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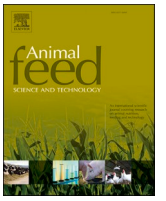

Impact of adding tannins or medium-chain fatty acids in a dairy cow diet on variables of *in vitro* fermentation using a rumen simulation technique (RUSITEC) system

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ABSTRACT

This work aimed to evaluate the effect of including 2 functional feed ingredients in a diet with a high proportion of pasture silage on in vitro ruminal fermentation, nutrient disappearances, and ruminal microbiomes. In a rumen simulation technique (RUSITEC) system (6 fermentation units), 4 treatments were evaluated: 1) CONTROL: a diet based on corn and grass silage, regular soybean meal (SBM), and corn; 178 g/kg of crude protein (CP) (used as negative control), 2) TSBM: Replacement of SBM by Mervobest® (used as positive control), 3) PHY (phytogenic mixture (tannins from quebracho and chestnut) at 10 g/kg dry matter (DM) replacing SBM) and 4) MCFA (mixture of medium-chain fatty acids, Aromabiotic Cattle® at 10 g/kg DM replacing SBM). Four runs were performed with 3 diets incubated simultaneously in duplicate using a balanced incomplete block design, lasting 17 days each. The inoculum was obtained from 3 rumen fistulated cows, and artificial saliva was infused at 650 mL/day. Samples were taken from each fermentation unit to determine the gas production, pH, NH_3-N, and volatile fatty acids concentrations. The apparent digestibility of diet compounds was determined. Microbial protein synthesis and microbial community diversity were estimated using ^{15}N as an external isotopic marker and by sequencing part of the 16 S rDNA gene, respectively. The TSBM and PHY treatments decreased NH_3-N concentrations ($P < 0.001$) and the DM disappearance ($P = 0.004$). The crude

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; ADIN, acid detergent insoluble N; ASVs, amplicon sequence variants; CONTROL, negative control (basal diet); CP, crude protein; DM, dry matter; EE, ether extract; LAB, liquid associated bacteria; MCFA, basal diet replacing SBM (10 g/kg DM) with a mixture of medium-chain fatty acids; MCFAs, medium-chain fatty acids; NAN, non-ammonia nitrogen; aNDF, neutral detergent fibre assayed with heat stable amylase and sodium sulfite and expressed inclusive of residual ash; NDIN, neutral detergent insoluble N; Non-CPC, non-crude protein compounds; OM, organic matter; PHY, basal diet replacing SBM (10 g/kg DM) with a mixture of phytogenic components; RUSITEC, rumen simulation technique; SAB, solid-associated bacteria; SBM, soybean meal; SEM, standard error of the mean; SP, soluble protein; TP, true protein; TSMB, positive control (basal diet replacing SBM with Mervobest®); VFA, volatile fatty acids.

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protein disappearance was the lowest for TSBM followed by PHY ($P < 0.001$). The true protein disappearance was lower for TSBM and PHY than for CONTROL and MCFA ($P < 0.001$). The isovaleric acid proportion was lower for TSBM, MCFA, and PHY than for CONTROL. Treatments TSBM and MCFA registered lower neutral detergent fibre disappearance ($P < 0.001$) than the other diets but without changes in the quantified fibrolytic genera in the ruminal microbiota. The PHY treatment increased the relative abundance of *Succinivibrio* spp. and reduced that of *Methanobrevibacter* relative to that of CONTROL and TSBM, which could be linked to lower methanogenesis. The MCFA treatment increased *Succinivibrio* compared with TSBM and *Succiniclasticum* spp. compared with CONTROL. These results indicate that PHY inclusion at 10 g/kg DM decreased rumen protein degradation, similar to TSBM; while MCFA decreased rumen protein degradation to a lesser extent. Furthermore, both additives generated changes in the ruminal microbiota that would be associated with lower methane production.

1. Introduction

The worldwide increase in milk production levels has led to an increase in nutritional requirements, including protein. Although ruminal microbiota can synthesize high-quality microbial protein from degradable proteins in the diet (Wallace, 1996), in high-producing animals, the amount of this protein is not enough to cover requirements. In addition, to reduce N excretion and limit its impact on the environment, a tendency has been observed to decrease the dietary crude protein (CP) content (Castillo et al., 2000; NRC, 2021). Thus, the use of low-degradable protein sources is a frequent practice to increase the amount of protein reaching the intestine to be digested and absorbed (Christensen et al., 1993; Bunglavan and Dutta, 2013; NRC, 2021).

To further increase the nondegradable but digestible protein fraction of feedstuffs, formaldehyde is one of the most common, effective, and inexpensive methods used to protect the protein from microbial degradation in the rumen. Formaldehyde modifies the structure of the protein by crosslink formation and making them covalently bonded, thereby increasing the resistance to proteolysis, which decreases ruminal degradability (Atole and Bestil, 2014; Kumar et al., 2015; Gadeyne et al., 2017). However, a high dose can have an adverse effect, thus making protein indigestible in the lower tract (Kumar et al., 2015; Mahima et al., 2015). This treatment has been questioned because of the cancer risk associated with formaldehyde use (McLaughlin, 1994; Checkoway et al., 2012; Swenberg et al., 2013), and it is not used in organic animal farming (IFOAM, 2006).

Numerous studies have demonstrated the benefits to animal nutrition of including natural alternative feed sources that are rich in bioactive phytonutrients, such as extracts and active components of plants (Cardozo et al., 2004; Almeida et al., 2021; Tilahun et al., 2022). Among these compounds, tannins are polyphenols with the ability to form complexes with proteins, which decreases the ruminal degradation of proteins (Patra and Saxena, 2011; Huang et al., 2017). Because of this property, tannins can also modulate the rumen microbial population and/or activity towards a reduction of both methane emissions from the rumen and the urinary excretion of urea N (Makkar, 2003; Bhatta et al., 2009). For this reason, tannins could be effective additives for regimes with high levels of fresh pasture or high-quality pasture silage which are commonly used in a great part of the southern hemisphere, given the excess of degradable protein they contain (Repetto et al., 2005; Keim et al., 2013). However, there are not many studies about the addition of tannins in these feeding conditions. Recently, Pozo et al. (2022) evaluated tannins from *Acacia mearnsii* as a strategy to increase N-use efficiency in grazing dairy cows. These authors concluded that tannins were effective in decreasing the excretion of urinary N without affecting dairy production. However, the pasture used in this work did not have as high protein levels to cause large excesses. This calls into question whether, with larger amounts of protein entering the rumen, the addition of tannins could be effective in controlling rumen-degraded N. Moreover, due to the known antimicrobial effects of tannins (Scalbert, 1991), it would be of interest to evaluate the effect of tannins on the rumen microbiota of animals consuming high-protein forages.

On the other hand, the use of fats in ruminant nutrition has gained renewed interest because fat might be a possible strategy for mitigating emissions of the greenhouse gas methane (Machmüller and Kreuzer, 2004). Medium-chain fatty acids (MCFAs) are oils that reduce H_2 producers (i.e., protozoa) and methanogen populations when added to ruminant diets (Almeida et al., 2021). A mixture of lauric and myristic acids was found to be the most effective at suppressing CH_4 emissions compared with the use of these fatty acids alone; nevertheless, the risk of adverse effects on fibre digestibility could restrict its use (Soliva et al., 2004; Almeida et al., 2021). However, Soliva et al. (2004), incubating in a RUSITEC system, a diet with 270 g/kg of neutral detergent fibre (NDF), using grass hay as the fibrous component, suggested that the desired effect of lower methanogenesis persists while the unfavorable effect of decreased ruminal fibre degradation seems to be reversible after some time. Even more, Machmüller (2006) suggested that supplementation with MCFAs causes negative or positive effects in ruminants depending on the dosage and frequency. Burdick et al. (2022) included MCFAs (0.0063 g/kg of dietary dry matter (DM)) in the total mixed ration diets of mid-lactation dairy cows (310 g/kg of NDF) and observed slight positive effects on rumen pH. These authors suggested that MCFAs may decrease the risk of subacute rumen acidosis with a greater inclusion rate. As far as we know, there are no studies about the impact on digestion and rumen microbiota when adding MCFAs to diets in which the main content of fibre and protein is provided by high-quality pasture or pasture- silages.

