

Artículo de investigación

Determination of volatile fatty acids clearance in intact ruminal digesta*Determinação do clearance de ácidos graxos voláteis na digesta ruminal intacta**Determinación del aclaramiento de ácidos grasos volátiles en digesta ruminal intacta*

João Chrysostomo de Resende Júnior ^{1*}  ^{MV, PhD}; João Luiz Pratti Daniel ² ^{MV, PhD}; André Rodrigues da Cunha Barreto-Vianna ¹ ^{MV, PhD}; Juliano Vogas Peixoto ¹ ^{MV, PhD}; Gregório Correia Guimarães ¹ ^{MV, PhD}; Suely de Fátima Costa ¹ ^{MV, PhD}; Ronaldo Francisco de Lima ³ ^{MV, PhD}; Fabiola da Cruz Meirelles ¹ ^{MV, PhDs}

Fecha correspondencia:

Recibido: 24 de octubre de 2018.
Aceptado: 15 de marzo de 2019.

Forma de citar:

Resende Júnior JC, Daniel JLP, Barreto-Vianna ARC, Peixoto JV, Guimarães GC, Costa SF, Lima RF, Meirelles, FC. Determination of volatile fatty acids clearance in intact ruminal digesta. Rev. CES Med. Zootec. 2019; Vol 14 (1): 8-17.

Open access

© Copyright

Creative commons

Ethics of publications

Peer review

Open Journal System

DOI: [http://dx.doi.org/10.21615/](http://dx.doi.org/10.21615/cesmvz.14.1.1)

cesmvz.14.1.1

ISSN 1900-9607

Comparte

**Abstract**

Cannulated cows were allocated to a cross-over design to evaluate the technique for determination of volatile fatty acid (VFA) clearance by infusion of marker solution into intact ruminal digesta, and verify the technique sensibility to the variation in the diet. Animals received diet of forage or forage plus concentrate and infusion of valeric acid and Cr-EDTA solution into intact or evacuated ruminal digesta. The total fractional rate of VFA clearance (FRVFAC) was estimated by the exponential decay of valerate over time. The passage FRVFAC to the omasum was assumed as the decay in ruminal Cr concentration and the absorption FRVFAC was estimated by the difference. The total FRVFAC and absorption did not differ between techniques. The passage FRVFAC, however, was lower when the marker solution was added into the evacuated digesta, probably reflecting the destabilization of the rumen environment during the evacuation and the largest volume of fluid observed in animals with evacuated digesta. In conclusion, the infusion of marker solution into intact digesta with homogenization performed by ruminal motility could be usable for the VFA ruminal clearance determination since it seems to cause lesser disturbance in the ruminal environment.

Keywords: *acidosis, Cr-EDTA, rumen physiology, stomach.*

Resumo

Bovinos canulados foram alocados em um delineamento cruzado para avaliar a técnica de determinação do clearance de ácidos graxos voláteis (VFA) por infusão de solução marcadora na digesta ruminal intacta, e verificado a sensibilidade da técnica à variação na dieta. Os animais receberam dieta de forragem ou forragem juntamente com concentrado e infusão de ácido valérico e solução de Cr-EDTA na digesta ruminal intacta ou evacuada. A taxa fracional total de depuração de AGV (FRVFAC) foi estimada pelo decaimento exponencial do valerato ao longo do tempo. A passagem FRVFAC para o omaso foi assumida como o decaimento na concentração ruminal

Filiación:

* Corresponding author. Tel.: +55 35 38291741; fax: +55 35 38291715. E-mail address: joacrcj@dmv.ufla.br (J.C.R. Júnior).

¹ Department of Veterinary Medicine, Federal University of Lavras, 37200-000, Lavras, MG, Brazil.

² Department of Animal Science, State University of Maringá, Av. Colombo, Bloco J45, Maringá, PR, Brazil.

