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Technical note: The persistence of microbial-specific DNA sequences through gastric digestion in lambs and their potential use as microbial markers[1]

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ABSTRACT: Two groups of 5 lambs were euthanized at the weaning (T45) and fattening stages (T90) to evaluate the use of microbial ribosomal DNA (rDNA) sequences as potential microbial markers in relation to purine bases (PB) as a conventional marker. Both microbial markers originated similar microbial N concentrations (mg/g of DM), although T45 showed decreased values compared with the T90 group when either PB or rDNA were considered ($P = 0.02$). The survival of microbial rDNA was determined in 3 digestive sites (omasum, abomasum, and duodenum), but no substantial differences were observed, indicating that rDNA maintains the molecular stability along the sampling sites analyzed. Contrarily PB concentration increased successively along the digestive tract ($P < 0.05$), likely as a consequence of the endogenous PB secretion. Undegraded milk PB may also explain the overestimation of the microbial N concentration (2.8 times greater) using PB than rDNA sequences. Abomasum was the sampling site where the best agreement between PB and rDNA estimations was observed. Protozoal N concentration was irrelevant in T45 animals, although substantial in T90 lambs (18% of microbial N). In conclusion, bacterial 16S and protozoal 18S rDNA sequences may persist through the gastric digestive tract and their utilization as a highly specific microbial marker should not be neglected.

Key words: bacteria, protozoa, purine base, quantitative polymerase chain reaction, ribosomal DNA, rumen

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INTRODUCTION

Rumen microorganisms represent the main source of protein in ruminants; therefore, the ability to discriminate between microbial N (**MN**) and nonmicrobial N may be regarded as a key measurement in ruminant nutrition. Rumen microbial yield is determined by combining postruminal sampling and microbial markers (Broderick and Merchen, 1992); nevertheless, inaccuracies can occur if the rumen-harvested microorganisms are not representative of the microbes reaching the postruminal absorptive sites. Moreover, the lack of protozoa specific markers means that their contribution to MN flow remains unknown and frequently ignored.

The existence of specific microbial ribosomal DNA (**rDNA**) sequences may help to overcome some of the limitations listed above (Sylvester et al., 2005). Such markers are highly specific and could allow a direct measurement of MN if rDNA escapes gastric digestion. Belanche et al. (2010a) demonstrated that microbial rDNA sequences persist in an in vitro simulation of abomasal digestion; therefore, it was hypothesized that rDNA can also be used in vivo. The aim of this preliminary study was to confirm the microbial rDNA persistence at different postruminal sites [before (omasum), during (abomasum), and after gastric digestion (duodenum)] and to compare MN concentrations derived from rDNA sequences with those derived from purine bases (**PB**) as a reference microbial marker.

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MATERIALS AND METHODS

Two groups of 5 Rasa Aragonesa singleton lambs were used and handled according to the Ethical Committee for Animal Research of the University of Zaragoza.

Animals, Diets, and Sampling Procedure

Lambs from the first group (**T45**) consumed milk (twice per day), as well as concentrate (Cebo Intensivo Retirada, NANTA, Grupo Nutreco Tres Cantos, Spain) and barley straw (both ad libitum) during the entire experiment. These animals were slaughtered at weaning age (45 d of age) with a BW of 15.2 ± 0.6 kg. The second group of animals (**T90**) was treated similarly to the former, but after weaning (at 45 d) the animals were fattened using the same solid diet and slaughtered at 90 d of age at 23.5 ± 1.1 kg of BW. Exactly 2 h after feeding, animals were anesthetized and euthanized by intramuscular administration of Xilacine (Xilagesic 2% Laboratorios Carlier S.A., Les Franqueses del Vallès, Spain, 0.3 mg/kg of BW) and Thiopental (Tiobarbital Braun, B. Braun Medical, S.A., Barcelona, Spain; 10 mg/kg of BW). The digestive tract was dissected and sampled as described by Askar et al. (2005). Total digesta contents were collected from the omasum, abomasum, and duodenum. Liquid-associated bacteria (**LAB**) were isolated from rumen liquor by differential centrifugation at 500 × *g* for 5 min followed by 2,000 × *g* for 20 min at 4°C (Pérez et al., 1998). Protozoal pellets were obtained by sedimentation in a glass separation funnel followed by sequential filtration (Yáñez-Ruíz et al., 2006) to remove plant material (100-μm nylon mesh) and most of the bacterial contamination (10-μm-diameter nylon mesh, Sefar Maissa S.A., Cardedeu, Barcelona, Spain).

