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Original article

Propionate uptake by rumen microorganisms: the effect of ruminal infusion

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Abstract — We assessed the ability of rumen microbes to significantly incorporate propionate when they are subjected in vivo to no infusion, long-term infusion of minerals, and short- and long-term infusion of high amounts of propionate. Four ruminally cannulated sheep fed 1000 g hay (8 meals per d) were used in a 4 × 4 Latin square design. The treatments consisted of no infusion (C), ruminal infusion of propionate (86 g·d^{-1}) for 1 (P1) and 7 d (P7), and of minerals for 7 d (M7). The infusion of propionate increased its ruminal molar percentage from 19 (C, M7) to 32% (P1, P7). Ruminal pH, osmolality, ammonia concentration, and protozoa counting were not or were poorly affected by the treatments. Rumen contents (100 mL liquid + 100 g solid) were incubated at 39 °C in anaerobic flasks containing artificial saliva, $(NH_4)_2SO_4$, ground hay, and 0.45 µCi [2-^{14}C] propionate. After 6 and 16 h, clarified fermenter fluid, liquid-associated protozoa, and liquid-associated bacteria considered as representative of total bacteria were separated by fractional centrifugations. Microbial pellets were washed with saline before ^{14}C determination. In flasks, pH, osmolality, gas, volatile fatty acid (VFA) production, ammonia concentration, protozoal counting and the amount of ^{14}C in clarified fermenter fluid, liquid-associated protozoa and liquid-associated bacteria were similar among the treatments. Between 6 h and 16 h, the amount of ^{14}C decreased in the clarified fermenter fluid, and increased in liquid-associated protozoa and liquid-associated bacteria. The amount of estimated microbial DM was 11.0 g per flask. After 6 h and 16 h incubation, the amount of ^{14}C incorporated into microbial fractions averaged 9.1 and 12.2% of the total amount of ^{14}C, and was estimated to account for 10.3% of propionate net production irrespective of the treatment and incubation time. It is concluded that the uptake of propionate by ruminal protozoa and bacteria is quantitatively significant, and may not be significantly affected when rumen microbes are submitted to minerals or propionate infusion. These results may explain the differences observed between the methods in the determination of the ruminal production rate of VFA.

sheep / rumen / microbe / propionate / infusion

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Résumé — Utilisation du propionate par les microorganismes du rumen : effet d'une infusion intra-ruminale. Nous avons étudié la capacité des microbes du rumen à incorporer significativement le propionate lorsqu'ils sont soumis ou non in vivo à des infusions à long terme de minéraux, et à court ou long terme d'une grande quantité de propionate. Quatre moutons fistulés du rumen, alimentés avec 1000 g de foin (8 repas par jour) ont subi 4 traitements, en carré Latin 4 × 4 : pas d'infusion (C), infusion de propionate (86 $g \cdot j^{-1}$) pendant 1 (P1) et 7 (P7) jours, infusion de minéraux pendant 7 jours (M7). Le pourcentage molaire de propionate dans le rumen augmente avec les infusions de propionate, de 19 (C, M7) à 32 % (P1, P7). Le pH, la pression osmotique, la concentration en ammoniaque, et le dénombrement des protozoaires dans le jus de rumen n'étaient pas ou peu différents entre traitements. Le contenu ruminal (100 mL phase liquide + 100 g phase solide) a été incubé à 39 °C dans des flacons anaérobies contenant de la salive artificielle, du sulfate d'ammonium, du foin broyé, et 0,45 µCi de [2-^{14}C] propionate. Après 6 et 16 h, les protozoaires et les bactéries libres – censées être représentatives des bactéries totales – ont été séparés du liquide ruminal par centrifugations différentielles. Les culots microbiens ont été rincés avec du sérum physiologique avant le comptage de ^{14}C. Dans les fermenteurs, le pH, la pression osmotique, la production de gaz et d'acides gras volatils (AGV), la concentration en ammoniaque, le dénombrement des protozoaires, et la quantité de ^{14}C dans le jus clarifié, les protozoaires et les bactéries libres étaient identiques entre traitements. Entre 6 h et 16 h, la quantité de ^{14}C diminuait dans le jus clarifié et augmentait dans les protozoaires et les bactéries libres. La quantité de microbes a été estimée à 9,6 g MS par fermenteur. Après 6 h et 16 h d'incubation, la quantité de ^{14}C incorporée dans les microbes atteignait 9,1 et 12,2 % de la quantité totale de ^{14}C, et était estimée représenter 10,3 % de la production nette de propionate, indépendamment du traitement et de la durée d'incubation. La captation du propionate par les protozoaires et les bactéries du rumen est donc quantitativement significative, et ne semble pas significativement modifiée quand les microbes du rumen sont soumis à des infusions de minéraux ou de propionate. Ces résultats pourraient expliquer les différences observées entre les méthodes de détermination de la production ruminale d'AGV.

