles in the rumen by leveraging the hydrolytic capacity of the rumen microbiome and increasing dry matter intake (**DMI**) as a consequence. However, the precise mechanisms that explain the observed results by Pittaluga and Relling (2023) are yet to be elucidated. Therefore, the objectives of this study were to evaluate the impact of dietary inclusion of an MFE on growth performance, nutrient digestibility, fermentation characteristics, and rumen microbiome composition of beef cattle fed forage-based diets. Our hypotheses were that including MFE to forage-based diets 1) increases the ruminal disappearance rate of the fibrous fraction of the diet and alters the composition of the rumen microbiome of beef cattle, and 2) improves the growth performance of beef steers.

Materials and Methods

All experimental procedures were approved by the Institutional Animal Care and Use committee of The Ohio State University (#2019A00000112-R1) and followed the guidelines recommended in the Guide for the Care and Use of Agricultural Animal in Agricultural Research and Teaching (FASS, 2010).

Experiment 1

The experiment was conducted at the Beef Research Center at the Wooster campus of The Ohio State University (Wooster, OH). To evaluate the effect of dietary inclusion of a MFE on in situ digestion, fermentation characteristics, and rumen microbiome composition, ruminally cannulated (n = 4; body weight [**BW**] = 569 ± 21 kg) Angus × SimAngus nonlactating, nonpregnant, mature cows were used in a randomized crossover design with two 21-d study periods. The two 21-d study periods were separated by a 23-d washout period to minimize carryover effects of treatments. Treatments consisted of a forage-based diet (Table 1) offered for ad libitum intake with (**WFE**) or without (**CON**) the inclusion of an MFE. The MFE (Biopremix Technologies LLC., Wilmington, DE) contained 1.65×10^9, 1.85×10^9, 2.50×10^9, 1.20×10^9 colony forming units/g of *Aspergillus oryzae*, *Aspergillus terreus*, *Trichoderma reesei*, and *Trichoderma viride*, respectively. The fungal extract was included at 0.04% of the diet DM following the manufacturer's recommendations and based on previous results (Pittaluga and Relling, 2023), where it was first incorporated into a mineral-vitamin premix and then mixed with the rest of the feed ingredients. Cows started consuming the forage-based diet 30 d prior to the beginning of the experiment. Subsequently, on day 1 of the first 21-d study period, the MFE began to be included in the basal diet. Cows were housed in individual pens (2.6 × 3.0 m) consisting of concrete slatted floors, with two 1.5-m long concrete feed bunks and ad libitum access to clean and fresh water. Cows were fed once daily at 0900 h.

Rumen in situ disappearance of DM, neutral detergent fiber (**NDF**), and acid detergent fiber (**ADF**) were determined by following the procedures described by Ciriaco et al. (2021). Briefly, dried and ground samples of the forage-based diet (2 mm) were weighed (10 g) into 10 × 20 cm Ankom in situ bags (R1020, Ankom Technology Corp., Macedon, NY) with a pore size of 50 ± 10 µm. The sample size to free bag surface area ratio was 50 mg/cm^2. Bags were heat-sealed and placed in duplicates (two bags/cow/incubation time) in zippered mesh bags attached to a rope and carabiner and soaked in warm (39 °C) water for 10 min to simulate the addition of saliva. Subsequently, mesh bags were incubated in the ventral sac of the rumen for 0 (water-washed, but not incubated in the rumen), 4, 8, 12, 24, and 36 h on three different instances (days 5 to 6, 10 to 11, and 20 to 21) throughout each 21-d study period. All bags were placed at the same time immediately before the morning feed. After removal from the rumen, all bags were immersed in ice water to halt fermentation,

Table 1. Ingredients and analyzed nutrient content of the diet fed to the ruminally cannulated beef cows

Item	
Ingredient, % of DM[1]	
DDGS[1]	5.000
Soy hulls	15.000
Corn silage	30.000
Grass hay	45.000
Supplemental Premix[2]	5.000
Analyzed composition, % of DM	
CP[1]	10.86
NDF[1]	48.13
ADF[1]	30.93
EE[1]	1.88
Ash	8.41

[1]Abbreviations: DM, dry matter; DDGS, dry distiller's grains with solubles; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extractable.
[2]7.50% urea, 50.702% soybean meal, 12.00% limestone, 14.00% NaCl, 14.00% dicalcium phosphate, 0.725% Se, 0.110% $CuSO_4$, 0.240% $ZnSO_4$, 0.176% $MnSO_4$, 0.002% $CoCO_3$, 0.140% vitamin A-30, 0.200% vitamin D-3, 0.003% vitamin E, 0.002% EDDI, 0.2% MGA 200 (Zoetis, Parsippany-Troy Hills, NJ).

subsequently rinsed with cold running tap water to remove adherent particles and bacteria, and stored at −80 °C for further analysis. At the end of the experiment, bags were thawed and then rinsed in a domestic washing machine using a cool-wash regular cycle (15 min at 30 °C) without soap. Rinsed bags were dried for 48 h at 55 °C and weighed to determine the remaining DM. After weighing, residues from ruminal incubations were composited by incubation time within cow and analyzed for DM, NDF, and ADF to estimate the ruminal disappearance rate of each fraction. The content of NDF and ADF were analyzed using an ANKOM 200 fiber analyzer (ANKOM Technology Corporation) according to the ANKOM Technology methods 5 and 6, respectively.

