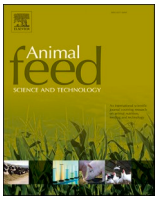
Animal Feed Science and Technology 309 (2024) 115900

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Comparison of ruminal fermentation characteristics of two common forages using a coupled *in vivo-in situ* approach and the *in vitro* rumen simulation technique RUSITEC

J.-H. Deitmers [a,1], T. Hartinger [b], N. Gresner [a,2], K.-H. Südekum [a,*]

[a] *Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany*
[b] *Institute of Animal Nutrition and Functional Plant Compounds, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria*

ARTICLE INFO

Keywords:
In sacco
Replacement method
Ruminant
Silage

ABSTRACT

The increasing demand for a reduction of animal experiments when studying rumen fermentation has led to the development of various *in vitro* techniques, such as the rumen-simulation technique (RUSITEC) system that is prominently applied in European ruminant research. Yet, comparability with the *in vivo* situation is rather less explored with a sparse data basis. Therefore, the present study aimed to directly compare the fermentation characteristics and degradability of two common forages, *i.e.*, grass silage (GS) and maize silage (MS), by parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. Both forages were incubated in the RUSITEC system as well as fed to rumen-cannulated sheep in six independent runs of 20 days in total with 14 days of adaptation and 6 days of sampling. The degradability coefficients of dry matter, organic matter and acid detergent fibre were affected by the method (each $P < 0.05$), while neutral detergent fibre (aNDFom) degradability was not different between RUSITEC and *in situ* measurements ($P = 0.10$). Likewise, Pearson correlation coefficients confirmed the comparability of *in vitro* and *in situ* values for aNDFom degradability, being 0.54 ($P = 0.04$) and 0.78 ($P = 0.02$) for GS and MS, respectively. Regarding the fermentation profile, total volatile fatty acid (VFA) concentrations were much higher *in vitro* than *in vivo* ($P < 0.01$), likely due to the missing absorptive capacity of the RUSITEC system. A comparison of absolute fermentation values between methods appears not feasible. However, the order of individual VFA proportions was similar between *in vivo* and *in vitro* and the correlations for both total and individual VFA further supported this congruency, especially for MS. The *in vitro* data appeared well comparable to the data from the coupled *in vivo-in situ* approach, especially for MS, with a high reproducibility in both methods. Therefore, the RUSITEC system may represent a sufficient replacement for laborious *in vivo* and *in situ* measurements when assessing nutrient degradability and general fermentation characteristics of feedstuffs. Adjustments in *in situ* incubation times as well as the frequently requested standardization of the operation of the RUSITEC

Abbreviations: ADF, acid detergent fibre; ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat-stable amylase and expressed exclusive of residual ash; ADL, acid detergent lignin; DM, dry matter; GS, grass silage; MS, maize silage; NDF, neutral detergent fibre; RUSITEC, rumen simulation technique; WSC, water-soluble carbohydrates; VFA, volatile fatty acid.

* Corresponding author.
E-mail address: ksue@itw.uni-bonn.de (K.-H. Südekum).
[1] Present address: Federal Office for Agriculture and Food (BLE), 53168 Bonn, Germany.
[2] Present address: Ministry of Agriculture and Consumer Protection, State of North Rhine-Westphalia, Stadttor 1, 40219 Düsseldorf, Germany.

https://doi.org/10.1016/j.anifeedsci.2024.115900
Received 7 August 2023; Received in revised form 17 January 2024; Accepted 23 January 2024
Available online 26 January 2024

system may further increase the significance of this *in vitro* method in the future. Likewise, further research on diurnal fermentation patterns is encouraged to substantiate the present findings.

1. Introduction

Due to ethical as well as workload reasons, various *in vitro* techniques that allow the reduction of animal experiments to study rumen fermentation have been developed and continuously improved over the last decades (GfE, 2017; Deitmers et al., 2022). However, comparability to *in vivo* results constitutes a prerequisite for the meaningfulness of any *in vitro* system, which yet often remains to be evaluated. The *in vitro* methods developed in the past comprise a large variety of systems with different purposes, including short-term batch culture systems such as the Hohenheim gas production test as well as elaborated continuous or semi-continuous culture systems that enable the long-term simulation of the rumen (Slyter et al., 1964; Weller and Pilgrim, 1974; Hoover et al., 1976; Czerkawski and Breckenridge, 1977). In contrast to *in vivo* experiments, compared to *in vivo* feeding trials, *in vitro* systems facilitate the investigation of a large number of treatments in a relatively short period of time. In particular complex long-term *in vitro* systems have received broader attention as they provide a closer and more realistic simulation of the *in vivo* situation that may in turn improve the significance of results obtained and their transferability to the *in vivo* situation in the rumen.