Although tannins protect dietary protein sources from ruminal breakdown, MCFAs, at least under certain conditions, generate a more favorable ruminal environment for microbiota, which could lead to greater production of microbial protein. Thus, taking in mind these different mechanisms of action, this study hypothesized that both functional feed ingredients added to a conventional dairy cow diet would increase protein flow to the duodenum similar to the frequently used formaldehyde-treated soybean meal (SBM).

Therefore, the objective of this work was to evaluate the effect of including 2 functional feed ingredients, namely a specific blend of

phytogenic components (basically tannins) and a mixture of medium-chain fatty acids (Aromabiotic® Cattle), on fermentation characteristics, ruminal microbiomes, and nutrient disappearance in a RUSITEC system using a diet with a high proportion of pasture silage as a substrate.

2. Materials and methods

This study followed the rules of the Bioethics Committee of the Veterinary Faculty (protocol number: CEUAFVET-642, UdelaR, Uruguay) and was previously approved.

2.1. Experimental diets

The experimental diets (Table 1) were formulated according to the NRC (2021) for a cow producing 30 kg/day of milk and consisted of a total mixed ration that included a ratio of 60:40 forage: concentrate based on 20 kg of DM/cow/day intake and a 169 g/kg CP level. The two additives were compared based on a diet containing regular SBM without any additives and a diet with the known commercial protein source additive Mervobest®, which is basically an SBM treated with formaldehyde to protect proteins against ruminal microbiota degradation (positive control) and is frequently used in dairy cows feeding. The treatments (experimental diets) were CONTROL: negative control (basal diet); TSBM: positive control (Mervobest®, Royal Agrifirm Group, The Netherlands; SBM treated with formaldehyde replacing regular SBM), PHY (10 g/kg DM of phytogenic components (tannins from quebracho (*Schinopsis balansae* Engl.) and chestnut (*Castanea sativa* Mill.), 61 g/kg tannic acid (ISO9648), replacing regular SBM); and MCFA (10 g/kg DM of MCFAs, Aromabiotic® Cattle, Royal Agrifirm Group, The Netherlands, replacing regular SBM). To reduce the effect of variability between batches, the soybean meals used in all treatments came from the same batch. As the commercial doses of the functional feed ingredients had not been established at the moment of this trial, the inclusion rates (10 g/kg) were defined in a previous assay using the same RUSITEC system, in which pH, ammonia nitrogen (NH_3-N), and disappearance of DM (dDM) were measured. Then, the doses used in this experiment were established as the maximum dose at which the aforementioned variables were not altered, to see the effects of the additives, if any.

2.2. In vitro fermentation experimental procedure

An in vitro fermentation study was carried out in a rumen simulation technique (RUSITEC) apparatus (Czerkawski and Breckenridge, 1977) provided with 6 fermentation units of 900 mL capacity. The diets were incubated in a balanced incomplete block design

Table 1
Ingredients and chemical composition (g/kg DM, if no other basis is specified) of the experimental diets.

	Diets[a]			
	CONTROL	TSBM	PHY	MCFA
Ingredients[b] *(g/kg DM)*				
Corn silage	300	300	300	300
Grass silage	300	300	300	300
Corn (grain)	285	285	285	285
Soybean meal	115	-	105	105
Mervobest®	-	115	-	-
Tannins	-	-	10	-
Aromabiotic® Cattle	-	-	-	10
Chemical Composition[c]				
DM (g/kg FM)	894	892	900	896
OM	901	905	913	920
aNDF	321	325	319	328
ADF	184	182	183	178
EE	30.4	30.4	30.4	30.5
CP	178	175	170	173
TP	114	116	108	111
SP	109	110	107	105
NDIN	13.6	15.2	13.7	13.1
ADIN	2.95	2.66	3.19	3.76

[a] CONTROL: control diet; TSBM: control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM; PHY: control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM, MCFA: control diet with a mixture of medium-chain fatty acids (Aromabiotic® Cattle) at 10 g/kg DM partially replacing regular SBM;

[b] Containing (g/kg of DM), corn silage: organic matter(OM), 951; neutral detergent fibre assayed with a heat stable amylase and sodium sulfite (aNDF), 416; acid detergent fibre (ADF), 216; ether extract (EE), 25.5; crude protein (CP),73.3; grass silage: OM, 893; aNDF, 464; ADF, 312; EE, 36.6; CP,206; corn grain: OM, 985; aNDF, 102; ADF, 51.1; EE, 32.0; CP, 90.3; SBM: OM, 928; aNDF, 111; ADF, 107; EE, 23.2; CP, 514.

[c] Average of the chemical analysis of each mixture (3 assays per diet, corresponding to each replication run), expressed as g/kg of DM unless otherwise stated, FM: fresh matter, DM: dry matter, TP: true protein, SP: soluble protein, NDIN: neutral detergent insoluble nitrogen and ADIN: acid detergent insoluble nitrogen.

(Montgomery, 2001), and 4 runs (blocks) were performed. Each run contained 3 randomly assigned diets (treatments), and each diet was randomly assigned to two units. With this design, each diet went through 3 runs, and all possible 4 by 3 combinations were included. Each run lasted 17 days, with 7 days to reach the equilibrium of the system (Martínez et al., 2011) and 10 days to perform measurements and sampling.

The inoculum (ruminal fluid) was obtained from 3 fistulated Holstein × Jersey cows (550 ± 25 kg of body weight) fed a diet consisting of grass silage (750 g/kg), corn grain (125 g/kg), and SBM (125 g/kg), at about 4 h after morning feeding. The ruminal fluid and the rumen content were separated by filtration through four layers of cheesecloth and transported to the laboratory in preheated thermal containers within 10 min.

The artificial saliva (McDougall, 1948) was continuously infused through a peristaltic pump at a renewal rate of 650 mL/day, which was prepared daily throughout the experimental phase. In addition, the effluent was collected in 2 L Kitasato flasks located in a cold chamber to avoid microbial growth. The gas produced (mL) was collected daily in hermetic bags (2 L capacity) with a one-way valve.

On the first day of each run, each fermentation unit was filled with a mixture of filtered ruminal fluid and artificial saliva, in a 0.7/0.3 ratio. Afterward, a nylon bag (R510, ANKOM Technology, Macedon NY, USA) containing 10 g DM of the corresponding experimental diet, and another bag with solid rumen content were placed in the fermentation unit. Before filling the bags, the ingredients of the experimental diets were dried at 60ºC and ground through a 2 mm sieve. After the first 24 h of incubation, the bag with solid rumen content was replaced by a bag filled with the experimental diet. From that moment on, the bag after 48 h of incubation was replaced daily by a new bag with the experimental diet.

2.3. pH, NH_3-N, and volatile fatty acid (VFA) concentrations in the RUSITEC fermenters

From days 8–17 of each run, the pH was measured using a digital pH meter (Oakton® eChem Instruments Pte. Ltd., Singapore). In addition, samples of the Rusitec medium from each fermentation unit were taken for VFA and NH_3-N analyses.

For the VFA analysis, 1 mL of the incubation medium was mixed with 1 mL of 0.1 M of perchloric acid, while for the NH_3-N analysis, 1 mL of the incubation medium was mixed with 0.02 mL of sulfuric acid (1:2 v/v). Next, the samples were stored at − 20 °C until analysis.