On day 20 of each 21-d study period, samples of ruminal fluid were collected at 0 (baseline), 4, 8, and 12 h after the morning feed to analyze the content and profile of short-chain fatty acids (**SCFA**). A representative 350 mL sample of ruminal fluid was collected from the cranial, mid, and caudal section of the ventral sac and strained through four layers of cheesecloth. A 10 mL sample of the strained ruminal fluid was placed into a 15-mL conical tube that contained 0.1 mL of a 20% (vol/vol) H_2SO_4 solution to immediately halt fermentation and preserve the SCFA. Samples were immediately placed on ice and stored at −80 °C for further analysis. At the end of the experiment, the concentrations of SCFA in the ruminal fluid samples were determined in a water-based solution using ethyl acetate extraction. After centrifuging the ruminal fluid samples for 10 min at 10,000 × *g*, the supernatant was mixed with a 5:1 (vol/vol) solution of meta-phosphoric and crotonic acid and frozen at −80 °C until analysis. On the day of the analysis, samples were thawed and centrifuged at 4 °C for 10 min at 10,000 × *g* and the supernatant was transferred into glass tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. Tubes were then vortexed, and the ethyl acetate fraction was transferred to vials. The content of SCFA was analyzed by gas chromatography (HP1850 series gas chromatography Hewlett-Packard, Wilmington, DE) on a glass column as described by Roman-Garcia et al. (2021) with modifications from Mitchell et al. (2023).

To analyze changes in the composition of the rumen microbiome, samples of ruminal solid material were collected immediately after removing the bags corresponding to each 24-h incubation time point. Samples of ruminal solid material were collected from the cranial, mid, and caudal section of the ventral sac and strained through four layers of cheesecloth. A 5 mL sample of the strained ruminal fluid was placed into a 15-mL conical tube that contained 3.0 mL of TRIzol reagent (95% 200 proof molecular grade ethanol, 5% Tri Reagent). Additional 2.0 mL of ruminal fluid was subsequently added by squeezing the samples of ruminal solids above the tube to obtain a sampling that represents the microbiome of the ruminal contents of the animal. The solution was homogenized by inverting the 15.0 mL conical tube several times, placed on ice, and subsequently stored at −80 °C for further DNA extraction. At the conclusion of the experiment, genomic DNA was extracted from aliquots (0.5 mL) of rumen fluid samples following the procedures described by Yu and Morrison (2004) and using the MagAttract PowerSoil DNA KF Kit (Qiagen, Hilden, Germany). Frozen DNA samples (≥200 ng for each sample) were submitted to Novogene (Novogene Co, Beijing, China) for the bacterial sequencing of the 16S rRNA gene (V4 region with primers 515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT) and fungal internal transcribed spacer region 1 (ITS1 with primers ITS5-1737F: GGAAGTAAAAGTCGTAACAAGG and ITS2-2043R: GCTGCGTTCTTCATCGATGC). The DNA sample quality check, PCR amplification, and library preparation were performed using standard procedures as outlined by the manufacturer. Amplicon sequencing by synthesis was conducted on an Illumina platform (NovaSeq PE250, Illumina Inc.) producing on average 93,500 (bacteria) and 62,800 (fungi) paired-end-reads of 250 bp per sample.

All statistical analyses were conducted using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC) and considering the animal as experimental unit. Ruminal disappearance rate of DM, NDF, and ADF were analyzed as repeated measurements. The model was fitted with individual animal data and included the MFE supplementation, incubation time, sampling day, and their interactions as fixed effects, as well as the animal as a random effect. Due to the lack of interactions ($P > 0.10$) with the main effects in the model and the minor relevance to the trial objectives, the period and incubation time effects are not reported. The most appropriate covariance structure was chosen as having the lowest Akaike Information Criterion. The ones tested were compound symmetry, composite symmetry, unstructured, autoregressive, and self-correcting structures. The first-order autoregressive covariance structure was used for the analysis of DM, NDF, and ADF in situ disappearance rate. The SCFA profile was analyzed as repeated measurements, and the model included MFE supplementation, sampling time, and their interaction as fixed effects, as well as the random effect of the animal. The LS-means were separated using the PDIFF and SLICE option of SAS. Differences were considered at $P \leq 0.05$; and tendencies at $P > 0.05$ and $P \leq 0.10$.

De-multiplexed and primer-adapters removed amplicon paired-end reads supplied by Novogene were processed using the R package DADA2 (Callahan et al., 2016). The reads underwent quality checking and filtering using parameters to remove any sequence with N bases, shorter than 50 bp or with a maximum expected error (EE = sum(10^(-Q/10))) (REF:) of 2 for each read in the pair. Merging overlapping forward and reverse reads (250 bp) to obtain full denoised sequences was done allowing 0 mismatches and a minimum overlapping length of 12 bp.

Amplicon sequence variants (**ASV**) were inferred and the abundance of each ASV across the samples was calculated after removing chimera sequences. Taxonomic assignment of ASV was performed using the Silva database (version 138.1) for bacterial 16S rRNA and the UNITE ITS database (version 9) for fungal ITS. To account for varying depths of coverage across samples, data were normalized using the cumulative sum scaling method implemented in the metagenomeSeq R package using a scaling factor of 0.5. Good's coverage was calculated to ensure that all samples reached a minimum value of 0.97. Effects of the treatment, sampling day, and their interaction on the bacterial and fungal composition of the rumen microbiome were estimated using the permutational multivariate analysis of variance (Vegan package). After grouping the ASV at the genus level and retaining only the most abundant taxa (>5%), differences in abundance of bacterial and fungal ASV between treatments were calculated using the edgeR package. Significance was

set at $P \leq 0.05$; and tendencies were determined at $P > 0.05$ and $P \leq 0.10$.

