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Growth and bacterial dynamics of beef calves during transition from milk/pasture to a high-concentrate diet added with tannins or medium-chain fatty acids

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Abstract

Context. Rumen fermentation modulation with feed additives during the transition period to high-concentrate diets might help to avoid bacterial dysbiosis.

Aims. Assessing the effects of the addition of tannins and medium-chain fatty acids (MCFA) to the adaptation diet of beef calves to a high-concentrate ration on the rate of intake, animal performance and rumen bacterial composition.

Methods. Eighteen 7-month-old beef calves were abruptly weaned and transitioned over a 28-day period from a milk/grass regime to one of the following diets: a non-supplemented high-concentrate diet plus wheat straw, both given *ad libitum* (C); C plus 20 g/kg of a 65:35 chestnut and quebracho tannin extract; and C plus 6 g/kg of a commercial mixture of MCFA. Concentrate and straw rate of intake were recorded. Rumen fluid was collected on Days 0, 1, 7, 14, 21 and 28 at 0, 3, 6 and 9 h after feeding to characterise rumen fermentation. Samples from 0 h were analysed to assess the bacterial population using Ion Torrent sequencing.

Key results. The rate of intake of concentrates and straw, as well as daily gains and final weights, were similar ($P > 0.05$) among diets. The addition of tannins or MCFA did not modify ($P = 0.98$) the rumen bacterial population, which was affected by sampling day ($P < 0.001$). The additives inclusion did not affect relative abundances of the main bacterial taxa ($P < 0.05$), most of them differing across days ($P < 0.001$). Diversity indexes (Shannon and richness) declined over sampling days ($P < 0.05$), although some genera emerged after concentrate inclusion.

Conclusions. At the doses used in the present experiment, tannins and MCFA did not exert any effect on intake, animal performance and bacterial population. Abrupt transition to high-concentrate diets modified the rumen environment and bacterial community, indicating bacterial adaptation to new environmental conditions.

Implications. Abrupt transition of 7-month-old calves from milk/pasture to a high-concentrate diet did not impair rumen microbiota or performance; therefore, the use of feed additives seems unnecessary.

Keywords: modulation of fermentation, rumen acidosis, rumen bacteria.

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Introduction

Conventional beef production in Spain is mainly performed under intensive conditions, with suckling calves reared with their dams on pasture for 6 months, and then abruptly weaned and switched to a high-grain ration. Initially, a 14–21-day transition period is applied, in which calves receive a compound feed to stimulate the rumen papillae development and to allow microbial adaptation to the fattening concentrate. Ruminal fermentation of these diets, rich in readily fermentable carbohydrates, yields a large amount of volatile fatty acids (VFA) and lactate, which may accumulate if production exceeds the rate of absorption. This situation is supposed to decrease rumen pH and increase the risk of acute or subacute ruminal acidosis (Krause and Oetzel 2006). Therefore, an inadequate adaptation to these high-concentrate diets might lead to depressed rumen health that would limit animal performance during the fattening period (Brown *et al*. 2006).

The use of feed additives has been proved to be useful for moderate rumen fermentation in high-concentrate fed cattle, especially during the transition period (González *et al*. 2012). Tannins and medium-chain fatty acids (MCFA) are known to interact with rumen microbiota, modifying the rumen environment (Henderson 1973; Makkar 2003) and slowing down rumen fermentation, preventing the onset of acidotic episodes. The information on the effect of tannins is not

consistent (Mueller-Harvey 2006), as their biological activity widely depends not only on their polyphenolic nature or plant sources (Amanzougarene *et al*. 2019), but also on the basal diet fed to the animals (Mueller-Harvey 2006). Reports on the effects of tannins in high-concentrate diets fed to beef cattle are limited, and results on rumen metabolism and animal performance are inconclusive (Krueger *et al*. 2010; Mezzomo *et al*. 2011). Additionally, studies on the effects of tannin inclusion on rumen microbial composition under these types of diets are scarce (Vasta *et al*. 2019), especially with beef calves during the transition period.

In contrast, MCFA are feed additives that have also been evaluated for their potential to interact with bacteria (Henderson 1973) and modulate rumen fermentation (Amanzougarene *et al*. 2017). MCFA have been extensively assessed for their inhibition of rumen methanogenesis (Machmüller 2006). Most studies have been performed with lauric acid (C_{12}) or with coconut oil as a source of MCFA, both *in vitro* (Dong *et al*. 1997; Patra and Yu 2013) and *in vivo* (Yabuuchi *et al*. 2007; Hristov *et al*. 2012) with adult cattle (steers or dairy cows). However, there are no studies of the adaptation of newly weaned beef calves. Moreover, many studies have been performed *in vitro*, focusing on the effect of other MCFA (e.g. caprylic -C_8 or capric-C_{10}) on rumen metabolism (Dohme *et al*. 2001). The *in vitro* experimental approach is not representative of *in vivo* dosing and rumen conditions, and the possible adaptation of microbiota might not be assessed. In any case, findings on the effects of MCFA on rumen fermentation and microbes diverge among studies due to the high variety of fatty acids and sources of oil, doses, and animal diets, as well as chemical forms of MCFA (Liu *et al*. 2011; Hristov *et al*. 2012).

Therefore, in view of the paucity of *in vivo* studies performed with beef calves during the transition period from milk/pasture to a high-concentrate feeding, and the inconclusive results obtained with the use of both additives, the aim of this study was to assess the effect of the inclusion of tannins and MCFA in the adaptation diet of beef calves on intake, growth performance and rumen bacterial composition. We hypothesised that tannins and MCFA would interact with rumen bacteria, and might attenuate rumen fermentation decreasing the risk of acidosis without greatly affecting the availability of energy by the host animal when added to a high-concentrate diet.

