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Evolution of fermentation parameters in Rusitec fermenters operated at different dilution rates and concentrate retention times

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Abstract. One 14-d incubation trial was carried out using 16 Rusitec fermenters to investigate the evolution of fermentation parameters with incubation day (ID) when fermenters were operated at different dilution rate (DR) and concentrate retention time (RT). The fermenters were fed a 30:70 alfalfa hay:concentrate diet, and the DR were 3.78 (LDR) and 5.42%/h (HDR). The concentrate RT was either 24 (T24) or 48 h (T48), and forage RT was 48 h in all fermenters. Significant ID x DR and ID x RT interactions (P<0.05) were observed for all the measured parameters with the exception of molar proportions of acetate. In general, fermenters operated at the HDR became stabilized before than LDR fermenters. Under the conditions of this study, 6 days were enough to reach steady values for most of the measured parameters.

Keywords. Rusitec – Fermentation parameters – Adaptation period – Dilution rate – Retention time.

Évolution des paramètres de fermentation dans des fermenteurs RUSITEC soumis à différents taux de dilution et temps de séjour du concentré

Résumé. *Un essai d'incubation de 14 jours a été mené en utilisant 16 fermenteurs Rusitec pour étudier l'évolution des paramètres de fermentation au cours du temps lorsque les fermenteurs sont soumis à différents taux de dilution (DR) et temps de séjour du concentré (RT). Les fermenteurs ont été alimentés avec un régime 30:70 foin de luzerne : concentré, et le DR étaient 3,78 (LDR) et 5,42%/h (HDR). Le RT était ou 24 (T24) ou 48 h (T48) et le fourrage RT 48 h dans tous les fermenteurs. Des interactions significatives (P<0,05) ID x DR et ID x RT ont été trouvées pour tous les paramètres mesurés à l'exception des proportions molaires d'acétate. Globalement, les fermenteurs opérés au plus haut HDR se sont stabilisés avant ceux LDR. Sous les conditions de cette étude, 6 jours ont été suffisants pour obtenir des valeurs stables dans la plupart des paramètres mesurés.*

Mots-clés. *Rusitec – Paramètres de fermentation – Période d´adaptation – Taux de dilution – Temps de séjour.*

I – Introduction

Several types of artificial rumen apparatus have been described in the literature, the semi-continuous flow Rusitec system being one of the most widely used (Czerkawski and Breckenridge, 1977). Although Rusitec fermenters are used in many laboratories, there is no standardization regarding operating conditions such as adaptation period, dilution rate or amount of feed delivered daily (Boguhn *et al.*, 2008; Carro *et al.*, 2009). Regarding adaptation period, Czerkawski and Breckenridge (1977, 1979) pointed out that it was possible to achieve steady-state conditions in 4-6 days when balanced diets were administered to the fermenters, but longer adaptation periods were required for low-quality diets. Our hypothesis was that operating conditions such as dilution rate (DR) and solids retention time (RT) might affect the length of the adaptation period necessary to reach steady-state conditions. The objective of this study was, therefore, to analyze the evolution of fermentation variables with time in Rusitec fermenters operated at different DR and concentrate RT.

II – Materials and methods

1. Apparatus, diet and experimental procedure

One 14-day incubation trial was carried out with 16 Rusitec fermenters (Czerkawski and Breckenridge, 1977) with an effective volume of 600 mL each. On day 0, each fermenter was inoculated with 300 ml of rumen liquor, 200 ml of artificial saliva (pH = 8.4) and 80 g of solid rumen contents. Ruminal contents were obtained from four rumen-fistulated Merino sheep fed the same diet received by the fermenters. Ruminal contents from each sheep were collected immediately before the morning feeding, mixed, strained through two layers of cheesecloth, and transferred to the fermenters within 30 minutes after collection.

