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Effect of variation in the proportion of solid- and liquid-associated rumen bacteria in duodenal contents on the estimation of duodenal bacterial nitrogen flow*

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ABSTRACT

The aim of this research was to examine to what extent variation in the relative proportions of solid- (SAB) and liquid-associated rumen bacteria (LAB) in duodenal bacteria have an impact on the estimation of duodenal flow of bacterial N. For this, four dairy cows were fed diets varying in forage: concentrate ratio (80:20, 65:35, 50:50 and 35:65). SAB and LAB were separated from rumen contents four h after the morning feeding. Adenine, cytosine and odd and branched-chain fatty acids were determined both in SAB and LAB and used to estimate bacterial N flow. Bacterial N flows were also calculated using a SAB:LAB ratio in duodenal bacteria, as estimated from the odd and branched-chain fatty acid pattern. Compared with calculations based on the estimated SAB:LAB ratio, estimations based on SAB or LAB only as a bacterial reference on average over- and underestimated bacterial N flow by 37 and 55 g N/d, respectively ($P<0.05$) when cytosine or adenine were used as bacterial marker. In contrast, due to the small differences in the OBCFA:N ratio between SAB and LAB, these differences were less than 15 g/d when OBCFA were used as bacterial marker. The results suggest that, depending on the marker used, changes in the proportions of SAB and LAB can have a substantial impact on estimated duodenal flow of bacterial N.

KEY WORDS: bacterial N, solid associated bacteria, liquid associated bacteria, rumen, odd- and branched-chain fatty acids

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INTRODUCTION

Estimates of rumen bacterial N synthesis (MN) have been calculated from marker:bacterial N ratios. These ratios have generally been established in bacteria isolated from the liquid phase of rumen digesta, and it has tacitly been assumed that the same relationship holds in the total population leaving the rumen. However, differences between liquid- (LAB) and solid-associated (SAB) bacteria for marker ratios have been reported (e.g., Dewhurst et al., 2000; Carro and Miller, 2002). The impact of using both SAB and LAB in the calculation of MN will depend on the relative contribution of both bacterial fractions to the duodenal flow of bacterial matter. Although there is little information about the contribution of SAB and LAB to total bacterial flow, the relative proportion of SAB and LAB in the rumen can be affected by dietary factors. Indeed, Faichney (1980) reported that the proportion of SAB reached 90% in sheep fed forage only whereas it declined to 50% for steers fed equal proportions of forage and concentrate (Merry and McAllan, 1983). Hence, variation in relative proportions of SAB and LAB could largely influence estimates of rumen microbial synthesis when either SAB or LAB marker:N ratios are used for calculations.

Recently, linear programming was used to partition N flowing to the duodenum into feed, bacteria, protozoa, and endogenous fractions (Shabi et al., 2000; Reynal et al., 2003). Using the same approach, we have calculated the relative proportions of SAB and LAB in duodenal content (Vlaeminck et al., 2006). For this, the odd and branched-chain fatty acids (OBCFA) pattern of SAB and LAB were used to estimate the relative proportions of both bacterial isolates in duodenal bacteria and found that the proportion of SAB ranged from 49.3 to 84.5%, and decreased with decreasing proportion of dietary forage (Vlaeminck et al., 2006). In the current paper, we used the latter results to illustrate the importance of this SAB:LAB ratio in estimating duodenal flow of MN.

MATERIAL AND METHODS

Experimental procedures were described previously (Moorby et al., 2006). Briefly, four rumen- and duodenal-fistulated dairy cows in mid-lactation were offered diets varying in forage:concentrate ratio (80:20, 65:35, 50:50, 35:65 on a DM basis; Moorby et al., 2006) in a 4 × 4 Latin square. Dietary treatments were based on *ad libitum* access to ryegrass silage and a standard dairy concentrate. Diet F:C ratios were achieved by measuring *ad libitum* silage DMI on a daily basis (silage was offered to allow at least 10% refusals), and allocating the appropriate amount of concentrate to each animal based on a rolling average of their silage

DMI from the previous 3 d of the experiment. Each experimental period lasted for 28 days of which the first 2 weeks for adaptation. Fresh forage was distributed daily at 09.00 h whereas concentrates were distributed twice daily in equal portions at milking (08.00 and 16.00 h).

Rumen and duodenal samples were taken during the final week of each experimental period. Rumen emptying was done by hand, on the final day of each period 4 h after feeding. Samples (5%) of rumen contents were taken throughout the emptying procedure for analysis and rumen contents were weighed and returned to the rumen within 30 min of commencement. The procedure for isolation of LAB and SAB is described in Vlaeminck et al. (2006). Duodenal sampling was performed over two consecutive days using the automated equipment described by Evans et al. (1981). Duodenal bacteria were separated from a reconstituted sample by differential centrifugation (Vlaeminck et al., 2006). Duodenal flows were determined based on the double marker technique as described by Faichney (1992). Cytosine, adenine and OBCFA were used as microbial marker and were determined in rumen bacteria and duodenal digesta. Cytosine and adenine were analysed using the method described by Cozzi et al. (1993) and OBCFA as described by Vlaeminck et al. (2006). Total bacterial N flow at the duodenum was calculated by dividing the marker flow by the marker:N ratio in mixed rumen bacteria. The latter was calculated as $(a \times marker_{SAB} + b \times marker_{LAB})/(a \times N_{SAB} + b \times N_{LAB})$ with a and b the relative proportion of SAB and LAB in duodenal content, respectively. The relative proportions of SAB and LAB in duodenal content were estimated using OBCFA of SAB and LAB isolated from rumen content and of duodenal bacteria as described in Vlaeminck et al. (2006). Differences in the calculated bacterial N flow using marker:N ratios of either SAB or LAB or the estimated ratios in mixed rumen bacteria were analysed according to:

$$Y_{ijkl} = \mu + T_i + P_j + C_k + B_l + TB_{il} + CB_{kl} + \varepsilon_{ijkl}$$

where Y_{ijk} is the individual observation, μ the overall mean, T_i the effect of dietary treatment, P_j the effect of experimental period, C_k the effect of cow, B_l the effect of bacterial reference, TB_{il} the interaction between treatment and bacterial reference, CB_{kl} the interaction between bacterial reference and cow and ε_{ijk} the residual error.