Experiment 2

The experiment was conducted at the Eastern Agricultural Research Station of The Ohio State University (Belle Valley, Noble County, OH). Eighty Angus × SimAngus-crossbred steers (10 steers/pen) with an initial BW of 370 ± 44 kg were used in a randomized complete block design to evaluate the inclusion rate of an MFE on growth performance and nutrient digestibility. Steers were blocked by initial BW and randomly assigned to 1 of 4 treatments (two pens/treatment). Treatments consisted of a grass hay-based diet (Table 2) offered for ad libitum intake during 112 d without the inclusion of MFE (**CON**) or with the inclusion of an MFE at 0.02% (**2MFE**), 0.04% (**4MFE**), and 0.08% (**8MFE**) of the diet DM. The MFE was first incorporated into the mineral-vitamin premix and then mixed with the rest of the feed ingredients from the grass hay-based diet. Pens were fed twice daily at 0900 and 1300 h. The diet was formulated to exceed requirements of vitamins and minerals for an estimated average daily gain (**ADG**) of 1.0 kg/d (NASEM, 2016).

After weaning, steers were fed soybean hulls and grass hay for 45 d, and subsequently group fed 3 to 3.5 kg/hd/d of a backgrounding diet (60% ground corn, 10% soybean meal, 28% soybean hulls, and 2% animal-vegetable blend fat) and ad libitum access to grass hay for 130 d. After a 7-d transition period, steers were offered the grass-hay based diet for ad libitum intake and began for a 21-d adaptation period to the GrowSafe bunks (GrowSafe, GrowSafe Systems Ltd., Airdrie, AB, Canada), after which the experiment was initiated. Steers were weighed on 2 consecutive days at the beginning and at the end of the experiment, and at 28-d intervals throughout the experimental period. Each weighing was performed before the morning feeding and without withholding steers from feed or water.

Steers were housed in pens (7.3 × 37.2 m) that included an area covered by a metal roof (7.3 × 8.5 m) and an outside loafing area (7.3 × 28.6 m). The flooring material under the covered space was comprised of crushed, compacted limestone (screenings), and the outside loafing area was concrete. Pens were divided by a 1.5 m high wood fence with a 10 cm separation between rectangular rails. Each pen contained 2 GrowSafe bunks (0.91 m ×0.53 m ×0.38 m). Each GrowSafe bunk allowed only one animal to eat at a time and recorded individual feed intake daily based on an electronic ear tag.

Table 2. Ingredients and analyzed nutrient content of the diet fed to beef steers

Item	
Ingredient, % of DM[1]	
DDGS[1]	14.000
Soy hulls	16.000
Grass hay	60.000
Supplemental Premix[2]	10.000
Analyzed composition, % of DM	
CP[1]	14.93
NDF[1]	55.77
ADF[1]	37.20
EE[1]	1.56
Ash	8.89

[1]Abbreviations: DM, dry matter; DDGS, dried distiller's grains with solubles; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extractable.
[2]5.07% urea, 60.615% soybean meal, 11.41% limestone, 10.14% NaCl, 0.44% Se, 0.05% $CuSO_4$, 0.121% $ZnSO_4$, 0.011% $MnSO_4$, 0.003% $CoCO_3$, 0.087% vitamin A-30, 0.125% vitamin D-3, 0.004% vitamin E, 0.004% EDDI, 11.92% Av fat blend.

A subset of 10 steers (five per pen) from each treatment were randomly selected for determination of ATTD of DM, crude protein (**CP**), NDF, and ADF. Beginning on days 20, 40, and 60, feed and fecal samples were collected on three consecutive days. Fecal samples were collected from the rectum once daily at 0700, 1100, and 1500 hours on the first, second, and third day, respectively, of each collection period. Samples of feed ingredients were collected twice daily after the morning and afternoon delivery. Feed and fecal samples were frozen immediately after collection and stored at −20 °C for further analysis. At the conclusion of the experiment, feed and fecal samples were analyzed for DM by oven-drying (48 h at 55 °C) and ground through a Wiley mill (1 mm screen, Arthur H. Thomas, Philadelphia, PA). Equal amounts of feed samples were composited within treatment, and equal amounts of feces were composited within steer to determine the concentration of nutrients and digestibility marker. This was based on the assumption that no feed sorting occurred in the GrowSafe bunks. Indigestible NDF (**iNDF**) was used as the digestibility marker.