The VFAs were analysed by high-performance liquid chromatography (HPLC, Dionex Ultimate® 3000, Waltham, MA, USA) at 210 nm using a 300 × 7.8 mm column (Acclaim, Rezex ROA-Organic Acid H + (8%), Phenomenex, USA) according to Adams et al. (1984). Total VFA concentrations were calculated as acetic + propionic + butyric + isobutyric + valeric + isovaleric acids, and each VFA was expressed as a molar proportion relative to the total VFAs (mol/100 mol). The NH_3–N concentration was analysed by colorimetry following the technique described by Weatherburn (1967) using a spectrophotometer (BEL Photonics®, S-2000, SP, Brazil).

2.4. Nutrient disappearance

The apparent disappearance of DM, CP, non-crude protein compounds (Non-CPC), true protein (TP), neutral detergent fibre (aNDF), and acid detergent fibre (ADF) (dDM, dCP, dNon-CPC, dTP, daNDF, and dADF, respectively) was determined from days 8–14. Bags were removed from each fermentation unit, rinsed with 100 mL of artificial saliva, and then stored at − 20 °C for subsequent analysis. To determine the dDM, each bag was dried at 60 °C for 48 h, and disappearance was calculated as dDM = ([DM incubated (g) − DM after incubation (g)]/ DM incubated). Then, a pool was made with the bag contents from days 8–14 of each fermentation unit and analysed for CP, Non-CPC, TP, aNDF, and ADF. These results were used to calculate the respective disappearance values according to the disappearance of daily DM (g).

2.5. Chemical composition analysis

The diets and fermentation residues were analysed for the DM, organic matter (OM), and N (CP = N × 6.25) contents according to the methods ID 934.01, ID 942.05, and ID 984.13, respectively (AOAC, 1990). The contents of aNDF and ADF were analysed according to Robertson and Van Soest (1981) in a nonsequential way using an ANKOM220 fibre analyser (ANKOM Technology Corp., Macedon, NY, USA), with thermostable amylase and without sodium sulfite for aNDF, and the values of both fractions were expressed inclusive of the residual ash content. Ether extract (EE) contents were analysed according to Nielsen (2003), using a Goldfisch fat extractor (Labconco 3500100, Kansas City, MO, USA) under a petroleum ether reflux at 180 °C for 3 h). The OM was calculated as 1 - ash and Non-CPC was calculated as DM - CP. In addition, the contents of TP (using trichloroacetic acid), soluble protein (SP), neutral detergent insoluble N (NDIN), and acid detergent insoluble N (ADIN) were determined according to Licitra et al. (1996).

2.6. Microbial protein synthesis

The synthesis of microbial protein in the solid and liquid phases of the system was determined from days 15–17. From day 8, a dose of ^{15}N ($^{15}NH_4Cl$, 98% enrichment; Sigma Chemical Co., Madrid, Spain) as a microbial marker was added daily to each fermenter via artificial saliva, according to the description of Martínez et al. (2009).

The nylon bag content (solid digesta) was extracted every day and then weighed and homogenized before a subsample (approximately 6 g) was collected and lyophilized (using an SP VirTis BenchTop Pro with Omnitronics; SP Industries, Inc., Missouri, USA lyophilizer) for 24 h before determining the DM content. Next, non-ammonia nitrogen (NAN) and ^{15}N enrichment (% atom of ^{15}N in

excess) were analysed by isotopic ratio mass spectrometry (IRMS; Delta V Advantage) coupled to an elementary analyzer for IRMS (Flash EA 2000), both by Thermo Fisher Scientific, Bremen, Germany. The rest of the solid content was subjected to treatment with a saline solution (9 g/L NaCl) to detach the solid-associated bacteria (SAB). The solution was first added at a 3 mL/gram residue rate, and then the mixture was homogenized for 3 min with a stomacher (BagMixer® 400 CC Click & Clean®, INTERSCIENCE) and finally filtered through a nylon cloth (2 layers, 40 μm). The total filtrate was used to isolate the SAB by differential centrifugation (20,000g, for 25 min). In this way, the microbial pellet for SAB was obtained, frozen, and lyophilized to analyse its content of DM, N, and ^{15}N enrichment.

The effluent collected from each flask during days 15–17 was weighed, and then the samples were frozen at − 20 °C for subsequent analysis. To isolate the liquid-associated bacteria (LAB) and obtain the LAB microbial pellet, the same procedure applied for the SAB was carried out except that the supernatant was retained instead of the pellet. Once the LAB pellet was obtained, its N content and ^{15}N enrichment were analysed.

To discount the ^{15}N incorporated in the NH_3-N dissolved in the effluent, a composite sample of effluent was prepared from each fermentation unit (flask), batch, and treatment (total: 24 pools). A NaOH solution (1 M, 1 mL/g of effluent) was added to each pool and left in an oven (105 °C) for 24 h to remove NH_3-N according to Firkins et al. (1992), and it was used for the subsequent analysis of the content of ^{15}N.

Daily SAB synthesis was estimated as the total NAN production in the nylon bag residues × (^{15}N: N in residues/^{15}N: N in SAB). Daily LAB synthesis was estimated as the total NAN production in the effluent × (^{15}N: N in effluent/15 N: N in LAB). The total daily microbial production was calculated as the sum of the flows of SAB and LAB. The efficiency of SAB, LAB, and total microbial synthesis was expressed as mg N per gram of incubated DM and per gram of disappeared DM.

2.7. Bacterial community

To analyse the bacterial community present in the fermenters, samples from days 8, 12, and 16 were kept at − 20 °C for later analysis. Samples were thawed at room temperature, and total microbial DNA extraction was performed using the ZR Faecal DNA MiniPrep™ kit (ZYMO Research, USA) following the manufacturer's protocol. The bead-beating step was performed using a FastPrep-24™ at 6 m s-1 for 40 s, and the extracted DNA was stored at − 20 °C. Library preparation and sequencing of the V4 region of the 16 S rDNA gene was performed at the Center for Sequencing and Genomic Analysis, University of Austin (UT GSAF, Texas, USA), with an Illumina MiSeq platform to generate paired readings of 250 bases in length. The generated data were processed with the R package dada2 (Callahan et al., 2016a) following the pipeline presented in the GitHub repository (https://benjjnn.g.gubub.io/dada2/tutorial.html) and in Callahan et al. (2016b). In summary, reads were trimmed and filtered using default parameters with truncLen=c (240, 240) and trimLeft= 10. Amplicon sequence variants (ASVs) were defined, and after removing the chimaeras (removeBimeraDenovo, method = "consensus"), the taxonomy was assigned using the Silva database (version 132). The ASVs with less than 10 reads in total were eliminated. When the analysis required it, counts were normalized by calculating relative abundances. DECIPHER was used to perform multiple alignments (Wright, 2015), and phangorn (Schliep, 2011) was used to construct a phylogenetic tree with the parameters recommended in Callahan et al. (2016b).

The R packages phyloseq (McMurdie and Holmes, 2013) and ampvis2 were used to visualise and analyse the sequencing data. Distance matrices were calculated using the Jaccard (presence/absence), Bray—Curtis (abundance) UniFrac (phylogenetic relationship), and Weighted UniFrac (abundance-weighted phylogenetic relationship) methods. Alpha-diversity parameters were calculated using the *estimate_richness* function, which was implemented in *phyloseq* with the functions of the *vegan* package (Oksanen et al., 2019).

2.8. Statistical analysis

The data were analysed using version 9.0 of SAS software (SAS Institute, Inc., Cary, NC, USA). The PROC MIXED procedure was used for repeated measurements, with the fermenter as the subject of the repeated measurements, according to the following model: $Y_{ijkl} = \mu + T_i + D_j + R_k + (T * D)_{ij} + \varepsilon_{ijkl}$, where Y_{ijkl} is the dependent variable, μ is the general mean, T_i is the fixed effect of the $_i^\circ$ treatment (CONTROL, TSBM, PHY or MCFA) observed in $_l^\circ$ replications (n = 6 fermentation units), D_j is the sampling time (7 days for pH, NH_3-N, VFA, and dDM), R_k is a random effect of the run, $(T * D)_{ij}$ is the interaction between treatment and sampling time and ε_{ijkl} is the residual error. For nutrient disappearances and ^{15}N-related variables, the model used was $Y_{ijk} = \mu + T_i + R_j + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the general mean, T_i is a fixed effect of the $_i^\circ$ treatment (CONTROL, TSBM, PHY or MCFA) observed in $_k^\circ$ replications (n = 6 fermentation units), R_j is a random effect of the run and ε_{ijk} is the residual error. The covariance structure used was autoregressive type 1. The means were compared by the least squares method using LS means, and the values were considered to differ at $P < 0.05$ and tended to differ at $0.05 \leq P \leq 0.10$.

