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Determination of the optimal priming interval of rumen fluids used as inocula for the *in vitro* digestibility trials through radial enzyme diffusion method

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Abstract

Context. Determination of the neutral detergent fibre digestibility is one of the important parameters to consider when formulating diets. However, the *in vitro* determination shows low repeatability because of the source of rumen-fluid inoculum. Priming of the rumen fluid inocula, obtained through an oesophageal probe, has been proposed to overcome this issue.

Aim. The objective of the study was to investigate the evolution of the microbial enzymatic activities of different rumen fluids during a priming procedure, to establish the fermentation interval that minimises the differences among rumen-fluid degradative potentials.

Methods. Three farms for each type of diet were involved in the study. Rumen fluids were obtained from dry and lactating cows fed the following four diet types: 100% hay or a diet with 80 : 20 forage : concentrate ratio (F : C) as dry-cow diets, and *ad libitum* hay and concentrate, or a total mixed ration (both at 60 : 40 F : C) as lactating-cow diets. On each farm, rumen fluid was collected from three Holstein cows by using an oesophageal probe, and mixed. Two aliquots of each rumen fluid mix were added to the medium containing the same priming substrate in an *in vitro* batch-fermentation system. During the incubation, the fermentation fluids were sampled in duplicate at 0-, 1-, 2-, 4-, 8-, 24- and 48-h intervals. Enzymatic activities of amylase, cellulase and xylanase were determined by radial enzyme diffusion method.

Key results. Initial enzymatic activities were quite variable and increased with an increasing incubation time. By 24 h, amylase showed similar values among high-concentrate diet fermentation fluids, and a lower data dispersion in comparison to the other intervals; cellulase was characterised by similar values in all the fermentation fluids derived from diets including concentrates, and xylanase showed similar activity in the fermentation fluids derived from high-concentrate diets. Development of the enzymatic activity of the fermentation fluids derived from the 100% hay diet differed from the others.

Conclusions. A 24-h priming procedure was needed to stabilise and equalise the enzymatic activity of the rumen fluid from cows fed high-concentrate diets. This was not observed in rumen fluid from cows fed hay-based diets.

Implications. The 24-h-primed rumen fluid can be used to increase the repeatability of neutral detergent fibre digestibility determination.

Keywords: dairy cows, *in vitro* digestibility, nutrient analysis.

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Introduction

Determination of the neutral detergent fibre digestibility (NDFD) is one of the important parameters for the evaluation of both feed and diet nutritional values. However, this technique has no standardised procedure, showing low repeatability (17% of variability of the NDFD at 30 h of fermentation) partially dependent on rumen-fluid consistency (Hall and Mertens 2012). The rumen-fluid activity can, in fact, be affected by several individual and dietary factors (Chaudhry 2008; Kim *et al*. 2019). Additionally, rumen fluid collected with an oesophageal probe contains variable amounts of saliva, which affects microbial dilution and content. To reduce the impact of the rumen-fluid variability on the NDFD assay, Goeser and Combs (2009) proposed priming (pre-incubation) of the rumen-fluid inoculum by using cellulose as substrate (priming substrate), demonstrating that this practice improves the repeatability of the *in vitro* NDFD assay; however, they

found a depression in fibre digestibility. A further experiment was conducted by Goeser *et al*. (2009), where rumen fluid was primed with a carbohydrate–urea mixture and fermented until a specific amount of gas was produced. They found a higher repeatability of the analysis, avoiding, in this case, the NDFD depression, probably as a result of a more complete priming diet. However, the incubation time in the latter experiment was unknown and the criteria used to standardise the rumen fluid was unrelated to a time-point and, consequently, difficult to apply in routine analysis. Moreover, the parameter tested was the cumulative gas production, which is, by definition, time-dependent and it is not a direct expression of the degradative potential of the rumen fluid. The latter is better described by the enzymatic activity (EA) of the rumen fluid, which, in turn, can be considered as a quantitative–qualitative reflection of rumen microbiota (Raghuvansi *et al*. 2007) and, on the basis of data from Dadvar *et al*. (2018) and Morgavi *et al*. (2000), a potential measure of its fermentative capacity.