Among these complex long-term *in vitro* systems, the rumen simulation technique (RUSITEC) of Czerkawski and Breckenridge (1977) represents a frequently used and well accepted method for pursuing research questions on rumen fermentation, especially in Europe (*e.g.*, Boguhn et al., 2006; Hildebrand et al., 2011a, 2011b; Hartinger et al., 2019a). However, studies directly comparing *in vivo vs. in vitro* , *i.e.* to the RUSITEC system, are yet sparse and the few available studies used either pooled inoculum for the *in vitro* incubation or varying numbers of animals for the *in vivo* and *in vitro* trials (Carro et al., 2009; Martínez et al., 2010). A direct animal-individual comparison with the RUSITEC system, which not only provides a comparison of *in vivo vs. in vitro* but also information on the variation of results, is lacking so far. Consequently, there is a clear demand for a reliable data basis and our objective was to directly compare the fermentation characteristics and degradability of two common forages by parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. We hypothesized that the *in vitro* RUSITEC system provides similar results in terms of fermentation characteristics and forage degradability as obtained in sheep. Further, as the experiment was designed as an animal-individual comparison with the RUSITEC system, we also expected a low variation within sheep and RUSITEC vessels.

2. Materials and methods

The experimental animals were kept according to the German Animal Welfare legislation at the Institute of Animal Science of the Faculty of Agriculture, University of Bonn in Germany. All experimental procedures were conducted in accordance with the German guidelines for animal welfare and were approved by the Animal Care Committee of the State of North Rhine-Westphalia in Germany (file number 81-02.05.40.18.008).

2.1. Experimental diets

Maize silage (MS) and grass silage (GS; pure stand of Italian ryegrass, *Lolium multiflorum*) used in this study originated from bunker

Table 1
Chemical composition of grass silage and maize silages (g/kg dry matter unless otherwise stated).

	Grass silage	Maize silage
Dry Matter, g/kg	357	377
Organic Matter	877	967
Crude protein	125	67.2
Ether extract	28.6	32.1
aNDFom[a]	469	325
ADFom[b]	312	185
ADL[c]	28.3	19.6
Starch	not analysed	451
WSC[d]	94.2	11.2
pH	4.37	3.64
Lactic acid	70.0	76.4
Acetic acid	20.5	13.4
Ammonia-N, g/kg N	125	167

[a] aNDFom = Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.
[b] ADFom = Acid detergent fibre expressed exclusive of residual ash.
[c] ADL = Acid detergent lignin.
[d] WSC = Water-soluble carbohydrates.

silos or round bales of the Campus Frankenforst of the Faculty of Agriculture, University of Bonn (Königswinter, Germany; 7° 12′ 22′ E; 50° 42′ 49′ N). After an ensiling period of 100 days and opening of bunker silos and round bales, the material was re-ensiled in 120 L plastic barrels for better handling during the trial period and to ensure the provision of unspoiled silage. The chemical composition of both forages is presented in Table 1. Due to a deficiency in crude protein concentration, the MS was supplemented with urea by addition of 15 g urea-N into the ration as well as by inclusion of 0.535 mg urea-N per mL into the buffer solution for the sheep and the RUSITEC system, respectively. Therefore, both forages were provided isonitrogenously.

2.2. Coupled in vivo-in situ approach

Six Merino- and Rhönschaf × Suffolk crossbreed sheep (mean body weight 85.1 ± 17.3 kg) fitted with permanent ruminal cannulas were used for the experiment and were fed at 120% of maintenance energy requirements. Drinking water were continuously available during the complete experiment. The experiment comprised six independent runs of 20 days in total with 14 days of adaptation period and 6 days of sampling period. Consequently, GS and MS were fed in three runs each in an alternating mode. In each run, GS or MS supplemented only with NaCl were offered to three sheep each twice a day for one hour (0730 h and 1930 h). The individual feed intake was calculated by weighing the feed before and after feeding for each animal.

During the complete experimental period, 10 mL of ruminal fluid was collected *via* the standardized procedure of Tafaj et al. (2001) from each sheep before the morning feeding to immediately measure the rumen pH *via* potentiometry (BlueLine 14 pH, SI Analytics, Mainz, Germany, and pH 315i, WTW, Weilheim, Germany). During the sampling period (experimental days 15 to 20), additional ruminal fluid samples were collected directly before the morning feeding, for VFA and ammonia-N analyses and directly stored at − 18 °C. On experimental days 16 and 18, *in situ* incubations were performed according to standardized protocol of Kirchhof (2007) and Südekum (2005). Briefly, three nylon bags (100 mm × 200 mm, 50 μm pore size, ANKOM Technology, Macedon, NY, USA) containing approximately 5 g DM of the respective silage were incubated for 48 h in the rumen of each sheep. Before insertion into the rumen, bags were incubated in a water bath at 39 °C for 15 min and then placed below the fibre mat in the rumen and fixed with a nylon cord to avoid displacement. After removal from the rumen after 48 h, bags were rinsed with cold water and stored at − 18 °C until further analysis.