³ Federal University of Western Pará, Institute of Biodiversity and Forest, 68040-260, Santarém, PA, Brazil.

de Cr e a absorção FRVFAC foi estimada pela diferença. A FRVFAC total e a absorção não diferiram entre as técnicas. A passagem FRVFAC, entretanto, foi menor quando a solução marcadora foi adicionada na digesta evacuada, provavelmente refletindo a desestabilização do ambiente ruminal durante a evacuação e o maior volume de líquido observado em animais com digesta evacuada. Em conclusão, a infusão da solução marcadora na digesta intacta com homogeneização realizada pela motilidade ruminal poderia ser utilizada para a determinação da depuração ruminal de VFA, uma vez que parece causar menor distúrbio no ambiente ruminal.

Palavras-chave: *acidose, Cr-EDTA, fisiologia ruminal, estômago.*

Resumen

Los bovinos canulados fueron asignados en un delineamiento cruzado para evaluar la técnica de determinación del clearance de ácidos grasos volátiles (VFA) por infusión de solución marcadora en la digesta ruminal intacta, y verificado la sensibilidad de la técnica a la variación en la dieta. Los animales recibieron dieta de forraje o forraje junto con concentrado e infusión de ácido valérico y solución de Cr-EDTA en la digesta ruminal intacta o evacuada. La tasa fraccional total de depuración de AGV (FRVFAC) fue estimada por el decaimiento exponencial del valerato a lo largo del tiempo. El pasaje FRVFAC para el omaso fue asumido como el decaimiento en la concentración ruminal de Cr y la absorción FRVFAC fue estimada por la diferencia. La FRVFAC total y la absorción no difirieron entre las técnicas. El paso FRVFAC, sin embargo, fue menor cuando la solución marcadora fue agregada en la digestión evacuada, probablemente reflejando la desestabilización del ambiente ruminal durante la evacuación y el mayor volumen de líquido observado en animales con digestión evacuada. En conclusión, la infusión de la solución marcadora en la digesta intacta con homogeneización realizada por la motilidad ruminal podría ser utilizada para la determinación de la depuración ruminal de la VFA, ya que parece causar menor disturbio en el ambiente ruminal.

Palabras clave: *acidosis, Cr-EDTA, fisiología ruminal, estómago.*

Introduction

Several techniques have been used to determine volatile fatty acids (VFA) absorption in vivo, such as the collection of portal blood (1); intraruminal infusion of radioactive (2) or stable VFA isotopes (3, 4); rumen evacuation and infusion of VFA into the washed ventral sac (5); continuous infusion of nonlabeled VFA into the intact ruminal digesta (6); and pulse-dose of valeric acid plus a marker of fluid passage (7) which may be mixed with the evacuated digesta (8) or mixed in locus (9). Although the last method has become common (infusion of markers in pulse-dose), there is a claim that this technique could alter the measurement of VFA clearance. If a large volume of marker solution is used to make homogenization easier (7), the higher volume of rumen fluid may decrease the clearance rate of VFA (5, 8). Another problem is the poor fitness of regressions describing the decay of markers concentrations over time, which ultimately reflect an incomplete homogenization in locus (2). On the other hand, if the ruminal digesta is evacuated, the markers can be satisfactorily mixed with the rumen content (8), but it is supposed to destabilize the ruminal environment, throughout disruption of the mat and loss of digesta stratification, increase of oxygen concentration, decrease of temperature, and decrease of blood flow and rumen motility.

Due to these concerns Daniel, de Resende Júnior (10) have proposed an alternative approach for infusion and collection of markers. The new technique is based on the natural mixing of markers with ruminal digesta because of rumen motility. A complete homogenization may be reached sometime after the pulse-dosing. At this time ruminal fluid sampling series can be started. Nonetheless, it is necessary to validate this technique. Thus, the objective of this study was to compare the approach proposed by Daniel, de Resende Júnior (10) with a technique which infusing markers into the evacuated digesta for measurements of ruminal clearance of VFA (8). Another aim was to verify the sensibility of the techniques to the variation of VFA absorption and passage rates induced by nutritional plans.

