Effect of diet and bacterial pellet on the bacterial N and amino acid flows from single-flow continuous-culture fermenters

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Effect of diet and bacterial pellet on the bacterial N and amino acid flows from single-flow continuous-culture fermenters

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Abstract. Six single-flow continuous-culture fermenters fed two different diets and with inoculum from goats or sheep were used in two 11-d incubation periods to investigate the effects of using different bacterial pellets on the estimation of bacterial N and amino acid (AA) flows. Diet AH consisted of 993 g of alfalfa hay and 7 g of a vitamin-mineral mixture per kg, and diet OLSUP was composed of 671 g of olive leaves, 228 g of barley grains, 94 g of faba beans and 7 g of a vitamin-mineral mixture per kg. On the last day of each run, solid- (SAB) and liquid- (LAB) associated bacteria were isolated from the content of each fermenter. Bacterial N and AA flows were estimated using SAB or LAB composition. Pellets isolated from AH-fed fermenters presented greater ($P < 0.001$) contents of purine bases (PB) either adenine or guanine, as well as greater PB:N ratios. Significant effects of the diet ($P < 0.05$) were found on 14 of 17 AA. LAB presented greater ($P < 0.05$) N and PB concentrations compared to SAB, although no differences ($P = 0.266$) were detected for PB:N ratio. The AA profile of SAB differed ($P < 0.05$) from LAB in 5 and 9 of 17 AA for diets AH and OLSUP, respectively, but the numerical differences were, in general, small. Estimates of bacterial N flow were not affected ($P = 0.125$) by the type of pellet used for calculations, but the flow of bacterial AA, estimated with LAB, was greater ($P < 0.05$) for 7 and 11 of 17 AA for AH and OLSUP diets, respectively, compared to that calculated with SAB. There were diet x bacterial pellet interactions ($P < 0.05$) for 6 AA, indicating that the bacterial pellet chosen to estimate the AA flow of bacterial origin could affect the interpretation of differences between diets.

Keywords. Ruminal bacteria – Bacterial pellets – Amino acid profile – Fermenters.

Effet du régime et du type de bactéries sur le flux d'ázote et des acides aminés bactériennes chez des fermenteurs à flux simple continu

Résumé. *Six fermenteurs à flux simple continu avec des inoculum obtenus des ovins ou des chèvres, nourris avec deux différents régimes, ont été utilisés dans deux périodes de 11-d d'incubation pour étudier les effets de l'utilisation de différentes fractions de bactéries sur l'estimation des fluxes d'azote et d'acides aminés (AA) bactériennes. Le régime AH était composé de 993 g de foin de luzerne et 7 g d'un mélange de vitamines et minéraux par kg et le régime OLSUP était composée de 671 g de feuilles d'olivier, 228 g de grains d'orge, 94 g de fabes et 7 g de un mélange de vitamines et minéraux par kg. Le dernier jour de chaque periode d'incubation, les bactéries associées au solide (SAB) et au liquide (LAB) ont été isolées à partir du contenu de chaque fermenteur. Le fluxes d'azote et des AA bactériennes ont été estimées en utilisant la composition des SAB ou LAB. Le fractions bactériennes isolées à partir des fermenteurs alimentés avec le régime AH ont présenté une plus grande (P <0,001) contenu d'adénine, guanine et bases puriques (PB), ainsi qu'une plus grande ratio PB: N. Des effets du régime (P <0,05) sur 14 des 17 AA ont été retrouvés. LAB a présenté une plus grande (P < 0,05) contenu en azote et PB par rapport à SAB, mais pas de différences (P = 0,266) ont été détectés dans les valeurs PB: N. Le profil des AA dans SAB était diffèrent (P < 0,05) de celui dans LAB en 5 et 9 des 17 AA pour les régimes AH et OLSUP, respectivement, mais les différences numériques sont, en général, petites. Les estimations du flux d'azote bactérienne n'ont pas été affectées (P = 0,125) par le type de bacteries utilisés pour les calculs, mais le flux d'AA bactériennes estimé en utilisant LAB a été plus grande (P < 0,05) pour 7 et 11 des 17 AA pour les régimes AH et OLSUP, respectivement, par rapport à celui calculé avec SAB. Il y avait une interaction régime x fraction bactérienne (P < 0,05) pour 6 AA, ce qui indique que les fractions de bactéries choisies pour estimer les flux d'AA d'origine bactérienne pourrait influer sur l'interprétation des différences entre les régimes alimentaires.*