It has been demonstrated that diet characteristics significantly affect the rumen microbiota and, consequently, the rumen enzymatic profile. Indeed, rumen-fluid carboxymethyl cellulase and xylanase activities have been shown to be lower, whereas the amylase expression is improved in dry cows and heifers fed a high-grain compared with a high-forage diet (Hristov *et al*. 1999). Moreover, an enhancement of the rumen carboxymethyl cellulase and xylanase activities of buffalo rumen fluid has been observed after increasing the concentration of roughages in their diets (Kamra *et al*. 2003).

The EAs can be evaluated by using different methods. Among them, the radial enzyme diffusion (RED) has been applied to quantify the exogenous cellulase, xylanase, amylase and protease in supplemental animal feedstuffs (Walsh *et al*. 2005). Furthermore, this method has been applied to the measurement of non-starch polysaccharide-degrading EAs in the rumen fluid of cows fed corn silage of different particle sizes (Zebeli *et al*. 2008). On the basis of the literature, it appears that RED could be a quick and reliable method for the evaluation of rumen-fluid EAs, allowing for the screening of the degradative capacity of the primed rumen fluids.

With this assumption, and considering the effect exerted by the diet on rumen-microbiota enzyme synthesis, we hypothesise that *in vitro* incubation of different rumen fluids on a common complete priming substrate can reduce the differences among their degradative potentials. Starting from this hypothesis, the present study was designed to investigate the evolution of the microbial EAs of different rumen fluids, obtained with an oesophageal probe, during a priming procedure, so as to establish the fermentation interval that minimises the differences among rumen-fluid degradative potentials.

Materials and methods

The animal procedures were conducted in accordance with the EU Directive 2010/63/EU (European Parliament and Council of the European Union 2010) for animal experiments. The trial involved three farms for each diet type. The rumen fluids were obtained from intact cows fed four type of diets, including two dry-cow diets and two lactating-cow diets (Table 1).

The dry-cow diets were a 100% forage diet (DTH) or a diet with a forage: concentrate ratio (F : C) of 80 : 20 (DFC).

Table 1. Estimated composition of the diets fed to the cows enrolled in the study and chemical composition of the priming diet (PTMR) used as a substrate for incubation

Diet compositions were estimated using the software NDS professional ver. 3.9.6.02 (Rum&n SAS, Reggio Emilia, Italy) on the basis of forages, feedstuffs and total mixed ration (TMR) analysis. Averages have been calculated as arithmetic means of the farm diets grouped under each diet and expressed as means ± s.d. Priming TMR dietary components (% of DM): alfalfa hay 30.07; ryegrass hay 15.06; cornmeal 23.61; corn flaked 7.38; soybean meal 6.72; whole soybean flacked 4.56; beet pulp 5.72; soybean hulls 3.80; wheat straw 1.08; mineral–vitamin supplement 2.00. ADF, acid detergent fibre; ADL, acid detergent lignin; NFC, non-fibre carbohydrates; NDFD24, neutral detergent fibre (NDF) digestibility at 24 h; F : C, forage to concentrate ratio in the diet; DTH, 100% hay fed to dry cows; DFC, 80 : 20 forage : concentrate ratio (F : C), fed separately to dry cows; LFC, 60 : 40 F : C, fed separately to lactating cows; LTMR, TMR fed to lactating cows

Item	DTH	DFC	LFC	LTMR	PTMR
DM (%)	84.98 ± 2.90	85.84 ± 3.02	85.98 ± 0.27	73.73 ± 7.24	63.4
			(% DM)		
Ash	8.71 ± 0.84	9.30 ± 0.77	8.63 ± 0.50	7.37 ± 0.72	8.61
Crude protein	9.93 ± 1.22	9.86 ± 0.59	15.23 ± 2.09	16.08 ± 0.54	16.67
Ether extract	1.56 ± 0.07	1.77 ± 0.04	3.65 ± 1.13	2.43 ± 0.74	3.23
Sugar+pectins	18.56 ± 3.33	15.7 ± 10.28	15.28 ± 0.59	16.98 ± 0.85	11.5
Starch	–	8.14 ± 1.62	17.14 ± 3.82	21.77 ± 3.44	22.77
NFC	18.56 ± 3.33	23.85 ± 1.35	32.42 ± 3.51	38.75 ± 4.24	34.27
NDF	61.23 ± 2.20	55.23 ± 2.05	40.08 ± 6.10	35.37 ± 3.19	37.22
ADF	39.74 ± 4.52	32.42 ± 1.31	25.25 ± 3.64	20.26 ± 3.81	20.28
ADL	7.93 ± 2.78	4.10 ± 1.00	4.07 ± 0.49	4.35 ± 0.16	3.55
Hemicellulose	21.49 ± 3.17	22.81 ± 0.92	14.83 ± 2.64	15.11 ± 0.85	16.94
Cellulose	31.81 ± 2.10	28.32 ± 0.58	21.18 ± 3.16	15.91 ± 3.84	16.73
NDFD24	33.10 ± 6.09	54.70 ± 0.91	47.15 ± 4.49	48.01 ± 1.96	70.83
F : C	100 : 0	80 : 20	60 : 40	60 : 40	