mouton / rumen / microbe / propionate / infusion

1. INTRODUCTION

Ruminal infusion of volatile fatty acids (VFA) is widely used to investigate ruminal production rate [11, 12] and metabolism during absorption [18, 28]. In these methods, it is assumed that VFA are not sequestrated into the ruminal microbial biomass. However, the incorporation of [1-^{14}C] acetate radioactivity into the microbial lipids has been demonstrated using whole rumen contents [10, 26] and rumen protozoa [8], although this de novo synthesis appears to be quantitatively low compared to the direct incorporation of fatty acids into the microbial lipids [5]. It has been recently quantified in vivo by Kristensen [17] that about 28% of the ^{13}C intraruminally infused as [2-^{13}C] acetate is incorporated into microbial matter (fatty acids and amino acids) or non-acetate VFA. This explained that the portal recovery of acetate in sheep was higher when the reticulorumen was temporarily isolated and washed (100%, [19]), than when ruminal infusions were performed in fed animals (67 to 82%, [25, 28]). Previous works suggest the ability of some rumen bacteria in pure cultures [9] and mixed rumen protozoa [8] to incorporate propionate into odd-numbered long-chain fatty acids. In studies focusing on the determination of ruminal production rate and metabolism during absorption, the infusion of propionate varies from low amounts and short-term infusions with isotope-dilution techniques [11, 24] to high amounts and short-term [12] to long-term [25] infusions with non-tracer methods. The aim of the present work was to assess (1) the ability of rumen microbes to significantly incorporate propionate, (2) the effect of long-term infusion of propionate or minerals, and

(3) the effect of the infusion length of propionate. Rumen contents were obtained in vivo from sheep submitted (or not) to ruminal infusions; the ability of microbes to take up propionate in vivo was revealed by in vitro incubations of the rumen contents with labelled propionate.

2. MATERIALS AND METHODS

2.1. Animals and treatments

Four adult (5 years) castrated Texel sheep weighing 67 (±3 kg) were used. They were surgically fitted with a rumen cannula in polyvinyl chloride (i.d. 75 mm). They were housed in individual boxes (1.5 m × 1 m) in an air-conditioned room with a controlled temperature (18 °C) and 24-h lighting. They were fed 1000 g DM per day of a first-cut of natural grassland hay (crude fibre 30.0%, crude protein 12.0% DM), covering maintenance requirements according to French energy and nitrogen systems [13]. No refusals were observed. The hay was chopped and given every 3 h in 8 equal meals beginning at 06:00, with an automatic feeder. The animals were allocated to a 4 × 4 Latin square design. Each experimental period lasted 7 days (Fig. 1). The treatments consisted of no infusion for 7 days (control, C), ruminal infusion of propionate for 1 day following 6 days without infusion (P1), ruminal infusion of propionate for 7 days (P7), and ruminal infusion of minerals for 7 days (M7). The comparison of C and M7 dealt with the specific effect of long term infusions, the comparison of M7 and P7 with the specific effect of propionate, and the comparison of P1 and P7 with the effect of infusion length. The infusion mixture of propionate consisted in 29.6 g propionate, 3.60 g NaOH and 1.68 g KOH, dissolved in 1 L water. The infusion mixture of minerals consisted in 5.26 g NaCl and 2.24 g KCl, dissolved in 1 L water. The actual daily infusion rates averaged 2.93, 2.87 and 2.90 litres for P1, P7 and M7, respectively. The animals had free access to water and salt blocks.

2.2. Rumen sampling and fermenter management

The measurements were performed on 2 animals per day on the 7th day of each period, which corresponded to the last day of the period for each animal. Rumen contents (about 850 g) were collected by hand at different places in the dorsal and the ventral sacs of each animal at 09:00 (just before feeding) and 16:00 (1 h after feeding). The rumen contents were separated in solid and liquid phases using a nylon tissue of 250 µm pore size for subsequent determination of physico-chemical parameters and in vitro incubations.

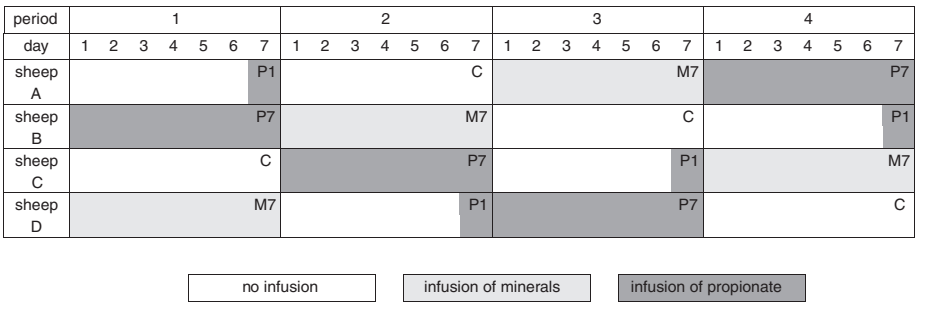

Figure 1. In vivo experimental design.

length of incubation	6 h (morning) or 16 h (evening)							
sheep	A - B				C - D			
flask	1	2	3	4	5	6	7	8
^{14}C in flask	no		yes		no		yes	
measurements	gas, physico-chemical parameters and protozoa		gas, ^{14}C		gas, physico-chemical parameters and protozoa		gas, ^{14}C	

Figure 2. In vitro experimental design.