2.3. In vitro RUSITEC system

In order to allow a comparison with the coupled *in vivo-in situ* approach, the same silage fed to sheep during one respective run was simultaneously incubated in the RUSITEC system. Therefore, again six independent runs of 20 days in total with 14 days adaptation period and 6 days sampling period were performed simultaneously in the RUSITEC system (Czerkawski and Breckenridge, 1977). Comprehensive information on setup, implementation and daily routine of the six-vessel RUSITEC system are given in detail in Hartinger et al. (2019a). In brief, each sheep was assigned to one vessel, and the liquid and solid inoculum was obtained individually from each sheep on day 0 before the morning feeding, strained through two layers of cheesecloth and immediately transferred to the corresponding vessels within 30 min. Each day, 14.6 g DM MS (chopped to 6–8 mm particle length) or 16.8 g DM GS (chopped to 5 cm particle length) filled in nylon bags (140 × 80 mm, 1000 μm pore size, Klein & Wieler oHG, Königswinter, Germany) were incubated in the vessels. The nylon bags were incubated for 48 h and washed with 50 mL pre-warmed buffer solution after removal. The liquid effluent was collected in cooled glass containers and volumes were measured daily.

During the sampling period, analogous to the coupled *in vivo-in situ* approach, 10 mL of fluid were taken *via* a 3-way-valve from each vessel prior to the nylon bag exchange. Then, pH was directly measured by potentiometry (BlueLine 14 pH, SI Analytics, Mainz, Germany, and pH 315i, WTW, Weilheim, Germany) and aliquots for VFA and ammonia-N analysis were stored at − 18 °C. Likewise, nylon bags with feed residuals of days 16 and 18 were stored at − 18 °C until further analysis. Again, pH was measured immediately and samples for VFA and ammonia-N were frozen at − 18 °C until further analysis.

2.4. Sample analyses

The silages and feedstuff residues of the *in situ* and RUSITEC incubations from days 16 and 18 were freeze-dried (P18K-E- 6, Piatkowski, Petershausen, Germany) and milled through a 3 mm screen (RETSCH SM 100, Retsch, Haan, Germany). Subsequently, samples were analysed according to German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA, 2012). The DM concentration was determined according to method 3.1 and the values were subsequently corrected for the loss of volatiles during drying using the equation of Weißbach and Strubelt (2008a) for GS and Weißbach and Strubelt (2008b) for MS. The ash and ether extract concentrations as well as the concentrations of neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom), acid detergent fibre expressed exclusive of residual ash (ADFom) and acid detergent lignin (ADL) were analyzed according to methods 5.1.2, 8.1, 6.5.1, 6.5.2 and 6.5.3, respectively. For the calculation of OM, the ash content was subtracted from the DM of the feed residues. Furthermore, pH as well as concentrations of water-soluble carbohydrates, lactic acid, acetic acid and ammonia-N were determined in cold-water extracts prepared from 50 g of fresh GS and MS as outlined in Hartinger et al. (2019b). The apparent disappearance of DM, OM, aNDFom and ADFom during *in situ* and *in vitro* incubation was calculated from the difference between the amount of DM, OM, aNDFom, ADFom before the incubation and the amount recovered in 48 h samples.

The analysis of VFA (*i.e.* acetate, propionate, n-butyrate, n-valerate, n-caproate, isobutyrate and isovalerate) was performed *via* gas chromatography (GC-2014, Shimadzu, Duisburg, Germany) as described in Hartinger et al. (2019a). Briefly, samples were thawed on ice and centrifuged at 20,000 × g for 15 min. The supernatant was transferred into a new tube, mixed with 100 μL of internal standard

(formic acid) and centrifuged again. Subsequently, VFA concentrations were determined in the clear supernatant. The ammonia-N concentration was determined by Kjeldahl method *via* automated distillation (Vapodest 50 s carousel, Gerhardt, Königswinter, Germany) according to Keay and Menage (1969).

2.5. Statistical analysis

One sheep was excluded from the trial due to impaired health condition before the experimental start and both, the *in vivo-in situ* and the *in vitro* data set, were therefore reduced. Data were checked for normal distribution using the Shapiro-Wilk test of the UNIVARIATE procedure in SAS v9.4 (SAS Institute Inc., Cary, NC, USA). Subsequently, datasets were statistically analysed using the repeated-measurements MIXED model procedure in SAS v9.4 to test for the effects of method and forage, the following model was used:

$$Y_{ijkl} = \mu + M_i + F_j + L_k + T_l + (M \times F)_{ij} + e_{ijkl}$$

where Y is the observed response, μ is the overall mean, M_i is the fixed effect of method (*in vivo-in situ* or *in vitro*), F_j is the fixed effect of forage (MS or GS), L_k is the random effect of experimental run, T_l is the random effect of vessel or animal and e_{ijkl} is the residual error. If significant interaction effects were observed, differences between forages within method were analysed using the post-hoc Tukey test. Significance was defined at $P < 0.05$ and a trend at $0.05 \leq P \leq 0.1$.