The lactating-cow diets were *ad libitum* hay administered separately from a concentrate distributed by an automatic feeder at an estimated F : C of 60 : 40 (LFC), or a total mixed ration (LTMR) at F : C of 60 : 40. On each farm, individual rumen fluids were collected from a total of three Holstein cows on each diet type, by using an oesophageal probe as described by Cinotti (1987), 3 h after the morning feed delivery. The individual rumen fluids maintained at 39°C were mixed in the laboratory in anaerobic conditions described by Simoni *et al*. (2020). Each rumen-fluid mix was blended, filtered through four layers of cheesecloth and divided into two aliquots. From each of these, a volume of 100 mL of rumen fluid was added at a ratio of 1 : 4 to a medium in a 1000-mL flask containing 5 g of TMR, ground to pass a 1-mm screen, used as a unique substrate (priming substrate). The TMR was a typical hay-based diet formulated for lactating cows employed in northern Italy, similar to those described by Righi *et al*. (2007, 2009) and Comino *et al*. (2015), the composition and formula of which are reported in Table 1.

Each flask containing the rumen-fluid inoculum, the medium and the priming substrate was incubated for 48 h by using an *in vitro* batch-fermentation system similar to that described by Goering and Van Soest (1970). Two 5-mL aliquots of the liquid phase (fermentation fluid) were sampled from each flask at 0, 1, 2, 4, 8, 24 and 48 h after inoculation and centrifuged at 5000*g* for 15 min at room temperature (around 20 to 24°C). The supernatant was collected, filtered through a 0.45-µm porosity PVDF (polyvinylidene fluoridone) syringe filter, and a volume of 0.125 µL of protease inhibitor (Protease Inhibitor Cocktail powder, code P2714-1BTL, Sigma Aldrich, Milano, Italy) was added to prevent enzyme degradation by protease, and the sample was then stored at –20°C for subsequent analysis.

Amylase (Amy), cellulase (Cell) and xylanase (Xyl) activities, as the main representatives of carbohydrate-degrading agents, were tested with the RED method, as described by Walsh *et al*. (2005). The method adopted was subjected to an internal variability evaluation, giving average coefficients of variation of 5.84%, 4.69% and 6.80% for Amy, Cell and Xyl respectively.

The Petri dishes used for the assay contained 0.5%, 0.5% or 0.1% (w/v) of cellulose (cellulose powder from cotton linter, code 22183, Fluka BioChemika, Buchs, Switzerland), starch (soluble starch, code 417585, Farmitalia Carlo Erba S.p.a, Milano, Italy) or xylan (AZCL-Arabinoxylan from wheat, cod. I-AZWAX, Megaxyme, Wicklow, Ireland), as substrates. Each enzyme substrate was solubilised with 1.5% (w/v) agar (Agar N°1, code LP001, Oxoid, Milan, Italy) in the proper buffer, represented by 100 mM Na-acetate, pH 5.0 for cellulase; 100 mM Na-acetate, pH 4.8 for α-amylase; and 100 mM Na-citrate, pH 5.3 for xylanase. Gelation was obtained by heating at 100°C for 12 min.

Four aluminium cylinders had been previously placed in each Petri dish, to create the wells for the fermentation-fluid inoculation. The gel was cooled to 50°C and a volume of 20 mL was poured into each Petri dish (90-mm diameter) while vigorously stirring, yielding a gel depth of 3 mm. Circular wells (diameter 10 mm) were obtained by removal of the aluminium cylinders from the solidified agar.