Determination of NDF and ADF followed the same procedures described previously in the materials and methods section of experiment 1. The concentration of iNDF in feed and feces was determined following the procedures described by Schulmeister et al. (2020). Briefly, 0.5 g of samples were weighed into Ankom F57 filter bags and then incubated at 39 °C using a 4:1 ratio of McDougall's buffer: ruminal fluid in a Daisy^{II} incubator (Ankom Technology Corporation) for 288 h for complete digestion of potentially digestible NDF. After incubation, samples were rinsed and then analyzed for NDF as previously described. Total nitrogen was analyzed using a LECO TruMac N Nitrogen Determinator (LECO Corporation, St. Joseph, MI) according to the AOAC method (AOAC, 1997; #990.03). The content of CP was calculated as N × 6.25. To calculate organic matter (**OM**), we first determined the ash content by ignition of samples at 600 °C for 2 h using a Thermolyte muffle oven Model F30420C (Thermo Scientific, Waltham, MA) according to the AOAC method (AOAC, 2005; #942.05). The ATTD of DM, OM, CP, NDF, and ADF were calculated as suggested by Ciriaco et al. (2022): using nutrient as an example:

$$\text{Nutrient digestibility (\%)} = 100 - \left\{100 \times \left[\left(\frac{\text{feed iNDF, \%}}{\text{feces iNDF, \%}}\right) \times \left(\frac{\text{feces nutrient, \%}}{\text{feed nutrient, \%}}\right)\right]\right\}$$

All statistical analyses were conducted using the MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Growth performance parameters and ATTD of nutrients were analyzed as a randomized complete block design with pen as the experimental unit. For the growth performance parameters, the model was fitted with individual animal data and included the MFE supplementation as a fixed effect, and the random effects of the BW block and pen within the BW block. The initial BW was used as a covariate to account for differences in the final

BW. For the ATTD of nutrients, the model included the MFE supplementation as a fixed effect, and the BW block and animal within the BW block as random effects. Mean treatment differences were separated using the polynomial (linear, quadratic, and cubic) contrast procedures. The LS-means were separated using the PDIFF option of SAS for those outcomes with quadratic and cubic effects. Differences were considered at $P \leq 0.05$; and tendencies at $P > 0.05$ and $P \leq 0.10$.

Results

Experiment 1

In situ digestion and ruminal fermentation

No three-way interactions were detected ($P \geq 0.68$; Table 3) for the ruminal disappearance rates of DM, NDF, and ADF. For DM disappearance, there was a tendency for MFE supplementation × sampling day interaction ($P = 0.06$). On day 10, adding the MFE to the diet increased the ruminal disappearance rate of the diet DM compared with CON. No other interactions were detected ($P \geq 0.98$) for the ruminal disappearance rate of the DM. For the NDF and ADF fractions of the diet, no interactions or treatment effects were detected ($P \geq 0.16$). Because there were no MFE supplementation × sampling time interactions for the SCFA analysis ($P \geq 0.24$; Table 4) and because of the minor relevance to the trial's objectives, the effect of sampling time was not reported. No treatment differences were observed for the total concentration of SCFA or the molar proportions of each SCFA in the rumen fluid ($P \geq 0.13$). In addition, there was no difference in the acetate:propionate ratio due to treatment ($P = 0.26$).

Rumen microbiome composition

The differential abundance analysis (CON:MFE) of the important ASV was summarized at the genus level for the bacterial (Figure 1) and fungal community (Figure 2). For the bacterial community of the rumen microbiome, no MFE supplementation × sampling d interaction was detected ($P = 0.46$), except for the *Fibrobacter* genus ($P = 0.01$), which on day 20 was less abundant (−3.74 Log2-fold change) in the rumen content of cows fed the MFE. The abundance of Ruminobacter at day 5 (1.799), Treponema at day 20 (−1.53) and Prevotella (day 5, 0.44 to day 20, −0.27) were not different ($P \geq 0.22$, Figure 1). Dietary inclusion of the MFE did not alter the differential abundance of ASV ($P \geq 0.34$) The differential abundance of bacterial community tended to fluctuate throughout the study with the greatest differences observed on day 10 ($P = 0.06$).

For the fungal community of the rumen microbiome, no MFE supplementation × sampling day interaction was detected ($P = 0.46$). Adding the MFE to the diet did not alter the differential abundance of ASV ($P \geq 0.12$) compared with CON. Additionally, the differential abundance of the ASV

Table 3. Effect of MFE supplementation on DM, NDF, and ADF in situ disappearance (%) after different incubation times in the rumen of ruminally cannulated beef cows

	Treatment[1]									
	Day 5		Day 10		Day 20			*P*-value[3]		
Item	CON	MFE	CON	MFE	CON	MFE	SEM[2]	Trt	d	Trt × d
n	4	4	4	4						
DM										
4	27.95	26.49	25.97[a]	28.82[b]	25.22	26.88	1.13			
8	27.74	29.70	27.49[a]	30.70[b]	28.66	27.38	1.13			
12	30.97	32.86	29.38[a]	32.34[b]	32.04	31.29	1.10	0.07	0.94	0.06
24	45.31	46.66	41.49[a]	48.80[b]	47.23	44.03	1.11			
36	54.57	54.91	54.85[a]	57.65[b]	56.13	56.61	1.18			
NDF[4]										
4	30.79	28.68	29.24	29.98	29.42	31.86	3.79			
8	29.12	31.40	31.21	31.32	32.54	31.64	3.80			
12	31.85	33.33	39.09	31.25	34.80	33.97	3.78	0.79	0.16	0.67
24	44.44	45.76	42.73	46.92	49.22	45.47	3.79			
36	55.20	54.36	49.69	57.90	58.79	57.70	3.81			
ADF[5]										
4	23.49	22.89	22.33	21.98	22.64	23.75	2.39			
8	21.90	25.02	24.62	23.64	26.55	24.03	2.40			
12	25.79	27.66	32.92	24.85	30.29	26.34	2.36	0.97	0.25	0.39
24	39.28	40.59	35.85	41.64	43.34	39.08	2.40			
36	51.23	49.90	44.49	53.78	55.83	54.85	2.41			

[1]CON = no inclusion of MFE in the diet; MFE = inclusion of MFEin the diet.
[2]Pooled standard error of treatments means.
[3]Trt = treatment; It = incubation time; d = sampling day; Trt × d = interaction between the treatment and day.
[4]NDF.
[5]ADF.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).
Significance was declared at $P \leq 0.05$; and tendencies were declared at $P > 0.05$ and $P \leq 0.10$.