Each fermenter received once daily 30 g of DM of a diet consisting of 30% alfalfa hay and 70% concentrate [dry matter (DM) basis]. Neutral-detergent fibre (NDF), acid-detergent fibre (ADF) and N of the diet was 359, 174 and 27.0 g/kg DM, respectively. Two nylon bags, one with 9 g of alfalfa hay and other with 21 g of concentrate, were incubated daily inside each fermenter. The experiment had a factorial design with two DR [low (LDR) and high (HDR)] and two concentrate RT of 24 (T24) and 48 h (T48). Forage RT was 48 h in all fermenters. Treatments were assigned randomly so that four fermenters received each of the treatments. Flow through the fermenters was maintained by continuous infusion of artificial saliva (pH = 8.4) at two different rates of 545 (LDR; 3.78 %/h) and 780 (HDR; 5.42 %/h) ml/d. These DR and solids RT were chosen to resemble values previously observed *in vivo* in sheep (Carro *et al.*, 2000). The HDR fermenters received diluted saliva (70%) in order to infuse daily the same amount of salts in all fermenters and maintain similar pH values. The general incubation procedure was as described by Carro and Miller *(*1999).

2. Sampling, analytical procedures and statistical analyses

Fermenters' fluid was sampled every day before the feeding and the pH was immediately measured. Liquid effluent was collected daily in flasks containing a solution of H_2SO_4 (20%; v:v) to maintain pH values below 2. Every day, total effluent was collected and the following samples were taken: 1 ml of effluent was added to 1 ml of deproteinizing solution (10% of metaphosphoric acid and 0.06% crotonic acid; wt/vol) for volatile fatty acid (VFA) determination, and 5 ml of effluent were stored at -20ºC for ammonia-N and total lactate determinations. Procedures for determination of DM, N, NDF, ADF, VFA, ammonia-N and lactate have been reported by Carro and Miller (1999).

Data were analysed as a repeated measures model using the Proc MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). Effects included in the model were DR, RT, incubation day (ID) and the interactions ID x DR, ID x RT, and DR x RT. When the effect of time was significant ($P<0.05$), differences between means were assessed by Tukey's test.

III – Results and discussion

Because significant ID x DR or ID x RT interactions ($P=0.01$ to <0.001) were observed for all analyzed parameters, with the exception of daily production of total VFA ($P=0.37$ and 0.25, respectively), the effects of ID were analyzed independently for each experimental treatment.

Previous studies have shown that modifications in the microbial populations are produced in fermenters over the incubation period (Slyter and Putnam, 1967; Prevot *et al.*, 1994). A period of time is required for adaptation of microbes to the incubated diet and conditions of the system before starting to determine the fermentation parameters. Different adaptation periods have been used in previous studies, most of them ranging from 5 to 11 days (Czerkawski and Breckenridge, 1977, 1979; Carro and Miller, 1999; Godoy and Meschy, 2001; Jalc *et al.*, 2006; Giraldo *et al.*, 2007; Carro *et al.*, 2009), but to date there is no standardization regarding the number of days neces-

sary for the microbial populations to be adapted to the fermenters' conditions. In this study, it was considered that steady-state conditions in the fermenters were reached when the daily output of fermentation products did not change significantly from day to day on several consecutive days.

As shown in Table 1, daily production of total VFA was affected by ID (P<0.001) for all experimental treatments. In all fermenters, total VFA production increased over the first days, but no differences (P>0.05) were found among the last 8 days of incubation indicating that steady state conditions were achieved after 6 days of incubation. For all experimental treatments daily VFA production by the end of the incubation run was greater (P<0.05) than that observed on day 1. Daily ammonia-N production changed over time (P=0.01 to <0.001) for all experimental treatments. In general, values increased over the first ID, but then decreased gradually and the values found on the last 8 incubation days were similar (P>0.05) to those measured on day 1 in all fermenters (excepting HDR-T48 fermenters, which had greater (P<0.05) values on days 13 and 14). Daily production of lactate changed (P=0.008 to <0.001) with time in LDR fermenters and HDR-T24 fermenters, but no changes (P=0.68) were detected in HDR-T48 fermenters. The production of lactate was rather stable over the last 8 days in all fermenters, and the values were similar (P>0.05) to those found on day 2.