Cellulase and Amy analyses were repeated in quadruplicate for each interval sample in different Petri plates, whereas Xyl was measured in duplicate. After the substrate and the Petri dish preparation, the frozen supernatant aliquots previously obtained were thawed at room temperature and a volume of 300 µL was poured in each well of the agar. The plates were then incubated for 16 h at 50°C for Cell and Amy measurements, and at 37°C for Xyl determination. Cell and Amy hydrolyses were shown by staining. Plates were flooded with 0.2% (w/v) I_2 in 2.0% KI staining solution for 15–20 min for Cell detection, and 1 : 40 diluted Lugol solution was put in contact with the plate for 5 s, so as to detect Amy; both procedures were followed by multiple rinses with water. No staining was needed for Xyl hydrolysis halo detection, since halos were already evident after incubation.

The halo of hydrolysis generated by the enzymes in the supernatant was measured through photographic digitalisation of the plate surface, followed by image measurement performed through the Meazure™ 2.0 software (C Thing Software, Sunnyvale, CA, USA). The well area was then subtracted from the total area of the halo circle, and the result was corrected accounting for the dry matter of the rumen fluid after filtration through the syringe filter. Results are, thus, expressed as the corrected area of the surface of the halo (mm^2).

All statistical analyses were performed using the SPSS for Windows software package (ver. 25.0; SPSS Inc., Chicago, IL, USA). The differences in Cell, Amy and Xyl EAs in the rumen fluid among the diet types were tested separately by using the repeated-measure procedure of the general linear model, with diet type as a fixed factor and priming intervals as repeated measures. Farm, which represented the experimental unit, was considered a random effect. Differences were declared significant at $P \leq 0.05$. Results are reported as least-square means.

Results

The EAs of the different fermentation fluids were quite variable at the beginning of the priming process (Table 2).

Similar concentrations of Amy were initially found in the DTH and DFC fermentation fluids; however, they were significantly ($P \leq 0.001$) lower than those in the LFC and LTMR fermentation fluids, whose Amy concentrations were, in turn, similar. All fermentation fluids completely differed for this EA after 1 h of fermentation with the priming diet. The LFC and LTMR fermentation fluids showed the peak of Amy activity between 2 and 4 h of incubation, while DTH and DFC fermentation fluids expressed the maximal Amy EA after 8 h from the starting point. By 24 h, a significant decline was observed, followed by a significant increase at 48 h of fermentation. Similarity in the maximal Amy EA among the fermentation fluids was observed after 8 h of incubation, when the concentration in the fermentation fluid from DTH was similar to that from the LFC, and that from the DFC fermentation fluid appeared similar to that from LTMR, and all values fell within a narrow range (from 233.37 to 272.11 mm^2). A 24-h priming procedure was needed to equalise the fermentation fluids from lactating-cow diets

Table 2. Development of the enzymatic activities (expressed as area of hydrolysis) of the rumen fluids (RFs) derived from cows fed four type of diets
DTH, 100% hay fed to dry cows; DFC, 80 : 20 forage : concentrate ratio (F : C), fed separately to dry cows; LFC, 60 : 40 F : C, fed separately to lactating cows; LTMR, total mixed ration (TMR) fed to lactating cows; I, interval. Means within a row without a common lower-case letter differ significantly at $P = 0.05$; means within a column without a common upper-case letter differ significantly at $P = 0.05$

RF	Interval (h)							s.e.m.	RF	*P*-value	
	0	1	2	4	8	24	48			I	RF × I
					Amylase						
DTH (mm^2)	135.61aA	197.81bB	220.65cB	231.88cdA	260.13eB	200.03bC	234.38dD	3.236	<0.001	<0.001	<0.001
DFC (mm^2)	135.84aA	180.91cA	183.75cA	216.99dA	233.3eA	141.54aA	153.15bA	3.203			
LFC (mm^2)	225.93cB	265.25dD	280.86efC	284.74fC	272.12deB	171.65aB	186.63bB	3.527			
LTMR (mm^2)	223.40cB	244.21dC	269.51eC	268.55eB	242.63dA	171.20aB	202.42bC	3.542			
s.e.m.	4.478	4.393	4.726	4.747	4.193	2.885	3.997				
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001				
					Cellulase						
DTH (mm^2)	202.79aA	356.85bA	397.45cA	352.34bA	353.05bA	415.26cB	504.15dC	7.767	<0.001	<0.001	<0.001
DFC (mm^2)	278.33aB	423.87deB	415.96dB	388.59cB	437.18eB	291.19aA	317.13bB	4.639			
LFC (mm^2)	371.42dC	476.51fC	479.53fC	403.13eB	350.46cA	290.91aA	325.31bB	5.031			
LTMR (mm^2)	499.92cdD	497.94cdD	550.85eD	509.27dC	481.88cC	306.67bA	286.23aA	6.246			
s.e.m.	9.917	5.301	5.866	5.760	6.665	8.519	9.716				
P-value	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001				
					Xylanase						
DTH (mm^2)	44.71aA	116.41bAB	100.24bA	123.48bA	119.42bA	254.15cC	338.86dB	11.974	<0.001	<0.001	<0.001
DFC (mm^2)	42.60aA	115.44bAB	176.52cdB	125.78bA	158.43cB	140.56bcA	191.56dA	7.016			
LFC (mm^2)	91.29aB	173.37bcC	220.19dC	198.02cdB	143.63bAB	188.14cdB	197.77cdA	5.698			
LTMR (mm^2)	120.54aC	149.44bBC	158.29bB	205.39cdB	190.73cC	176.37bcAB	190.85bcA	7.493			
s.e.m.	8.302	7.914	10.518	8.640	7.864	10.140	14.686				
P-value	≤0.001	0.002	≤0.001	≤0.001	≤0.001	≤0.001	≤0.001				

