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Applications of mass spectrometry for the determination of the microbial crude protein synthesis in ruminants

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Abstract

The importance of quantifying ruminal microbial crude protein synthesis has promoted the development and comparison of several different methods for precise determination of both the amount and rate of synthesis. One major challenge is in estimating and differentiating protein in the rumen between microbial, dietary, and endogenous fractions, and to correctly isolate the microbial fraction of the solid and liquid fractions of rumen contents. This is further complicated by the goal of using non-invasive methods as much as is feasible, such as avoiding the use of fistulated animals; as well as the selection of an appropriate microbial marker, specifically one that behaves similarly in the solid-associated and liquid-associated microbial fractions. It is also vital to be able to accurately estimate the contribution of microbial protein to overall nitrogen used by the animal, which can be accomplished by the use of ^{15}N labeled, as assimilated by ruminal bacteria, and by the quantification of labeled nitrogen via mass spectrometry ($^{15}N/^{14}N$). This review focuses on discussion of these challenges regarding accurate quantification of microbial crude protein synthesis in the rumen, as well as providing the methodology for quantification using the ^{15}N marker. This review is based on the

collection of scientific papers from the main research groups in feed and animal nutrition in ruminants.

Keywords: ^{15}N; Microbial protein; endogenous excretion; ruminants; Purine derivatives

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Introduction

Ruminants are inefficient at utilizing dietary nitrogen, so they have to use microbial protein (MP) to meet their metabolizable protein requirements [1,2]. In order to know the crude protein (CP) requirements of ruminants and improve their efficiency, microbial crude protein (MCP) synthesis is used. Menezes et al. [3] mentions that if more CP is given than required by the ruminant, the excreted Nitrogen (N) increases instead of improving their performance.

The importance of quantifying MCP in the rumen has spurred the development and comparison of several different methods of analysis. However, these methods do not always satisfy the required scientific needs for specificity, efficiency, or cost, nor do they always address concerns regarding quantitative inconsistency in replicating this technique for estimation. Some of the major challenges facing these methods focus on 1) how to estimate and differentiate protein of microbial, dietary, and endogenous origins; 2) the proper isolation of the microbial fraction in the particulate and fluid fractions of the rumen; 3) how to accurately quantify microbial protein in a minimally invasive manner, such as without the use of fistulated animals (ruminally and duodenally); and 4) the choice of an appropriate microbial marker that behaves similar in both solid and liquid fractions [4, 5]. Regarding this final challenge, a currently used microbial marker is the labeled nitrogen ^{15}N, which is one of the most reliable and recommended methods currently used, unlike other methods which often overestimate or underestimate MCP [6,7,8,9,10].This review focuses on discussion of these challenges regarding accurate quantification of MCP synthesis in the rumen, as well as providing the methodology for quantification using the ^{15}N marker. This review was

based on the collection of scientific papers from the main research groups in feed and animal nutrition in ruminants.

Microbial crude protein synthesis

Nutrition studies of ruminants are aimed at the selection of feeds based on high efficiency of MCP synthesis in the rumen along with the available N sources and energy support. A key strategy for improving production has therefore been to maximize the efficiency of utilization of available feed resources in the rumen by providing optimum conditions for microbial growth and thereby supplementing dietary nutrients to complement and balance the products of rumen digestion to the animal's requirement. Supplementation with rumen protected lysine and methionine can improve N use efficiency, decreasing the CP requirement from 18 to 15% in dairy cattle, without affecting milk yield production or animal performance [11].

Feed consumed by the ruminant, such as forage and cereals, enters the rumen and is available for degradation by rumen microbes. One fraction is rapidly degraded (Fraction A), while another is degraded more slowly (Fraction B). Both fractions are then used for microbial growth and synthesis of MCP (Figure 1a, [12]). This process depends on several factors, such as type of feed, the ratio of forage: concentrate in the diet, the fractional rate of degradation in the rumen, the physiological stage of the animal, the presence of secondary compounds (saponins, tannins, polyphenols, etc.), as well as the use of additives such as enzymes or ionophores, all of which can affect digestion and microbial kinetics.