Material and methods

Animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. Care and management of animals were performed according to the Spanish Policy for Animal Protection RD 1201/05, which meets the EU Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Animals, dietary treatments and experimental design

The experimental design and dietary treatments are described by Yuste *et al*. (2019). Briefly, 18 7-month-old (224 ± 54.3 days) weighing 212 ± 27.0 kg liveweight (LW) Limousin crossbred male calves reared with their dams on pasture (free suckling and grazing) were abruptly weaned and received grass hay *ad libitum* until the start of the experiment 3 weeks later. Calves were fistulated in the dorsal sac of the rumen with a 150-mm long, 15-mm i.d. cannula 1 week after weaning and allowed to recover from surgery for 2 weeks. Then, calves were abruptly switched to a ration consisting of a cereal and soybean meal-based concentrate (details in Yuste *et al*. 2019) plus wheat straw, both given *ad libitum*. The three dietary treatments were: a non-supplemented diet, as control (C); C plus 20 g/kg (as fed) of a commercial 65:35 chestnut (*Castanea* spp.) and quebracho (*Schinopsis* spp.) tannin extract containing >0.65 g tannins/g (T); and C plus 6 g/kg (as fed) of a commercially available mixture of MCFA (M). The experiment was performed for 28 days. Concentrate was offered once daily at 0800 hours, and straw was offered three times daily to ensure *ad libitum* consumption.

The following procedures were not included in the previous paper (Yuste *et al*. 2019).

Animals were weighed weekly, before feed distribution, and the average daily gain (ADG) was obtained by linear regression of weight on time. The feed conversion ratio was calculated as the ratio between the total DM intake (DMI; concentrate plus straw) and weight gained throughout the study.

The rate of intake of concentrate and straw was measured on Days 6, 13, 20 and 27, recording concentrate intake at 2-h intervals from 0800 to 2000 hours, and from 2000 to 0800 hours of the next day. The rate of intake of straw was recorded every 4 h from 0800 to 2000 hours, and from 2000 to 0800 hours of the next day.

Rumen fluid samples were taken at 0 h (before feeding) on Day 0, and at 0, 3, 6 and 9 h after feeding on Days 1, 7, 14, 21 and 28. Yuste *et al*. (2019) only used the first sample (0 h, Day 0), but here, we considered that the average daily values would be more representative of rumen conditions. These results were used to establish correlations between relative abundances of the bacterial taxa, diversity indexes and rumen fermentation variables (see below). Approximately 200 mL were removed from the rumen of each animal on each sampling time. A 5-mL subsample was taken and immediately frozen in liquid nitrogen at –80°C until analyses of bacterial DNA were performed. Then, the protocol described by Yuste *et al*. (2019) was followed.

Chemical analyses

Feeds and refusals, concentration of total VFA (TVFA), ammonia and lactate in rumen samples, and the concentration of individual MCFA in the tested additive were analysed as described in Yuste *et al*. (2019). Metabolisable energy (ME) of wheat straw was calculated from acid detergent fibre content according to the equation proposed by Mertens (1983) (NE = 2.469–0.0351 × %acid detergent fibre; R^2 = 0.849; ME = NE/0.61), whereas ME of concentrates was calculated taking into account the ingredient composition and ME values from FEDNA (2010).

DNA extraction and Ion Torrent sequencing

Rumen samples taken before feeding (0 h) on Days 0, 14, 21 and 28 were chosen for sequencing analyses using Ion Torrent

next-generation sequencing. Samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater; Biospec Products, Bartlesville, OK, USA). The microbial DNA was extracted using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen, West Sussex, UK) following the manufacturer's recommendations, except that samples were initially heated at 95°C for 5 min to maximise bacterial cell lysis. Concentration and purity of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA). Sequencing of the 16S rRNA gene was conducted following the procedure described by de la Fuente *et al*. (2014) and Schauf *et al*. (2018). Briefly, the bacterial V1-V3 hypervariable region was amplified by PCR using barcoded fusion primer pairs 27F and 338R (Wang *et al*. 2014). Sequencing of the PCR products was performed in the Ion Torrent Personal Genome Machine system (Life Technologies, Carlsbad, CA, US) using the Ion Personal Genome Machine Sequencing 200 kit v2 (Life Technologies). Following sequencing, data were combined and sample identification numbers assigned to multiplexed reads using Ion Reporter™ 5.10. Software (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing amplicon reads were subjected to trimming, denoising and chimera removal, and clustered into operational taxonomic units at 0.97 identity using UPARSE pipeline (Edgar 2013). Reads were subjected to quality filtering (quality score of 20 in a 1–40 scale) and trimmed at a maximum length of 250 bp. Taxonomic assignment of 16S rRNA sequences was established by comparison against the Ribosomal Data Project II database (Cole *et al*. 2003), considering a bootstrap value of 0.80 for annotation, leaving successive taxon levels as unclassified. To maximise the comparability across samples, the number of reads was manually normalised to the sample with the lowest number of reads (15 935). Numbers of reads of each microbial taxon were $\log_{10}$-transformed (number of reads + 1) before statistical analysis to assume normality; however, for a better interpretation, relative abundances of bacterial taxa are presented as proportion (mean ± s.e.m.) in the results section. Three measures of diversity were calculated: β-diversity, Shannon index and genera richness using R software.

Statistical analyses

Analysis of concentrate and straw DMI, rate of intake of concentrate and straw, ADG, rumen fermentation variables, bacterial taxa abundance (excluding those with relative abundances lower than 0.1% of total sequences), different ratios between phyla, and diversity indexes were analysed as repeated measures with the MIXED procedure of SAS (v 9.4; SAS Institute, Cary, NC, USA), considering the experimental diet, the sampling day and their interaction as fixed effects, and the animal within diet as the random effect. Sampling time after feeding within day or sampling day were considered as repeated measures, as appropriate. Analysis of final LW, total concentrate DMI and feed conversion ratio were performed with the PROC GLM, with the diet as the fixed effect and the animal as the random effect. Initial age and bodyweight were included in the model as covariates for analysis of DMI, final LW and ADG. For bacterial taxa abundance, treatment means were compared under a conservative Bonferroni adjustment. The variance–covariance structure was selected based on the lowest Akaike information criterion. For sequencing data, multivariate statistical analyses were performed using package 'vegan' from the R statistical program. To determine the impact of the diet and time on overall microbial community structure, a non-parametric permutational multivariate analysis of variance (PERMANOVA) was conducted based on the Bray–Curtis dissimilarity. Non-metric multidimensional scaling plot was generated representing the Bray–Curtis distance. To evaluate differences in community structure (β-diversity), an ANOVA was conducted on the distances to the centroids for each day or in each diet. Spearman correlation coefficients were calculated to assess the relationships between the ruminal fermentation characteristics at 0 h and the log-transformed data of the main bacterial taxa concentrations, different ratios between phyla, and diversity indexes using R software. Only Spearman correlations with $r > 0.50$ or $r < -0.50$ and $P < 0.05$ are shown. For all data, differences were considered significant if $P < 0.05$, whereas differences were considered to indicate a trend to significance when $0.05 \leq P \leq 0.10$

Results

Results of feed intake (except ME) and rumen fermentation variables only at 0 h have been published in a previous paper (Yuste *et al*. 2019).