The pH was immediately measured on rumen fluid using a combination electrode. The osmotic pressure was determined with a vapour pressure osmometer (Knauer D1000, Berlin, Germany). After adding 0.1 volume of orthophosphoric acid (5% v/v) for conservation, VFA were determined by gas chromatography using 4-methylvaleric acid as an internal standard [14] and ammonia was determined colorimetrically by the phenol-hypochlorite automated method [31]. Since no effect of sampling time was observed on most rumen variables, protozoa concentrations were determined only in samples withdrawn at 09:00, according to Jouany and Senaud [15].

The closed anaerobic system described by Jouany and Thivend [16] and detailed by Doreau et al. [7] was used for in vitro incubations. In order to reduce variability due to uncontrolled differences in the dry matter percentage of rumen contents from one sample to another, the same amount of solid and liquid phases were incubated. Eight flasks (1 L) were simultaneously maintained in a common lateral shaking water bath kept at 39 °C. The fermentation medium in each flask was made of 100 g solid phase of rumen content, 100 mL rumen liquid, 200 mL artificial saliva[1], N source and energy source. The N source consisted of 5 mL of $(NH_4)_2SO_4$ solution at 17.6% (w/v). The energy source consisted of 17 g DM of a first-cut cocksfoot hay (crude fibre 38.9%, crude protein 7.3%), ground through a 0.8 mm sieve.

For each period, the incubations lasted 6 h (morning series with rumen samples withdrawn at 09:00) or 16 h (evening series with rumen samples withdrawn at 16:00), with 4 flasks per animal and per series, two of them containing 0.45 µCi [2-^{14}C] propionate (Fig. 2). The gas produced by the fermentation was evacuated in a test-tube, and the volume was read after 1, 2, 3, 4, 5 and 6 h incubation for the morning series, and 16 h incubation for the evening series (4 flasks / animal / period / series). For each animal / period / series, liquid samples from the two non-labelled flasks were collected with a syringe after 1, 2, 3, 4, 5 and 6 h incubation for the morning series, and after 16 h incubation for the evening series for pH measurement. Other liquid samples from one non-labelled flasks were taken after 6 h (morning series) and 16 h (evening series) of incubation for determination of osmotic pressure, volatile fatty acids, ammonia, and protozoa concentration, by the methods described above.

2.3. Isolation procedures

At the end of the incubations, the total content of the two labelled flasks and of one non-labelled flask / animal / period / series were filtrated through a nylon tissue of 150 µm pore size. The filtrate was weighed

[1] NaCl = 2.35 g; KCl = 2.25 g; $MgCl_2$, 6 H_2O = 0.43 g; $CaCl_2$, 2 H_2O = 0.36 g; $NaHCO_3$ = 92.40 g; Na_2HPO4, 12 H_2O = 71.24 g dissolved with water to a total volume of 5 litres, and saturated with CO_2.

then centrifuged (10 min, 4 °C, 800 × *g*) to separate small particles and protozoa from the supernatant [22]. The pellet of the protozoa was washed twice with saline (9 g $NaCl \cdot L^{-1}$). The pellet of the small particles was washed with saline. The supernatant was weighed then centrifuged (20 min, 4 °C, 27000 × *g*) to separate the liquid-associated bacteria from the clarified fermenter fluid. The pellet of the bacteria was washed with saline. Each of the 3 fractions, i.e. liquid-associated protozoa, liquid-associated bacteria and clarified fermenter fluid from both labelled flasks were pooled and frozen (–20 °C) till ^{14}C analyses.

2.4. ^{14}C analytical procedures

^{14}C was determined by liquid scintillation counting (Packard-Tri-Carb-1600 TR). Two volumes of H_2O_2 were added to one volume of the clarified fermenter fluid for decolourisation two days before counting to allow a decrease in luminescence. After lyophilisation and grinding, liquid-associated protozoa and liquid-associated bacteria were solubilised in NaOH 0.3M (8 mL for 100 mg dry microbial matter) for 45 minutes in a water bath at 37 °C. The samples were introduced in a counting flask then diluted with water, with 3 mL H_2O for 2 mL decolorised clarified fermenter fluid, and 4 mL H_2O for 2 mL solubilised liquid-associated protozoa or liquid-associated bacteria. All samples were then added to 10 mL of scintillant solution (Quick Safe A), then analysed immediately (clarified fermenter fluid) or conserved at 4 °C for 4 days (liquid-associated protozoa and liquid-associated bacteria) before ^{14}C determination, to allow equilibrium between NaOH and the scintillant solution. Corrections for quenching were made for each sample by counting the paired-samples prepared with the same procedure, except that 1 mL H_2O was substituted by 1 mL internal standard (525.5 $dpm \cdot mL^{-1}$ for the clarified fermenter fluid, 210.2 $dpm \cdot mL^{-1}$ for the liquid-associated protozoa and liquid-associated bacteria). The internal standard was prepared by diluting the calibrated ^{14}C internal standard capsules in NaCl 0.9%. The ^{14}C enrichment was determined by counting the cpm including a correction for luminescence and colouration.