To study the microbial community, the calculated alpha-diversity parameters of the treated groups were compared with the Kruskal—Wallis test (*P value* set at 0.05). To assess the effect of treatment, day, and experimental instance on the bacterial community, a multivariate analysis of variance with permutations (PERMANOVA) was performed with the *adonis* function (*vegan* package) using the Weighted UniFrac distance matrix. Then, pairwise comparisons were performed between the control groups and each treatment group. The functions *betadisper* and *permutest* were used to check the homogeneity of variance (*permutations* = 1000). The SIMPER analysis (*vegan* package) was performed to determine the genera that contributed significantly to dissimilarities between treatments (Clarke, 1993), and then the relative abundances of those genera were compared using the Kruskal—Wallis test (*P value* set at 0.05).

3. Results

3.1. pH and NH_3-N and VFA concentrations in RUSITEC fermenters

There was no interaction between treatment and sampling day for pH, NH_3-N, and VFA concentrations ($P > 0.1$), despite differences between days being detected (data not shown in tables).

Treatments TSBM and PHY resulted in lower NH_3-N concentrations than CONTROL (TSBM vs CONTROL, $P < 0.05$; PHY vs CONTROL, $P < 0.05$) and MCFA treatments (TSBM vs MCFA, $P < 0.05$; PHY vs MCFA, $P < 0.05$; Table 2). pH, gas production, and total VFA concentration were not affected by the treatments ($P > 0.1$), but MCFA showed a higher butyric acid proportion than TSBM ($P = 0.007$) and PHY ($P = 0.001$). Also, PHY showed a lower butyric acid proportion than CONTROL ($P = 0.031$). The TSBM, PHY, and MCFA treatments showed similar isovaleric acid proportions but lower than CONTROL (TSBM vs CONTROL, $P = 0.016$; PHY vs CONTROL, $P = 0.007$; MCFA vs C, $P = 0.002$). The acetic:propionic ratio was higher for TSBM than the other treatments ($P = 0.005$).

3.2. Nutrient disappearance

The TSBM and PHY treatments decreased the disappearance of nutrients (DM, CP, TP, Non-CPC, and aNDF) compared with the CONTROL and MCFA treatments (Table 3). Treatments TSBM and PHY showed lower dDM than CONTROL and MCFA ($P = 0.004$). The TSBM treatment showed the lowest dCP followed by PHY and MCFA, and CONTROL showed the highest dCP ($P < 0.001$). Treatments TSBM and PHY showed lower dTP than CONTROL and MCFA ($P < 0.001$). Treatments TSBM, PHY, and MCFA showed lower dNon-CPC than CONTROL ($P < 0.001$). The TSBM and MCFA treatments showed lower daNDF than PHY, and the highest daNDF was for CONTROL ($P < 0.001$). For dADF, MCFA presented the lower value and CONTROL presented the higher value, PHY and TSBM did not differ from the formers.

3.3. Microbial protein synthesis

The estimated synthesized SAB, LAB, and total microbial N in the fermenters (Table 4) did not show differences between treatments ($P>0.1$). Neither SABs nor total microbial N synthesis efficiency was different between treatments. However, the microbial N synthesis efficiency of LABs, expressed as mg of N/g of incubated DM, was higher for TSBM than of the CONTROL and PHY ($P = 0.004$ and $P = 0.009$, respectively) and similar to that of MCFA. When the microbial N synthesis efficiency of LABs was expressed as mg of N/g of disappeared DM, it was higher for TSBM than of the CONTROL and PHY ($P = 0.001$ and $P = 0.005$, respectively) and similar to that of MCFA.

3.4. Bacterial community

After filtering by quality and size and eliminating singletons and chimaeras, 83.3% of the initial reads were retained, leaving an average of 24,124 (± 6066) reads per sample. Phyla with less than 2% relative abundance and ASVs in a low proportion (fewer than

Table 2
Fermentation characteristics of the treatments diets in a RUSITEC system.

	Treatments[a]				SEM[b]	P^c
	CONTROL	TSBM	PHY	MCFA		
pH	7.12	7.14	7.16	7.15	0.01	0.139
Gas, mL/d	678	701	693	675	48.4	0.686
NH_3-N, mg/dL	8.42^a	7.24^b	6.72^b	8.07^a	0.54	< 0.001
Lactic, mM	0.15	0.12	0.13	0.13	0.028	0.729
Total VFA, mM[d]	23.36	25.55	21.72	23.71	1.969	0.187
VFA proportion, mol/100 mol[e]						
Acetic	41.73	44.93	43.31	42.28	1.234	0.155
Propionic	27.29	26.30	28.95	27.81	0.763	0.114
Butyric	16.25^{ab}	14.31^{bc}	13.57^c	17.59^a	1.320	0.004
Isobutyric	3.13	4.58	3.96	3.09	0.859	0.536
Valeric	4.73	4.94	5.01	5.09	0.709	0.966
Isovaleric	6.67^a	5.12^b	4.90^b	4.64^b	0.472	0.008
Acetic:propionic ratio	1.56^b	1.71^a	1.53^b	1.56^b	0.064	0.005

[a] CONTROL: control diet; TSBM: control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM; PHY: control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM, MCFA: control diet with a mixture of medium-chain fatty acids (Aromabiotic® Cattle) at 10 g/kg DM partially replacing regular SBM;

[b] Standard error of the means.

[c] Level of significance of the effect of the treatment (T). The means within the same row with different superscripts (a, b, c) are significantly different ($P < 0.05$).

[d] Acetic+propionic+butyric+isobutyric+valeric+isovaleric.

[e] Molar proportion of individual VFA to total VFA.

Table 3
Apparent disappearance of nutrients[a] of the treatments diets in a RUSITEC system.

	Treatments[b]				SEM[c]	*P*[d]
	CONTROL	TSBM	PHY	MCFA		
dDM	0.450[a]	0.425[b]	0.429[b]	0.444[a]	0.012	0.004
dCP	0.503[a]	0.416[d]	0.454[c]	0.478[b]	0.010	< 0.001
dTP	0.294[a]	0.176[b]	0.184[b]	0.282[a]	0.020	< 0.001
dNon-CPC	0.402[a]	0.359[b]	0.371[b]	0.367[b]	0.007	< 0.001
daNDF	0.206[a]	0.124[c]	0.176[b]	0.145[c]	0.015	< 0.001
dADF	0.128[a]	0.117[ab]	0.119[ab]	0.106[b]	0.012	0.014

[a] dDM: disappearance of dry matter, dCP: disappearance of crude protein, dTP: disappearance of true protein, dNon-CPC: disappearance of non crude protein compounds (DM – CP), daNDF: disappearance of neutral detergent fibre, dADF: disappearance of acid detergent fibre.
[b] CONTROL: control diet; TSBM: control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM; PHY: control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM, MCFA: control diet with a mixture of medium-chain fatty acids (Aromabiotic® Cattle) at 10 g/kg DM partially replacing regular SBM;
[c] Standard error of the means.
[d] Level of significance of the effect of the treatment (T). The means within the same row with different superscripts ([a, b, c]) are significantly different ($P < 0.05$).

Table 4
Microbial synthesis and its efficiency of the treatments diets in a RUSITEC system.