In addition to the factors mentioned above, the synthesis of MCP is dependent on the energy supplied, which averages 35 g MCP/Mcal of metabolizable energy (ME) intake across particulate associated bacteria (PAB) and liquid associated bacteria (LAB). If the

diet is energy deficient, there will be a corresponding reduction in MCP synthesis and ammonia from breakdown of amino acids and NPN will be instead absorbed into the bloodstream rather than being used for formation of MCP [13]. If the diet is too high in protein excess protein will also be converted to ammonia and absorbed across the rumen wall into the bloodstream [14].

Once, this MCP has been synthesized in the rumen, depending on the method of estimation [15], 75% is true microbial protein, the rest (25%) are nucleic acids, 85% is true digestible microbial protein, (15% are undegradable amino acids) so, for every 100 g of MP synthesized in the rumen, only 64 g will reach to the duodenum in the form of true digestible MCP (Figure 1b). Dietary protein undegradable in the rumen (RUP), will exit to the duodenum. The amount of true RUP varies depending on diet and processing methods, though the calculation of RUP includes Acid detergent insoluble nitrogen (ADIN). Protein reaching the duodenum is of both sources, food and microbial origin, and is called protein digestible in the intestine (PDI). However, as discussed above, the amount of MCP varies depending on the factors discussed above, and hence it is important to quantify the protein flow entering the duodenum, and then to differentiate into that of microbial or food origin, combined with the Non Ammonia Nitrogen(NAN) that reaches the duodenum, and it is recycled by the ruminant[15,16, 17].

Dietary nitrogen fractions and their potential use by ruminants

The Cornell Net Carbohydrate and Protein System (CNCPS) is made up of a series of sub-models that assess, respectively, the carbohydrate and protein content available in the diet [18], the processes of fermentation and MP synthesis [19], the energy and protein requirements of cattle [20] and needs of essential amino acids [21] (i.e. lysine and methionine), this CNCPS predict requirements, feed utilization, animal performance and nutrient excretion for dairy and beef cattle using accumulated

knowledge about feed composition, digestion, and metabolism in supplying nutrients to meet requirements.

Dietary protein is traditionally divided up into true protein (amino acids) and non-protein nitrogen (NPN), but Licitra et al. [22] developed a new concept to differentiate nitrogen fractions present in feed and their potential uses by ruminants (Table 1). In the CNCPS, three nitrogen fractions are differentiated: non-protein N (NPN, fraction A), which is used exclusively in the form of $N\text{-}NH_3$, the potentially degradable true protein (fraction B) and the protein bound to the detergent acid fiber (fraction C), which is not digestible in the intestines. In turn, fraction B is subdivided into three others that are characterized by their different degradation rates, as indicated in Table 1. When considering the degradation (k_d) and passage (k_p) rates, this allows for estimation of the N contribution available to microorganisms in the form of $N\text{-}NH_3$ and peptides or amino acids, as well as the proportion of protein that escapes without being degraded (UIP).
As mentioned earlier, there is a first fraction that quantifies the non-protein nitrogen (NPN, Fraction A) and the true protein; true protein is divided into a rapidly soluble (BSP, Fraction B_1) and another that is insoluble but potentially degradable (smaller particles) which is called fraction B. Of the N fractions that are bound to dietary fiber (hemicellulose, cellulose and lignin), Fraction B_2 is linked to Neutral Detergent Fiber, fraction B_3 is soluble in Acid Detergent Fiber and finally there is a fraction insoluble in acid detergent (Fraction C), which is known as ADIN. The rapidly soluble fractions (Fraction A), together with the potentially degradable fractions in rumen (Fraction B), can be potentially degraded by rumen bacteria and synthetize MCP.

Methods of directly and indirectly estimating microbial protein synthesis

Interest in estimation of MCP synthesis goes back to the study of Faichney in 1975 [14] whose estimates the duodenal flow of purine bases (i.e, adenine and guanine), from the

content of nucleic acids present in bacteria (DNA, RNA), which contain purine bases (PB).Other authors used some markers such as diaminopimelic acid (DAPA) and D-Alanine, which are contained in the bacterial fraction of the rumen, while other authors calculated the PB / N ratio, based on PAB and LAB, with variable results[23,24]. Therefore, in recent years, the isotopic enrichment of ruminal bacteria and purine bases with $(^{15}NH_4)_2SO_4$ and $^{35}SO^{4}{}_{-2}$ was chosen for more general use, due to its improved accuracy [25; 5].