DNA Extraction and Quantitative PCR

Total DNA was extracted in duplicate from frozen samples using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK), and total DNA concentration was measured by spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, DE). Bacterial and protozoal rDNA concentrations were determined by quantitative PCR (**qPCR**) using primers developed by Maeda et al. (2003): forward 5′GTGSTGCAYGGYTGTCGTCA3′, reverse 5′ACGTCRTCCMCACCTTCCTC3′ and Sylvester et al. (2005): forward 5′GCTTTCGWTGGTAGTGTATT3′, reverse 5′CTTGCCCTCYAATCGTWCT3′, respectively. The DNA extracted from LAB and protozoal pellets was serially diluted and used as a standard to quantify bacterial and protozoal rDNA concentrations, respectively, assuming a constant DNA/rDNA ratio. The cross-contamination of bacterial rDNA in the protozoa or protozoa rDNA in the bacterial extracts was measured by qPCR and subtracted from the concentration determined by spectrophotometry. The qPCR was performed using an ABI Prism 7000 (Certified GeneTool Inc., Pleasanton, CA). Extracted DNA (1 μL) was added to the amplification reaction (25 μL of total volume) containing 25 pmol of each primer, 12.5 μL of Platinum SYBR Green (Invitrogen, Madrid, Spain) qPCR SuperMix-UDG, and 0.5 μL of ROX Reference Dye (Invitrogen, Paisley, UK). Cycling conditions for bacterial PCR were 95°C for 10 min and 30 cycles of 95°C for 15 s, 61°C for 30 s, and 72°C for 30 s, whereas for the protozoal PCR were 95°C for 10 min and 40 cycles of 94°C for 10 s, 55°C for 20 s, and 72°C for 30 s. Primers specificity was confirmed after the amplification by a single sharp peak in the melting analysis. Samples were analyzed in duplicate, and a negative control was used to screen possible contamination.

Chemical and Statistical Analysis

Nonammonia N was determined after removing ammonia using the procedure of Firkins et al. (1992). Total N and nonammonia N were determined by the Kjeldahl technique using selenium as a catalyst. Purine bases (**PB**) were analyzed as described by Balcells et al. (1992) with the modification proposed by Martín-Orúe et al. (1996). The proportion of MN in the digesta was determined using PB (MN_{PB}) as microbial marker and LAB as a reference microbial pellet as follows: $[MN_{PB} = PB_{digesta}/(PB/N)_{LAB}]$, where the microbial origin of all PB was assumed. Bacterial and protozoal N concentration in the digesta were determined using 16S and 18S rDNA as specific microbial markers, and MN content was estimated as follows: [MN_{rDNA} = bacterial N + protozoal N], [bacterial N = $(16S\ rDNA)_{digesta}/(16S\ rDNA/N)_{LAB}$], and [protozoal N = $(18S\ rDNA_{digesta}/(18S\ rDNA/N)_{protozoa}$]. The 18S rDNA/N ratio in protozoal pellets was corrected based on their bacterial contamination assuming an rDNA/N ratio equal to LAB.

Unfortunately, after slaughter, the omasums of T45 lambs did not yield sufficient samples to allow accurate determinations. Therefore, statistical analysis was carried out as 2 independent analyses. Data were analyzed using Proc MIXED (SAS Inst. Inc., Cary, NC) according to the following model: $Y_{ijk} = \mu + D_i + S_j + (D \times S)_{ij} + e_{ijk}$, where Y_{ijk} is the dependent, continuous variable; μ is the overall mean; D_i is the fixed effect of the gut development (T45 vs. T90); S_j is the fixed effect of the sampling site (abomasum vs. duodenum); D × S is the interaction between D_i and S_j; and e_{ijk} is the residual error considering the animal as random effect. The effect of the 3 sampling sites within the T90 group (omasum vs. abomasum vs. duodenum) was determined by an additional analysis: $Y_{jk} = \mu + S_j + e_{jk}$, where factors are the same as described above. Means separation was conducted using Fisher's protected least significant difference test, with significance declared at $P < 0.05$ and tendencies at $P < 0.15$. The microbial marker effect (PB vs. rDNA) was analyzed within each experimental group and sampling site using the Wil-

coxon matched nonparametric pair test after checking the homogeneity of variance.