Table 1. Effects of incubation day (ID) on daily production of volatile fatty acids (VFA), ammonia-N and lactate in Rusitec fermenters fed a 30:70 alfalfa hay:concentrate diet and operated at high (HDR) or low (LDR) dilution rate and concentrate retention times of 24 (T24) or 48 (T48) h

ID	VFA (mmol/d)				Ammonia-N (mg N/d)				Lactate (mg/d)			
	HDR		LDR		HDR		LDR		HDR		LDR	
	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48
1	69.0^{a}	63.1^{a}	61.1^{a}	56.7^{a}	191^{a}	180^{a}	173^{a}	162^{a}	14.2^{b}	10.8	12.4^{b}	7.87^{ab}
2	78.4^{ab}	81.2^{ab}	66.5^{ab}	69.4^{a}	254^{b}	268^{b}	231^{b}	229^{b}	8.56^{a}	10.0	7.45^{a}	6.09^{ab}
3	78.9^{abc}	84.2^{bc}	77.8^{abc}	89.1^{b}	246^{b}	286^{c}	237^{b}	238^{b}	7.31^{a}	7.99	5.37^{a}	9.88^{ab}
4	81.0^{abc}	99.3^{bc}	81.3^{bc}	89.3^{b}	235^{b}	266^{bc}	225^{b}	230^{b}	12.4^{ab}	9.46	7.80^{a}	8.88^{ab}
5	86.8^{bc}	87.9^{bc}	82.0^{bc}	87.8^{b}	241^{b}	237^{abc}	210^{ab}	219^{b}	11.2^{ab}	9.86	9.32^{ab}	8.84^{ab}
6	83.8^{abc}	92.3^{bc}	78.2^{bc}	87.1^{b}	228^{b}	224^{abc}	187^{ab}	217^{b}	9.66^{ab}	10.6	6.55^{a}	7.51^{ab}
7	95.8^{d}	94.6^{bc}	82.5^{bc}	91.0^{b}	215^{b}	222^{ab}	190^{ab}	211^{b}	9.36^{ab}	10.5	6.35^{a}	5.83^{ab}
8	95.0^{cd}	102^{c}	87.5^{c}	87.7^{b}	223^{b}	253^{bc}	183^{a}	204^{ab}	10.3^{ab}	10.8	5.52^{a}	5.70^{a}
9	95.1^{cd}	100^{c}	83.1^{bc}	89.6^{b}	249^{b}	263^{bc}	172^{a}	201^{a}	8.74^{a}	9.29	6.56^{a}	6.86^{ab}
10	94.8^{cd}	98.3^{bc}	83.4^{bc}	88.3^{b}	218^{b}	237^{abc}	173^{a}	189^{a}	10.8^{ab}	11.3	8.88^{ab}	7.14^{ab}
11	96.0^{d}	97.6^{bc}	86.3^{c}	90.2^{b}	235^{b}	229^{abc}	185^{a}	191^{ab}	9.99^{ab}	9.94	9.52^{ab}	9.05^{ab}
12	94.3^{cd}	100^{c}	85.4^{bc}	92.9^{b}	238^{b}	223^{ab}	168^{a}	179^{a}	9.30^{ab}	9.07	9.08^{ab}	8.74^{ab}
13	92.9^{bcd}	95.3^{bc}	83.8^{bc}	88.6^{b}	223^{b}	241^{b}	174^{a}	184^{a}	8.92^{ab}	8.91	8.85^{ab}	8.39^{ab}
14	93.1^{bcd}	97.0^{bc}	87.4^{c}	92.2^{b}	215^{b}	253^{b}	176^{a}	183^{a}	9.60^{ab}	9.12	7.78^{a}	8.21^{ab}
SEM†	3.37	3.84	3.97	3.13	15.0	15.0	13.0	9.4	1.050	1.041	0.921	0.822
P	<0.001	<0.001	<0.001	<0.001	0.01	<0.001	<0.001	<0.001	0.008	0.68	<0.001	0.01