Rate of intake and animal performance

Considering the average DMI of concentrate and straw already given by Yuste *et al*. (2019), and the estimated metabolisable energy of straw (6.40 MJ/kg DM) and concentrates given in C (11.41 MJ/kg DM), T (11.39 MJ/kg DM) and M (11.40 MJ/kg DM) diets, the estimated average ME intake for the whole experimental period was 54.6 MJ/day. The rate of intake of concentrate, pooled in 4-h intervals for an easier comparison with results of rate of intake of straw, is given in Table 1. There were no differences among treatments ($P = 0.80$) or sampling days ($P = 0.56$). However, intake was affected by the time interval after feeding ($P < 0.001$), and calves showed the highest intake of concentrate during the first 4 h after feeding, consuming on average 30% of the concentrate. Similarly, the rate of intake of straw was neither affected by diet ($P = 0.83$) nor by day ($P = 0.25$). However, it was affected by sampling time interval ($P < 0.001$), and calves exhibited the highest straw intake 8–12 h after feeding (Table 1), averaging 32% of total straw intake. Daily weight gain during the transition period was not affected by the inclusion of additives ($P = 0.98$), and averaged 0.85 ± 0.073 kg/day throughout the trial, increasing over time ($P < 0.001$) from 0.78 ± 0.038 kg/day in the first week to 0.95 ± 0.053 kg/day in the last week of the experiment. Likewise, no diet effect was found on final LW (248 ± 4.24 kg, $P = 0.83$) or on feed conversion ratio (4.48 ± 0.038, $P = 0.87$).

Table 1. Average intake of concentrate and straw (g DM/h) of beef calves fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 0.65 of tannins, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) when the rate of intake was recorded

Average daily intake of concentrate on the days when the rate of intake was measured was 4.89 kg. Average daily intake of straw on the days when the rate of intake was measured was 0.88 kg. Sampling intervals start at feeding. The order of the sampling intervals was S1 > S3 > S2 > S4 for concentrate, and S3 > S1 = S2 > S4 for straw

		Sampling interval (S)					*P*-value		
	Diet (D)	0–4 h	4–8 h	8–12 h	12–24 h	s.e.m.	D	S	D × S
Concentrate	C	422	226	268	114	8.3	0.80	<0.001	0.23
	T	316	191	284	114				
	M	340	208	295	124				
Straw	C	46.3	34.9	82.1	19.3	7.15	0.83	<0.001	0.52
	T	48.7	43.4	69.6	24.2				
	M	48.0	38.1	60.2	21.2				

Table 2. Rumen fermentation characteristics at 6 h after feeding of beef cattle fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 0.65 of tannins, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) during the transition to a high-concentrate diet

Probability of effects of diet (D), sampling day (S) and their interaction is also included. Different lowercase letters within a row indicate differences between sampling days at $P < 0.05$

		Sampling day						*P*-value	
	Diet	1	7	14	21	28	s.e.m.	D	S	D × S
Rumen pH	C	5.92c	6.64a	6.65a	6.48ab	6.04bc	0.176	0.95	<0.001	0.42
	T	5.85c	6.87a	6.47ab	6.27bc	6.16bc				
	M	6.14ab	6.56a	6.48ab	6.08b	6.25ab				
Lactate	C	0.52	1.57	1.08	0.89	1.53	1.297	0.27	0.56	0.64
(mmol/L)	T	0.99	4.76	0.86	3.79	0.84				
	M	1.23	0.77	0.68	1.13	1.52				
Ammonia	C	153a	74b	93b	60b	73b	21.2	0.34	<0.01	0.48
(mg/L)	T	93	67	84	43	44				
	M	144a	75b	72b	78b	55b				
VFA	C	113a	92ab	83ab	76b	103ab	11.8	0.60	<0.001	0.92
(mmol/L)	T	117a	70b	80b	69b	99ab				
	M	108ab	79b	89ab	86ab	113a				
Acetate	C	0.576ab	0.599a	0.507bc	0.481c	0.512bc	0.0314	0.77	0.002	0.20
(mmol/mmol VFA)	T	0.575a	0.591a	0.519ab	0.474b	0.578a				
	M	0.591a	0.483b	0.535ab	0.490b	0.566ab				
Propionate	C	0.253	0.173	0.218	0.275	0.247	0.0304	0.24	0.07	0.69
(mmol/mmol VFA)	T	0.272	0.170	0.203	0.222	0.221				
	M	0.264	0.258	0.232	0.272	0.226				
Butyrate	C	0.141	0.157	0.191	0.180	0.182	0.0247	0.52	<0.01	0.33
(mmol/mmol VFA)	T	0.129b	0.178ab	0.216a	0.236a	0.149b				
	M	0.115b	0.201ab	0.167ab	0.161ab	0.152ab				

Rumen fermentation variables

Even though the statistical analyses performed here and by Yuste *et al*. (2019) were different, the results were roughly the same, so the reader is referred to the above-mentioned paper to check for the diet effects. Of the sampling hours, Hour 6 was chosen as representing the maximum fermentation, as animals consumed the greatest amount of concentrate in the first four hours. The lowest rumen pH was also found at that time. No effect of diet was found in any of the rumen characteristics (Table 2), and a significant effect of the sampling day was found in all variables except for propionate and lactate.