RESULTS AND DISCUSSION

Representativeness of the Microbial Sample

Average DMI during the 7 d before euthanasia was 522 (SD 88) g/d (28% milk, 67% compound feed, and 5% straw) for T45 lambs and 1,021 (SD 142) g/d (90% compound feed and 10% straw) for T90 lambs. To avoid large changes in the digesta, animals were fed ad libitum (T45 also received milk twice a day) and total digesta was collected to maximize its representativeness. Liquid-associated bacteria have been used as a single bacterial reference in growing lambs (Askar et al., 2005), although the authors are aware of a slight underestimation of MN flow that can occur when solid-associated bacteria are not considered (Pérez et al., 1998). Under our experimental conditions no diet (or gut development) effect was observed in N, PB, and PB/N ratio in the bacterial pellet (Table 1); however, bacterial extracts obtained from T45 lambs tended to have greater rDNA concentration than T90 lambs ($P = 0.068$), which led to a greater rDNA/N ratio ($P = 0.045$). Cross-contamination of LAB with protozoa DNA was negligible ($<2 \pm 0.1\%$). However, to our knowledge any procedure to isolate protozoal cells without bacterial contamination has not been described yet. A sedimentation-filtration protocol was used as the most efficient procedure to remove bacterial contamination (Sylvester et al., 2004), but a substantial fraction of bacteria still remained attached to the protozoa ($39 \pm 6.6\%$). These contamination values agree with other authors and isolation procedures; 23 to 60% by sedimentation (Volden et al., 1999) and 7.4% (Yáñez-Ruíz et al., 2006) or 4.37% (Sylvester et al., 2005) by filtration procedures. Only T90 lambs had a consistent protozoal population and its average composition (after correction) was 50.1 mg of N/g of OM, 6.6 mg of rDNA/g of OM, and 0.13 rDNA/N ratio. This rDNA/N ratio values are substantially less than observed in literature [from 1.06 to 1.15 (Sylvester et al., 2005) or from 0.28 to 0.34 (Yáñez-Ruíz et al., 2006)], possibly explained by their significant bacterial contamination.

Microbial Synthesis Based on PB

In the present experiment PB were used as a reference to validate rDNA as a microbial marker. Both approaches quantify microbial nucleic acid; thus, analogous kinetics and metabolism through the digestive tract were hypothesized. Such assumptions raise a basic question: do all digesta PB have a microbial origin? This question is open to discussion. Although milk contains a significant concentration of purine compounds (323 ± 9.3 μM; Gonzalez-Ronquillo et al., 2003), most of them (about 90%) are in free and oxidized forms, as allantoin or uric acid (Martín-Orúe et al., 1996). Nevertheless, persistence of esophageal reflex allows milk PB to pass directly from the esophagus to the abomasum without undergoing rumen degradation, promoting an overestimation of the MN concentration (in abomasum and duodenum; $P = 0.008$) when PB were used as microbial marker rDNA sequences in T45 lambs. Group T90 tended to have a greater PB concentration ($P = 0.11$) and a significantly greater MN concentration ($P = 0.02$) than in the T45 using PB as a microbial marker. However, this PB concentration and its MN-derived data were greatly affected by the sampling site, showing a progressive increment along the digestive tract in both experimental groups ($P = 0.03$). Askar et al. (2005), using a similar protocol, but labeling microbial-PB with ^{15}N, demonstrated that nonmicrobial PB fraction in abomasal samples accounted for 22% [attributed mostly to dietary origin, Pérez et al. (1996)], whereas in the duodenum nonmicrobial PB (including dietary and endogenous) increased the total to 53% of the total PB concentration, suggesting the presence of a significant component of endogenous PB (Mota et al., 2008). Therefore, the presence of endogenous PB in duodenal samples derived from sloughed cells and bile secretion may explain the greater PB concentration (and subsequent MN concentration) in duodenum than in abomasum in T45 lambs ($P = 0.03$) or in omasum in T90 lambs ($P = 0.02$). Despite this, the reduced PB concentration in T90 omasal samples is still difficult to explain, and more sophisticated studies based on an accurate estimation of digesta flow are required.

Microbial Synthesis Based on rDNA Sequences

In a recently published study, Belanche et al. (2010b) described how rumen maturity increases the rumen microbial mass. Our results show that this gut development promoted a trend to increase microbial rDNA concentration ($P = 0.14$) and a substantial increase of the MN concentration ($P = 0.02$) between T45 and T90 lambs, in agreement with the PB estimations. Moreover, similar absolute MN concentrations were obtained with PB and rDNA in T90 lambs (21.1 vs. 21.0 mg of N/g of DM, respectively). In agreement with our results, milk diet supplemented with concentrate seems

Table 1. Chemical composition of the rumen liquid-associated bacteria isolated from lambs that were 45 (T45) and 90 d old (T90)

Item	T45	T90	SED	*P*-value
N, mg/g of OM	73.1	85.2	10.00	0.26
PB,[1] μmol/g of OM	195	192	42.6	0.94
rDNA,[2] mg/g of OM	14.5	9.29	2.47	0.07
PB/N, μmol/g	2.61	2.19	0.303	0.20
rDNA/N, mg/mg	0.21	0.12	0.037	0.04

[1]PB = purine bases.

[2]rDNA = ribosomal DNA.

