Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents.

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Influence of Sampling Time and Diet on Amino Acid Composition of Protozoal and Bacterial Fractions from Bovine Ruminal Contents

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ABSTRACT: Four ruminally cannulated cows were used to compare amino acid (AA) composition of protozoal and bacterial fractions as affected by sampling time and diet. Cows were given once a day restricted feed (80% of ad libitum intake) of 7 kg DM with two successive diets. Diet HB was 65% Cocksfoot hay and 35% pelleted ground barley, and Diet H was 100% Cocksfoot hay. Samples of whole ruminal contents were taken 2, 5, 8, 11, and 23 h after feeding for Diet HB and 2 h after feeding for Diet H to isolate the liquid-associated protozoa and bacteria (LAP, LAB) and particle-associated bacteria (PAB). At each sampling time, the AA compositions of the different microbial populations were determined. The AA profiles of the LAP were different from those of the bacteria for 13 AA out of 17 studied. Differences between AA compositions of LAB and PAB were also observed for 10 AA out of 17 studied. Irrespective of the microbial population, AA composition did not vary with sampling time after feeding diet HB ($P > .05$; except for arginine, glutamate, and glycine). The AA contents of none of the three microbial populations were affected ($P > .05$) by the diet except for leucine and glutamate ($P < .01$). The differences in AA profiles between LAP and bacteria and between LAB and PAB confirm the importance of the representativeness of the microbial reference sample for correctly estimating microbial AA flow into the small intestine.

Key Words: Rumen Microorganisms, Protozoa, Bacteria, Amino Acids

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Introduction

The intestinal supply of amino acids (AA) in ruminants is derived from microbial protein, endogenous protein, and dietary protein that escapes ruminal fermentation. Because protein of microbial origin usually accounts for a substantial portion (50% or more) of the total AA entering the small intestine, AA composition of the microorganisms must be determined. During the last two decades, the method largely used has been the estimation of microbial protein yield leaving the rumen using AA composition of ruminal free-floating bacteria. Doubts about this method can be expressed because of differences in composition between fluid and particulate bacteria (Legay-Carmier and Bauchart, 1989) and protozoa (Martin et al., 1994). Indeed, the relative contributions of the protozoa and bacteria to microbial matter flow into the small intestine are not clear (Punia et al., 1992). The recent review of Williams and Coleman (1991) indicates that protozoal N may represent up to 50% of the microbial N reaching the lower gut. Moreover, variations in chemical composition (OM, N, DAPA, ^{15}N) of the protozoa and bacteria with sampling time after feeding was observed in a previous paper (Martin et al., 1994), but the postprandial effect on AA composition of these microbial fractions has not been studied. Therefore, large errors may result in the estimation of the quantity of microbial AA that pass to the intestines if the AA composition of the microbial sample is not representative of what leaves the rumen. The aim of the present study was to compare the AA composition of ruminal protozoal and bacterial fractions at various times postfeeding. The effect of diet on AA composition of these different microbial populations also was examined.

Materials and Methods

Four nonlactating adult Jersey cows (average BW 375 ± 28 kg) fitted with permanent ruminal cannulas made of polyamide and polyvinylchloride (Synthesia, Nogent sur Marne, France) were used in this experiment as donor animals for ruminal contents. Surgery was performed in a sterile environment under general

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Table 1. Chemical composition of experimental feeds and diets

Item	OM	CP	NDF	ADF	Starch
	% DM				
Feeds					
Hay	92.3	17.6	60.9	30.3	—
Barley	97.3	11.6	17.2	5.7	60.2
Diets					
Hay	91.3	17.8	60.4	30.8	—
Hay + barley (65/35)	94.1	15.2	44.8	20.6	21.8

anesthesia (Halothane, ICIU Pharma-vétérinaire, Paris, France). The cows received antibiotic treatment for 4 d after surgery. Cows were housed in individual metabolism cages in an air-conditioned room (maximum daily temperature 20 ± 2.7°C, minimum daily temperature 14 ± 1.7°C) with free access to water and mineralized salt blocks (38% Na, .9% Zn, .075% Mn, .15% Cu, .009% I, and .0003% Co), and received two successive diets. The first diet (**HB**) was 65% unchopped Cocksfoot hay (*Dactylis glomerata*; second cutting) and 35% pelleted ground barley (3-mm screen) on a DM basis. The second diet (**H**) was 100% of the same hay. Cows were fed once daily (0800) to increase the depressive effect of barley and study this effect over time after feeding. Feed availability was restricted (80% of ad libitum hay intake) to 7 kg of DM per day to ensure that the diet was ingested quickly and without orts. Moreover, a constant intake between diets allowed us to study differences in AA composition of the microorganisms resulting from the diet. The chemical compositions of experimental feeds and diets are given in Table 1. Six-week adaptation periods were allowed for each diet followed by a 1-wk measurement period.