Additionally, Pearson correlation coefficients between *in vivo-in situ* and *in vitro* data sets were calculated using proc CORR in SAS v9.4 (SAS Institute Inc., USA). The coefficients were ranked according to a scale presented by Akoglu (2018) to enable a uniform interpretation of their strengths. Strong and moderate correlations are indicated by absolute values ranging from 0.7 to 0.9 and 0.4 to 0.6, respectively,

3. Results

3.1. Fermentation profile

As presented in Table 2, incubation of GS in the RUSITEC system resulted in a lower pH than when fed to sheep, while the *in vivo* pH was lower with MS than with GS. This was not the case *in vitro* ($P < 0.001$), which is indicated by a significant method x silage interaction effect. The ammonia-N concentration was higher *in vitro* than *in vivo* ($P < 0.001$) and also affected by forage with higher ammonia-N concentrations with MS than with GS ($P < 0.001$).

We observed an interaction of method and silage for n-valerate and n-caproate (each $P < 0.001$) with higher proportions *in vitro* during GS incubation compared to MS incubation, whereas no difference between GS and MS feeding was present *in vivo*. The method showed an effect and revealed higher proportions *in vitro* than *in vivo* for total VFA as well as all individual VFA (each $P < 0.001$), except propionate ($P = 0.022$) and isobutyrate ($P = 0.496$). Moreover, silage type influenced the proportions of propionate and n-caproate (each $P < 0.001$) were propionate proportions were higher with MS than with GS, and also total VFA tended to be higher with MS than with GS ($P = 0.084$).

3.2. Degradability of DM, OM, aNDFom and ADFom

Table 3 illustrates the *in vitro* and *in situ* rumen degradability coefficients for DM and OM as well as the fibre fractions aNDFom and ADFom. The data revealed interactions of method and silage for all variables. Thereby, DM ($P = 0.030$) and OM ($P = 0.015$)

Table 2
Daily fermentation characteristics in *in vitro* RUSITEC fermenters and *in vivo* sheep rumen with grass silage (GS) or maize silage (MS).

	In vitro		*In vivo*			P-value		
	GS	MS	GS	MS	SEM	Method	Silage	Method × silage
pH	6.59^{B}	6.58	6.82^{aA}	6.57^{b}	0.032	< 0.001	< 0.001	< 0.001
Ammonia-N, mg/L	140.2	260.9	91.9	187.8	12.30	< 0.001	< 0.001	0.239
Volatile fatty acids								
Total, mmol/L	112.2	113.8	61.5	69.2	4.90	< 0.001	0.084	0.233
Proportion of total (mol/mol)								
Acetate	0.510^{aA}	0.544^{b}	0.690^{aB}	0.617^{b}	0.009	< 0.001	0.037	< 0.001
Propionate	0.182	0.206	0.194	0.246	0.010	0.002	< 0.001	0.077
n-Butyrate	0.131	0.152	0.078	0.090	0.008	< 0.001	0.028	0.532
n-Valerate	0.051^{aA}	0.029^{bA}	0.012^{B}	0.014^{B}	0.003	< 0.001	0.001	< 0.001
n-Caproate	0.037^{aA}	0.013^{b}	0.005^{A}	0.006	0.003	< 0.001	< 0.001	< 0.001
Isobutyrate	0.009	0.005	0.008	0.009	0.002	0.496	0.401	0.312
Isovalerate	0.080^{aA}	0.050^{bA}	0.012^{B}	0.016^{B}	0.005	< 0.001	0.013	0.001
Acetate:propionate	2.94	2.67	3.65	2.56	0.20	0.471	< 0.001	0.075

Capitalized superscript letters indicate differences between methods within the same forage ($P < 0.05$).
Lowercase superscript letters indicate differences between forages within the same method ($P < 0.05$).

degradability coefficients were lower in GS than in MS for the *in vitro* incubation, but not differing for the *in situ* approach. In contrast, aNDFom and ADFom degradability coefficients (both $P < 0.001$) were lower in MS than in GS for the *in situ* incubation, but not differing for the RUSITEC data.

Regarding main effects, applied method affected the degradability coefficients of DM ($P < 0.001$), OM ($P < 0.001$) and ADFom ($P < 0.043$) with lower values *in vitro* than *in situ*, whereas aNDFom degradability was not different between the incubation techniques. In addition, silage type had an impact on the fibre degradabilities (both $P < 0.001$), being lower for MS than for GS.