Likewise, indirect non-invasive methods have been developed that allow for the estimation of MCP synthesis from the excretion of purine derivatives (PD, i.e. Xanthine, Hypoxanthine, Uric acid and Allantoin) in urine and milk [26] with favorable results in urine (Figure 2).

Characteristics and uses of ^{15}N

Nitrogen labeled as ^{15}N is an example of an *external marker*, which are administered to animals in an inorganic form. These inert markers are then incorporated into the microbial mass in the rumen, allowing for the differentiation the dietary, endogenous, and microbial fractions of rumen contents [26].According to Carro [27] and Broderick and Merchen [23], these markers are easy to administer to the animal, and provide a constant enrichment rate, without risks of contamination or radioactivity – they specifically enrich ruminal microbes, rather than marking feed particles, and allow for the differentiation of microbial versus dietary protein. Such labeled nitrogen is directly incorporated into the rumen's bacterial populations, and indirectly into rumen protozoa during protozoal predation of other rumen microorganisms [23]. These specific characteristics allow for the use of external markers such as ^{15}N in *in vivo* or *in vitro* studies, with a high reliability rate.

Common uses of ^{15}N

The labeled nitrogen ^{15}N has been used in studies focusing on microbial protein synthesis as an indicator of efficiency on diets that promote optimal maintenance and health of rumen microorganisms [10, 28, 29, 30]. This aspect is particularly important, as microbial protein is the major source of protein available to the ruminant, accounting for 60 to 90% of protein entering the small intestine, in contrast to dietary protein [28,1], though this proportion is not constant and varies depending on a combination of factors, both dietary and animal.

Another alternative for the administration of ^{15}N to ruminants is from the production of ^{15}N labelled feed during growth (forages). For this purpose, $^{15}NH_4{}^{15}NO_3$ (Larodan Fine Chemicals AB, Malmö, Sweden) containing 2% of $^{15}N/^{14}N$, $(NH_4)_2SO_4$ with 10% enrichment of $^{15}N/^{14}N$ (Isotec, Miamisburg, OH) is used. The animals then are fed with this forage both silage and hay enriched with ^{15}N [31,32,33].

^{15}N-labelled forage

For the cultivation of grasses (i.e. Alfalfa, Timothy grass) labeled with ^{15}N are fertilized with 1,100 g of the $(NH_4)_2SO_4$ label. The grass is then cut, withered for 3 hours at 20°C and subsequently ensiled, in addition to using formic acid (5 mL/kg) as a preservative. The silage is defrosted at room temperature and separated into fractions enriched with soluble and insoluble substances. Portions of 20 g are suspended in 400 mL of ultrapure water (Milli-Q, Merck Millipore Corporation, Darmstadt, Germany) and stirred for 1 h at 39°C. Then, the suspension is centrifuged at 15 000 x g for 15 min (Avanti J26S XP, Beckman Coulter, Inc. Brea, CA, USA) and filtered through Whatman no. 1 filter paper.

198 The filtrate consisting of the soluble fraction, subsequently frozen at -20 °C and freeze-
199 dried (Figure 3[32]) [32,33].
200 Two samples of the soluble fraction are taken for chemical analysis. The first 15 mL
201 sample is treated with 0.3 mL of 50% (vol/vol) H_2SO_4 to determine the concentration of
202 ammonia N (AN) marked with ^{15}N and the excess ammonia N (APE) at 15%. A further
203 200 g of soluble fraction is treated with 5 mL of saturated $HgCl_2$ solution for the
204 analysis of total soluble N, and the APE of total soluble N. To calculate the APE in each
205 fraction, the ^{15}N-atom % background is determined from unlabeled samples. All
206 samples are stored at -20°C until further analysis.
207 Forage labelled ^{15}N is administered to ruminants on days (i.e. day 10 and 11) of the
208 experiment. For ruminal metabolism of ^{15}N fractions, samples are taken from ruminal
209 digestion at different times,0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 11, 14, 17, 22, 27, 33,
210 39, 47, 55, 63, and 72 h after administration of N-labelled sources. For more detail
211 information see Ahvenjärvi et al. [33].
212
213 ***Ammonium sulfate & ammonium chloride***