Mots-clés. *Bactéries du rumen – Fractions bactériennes – Profil d'acides aminés – Fermenteurs.*

I – Introduction

Ruminal microorganisms make a considerable contribution to the organic matter and N entering the duodenum of the ruminant and thus of nutrients available for maintenance and productive purposes. Therefore, in terms of meeting ruminant nutrient requeriments, it is important a good knowledge of the chemical composition of ruminal bacteria, which is affected by a range of dietary factors (Yang *et al*., 2001; Molina-Alcaide *et al*., 2009a). Furthermore, previous studies have shown differences between solid- (SAB) and liquid- (LAB) associated bacteria in their chemical composition. The aim of this work was to investigate the effects of using different bacterial pellets (SAB and LAB) on the estimation of bacterial N and AA flows in single-flow continuous-culture fermenters fed a standard diet (alfalfa hay) or a diet based on a very important by-product in the Mediterranean such as olive leaves.

II – Materials and methods

Four rumen-fistulated Segureña wethers and 4 rumen-fistulated Granadina goats were used as donors of ruminal contents to inoculate six single-flow continuous-culture fermenters. Two experimental diets were formulated. Diet AH consisted of 993 g of alfalfa hay and 7 g of a vitamin-mineral mixture per kg and diet OLSUP was composed of 671 g of olive leaves, 228 g of barley grains, 94 g of faba beans and 7 g of a vitamin-mineral mixture per kg. Two identical 21-d trials were carried out with days 1 to 10 for animal adaptation to the diets, and d 11 to 21 for incubation runs. In each trial, 2 animals of each species received each of the experimental diets. Ruminal contents were collected on d 11 from each animal 2 h after feeding, pooled by animal species and diet, and used to inoculate the fermenters. In each incubation run, 3 fermenters were inoculated with ruminal fluid from wethers and the other 3 with ruminal fluid from goats. Experimental treatments were assigned randomly within each incubation run, so that each treatment (diet and inoculum source) was conducted in triplicate. Each fermenter received daily 50 g of dry matter (DM) of the corresponding diet in two equal portions at 09:00 and 16:00 h. The complete experimental procedures were described in detail in Molina-Alcaide *et al*. (2009b). The period from d 11 to d 19 was for adaptation, and on d 20 and 21 total effluent was collected and a pooled sample used for N, purine bases (PB) and AA analyses. On d 21 the content of each fermenter flask was strained through four layers of cheesecloth; the solid residue was washed with cold NaCl solution and strained again through four layers of cheesecloth. The final filtrate was centrifuged at 800 *x g* for 10 min at 4ºC to remove feed particles, and the supernatant fraction was centrifuged at 27,800 *x g* for 10 min at 4ºC to isolate a pellet of LAB. For isolation of SAB, the solid fraction was resuspended in cold NaCl solution (2.5 ml/g), subjected to vigorous mechanical pummeling, and strained through four layers of cheesecloth. The solid was washed with cold NaCl solution, strained and the filtrate added to the one obtained previously. The final filtrate was differentially centrifuged as described for LAB to isolate a SAB pellet. All pellets were lyophilised and ground to a fine power with a mortar and pestle before analyses. Total N was analysed by following the Kjeldahl procedure (AOAC, 2005). The AA-N content in samples of feeds, effluents and bacterial pellets was determined by HPLC using the Waters® Pico-Tag method (Fernández-Fígares *et al*., 1997). Purine bases (PB) content in feeds, effluents and bacterial pellets were determined by reverse-phase HPLC following the procedures described by Balcells *et al*. (1992).