Rumen bacterial populations

Collectively, four phyla represented >97% of the total sequences: Bacteroidetes (43.5 ± 1.21%), Firmicutes (38.3 ± 1.49%), Proteobacteria (11.3 ± 1.98%) and Actinobacteria (4.4 ± 0.80%), which, together with five other phyla (Table 3), represented on average 99.9% of total sequences. Of the two major phyla, Bacteroidetes was dominated by a single genus (*Prevotella*), whereas Firmicutes comprised several families and genera (Table 3). The PERMANOVA showed no differences among feed additives ($P = 0.92$, $R^2 = 0.007$), whereas day

Table 3. Effect of the diet and day of sampling on the relative abundance of the main bacterial taxa, and on diversity indexes of beef calves fed different diets (C, T and M; for diet composition see Table 1) during the transition period (28 days) from milk and pasture diet to a high-concentrate ration

The number of reads was normalised to 15 935 reads and $\log_{10}$-transformed (number if reads + 1). Only bacteria taxa that represented on average >0.1% of total sequences are shown. Proteobacteria ratio was calculated as the abundance of Proteobacteria sequences divided by the sum of the abundance of Firmicutes and Bacteroidetes sequences. Different lowercase letters within a row indicate differences between treatments or between days at ($P < 0.05$), as obtained using Bonferroni's test

			Diets				Day					*P*-values		
Phylum	Family	Genus	C	T	M	s.e.m.	0	7	14	28	s.e.m.	Diet	Day	D × D
Actinobacteria			2.52	2.59	2.38	0.094	2.30bc	2.08c	2.93a	2.69ab	0.108	0.30	<0.001	0.98
	Coriobacteriaceae		2.14	2.20	2.09	0.092	2.14	2.03	2.25	2.17	0.107	0.70	0.520	0.84
		Olsenella	1.66	1.92	1.78	0.119	1.61	1.62	1.95	1.98	0.137	0.31	0.102	0.96
		Atopobium	1.09	1.06	1.06	0.132	1.55a	1.32ab	0.40c	1.01b	0.130	0.98	<0.001	0.51
	Bifidobacteriaceae		1.31	1.30	1.15	0.137	0.03b	0.06b	2.73a	2.19a	0.158	0.64	<0.001	0.79
		Bifidobacterium	1.21	1.18	1.03	0.110	0.03c	0c	2.68a	1.86b	0.128	0.48	<0.001	0.70
Bacteroidetes (B)			3.81	3.83	3.85	0.022	3.89a	3.82ab	3.83ab	3.77b	0.025	0.55	0.012	0.97
	Prevotellaceae		3.66	3.66	3.70	0.028	3.62b	3.66ab	3.75a	3.67ab	0.030	0.57	0.017	0.98
		Prevotella	2.73	2.78	2.80	0.079	2.57b	2.76ab	2.95a	2.79ab	0.091	0.83	0.045	0.73
Chloroflexi			0.77	0.70	0.86	0.091	1.64a	1.10b	0.22c	0.15c	0.105	0.45	<0.001	0.60
Fibrobacteres			0.98	1.27	1.35	0.135	2.26a	0.85b	1.03b	0.64b	0.156	0.14	<0.001	0.20
	Fibrobacteraceae		0.98	1.27	1.35	0.135	2.27a	0.85b	1.03b	0.64b	0.156	0.14	<0.001	0.20
		Fibrobacter	0.85	1.14	1.20	0.130	2.15a	0.67b	0.91b	0.54b	0.150	0.13	<0.001	0.29
Firmicutes (F)			3.76	3.76	3.77	0.030	3.81a	3.7a	3.81a	3.63b	0.033	0.99	<0.001	0.70
	Clostridiaceae		2.98	3.05	2.95	0.051	3.19a	3.03a	2.98ab	2.77b	0.056	0.38	<0.001	0.96
		Clostridium	1.94	1.87	1.86	0.079	2.05a	2.01a	2.13a	1.38b	0.085	0.75	<0.001	0.26
	Eubacteriaceae		2.44	2.50	2.41	0.067	2.78a	2.63a	2.08b	2.32b	0.077	0.62	<0.001	0.98
		Eubacterium	1.57	1.56	1.63	0.156	2.00a	1.60a	1.16b	1.59a	0.128	0.95	<0.001	0.78
	Lachnospiraceae		3.16	3.11	3.19	0.045	3.14ab	3.15a	3.24a	3.09b	0.041	0.44	0.045	0.33
		Butyrivibrio	2.04	2.11	1.72	0.114	2.56a	2.52a	1.82b	0.94c	0.132	0.05	<0.001	0.15
		Blautia	1.13	0.99	0.99	0.138	0.90bc	1.20ab	1.41a	0.63c	0.120	0.72	<0.001	0.25
		Catonella	1.08	1.11	1.32	0.097	1.12ab	1.15ab	1.45a	0.97b	0.103	0.19	0.012	0.16
		Coprococcus	0.97	1.15	1.08	0.129	1.67a	1.54a	0.60b	0.46b	0.129	0.62	<0.001	0.81
		Lactonifactor	0.54ab	0.93a	0.48b	0.117	0.28b	0.65ab	1.06a	0.61b	0.117	0.03	<0.001	0.27
		Pseudobutyrivibrio	1.05	1.14	0.97	0.090	1.64a	1.34a	0.66b	0.59b	0.103	0.44	<0.001	0.28
		Roseburia	0.68	0.55	0.82	0.145	0.03b	0.18b	1.26a	1.26a	0.168	0.43	<0.001	0.22
	Lactobacillaceae		1.30	1.52	1.63	0.137	1.71a	1.08b	1.5ab	1.64ab	0.150	0.26	0.019	0.51
		Lactobacillus	0.29	0.36	0.49	0.131	0b	0.02b	0.69a	0.81a	0.135	0.58	<0.001	0.46
	Veillonellaceae		2.02	2.00	2.11	0.106	1.81b	1.78b	2.33a	2.26a	0.113	0.26	0.019	0.51
		Selenomonas	1.46	1.60	1.46	0.152	1.07c	1.28bc	2.04a	1.63ab	0.141	0.77	<0.001	0.77
		Schwartzia	0.31b	0.56ab	0.79a	0.113	0.54ab	0.09b	0.80a	0.78a	0.130	0.02	<0.001	0.70
		Anaerovibrio	0.88	1.11	1.11	0.117	0.36b	1.03a	1.42a	1.32a	0.135	0.29	<0.001	0.90
	Ruminococcaceae		2.83	2.74	2.83	0.096	2.78	2.78	2.98	2.65	0.111	0.76	0.216	0.79
		Ruminococcus	2.11	1.97	2.13	0.127	2.26a	1.78b	2.55a	1.69b	0.146	0.63	<0.001	0.80
	Streptococcaceae		1.48	1.33	1.50	0.123	1.78	2.45	0.96	0.56	0.142	0.57	<0.001	0.70
		Streptococcus	1.42	1.40	1.20	0.123	1.54b	2.42a	0.94cd	0.47d	0.142	0.38	<0.001	0.66
	Acidaminococcaceae		2.22	2.24	2.21	0.051	2.18b	2.43a	2.12b	2.16b	0.059	0.86	<0.01	0.73
		Succiniclasticum	1.70	1.91	1.75	0.102	1.73ab	2.13a	1.70ab	1.57b	0.118	0.32	<0.01	0.80
	Erysipelotrichaceae		2.10	2.35	2.16	0.098	2.63a	2.23b	1.71c	2.23b	0.096	0.21	<0.001	0.32
		Sharpea	0.39	0.38	0.53	0.160	0b	0b	0.85a	0.88a	0.185	0.77	<0.001	1.00
Proteobacteria (P)			2.65	2.62	2.70	0.172	2.44b	3.23a	1.70c	3.27a	0.178	0.95	<0.001	0.95
	Succinivibrionaceae		1.60	1.92	1.77	0.190	1.04b	2.62a	0.87b	2.53a	0.215	0.51	<0.001	0.68
		Ruminobacter	0.65	0.91	0.75	0.168	0.11b	2.31a	0.13b	0.54b	0.181	0.56	<0.001	0.59
		Succinivibrio	0.81	1.09	0.94	0.156	1.00b	0.45b	0.37b	1.97a	0.180	0.47	<0.001	0.76
Spirochaetes			0.98	0.90	1.19	0.167	1.82a	1.17b	0.38c	0.73bc	0.167	0.48	<0.001	0.91
Synergistetes			1.20	1.18	1.13	0.099	1.84a	1.76a	0.53b	0.55b	0.114	0.90	<0.001	0.58
Tenericutes			1.78	1.71	1.79	0.124	2.42a	2.06a	1.40b	1.15b	0.143	0.87	<0.001	0.45
Shannon index			1.96	2.06	1.99	0.078	2.43a	1.93b	1.81b	1.84b	0.092	0.58	<0.001	0.23
Richness			25	27	27	0.91	28a	26b	25b	25b	0.88	0.49	0.01	0.38
Ratio F/B			1.02	0.86	0.94	0.097	0.83	1.04	1.09	0.80	0.116	0.54	0.48	0.45
Ratio P			0.23	0.17	0.20	0.066	0.03b	0.20b	0.07b	0.48a	0.078	0.83	<0.001	0.80