(LFC and LTMR), while it failed to equalise the other two types of fermentation fluids ($P \leq 0.001$). At 48 h, all fermentation fluids showed different Amy concentrations and both DTH- and DFC-derived fermentation fluids showed a higher Amy concentration than was the initial value, whereas LFC and LTMR fermentation fluids showed a lower Amy concentration than in the initial inoculum ($P \leq 0.001$).

Initial values of Cell activity differed among rumen fluids, with values increasing from DTH to LTMR ($P \leq 0.001$). A peak of Cell activity was generally found after 2 h of incubation, and a further rise was observed at 8 h for the DFC diet. A subsequent general decrease from the initial values was observed in DFC and LFC fermentation fluids. An opposite trend was found for Cell activity of the fermentation fluid from the DTH diet, which increased over time and was similar to the value of the fermentation fluid from the LTMR by the end of the treatment. The initial differences among the rumen fluids were maintained up to a 2-h interval, while starting from the 4 h, at least two fermentation fluids per interval were similar for Cell activity. At 24 h of fermentation, the Cell concentration appeared similar for the DFC, LFC and LTMR diets, which all differed from that of the DTH ($P \leq 0.001$), which showed the highest value. This difference was maintained at 48 h, when also the reduction in Cell EA became significant for the fermentation fluid from the LTMR-fed cows.

The Xyl EA of DTH and DFC fermentation fluids was initially similar, but significantly ($P \leq 0.001$) lower than that of the LFC and LTMR fermentation fluids, which also differed from each other ($P \leq 0.001$). The general trend was for an increase of the activity of Xyl during the experiment; so, at the end of the trial, Xyl concentrations of all fermentation fluids were higher ($P \leq 0.001$). A peak of Xyl activity was found between 1 and 4 h of fermentation in all cases. Of interest, from 24 h onward, the rumen fluid from DTH-fed cows showed a higher production of Xyl than did the other fermentation fluids ($P \leq 0.001$). The fermentation fluids from the two lactating-cow diets were equal at 24 h. At 48 h of priming, DFC-, LFC- and LTMR-derived fermentation fluids were almost identical for Xyl activities, with values of ~190 mm^2. A higher level of EA of the DTH fermentation fluid was observed with Cell as well, at the same intervals.

Discussion

The present work is focused on establishing a procedure to standardise rumen fluid for use in forage-digestion studies, especially rumen fluid obtained through the oesophagus. This procedure becomes necessary where no information is provided about both the donor-cow diet and the animals themselves, or when an approved donor cow is not available as a result of the legal constraints on animal welfare (Directive 2010/63/UE).

The priming procedure has been demonstrated to be promising, but several aspects still need to be clarified. Among them, the optimal priming time needs to be estimated. The time period evaluated in the present study was based on the measurement of the degradative potential of the rumen fluid as a preliminary step for further studies involving the determination of the NDFD repeatability.

Consistent with literature, in the present trial, rumen fluids derived from the different diets were characterised by different initial EAs, and underwent subsequent modifications due to the

adaptation to the priming diet. Similar results have been found in dry cows and heifers (Hristov *et al*. 1999), wethers (Wolff *et al*. 2017) and buffaloes (Kamra *et al*. 2003; Agarwal *et al*. 2004). In contrast, no significant observations were found in rumen-fluid EAs by Sinha *et al*. (2017), who changed the ratio of the concentrate mixture to wheat straw and green maize from 60 : 20: 20 to 40 : 30 : 30 and to 20 : 40 : 40 in the diets of buffalo and cross-bred cattle respectively. However, it should be noted that the EAs were tested in the latter study after a long adaptation period to a new diet, which could have allowed for the re-equilibration of the ruminal microbiota and, consequently, of the EAs.