Material and methods

Experimental design

This study was approved by the Committee of Ethics in Animal Experimentation of the Federal University of Lavras under protocol no. 001/2008. Three Jersey and one Nellore nonlactating and nonpregnant cows were surgically adapted with rumen cannula (10.2 cm diameter) and randomly allocated in a cross-over design, with 18-d periods. Animals were housed in a tie-stall barn with sand beds. Feeds, mineral-vitamin mix, and fresh water were available ad libitum. The four treatments comprised a combination of two factors arranged in split-plot:

- a. 100% forage diet (maize silage) and marker infusion to intact digesta.
- b. 100% forage diet and marker infusion to evacuated digesta.
- c. 50% forage plus 50% concentrate diet and marker infusion to intact digesta.
- d. 50% forage plus 50% concentrate diet and marker infusion to evacuated digesta.

Diets were whole-plots whereas techniques to measure VFA clearance were sub-plots. Diets were composed of maize silage and a commercial concentrate based on ground corn and soybean meal. Cows were fed once daily at 8:00 h and feed intake was determined by the difference between the amounts of feed offered and refused.

Measurements of VFA absorption

Measurements of VFA absorption were performed on days 16 and 18 of each period, 6 h after feeding (14:00 h). During the collection, water and feed were suspended to avoid interfering with rumen volume.

Marker solutions contained 340 g of valeric acid (8) and Cr-EDTA (14.2 g of Cr) (11), with pH adjusted to 6.5 in a total volume of 4 L. Solutions were stored in plastic bottles and kept refrigerated until usage. For measuring VFA clearance with intact digesta, marker solution was infused into the reticulorumen using a funnel attached to a rigid PVC tube (3/4 inch), with several holes in the distal third. Immediately before infusion, the solution was heated to 37 °C and the total volume was divided into four equal parts. One liter was infused into the cranial sac and other three liters were infused in different places of the rumen ventral sac (10).

For the standard technique, the ruminal digesta was manually evacuated and transferred to buckets (capacity of 50 L) for the addition and mixing of the marker solution, as proposed by (8). A representative sample of rumen content was collected for

dry matter (DM) determination. During the evacuation, the solution of markers was intermittently added into the digesta to facilitate and ensure good homogenization. The digesta was weighted, intensely homogenized, and returned to the reticulorumen. Samples of approximately 100 mL of rumen fluid were collected from the ventral sac using the rigid PVC tube (7.2 cm in diameter) with several holes in the distal third coupled to a tube and a plastic bottle for suction. Immediately after collection, pH was determined and aliquots were frozen at $-20\text{ }^{\circ}\text{C}$ for determination of VFA and Cr concentrations.

For the intact digesta, samples of rumen fluid were collected at 90, 105, 120, 150, 210, and 330 min after infusion. Sampling sequence started 90 minutes after infusion in order to wait the ruminal motility to perform the homogenization of the markers with the digesta, as proposed by Daniel, de Resende Júnior (10). For the evacuated digesta, the samples were collected at the following times: zero (immediately after the return of digesta to the rumen), 15, 30, 60, 120 and 240 min (8).

After thawing, rumen fluid was centrifuged (8.855 g for 15 min) to obtain the supernatant, which was analyzed for chromium by atomic absorption spectrophotometry (Varian Spectr AA-100, Varian Australia Pty Ltd, Victoria, Australia) and VFA by gas-liquid chromatography (CP-3800 Gas Chromatograph Varian, Varian Chromatography Systems, California, USA). For VFA analysis, 250 μL of the supernatant were added with 150 μL of caproic acid as an internal standard (N Caproic Acid, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 100 μL of distilled water. As external standard, a composed solution of acetic, propionic, butyric and valeric acid was used. The capillary column of composite phase had 25 m of length, 0.25 mm internal diameter and 2 mm thick film (CP-Wax 58 - FFAP- CB, Varian Analytical Instruments, California, USA). A temperature cycle was used in the column oven. It was $65\text{ }^{\circ}\text{C}$ in the first 30 seconds; in the next three minutes it was increasing at $20\text{ }^{\circ}\text{C}$ per minute up to $125\text{ }^{\circ}\text{C}$, and then the temperature was rising $50\text{ }^{\circ}\text{C}$ per minute to reach $170\text{ }^{\circ}\text{C}$. The total time of analysis was 4.9 minutes.