Representative samples of ruminal digesta (3 kg) were taken manually from different parts of the rumen to isolate microbial samples. Ruminal contents were withdrawn 2, 5, 8, 11, and 23 h after feeding Diet HB; these samples had large postprandial variations in ruminal pH (> 1 pH unit). Ruminal contents were sampled at only 2 h after feeding for Diet H because of low postprandial variations in ruminal pH measured for this diet (< .5 pH unit) (Martin et al., 1994). To avoid any disturbing effect on rumen function of altering ruminal volume during the collection period, sampling was spread over 5 d to obtain one sample per cow per day. Ruminal contents were strained through a 100-μm nylon filter to separate a liquid phase (**LP**) and a solid phase (**SP**), from which the different microbial populations were subsequently isolated under anaerobic conditions according to the method detailed by Martin et al. (1994). After dilution of LP (500 mL) with prewarmed (39°C) salt solution (vol/vol; Coleman, 1978) and incubation for 30 to 60 min, the layer of flocculent feed particles created by the microbial fermentative activity in the liquor was removed by suction. The protozoal pellet (**LAP**) was recovered by centrifugation of the clarified fluid (1,000 × *g*, 10 min, room temperature) and was washed on a 20-μm nylon filter with Coleman buffer (1 L at 39°C) to minimize contamination by bacteria and plant residues. The insignificant contamination of protozoal sample by plant material and bacteria was confirmed by microscopic examination and 2,6-diaminopimelic acid (**DAPA**) measurements, respectively (Martin et al., 1994). And the protozoa lost in the washing buffer were controlled by counting using a Dolfuss cell (Jouany and Senaud, 1982). They represented only a minor proportion (< 4%) of the protozoa present in the ruminal liquor. The bacterial population associated with the liquid phase (**LAB**) was obtained by centrifuging the protozoa-free supernatant fluid at 15,000 × *g* for 20 min at 4°C. The SP (200 g) was washed with pre-warmed (39°C) Coleman buffer (800 mL) to remove the nonadherent population associated with the solid phase and squeezed in a 100-μm nylon filter. The filtrate was centrifuged at 1,000 × *g* for 10 min at room temperature to recover a pellet of small particles that was added to the large particles retained on the filter. The combined solid material was ground and pummeled in pre-cooled (4°C) buffer (1 g solid/4 mL) to dislodge particle-associated bacteria and any fungi and protozoa trapped within the particulate matter. Because the extraction yield with treatment was lower than 50% (Legay-Carmier and Bauchart, 1989), the sample extracted has to be considered as representing the total attached microbial population. After filtration (100 μm) and rinsing of the homogenate, the bacterial population associated with particles (**PAB**), even though not made up solely of bacteria, was isolated by centrifuging (27,000 × *g* for 30 min at 4°C) the supernatant obtained after a first centrifugation (1,000 × *g* for 10 min at 4°C). The different microbial populations isolated (LAP, LAB, PAB) were stored at –20°C before they were freeze-dried and analyzed.

Amino acid composition of the microbial isolates was measured after hydrolysis of dried samples (150 to 170 mg) with 6 *N* HCl at 120°C for 24 h. The hydrolysate was then evaporated and suspended in AA sample dilution buffer of lithium citrate (Beckman, 338084, pH 2.2). Because sulfur-containing AA (cystine and methionine) are degraded under these conditions, dried samples (150 to 170 mg) were also

oxydated with formic acid-water-phenol (88.6:10.9:.5%) at 4°C for 4 h before acid hydrolysis. Cystine and methionine were also measured as cysteic acid and methionine sulfone, respectively. Amino acid analyses were performed by ion-exchange chromatography on the physiological column of a Beckman Auto Analyzer (Model 6300, Beckman Instruments, Palo Alto, CA).