The initial higher Amy concentration found in fermentation fluids from cows fed concentrate diets is consistent with the literature, since higher Amy concentrations have been found in dairy cattle fed high-grain than in those fed high-roughage diets (Huhtanen and Khalili 1992; Martin and Michalet-Doreau 1995; Hristov *et al*. 1999). The same concept applies longitudinally during the fermentation period, with a general increase in Amy EA in the initial phases of fermentation being related to a higher starch content of the priming diet than that of the diets fed to the donor cows. The rise in the Amy activity was more pronounced and delayed in DTH- and DFC-derived fermentation fluids, probably because of the initial lower abundance of amylolytic bacteria. In addition, the change in the activity of Amy was less pronounced in the fermentation fluids from LFC- and LTMR-fed cows, and this is consistent with a possible higher abundance of amylolytic bacteria in the initial inoculum. The subsequent dynamic of Amy can be attributable to the gradual decrease in the amount of rapidly degradable starch in the system, followed, at 48 h of fermentation, by the degradation of the most crystallised forms of starch (Trináctý *et al*. 2016). On the basis of the least-square mean values, it seems that the maximum similarity was at 8 h, while the lower variability was measured at 24 h.

The initial Cell EA showed an increase from the DTH to the LTMR diet, indicating a relationship between the Cell EA and the increased amount of dietary non-fibre carbohydrates and concentrates fed to the donor cows. This is in agreement with findings from Ding *et al*. (2014) who observed that incrementally increasing the proportion of concentrates in steer diets (from 30% to 90%) induced a linear increase in both carboxymethyl cellulase and xylanase activities in the steer rumen fluids. Furthermore, a tendency for higher activities in both carboxymethyl cellulase and xylanase enzymes was observed in the rumen of the steers fed the TMR diet, when compared with those fed a diet where roughages and concentrates were fed separately (Ding *et al*. 2014). This probably relates to the fact that the TMR system improves ruminal environment with higher activities of fibrolytic (Cell and Xyl) enzymes (Li *et al*. 2003). Cellulase and hemicellulase are released in the rumen from fungi, protozoa and bacteria, which have generation times of 24–32 h, 8–36 h and ~20 min respectively (Hobson and Stewart 1997). On the basis of this information and the observations from Wang and McAllister (2002), the first peak of Cell activity can be attributed to bacterial enzymatic production, while the second can be connected to the proliferation and activity of protozoa and other slow-growing microorganisms in the rumen, generating the two waves of Cell activity clearly evidenced with the DFC diet. Moreover, on the basis of Belanche *et al*. (2012), consumption of high-fibre diets increases the bacterial and fungal number and diversity, with a consequent high concentration of cellulolytic microorganisms. This is probably the case of the DTH-derived fermentation fluids which, incubated in the buffered environment of the fermenters with a highly digestible complete diet as substrate, could have found the better conditions to release a higher amount of Cell and Xyl, as evidenced by the concentrations of these enzymes at 24 and 48 h. This is also supported by the study from Baba *et al*. (2017), who showed that the relative abundance of *Ruminococcus albus* and *flavefaciens*, known as cellulolytic, cello-oligosaccharolytic and xylanolytic bacteria, increased, improving the cellulose and hemicellulose degradation rates.