of sampling exerted a significant effect on overall microbial composition ($P < 0.001$; $R^2 = 0.32$). The non-metric multidimensional scaling plot representing Bray–Curtis distance showed that bacterial communities did not cluster by diet; however, it revealed a slight clustering by sampling days, separating Days 0 and 7, whereas samples from Days 14 and 28 exhibited higher heterogeneity (Fig. 1). Analysis of β-diversity showed that dispersion within each dietary group was homogeneous ($P = 0.12$). However, dispersion to the centroid differed among days ($P = 0.011$),

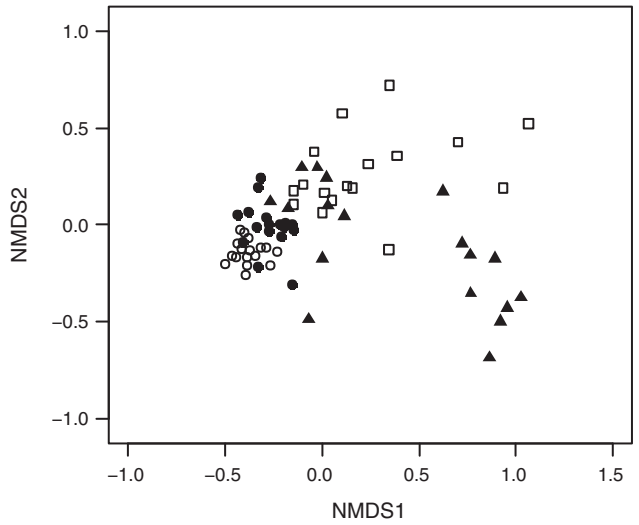

Fig. 1. Non-metric multidimensional scaling (NMDS) ordination plot based on Bray–Curtis dissimilarity showing the relationship among bacterial populations in the rumen of beef calves fed different diets during the transition. Days 0 (○), 7 (●), 14 (□) and 28 (▲) after concentrate inclusion.

and the pairwise comparison showed that dispersion of Day 0 versus Day 28 differed significantly.

The addition of tannins or MCFA did not show any effect on the abundance of the analysed taxa or diversity indexes ($P > 0.10$; Table 3), and, therefore, only differences among days will be mentioned. One week after the shift to a concentrate diet, the most noticeable change compared with Day 0 was an increase of the abundance of Proteobacteria from 2.6 to 15%, with a significant increase of the genus *Ruminobacter* within this phylum (from <0.1 to 6.32%); also, there was a manifest increase of *Streptococcus* on Day 7 (from 0.28 to 5.28%) that recovered initial abundance thereafter. Genera *Bifidobacterium*, *Lactobacillus* and *Sharpea* emerged 14 days after concentrate inclusion irrespective of diet.

Comparing Day 0 with Day 28, the most noticeable change was the decrease of relative abundance of the two major phyla (Bacteroidetes and Firmicutes) with an increase of Proteobacteria and, to a lesser extent, Actinobacteria. In addition, the minor phyla Chloroflexi, Spirochetes, Synergistetes and Tenericutes also decreased ($P < 0.001$) at the end of the study. Relative abundance of Proteobacteria differed across sampling days, showing the highest numerical values on Day 28 (23 ± 5.0%; $P < 0.001$; Table 3). The ratio Proteobacteria (calculated as Proteobacteria/(Firmicutes + Bacteroidetes)) was constant until Day 14, increasing on the last sampling day (Day 28; $P < 0.001$; average value of 0.48; Table 3). The ratio Firmicutes/Bacteroidetes remained stable over time ($P = 0.18$), and was not affected by diet ($P = 0.54$). Compared with Day 0, when animals had been fed only on milk and pasture, at the end of the study there was a different genera composition irrespective of the tannins or MCFA inclusion; for instance, there was a substantial ($P < 0.001$) increase of *Succinivibrio*, *Bifidobacterium*, *Lactobacillus*, *Roseburia*, *Anaerovibrio* and *Sharpea*, and a decrease ($P < 0.001$) in *Butyrivibrio*, *Pseudobutyrivirio*, *Clostridium*, *Coprococcus* and *Fibrobacter*. The Shannon index and richness were only affected by sampling day, and decreased over time ($P < 0.001$; Table 3).