214 Ammonium sulfate($^{15}NH_4SO_4$) and ammonium chloride ($^{15}NH_4Cl$) are the most
215 commonly used forms of ^{15}N used for direct applications [23], with ammonium sulfate
216 being used more frequently [10, 29, 31, 34, 35]. The typical procedure for use of ^{15}N
217 involves the administration of the labeled compound via continuous ruminal infusion,
218 allowing for uniform enrichment of rumen microbes. The advantage offered by these
219 compounds is that they dissolve rapidly in water, allowing for the integration of the ^{15}N
220 over a short time of approximately 3 days [29, 31, 35].
221
222 ***Omasal sampling and analysis***

223 The omasal sampling technique can be an alternative for the calculation of the MCP
224 synthesis, being a less invasive method and allowing to investigate in more detail the
225 flow of soluble quantify microbial Non-Ammonia N (NAN) components in the rumen,
226 if duodenal cannulated animals are not available [36].
227 This procedure is done during the last week of each experimental period (when you are
228 sampling omasal samples for MCP synthesis) using the techniques developed by
229 Huhtanen et al. [37] and Ahvenjärvi et al. [38], as adapted by Reynal and Broderick [1],
230 to quantify digesta flow from the rumen. Indigestible NDF [39], $YbCl_3$[40], and Co-
231 EDTA [41]. Approximately 500 mL of omasal digesta is sampled at each sampling
232 point (n= 3). A sub-sample (70 mL) is used to obtain the LAB and the PAB. Another
233 sub-sample (approximately 400 mL) is frozen (−20 °C). This sample is filtered in
234 cheesecloth and washed with 1 L of 0.9% NaCl solution. The solids retained in the
235 cheesecloth which are mainly associated with the large particle (LP). The filtrate is
236 centrifuged (1000Å~g for 10 min at 5 °C) and the precipitate is considered small
237 particle (SP), whereas supernatant is designated fluid phase (FP) of digesta [37],
238 respectively, are used as flow markers in omasal or duodenal contents. Indigestible NDF
239 is determined in LP, SP and total mixed ration (TMR) but not in FP [38]. During the
240 determination of indigestible NDF, samples (0.35g) are weighed into duplicate 5 x 10-
241 cm dacron bags with 6-µm pore size, incubated in the rumen of two or three dairy cows
242 for 12 days, rinsed with water, then subjected to NDF analysis as described below. The
243 external microbial marker ^{15}N is used to quantify NAN flow from the rumen.
244 The triple marker technique [42]; France and Siddons [43] is used to determine the
245 proportions with which to recombine the 3 phases of rumen contents to quantify omasal
246 true digesta (OTD). Before marker infusion begins, whole ruminal contents are taken
247 from each cow to determine the background ^{15}N abundance (Natural abundance). Means

248 of the total observations of background ^{15}N abundance vary (i.e. 0.3681% of N). Cobalt-
249 EDTA, $YbCl_3$, and $^{15}NH_4SO_4$ containing 10% atom excess ^{15}N (i.e. Isotec, Miamisburg,
250 OH) are dissolved in distilled water and continuously infused into the rumen at rates of
251 2.0 g of Co EDTA, 3.0 g of Yb, and 70 mg of ^{15}N per day for approximately 6 days.
252 Markers are continuously infused for 6 days using a peristaltic pump. After 3 days of
253 infusion, ruminal and omasal samples are collected at 2-h intervals over a 3-d period to
254 represent the 24-h day flows. The sampling protocol includes confirming that sample
255 tubes are correctly positioned in the omasal canal, sampling times and volumes, sample
256 processing, isolation of LAB and PAB, digesta marker analyses, and preparation of
257 omasal true digesta as described by Reynal and Broderick [1] and Brito et al. [44],
258 except that ammonia and protozoa are not isolated for determination of ^{15}N enrichment.
259 Samples of OTD are analyzed for total N, DM, OM, NDF, ADF, NPN and ADIN.
260 Samples of OTD and isolated bacteria are treated with K_2CO_3[44] to remove residual
261 ammonia and analyzed for total N (equivalent to NAN) and for ^{15}N abundance using an
262 elemental analyzer (i.e. Costech Analytical Technologies Inc., Valencia, CA) interfaced
263 to a Thermo-Finnigan Delta-Plus Advantage isotope ratio mass spectrometer (i.e.
264 Thermo-Electron GmbH, Bremen, Germany). Equations used to compute flows of
265 nutrients of dietary and microbial origin, and extents of ruminal digestion are detailed
266 by Brito et al. [44].
267 Feed samples, ruminal content and OTD from any experiment in order to determine
268 MCP synthesis in ruminants are determined by drying at 60 ° C (forced-air oven) for 48
269 h. Feed samples, ruminal content and OTD are ground to pass a 1-mm Wiley mill
270 screen, and analyzed later for total N (i.e. Leco FP-2000 Nitrogen Analyzer, Leco
271 Instruments Inc., St. Joseph, MI), DM, OM [45], and then sequentially for NDF, ADF