The daily bacterial N flow was estimated in each fermenter from the PB:N ratios in the effluent and in the bacterial pellets. Because the inoculum source (goats vs. wethers) did not promote differences ($P > 0.05$) for any measured variable and no significant ($P > 0.05$), inoculum source x diet or inoculum source x bacterial pellet interactions were detected data were pooled and results presented as the mean of six values for each treatment. Data were analyzed using the Proc Mixed procedure of the Statistical Analysis Systems (version 8.02; SAS Institute, Cary, NC, USA). The effects of diet, bacterial pellet and diet x bacterial pellet interaction were considered fixed, and fermenter and run as random effects. Mean effects were declared significant at $P < 0.05$.

III – Results and discussion

Pellets isolated from AH-fed fermenters presented greater ($P < 0.001$; Table 1) contents of adenine, guanine and PB, as well as greater PB:N ratios compared to those from OLSUP-fed fermenters. Total AA content in the bacterial pellets varied between 39.0 and 70.3 g/100 g total N, with greater ($P < 0.001$) values in pellets isolated from AH-fed fermenters compared to those from fermenters fed OLSUP. These values were within the range of those previously reported in the literature (Molina-Alcaide *et al*., 1996; Rodríguez-Prado *et al*., 2004; Boguhn *et al*., 2006). Although the AA profile of ruminal microbes has been reported to be relatively constant (Ørskov, 1988; Martin *et al*., 1996), in the present study it was modified ($P < 0.05$) by the diet in 14 of 17 AA in agreement with Calsamiglia *et al*. (1995) and Boguhn *et al*. (2006), who found significant effects of the diet on the AA profile of bacteria isolated from dual-flow continuous-culture and Rusitec fermenters, respectively. Differences in AA profile have been attributed to the substrate availability and bacterial species (Czerkawski, 1976).

In accordance with others (Carro and Miller, 2002; Molina-Alcaide *et al*., 2009a), LAB presented greater ($P < 0.05$) N and PB concentrations compared to SAB, but no differences ($P = 0.266$) were detected in PB:N ratio. The lower content of PB found in SAB might stem from different bacterial species and a lower growth rate of these populations. In agreement with Rodríguez-Prado *et al*. (2004) and Boguhn *et al*. (2006), comparisons of SAB and LAB indicated differences ($P < 0.05$) in the concentrations of 5 and 9 of 17 AA for AH and OLSUP diets, respectively, although the numerical differences were, in general, small.

Estimations of bacterial N flow were not dependent ($P = 0.125$) on the type of pellet used for calculations, and values were greater ($P < 0.001$) for AH compared to OLSUP (Table 2). The flow of bacterial AA was 2.3 and 1.7 times greater for diet AH compared to OLSUP diet when SAB or LAB were used as reference, respectively (Table 2). Diet AH promoted greater ($P < 0.001$) bacterial AA flow for all analysed AA in comparison to OLSUP diet. The bacterial AA flow estimated with LAB was greater ($P < 0.05$) for 7 and 10 of 17 AA for AH and OLSUP diets, respectively, compared to that calculated with SAB. There were significant ($P < 0.05$) diet x bacterial pellet interactions for 6 AA (glutamic acid, serine, glycine, alanine, histidine and tyrosine), indicating that the bacterial pellet chosen to estimate the AA flow of bacterial origin could affect the interpretation of differences between diets. Bacterial protein usually contributes to a large proportion of total AA leaving the rumen (Clark *et al*., 1992). In the present study, total AA flow was 646 and 362 mg/d for AH and OLSUP diets, respectively (Molina-Alcaide *et al*., 2009b), and bacterial AA contributed to 0.67 and 0.52 for AH and OLSUP diets, respectively, when SAB were used as reference, and to 0.73 and 0.78 when LAB were used. These values were in the range of those reported by others (Molina-Alcaide *et al*., 1996; Rodriguez-Prado *et al*., 2004) in continuous-culture fermenters fed diets of variable composition. There were no differences ($P = 0.141$) between SAB and LAB for estimating the contribution of bacterial EAA to total EAA flow for diet AH (0.52 and 0.55, respectively; results not shown), but for diet OLSUP the proportion was greater ($P < 0.001$) for SAB compared to LAB (0.57 and 0.52, respectively) indicating that using SAB or LAB for calculations can influence interpretation of results.