Certain bacterial genera were found to be ubiquitous across all samples throughout the experiment, and therefore, were defined as belonging to the 'bacterial core'. Comparisons were also performed among diets, but there were no differences regarding core taxa. The abundance of the shared taxa in the overall bacterial community was highly diverse, ranging from 0.08 to 64% of total bacteria.

Correlation analysis between relative abundances of the bacterial taxa, diversity indexes and rumen fermentation variables were not consistent across diets (Table 4), except from the Shannon index that was negatively correlated with lactate concentration under the three diets. Interestingly, diets T and M showed similar correlations. Although it did not meet our criteria of considering a valid correlation when $r > 0.50$ or $r < –0.5$, the Proteobacteria ratio showed significant ($P < 0.05$) correlations under the three diets with TVFA ($r = 0.28$, $P = 0.02$), propionate concentration ($r = 0.28$, $P = 0.02$) and rumen pH ($r = –0.32$, $P = 0.01$).

Discussion

Overall, no major effect of the tested additives was found on any of the studied traits. Despite the high fermentability of the diet and the abrupt concentrate inclusion, none of the animals showed pH values <5.5 or lactate concentrations >50 mmol/L that could be indicative of subacute acidosis (Krause and Oetzel 2006). In contrast, irrespective of the diet, concentrate inclusion strongly altered the composition of bacterial population and rumen fermentation. At the end of the study, the rumen ecosystem significantly differed from that at the beginning. These findings agree with other reports on the dynamics of rumen microbial populations in cattle transitioned from high-forage to high-concentrate diets (Fernando *et al*. 2010; Petri *et al*. 2013).

The ratio of Proteobacteria is an indicator of microbial disturbance or dysbiosis when it is >0.19, and it is generally observed after changes in ruminal VFA potentially associated with low rumen pH (Auffret *et al*. 2017). Accordingly, we found that the Proteobacteria ratio was correlated with TVFA concentration ($r = 0.28$) and rumen pH ($r = –0.32$). In addition, irrespective of diet, 39% and 56% of the animals showed a Proteobacteria ratio >0.19 on Days 7 and 28, respectively. Therefore, although animals did not reach acidotic conditions, there was an unbalanced rumen condition that could indicate a selection of other bacteria involved in starch metabolism to adapt to the new rumen environment.

In fact, regardless of the inclusion of additives, and even though diversity decreased, some amylolytic genera emerged after concentrate inclusion. In relation to this, ME intake averaged 54.6 MJ/day, and net energy maintenance requirements for 236 kg (average between initial and final LW) were estimated to be 22.6 MJ/day (ARC 1980). The average metabolisability of the diet was 0.57, hence the efficiency of use of ME for maintenance was calculated as

Table 4. Spearman correlations between bacterial taxa (P, phylum; F, family; G, genus) and Shannon Index, and rumen pH, concentrations of lactate (mmol/L), ammonia (NH_3; mg/L), volatile fatty acids (VFA; mmol/L) and the main individual VFA (mmol/L) in beef calves fed different diets (C, T and M; for diet composition see Table 1) during the transition period (28 days) from milk and pasture diet to a high-concentrate ration

Only Spearman correlations with r > 0.50 and $P < 0.05$ are shown ($n = 72$)

	pH			Lactate			NH_3			Total VFA			Propionate			Butyrate			Valerate			Ratio acetate/propionate		
	C	T	M	C	T	M	C	T	M	C	T	M	C	T	M	C	T	M	C	T	M	C	T	M
G. olsenella		–0.57																						
G. atopobium																								
F. bifidobacteriaceae				0.55												0.50			0.64	0.53	0.62			
G. bifidobacterium				0.59															0.56		0.65			
P. bacteroides			0.55				0.52																	
F. prevotellaceae										–0.54														
P. chloroflexi		0.51			–0.52	–0.61														–0.77	–0.72		0.52	
P. fibrobacteres					–0.62													–0.56		–0.50	–0.61			
P. fibrobacteraceae					–0.62													–0.56		–0.50	–0.61			
G. fibrobacter			0.50		–0.63	–0.51												–0.59		–0.52	–0.60			
P. firmictes								0.62																
F. clostridiaceae								0.51										–0.58		–0.50				
G. clostridium		0.60	0.55															–0.64		–0.51				
G. eubacterium																					–0.52			
G. butyrivibrio		0.68	0.73			–0.60														–0.61	–0.79			
G. blautia			0.56									–0.50												
G. coprococcus		0.66	0.60		–0.55	–0.74												–0.63		–0.74	–0.81			
G. lactonifactor														–0.51										
G. pseudobutyrivibrio		0.60		–0.57												–0.50				–0.55				
G. roseburia	–0.56	–0.63														0.51			0.67	0.58				
G. lactobacillus																			0.51		0.60			
F. veillonellaceae																						–0.51		
G. selenomonas	–0.59			0.64												0.54								
G. anaerovibrio																			0.51			–0.53		
F. streptococcaceae			0.52			–0.54															–0.65			
G. streptococcus																					–0.53			
F. erysipelotrichaceae	0.51			–0.68																				
G. sharpea		–0.53												0.53		0.54				0.56	0.54			
P. spirochaetes					–0.60		0.52													–0.57	–0.69			
P. synergistetes			0.51	–0.51		–0.67														–0.51	–0.78			
P. tenericutes	0.60		0.69	–0.64		–0.83										–0.70		–0.51			–0.69			
Shannon index				–0.50	–0.57	–0.55																		

0.7, which means that only 22.3 MJ of the ingested ME were used for growth. The efficiency of use of ME for growth was estimated to be 0.45, hence only 10.0 MJ of net energy were retained as gains. Considering the net energy retained per kg of gain for the type of animals used in the present experiment (ARC 1980), the predicted ADG was 0.75 kg. Our results showed a higher average value (0.85 kg), which was considered acceptable taking into account a security factor of 10% to be applied to the ARC (1980) predictions. The only reason for these low values was the low intake reached, a typical defence strategy in animals affected by the unbalanced rumen conditions referred to above (Krause and Oetzel 2006; González *et al*. 2012).