2.5. Calculations and statistics

For each sample, the amount of disintegration per minute (dpm S) was determined as follows:

$$dpm\ S = cpm\ S / [\ (cpm\ SI - cpm\ S) / dpm\ I\ ]$$

where cpm S and cpm SI are counts per minute in samples without and with internal standard, respectively, and dpm I is the disintegration per minute in the internal standard.

Data were analysed by analysis of variance with the animal (1 to 4), period (1 to 4), and treatment (C, M7, P1, P7) as factors with the repeated time procedure of GLM of SAS [30], except for protozoa in the rumen fluid which were determined only in 09:00 samples. Analysis of protozoal counts was performed on concentrations without log transformation prior to statistical analysis. For each time, the mean per animal and per treatment was considered as the experimental unit. When the analysis of variance was significant, differences between treatments were assessed by the Student-Newman-Keuls t-test. Significance was declared at $P < 0.05$, and a trend was considered at $P < 0.10$.

3. RESULTS

3.1. Rumen physico-chemical parameters and protozoa

The infusion of minerals for 7 days tended to increase ruminal pH from 6.28 (SE = 0.09) to 6.46 (SE = 0.12), and to decrease total VFA concentration ($P < 0.10$)

Table I. The effects of infusions and sampling time on physico-chemical parameters in the rumen fluid.

	Treatment [1]					Effects		
	C	M7	P1	P7	SE	Treatment	Time	Treatment × Time
pH [2]	6.28	6.46	6.27	6.41	0.07	NS	NS	NS
Osmolality (mosm·L^{-1})	284	252	253	259	33	NS	NS	NS
Ammonia (mM)	5.08 ^{a}	5.10 ^{a}	4.79 ^{a}	3.62 ^{b}	0.38	*	NS	NS
VFA (mM)	101.9 ^{ab}	93.3 ^{b}	113.5 ^{a}	104.8 ^{ab}	4.8	*	NS	NS
Acetate	70.3	64.7	66.0	61.4	3.5	NS	*	NS
Propionate	19.9 ^{a}	18.0 ^{a}	36.9 ^{b}	33.6 ^{b}	1.7	***	NS	NS
Isobutyrate	0.81	0.83	0.74	0.87	0.07	NS	NS	NS
Butyrate	9.29	8.24	8.31	7.39	0.84	NS	NS	NS
Isovalerate	0.84	0.90	0.85	0.85	0.08	NS	**	NS
Valerate [3]	0.65	0.58	0.72	0.68	0.04	NS	NS	NS

[1] C: control; M7: infusion of minerals for 7 days; P1: infusion of propionate for 1 day; P7: infusion of propionate for 7 days. Each value consists in the mean of morning and evening samples.
[2] Non-significant trend: $P = 0.082$ for treatment effect.
[3] Non-significant trend: $P = 0.090$ for treatment effect.
[a, b] Means within the same row with different superscripts differ ($P < 0.05$).
NS = non significant ($P > 0.05$); *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

from 101.9 (SE = 2.8) to 93.3 (SE = 4.7) mM (Tab. I). This decrease was mainly due to acetate from 70.3 (SE = 2.6) to 64.7 (SE = 2.5) mM, although it failed statistical significance. Ruminal osmolality, ammonia, and other individual VFA concentrations were not affected by the infusion of minerals for 7 days. The infusion of propionate for 7 days tended to increase ruminal pH from 6.28 (SE = 0.09) to 6.41 (SE = 0.04), and induced a decrease in ammonia concentrations from 5.08 (SE = 0.52) to 3.62 (SE = 0.33) mM, and an increase in propionate concentration from 19.9 (SE = 0.5) to 33.6 (SE = 1.0) mM concomitant to a decrease in acetate concentration from 70.3 (SE = 2.6) to 61.4 (SE = 2.7) mM, which failed statistical significance. The ruminal osmolality, total VFA, isobutyrate, butyrate, isovalerate and valerate concentrations were not affected by the infusion of propionate for 7 days. The concentrations of ammonia were higher, 4.79 (SE = 0.33) vs. 3.62 (SE = 0.33) mM, whereas pH tended to be lower, 6.27 (SE = 0.06) vs. 6.41 (SE = 0.04), after 1 day than after 7 days of propionate infusion. Osmolality, total and individual VFA concentrations were similar between P1 and P7.

The acetate and isovalerate concentrations were higher in the morning samples used for 6-h in vitro fermentations, than in the evening samples used for 16-h in vitro fermentations, 66.8 (SE = 2.6) vs. 64.4 (SE = 2.6) mM for acetate, and 0.92 (SE = 0.06) vs. 0.80 (SE = 0.06) mM for isovalerate, respectively. No significant effect of sampling time was observed on pH, osmolality, ammonia and the concentration of total and other individual VFA. The interaction between treatment and sampling time was not significant for all ruminal parameters.