Feed samples were also collected during sampling periods and oven-dried at $60\text{ }^{\circ}\text{C}$ for 72 h. Dried samples were ground through a 1-mm screen (Wiley mill) and sub-samples were analyzed for the following: DM in an air-forced oven at $105\text{ }^{\circ}\text{C}$ for 24 h; crude protein (CP), ether extract (EE) and ash, and neutral detergent fiber (NDF). The nonfiber carbohydrates fraction was computed as: $\text{NFC} = 100 - \text{CP} - \text{NDF} - \text{EE} - \text{ash}$.

For the measurements with the evacuated digesta, the rumen fluid volume was estimated by multiplying the weight of evacuated digesta by moisture content. Another estimation of the rumen fluid volume was done by the dilution of Cr at time zero of collection, computed as the intercept of the curve describing the decay of Cr concentration over time. For the infusions with intact digesta, the rumen fluid volume was estimated only by the dilution of Cr 90 min after pulse-dosing which was considered as time zero.

A simple exponential equation describing the change in the valerate concentration over time was used to calculate the fractional clearance rate: $R_t = R_0 \times e^{-k \times t}$ (8). The disappearance of valerate by passage with the rumen fluid was assumed equivalent to the ruminal fluid passage rate, determined by the exponential decay of Cr concentration over time. The fractional rate of valerate absorption was calculated by difference.

Statistical analysis was performed by the Mixed procedure of SAS (12) using the following model: $Y_{ijkl} = \mu + P_i + C_j + D_k + C(D)_{j(k)} + I_l + DI_{kl} + e_{ijkl}$; where μ : overall mean, P_i : random effect of period ($i = 1$ or 2), C_j : random effect of cow ($i = 1$ to 4), D_k : fixed effect of diet ($k =$ forage or forage plus concentrate), $C(D)_{j(k)}$: error term to test whole-plot effects, I_l : fixed effect of infusion technique ($l =$ intact or evacuated), DI_{kl} : interaction of diet and infusion technique, e_{ijkl} : residual error. Feed intake and digesta weight were analyzed with period, cow, and diet effects in the model.

Results

The concentrate supplementation decreased the content of NDF and increased the content of NFC (Table 1). Due to the lower NDF content (13), the diet comprising forage plus concentrate increased food intake (+43%, $P = 0.02$).

Table 1. Chemical composition of experimental diets

Nutrient (% dry matter)	100% Maize silage	50% Maize silage + 50% concentrate[†]
Dry matter, % as fed	39.3	47.1
Crude protein	9.2	11.1
Ether extract	3.9	2.7
Ash	3.8	4.0
Neutral detergent fiber	47.0	36.8
Nonfiber carbohydrates	36.1	45.4

[†]Commercial concentrate based on ground corn and soybean meal.

The volume of rumen fluid was greater when cows fed only forage than for those fed forage plus concentrate when the calculation was based on the moisture content of rumen digesta (Figure 1). However, there was interaction between diet and calculation method, so when the calculation was done by the exponential curve of chrome dispersion in the rumen fluid the amount of rumen liquid was greater in the concentrate containing diet than in the forage diet.