and ADIN using heat-stable α-amylase and Na_2SO_3[46] as per Hintz et al., [47] , as well as for NPN without use of Na_2SO_3[22].

We need 0.5 g sample of OM truly digestible in the rumen (OMTDR) from each cow or any ruminant content which is extracted in 10 ml of citrate buffer (77.5 mM adjusted to pH 2.2 with HCl) for 30 min at 39 °C, and centrifuged at 15,000x g at 4 °C for 15 min. Broderick et al. [48] conducted a meta-analysis of the omasal sampling technique finding that models based on OM intake are better predictors of microbial N flow.

Ruminal and duodenal samples

To accurately estimate microbial protein synthesis, it is necessary to calculate the flow of total nitrogen, food nitrogen, microbial N flow (non-ammoniacal non-microbial, N-NANM) in the duodenum [15, 49, 50].This typically involves the use of ruminally and duodenally cannulated animals, from which samples of the microbial fractions are taken, as well as from of the omasal canal (Rumen) if we don't have duodenal cannulas [1] (See above) or in the small intestine at the duodenal level [26] with either T-shaped duodenal cannula, and the use of flow markers (Cobalt-EDTA, Cr-EDTA, $YbCl_3$, Titanium oxide, NDF indigestible) [26,51].

After samples of digesta are obtained [52] the next step is to differentiate nitrogen by its origin into microbial, dietary and endogenous pools. For this, the marker / nitrogen ratio in duodenal flow, as well as in ruminal microorganisms present in the PAB and LAB are calculated.

When performing this procedure, there are differences in the relationships between bacteria associated with PAB and LAB of the ruminal content [1 ,25, 34, 53,54]. The relationship between both values (marker-nitrogen / duodenal flow and marker-nitrogen / bacteria (LAB and PAB)), represents the nitrogen fraction of the flow that is of

297 microbial origin. The labeled ^{15}N is commonly used for these procedures, as it is highly
298 recommended for its accuracy and application [55, 56].

299 The *in vivo* techniques for calculating microbial N flow as described above have major
300 disadvantages and are also controversial for animal welfare reasons, since cannulated
301 animals are needed, which generate labor costs at the time of surgery and maintenance
302 of the animals [7].

303 ***Liquid-associated and solid-associated microbes in the rumen***

304 As previously discussed, the bacteria present in the rumen are associated with the liquid
305 (LAB) and particulate (PAB) fractions, so these fractions must be separated and
306 quantified (Figure 4). This procedure is performed via filtration and rinsing with
307 solutions based on sodium chloride (NaCl) and is performed at low temperatures (2-3
308 °C) [34]. To separate the supernatant of ruminal fluid and rinse solution from the
309 bacterial component, various centrifugation techniques are used to reduce bacterial cell
310 rupture [10, 35, 34]. Finally, the bacterial sediment (pellet) passes through a lyophilizate
311 to be analyzed [10, 34]. Mass spectrometry is one of the most commonly used methods
312 to analyze the enrichment of ^{15}N within the bacterial population [10, 57].
313

314 ***Endogenous excretion of purine derivatives***

315 Now once the N is absorbed in the small intestine, and it is fixed in the tissues, or it is
316 synthesized to produce milk, a part of this N that it is not used, is excreted in the urine.
317 Absorbed nitrogen that is not retained nor excreted in milk is excreted as waste.
318 However, waste also contains nitrogen from endogenous sources, which is difficult to
319 quantify.