3.3. Correlation analysis

All correlations coefficients and associated P-values are presented in Table 4. The aNDFom degradability between *in situ* and *in vitro* measurements revealed a moderate ($r = 0.54$; $P = 0.04$) and strong ($r = 0.78$; $P = 0.02$) correlation for GS and MS, respectively. For MS, a strong correlation between *in situ* and *in vitro* data was also present for ADFom degradability, *i.e.* $r = 0.85$ and $P = 0.01$. Similar to aNDFom degradability, total VFA concentration was moderately ($r = 0.55$; $P = 0.03$) and highly (0.74; $P = 0.02$) correlated for GS and MS, respectively. At the individual VFA level, *in vivo* and *in vitro* data showed moderate correlations of 0.62 ($P = 0.01$) for propionate as well as of 0.57 ($P = 0.03$) for n-butyrate concentrations. Additionally, caproate tended to be moderately correlated between *in vivo* and *in vitro* data, *i.e.* $r = 0.48$ and $P = 0.07$. Regarding MS, concentrations of acetate and propionate were strongly correlated between *in vivo* and *in vitro* with 0.72 ($P = 0.03$) and 0.75 ($P = 0.02$), respectively.

4. Discussion

The present study provides a direct comparison of the fermentation characteristics and degradability of two common forages by parallel application of a coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as the *in vitro* RUSITEC system. Thereby, the hypothesis was set that the *in vitro* RUSITEC system provides similar results in terms of fermentation characteristics and forage degradability as obtained in sheep rumen. Secondly, we expected a low variation within sheep and RUSITEC vessels in the animal-individual comparison.

The degradability data revealed an impact of methodology on degradabilities of DM, OM and ADFom measured by *in situ* or *in vitro* procedure. However, no difference was found for the aNDFom fraction and our correlation analysis indeed showed a moderate and even strong correlation for aNDFom degradability of GS and MS, respectively. Consequently, it appears that RUSITEC-derived degradability coefficients can be sufficient proxies for *in situ* measurements, representing a much less laborious and more cost-effective strategy to assess ruminal degradability of total structural carbohydrates, *i.e.*, aNDFom comprising hemicelluloses, cellulose and lignin. Comparing fibre degradabilities obtained *in situ* to those received from a short-term *in vitro* system, Trujillo et al. (2010) also observed a consistent ranking of feedstuffs with both methods, which supports the general feasibility of reducing *in situ* measurements by *in vitro* procedures for assessing ruminal fibre degradability. In addition, *in vitro* ADFom degradability of MS was strongly correlated with the *in situ* measurements, therefore indicating that comparability of methods may depend on the forage type, which became also apparent from the significant interactions found for degradability coefficients.

In this context, we believe that the overall satisfying consistency in *in situ* and *in vitro* fibre degradability coefficients may be further improved by adapting the incubation time of nylon bags into the rumen. The 48-h incubation time, which was chosen for both methods in the present study, is a fixed figure in the RUSITEC system, but can be flexibly designed with the *in situ* method. Thereby, an adaptation of the *in situ* incubation time may translate into improved concordance between *in situ* and *in vitro* degradabilities for fibre fractions but also DM, OM and other nutrients. In fact, a reduction of incubation time should more closely resemble the true *in vivo* rumen scenario, where 48 h retention time of forages is rather unlikely, especially in the case of MS or high feed intake levels (Krämer et al., 2013). A follow-up comparison study with several time points for the *in situ* incubations, analogous to the determination of ruminal degradation kinetics (Kirchhof, 2007), may then provide information on a suitable incubation time for a specific comparison with RUSITEC data. For a comparison of RUSITEC data across studies, however, a standard incubation time is required (Deitmers et al., 2022).

Regarding ruminal fermentation, concentrations of total VFA were on a substantially higher level *in vitro* than *in vivo*, which can be explained by the lack of any absorptive capacity in the RUSITEC system (Carro et al., 2009). The fermentation end products are only removed *via* the effluent flow, *i.e.* simulating the outflow to the lower digestive tract, whereas direct absorption constitutes a dominant

Table 3
Degradability coefficients of grass silage (GS) and maize silage (MS) during *in situ* or *in vitro* incubation for 48 h.

	In vitro		*In situ*			P-values		
	GS	MS	GS	MS	SEM	Method	Silage	Method × silage
Dry matter	0.593[b]	0.613[a]	0.729	0.708	0.011	< 0.001	0.916	0.030
Organic matter	0.581[b]	0.617[a]	0.716	0.705	0.011	< 0.001	0.209	0.015
aNDFom[1]	0.400	0.353	0.561[a]	0.251[b]	0.019	0.104	< 0.001	< 0.001
ADFom[2]	0.390	0.343	0.596[a]	0.216[b]	0.022	0.043	< 0.001	< 0.001

Lowercase superscript letters indicate differences between forages within the same method ($P < 0.05$).
[1] aNDFom = Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash.
[2] ADFom = Acid detergent fibre expressed exclusive of residual ash.

Table 4
Pearson correlation coefficients for variables measured *via* the coupled *in vivo-in situ* approach and *in vitro* for grass silage and maize silage separately. Significant correlations (P < 0.05) are marked in bold print.