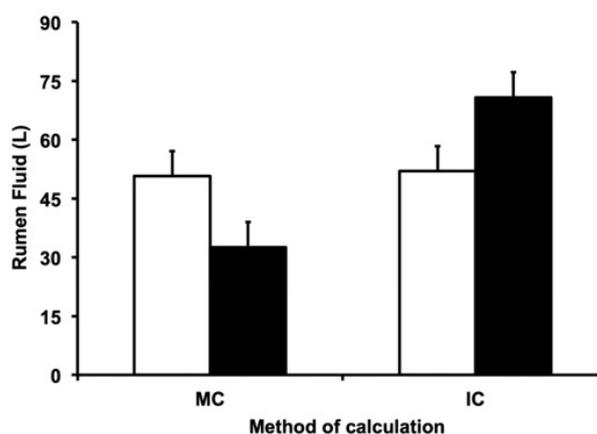


Figure 1. Volume of rumen fluid. Volume of rumen fluid estimated from the moisture content (MC) of the ruminal digesta or calculated from the exponential curve of chrome dispersion in ruminal fluid (IC) when the markers were added into evacuated digesta. Diets contained 100% forage (□) or 50% forage plus 50% concentrate (■). $P = 0.02$ for effect of calculation method; $P = 0.96$ for effect of diet; $P = 0.03$ for interaction of diet and calculation method.

When evaluating the ruminal pH, we verified that the pH was higher in forage diet than in the concentrate containing diet (Figure 2). We also found that supplementation with concentrate resulted in higher concentration of total VFA, acetate, propionate and butyrate (Figure 3).

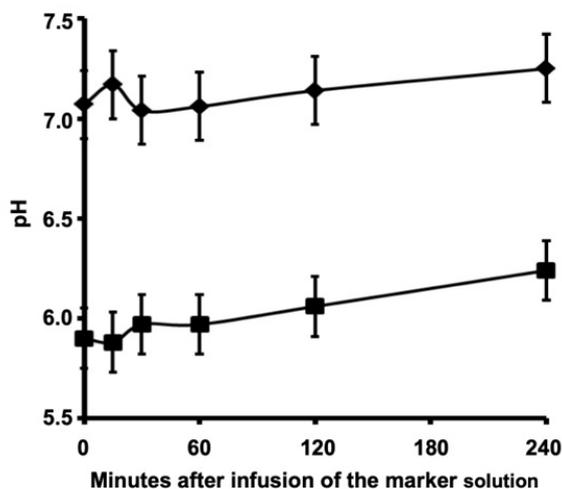


Figure 2. Rumen fluid pH during sampling. Diets contained 100% forage (◆) or 50% forage plus 50% concentrate (■). $P = 0.01$ for diet effect; $P = 0.06$ for collection time effect.

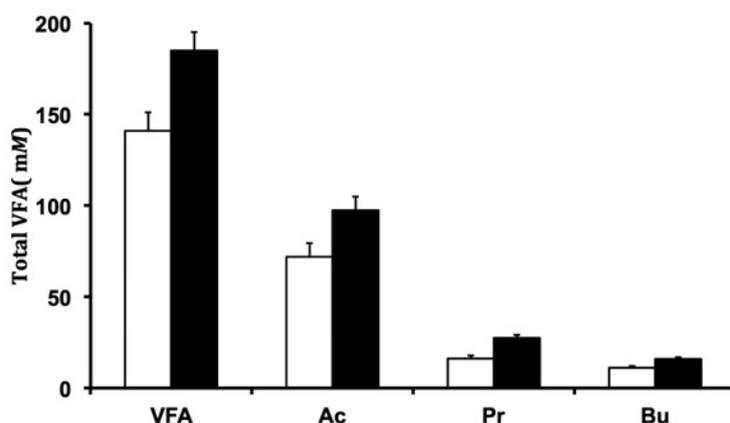


Figure 3. Total VFA. Acetate (Ac), propionate (Pr), and butyrate (Bu) concentrations in the rumen fluid when the marker solution was infused into the intact digesta diets contained 100% forage (□) or 50% forage plus 50% concentrate (■). $P = 0.03$ for the diet effect.

The fractional clearance rate of total rumen VFA (Table 2) was similar between the two methods of markers infusion. In the current study, the R^2 of the regressions which described the decline in valerate and Cr concentration over time did not differ across methods of infusion and were satisfactory high (Table 2). The R^2 of the regressions describing the decline in Cr concentration over time (Table 2) was high for both techniques and numerically ($P = 0.22$) higher in the technique of infusion of markers into intact digesta.