Surprisingly, the high-forage diets (DTH and DFC) induced a lower initial Xyl activity than did the diets including more concentrates in their formula (LFC and LTMR). These results are in contrast with data from Martin and Michalet-Doreau (1995), who found significantly higher ruminal carboxymethyl cellulase and xylanase activities in cows fed *Dactylis glomerata* hay than in those receiving 65% hay and 35% pelleted ground barley. Moreover, Xyl activity in ruminal content from cattle fed a high-forage diet was 2.5 times higher than that in the rumen content from cattle fed a high-grain diet also in the study from Hristov *et al*. (1999). During the priming fermentation, an increase in Xyl activity was observed in all cases. Of interest was the higher activity level of both Xyl and Cell found in the DTH-derived fermentation fluid after 24 h of incubation. This can be related to the potential prevalence of fibrolytic bacteria in the initial phase of fermentation, given the complete absence of concentrates in the DTH diet, and to the increase in the NDFD, to which the rumen fluid was exposed moving from the DTH to the priming diet. The lack of non-fibre carbohydrate bacteria could have induced a faster growth of the fibrolytic bacteria on the priming diet that likely generated higher activity levels of Cell and Xyl. Similarly, higher carboxymethyl cellulase and xylanase activities have been observed *in vitro* by Agarwal *et al*. (2004), when maize was used as an alternative substrate to lucerne, probably due to its higher digestible-fibre content. In reference to the latter study, both carboxymethyl cellulase and xylanase activities in sheep rumen fluid increased significantly when forages transitioned from alfalfa hay to corn stover (Xie *et al*. 2018). Moreover, consistent with our *in vitro* observations from the DTH diet, both carboxymethyl cellulase and xylanase activities were greater 23 h after the meal in a study of Martin and Michalet-Doreau (1995). Effective degradation of plant cell-wall polysaccharides occurs at the later stages of the post-prandial period (Argyle and Hespell 1987), after the soluble carbohydrate utilisation and the build-up of the fibrolytic population. The DFC, LFC and LTMR fermentation fluids showed similar activities for Xyl after 48 h of incubation, while those derived from the DTH diet showed a completely different activity for the Xyl, which increased continuously during the fermentation period.

Concerning the ideal priming fermentation interval, the results showed that especially when the rumen fluids were derived from cows fed diets with at least 40% of concentrate, 24-h interval is needed for a complete equalisation of the EAs. At this time point, Amy activity showed similar values among the fermentation fluids derived from cows fed high-concentrate diets, and a lower data dispersion; Cell was characterised by similar values in all fermentation fluids from cows fed diets including concentrates (DFC, LFC and LTMR) and Xyl showed similar activity in the fermentation fluids derived from cows fed high-concentrate diets, which were comparable to the DFC-derived fermentation fluid (20% of concentrate in the ration). It should be highlighted that there was a clear difference between the EA evolution of the fermentation fluid derived from the cows fed diet comprising only hay (DTH) and that derived from cows fed diets including concentrates. The priming procedure was able to equalise the EAs of rumen fluids originating from diets containing at least 20% concentrate, while it failed to equalise rumen fluids from total hay-based diets. However, so as to comply with rules on animal welfare, several laboratories collect rumen fluids at the slaughterhouses that usually receive cows that are fed diets containing concentrates. High-energy diets are, in fact, needed to satisfy the high nutrient requirements of the high-producing dairy cows; so, it is rare that slaughtered cows would be fed only hay and, thus, our results can have a direct application in the real situation. Moreover, from a practical point of view, the 24 h of fermentation represent also a favourable interval for the laboratory routine, since it allows for a distribution of the work over 2 days when a digestion trial has to be started after priming.

Further studies should be conducted to test the fermentation fluids primed for 24 h as inocula for *in vitro* digestibility, so as to verify the actual impact of the priming procedure on the NDFD assay in terms of lag time, digestibility at specific intervals and repeatability. Moreover, it will be important to test the real relationship between the primed fermentation fluid EAs and their content of active microbes available for colonising other samples. In the standardised fermentation fluid, microbes could be attached to the priming substrate and, after filtration, the ability of measuring NDFD would rely on the presence of daughter cells to be harvested within the liquid phase. High enzymatic concentration suggests a high and healthy rumen microbe population, but it might not demonstrate the availability of active bacteria able to colonise the samples to be tested for NDFD.

Conclusions

The rumen fluids of different dietary origin can significantly modify their EAs during the *in vitro* priming procedure. These variations are dependent on the characteristics of the diet from which the rumen fluids originate and the fermentation interval considered. In general, using a complete diet as a priming substrate, the fermentation fluids derived from high-fibre diets improved their EAs during the fermentation, while fermentation fluids derived from cows fed low-fibre diets showed decreasing EAs during the incubation. Xyl was improved by the priming process in all cases.

The priming procedure was able to equalise the EAs of rumen fluids originating from diets containing at least 20% of concentrate, while it failed to equalise rumen fluid from total hay-based diets. Despite some similarities in the degradative potential of the different fermentation fluids, the 24-h priming interval is needed for a complete equalisation of the EAs of the fermentation fluids derived from diets containing concentrates.

Conflicts of interest

The authors declare no conflicts of interest.

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