Variable	Grass silage		Maize silage	
	Coefficient	P-value	Coefficient	P-value
DM degradability	0.33	0.23	0.56	0.12
OM degradability	0.41	0.13	0.55	0.12
aNDFom degradability	**0.54**	**0.04**	**0.78**	**0.02**
ADFom degradability	0.14	0.61	**0.85**	**0.01**
Ammonia-N	0.42	0.12	-0.54	0.13
Total VFA	**0.55**	**0.03**	**0.74**	**0.02**
Acetate	0.41	0.13	**0.72**	**0.03**
Propionate	**0.62**	**0.01**	**0.75**	**0.02**
n-Butyrate	**0.57**	**0.03**	0.45	0.22
n-Valerate	-0.27	0.32	0.31	0.42
n-Caproate	0.48	0.07	0.47	0.21
Isobutyrate	-0.04	0.87	-0.37	0.33
Isovalerate	0.13	0.64	0.25	0.52
pH liquid phase	-0.01	0.87	0.08	0.28

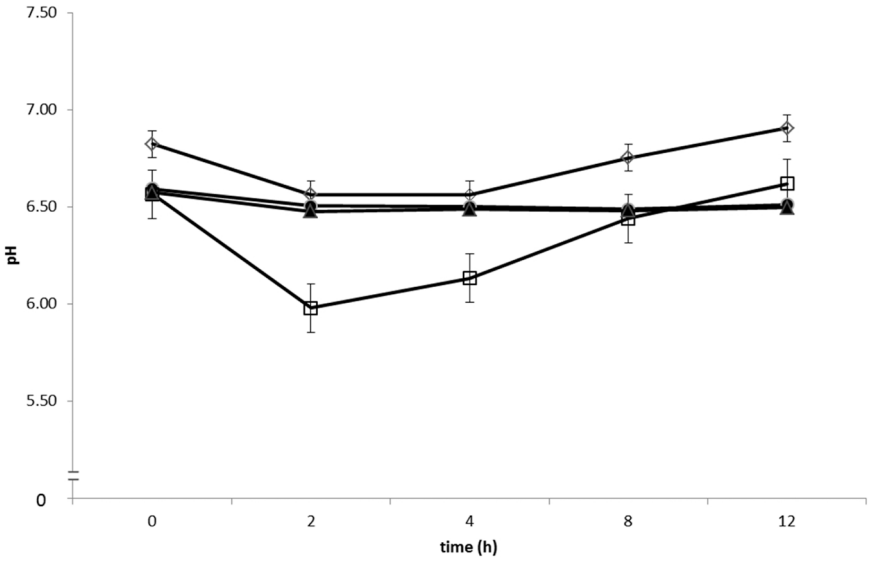


Fig. 1. Diurnal pH pattern in the rumen of sheep and RUSITEC fermenter receiving the same silage, *i.e.*, maize silage and grass silage. Sheep were fed at 0 h and 12 h and RUSITEC fermenters were incubated with fresh forage once daily at 0 h. Error bars represent SEM. (▲fermenter incubated with maize silage, ● fermenter incubated with grass silage, □ sheep fed maize silage, ◊ sheep fed grass silage).

mechanism of VFA removal in the rumen (Aschenbach et al., 2011). Likewise, this may also serve as an explanation for the higher ammonia-N concentrations found *in vitro* compared to *in vivo*. Notably, the high ammonia-N concentrations observed with MS both in sheep and in the RUSITEC system should derive from the urea supplementation as MS is typically low in nitrogen compounds and therefore explains the discrepancy with GS richer in crude protein (Boguhn et al., 2013). Consequently, a direct comparison of absolute fermentation values originating from *in vivo* and the RUSITEC system appears not feasible. In this context, we also observed an impact of method on proportions of individual VFA. Therefore, not only absolute but also relative VFA concentrations varied between *in vitro* and *in vivo*, which deserves attention in future studies. Presumably, differences in the microbial community and its metabolic activity could be causative (Martínez et al., 2010). Nevertheless, the order of individual VFA proportions was similar between *in vivo* and *in vitro* and the calculated correlations for both total and major individual VFA further supported this congruency, which was especially true for MS. Similarly, Yanza et al. (2022) observed a consistent effect of *Coleus amboinicus* Lour polyphenols on the final products of ruminal fermentation in both *in vitro* and *in vivo* experiments.

The RUSITEC system did not reproduce the diurnal pH pattern and so appeared less suitable for replacing *in vivo* measurements. This *in vitro* system possesses a high buffering capacity when run with the typically used buffer solution (Khiaosa-Ard et al., 2020; Deitmers et al., 2022). Coupled with its missing absorption function that led to higher VFA concentrations in the fermenters (Carro et al., 2009), it may explain the absence of any pH drop in the RUSITEC after feedbag exchange that, however, was clearly observed 2–4 h after feeding MS to the sheep. Moreover, the diurnal pH patterns were similar with both forages in the RUSITEC, which was not

the case for the *in vivo* situation and due to the distinct chemical composition of GS and MS, predominantly the starch content, also not expected. Thus, our hypothesis had to be rejected since the acidotic impact of the present MS was blurred *in vitro* and eventually revealed this limitation of the RUSITEC system – at least for starch-rich feedstuffs.