Table II. The effect of infusions on the number and composition of the protozoal population in the rumen fluid.

	Treatment [1]					Effect
	C	M7	P1	P7	SE	Treatment
Number of protozoa ($\times 10^3 \cdot mL^{-1}$)	105.0	80.3	73.3	71.8	14.6	NS
Protozoal genera population (%)						
Isotricha	2.3	2.0	3.1	2.3	0.5	NS
Dasytricha	4.9	5.7	7.7	5.7	0.7	NS
Entodinium	88.3	89.1	86.4	90.9	1.7	NS
Diplodinium	2.9	1.9	1.9	1.3	0.9	NS
Eudiplodinium	0.8	0.8	0.4	0.4	0.2	NS
Polyplastron	0.8	0.6	0.5	0.3	0.2	NS

[1] C: control; M7: infusion of minerals for 7 days; P1: infusion of propionate for 1 day; P7: infusion of propionate for 7 days.
NS = non significant ($P > 0.05$).

The amount of protozoa in the rumen did not significantly differ between treatments (Tab. II), averaging $82.6 \times 10^3 \cdot mL^{-1}$. The percentage of each genus was similar between treatments, averaging 2.4, 6.0, 88.7, 2.0, 0.6, and 0.6% for *Isotricha, Dasytricha, Entodinium, Diplodinium, Eudiplodinium*, and *Polyplastron*, respectively.

3.2. In vitro fermentations

The pH, osmolality, ammonia and total VFA concentrations, propionate and total VFA net production, and gas production in the fermenters were similar among treatments (Tab. III). The ruminal concentration of propionate was higher with treatments P1 and P7 than with treatments C and M7.

The pH and ammonia concentration decreased whereas the osmolality, the propionate and total VFA concentrations and net production, and the gas production increased with the incubation time (on average, 6.71 (SE = 0.04) vs. 5.86 (SE = 0.09) for pH, 19.2 (SE = 0.7) vs. 13.9 (SE = 1.2) mM ammonia, 382 (SE = 42) vs. 440 (SE = 51) mOsm, 30.0 (SE = 1.7) vs. 54.3 (SE = 1.0) mM propionate, 116 (SE = 6.1) vs. 198 (SE = 3.9) mM total VFA, 6.6 (SE 0.71) vs. 15.5 (SE 0.30) mmol propionate, 25.2 (SE = 2.5) vs. 56.3 (SE = 1.6) mmol total VFA, 1157 (SE = 132) vs. 2176 (SE = 19) mL gas, after 6 h and 16 h, respectively). The interaction between treatment and incubation time was not significant for pH, osmolality, ammonia, propionate and total VFA concentration and net production, and gas production.

The amount of protozoa (Tab. III), and most protozoal genera in fermenters (data not shown) were not affected by the treatments, but the percentage of *Eudiplodinium* was higher with C and M7 (averaging 1.1%) than with P1 and P7 (averaging 0.4%). The amount of protozoa in the fermenters did not vary with the incubation time, but the percentage of *Dasytricha* increased from 5.0 (SE = 0.9) to 8.5 (SE = 2.0) % between 6 and 16 h incubation time, whereas the percentage of *Entodinium* and *Polyplastron* decreased from 91.3 (SE = 1.3) to 88.5 (SE = 2.3), and 0.3 (SE = 0.1) to 0.1 (SE = 0.1)%, respectively. The percentage of *Diplodinium* and *Eudiplodinium* also tended to decrease with the incubation

Table III. The effects of infusions and incubation time on physico-chemical parameters, VFA and gas production, and the number of protozoa in the fermenters.

	Treatments [1]					Effects		
	C	M7	P1	P7	SE	Treatment	Time	Treatment × Time
pH								
6 h	6.70	6.79	6.66	6.68				
16 h	5.84	5.89	5.82	5.88	0.08	NS	***	NS
Osmolality (mOsm·L^{-1})								
6 h	416	337	395	381				
16 h	442	420	466	433	58	NS	*	NS
Ammonia (mM)								
6 h	19.4	18.4	19.6	19.5				
16 h	14.3	14.8	13.4	12.9	1.1	NS	***	NS
Propionate (mM)								
6 h	25.5 [a]	26.1 [a]	36.4 [b]	32.0 [ab]				
16 h	50.7 [a]	50.7 [a]	58.1 [b]	57.7 [b]	1.6	**	***	NS
Total VFA (mM)								
6 h	111.4	112.3	125.4	114.2				
16 h	192.7	196.2	202.7	200.7	6.5	NS	***	NS
Propionate net production (mmol)								
6 h	6.1	6.7	7.3	6.2				
16 h	15.5	15.8	15.2	15.6	0.7	NS	***	NS
Total VFA net production (mmol)								
6 h	23.3	25.7	27.2	24.6				
16 h	55.1	57.3	56.1	56.8	2.7	NS	***	NS
Gas production (mL)								
6 h	1138	1174	1199	1115				
16 h	2241	2126	2168	2168	107	NS	*	NS
Number of protozoa (× 10^3·mL^{-1}) [2]								
6 h	87.1	52.7	63.1	47.6				
16 h	70.8	68.3	44.8	49.4	11.9	NS	NS	NS