Variance of the data was analyzed using the GLM procedure of SAS (1985). In a first analysis, the effect of time after feeding on the AA composition of the different microbial populations with the mixed diet was studied. Animals and microbial population were the two main factors, and the effect of time postfeeding was taken into account in a repeated measures analysis of variance. In a second analysis, we studied the effect of diet on the AA composition of the different microbial populations. The diet was the whole-plot treatment and tested using animal × diet interaction as the error term. Microbial population was the subplot treatment and was tested using the residual error of the model. Diet and period effects were confounded in this experiment, but because animals were adult, nonlactating, nonpregnant, restricted-fed, and maintained in a controlled environment, any period effect was assumed to be negligible. For each statistical analysis, the single degree of freedom orthogonal comparisons were 1) protozoa vs bacteria and 2) one bacterial population vs the other.

Results and Discussion

Amino acid compositions of the different microbial populations (LAP, LAB, PAB) for cows fed a mixed diet are presented in Table 2. Mean AA composition of the protozoal and bacterial fractions were different for 13 AA out of 17 studied. The LAP contained more isoleucine, lysine, aspartate, glutamate ($P < .001$), cystine, phenylalanine ($P < .01$), tyrosine ($P < .05$) and less threonine, arginine, glycine, alanine ($P < .001$), valine ($P < .01$) and serine ($P < .05$) than the bacterial fractions. Czerkawski (1976) and Chamberlain et al. (1986) observed also that protozoal protein was lower in alanine than liquid-associated bacterial protein. Moreover, the lower alanine content of the protozoa compared to the liquid- and solid-associated bacteria (45% and 38% lower, respectively) observed in this experiment is consistent with results (45% and 37% lower, respectively) reported by Tanan (1994). This AA is an essential component of the bacterial cell wall (Hoogenrad, 1970; Ling, 1990). Differences in structures of bacterial and protozoal cell walls may explain, at least in part, the lower alanine content of the protozoal protein. The higher mean glutamate and lysine contents of the protozoal protein (14.1% and 11.5% of total AA, respectively) compared with the bacterial fractions (12.8% and 8.8% of total AA, respectively) has been reported by other authors (reviews of Storm and Ørskov, 1983; Bonhomme, 1990). High lysine content of the protozoal protein is consistent with the finding that the amount of lysine in the ruminal contents of faunated animals is higher than in samples from defaunated animals (Williams and Coleman, 1991). Several authors (Onodera, 1986; Masson et al., 1991) have reported that the protozoa may synthesize lysine from DAPA contained in the mucopeptides of bacterial cell walls.

Amino acid composition of the LAB differed from that of the PAB for 10 AA out of 17 studied (Table 2). Essential and semi-essential AA contents (i.e., leucine, phenylalanine [$P < .001$], histidine, arginine, cystine [$P < .01$], and methionine [$P < .05$]) were lower in the LAB, whereas threonine ($P < .01$) and nonessential AA contents (i.e., alanine [$P < .001$], glycine [$P < .01$], and aspartate [$P < .05$]) were lower in the PAB. Very few comparisons of relative AA compositions of free-floating and attached bacteria are available in the literature. This may be due to lack of data concerning protein AA content of the solid-adherent bacteria compared with the liquid-associated bacteria, which are commonly used as a microbial reference sample in estimating duodenal flow of microbial compounds. In addition, differences in techniques used to isolate the different bacterial fractions from the ruminal contents can lead to differences in AA composition of the microorganisms and so the scant data available in the literature are difficult to compare. Differences in AA composition between the liquid- and solid-associated bacteria may reflect different species present in the liquid and solid phase of the ruminal contents. Large differences in glycolytic activity of the liquid- and solid-associated bacteria have already been reported (Martin et al., 1995). However, differences in AA composition between these two bacterial fractions may be more probably related to higher feed particle contamination for the solid- than for the liquid-associated bacteria. Indeed, lower threonine and lysine content of barley and forages and higher leucine, phenylalanine, and arginine content than isolated ruminal bacteria (Schwab, personal communication) supports this hypothesis and may explain our results. The differences in AA profiles between LAP and bacteria, on the one hand, and between LAB and PAB, on the other hand, are noteworthy. They resulted in different estimates of microbial AA flow to the intestines, depending on microbial reference sample choice. As proposed by Cecava et al. (1990), for N:purine ratio, a blended sample of the different ruminal microbial populations may be a solution to estimate representative microbial AA flow to the duodenum, but the relative proportions of the different microbial populations to the intestines have to be determined.