Similarly, Hildebrand et al. (2011a) obtained uniform pH patterns when incubating GS- and MS-based diets in the RUSITEC, suggesting that this limitation of the RUSITEC is not only restricted to the incubation of pure forage diets but seems to be a general feature of this *in vitro* system. Yet, adaptations in buffer solution and/or infusion rate may enable a better accordance of *in vivo* fermentation patterns with those from the RUSITEC system, which then would further increase the significance of *in vitro* systems and should be pursued in future research. However, despite all efforts to simulate *in vivo* conditions as closely as possible, a meta-analysis by Hristov et al. (2012) revealed that RUSITEC and continuous culture systems are characterized by lower total volatile fatty acid (VFA) and acetate concentrations, low counts or lack of ruminal protozoa, and lower organic matter and NDF ruminal degradability. In addition, the variability of data obtained by using RUSITEC and continuous culture systems is much higher than for *in vivo* experiments (Hristov et al., 2012). Worthy of remark, however, the repeatability of our findings with the coupled *in vivo-in situ* approach in rumen-cannulated sheep as well as with the *in vitro* RUSITEC system was indeed given, as evidenced by the hypothesized low variation within sheep and RUSITEC vessels, respectively.

5. Conclusions

Our study investigated the comparability of fermentation characteristics and degradability of GS and MS in a coupled *in vivo-in situ* approach and *in vitro* RUSITEC system. We observed a high congruence of degradability values of both methods, especially the aNDFom degradability showed high similarity. Still, a forage-dependent impact was indicated by higher correlations coefficients found for MS than for GS. The comparability of VFA profiles between the approaches was not given, likely due to a lack of absorption capacity in the RUSITEC system. Still, we observed strong correlations for total VFA and the predominant individual VFA between *in vitro* and *in vivo*. Therefore, the *in vitro* data seemed well comparable to the data derived from the coupled *in vivo-in situ* approach. The RUSITEC system constituted a sufficient replacement for more laborious *in vivo* and *in situ* measurements when investigating nutrient degradability and ranking general fermentation characteristics of feedstuffs. For the purpose of studying diurnal fermentation patterns, however, the RUSITEC system may have limitations in resembling the *in vivo* situation that need to be acknowledged. However, further investigations on diurnal fermentation patterns are requested to support the present results and extent the knowledge.

CRediT authorship contribution statement

Jan-Helge Deitmers: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Karl-Heinz Südekum:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Thomas Hartinger:** Writing – review & editing, Validation, Formal analysis. **Nina Gresner:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors have no conflict of interest.

Acknowledgements

We thank Walter Diefenthal and Viktor Braun, University of Bonn, for their support in care of animals. We thank Petra Jacquemien, University of Bonn, and Dr Kirsten Weiß and her team, Central Analytical Laboratory, Humboldt University Berlin, for skilled support in chemical analyses. Bernd Hilgers and Karolin Skiba, University of Bonn, are acknowledged for their assistance with silage production and implementation of the RUSITEC system.

References

Akoglu, H., 2018. User's guide to correlation coefficients. Turk. J. Emerg. Med. 18, 91–93. https://doi.org/10.1016/j.tjem.2018.08.001.

Aschenbach, J.R., Penner, G.B., Stumpff, F., Gäbel, G., 2011. Ruminant nutrition symposium: role of fermentation acid absorption in the regulation of ruminal pH. J. Anim. Sci. 89, 1092–1107. https://doi.org/10.2527/jas.2010-3301.

Boguhn, J., Rodehutscord, M., Kluth, M., 2006. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. J. Dairy Sci. 89, 1580–1591.

Boguhn, J., Zuber, T., Rodehutscord, M., 2013. Effect of donor animals and their diet on in vitro nutrient degradation and microbial protein synthesis using grass and corn silages. J. Anim. Physiol. Anim. Nutr. 97, 547–557. https://doi.org/10.1111/j.1439-0396.2012.01295.x.

Carro, M.D., Ranilla, M.J., Martín-García, A.I., Molina-Alcaide, E., 2009. Comparison of microbial fermentation of high- and low-forage diets in Rusitec, single-flow continuous-culture fermenters and sheep rumen. Animal 3, 527–534. https://doi.org/10.1017/S1751731108003844.

Czerkawski, J.W., Breckenridge, G., 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br. J. Nutr. 38, 371–384. https://doi.org/10.1079/BJN19770102.

Deitmers, J.-H., Gresner, N., Südekum, K.-H., 2022. Opportunities and limitations of a standardisation of the rumen simulation technique (RUSITEC) for analyses of ruminal nutrient degradation and fermentation and on microbial community characteristics. Anim. Feed Sci. Technol. 289, 115325 https://doi.org/10.1016/j.anifeedsci.2022.115325.

GfE, 2017. Stellungnahme zur Unerlässlichkeit von Tierversuchen und zur Eignung von Ersatzmethoden in der Tierernährungsforschung. Proc. Soc. Nutr. Physiol. 26, 218–224.