[1] C: control; M7: infusion of minerals for 7 days; P1: infusion of propionate for 1 day; P7: infusion of propionate for 7 days.
[2] Non-significant trend: $P = 0.084$ for treatment × time effect.
[a, b] Means within the same row with different superscripts differ ($P < 0.05$).
NS = non significant ($P > 0.05$); *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

Table IV. The effects of infusions and incubation time on ^{14}C in the clarified fermenter fluid, liquid-associated-protozoa and liquid associated bacteria from the fermenters.

	Treatments [1]					Effects		
	C	M7	P1	P7	SE	Treatment	Time	Treatment × Time
^{14}C in clarified fermenter fluid (dpm·mL^{-1})								
6 h	1850	1667	1783	1700				
16 h	1416	1342	1571	1473	83	NS	***	NS
^{14}C in liquid-associated bacteria (dpm·mg^{-1} DM)								
6 h	7.67	8.46	7.30	5.57				
16 h	12.89	11.05	13.23	8.98	2.27	NS	***	**
^{14}C in liquid-associated protozoa (dpm·mg^{-1} DM) [2]								
6 h	7.77	7.71	7.41	9.21				
16 h	9.09	13.22	7.86	8.02	2.14	NS	NS	NS

[1] C: control; M7: infusion of minerals for 7 days; P1: infusion of propionate for 1 day; P7: infusion of propionate for 7 days.
[2] Non-significant trends: $P = 0.057$ for time effect, $P = 0.059$ for treatment × time effect.
[a, b] Means within the same row with different superscripts differ ($P < 0.05$).
NS = non significant ($P > 0.05$); **: $P < 0.01$; ***: $P < 0.001$.

time, from 1.4 (SE = 0.5) to 1.0 (SE = 0.2) and from 0.8 (SE = 0.2) to 0.5 (SE = 0.1)%, respectively.

3.3. ^{14}C distribution

The amount of radioactivity recovered in the clarified fermenter fluid, liquid-associated bacteria and liquid-associated protozoa did not vary with the infusion of minerals or propionate (Tab. IV). Between 6 and 16 h incubation time, the radioactivity decreased in the clarified fermenter fluid, from 1750 (SE = 78) to 1450 (SE = 59) dpm·mL^{-1}, whereas it increased in liquid-associated bacteria, from 7.25 (SE = 1.84) to 11.54 (SE = 1.93) dpm·mg^{-1} DM. The same trend was observed in liquid-associated protozoa, from 8.03 (SE = 2.12) to 9.55 (SE = 1.55) dpm·mg^{-1} DM. The increase in radioactivity in liquid-associated microbes with incubation time was more marked with treatments C and P1 for bacteria and with treatment M7 for protozoa.

4. DISCUSSION

4.1. Ruminal parameters

The main assumptions in techniques based on ruminal infusions are that infusions do not cause disturbance in digestive and absorptive processes. In the present work, the infusion of propionate (50 mmol·day^{-1}·kg BW$^{-0.75}$) tended to decrease acetate ruminal concentration. The decrease was not attributable to propionate but to the high level of infusion by itself, since a similar trend was observed following the infusion of minerals. This is consistent with previous studies who reported no significant changes in acetate concentration between the control treatment consisting of the infusion of minerals and the experimental treatments consisting of the infusion of 13 to 72 mmol propionate·day^{-1}·kg BW$^{-0.75}$ [20, 29]. This shows the need to compare the infusion of VFA with the infusion of minerals. The ruminal pH, osmolality, concentrations of

ammonia and minor VFA were not or were to a low extent affected by the infusion of minerals or propionate. In the same way, the number and composition of the protozoal population in the rumen were not modified by the infusions, as also reported by Martin et al. [24]. These results support the hypothesis that the cellulolytic microbial activity was not affected, and were in agreement with Nozière et al. [25] who reported that long-term ruminal infusion of VFA in sheep fed hay did not affect the digestibility of the diet. The low decrease in ruminal concentrations of VFA following infusion may thus be related to small changes in the dynamics of ruminal fluid (volume, turnover rate) induced by infusion or water intake, rather than to a decrease in VFA production. The differences in acetate and isovalerate concentrations between the morning and evening samples were low, and no time effect was observed on the other physico-chemical ruminal parameters. This was related to both the continuous ruminal infusions and the semi-continuous distribution of hay, as also previously reported [25]. This resulted in the lack of an inoculum effect between the in vitro morning and evening series.