In contrast, we identified some bacterial taxa that were ubiquitous in all calves representing the rumen core (Bacteroidetes (including families: Porphyromonadaceae, Flavobacteriaceae, Sphingobacteriaceae, Cytophagaceae and the genus *Prevotella*), Firmicutes (families: Erysipelotrichaceae, Paenibacillaceae, Acidaminococcaceae, Peptostreptococcaceae, Syntrophomonadaceae, Lactobacillaceae, Bacillaceae, Peptococcaceae, Marinilabiliaceae; and the genera *Blautia*, *Butyrivibrio*, *Catonella*, *Clostridium*, *Eubacterium*, *Lactonifactor*, *Moryella*, *Ruminococcus*, *Schwartzia*, *Selenomonas*, *Streptococcus*, *Succiniclasticum* and *Syntrophococcus*), Actinobacteria (genera *Atopobium* and *Olsenella*) and Proteobacteria (family Desulfovibrionaceae, and genus *Succinivibrio*). From minor phyla: Fibrobacteres (genus *Fibrobacter*), Tenericutes (families Spiroplasmataceae and Anaeroplasmataceae), Spirochaetes (genus *Treponema*) and Synergstetes (family Synergistaceae) were also part of the rumen core), which agreed with the bacterial core reported by other authors (Petri *et al*. 2013; Mannelli *et al*. 2019).

Effects of tannin inclusion

Tannins are a very diverse and complex group of polyphenolic plant secondary metabolites that are classically classified into condensed (CT) and hydrolysable (HT), but the variable magnitude of their response irrespective of their chemical structure has created controversy (Mueller-Harvey 2006). Tannins possess the capacity to form complexes with proteins, and to a lesser extent with other macromolecules, such as fibre, starch, nucleic acids, minerals and so on (Makkar 2003; Mueller-Harvey 2006).

In the current study, we did not observe differences in the rate of feed intake or daily gains with the inclusion of 20 g/kg of tannin extract (99 g tannins extract ingested per day) compared with the control diet, although the rate of concentrate intake in the first 4 h after feeding was numerically lower with the tannins diet (422 vs 316 g DM/h for C and T; Table 1). Similar feed intake was observed in steers fed high-concentrate diets with mimosa or chestnut tannins extracts included at 14.9 g/kg DM (Krueger *et al*. 2010) or when quebracho was added at 4 g/kg DM on the concentrate (Mezzomo *et al*. 2011).

By contrast, Rivera-Méndez *et al*. (2017) observed increased intake and ADG in finishing steers supplemented with 6 g tannins/kg DM, regardless of the type of tannins (CT, HT or mixture). Available information with respect to the effects of tannins on rumen fermentation is not consistent, as responses widely differ among plant sources, types (CT or HT) and doses (Mueller-Harvey 2006; Amanzougarene *et al*. 2019). Further, Martínez *et al*. (2006) found different responses to tannin inclusion (50 g/kg DM) in diets with corn or wheat, suggesting that the effect of tannin addition was grain-type dependent (according to the different endosperm architecture). Studies with forage-fed animals including *ca.*20 g CT/kg DM resulted in lower ammonia and branched-chain fatty acids concentration (Carulla *et al*. 2005; Beauchemin *et al*. 2007), and lower VFA concentration (Carulla *et al*. 2005), whereas Díaz Carrasco *et al*. (2017), using a lower dose (2 g/kg of feed), found higher rumen pH in steers supplemented with a blend of HT and CT tannins.

In this and the previous results (Yuste *et al*. 2019), we did not find changes in the ruminal fermentation pattern in response to tannin addition. Similarly, in steers fed high-concentrate diets, the inclusion of 14.9 g/kg DM chestnut or acacia tannins (Krueger *et al*. 2010) or 4 g CT/kg DM (Mezzomo *et al*. 2011) did not exert any effect on rumen fermentation. Interestingly, Salami *et al*. (2018) pointed out that the observed difference in the magnitude of the response between concentrate and forage-based diets on digestion kinetics is one factor to take into consideration, as concentrate diets are rapidly digested compared with forage diets, and therefore, formation of tannin complexes can be limited by time.

Regarding bacterial population, tannins are generally regarded as bacteriostatic and bactericide compounds (Jones *et al*. 1994; Smith *et al*. 2005). Tannin–bacterial interactions are strongly dependent on the type and chemical structure of tannins. In this sense, CT possess stronger inhibition activity than HT (Costa *et al*. 2018; Salami *et al*. 2018). Nevertheless, bacteria can hydrolyse both types of tannins, especially HT (Smith *et al*. 2005), and some bacteria are able to develop resistance mechanisms, such as production of extracellular glycocalyx or an overproduction of tannin-degrading enzymes (McSweeney *et al*. 2001; Smith *et al*. 2005).

Tannins generally inhibit either directly or indirectly proteolytic and fibrolytic bacteria due to their binding capability (Jones *et al*. 1994; McSweeney *et al*. 2001). Jones *et al*. (1994) studied *in vitro* bacterial growth with different levels of sainfoin CT (100–600 μg CT/mL), and observed an inhibition of some proteolytic bacteria (*Butyrivibrio fibrisolvens* and *Streptoccocus bovis*); however, *Prevotella ruminicola* was found to be tolerant, because it developed some mechanisms against tannins.

In the current study, the addition of tannins did not affect bacterial composition, which is directly linked to the lack of effects on rumen fermentation. Some studies with chestnut tannins reported a lack of profound changes or disturbance on the overall structure of bacterial community. For example, Díaz Carrasco *et al*. (2017) with a mixture of 66:33 chestnut and quebracho tannin extract (similar to that used in the present experiment) reported a decreased richness, but intact Shannon index, which indicates that the structure of the community was not affected by tannins. In addition, these authors reported increases in some genera, such as *Succiniclasticum*, *Selenomonas* and *Anaerovibrio.* Further, Mannelli *et al*. (2019) included 16 g/kg chestnut tannins in a forage-based diet for ewes and reported only slight changes

in microbial composition (e.g. higher *Anerovibrio*, *Streptoccocus*, *Pseubutyrivibrio*).