320 Various techniques have been proposed to help quantify endogenous nitrogen
321 excretions, among them the proposed fasting of the animals [58,59], or making these
322 animals artificially “non-ruminant”, by feeding them on milk. However, the latter
323 method presents difficulty in estimation, because part of this milk ferments in the
324 rumen. Other methods used for quantification of endogenous nitrogen are based on
325 intragastric infusion of nucleic acids [26], or the replacement of duodenal content [60],
326 in animals fistulated in the duodenum with reentry cannulas, which ultimately affect the
327 normal physiology of the animal. Endogenous nitrogen excretion values have been
328 determined in intra-gastrically fed animals [61,62], in animals whose duodenal digesta
329 was substituted with an infusion solution containing free purine bases[63], as described
330 in Gonzalez Ronquillo et al,[26] or following rumen emptying [64]. It is intrinsically
331 assumed in existing reports that endogenous PD losses are equivalent to the basal
332 excretion when there is no exogenous PB absorption from the small intestine. Therefore,
333 it is possible to opt for the continuous infusion of ^{15}N-NH_3provided naturally, labeled
334 PB in the digesta leaving the rumen in cattle and with it the quantification of the
335 endogenous N fraction of the animals.
336 In theory, microbial PB absorbed from the small intestine are metabolized and excreted
337 in urine proportionate to their rate of flow into the small intestine. However, the
338 relationship between duodenal input and urinary output of purine compounds is masked
339 by the presence of an endogenous fraction originating from the animals via their own
340 nucleic acid turnover. Therefore, methods based on the flow of PB to the intestine have
341 been used to quantify the urinary endogenous excretion of PD.
342 The endogenous urinary PD fraction is determined from the isotopic enrichment of
343 urinary PD and that of duodenal PB (Figure 5). Duodenal PB are labeled by continuous
344 infusion of the ^{15}N supplied as ammonium phosphate. Duodenal flow of PB is

345 determined using a double [65] or triple marker system [39] using Ytterbium acetate
346 and Cr-EDTA, or any other particulate and liquid markers, respectively.
347 To do this, once $(^{15}NH_4)_2SO_4$ has been infused for five days at least, urine samples and
348 rumen and /or omasal contents are taken from day 6 to 8, and samples of duodenal
349 digesta (250 ml) are collected at 6 hourly intervals for 48 h, refrigerated and pooled on
350 an individual basis at the end of the collection period. Samples of whole and solid
351 digesta are then freeze-dried for subsequent analyses [26]. Urine is collected daily into
352 200 ml of sulphuric acid solution (10% v/v) by means of external separators glued to the
353 vulva of the dairy cattle or using urethral catheters. Urine is weighed and specific
354 gravity is recorded or weighted, pooled and stored at –20 °C in order to proceed with
355 the determination of Purine derivatives (PD) and N content. PD in acidified urine is
356 determined by HPLC [66]. Adenine and guanine in duodenal digesta samples (40 mg)
357 are determined by HPLC technique, after acid hydrolysis [67].
358

359 ***Extraction of Purine Bases and Purine Derivatives***

360 Purine bases in duodenal digesta are extracted by specific precipitation with silver ion
361 as an Ag complex following the procedure of Kerr and Seraidarian [67] and modified by
362 Aharoni and Tagari [30]. In this modification acetic acid is substituted for ammonium-
363 phosphate buffer in order to avoid $^{14}N/^{15}N$ contamination. The purity of the precipitate
364 must be tested by HPLC [66] by screening the effluent from 205 to 300 nm wavelength.

365 ***Extraction of allantoin or allantoic acid***

366 Urinary allantoin is extracted by ion-exchange chromatography after its conversion to
367 allantoic acid by alkali hydrolysis. The extraction procedure is as follows. Urine (2
368 ml)is mixed with 0.5 ml of 0.5 M NaOH and heated on a boiling water bath for 20 min.