$^{a, b, c, d}$ Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

The molar proportions of acetate decreased (P<0.05) over the first 2 ID, but values were stable (P>0.05) from day 3 to 14 (Table 2). In general, the molar proportions of propionate did not change (P>0.05) during the first 4 ID, but then decreased and became stable after 6 ID with the exception of LDR-T48 fermenters which needed 7 days to show steady values. The molar proportions of butyrate in HDR fermenters reached steady values (> 0.05) after 3 - 4 days, but a longer period of 7 - 8 days was required in LDR fermenters.

Table 2. Effects of incubation day (ID) on molar proportions of acetate, propionate and butyrate in Rusitec fermenters fed a 30:70 alfalfa hay:concentrate diet and operated at high (HDR) or low (LDR) dilution rate and concentrate retention times of 24 (T24) or 48 (T48) h

ID	Acetate (mol/100 mol)				Propionate (mol/100 mol)				Butyrate (mol/100 mol)			
	HDR		LDR		HDR		LDR		HDR		LDR	
	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48
1	51.9^{c}	52.4^{b}	53.8^{c}	53.9^{d}	23.6^{c}	23.8^{c}	23.7^{cd}	22.9^{d}	18.7^{ab}	18.0^{ab}	16.8^{a}	17.1^{a}
2	50.0^{bc}	47.9^{ab}	50.0^{bc}	48.9^{c}	24.2^{c}	25.8^{c}	24.4^{cd}	24.0^{de}	18.2^{a}	17.5^{ab}	17.5^{a}	17.6^{a}
3	47.0^{abc}	45.1^{a}	46.8^{ab}	44.6^{bc}	24.2^{c}	26.4^{c}	26.0^{d}	25.6^{e}	17.8^{a}	16.7^{a}	17.6^{a}	17.6^{a}
4	46.9^{abc}	44.2^{a}	47.3^{ab}	43.1^{b}	23.5^{bc}	24.1^{c}	24.3^{cd}	23.5^{d}	17.7^{a}	18.3^{ab}	17.0^{a}	18.5^{a}
5	46.7^{abc}	43.4^{a}	45.8^{ab}	41.5^{ab}	21.5^{b}	20.8^{b}	21.0^{bc}	20.3^{c}	18.4^{ab}	19.4^{ab}	18.3^{ab}	19.9^{b}
6	46.3^{ab}	45.5^{a}	45.5^{a}	46.6^{bc}	18.9^{a}	17.2^{a}	17.9^{ab}	15.2^{b}	19.8^{ab}	19.6^{ab}	20.8^{bc}	18.3^{a}
7	45.2^{ab}	45.8^{a}	46.4^{ab}	46.0^{bc}	18.4^{a}	16.8^{a}	15.8^{a}	12.7^{a}	21.0^{ab}	20.2^{ab}	21.2^{c}	19.4^{b}
8	46.0^{ab}	45.0^{a}	44.9^{a}	45.6^{bc}	18.5^{a}	16.0^{a}	16.0^{a}	12.2^{a}	20.8^{ab}	20.7^{b}	21.6^{c}	20.3^{b}
9	47.0^{abc}	45.2^{a}	43.0^{a}	45.6^{bc}	18.5^{a}	16.8^{a}	16.5^{a}	12.9^{a}	20.3^{ab}	20.1^{ab}	21.7^{c}	20.0^{b}
10	44.0^{a}	45.5^{a}	44.0^{a}	45.9^{bc}	20.8^{b}	17.8^{a}	17.2^{ab}	13.0^{a}	21.4^{ab}	19.5^{ab}	21.0^{b}	20.4^{b}
11	43.0^{a}	44.3^{a}	44.0^{a}	45.6^{bc}	21.2^{b}	16.7^{a}	17.7^{ab}	12.5^{a}	22.0^{b}	20.5^{b}	22.1^{c}	21.5^{b}
12	44.4^{a}	44.6^{a}	45.2^{a}	45.3^{bc}	19.9^{ab}	16.2^{a}	17.9^{ab}	13.2^{a}	21.4^{ab}	20.4^{b}	20.8^{b}	21.8^{b}
13	44.9^{ab}	44.8^{a}	44.5^{a}	46.2^{bc}	18.9^{a}	15.6^{a}	17.5^{ab}	12.8^{a}	21.4^{ab}	19.1^{ab}	19.8^{ab}	20.3^{b}
14	44.3^{a}	46.0^{a}	43.0^{a}	45.6^{bc}	19.0^{a}	16.2^{a}	18.0^{ab}	13.2^{a}	21.8^{b}	19.1^{ab}	20.4^{b}	21.3^{b}
SEM†	1.06	1.07	0.88	0.97	0.41	0.58	0.79	0.39	0.75	0.73	0.63	0.48
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	<0.001	<0.001