4.2. Validation of in vitro fermentations

The net production of total VFA, propionate and gas in fermenters were similar among treatments. These results, together with those of ruminal parameters, confirm that the hydrolytic and fermentative activities of microbes in the rumen were not affected by minerals and propionate infusions, and suggest that the higher concentration of propionate in the rumen fluid introduced in the flasks with treatments P1 and P7 did not affect the in vitro fermentations. In the present work, it was of importance that the fermentation rate of the substrate be high and long enough to allow the potential incorporation of ^{14}C into the microbes, and that the protozoal population outlive the conditions of fermentation. These objectives were achieved, as shown by the increase ($P < 0.001$) in the protozoal population in the fermentation medium from $39 \times 10^3 \cdot mL^{-1}$ at 0 h, to 63×10^3 and $58 \times 10^3 \cdot mL^{-1}$ after 6 and 16 h, respectively, on average for the 4 treatments. These results were consistent with the minimum doubling time reported for *Entodinium* spp., as reviewed by Williams and Coleman [33]. The survival of protozoa was related to an adequate buffering of the media, since the pH never decreased under 5.47. In the same way, the amount of VFA and the molar percentage of individual VFA produced between 0 and 6 h and between 6 and 16 h incubation time were similar. Acetate, propionate, butyrate, valerate and isoacids accounted on average for 61, 28, 9.5, 1.1, and 0.8% of the 25.2 and 56.3 mmol of total VFA produced after 6 and 16 h. These patterns were consistent with normal ruminal fermentations, indicating that the microbial metabolism was not modified by the accumulation of end products of fermentation in the flasks. The osmolality in the fermenters reached high values, averaging 382 and 440 $mOsm \cdot L^{-1}$ after 6 and 16 h incubation time. However these values remained consistent with the physiological conditions and with the tolerance of rumen microbes to hypertonicity, until 500 $mOsm \cdot L^{-1}$, as reviewed by Carter and Grovum [3].

4.3. ^{14}C distribution

In the present experiment, it was assumed that the ability of microbes to take up ^{14}C in vitro reflected their ability to take up propionate in vivo. Our results evidence a significant incorporation of ^{14}C from propionate into both bacterial and protozoal populations. This is in line with previous works which evidenced the ability of some rumen bacteria in pure cultures [9] and mixed rumen protozoa [8] to incorporate propionate into odd-numbered long-chain fatty acids. The incorporation into amino acids, clearly demonstrated for acetate [17, 27], remains questionable for propionate.

Since it was not possible to isolate pure microbial fractions in totality, the counting was performed on samples previously purified by washing. The enrichment was similar in both liquid-associated bacteria and liquid-associated protozoa, averaging 8.2 and 11.1 dpm·mg^{-1} microbial DM after 6 and 16 h incubation time, respectively. The total amount of radioactivity taken up by the microbes in the flasks was estimated. For this estimation it was assumed that 1) each flask contained 170 mL rumen liquid (95 mL from the liquid phase + 75 mL from the solid phase) with 12 g microbial DM·L^{-1}, and 30 g DM solid phase (5 d from the liquid phase + 25 g from the solid phase) with 300 g microbial DM·kg^{-1} [21], thus a total amount of 11.0 g microbial DM; 2) ^{14}C enrichment of liquid-associated microbes was representative of the enrichment of solid-associated bacteria, although these two populations differ in lipid and N content [1, 4] and AA composition [23]. The calculation of the amount of ^{14}C incorporated into the total microbial matter after 6 h and 16 h incubation time averaged 91 000 and 122 100 dpm per flask, respectively, i.e. 9.1 and 12.2% of total amount of ^{14}C incubated. These calculations also indicate that the percentage of ^{14}C incubated which was not detected in the clarified fermenter fluid or microbes averaged 26 and 34% after 6 h and 16 h incubation time, respectively. Although it depends on the estimations of the volume of fluid and the amount of microbial matter in the flasks, the high percentage of non-recovered ^{14}C cannot be entirely attributable to the lack of accuracy of these estimations. Other factors may be involved. Although labelled propionate used in this study was [2-^{14}C] propionate, a part of non-recovered radioactivity may have been lost into the gas, CO_2 or CH_4. Indeed, an anaerobic degradation of propionate by syntrophic consortia of acetogenic and methanogenic bacteria has been evidenced [2, 6]. Since the turnover of ruminal bacteria is quick [32], it may also induce a significant loss of radioactivity through the metabolism of propionate into CO_2. Another part of this loss can be attributed to methodological limits in the correction of quenching although one correction was made for each sample. The determination of total recovery using the whole content of the incubation flask, non incubated samples or samples incubated with rumen fluid without microbial activity, would have provided additional information compared to the recovery calculated from the separate fractions.