Table 4. Effect of MFE supplementation on total SCFA and the molar proportions of SCFA in the rumen fluid of ruminally cannulated beef cows

	Treatment[1]			
Item	CON	MFE	SEM[2]	*P*-value
n	4	4		
SCFA, mol/100 mol				
Acetate	51.6	50.2	2.2	0.65
Propionate	16.5	15.3	1.0	0.43
Butyrate	9.8	8.9	0.7	0.29
Isobutyrate	0.73	0.68	0.06	0.13
Isovalerate	0.59	0.54	0.05	0.34
Valerate	1.22	1.16	0.11	0.68
Total SCFA, m*M*	81.2	77.6	3.7	0.51
Acetate:propionate	3.2	3.4	0.1	0.26

[1]CON = no inclusion of MFE in the diet; MFE = inclusion of MFE in the diet.
[2]Pooled standard error of treatments means.
Significance was declared at $P \leq 0.05$; and tendencies were declared at $P > 0.05$ and $P \leq 0.10$.

changed ($P < 0.01$) throughout the study with the greatest differences observed on day 5 for Penicillium (0.75 fold-change) and for Cyllamyces (–0.66 fold-change).

Experiment 2

Dietary inclusion of the MFE resulted in a cubic change ($P \leq 0.04$; Table 5) of the final BW and ADG. Compared to 2MFE steers, 4MFE steers had a lower ADG during the 112-d feeding period and were lighter at the end of experiment but all MFE treatments did not differ from CON. No other differences were observed for the ADG and final BW between treatments. Supplementing steers with the MFE resulted in a cubic change ($P < 0.01$) of the gain-to-feed ratio (G:F), where steers fed the diet where 2MFE steers had the greatest G:F among the MFE-supplemented groups, which all did not differ from CON. Additionally, dietary inclusion of the MFE did not affect the DMI of steers ($P \geq 0.37$). For the ATTD of nutrients, adding the MFE to the diet led to a tendency for a quadratic increase in NDF digestibility (linear, $P = 0.05$; quadratic, $P = 0.09$; Table 6) with no differences being observed between the MFE-supplemented groups. The ATTD of the ADF fraction, adding the MFE to the diet led to a tendency for a cubic increase in ADF digestibility (quadratic, $P = 0.03$; cubic, $P = 0.07$) where steers supplemented with MFE at 0.04% had the greatest ADF digestibility among dietary treatments. No linear, quadratic, or cubic responses to MFE supplementation were observed for the ATTD of DM and CP ($P \geq 0.48$).

Discussion

Feeding diets whereby the energy is predominantly supplied by structural carbohydrates, limits growth performance of beef cattle due to the relatively low digestibility of the complex polymers found in the plant cell wall. Furthermore, rumen distension-based decreases in DMI as a result of a restricted fractional rate of passage of digesta from the rumen (Allen, 1996; Villalba et al., 2009) seems to be the primary constraint to growth performance of cattle consuming forage-based diets. Since ruminal pool size accelerates digesta flow from the rumen (Firkins, 2021; Firkins and Mitchell, 2023), and the fractional rate of passage from the rumen strongly decreased the extent of ruminal degradation (Firkins, 1997), dietary interventions aimed to enhance fiber digestibility might improve growth performance by promoting greater DMI (Church and Kellems, 1998). Beauchemin et al. (1995), investigated the effect of including FFE in diets with varying forage sources and reported increases in DMI and ADG of beef cattle when feeding either alfalfa or timothy hay. Similarly, McAllister et al. (1999) added FFE to a barley silage-based diet and observed growth performance improvements of beef cattle by increasing DMI. Krueger et al. (2008) fed cattle a Bermudagrass hay-based diet and reported that incorporating an FFE in the diet increased DMI but had no impact on growth performance. Other researchers working with dairy cattle also reported a greater DMI as a result of dietary inclusion of fungal feed additives. Lewis et al. (1999) fed a total mixed ration based on alfalfa hay and silage to midlactation Holstein cows and reported that including FFE in the diet enhanced DMI and improved lactational performance. However, ZoBell et al. (2000) observed no results in feed intake or growth performance of beef cattle when including FFE to a corn silage and alfalfa hay-based diet. In agreement, Eun et al. (2009) reported no differences when investigating the effect of dietary inclusion of FFE to a corn silage-based diet on growth performance of beef steers. While the precise mechanism by which FFE might promote DMI remains unclear, it has been suggested that the fibrolytic enzymes might increase the digestion rate of feed particles in the rumen by leveraging the hydrolytic capacity of the rumen microbiome and increasing feed intake as a consequence (Beauchemin et al., 2004). More specifically, FFE might facilitate the attachment of cellulolytic bacteria to feed particles in the rumen through direct hydrolysis of the surface structure or by chemotactic attraction (Newbold 1997; Morgavi et al., 2004). Consequently, the flow of digesta from the rumen to the lower tract is accelerated and the rumen distension-based DMI limitation is alleviated. However, faster passage rates might counteract potential increases in the extent of digestion due to a briefer exposure of particles to microbial digestion. Faster degradation rates of feed particles in the rumen without alterations of the extent of digestion have been reported for in situ trials (Feng et al., 1996; Hristov et al., 1996; Pinos-Rodríguez et al., 2002; Chaucheyras-Durand et al., 2012).