$^{a, b, c}$ Mean values within a column with unlike superscripts differ (P<0.05).

† Standard error of the mean.

As shown in Table 3, acetate/propionate ratios were affected by ID (P<0.001) for all experimental treatments. The acetate/propionate ratios over the last 5 ID did not differ (P>0.05) from those on day 1 for T24 fermenters, but the values for T48 fermenters were greater (P<0.05) than those found on day 1. Over the first 4 ID, the molar proportions of other VFA (calculated as the sum of isobutyrate, isovalerate and valerate) increased (P<0.001) by about twice the values on day 1 for all treatments, but values became stable (P>0.05) by day 5 and 9 for HDR and LDR fermenters, respectively. The higher molar proportions of other VFA compared with the proportions usually found *in vivo* is in agreement with the results obtained previously in our laboratory (Gómez *et al.*, 2005; Giraldo *et al.*, 2007; Carro *et al.*, 2009).

The molar proportions of caproate increased rapidly over the first ID, but became stable after 5 days in all fermenters. The values found over the last 4 ID were 6.3, 13.9, 13.3 and 15.5 times greater than those observed on day 1 for HDR-T24, HDR-T48, LDR-T24 and LDR-T48 fermenters, respectively. These results seem to indicate that the unphysiologically high caproate concentrations found in Rusitec fermenters fed high-concentrate diets (Gómez *et al.*, 2005; Carro *et al.*, 2009) might have been due to the high RT of concentrate feeds (48 h in all the cited experiments) and to the low DR (about 3.8%/h). This would indicate that using higher DR and shorter concentrate RT than those typically used in Rusitec fermenters would contribute to reduce this abnormally high production of caproate.

Table 3. Effects of incubation day (ID) on acetate:propionate ratio (Ac/Pr) and molar proportions of other VFA† and caproate in Rusitec fermenters fed a 30:70 alfalfa hay:concentrate diet and operated at high (HDR) or low (LDR) dilution rate and concentrate retention times of 24 (T24) or 48 (T48) h