Aftercooling in running tap water, the pH of the solution is adjusted to 9.0 and injected into the ion-exchange column (12 x 2 cm; Amberlite IRA-400, Sigma Co., Ma, USA) that has been conditioned previously for 2 h with boric acid buffer (0.05 M boric acid adjusted to pH 9.0 with NaOH).The column, then is washed with the same buffer for 2 h and adjusted to pH 6.0 with$(NH_4)H_2PO_4$ (0.05 M, pH 6.0). Ammonium phosphate buffer is allowed to flow for 45 min approximately (depending of the fluid rate) and allantoic acid is eluted using NaCl (1M) in 2 ml-fractions. Chromatography is carried out at room temperature and at a flow rate of 0.6 ml/min (recommended). Allantoic acid is eluted after 25 min of saline elution and its presence is colorimetrically detected using 0.5 ml sub-samples [68]. Allantoic acid fractions are mixed (6 or 8 ml), freeze-dried and stored until^{15}N analysis. Purity of the eluted samples is tested by re-diluting samples of the freeze-dried material, injecting the resultant solution on the HPLC system [66] and screening for the presence of interfering compounds from 205 to 320 nm wavelength.

Isotope abundance

The^{15}N abundance in samples (Urine, LAB, PAB, Duodenal content or omasal content) is determined using a gas isotope-ratio mass spectrophotometer (VG PRISM II, IRMS) in series with a Dumas-style combustion N analyzer (EA 1108 CARLO ERBA). Enrichment of urinary and duodenal PB during the period of ^{15}N infusion is corrected for background enrichment in similar samples collected before the infusion was started.

The endogenous fraction is determined by difference in the isotopic enrichment between duodenal PB and urinary PD. Duodenal PB are labelled by continuous infusion of the isotope (^{15}N) supplied as ammonium sulphate (Figure 6). The time necessary for equilibrium of labelled purine compounds through the digestive and metabolic pathways should be determined previously, assuming that 48-72 h would be needed to obtain a

393 homogeneous distribution of the isotope within the rumen ecosystem and that a further
394 24 h would be required for urinary PD enrichments to equilibrate with duodenally
395 absorbed PB [34]. The percentage of urinary PD coming from tissue nucleic acids at
396 steady state conditions is calculated as follows:

397 Endogenous excretion of PD (%) = (1 – [^{15}N urinary PD enrichment (allantoin or
398 allantoin precursors) /^{15}N duodenal PB enrichment]) × 100 (eq.1)

399 % Microbial N = (^{15}N duodenal PB enrichment - natural ^{15}N duodenal PB)/(^{15}N
400 microbial enrichment - ^{15}N microbial natural)) x 100 (eq.2).

401 For calculate the Rumen Pool Sizes, Rumen soluble N (RSN) pool size is calculated
402 based on the concentration of soluble N in rumen digesta grab samples and rumen fresh
403 matter pool size determined as a mean of three rumen equations:

404 Rumen soluble N pool size (g) = rumen fresh matter pool size (kg) × soluble N
405 concentration in rumen digesta (g/kg). (eq.3)

406 Rumen pool size of AN and fraction B_1 are calculated similar to Equation 3. Rumen
407 Soluble Nitrogen Ammonia nitrogen (SNAN) pool size is calculated as a difference
408 between rumen soluble N pool size and rumen AN pool size.

409 ***Rumen ^{15}N Pool Sizes in Excess of Background Levels.***

410 The background ^{15}N-atom% observed in rumen microbial and digesta samples taken
411 before dosing ^{15}N are used to calculate APE in different N fractions. Pool size of rumen
412 soluble ^{15}N in excess of background levels (^{15}NEP) is calculated as:

413 Rumen ^{15}NEP of soluble N (mg)= rumen soluble N pool size (mg) × ^{15}N excess atom %
414 in rumen soluble N/100. (eq. 4)

Rumen ^{15}NEP of AN and fraction $B_1$is calculated similar to Equation 4, and ^{15}NEP of rumen SNAN is calculated by difference between ^{15}NEP of soluble N and AN.

Rumen insoluble N pool size (bacteria N + solids N) is calculated based on rumen insoluble DM pool size determined as a mean of rumen (equation 3) and insoluble N concentrations analyzed from rumen samples.

The APE of rumen insoluble N is not analyzed directly, but instead is calculated as a weighted mean of APE of bacteria N and solids N isolated from rumen digesta grab samples. The fractional proportions of unlabeled feed N, bacterial N, and protozoal N in rumen insoluble N pool size are predicted based on an assumption that APE of rumen insoluble N is a weighted mean of each sub-fraction contributing to APE.