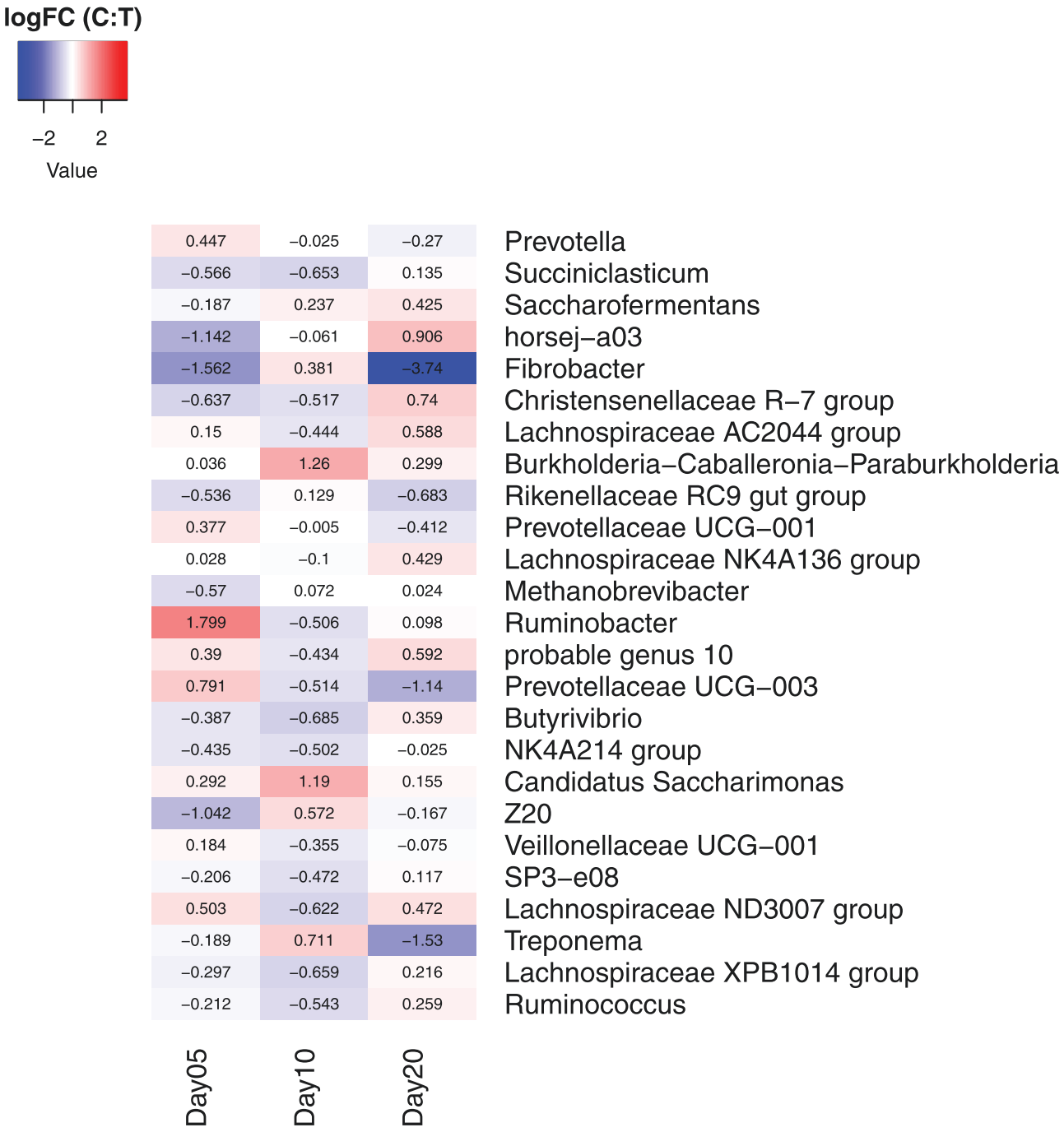

Figure 1. The differential abundances of ASV on the bacterial community of the rumen content of beef cows fed a forage-based diet with (WFE) or without (CON) the inclusion of an MFE. Based on their overall differential abundance profile (positive change vs. negative change), ASV were grouped at the genus level retaining only the most abundant taxa (>5%). Log2-fold changes represent the mean logarithm abundance difference of CON:MFE at the ASV level. There was a tendency for an MFE supplementation × day interaction ($P = 0.06$) on the bacterial community structure of the rumen microbiome, where on day 20, adding the MFE to the diet decreased the relative abundance (Log2-fold change) of the *Fibrobacter* genus.