ID	Ac/Pr (mol/mol)				Other VFA (mol/100 mol)				Caproate (mol/100 mol)			
	HDR		LDR		HDR		LDR		HDR		LDR	
	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48	T24	T48
1	2.20^{abc}	2.20^{ab}	2.28^{abc}	2.35^{b}	5.05^{a}	5.15^{a}	5.02^{a}	5.27^{a}	0.67^{a}	0.71^{a}	0.71^{a}	0.82^{a}
2	2.07^{ab}	1.87^{a}	2.05^{ab}	2.04^{ab}	5.74^{ab}	6.42^{ab}	5.91^{ab}	6.87^{ab}	1.98^{ab}	2.43^{ab}	1.88^{ab}	2.62^{ab}
3	1.95^{a}	1.71^{a}	1.80^{a}	1.74^{a}	7.51^{c}	7.87^{bc}	6.78^{bc}	8.05^{bc}	3.54^{bc}	3.89^{bc}	2.87^{abc}	4.19^{abc}
4	2.00^{ab}	1.84^{a}	1.95^{ab}	1.84^{ab}	8.37^{cd}	8.87^{cd}	7.85^{cd}	9.25^{cd}	3.64^{c}	4.58^{bcd}	3.56^{abcd}	5.64^{bc}
5	2.18^{abc}	2.09^{ab}	2.19^{abc}	2.05^{ab}	9.57^{de}	10.5^{e}	9.73^{e}	10.5^{de}	3.87^{c}	5.90^{cde}	5.17^{abcd}	7.75^{cd}
6	2.46^{cd}	2.66^{bc}	2.56^{c}	3.08^{c}	10.6^{e}	10.6^{e}	9.71^{e}	10.2^{de}	4.54^{c}	7.13^{def}	6.07^{bcde}	9.68^{de}
7	2.47^{cd}	2.78^{c}	2.95^{cd}	3.62^{cd}	10.6^{e}	9.79^{de}	9.29^{de}	9.88^{de}	4.85^{c}	7.39^{ef}	7.40^{cde}	12.0^{e}
8	2.49^{cd}	2.85^{c}	2.85^{cd}	3.74^{d}	10.3^{e}	10.4^{de}	9.63^{e}	9.70^{de}	4.39^{c}	7.96^{ef}	7.91^{de}	12.2^{e}
9	2.56^{cd}	2.73^{c}	2.62^{c}	3.61^{cd}	10.1^{e}	9.80^{de}	9.34^{de}	8.74^{cd}	4.13^{c}	8.20^{ef}	9.57^{de}	12.7^{e}
10	2.12^{ab}	2.59^{bc}	2.57^{c}	3.54^{cd}	10.0^{e}	9.40^{cde}	8.28^{cd}	8.20^{bc}	3.85^{c}	8.05^{ef}	9.48^{de}	12.5^{e}
11	2.03^{a}	2.66^{bc}	2.54^{bc}	3.66^{d}	10.1^{e}	9.62^{de}	7.79^{cd}	8.11^{bc}	3.68^{c}	8.87^{fg}	8.35^{de}	12.4^{e}
12	2.23^{abc}	2.76^{c}	2.57^{c}	3.44^{c}	10.3^{e}	9.13^{cde}	7.62^{cd}	7.34^{bc}	3.93^{c}	9.65^{fg}	8.58^{de}	12.4^{e}
13	2.37^{bcd}	2.88^{c}	2.59^{c}	3.62^{cd}	10.2^{e}	9.21^{cde}	8.92^{de}	7.48^{bc}	4.62^{c}	11.3^{g}	9.83^{de}	13.3^{e}
14	2.33^{bcd}	2.84^{c}	2.42^{bc}	3.47^{d}	10.3^{e}	9.24^{cde}	8.41^{de}	7.08^{bc}	4.68^{c}	9.53^{fg}	10.1^{e}	12.9^{e}
SEM††	0.075	0.118	0.126	0.105	0.268	0.334	0.317	0.358	0.326	0.515	0.946	0.729
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

$^{a, b, c, d, e, f, g}$ Mean values within a column with unlike superscripts differ (P<0.05).

† Calculated as the sum of isobutyrate, isovalerate and valerate.

†† Standard error of the mean.

IV – Conclusions

Under the conditions of the present study, most of the fermentation parameters reached steady values after 6 days of incubation, and the values for the majority of them were different from those observed in the fermenters over the first 2 days of incubation. In general, the fermenters operated at the high DR became stabilized before than those operated at the low DR. The results indicate that using higher DR and shorter concentrate RT than those typically used in Rusitec fermenters would contribute to reduce the acetate/propionate ratio and the production of caproate.

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