Rumen insoluble N (APE) = a × feed N (APE) + b× bacterial N (APE) + c × protozoal N (APE), (eq. 5)

where a, b, and c are fractional proportions of feed N, bacterial N, and protozoal N in rumen insoluble N pool size. The sum of a + b + c is constrained to 1. Because all APE given to the cows is soluble, APE of insoluble N of feed origin should be 0 at all sampling times, and consequently all APE of the insoluble N should therefore originate from bacterial and protozoal pools. The regression coefficients a, b, and c are iteratively adjusted using the Solver tool in MS Excel (Microsoft Corp., Redmond, WA) until the minimum of sum of squares for the difference between observed and predicted APE of rumen insoluble-N (SS_{diff}) is reached.

SS_{diff} = Σ (observed APE in rumen insoluble Nat time t − predicted APE in rumen in soluble N at time t)^2 (eq.6).

Rumen ^{15}NEP of bacterial and protozoal N is calculated based on the regression coefficients, rumen insoluble N pool size, and APE in bacterial and protozoal N:

Rumen ^{15}NEP of bacterial N (mg) = proportion of rumen bacteria in rumen insoluble N× rumen insoluble N pool size (mg) × APE in bacterial N/100, (eq. 7)

Rumen ^{15}NEP of protozoal N (mg) = proportion of rumen protozoa in rumen insoluble N× rumen insoluble N pool size (mg) × APE in protozoal N/100 (eq.8)[33].

Also, the ^{15}N enrichment and the PB concentration in the samples can used to estimate the microbial synthesis. The flow of NAN associated with liquid associated bacteria (LAB-NAN, g/d) and particles adhered bacteria (PAB-NAN, g/d) is calculated as:

$$LAB - NAN = FP_{flow} \times NAN\ FP \times (FP_{marker} / LAB_{marker})$$

$$PAB - NAN = SP\ flow \times NAN\ SP \times (SP_{marker} / PAB_{marker}) + LP_{flow} \times NAN_{LP} \times (LP_{marker} / PAB_{marker})$$

Where FP_{flow}, SP_{flow}, and LP_{flow} are the flows of omasum phases (g/d); NAN_{FP}, NAN_{SP}, and NAN_{LP} are the non-ammonia nitrogen of omasum phases (g/g); FP_{marker}, SP_{marker}, LP_{marker}, LAP_{marker}, and PAB_{marker} are the microbial markers of omasum phases and bacterial pellets.

Estimates of the endogenous contribution to PD excretion were obtained as following [26, 29]:

Endogenous contribution = { 1 - Urine PD ^{15}N enrichment/ Omasum PB ^{15}N enrichment}

CONCLUSIONS

The use of ^{15}N as a microbial marker has allowed a more precise quantification of microbial protein synthesis in particulate and fluid associated fractions, and the amount of protein of microbial origin that reaches to the duodenum versus that of food origin and non-ammonia nitrogen. Likewise, the estimation of endogenous N excretion from the excretion of purine derivatives in urine allows for exploration of the relation of endogenous N from bacteria and the total N excreted by the in a noninvasive manner. However, techniques for direct determination of the microbial synthesis in the rumen are laborious, expensive and even difficult to understand, so more articles are needed that address the entire procedure for calculating the MCP in ruminants in a didactic way.

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest.

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Figure 1a. Ruminal degradable fractions, and microbial crude protein synthesis into the rumen (adapted from AFRC, [12])

Figure 1b. Microbial crude protein synthesis into the rumen and the estimation of microbial protein through purine derivatives excretion in dairy cattle

Figure 2. Estimation of microbial protein through purine derivatives excretion in dairy cattle

Figure 3. Procedures used for extracting forage N fractions from silages and dried grass (adapted from Vaga et al. [32]).

Figure 4. Isolation of liquid (LAB) and particulate associated bacteria´s (PAB) fractions in ruminal content.

Figure 5. The endogenous urinary purine derivatives fraction is determined from the isotopic enrichment (^{15}N) of duodenal purine bases enrichment and urinary purine derivatives excretion in urine.

Figure 6. Endogenous urinary purine derivatives fraction, determined from the isotopic enrichment (^{15}N) of urinary PD (allantoic acid-^{15}N) in urine.