Amino acid composition of the different microbial populations as affected by sampling time after feeding diet HB was studied (Table 2). Only arginine ($P < .001$), glutamate, and glycine contents of the ruminal

Table 2. Amino acid (AA) composition of protozoal and bacterial fractions[a] in response to time after feeding

Amino acid and time after feeding, h	Hay + barley (65/35)			SEM	Effect[b]				
	LAP	LAB	PAB		LAP vs Bact	LAB vs PAB	Time	LAP vs Bact × time	LAB vs PAB × time
	% Total AA								
Essential and semi-essential AA									
Threonine									
2	5.8	6.4	6.0						
5	5.8	6.5	6.5						
8	5.9	6.7	6.2						
11	5.9	6.7	6.4						
23	6.0	6.6	6.4						
Mean	5.9	6.6	6.3	.2	***	**	NS	NS	NS
Valine									
2	5.2	6.5	6.2						
5	5.5	6.6	6.3						
8	5.1	6.3	6.6						
11	5.3	6.6	6.5						
23	5.2	6.3	6.0						
Mean	5.3	6.5	6.3	.8	**	NS	NS	NS	NS
Isoleucine									
2	6.3	5.4	5.5						
5	6.1	5.4	5.6						
8	6.2	5.5	5.5						
11	6.2	5.4	5.9						
23	6.1	5.6	5.6						
Mean	6.2	5.5	5.6	.2	**	NS	NS	NS	NS
Leucine									
2	7.9	7.4	8.4						
5	7.7	7.3	8.5						
8	7.8	7.5	8.2						
11	7.8	7.3	8.6						
23	7.7	7.7	8.2						
Mean	7.8	7.4	8.3	.3	NS	***	NS	NS	NS
Tyrosine									
2	4.9	4.8	4.5						
5	5.2	4.9	4.5						
8	5.0	5.0	4.8						
11	5.0	4.8	4.9						
23	5.2	5.0	4.9						
Mean	5.1	4.9	4.7	.4	*	NS	NS	NS	NS
Phenylalanine									
2	5.8	5.1	5.7						
5	5.6	5.1	5.4						
8	5.7	5.1	5.5						
11	5.7	5.0	5.7						
23	5.6	5.3	5.7						
Mean	5.7	5.1	5.6	.2	**	***	NS	NS	NS
Lysine									
2	11.5	9.0	8.2						
5	11.5	9.4	8.4						
8	11.4	9.0	8.7						
11	12.0	9.5	8.8						
23	11.3	8.5	8.3						
Mean	11.5	9.1	8.5	.9	***	NS	NS	NS	NS
Histidine									
2	1.7	1.7	1.9						
5	1.7	1.6	1.9						
8	1.7	1.6	1.8						
11	1.7	1.6	1.8						
23	1.8	1.6	1.8						
Mean	1.7	1.6	1.8	.1	NS	**	NS	NS	NS
Arginine									
2	4.6	5.0	5.4						
5	4.6	5.0	5.5						
8	4.6	5.0	5.2						
11	4.5	4.8	5.2						
23	4.5	4.8	5.0						
Mean	4.6	4.9	5.3	.3	***	**	***	NS	*

continued

Table 2 *(continued).* Amino acid (AA) composition of protozoal and bacterial fractions[a] in response to time after feeding