Table 1. Composition of solid- (SAB) and liquid- (LAB) associated bacteria isolated from single-flow continuous-culture fermenters fed two different diets (n=6)[†]

Item	AH		OLSUP			Significance of effects (*P*)		
	SAB	LAB	SAB	LAB	SEM	Diet	Bacterial pellet	Diet × Bacterial pellet
Total N, mg/g DM	36.3[a]	39.0[b]	39.5[a]	46.9[b]	0.80	<0.001	<0.001	0.012
Adenine, µmol/g DM	16.8[a]	18.2[b]	12.0	12.2	0.32	<0.001	0.028	0.066
Guanine, µmol/g DM	26.3	27.0	19.3[a]	22.6[b]	0.45	<0.001	<0.001	0.014
Purine bases, µmol/g DM	43.0[a]	45.2[b]	31.3[a]	34.7[b]	0.76	<0.001	0.003	0.416
Purine bases/N, µmol/mg N	1.19	1.16	0.80	0.74	0.037	<0.001	0.266	0.705
Total AA, mg/g N	65.1[a]	70.3[b]	39.0[a]	54.9[b]	1.91	<0.001	<0.001	0.014
AA, g/100 g AA								
aspartic acid	8.91	8.85	9.02	9.39	0.491	0.514	0.753	0.665
glutamic acid	8.52	6.23	8.19	9.90	0.412	0.001	0.490	<0.001
serine	5.54	5.86	4.07[a]	5.53[b]	0.188	<0.001	<0.001	0.009
threonine	5.95[a]	6.66[b]	5.75[a]	6.05[b]	0.098	0.001	<0.001	0.056
glycine	8.06[b]	7.61[a]	5.44[a]	6.34[b]	0.049	<0.001	<0.001	<0.001
alanine	9.41[b]	9.08[a]	8.36[a]	9.17[b]	0.070	<0.001	0.004	<0.001
arginine	13.2[a]	14.6[b]	12.9[a]	13.5[b]	0.149	<0.001	<0.001	0.025
proline	3.68	3.61	3.01	2.69	0.112	<0.001	0.103	0.281
valine	5.39	6.22	8.19	6.70	0.227	<0.001	0.173	<0.001
methionine	0.59	0.58	0.88[b]	0.65[a]	0.034	<0.001	0.004	0.006
isoleucine	4.64	5.35	5.73	5.37	0.251	0.054	0.501	0.051
leucine	5.36	5.60	7.89	7.04	0.258	<0.001	0.256	0.053
phenylalanine	3.36	3.48	4.61	3.70	0.255	0.012	0.141	0.065
lysine	8.63	8.95	8.49[b]	6.47[a]	0.296	<0.001	0.012	0.001
histidine	4.41[b]	3.33[a]	2.80[a]	3.21[b]	0.114	<0.001	0.010	<0.001
tyrosine	3.58	3.41	3.63	3.60	0.068	0.095	0.151	0.321
cysteine	0.69	0.60	1.09[b]	0.75[a]	0.050	<0.001	<0.001	0.024
EAA[††]	51.4	54.7	57.3	52.7	0.74	0.021	0.353	<0.001
NEAA[††]	48.6	45.3	42.7	47.3	0.74	0.021	0.353	<0.001

[†] AH = 993 g of alfalfa hay and 7 g of a vitamin-mineral mixture per kg (as-fed basis); OLSUP = 671 g of olive leaves, 228 g of barley grains and 94 g of faba beans and 7 g of a vitamin-mineral mixture per kg (as-fed basis).
[††] EAA = Essential AA (threonine, arginine, valine, metionine, isoleucine, leucine, phenylalanine, lysine and histidine); NEAA = Non-essential AA (alanine, aspartic acid, glutamic acid, glycine, proline, serine, tyrosine and cysteine).
[a, b] Within a variable and diet, means with different superscripts differ (P<0.05).