In contrast to FFE, fungal extracts containing cells and fermentation products in addition to fibrolytic enzymes have not been extensively studied, particularly in beef cattle. Few studies that investigated the impact of supplementing beef cattle with fungal extracts added an *Aspergillus oryzae*-derived extract to sorghum silage or corn stalks-based diets and could not demonstrate significant benefits (Kreikemeier et al., 1997; Podversich et al., 2023). However, Podversich et al. (2023) reported feed efficiency improvements when including the *Aspergillus oryzae*-derived extract to a byproduct-based diet suggesting a diet-dependent effect. In the present experiment, we aimed to shed light on the mode of action of a MFE. Based on the DMI-driven improvements on growth performance of beef cattle previously observed by Pittaluga and Relling (2023) when adding an MFE to a forage-based diet, it seemed reasonable to assume that, similar to what was suggested for FFE (Newbold 1997; Beauchemin et al., 2004), the fibrolytic enzymes contained in the MFE might enhance the hydrolytic capacity of the rumen microbiome and accelerate the

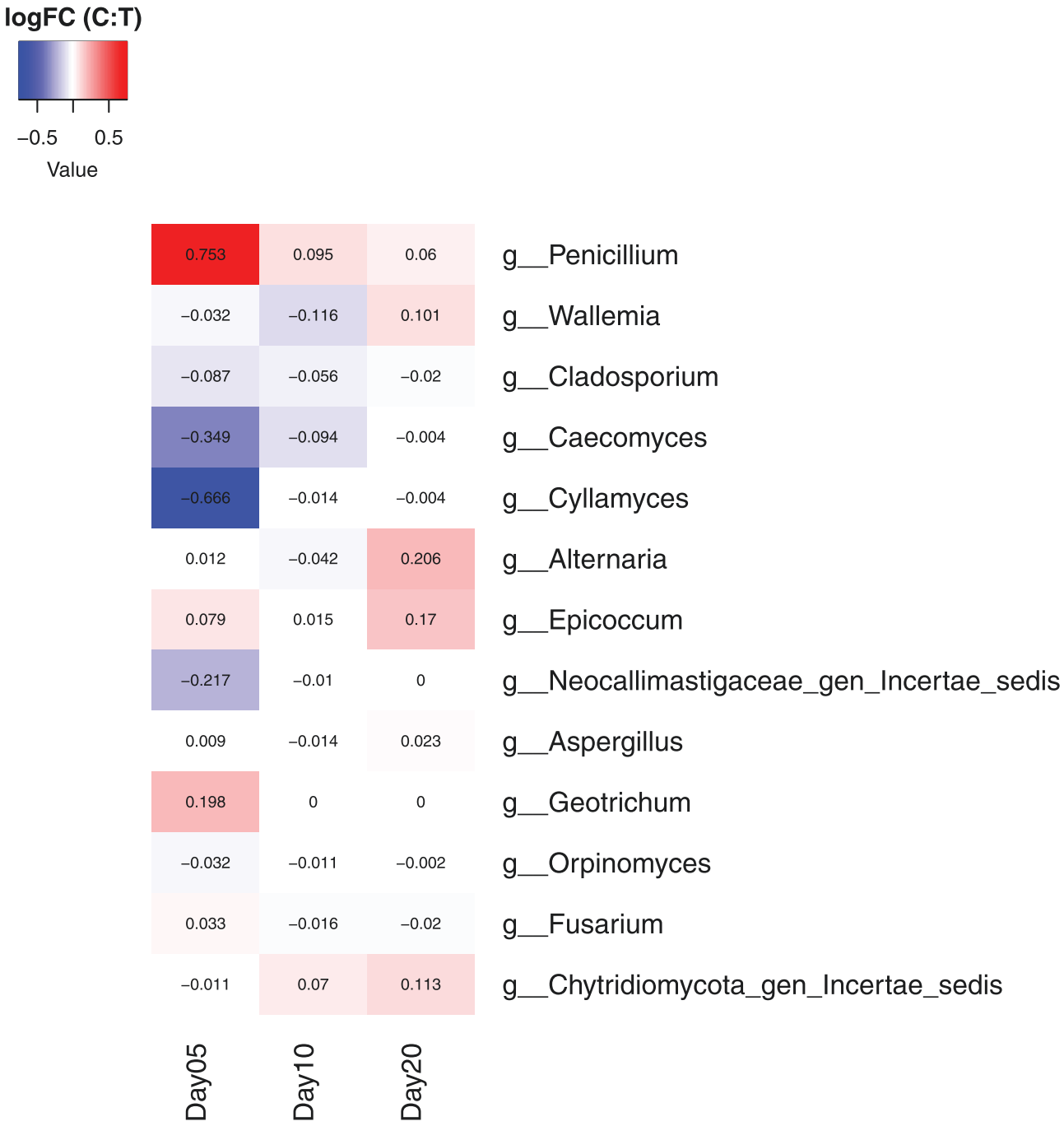

Figure 2. The differential abundances of ASV on the fungal community of the rumen content of beef cows fed a forage-based diet with (WFE) or without (CON) the inclusion of an MFE. Based on their overall differential abundance profile (positive change vs. negative change), ASV were grouped at the genus level retaining only the most abundant taxa (>5%). Log2-fold changes represent the mean logarithm abundance difference of CON:MFE at the ASV level.

ruminal disappearance rate of the diet. Nonetheless, growth factors in the MFE could also promote the growth of cellulolytic bacteria. The increased supply of growth factors like B vitamins and branched chain volatile fatty acids has been identified as partially responsible for increases in the activity and growth rates of ruminal cellulolytic bacteria when evaluating fungal extracts (Beharka and Nagaraja, 1998). For reasons that are not clear to the authors, the MFE only increased the disappearance rate of the diet DM on d 10. Although microbial activity was not measured, the lack of differences in the relative abundance of most bacterial genera and all fungal genera suggests that MFE supplementation does not seem to alter the rumen fibrolytic activity by changing the composition of the rumen microbiome. The absence of treatment differences on the total content and molar proportions of SCFA in the rumen fluid agrees with data reported from previous trials that evaluated the effect of adding fungal extracts to forage-based diets (Caton et al., 1993; Kreikemeier et al., 1997). In the current experiment, because samples of rumen fluid for SCFA analysis were collected on day 20, when there were no differences in feed degradability, it seems reasonable to interpret that modifications in the total content and profile of SCFA should not be anticipated.