Amino acid and time after feeding, h	Hay + barley (65/35)			SEM	Effect[b]				
	LAP	LAB	PAB		LAP vs Bact	LAB vs PAB	Time	LAP vs Bact × time	LAB vs PAB × time
	% Total AA								
Cystine									
2	1.3	0.9	1.2						
5	1.3	0.9	1.1						
8	1.4	0.9	1.3						
11	1.2	1.0	1.0						
23	1.4	1.1	1.3						
Mean	1.3	1.0	1.2	.2	**	**	NS	NS	NS
Methionine									
2	1.7	1.6	1.9						
5	2.0	1.6	1.5						
8	1.9	1.5	2.3						
11	1.7	1.7	1.8						
23	1.9	1.6	1.9						
Mean	1.8	1.6	1.9	.3	NS	*	NS	NS	NS
Non-essential AA									
Aspartate									
2	12.9	11.7	11.1						
5	12.9	11.8	11.4						
8	12.8	11.9	11.3						
11	12.8	12.0	11.7						
23	13.1	12.0	11.8						
Mean	12.9	11.9	11.5	.5	***	*	NS	NS	NS
Serine									
2	4.2	4.6	4.7						
5	4.2	4.5	4.8						
8	4.2	4.5	4.6						
11	4.2	4.6	4.8						
23	5.0	4.6	4.7						
Mean	4.4	4.6	4.7	.3	*	NS	NS	NS	NS
Glutamate									
2	14.4	12.6	13.6						
5	14.1	12.7	13.0						
8	14.1	12.7	12.4						
11	14.1	12.7	12.8						
23	14.0	12.6	12.6						
Mean	14.1	12.7	12.9	.5	***	NS	*	NS	*
Glycine									
2	4.9	6.6	6.1						
5	4.8	6.4	6.0						
8	4.9	6.5	6.1						
11	4.9	6.4	6.5						
23	5.1	6.6	6.2						
Mean	4.9	6.5	6.2	.3	***	**	*	NS	*
Alanine									
2	3.4	6.3	5.2						
5	3.3	6.3	5.5						
8	3.5	6.2	5.4						
11	3.5	6.2	5.8						
23	3.5	6.2	5.6						
Mean	3.4	6.2	5.5	.4	***	***	NS	NS	NS
Proline									
2	3.5	3.7	4.4						
5	3.6	3.4	4.1						
8	3.7	3.4	3.8						
11	3.5	3.3	3.9						
23	3.4	3.4	3.8						
Mean	3.5	3.4	4.0	.8	NS	NS	NS	NS	NS

[a]LAP = liquid-associated protozoa; LAB = liquid-associated bacteria; PAB = particle-associated bacteria; Bact = (LAB + PAB).
[b]Effect: NS = not significant; *P < .05; **P < .01; ***P < .001.

Table 3. Amino acid (AA) composition of protozoal and bacterial fractions[a] in response to diets

Item	Hay			Hay + barley (65/35)			SEM	Effect[b]				
	LAP	LAB	PAB	LAP	LAB	PAB		LAP vs Bact	LAB vs PAB	Diet	LAP vs Bact × diet	LAB vs PAB × diet
	% Total AA											
Essential and semi-essential AA												
Threonine	5.8	6.5	6.5	5.8	6.4	6.0	.3	***	NS	NS	NS	NS
Valine	5.5	6.8	6.2	5.2	6.5	6.2	.3	***	**	NS	NS	NS
Isoleucine	6.3	5.5	5.5	6.3	5.4	5.5	.2	***	NS	NS	NS	NS
Leucine	7.9	7.4	8.8	7.9	7.4	8.4	.2	NS	***	**	NS	NS
Tyrosine	4.8	4.7	4.9	4.9	4.8	4.5	.2	NS	NS	NS	NS	*
Phenylalanine	5.5	5.1	6.0	5.8	5.1	5.7	.2	NS	***	NS	NS	NS
Lysine	11.9	9.4	9.4	11.5	9.0	8.2	.8	***	NS	NS	NS	NS
Histidine	1.7	1.5	1.7	1.7	1.7	1.9	.1	NS	***	NS	NS	NS
Arginine	4.5	4.8	5.1	4.6	5.0	5.4	.3	***	*	NS	NS	NS
Cystine	1.1	1.0	1.2	1.3	.9	1.2	.2	NS	NS	NS	NS	NS
Methionine	1.5	1.6	2.0	1.7	1.5	1.9	.3	NS	**	NS	NS	NS
Non-essential AA												
Aspartate	13.4	11.6	11.7	12.9	11.7	11.1	.5	***	NS	NS	NS	NS
Serine	4.2	4.4	4.8	4.2	4.6	4.7	.1	***	***	NS	NS	**
Glutamate	14.1	12.0	12.1	14.5	12.6	13.6	.5	***	NS	**	NS	NS
Glycine	5.0	6.5	6.0	4.9	6.6	6.1	.2	***	***	NS	NS	NS
Alanine	3.5	6.3	5.2	3.4	6.3	5.2	.2	***	***	NS	NS	NS
Proline	3.2	3.5	3.9	3.5	3.7	4.4	.9	NS	NS	NS	NS	NS