In the present study, the lack of effect of tannins may indicate bacterial hydrolysis of tannins, as HT are more susceptible (Mueller-Harvey 2006) and our extract contained 65% HT, an adaptation of rumen bacteria to tannins (McSweeney *et al*. 2001) or a reduced dose of tannins. The dose used in the present experiment was within the range of doses used in the experiments mentioned above, and the adaptation could be discarded because there were no differences in rumen fermentation compared with the control during the initial days, so a plausible explanation might be the degradation of the tannin source. This should be supported by the lower fermentative effect observed with chestnut tannins compared with other sources (Amanzougarene *et al*. 2019).

Effects of MCFA inclusion

Certain medium-chain fatty acids have been extensively assessed for their potential to interact with rumen microbiota reducing their fermentative activity (Henderson 1973; Dohme *et al*. 2001) and, thus, are used in ruminant diets for preventing ruminal acidosis. The mechanisms for their antimicrobial activity have been suggested to be due to changes in permeability leading to a membrane destabilisation, suppression of enzymatic activities and nutrient intake, and even cell death (Desbois and Smith 2010).

In ruminant feeding, most studies assessing the effect of MCFA on rumen metabolism and microbes have used coconut oil as the most common and practical source of MCFA containing 7.4% (of total fatty acids) of C_8, 6.2% of C_{10} and 47.1% of C_{12} (Dohme *et al*. 2000), rather than individual MCFA. In the present study, diet M contained 6 g of MCFA per kg of feed that resulted in the ingestion of *ca*.31 g of MCFA/d per animal (3.1 g of C_6, 6.2 g of C_8, 6.2 g of C_{10} and 15.5 g of C_{12}). No differences in intake were observed in group M compared with the control (Yuste *et al*. 2019). When coconut oil was included as a source of MCFA in the ration of sheep, Liu *et al*. (2011) observed no change in intake with 25 g/kg (or 37.58 g/day), but decreased intake was noted in beef heifers supplemented with 125, 250 or 375 g/day (Jordan *et al*. 2006), and Machmüller and Kreuzer (1999) also reported decreased intake in sheep when including MCFA at 70 g/day. Intake depression can be due to reduced palatability and/or lower organic matter and neutral detergent fibre (ashes excluded) digestibility. In our study, following the manufacturer recommendations, the dose used was far below those used in the mentioned studies, which can explain the lack of effect.

The effects on rumen fermentation have been related to the type of fatty acid (Dohme *et al*. 2001), doses, experimental approach and nature of diet (forage- vs concentrate- based diets; Machmüller 2006). Hristov *et al*. (2004*a*) reported a decrease in ammonia concentration as a result of MCFA addition to the diet, which might be in part linked to the suppressive effect of MCFA on rumen protozoa. In an *in vitro* study of the effect of the same additive used in the present experiment (at 2, 4 and 6 g/kg DM), Amanzougarene *et al*. (2017) reported a tendency to increased rumen pH, without adverse effects on VFA, after 24 h of incubation of barley. Ajisaka *et al*. (2002), however, reported decreased rumen pH with C_8, C_{10} and C_{12} (0.17–0.67 mg of fatty acid per mL of incubation liquid) compared with the control. The effects of MCFA on total VFA concentration and molar proportions of the main VFA are not consistent. For instance, Dohme *et al*. (2001) evaluated *in vitro* the effect of pure individual MCFA (C_8–C_{16}) at 50 g/kg DM, and the responses on fermentation characteristics differed depending on the type of fatty acid, indicating that each fatty acid interacts differently with microbes, and the same was reported by Hristov *et al*. (2004*b*) with C_6, C_8 and C_{10} (0.625, 1.25 and 2.5 g/L of incubation liquid) and C_{12} (2.5, 5.0 and 10 g/L). *In vivo* results are even more inconsistent due to the different nature of diets, and most of the studies have been performed with the addition of coconut oil or C_{12} (Liu *et al*. 2011; Hristov *et al*. 2012).

In the present study, the addition of a MCFA mixture did not affect the structure of the bacterial population or any bacteria taxon, which is consistent with the lack of effect on rumen fermentation pattern. However, in a parallel study (Yuste *et al*. 2019), we observed that MCFA exerted a transient inhibitory effect on rumen protozoa and, although some studies (Machmüller and Kreuzer 1999) reported that total bacterial counts increase with MCFA, this might be a result of a decrease in protozoal numbers. Nevertheless, the additive used here had 50% of C_{12} (i.e. 15 g C_{12}/day), which is lower than doses used by others, resulting in a depressive effect on rumen bacteria (Hristov *et al*. 2012).

Henderson (1973) and Patra and Yu (2013) reported that MCFA are inhibitory for cellulolytic bacteria, although this effect can be diet-dependent, as found by Dong *et al*. (1997), who observed that cellulolytic activity was not inhibited in concentrate diets, even with 10% coconut oil. In any case, there is no consensus for the general effects of MCFA on bacterial composition. For instance, Hristov *et al*. (2012) intraruminally dosed 240 g/day of C_{12} and reported significant changes within the phylum Firmicutes, and a decrease in *Prevotella*, whereas Yabuuchi *et al*. (2007) did not observe differences in bacteria population with the inclusion (25 or 50 g/day) of C_{12} in a high-concentrate diet for steers. The lack of effect in our study could have been due to microbial adaptation, as some microbes can adapt to MCFA by upregulating the expression of genes encoding proteins involved in cell wall synthesis (Desbois and Smith 2010). However, the lack of effect on rumen fermentation since Day 1 might denote that this MCFA mixture given at 6 g/kg feed was innocuous for bacteria.

Conclusions

An abrupt change from a milk and grass feeding regime to a high-concentrate diet did not impair animal performance or rumen health of beef calves, but concentrate inclusion decreased bacterial diversity, and strongly altered bacterial composition towards increased amylolytic and acid-tolerant species. This fact, together with the microbial adaptation to the new rumen environmental conditions, was likely of vital importance for the animals to cope with the abrupt dietary

shift without going through acidotic episodes. At the doses used in the present experiment, the addition of tannins and medium-chain fatty acids in the adaptation diet of newly weaned beef calves did not negatively affect productivity and did not result in major effects on rumen fermentation or rumen bacterial populations.

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Conflicts of interest

The authors declare no conflicts of interest.

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