	Treatments[a]				SEM[b]	*P*[c]	
	CONTROL	TSBM	PHY	MCFA			
Solid-associated bacteria (SAB)							
n[d]	6	6	6	6			
N, mg/d	40.7	43.8	44.6	46.4	6.77	0.834	
N, mg/g of incubated DM	4.24	4.57	3.91	4.40	0.642	0.601	
N, mg/g of disappeared DM	9.99	10.15	9.71	11.63	1.483	0.610	
Liquid-associated bacteria (LAB)							
n	6	6	5	6			
N, mg/d	19.9	39.3	21.2	30.7	6.47		0.116
N, mg/g of incubated DM	1.74[b]	4.21[a]	1.88[b]	3.29[ab]	0.684	0.014	
N, mg/g of disappeared DM	3.70[b]	9.88[a]	4.28[b]	6.79[ab]	1.595	0.007	
Total microbial synthesis (SAB + LAB)							
n	6	5	5	5			
N, mg/d	59.7	77.6	67.5	72.9	9.73	0.408	
N, mg/g of incubated DM	5.71	7.42	6.48	7.01	0.930	0.401	
N, mg/g of disappeared DM	12.8	17.5	15.2	16.0	2.231	0.268	

[a] CONTROL: control diet; TSBM: control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM; PHY: control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM, MCFA: control diet with a mixture of medium-chain fatty acids (Aromabiotic® Cattle) at 10 g/kg DM partially replacing regular SBM;
[b] Standard error of the means.
[c] Level of significance of the effect of the treatment (T). The means within the same row with different superscripts ([a, b]) are significantly different ($P < 0.05$).
[d] Data included in the analysis.

10 total reads) were eliminated. A total of 211 genera and 3036 different ASVs were retained. A total of 26.0% of the ASVs could not be classified at the genus level. The most abundant phyla were Firmicutes, Bacteroidota, Proteobacteria, Actinobacteriota, and Euryarchaeota, which together represented 78–97% of the total microbiota (Supplementary Figure 1, S1). The most abundant genera across all samples were *Lactobacillus*, *Rikenellaceae_RC9_gut_group, Succinivibrio, Methanobrevibacter, Christensenellaceae_R-7_group*, *Bifidobacterium* and *Prevotella* (Supplementary Figure 2, S2).

Alpha diversity was not affected by the treatment (Supplementary Figure 3, S3), but it was affected by sampling time, with a loss of richness observed throughout the experimental period. The total microbial composition was affected by treatment (Table 5), as also

Table 5
PERMANOVA model for total microbial composition analysis.

	DF	Sum of Sqs	R^2	F. model	*P*
Treatment	3	0.11110	0.10938	2,7205	0.005
Day	2	0.08944	0.08806	3,2853	0.005
Treatment: Day	6	0.03929	0.03868	0481	0.995
Residual	57	0.77592	0.76389		
Total	68	101.575	1		

revealed by SIMPER analysis (Fig. 1). The SIMPER analysis revealed that several bacterial genera were affected by treatment (Fig. 1). In fact, PHY increased *Succinivibrio* spp. and reduced *Methanobrevibacter* spp. compared with that of CONTROL and TSBM, and MCFA increased *Succiniclasticum* spp. and *Succinivibrio* spp. compared with that of CONTROL and TSBM, respectively. *Prevotella* spp. abundance was higher for MCFA compared with the CONTROL fermenters and for TSBM compared with the CONTROL fermenters. Meanwhile, *Fibrobacter* spp. was similar between treatments. Known proteolytic genera (*Selenomonas, Bacteroides, Eubacterium, Bacillus, Fusobacterium, Clostridium_sensu_stricto_1, Streptococcus*) represented 0.5–8.3% of the total community. A comparison of this whole group revealed a higher relative abundance in PHY compared with that in the control and MCFA and a higher abundance in

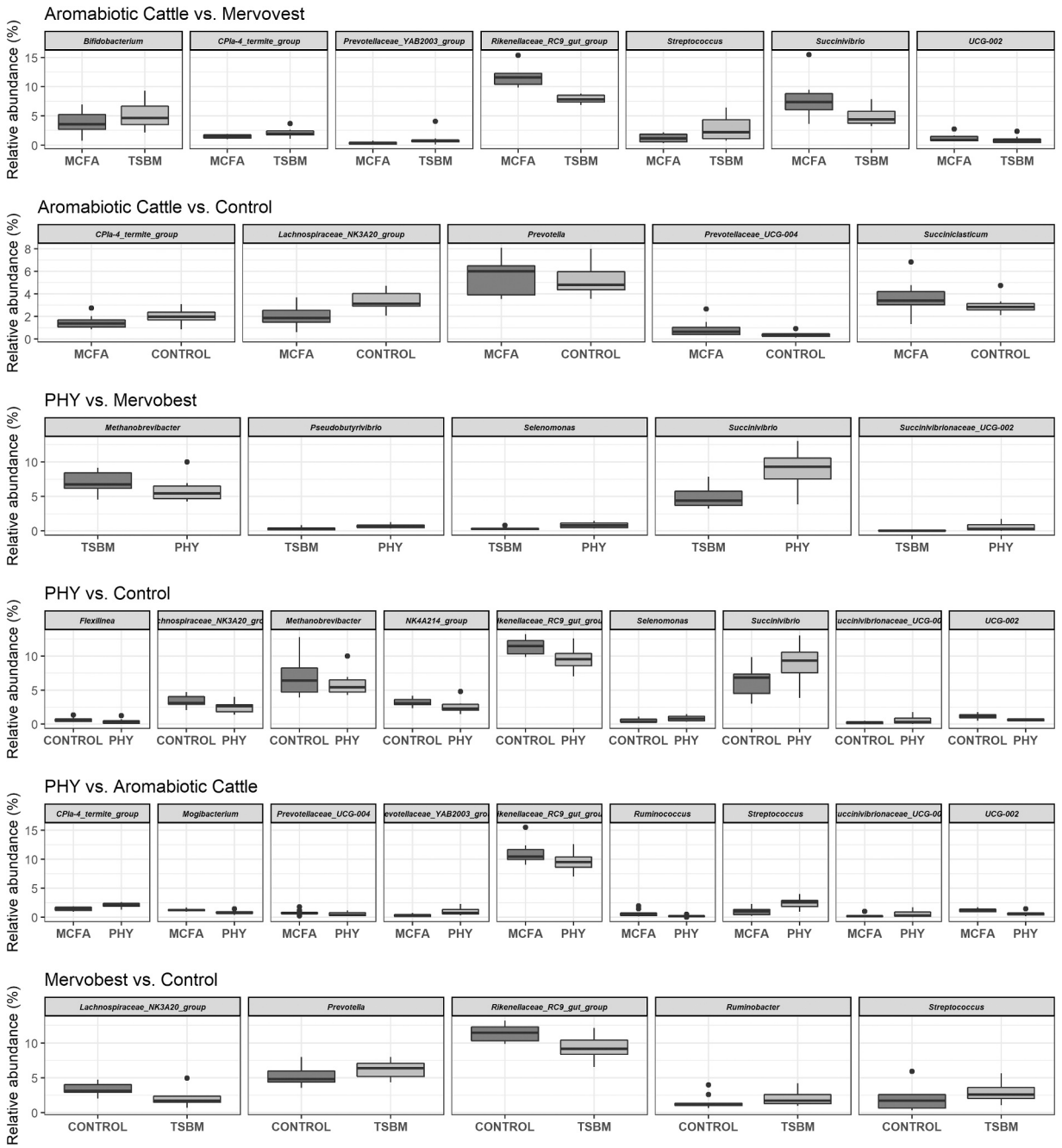

Fig. 1. Relative abundance of bacterial phyla in RUSITEC fermenters that were significantly different between treatments (SIMPER analysis) for the control diet (CONTROL), control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM (TSBM), control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM (PHY) or control diet with a mixture of medium-chain fatty acids at 10 g/kg DM partially replacing regular SBM (MCFA).