[a]LAP = liquid-associated protozoa; LAB = liquid-associated bacteria; PAB = particle-associated bacteria; Bact = (LAB + PAB).
[b]Effect: NS = not significant; *P < .05; **P < .01; ***P < .001.

microorganisms varied (P < .05) with time of sampling after feeding (Table 2). This was a consequence of postprandial variation in composition of particle-associated bacteria (LAB vs PAB × time, P < .05). For example, glutamate content of the LAB and PAB, as expressed in percentage of total AA, was respectively equal to 12.6% for the LAB but decreased from 13.6 to 12.6% for the PAB between 2 and 23 h after feeding. For all the other AA, concentration in the different microbial fractions was similar irrespective of sampling time (P > .05) and thus irrespective of the ruminal pH, which varied considerably in our study with a mixed diet given once daily (Martin et al., 1994). To our knowledge no data on the postprandial variation in AA composition of the different microbial populations of the ruminal ecosystem have been published. This relative constancy in AA composition of ruminal microbial samples over time after feeding suggests that AA composition of the microorganisms would not be related to their metabolic activity, but may be rather an intrinsic characteristic of each microbial population. Consequently, for animals fed infrequently, it would not be necessary to isolate microbial samples from the rumen at various times after feeding for obtaining representative AA composition of microorganisms. Amino acid composition of the different microbial populations isolated from a single ruminal sample should be meaningful in the calculation of duodenal microbial AA flow. However, more information about the relative contributions of the different microbial populations to the small intestine is needed.

Because the AA composition of the ruminal microorganisms did not vary with sampling time, isolation of the different microbial populations was performed only 2 h after feeding with diet H, and their AA compositions were compared with those isolated from diet HB at the same sampling time (Table 3). The diets studied did not influence AA profiles of either ruminal protozoal or bacterial fractions. Lysine contents for diets H and HB were 11.5 and 11.9%, respectively, of total AA for the LAP, 9.0 and 9.4% of total AA for the LAB, and 8.2 and 9.4% of total AA for the PAB. More data, compared with data for postprandial effects, are available in the literature concerning the effect of diet on AA composition of ruminal microorganisms. Several authors (Storm and Ørskov, 1983; Clark et al., 1992), who have compiled results of different trials, observed a diet effect on AA composition of ruminal microorganisms. However, a large part of this effect may be attributed to differences in techniques used between trials to isolate the different microbial fractions and to measure their AA composition. Indeed, within the same experiment, and in agreement with our study, AA profiles of the protozoa (Czerkawski, 1976; Chamberlain and Thomas, 1979; Syvoaja and Kreula, 1979) and the liquid- (Meyer et al., 1967; Chamberlain et al., 1986; Hvelplund, 1986) or solid-associated bacteria (Lallès et al., 1992) were not affected by the different experimental diets fed.

Therefore, it seems that AA composition of the microorganisms is independent of diets fed to animals. This would be consistent with the hypothesis previously expressed that AA composition of microbial protein would be an intrinsic characteristic of each group of microorganisms. However, these observations should be confirmed, especially for the solid-associated bacteria, which represent the most important microbial biomass of the ruminal ecosystem, and for which only a few data are available in the literature, compared with the relatively large body of data on liquid-associated bacteria.

Implications

Amino acid composition of the protozoal and bacterial fractions did not vary with sampling time after feeding and diet. This would suggest that the quality of the microbial protein is independent of the physicochemical conditions of the rumen (sampling time or diet). However, differences were observed between amino acid profiles of protozoa and bacteria, on the one hand, and between amino acid profiles of the liquid- and solid-associated bacteria, on the other. Therefore, further research on the relative contributions of these different microbial fractions to amino acid passage to the small intestine is required if accurate measurements of microbial amino acid flow are to be obtained.

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