Table 2. Clearance of volatile fatty acids (VFA), volume of rumen fluid, and determination coefficient of the exponential regressions describing the decay of ruminal valerate (R^2 Val) and chrome (R^2 Cr) concentrations over time

Item	Infusion method (M)			Diet (D)			Interaction (M × D)					p-value		
	I	E	SEM [†]	F	C	SEM	F×I	F×E	C×I	C×E	SEM	M	D	M × D
Fractional rate of VFA clearance (% h ⁻¹)	37.8	30.5	4.6	28.3	40.0	4.6	29.2	27.4	46.5	33.5	6.4	0.30	0.17	0.41
Fractional rate of VFA passage (% h ⁻¹)	11.8	9.4	1.4	11.4	9.8	1.8	12.5	10.4	11.2	8.4	2.0	0.06	0.58	0.79
Fractional rate of VFA absorption (% h ⁻¹)	26.0	21.1	5.1	16.9	30.2	5.1	16.7	17.1	35.3	25.0	7.2	0.52	0.16	0.48
Volume of rumen fluid ‡ (L)	52	61	6	47	66	7	41	52	62	71	7.3	<0.01	0.08	0.51
R ² Val	0.93	0.93	0.02	0.92	0.94	0.02	0.91	0.93	0.94	0.94	0.03	0.84	0.46	0.65
R ² Cr	0.92	0.89	0.02	0.90	0.91	0.02	0.90	0.89	0.93	0.88	0.02	0.22	0.61	0.44

[†] Standard error of the mean.

[‡] Volume of rumen fluid calculated from the intercept of the exponential regression describing the decay of chrome concentration over time.

The fractional rates of absorption of VFA (Table 2) were similar between the two infusion methods and the fractional rate of total clearance ($P = 0.17$) and of absorption ($P = 0.16$) tended to be higher for the concentrate containing diet (Table 2).

Over the sampling period, there was a pH increase (Figure 2) and VFA concentration decrease (Figure 4) in both diets, but with different magnitudes.

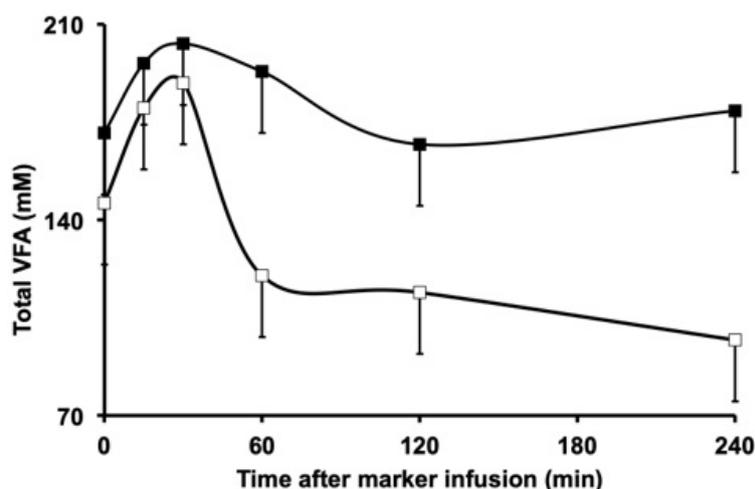


Figure 4. Total VFA concentration in the rumen fluid during sampling when the marker solution was infused into the intact digesta. Diets contained 100% forage (□) or 50% forage plus 50% concentrate (■). $P = 0.03$ for diet effect, $P = 0.12$ for time effect, $P = 0.39$ for interaction of diet and time.

Discussion

As expected, the increased in food intake showed that concentrate supplementation was effective to drive higher energy intake compared with the forage based diet.