Table 2. Effect of lamb gut development and sampling site on microbial N content[1]

	T45		T90					*P*-value			
Item	Abo	Duo	Oma	Abo	Duo	SED[2]	SED[3]	D	Sam	D × Sam	Sam_{T90}
Concentration, mg/g of DM											
Nonammonia N	26.2	40.0	37.1	34.9	36.8	4.29	5.12	0.46	0.14	0.26	0.95
Purine bases, mmol/g of DM	26.7	54.5	25.6	53.8	53.3	9.12	8.93	0.11	0.04	0.03	0.02
Microbial rDNA	0.85	1.22	2.26	1.94	2.02	0.670	0.410	0.14	0.50	0.67	0.72
Bacterial rDNA	0.85	1.22	1.65	1.59	1.59	0.510	0.440	0.20	0.56	0.57	0.99
Protozoal rDNA	0.001	0.001	0.61	0.35	0.43	0.260	0.200	0.16	0.66	0.66	0.44
Microbial N, mg/g of DM											
Using purine bases[4]	10.2[a]	21.1[a]	11.8[b]	26.0	25.1	2.95	2.85	0.02	0.06	0.03	0.01
Using rDNA[4]	4.4[b]	6.8[b]	23.8[a]	20.8	18.7	4.65	4.88	0.02	0.98	0.63	0.75
Bacterial N	4.4	6.8	19.2	18.2	15.5	4.79	4.91	0.06	0.97	0.58	0.86
Protozoal N	0.01	0.01	4.6	2.6	3.2	1.41	1.08	0.16	0.66	0.67	0.44

[a,b]Within a column, means with a different superscript differ ($P < 0.05$; n = 5).

[1]Lambs slaughtered at 45 (T45) and 90 d old (T90); Oma, omasum; Abo, abomasum; Duo, duodenum; rDNA = ribosomal DNA.

[2]Error term to study the effect of gut development (D; T45 vs. T90), sampling site (Sam; Abo. vs. Duo), and its interaction (D × Sam). Omasum data from the T90 group were not included.

[3]Error term to study the effect of sampling site (Oma vs. Abo vs. Duo) within T90 group.

[4]Comparison between microbial markers (purine bases vs. rDNA) was analyzed within each experimental group and sampling site using the Wilcoxon matched nonparametric pair test.

to develop ruminant conditions that are not compatible with considerable protozoal growth (protozoa represented about 0.2% of MN), whereas a solid diet (even if the concentrate represents 90% of the diet) seems to promote a ruminal environment more favorable for protozoal growth and protozoal N represented 18% of total MN. These postruminal concentrations of protozoal N were consistent with counts of protozoa cells in the rumen ($6.5 \times 10^2 \pm 2.4 \times 10^2$ vs. $1.5 \times 10^6 \pm 0.9 \times 10^6$ protozoa/mL in T45 and T90) and agree with the protozoal N contribution proposed in Holstein cows (5.9 to 11.9%, Sylvester et al., 2005) and steers (21 to 25%, Yáñez-Ruíz et al., 2006) fed different diets.

The possible rDNA degradation as it passes through the digestive tract represents a potential limitation; therefore, the sampling site is the key aspect to validate the use of rDNA as a microbial marker. Three sampling sites were considered in this work: before gastric digestion (omasum), at the likely digestion site (abomasum), and after gastric digestion (duodenum). In vitro simulation of gastric digestion suggested that, after an incubation at pH 2.3 during 40 min in the presence of cellulose, up to 95% of the bacterial rDNA sequences passed through the abomasum without suffering any damage, whereas protozoa rDNA was slightly more vulnerable, and only around 78% was recovered after the abomasal digestion (Belanche et al., 2010a). The present in vivo data confirmed those reported in vitro, and neither bacterial nor protozoal rDNA concentration was modified by the sampling site (Table 2), suggesting that rDNA maintains its molecular stability along the sampling sites analyzed. Microbial N concentration was also not modified by the sampling site ($P > 0.05$), showing a low interference of the endogenous secretions when rDNA is used as microbial marker in both experimental groups. This observation suggests that microbial rDNA could potentially be used as microbial markers to estimate rumen microbial yield or production.

Conclusions

This preliminary study suggests that microbial rDNA sequences could be considered as potential internal markers to determine microbial synthesis in vivo. This procedure gave similar results of microbial synthesis as the PB method, but rDNA showed a greater specificity. This specificity allows the origin of the microbial population to be distinguished in digesta (bacterial vs. protozoa); moreover, the rDNA procedure was less affected by the presence of endogenous and dietary fractions than the PB method. However, these observations should be carefully interpreted as a consequence of the high variability observed. More accurate studies using cannulated animals and flow markers must be done to confirm the validity of these findings in adult ruminants fed conventional diets.

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