4.4. Estimation of propionate uptake by microbes

Assuming that all ^{14}C remained in propionate, the ^{14}C enrichment in propionate (dpm·µmol^{-1}) was calculated as the ratio between the radioactivity in the fermenter fluid (dpm·mL^{-1}) and the concentration of propionate in the fermenter fluid (µM), and the statistics were performed on these calculated data (Tab. V). In the beginning of incubation, the enrichment of propionate was higher for C and M7 (averaging 314 dpm·µmol^{-1}) where propionate concentrations were low (averaging 8.8 mM), than for P1 and P7 (averaging 169 dpm·µmol^{-1}) where propionate concentrations were high (averaging 16.5 mM). The enrichment decreased during incubation when the propionate concentration increased. It was similar among treatments, averaging 61 and 27 dpm·µmol^{-1} after 6 and 16 h incubation, respectively. Between 0 and 16 h, the average enrichment of propionate was higher for C and M7 (averaging 132 dpm·µmol^{-1}) than for P1 and P7 (averaging 90 dpm·µmol^{-1}), and between 6 and 16 h, it was similar among treatments, averaging 39 dpm·µmol^{-1}.

The amount of propionate taken up by the microbial fractions (µmol·mg^{-1} microbial DM) after 6 and 16 h incubation time was estimated according to these calculations, assuming a similar enrichment in both liquid-associated bacteria and

liquid-associated protozoa, and the statistics were performed (Tab. V). The estimated propionate uptake was similar between the treatments after 6 h incubation time, averaging 75 µmol propionate·g^{-1} microbial DM. It increased with time, averaging 155 µmol propionate·g^{-1} microbial DM after 16 h incubation, where it tended to be lower with P7 than with the other treatments. The estimated propionate uptake averaged 10.3% of propionate net production, and this value was similar among incubation time and treatments. This was the first estimation of propionate uptake by the complex microbial biomass in the rumen. As expected, this value was lower than the one estimated for acetate from data reported by Kristensen [17], which suggested that nearly 20% of ^{13}C ruminally infused in [2-^{13}C] acetate was absorbed before the duodenum as non-acetate in cows.

4.5. Implication on the determination of VFA production rate in the rumen

The uptake of propionate was not directly measured by this experimental design, but the ability of microbes to take up

Table V. The effects of the infusions and incubation time on the estimated propionate enrichment in the fermenter fluid and microbial uptake.

	Treatments [1]					Effects		
	C	M7	P1	P7	SE	Treatment	Time	Treatment × Time
Estimated propionate enrichment [2] (dpm·µmol^{-1})								
0 h	295 [a]	332 [a]	161 [b]	176 [b]				
6 h	76 [a]	65 [a]	49 [a]	54 [a]				
16 h	28 [a]	26 [a]	27 [a]	26 [a]	8	***	***	***
Estimated mean propionate enrichment [3] (dpm·µmol^{-1})								
0 to 6 h	134 [a]	130 [a]	85 [b]	94 [b]				
6 to 16 h	43 [a]	39 [a]	36 [a]	36 [a]	7	*	***	**
Estimated propionate uptake [4] (µmol·g^{-1} microbial DM)								
6 h	64 [a]	72 [a]	85 [a]	80 [a]				
16 h	168 [a]	167 [a]	175 [a]	111 [b]	8	*	**	NS
Estimated propionate uptake / propionate net production (%)								
6 h	8.9	9.6	12.7	12.9				
16 h	10.1	10.0	11.2	6.7	1.5	NS	NS	NS

[1] C: control; M7: infusion of minerals for 7 days; P1: infusion of propionate for 1 day; P7: infusion of propionate for 7 days.
[2] Propionate enrichment = radioactivity/propionate, in fermenter fluid. For T = 0 h, radioactivity added in fermenters was 2700 dpm·mL^{-1}.
[3] Mean propionate enrichment between Ti and Tf = [enrichment at Ti × amount of propionate at Ti + enrichment at Tf × amount of propionate at Tf] / [amount of propionate at Ti + amount of propionate at Tf].
[4] Propionate uptake at Tf = propionate uptake at Ti + [increase in ^{14}C in microbes between Ti and Tf] / [mean propionate enrichment between Ti and Tf], with Ti = 0 when Tf = 6 h, Ti = 6 h when Tf = 16 h. ^{14}C in microbes is the mean of measurements in bacteria and protozoa.
[a, b] Means within the same row with different superscripts differ ($P < 0.05$).
NS = non significant ($P > 0.05$); *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

propionate was investigated. The ability of ruminal protozoa and bacteria to take up propionate is demonstrated in this study, although its importance in vivo cannot be quantified by this in vitro approach. This uptake may be responsible for the overestimation of ruminal production rate of VFA measured by the isotope-dilution techniques, as suggested by Martin et al. [24]. In the non tracer method based on regression approach after short-term ruminal infusions of increasing levels of VFA into the rumen [12], the production rate is given by the ratio intercept/slope of the relationship between the infusion rate and ruminal concentration. Our estimations indicated that the ability of microbes to take up propionate is not modified after they have been submitted to short-term infusions of high amounts of propionate. Although these estimations have to be carefully interpreted due to the lack of steady state and the complexity of tracer kinetics under in vitro incubations, they suggest that the determination of the VFA production rate by the regression approach is not significantly biased by the incorporation of propionate into microbes. This is in line with the results of Martin et al. [24] who reported that the rumen production rate of individual VFA determined by the regression approach was consistent with stoechiometry.

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