The estimation of fluid volume from the moisture of rumen content includes the water contained inside the feed particles, it is expected that this method should provide

a higher amount of fluid for the forage than for concentrate based diet, due to the concentrate retains less water inside of its particles. When the calculation was done from the dispersion curve of chromium, the water inside the particles is not considered. Actually, it was expected that the amount of fluid out of the food particles should be greater in the concentrate plus forage than the forage diet, mainly due to the higher production of VFA by rumen microorganisms which could lead to higher osmotic pressure of rumen content. Higher osmotic pressure was associated to increasing in rumen liquid content (14).

The ruminal pH was higher in forage diet than in the concentrate containing diet. This difference in pH was expected since the pH is inversely related to the concentrations of VFA present in the rumen (8).

The peak of VFA production occurs several hours after feed intake (15). The sampling series began 6 and 7.5 h after feeding and this moment was closer to the VFA production peak. Since feed was withdrawn during sampling period, VFA formation was lower than the clearance, which led to decreased concentrations of VFA across sampling. Thus, the pH was expected to rise during the 240 min of sampling.

The approach developed by Daniel, de Resende Júnior (10) seems to be reliable for estimating the clearance of VFA because its estimates are consistent with those obtained by the technique of adding markers into the evacuated digesta (8).

One of the advantages of the infusing of markers into evacuated digesta is the homogenization, because the rumen content can be easily manipulated out the rumen, allowing an excellent mixture of the markers. This was demonstrated by Júnior, Pereira (8), who achieved high values of R² of the non-linear regressions that described the decay of markers over time. In the current study, the R² of the regressions which described the decline in valerate and Cr concentration over time did not differ across methods of infusion and were satisfactory high. The homogenization of digesta by rumen motility is too efficient as that performed by hand in the evacuated digesta.

The fractional passage rate of ruminal fluid to the omasum tended to be higher when the marker was infused into intact digesta, probably because of the rumen environment was not destabilized, especially by not disrupting the mat, so that the rumen motility may be maintained without interruption. Thus, the infusion of markers into intact digesta seems to be more efficient than infusion into evacuated digesta, because the rumen physiology is better preserved. In this experiment, the lower fractional passage rate when the marker was infused into evacuated digesta was associated with higher rumen fluid volume, confirming what was reported by Júnior, Pereira (8) who found a negative correlation between the volume of rumen fluid and the fractional rate of passage.

The R² of the regressions was high for both techniques and numerically higher in the technique of infusion of markers into intact digesta, providing more evidences that markers can be better homogenized by natural rumen motility than by hand in the evacuated digesta, indicating once again that the method proposed by Daniel, de Resende Júnior (10) seems to be superior to the method of adding markers to the evacuated digesta. Other authors used infusion of markers without rumen evacuation. However, they used larger volume of solution (7), which also interferes with the clearance of VFA, or they reported problems related to the homogenization (2). This demonstrates that the technique proposed by Daniel, de Resende Júnior (10) also

appears to be superior to other techniques reported for infusion of tracers in pulse dose, without rumen evacuation.

The fractional rates of absorption of VFA were similar between the two infusion methods, demonstrating again that it is secure estimating fractional rates of clearance using the technique of infusion of markers into intact digesta, as proposed by Daniel, de Resende Júnior (10).

The fractional rate of total clearance and of absorption ($P = 0.16$) tended to be higher for the concentrate containing diet. This phenomenon has biological coherence whereas the areas of the rumen papillae were largest and the epithelium had higher mitotic index in animals fed forage plus concentrate (data not showed).

The presence of a higher amount of rapidly fermentable nutrients and higher VFA concentration stimulated the cell proliferation leading to the rumen adaptation to the larger amount of VFA as evidenced by the clearance and absorption rate when animals were fed the more energetic diet. Melo, Costa (16) found a direct association between ruminal epithelium surface and the fractional rate of VFA clearance and absorption, confirming what was reported by (17).

The infusion of markers into intact digesta proved to be effective for determining the clearance of ruminal VFA. The homogenization of the marker solution into the intact digesta promoted by rumen motility seems to be more efficient than the one done manually into the evacuated digesta. This result has direct applicability in improving the productive efficiency of livestock, since it is fast, low cost and requires no experience beyond that required by the commonly used techniques.