The effect of bacterial marker was analysed according to:

$$Y_{ijkl} = \mu + T_i + P_j + C_k + M_l + TM_{il} + CM_{kl} + \varepsilon_{ijkl}$$

with M_l the effect of bacterial marker and other abbreviations as before.

All statistical analyses were performed using SPSS 12.0 (SPSS software for Windows, release 12.0., SPSS, Inc., USA).

RESULTS AND DISCUSSION

The effect of dietary forage:concentrate ratio on MN was discussed in detail previously (Moorby et al., 2006). The overall estimated duodenal flow of MN was on average 168, 222 and 197 g/d using marker : N ratios of LAB and cytosine, adenine and OBCFA as bacterial marker, respectively (Table 1). When

Table 1. Estimated bacterial N flow to the duodenum using marker:N ratios of mixed duodenal bacteria, as estimated from the ratio solid (SAB) to liquid-associated rumen bacteria (LAB) (Vlaeminck et al., 2006), vs marker:N ratios of SAB or LAB only for three different bacterial markers

Marker	SAB : LAB ratio			SEM[2]	P
	calculated[1]	1:0	0:1		
Cytosine	209^{b,A}	246^{c,A}	168^{a,A}	7.2	0.001
Adenine	283^{b,C}	335^{c,B}	222^{a,B}	9.1	0.001
OBCFA	233^{b,B}	239^{b,A}	220^{a,B}	2.5	0.040
SEM[1]	7.4	10.6	5.3		
P	0.001	0.001	0.002		

[1] SAB : LAB ratio in duodenal bacteria calculated from odd and branched-chain fatty acid pattern (Vlaeminck et al., 2006)

[2] standard error of the mean

$^{a, b, c}$ mean values lacking a common superscript within a row differ significantly ($P<0.05$)

$^{A, B, C}$ mean values lacking a common superscript within a column differ significantly ($P<0.05$)

using the SAB reference ratios for cytosine, adenine and OBCFA as bacterial marker, daily duodenal flows of MN were on average 77.6, 112.8 and 18.7 g higher, respectively (Table 1). The large impact of bacterial reference (SAB vs LAB) on estimated duodenal flow of MN is well known and is related to differences in the marker : N ratio in the different bacterial fractions (Dewhurst et al., 2000). Indeed, the respective marker : N ratios for purine and pyrimidinic bases in SAB were 32 and 29% lower compared to LAB (Table 2), possibly

Table 2. Bacterial content of N, cytosine, adenine and odd and branched-chain fatty acids (mg/g DM) and marker:N ratio (g/g) of solid- (SAB) and liquid-associated rumen bacteria (LAB)

Item	LAB	SAB	SEM[1]	P
N	78.5	86.7	1.42	0.001
Cytosine	6.08	3.37	0.176	0.001
Adenine	8.60	4.60	0.203	0.001
OBCFA[2]	9.57	7.01	0.172	0.001
Cytosine:N	0.062	0.043	0.003	0.001
Adenine:N	0.087	0.059	0.003	0.001
OBCFA:N	0.096	0.090	0.003	0.038

[1] standard error of the mean differences between SAB and LAB

[2] odd and branched-chain fatty acids

reflecting differences in growth rate, nutrient availability and/or bacterial species (Volden et al., 1999; Carro and Miller, 2002). Interestingly, the ratio OBCFA:N in SAB was only 6 % lower compared with LAB.

From the above, it is clear that changes in the proportions of SAB and LAB can have a substantial impact on the estimated duodenal flow of MN. Rodríguez-Prado et al. (2004) suggested that many of the estimates of MN changed more because of the bacterial isolate considered than because of the main dietary factors studied. In addition, Martin et al. (1996) stated that information about the relative contributions of the different microbial populations to the small intestine is needed if accurate measurements of MN flow are to be obtained. In the current experiment, the relative proportions of SAB in duodenal content were estimated using a linear programming approach and ranged from 49.3 to 84.5% (Vlaeminck et al., 2006). Irrespective of the marker used, MN yield based on the estimated ratio of SAB:LAB was different from estimations based on marker:N ratios of SAB or LAB (Table 1). Although duodenal flow of MN based on measured SAB:LAB ratio was highly correlated with MN yield based on SAB or LAB ($r_{pearson}$ > 0.850), the lower accuracy resulted in a mean error of more than 35 and 50 g N/d with cytosine and adenine as microbial marker, respectively. In contrast, due to the small difference in OBCFA:N between SAB and LAB, differences between flow of MN based on estimated SAB:LAB ratios and MN yield based on SAB or LAB was less than 15 g N/d.

Strikingly, differences in MN yield between the three markers were as high as changes brought about by the bacterial reference (Table 1). Hence, despite the substantial impact of the bacterial isolate on the estimated duodenal flow of MN, adjusting the latter using SAB:LAB ratios estimated from OBCFA profiles (Vlaeminck et al., 2006) seems of little value when observing these large differences among markers. Although it is impossible to state which marker method provides the most accurate estimate of MN flow (Stern et al., 1994), the current results suggest OBCFA to be more appropriate as bacterial marker compared with cytosine and adenine because of the small differences in OBCFA:N ratio between SAB and LAB.

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