TSBM compared with that in MCFA (Fig. 2).

4. Discussion

In the present work, nutrient disappearances and VFA concentrations were particularly low, even considering that the experimental diets were based on forages. However, VFA results are in agreement with those reported in previous works. Using the same type of in vitro system, Romero-Pérez et al. (2015) and Öztürk et al. (2015), reported similar total VFA concentrations, while Khiaosa-ard et al. (2015) and García-Rodríguez et al. (2020) reported similar individual VFA proportions. According to Hristov et al. (2012), in RUSITEC systems values are usually lower than those obtained in vivo, which can explain the low values observed in the present study. It should be noted that VFA results are consistent with the low apparent disappearances, especially for the aNDF and TP. Concerning aNDF, its low values and high variability were already communicated in the meta-analysis of Hristov et al. (2012). Meanwhile, TP is a fraction composed of amino acid chains precipitated by trichloroacetic acid (Licitra et al., 1996). This fraction is of low degradation in some feedstuffs, which could explain the low apparent disappearance observed in this study. However, we cannot discard that the values could be, at least in part, affected by microbial contamination of the residual samples, as microbes are composed of a high proportion of true protein (Clark et al., 1992).

As expected, the use of a protected protein source (Mervobest®) decreased protein degradation (Ferguson, 1975; Waltz and Stern, 1989; Gulati et al., 2005). This fact was evidenced by lower concentrations of final products of protein fermentation (NH_3-N and isovaleric acid; El Shazly, 1952) and by reductions of CP and TP disappearances, which could lead to an increase in bypass protein. Despite these observations, a higher abundance of the proteolytic microbial group with the use of Mervobest® was found. This finding could not be clearly explained, but it must be considered that only some of the genera with proteolytic activity were quantified. Even though the higher acetic:propionic ratio showed by TSBM, the absolute difference between treatments is low to be important from a biological point of view.

The partial replacement of regular SBM for phytogenic components (10 g/kg DM; PHY treatment), showed a dietary protein protection similar to the use of treated SBM. Thus, similar reductions in NH_3-N concentrations were obtained with PHY and Mervobest®, decreasing 23% and 19% compared to the CONTROL concentrations, respectively. Similar decreases have been observed in in vitro batch culture trials. Among them, Cardozo (2005) evaluated the effects of natural plant extracts addition to a high-concentrate diet, and Busquet et al. (2006) added different plant extracts and secondary plant metabolites to a 50:50 forage:concentrate diet. Using in vivo approaches, Matloup et al. (2017), evaluated the effects of coriander oil, and Tilahun et al. (2022), assessed fresh fruit rich in phenolics. Both studies reported a decrease in ruminal NH_3-N concentration as in the present experiment. The in vitro apparent disappearance of CP was also reduced with PHY but at a lower magnitude than Mervobest® (9.9 vs. 17% regarding CONTROL). In addition to this difference in the dCP, the decrease in the dTP was similar between both treatments and of considerable magnitude (39%). This shows that phytogenic components used in the PHY treatment specifically decrease protein degradability and could be related to its high content of tannins (61 g/kg tannic acid), compounds known by this effect (McMahon et al., 2000). Also, tannins have been associated with a reduction in rumen methanogenesis (Bhatta et al., 2009; Cieslak et al., 2014; Jolazadeh et al., 2015); hence, the observed reduction in *Methanobrevibacter* and the increase in the abundance of succinate-producing microorganisms (*Selenomonas* spp. and *Succinivibrio* spp.) could also be associated with the tannin content of PHY. A recent study (Xue et al., 2022) reported a positive interaction between *Selenomonas* spp. and *Succinivibrio* spp., to improve the rumen function of high-efficiency dairy cattle. Thus, the higher abundance of these microbial groups observed with PHY could be linked to an extra improvement in feed efficiency.

Contrary to Hristov et al. (2009), who reported a 30% reduction in NH_3–N concentration with the addition of lauric acid and coconut oil in an in vitro system, the partial replacement in the control diet of regular SBM with MCFA (10 g/kg DM) did not change the NH_3–N concentration. Nevertheless, a slight decrease of dCP and the decrease in the proportion of isovaleric acid indicated less protein ruminal degradation. The MCFAs are known to have toxic effects on ruminal microorganisms, mainly on fibrolytic bacteria (Patra and Yu, 2013); however, no effects of MCFA treatment were detected in the quantified fibrolytic genera, such as *Fibrobacter* or *Ruminococcus*. Although MCFA led to lower daNDF and dADF than CONTROL, this was not reflected in the final fermentation products (i.e., VFA profile) and pH as described by other authors (Mould and Ørskov, 1983; Cardozo et al., 2002; Calsamiglia et al., 2002). In this case, the negative effect of fatty acids on fibre degradation was not evidenced, possibly due to the low EE level of the diets. At the doses of MCFA used, no differences were detected in the total microbial synthesis, although MCFA presented higher absolute values of microbial synthesis than CONTROL and PHY. The high SEM observed (more than 10% of the mean) would have interfered with the visualisation of the response, and there were only treatment effects on the efficiency of microbial synthesis of the LAB fraction. It is known that the treatment performed on the samples to detach biomass from digesta particles, cannot detach tightly attached bacteria from solid digesta (Ramos et al., 2009). This is considered a methodology limitation. It is necessary to point out that the absolute values of SAB and LAB can be affected by this fact, and this could also explain the low apparent disappearance values observed. However, as the same methodology was used for all samples, after Ramos et al. (2009), we can conclude about treatment differences. The higher abundance of succinate producers (*Prevotella* and *Succiniclasticum)* in MCFA treatment than in CONTROL, suggests that MCFA would be associated with lower methane emissions, as previously described when using MCFAs for supplementation (Patra and Yu, 2013). Also, Aguilar-Marin et al. (2020) associated the abundance of *Prevotella* with decreases in methanogenesis.

5. Conclusions

The inclusion of both functional feed ingredients (the phytogenic additive mixture of quebracho and chestnut tannins, and the medium-chain fatty acids), protected diet protein from ruminal degradation in a slightly smaller magnitude than treating the soybean

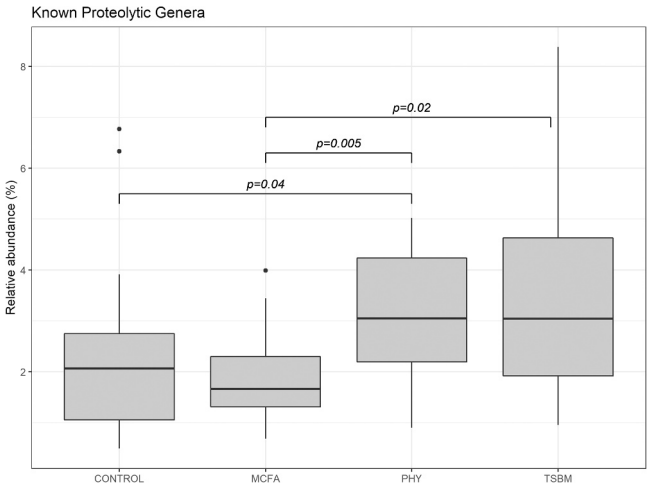

Fig. 2. Relative abundance (%) of known proteolytic genera (*Selenomonas, Bacteroides, Eubacterium, Bacillus, Fusobacterium, Clostridium_sensu_stricto_1, Streptococcus*) in RUSITEC fermenters for the control diet (CONTROL), control diet with formaldehyde-treated SBM (Mervobest®) replacing the regular SBM (TSBM), control diet with a mixture of tannins at 10 g/kg DM partially replacing regular SBM (PHY) or control diet with a mixture of medium-chain fatty acids at 10 g/kg DM partially replacing regular SBM (MCFA).

meal. Hence, they could be used as alternatives to hazardous formaldehyde treatment. Both ingredients enhanced feed efficiency as they increased the succinate-producing microorganisms, and the phytogenic, decreased one of the methane-producing genus of archaea studied. These effects are especially interesting for diets based on fresh or preserved high-quality forages.