References

1. Huntington G, Reynolds P, Tyrrell H. Net Absorption and Ruminal Concentrations of Metabolites in nonpregnant Dry Holstein Cows before and after Intraruminal Acetic Acid Infusion1. *Journal of dairy science*. 1983;66(9):1901-8.
2. Sutton JD, Dhanoa MS, Morant SV, France J, Napper DJ, Schuller E. Rates of production of acetate, propionate, and butyrate in the rumen of lactating dairy cows given normal and low-roughage diets. *J Dairy Sci*. 2003;86(11):3620-33.
3. Noziere P, Martin C, Remond D, Kristensen NB, Bernard R, Doreau M. Effect of composition of ruminally-infused short-chain fatty acids on net fluxes of nutrients across portal-drained viscera in underfed ewes. *Br J Nutr*. 2000;83(5):521-31.
4. Kristensen NB. Rumen microbial sequestration of [2-13C] acetate in cattle. *Journal of animal science*. 2001;79(9):2491-8.
5. Dijkstra J, Boer H, Van Bruchem J, Bruining M, Tamminga S. Absorption of volatile fatty acids from the rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid volume. *British Journal of Nutrition*. 1993;69(2):385-96.
6. Peters JP, Shen RY, Robinson JA, Chester ST. Disappearance and passage of propionic acid from the rumen of the beef steer. *J Anim Sci*. 1990;68(10):3337-49.

7. Allen M, Armentano L, Pereira M, Ying Y, Xu J, editors. Method to measure fractional rate of volatile fatty acid absorption from the rumen. Proc 25th Conf Rumen Function, Chicago, IL; 2000.
8. Júnior JR, Pereira M, Boer H, Tamminga S. Comparison of techniques to determine the clearance of ruminal volatile fatty acids. *Journal of dairy science*. 2006;89(8):3096-106.
9. Voelker JA, Allen MS. Pelleted beet pulp substituted for high-moisture corn: 3. Effects on ruminal fermentation, pH, and microbial protein efficiency in lactating dairy cows. *J Dairy Sci*. 2003;86(11):3562-70.
10. Daniel JLP, de Resende Júnior JC, de Melo LQ, de Souza RLM, Guerrero MC. Funcionalidade do sulco do retículo em bovinos adultos e avaliação de técnicas alternativas para preparação, infusão e coleta de marcadores da fase fluida ruminal. *Ciênc Agrotec*. 2007;31:1850-6.
11. Binnerts W, Van't Klooster AT, Frens A. Soluble chromium indicator measured by atomic absorption in digestion experiments. *Veterinary Record*. 1968;82.
12. Institute S. SAS/STAT user's guide: version 6: Sas Inst; 1990.
13. Mertens D, FAHEY JR G, Collins M, Mertens D. Forage quality, evaluation and utilization. *Forage quality, evaluation and utilization*. 1994.
14. Lopez S, Hovell FD, MacLeod NA. Osmotic pressure, water kinetics and volatile fatty acid absorption in the rumen of sheep sustained by intragastric infusions. *Br J Nutr*. 1994;71(2):153-68.
15. Pereira MN, Armentano LE. Partial replacement of forage with nonforage fiber sources in lactating cow diets. II. Digestion and rumen function. *J Dairy Sci*. 2000;83(12):2876-87.
16. Melo LQ, Costa SF, Lopes F, Guerreiro MC, Armentano LE, Pereira MN. Rumen morphometrics and the effect of digesta pH and volume on volatile fatty acid absorption. *J Anim Sci*. 2013;91(4):1775-83.
17. Dirksen G, Liebich H, Brosi G, Hagemester H, Mayer E. Morphologie der pansenschleimhaut und fettsäureresorption beim rind—Bedeutende faktoren für gesundheit und leistung. *Transboundary and Emerging Diseases*. 1984;31(1-10):414-30.