Table 5. Effect supplementing beef steers with varying doses of an MFE on growth performance

	Treatment[1]					*P*-value		
Item	CON	2MFE	4MFE	8MFE	SEM[2]	Linear	Quadratic	Cubic
n	20	19	20	20				
IBW[3], kg	371	372	374	365	36	0.47	0.45	0.80
FBW[4], kg	460[a,b]	466[b]	453[a]	460[a,b]	7	0.61	0.49	0.03
ADG[5], kg/d	0.79[a,b]	0.85[b]	0.73[a]	0.79[a,b]	0.04	0.52	0.57	0.04
DMI[6], kg/d	9.32	9.26	9.35	9.56	0.31	0.37	0.68	0.86
G:F[7]	0.086[a,b]	0.092[a]	0.078[b]	0.083[b]	0.004	0.17	0.59	<0.01

[1]CON = no inclusion of MFE in the diet; MFE = inclusion of multispecies fungal feed additive at 0.02, 0.04, and 0.08% of the diet DM.
[2]Pooled standard error of treatments means.
[3]Initial BW.
[4]Final BW.
[5]ADG.
[6]DMI.
[7]Gain to feed ratio.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).
Significance was declared at $P \leq 0.05$; and tendencies were declared at $P > 0.05$ and $P \leq 0.10$.

Table 6. Effect supplementing beef steers with varying doses of an MFE on apparent total tract digestibility

	Treatment[1]					*P*-value		
Item	CON	2MFE	4MFE	8MFE	SEM[2]	Linear	Quadratic	Cubic
n	10	10	10	10				
Digestibility, %								
DM	53.2	52.4	52.5	52.3	0.7	0.45	0.60	0.66
NDF	41.6[a]	43.2[b]	43.7[b]	43.6[b]	0.6	0.05	0.09	0.74
ADF	45.1[a]	45.4[a]	47.9[b]	46.0[a]	0.7	0.24	0.03	0.07
CP	54.2	53.6	55.2	54.3	1.5	0.82	0.77	0.48

[1]CON = no inclusion of MFE in the diet; MFE = inclusion of MFE in the diet.
[2]Pooled standard error of treatments means.
[3]Initial BW.
[4]Final BW.
[5]ADG.
[6]DMI.
[7]Gain to feed ratio.
[a,b]Within a row, means without a common superscript differ ($P \leq 0.05$).
Significance was declared at $P \leq 0.05$; and tendencies were declared at $P > 0.05$ and $P \leq 0.10$.

In a previous experiment performed by our lab (Pittaluga and Relling, 2023), we reported that dietary inclusion of an MFE to a forage-based diet at 0.04% of the diet DM enhanced DMI and ADG of beef cattle without modifying feed efficiency. In the current study, for reasons that are yet to be elucidated, including the same MFE to a similar forage-based diet did not improve the growth performance of beef steers. Moreover, we did not expect that steers supplemented with the lowest dose of MFE (0.02%) were going to be more efficient than the remaining MFE-supplemented groups. Possibly, MFA doses above 0.02% of the diet DM might have been high enough to create competition with the rumen microbial enzymes for binding sites on feed particles, as has already been reported for FFE (Beauchemin et al., 2004). Improvements in NDF and ADF digestibility due to MFE supplementation evidenced herein agrees with previous trials (Beharka and Nagaraja, 1993; Podversich et al., 2023). However, such improvements in the ATTD digestibility of the fibrous fraction of the diet were probably not enough to elicit growth performance improvements relative to CON.

Conclusions

Dietary inclusion of an MFE to a forage-based diet transiently increased the DM disappearance rate from the rumen, which diminished as the experiment progressed. Most bacterial genus and all fungal community of the rumen microbiome were not modified due to MFE supplementation. However, intriguingly, relevant fibrolytic bacteria from the genus *Fibrobacter* decreased on day 20 when adding the MFE to the diet. Fiber digestibility was improved as a result of MFE supplementation but was not enough to elicit growth performance improvements of beef steers fed forage-based diets. To further elucidate the mode of action of fungal extracts, more research is needed to determine optimal inclusion rates and evaluate alternative biological mechanisms by which these additives might benefit productivity and efficiency of the beef industry.

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Conflict of interest statement

Alejandro M. Pittaluga, Florencia E. Miccoli, Leandro D. Guerrero, and Alejandro E. Relling have no conflicts of interest to declare.

Author Contributions

Alejandro Pittaluga (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing—original draft), Florencia Miccoli (Data curation, Methodology, Writing—review & editing), Leandro Guerrero (Data curation, Formal analysis, Methodology, Software, Writing—review & editing), and Alejandro Relling (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision)

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