It would be interesting to evaluate whether these compounds have similar effects when used in commercial conditions and if these differences are reflected in milk production.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest except for Mariana Bustos and Frederik Gadeyne, who declare that they have a conflict of interest because they work in the company that provided the tested additives and funded this project.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115763.

References

A.O.A.C, 1990. Association of Official Analytical Chemists. Official Methods of Analysis, 15th ed. AOAC, Arlington VA.
Adams, R.F., Jones, R.L., Conway, P.L., 1984. High-performance liquid chromatography of microbial acid metabolites. J. Chromatogr. 336, 125–137.
Aguilar-Marin, S.B., Betancur-Murillo, C.L., Isaza, G.A., Mesa, H., Jovel, J., 2020. BMC Microbiol. 20, 364. https://doi.org/10.1186/s12866-020-02037-6.
Almeida, A.K., Hegarty, R.S., Cowie, A., 2021. Meta-analysis quantifying the potential of dietary additives and rumen modifiers for methane mitigation in ruminant production systems. Anim. Nutr. 7, 1219–1230.
Atole, A.F.F., Bestil, L.C., 2014. Extrapolating bypass protein potential of treated soybean meal by degradation in rumen-fistulated Brahman cattle. Ann. Trop. Res. 36 (1), 50–62.
Bhatta, R., Uyeno, Y., Tajima, K., Takenaka, A., Yabumoto, Y., Nonaka, I., Enishi, O., Kurihara, M., 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. J. Dairy Sci. 92, 5512–5522.
Bunglavan, S.J., Dutta, N., 2013. Use of tannins as organic protectants of proteins in digestion of ruminants. J. Livest. Sci. 4, 67–77.
Burdick, M., Zhou, M., Guan, L.L., Oba, M., 2022. Effects of medium-chain fatty acid supplementation on performance and rumen fermentation of lactating Holstein dairy cows. Animal 16 (4), 100491. https://doi.org/10.1016/j.animal.2022.100491.
Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2006. Plant extracts affect in vitro rumen microbial fermentation. J. Dairy Sci. 89, 761–771.
Callahan, B., McMurdie, P., Rosen, M., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016a. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583.
Callahan, B., Sankaran, K., Fukuyama, J., McMurdie, P., Holmes, S., 2016b. Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. F1000Res 5, 1492. https://doi.org/10.12688/f1000research.8986.2.
Calsamiglia, S., Ferret, A., Devant, M., 2002. Effect of pH and pH fluctuations on microbial fermentation and nutrient flow in a dual-flow continuous culture system. J. Dairy Sci. 85, 574–579.
Cardozo, P.W., 2005. Efecto de los extractos de plantas sobre las características de fermentación microbiana ruminal en sistemas in vitro e in vivo (PhD Thesis). Universitat Autònoma de Barcelona, Departament de Ciència Animal i dels Aliments, Barcelona, Spain.
Cardozo, P.W., Calsamiglia, S., Ferret, A., 2002. Effects of pH on nutrient digestion and microbial fermentation on a dual flow continuous culture system fed a high concentrate diet. J. Dairy Sci. 85 (1), 182.

Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2004. Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. J. Anim. Sci. 82, 3230–3236.
Castillo, A.R., Kebreab, E., Beever, D.E., France, J., 2000. A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. J. Anim. Feed Sci. 9 (1), 1–32.
Checkoway, H., Boffetta, P., Mundt, D.J., Mundt, K.A., 2012. Critical review and synthesis of the epidemiologic evidence on formaldehyde exposure and risk of leukemia and other lymphohematopoietic malignancies. Cancer Causes Control 23, 1747–1766.
Christensen, R.A., Cameron, M.R., Klusmeyer, T.H., Elliott, J.P., Clark, J.H., 1993. Influence of amount and degradability of dietary protein on nitrogen utilization by dairy cows. J. Dairy Sci. 76, 3497–3513.
Cieslak, A., Zmora, Pawel, Pers-Kamczyc, E., Stochmal, A., Sadowinska, A., Salem, A.Z.M., Kowalczyk, D., Zbonik, P., Szumacher-Strabel, M., 2014. Effects of two sources of tannins (Quercus L. and Vaccinium Vitis Idaea L.) on rumen microbial fermentation: an in vitro study. Ital. J. Anim. Sci. 13, 290–294.
Clark, J.H., Klusmeyer, T.H., Cameron, M.R., 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75, 2304–2323.
Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. 18, 117–143.
Czerkawski, J.W., Breckenridge, G., 1977. Design and development of a long-term rumen simulation technique (RUSITEC). Br. J. Nutr. 38, 371–384.
El Shazly, K., 1952. Degradation of protein in the rumen of sheep. Biochem. J. 51, 647–653.
Ferguson, K.A., 1975. The protection of dietary proteins and amino acids against microbial fermentation in the rumen. In: McDonald, I.W., Warner, A.C.I. (Eds.), Digestion and Metabolism in the Ruminant. University of New England Publishing Unit, Armidale, Australia, pp. 448–464.
Firkins, J.L., Weiss, W.P., Piwonka, E.J., 1992. Quantification of intraruminal recycling of microbial nitrogen using nitrogen- 15. J. Anim. Sci. 70, 3223–3233.
Gadeyne, F., De Neve, N., Vlaeminck, B., Fievez, V., 2017. State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods. Eur. J. Lipid Sci. Technol. 119, 1600345. https://doi.org/10.1002/ejlt.201600345.
García-Rodríguez, J., Saro, C., Mateos, I., González, J.S., Carro, M.D., Ranilla, M.J., 2020. Effects of replacing extruded maize by dried citrus pulp in a mixed diet on ruminal fermentation, methane production, and microbial populations in RUSITEC fermenters. Animals 10, 1316. https://doi.org/10.3390/ani10081316.
Gulati, S.K., Garg, M.R., Scott, T.W., 2005. Rumen protected protein and fat produced from oilseeds and/or meals by formaldehyde treatment; their role in ruminant production and product quality: a review. Aust. J. Exp. Agric. 45, 1189–1203.
Hristov, A.N., Vander Pol, M., Agle, M., Zaman, S., Schneider, C., Ndegwa, P., Vaddella, V.K., Johnson, K., Shingfield, K.J., Karnati, S.K., 2009. Effect of lauric acid and coconut oil on ruminal fermentation, digestion, ammonia losses from manure, and milk fatty acid composition in lactating cows. J. Dairy Sci. 92, 5561–5582.
Hristov, A.N., Lee, C., Hristova, R., Huhtanen, P., Firkins, J.L., 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. J. Dairy Sci. 95, 5299–5307.
Huang, Q., Liu, X., Zhao, G., Hu, T., Wang, Y., 2017. Potential and challenges of tannins as an alternative to in-feed antibiotics 2 for farm animal production. Anim. Nutr. J. 187 https://doi.org/10.1016/j.aninu.2017.09.004.
International Federation of Organic Agriculture Movements (IFOAM), 2006. Proceedings of the First International Conference of Animal in Organic Production. Minnesota University, St. Paul, Minnesota, USA. August 23–25.
Jolazadeh, A.R., Dehghan-banadaky, M., Rezayazdi, K., 2015. Effects of soybean meal treated with tannins extracted from pistachio hulls on performance, ruminal fermentation, blood metabolites and nutrient digestion of Holstein bulls. Anim. Feed Sci. Technol. 203, 33–40.
Keim, J.P., Valderrama, X., Alomar, D., López, I.F., 2013. In situ rumen degradation kinetics as affected by type of pasture and date of harvest. Sci. Agric. 70 (6), 405–414.
Khiaosa-ard, R., Metzler-Zebeli, B.U., Ahmed, S., Muro-Reyes, A., Deckardt, K., Chizzola, R., Böhm, J., Zebeli, Q., 2015. Fortification of dried distillers grains plus solubles with grape seed meal in the diet modulates methane mitigation and rumen microbiota in RUSITEC. J. Dairy Sci. 98, 2611–2626.
Kumar, S., Kumari, R., Kumar, K., Walli, T.K., 2015. Roasting and formaldehyde method to make bypass protein for ruminants and its importance: A review. Indian J. Anim. Sci. 85 (3), 223–230.
Licitra, G., Hernández, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347–358.
Machmüller, A., 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agric., Ecosyst. Environ. 112, 107–114.
Machmüller, A., Kreuzer, M., 2004. Impact of the methane-suppressing feed additive myristic acid on energy and calcium balance of sheep. J. Anim. Feed Sci. 13 (1), 151–154.
Mahima, Kumar, V., Tomar, S.K., Roy, D., Kumar, M., 2015. Effect of varying levels of formaldehyde treatment of mustard oil cake on rumen fermentation, digestibility in wheat straw based total mixed diets in vitro. Vet. World 8 (4), 551–555.
Makkar, H.P.S., 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rum. Res. 49, 241–256.
Martínez, M.E., Ranilla, M.J., Ramos, S., Tejido, M.L., Saro, C., Carro, M.D., 2009. Evaluation of procedures for detaching particle-associated microbes from forage and concentrate incubated in RUSITEC fermenters: efficiency of recovery and representativeness of microbial isolates. J. Anim. Sci. 87, 2064–2072.
Martínez, M.E., Ranilla, M.J., Ramos, S., Tejido, M.L., Carro, M.D., 2011. Evolution of fermentation parameters in RUSITEC fermenters operated at different dilution rates and concentrate retention times. In: Ranilla, M.J., Carro, M.D., Ben Salem, H., Morand-Fehr, P. (Eds.), 'Challenging Strategies to Promote the Sheep and Goat Sector in the Current Global Context'. CIHEAM, Universidad de León, Zaragoza, Spain, pp. 121–126.
Matloup, O.H., Abd El-Tawab, A.M., Hassan, A.A., Hadhoud, F.I., Khattab, M.S.A., Khalel, M.S., Sallam, S.M., Kholif, A.E., 2017. Performance of lactating Friesian cows fed a diet supplemented with coriander oil as an alternative to antibiotic feed additives: Feed intake, nutrient digestibility, ruminal fermentation, blood chemistry, and milk production. Anim. Feed Sci. Technol. 226, 88–97.
McDougall, E.I., 1948. Studies on ruminant saliva. I. The composition and output of sheep's saliva. Biochem. J. 43, 99–109.
McLaughlin, J.K., 1994. Formaldehyde and cancer: a critical review. Int. Arch. Occup. Environ. Heath 66, 295–301.
McMahon, L.R., McAllister, T.A., Berg, B.P., Majak, W., Acharya, S.N., Popp, J.D., Coulman, B.E., Wang, Y., Cheng, K.J., 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Can. J. Plant. Sci. 80, 469–485.
McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8 (4), e61217. https://doi.org/10.1371/journal.pone.0061217.
Montgomery, D.C., 2001. Design and Analysis of Experiments, fifth ed. John Wiley & Sons Ltd., New York.
Mould, F.L., Ørskov, E.R., 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digest. Anim. Feed Sci. Technol. 10, 1–14.
National Research Council (NRC), 2021. Nutrient Requirements of Dairy Cattle, 8th ed. Nat. Acad. Press, Washington, DC.
Nielsen, S.S., 2003. Food Analysis Laboratory Manual (Food science texts series), third ed. Kluwer Academic/Plenum Publishers, New York.
Oksanen, J., Blanchet, F., Friendly, M., 2019. Vegan: community ecology package. R package version 2.5–4.
Öztürk, H., Salgirli Demïrbaş, Y., Aydin, F.G., PİŞKİN, İ., Ünler, F.M., Emre, M.B., 2015. Effects of hydrolyzed and live yeasts on rumen microbial fermentation in a semicontinuous culture system (RUSITEC). Turk. J. Vet. Anim. Sci. 39, 556–559.
Patra, A.K., Saxena, J., 2011. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91, 24–37. https://doi.org/10.1002/jsfa.4152.
Patra, A.K., Yu, Z., 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations in vitro. J. Dairy Sci. 96, 1782–1792.
Pozo, C.A., Kozloski, G.V., Cuffia, M., Repetto, J.L., Cajarville, C., 2022. Changing the grazing session from morning to afternoon or including tannins in the diet was effective in decreasing the urinary nitrogen of dairy cows fed a total mixed ration and herbage. J. Dairy Sci. 105 https://doi.org/10.3168/jds.2021-21149.
Ramos, S., Tejido, M.L., Ranilla, M.J., Martínez, M.E., Saro, C., Carro, M.D., 2009. Influence of detachment procedure and diet on recovery of solid-associated bacteria from sheep ruminal digesta and representativeness of bacterial isolates as assessed by automated ribosomal intergenic spacer analysis-polymerase chain reaction. J. Dairy Sci. 92, 5659–5668.

Repetto, J.L., Cajarville, C., D'Alessandro, J., Curbelo, A., Soto, C., Garín, D., 2005. Effect of wilting and ensiling on ruminal degradability of temperate grass and legume mixtures. Anim. Res. 54, 73–80.
Robertson, J.B., Van Soest, P.J., 1981. The detergent system of analysis and its application to human foods. In: James, W.P.T., Theander, O., Dekker, M. (Eds.), The Analysis of Dietary Fiber in Food. Marcel Dekker, New York, NY, USA, pp. 123–158.
Romero-Pérez, A., Okine, E.K., Guan, L.L., Duval, S.M., Kindermann, M., Beauchemin, K.A., 2015. Effects of 3-nitrooxypropanol on methane production using the rumen simulation technique (RUSITEC). Anim. Feed Sci. Technol. 209, 98–109.
Scalbert, A., 1991. Antimicrobial properties of tannins. Phytochemistry 30 (12), 3875–3883.
Schliep, K.P., 2011. Phangorn: phylogenetic analysis in R. Bioinformatics 27, 592–593.
Soliva, C.R., Meile, L., Cieślak, A., Kreuzer, M., Machmüller, A., 2004. Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. Br. J. Nutr. 92, 689–700.
Swenberg, J.A., Moeller, B.C., Lu, K., Rager, J.E., Fry, R.C., Starr, T.B., 2013. Formaldehyde carcinogenicity research: 30 years and counting for mode of action, epidemiology, and cancer risk assessment. Toxicol. Pathol. 41 (2), 181–189.
Tilahun, M., Zhao, L., Guo, Z., Shen, Y., Ma, L., Callaway, T.R., Xu, J., Bu, D., 2022. Amla (Phyllanthus emblica) fresh fruit as new feed source to enhance ruminal fermentation and milk production in lactating dairy cows. Anim. Feed Sci. Technol. 283. https://doi.org/10.1016/j.anifeedsci.2021.115160.
Wallace, R.J., 1996. Ruminal microbial metabolism of peptides and amino acids (Conference: Altering ruminal nitrogen metabolism to improve protein utilization). J. Nutr. 126, 1326s–1334s.
Waltz, D.M., Stern, M.D., 1989. Evaluation of various methods for protecting soya-bean protein from degradation by rumen bacteria. Anim. Feed Sci. Technol. 25, 111–122.
Weatherburn, M.W., 1967. Phenol-Hypochlorite reaction for determination of ammonia. Anal. Chem. 39, 971–974.
Wright, E.S., 2015. Decipher: harnessing local sequence context to improve protein multiple sequence alignment. BMC Bioinforma. 16, 1–14.
Xue, M.Y., Xie, Y.Y., Zhong, Y., 2022. Integrated meta-omics reveals new ruminal microbial features associated with feed efficiency in dairy cattle. Microbiome 10, 32. https://doi.org/10.1186/s40168-022-01228-9.