Table 2. Bacterial N and amino acid (AA) flows in single-flow continuous culture fermenters fed two different diets as determined using solid- (SAB) and liquid- (LAB) associated microbes as bacterial reference (n=6)†

Item	AH		OLSUP			Significance of effects (*P*)		
	SAB	LAB	SAB	LAB	SEM	Diet	Bacterial pellet	Diet × Bacterial pellet
Bacterial N, mg/d	662	669	480	515	13.0	<0.001	0.125	0.299
Bacterial AA, mg/d								
aspartic acid	38.2	41.5	16.9	26.5	2.13	<0.001	0.009	0.161
glutamic acid	36.6	28.9	15.2	28.2	1.42	<0.001	0.081	<0.001
serine	24.1^{a}	27.7^{b}	7.4^{a}	15.6^{b}	1.08	<0.001	<0.001	0.049
threonine	25.9^{a}	31.5^{b}	10.7^{a}	17.0^{b}	1.18	<0.001	<0.001	0.773
glycine	34.8	35.8	10.3^{a}	18.0^{b}	1.03	<0.001	<0.001	0.006
alanine	40.7	42.8	15.9^{a}	26.1^{b}	1.36	<0.001	<0.001	0.010
arginine	57.3^{a}	68.6^{b}	24.6^{a}	38.3^{b}	2.13	<0.001	<0.001	0.570
proline	15.9	17.0	5.8^{a}	7.6^{b}	0.61	<0.001	0.030	0.551
valine	23.1^{a}	29.2^{b}	15.4^{a}	18.9^{b}	1.23	<0.001	0.002	0.311
methionine	2.5	2.7	1.6	1.9	0.14	<0.001	0.169	0.859
isoleucine	19.6^{a}	24.8^{b}	10.6^{a}	15.4^{b}	1.00	<0.001	<0.001	0.862
leucine	23.2^{a}	26.4^{b}	15.0^{a}	19.8^{b}	1.20	<0.001	0.005	0.514
phenylalanine	14.6	16.4	8.8	10.3	0.92	<0.001	0.084	0.863
lysine	37.4^{a}	42.3^{b}	15.7	18.1	1.58	<0.001	0.039	0.445
histidine	19.1	15.7	5.2	9.1	0.64	<0.001	0.670	<0.001
tyrosine	15.5	16.0	6.8^{a}	10.2^{b}	0.65	<0.001	0.009	0.042
cysteine	3.0	2.8	2.0	2.1	0.17	<0.001	0.872	0.431
EAA††	223^{a}	258^{b}	107^{a}	148^{b}	8.8	<0.001	<0.001	0.725
NEAA††	209	212	81^{a}	135^{b}	6.4	<0.001	<0.001	0.002
Total	432^{a}	470^{b}	188^{a}	283^{b}	14.2	<0.001	<0.001	0.066

† AH = 993 g of alfalfa hay and 7 g of a vitamin-mineral mixture per kg (as-fed basis); OLSUP = 671 g of olive leaves, 228 g of barley grains and 94 g of faba beans and 7 g of a vitamin-mineral mixture per kg (as-fed basis).

†† EAA = Essential AA (threonine, arginine, valine, metionine, isoleucine, leucine, phenylalanine, lysine and histidine); NEAA = Non-essential AA (alanine, aspartic acid, glutamic acid, glycine, proline, serine, tyrosine and cysteine).

$^{a, b}$ Within a variable and diet, means with different superscripts differ (P<0.05).

IV – Conclusions

The AA profile of SAB and LAB was affected by the diet, and each bacterial fraction promoted different responses to diet. The isolation of different bacterial pellets may therefore have an influence on the estimation of bacterial AA flow in fermenters and can change the interpretation of results.

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