Hartinger, T., Gresner, N., Südekum, K.-H., 2019a. Effect of wilting intensity, dry matter content and sugar addition on nitrogen fractions in lucerne silages. Agriculture 9, 11. https://doi.org/10.3390/agriculture9010011.

Hartinger, T., Gresner, N., Südekum, K.-H., 2019b. In vitro ruminal fermentation characteristics of alfalfa silages in response to different pre-ensiling treatments. Anim. Feed Sci. Technol. 258, 114306 https://doi.org/10.1016/j.anifeedsci.2019.114306.

Hildebrand, B., Boguhn, J., Rodehutscord, M., 2011a. Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. Animal 5, 537–546. https://doi.org/10.1017/S1751731110002156.

Hildebrand, B., Boguhn, J., Rodehutscord, M., 2011b. Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation in vitro. Animal 5, 528–536. https://doi.org/10.1017/S1751731110002211.

Hoover, W.H., Crooker, B.A., Sniffen, C.J., 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continous cultures of rumen contents. J. Anim. Sci. 43, 528–534. https://doi.org/10.2527/jas1976.432528x.

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. https://doi.org/10.3168/jds.2012-5533.

Keay, J., Menage, P., 1969. Automated distillation procedure for the determination of nitrogen. Analyst 94, 895–899.

Khiaosa-Ard, R., Mahmood, M., Lerch, F., Traintinger, F.-P., Petri, R.M., Münnich, M., Zebeli, Q., 2020. Physicochemical stressors and mixed alkaloid supplementation modulate ruminal microbiota and fermentation in vitro. Anaerobe 65, 102263. https://doi.org/10.1016/j.anaerobe.2020.102263.

Kirchhof, S., 2007. Kinetik des ruminalen in situ-Nährstoffabbaus von Grünlandaufwüchsen des Alpensraumes unterschiedlicher Vegetationsstadien sowie von Maissilagen und Heu - ein Beitrag zur Weiterentwicklung der Rationsgestaltung für Milchkühe. Dissertation. Kiel 146.

Krämer, M., Lund, P., Weisbjerg, M.R., 2013. Rumen passage kinetics of forage- and concentrate-derived fiber in dairy cows. J. Dairy Sci. 96, 3163–3176. https://doi.org/10.3168/jds.2012-6146.

Martínez, M.E., Ranilla, M.J., Tejido, M.L., Ramos, S., Carro, M.D., 2010. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. J. Dairy Sci. 93, 3684–3698. https://doi.org/10.3168/jds.2009-2933.

Slyter, L.L., Nelson, W.O., Wolin, M.J., 1964. Modifications of a device for maintenance of the rumen microbial population in continuous culture. Appl. Microbiol. 12, 374–377.

Südekum, K.-H., 2005. Möglichkeiten und Grenzen einer Standardisierung der in situ-Methodik zur Schätzung des ruminalen Nährstoffabbaus. Übers. Tierernähr, 33, 71–86.

Tafaj, M., Maulbetsch, A., Junck, B., Steingass, H., Drochner, W., 2001. Research note: a method for studying local differences in ruminal fermentation in dairy cattle. Arch. Anim. Nutr. 54, 341–347. https://doi.org/10.1080/17450390109381990.

Trujillo, A.I., Marichal, M.J., Carriquiry, M., 2010. Comparison of dry matter and neutral detergent fibre degradation of fibrous feedstuffs as determined with in situ and in vitro gravimetric procedures. Anim. Feed Sci. Technol. 161, 49–57. https://doi.org/10.1016/j.anifeedsci.2010.08.001.

Weißbach, F., Strubelt, C., 2008a. Correcting the dry matter content of grass silages as a substrate for biogas production. Landtechnik 63, 210–211.

Weißbach, F., Strubelt, C., 2008b. Correcting the dry matter content of maize silages as a substrate for biogas production. Landtechnik 63, 82–83.

VDLUFA, 2012. Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch): Band III. Die chemische Untersuchung von Futtermitteln, third ed. VDLUFA-Verlag, Darmstadt.

Weller, R.A., Pilgrim, A.F., 1974. Passage of protozoa and volatile fatty acids from the rumen of the sheep and from a continuous in vitro fermentation system. Br. J. Nutr. 32, 341. https://doi.org/10.1079/BJN19740087.

Yanza, Y.R., Szumacher-Strabel, M., Lechniak, D., Ślusarczyk, S., Kolodziejski, P., Patra, A.K., Váradyová, Z., Lisiak, D., Vazirigohar, M., Cieslak, A., 2022. Dietary *Coleus amboinicus* Lour. decreases ruminal methanogenesis and biohydrogenation, and improves meat quality and fatty acid composition in longissimus thoracis muscle of lambs. J. Anim. Sci. Biotechnol. 13, 5 https://doi.org/10.1186/s40104-021-00654-3.