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Review

Factors affecting the ruminal microbial composition and methods to determine microbial protein yield. Review

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Abstract:

Microbial protein synthesized in the rumen is a major contributor of metabolizable protein. Thus, accurate estimation of microbial protein is essential in ruminant nutrition. The objective of this review is to describe the microbial composition, major factors affecting its yield and methods to estimate microbial protein flow to the intestine. The use of novel molecular techniques to elucidate the ruminal microbiome and improve methods for estimating microbial protein are discussed. Bacteria, protozoa, fungi and archaea compose the ruminal microbiome. Main factors affecting microbial protein synthesis are availability of carbohydrates, ruminally degradable protein, dietary fat, and ruminal pH. Major microbial markers used to estimate microbial protein synthesis are total purines, diaminopimelic acid and labeled nitrogen; in addition, DNA through real-time PCR is being tested for the estimation of bacterial, protozoal and yeast protein separately. The main difficulty in the estimation of microbial protein flow is obtaining representative microbial pellets from the rumen, which are used as reference to establish the ratio of marker/nitrogen. Detailed phylogenetic analysis using High-throughput DNA sequencing has recently revealed drastic

taxonomic differences between fluid-associated bacteria and bacteria from whole intestinal digesta contents. For example, ruminal fluid contains less *Fibrobacteres* and *Proteobacteria*, but more *Firmicutes* compared to whole intestinal digesta. This demonstrates the need of developing effective bacterial collection procedures for obtaining representative ruminal microbial reference pellets to prevent bias on the estimation of the contribution of microbial protein to the intestinal supply of metabolizable protein.

Key words: Microbial protein, Metabolizable protein, Marker, Rumen, DNA.

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Introduction

Metabolizable protein is the true protein absorbed by the intestine supplied by microbial protein, rumen undegradable protein and, to a minor extent, sloughed (endogenous) protein[1]; with microbial protein usually representing the largest source of the metabolizable protein supply[2,3]. When absorbed, this protein may be utilized for maintenance, growth, reproduction or lactation. Therefore, it is important for nutritionists to understand the nature, factors affecting, and appropriate methods for estimating the flow of microbial protein to the small intestine. The estimation of microbial protein synthesis has been carried out by a variety of methods, including the purine analysis[4]; the diaminopimelic acid method[5], and isotope incorporation into the microbial cells[6]. Recent advances in molecular techniques have allowed estimation of microbial protein using microbial DNA through real-time PCR[7,8,3], which is particularly important when rations include ingredients containing yeast DNA from *Saccharomyces cerevicieae*, such as those from the ethanol industry[9] or when researchers need to estimate the contribution of protozoa to total microbial protein[7].

Quantification of microbial protein requires the isolation of microbial pellets from the rumen, which are used as reference to establish de ratio microbial marker/nitrogen. However, if the isolated reference pellets are not representative of whole ruminal contents, the estimation will be biased[10]. Differences between solid-associated bacteria and liquid-associated bacteria

have been assumed longtime ago[11]; however, detailed phylogenetic differences between these fractions have remained largely unknown. The use of cutting-edge technology such as high-throughput microbial DNA sequencing in combination with bioinformatics[12,13] has provided new insights into the ruminal microbiome and have revealed drastic differences between liquid- and solid-associated bacteria[14].

Studies have compared the use of conventional microbial markers[4,11,15] or factors affecting microbial growth. In addition, recent reports have evaluated equations to predict postruminal microbial protein flow[16,17,18]. However; to the authors' knowledge, there is a lack of studies integrating recent advances and findings from the use of molecular techniques in ruminal microbiology, to improve the understanding on factors affecting and appropriate procedures to quantify microbial protein synthesis and its contribution to metabolizable protein.

Therefore, the objective of this review is to describe the microbial composition, major factors affecting its yield and methods to estimate microbial protein flows to the intestine. In addition, the use of high-throughput DNA sequencing to improve our understanding on the microbiome and factors affecting quantification of ruminal microbial protein and its flow to the small intestine is discussed.

Ruminal microorganisms and their importance

The reduced, anaerobic environment in the rumen allows the development of different kinds of microbes composed primarily of bacteria, protozoa, fungi and archaea[19,20].

Bacteria

In 1994[20] around 200 species of bacteria had been cultured. Recent reports using high-throughput DNA sequencing have revealed the presence of 13 major bacterial phyla in the rumen, that include 40 bacterial orders, around 80 bacterial classes, at least 180 bacterial families, around 320 bacterial genera and more than 2,000 bacterial operational taxonomic units[21,14]. Bacterial density in the rumen is found in the range of 10^7 to 10^{10} cells/mL of ruminal fluid. The most abundant bacterial phyla are *Bacteroidetes* and *Firmicutes*, which

account for at least 75 % of total bacterial population. The most abundant ruminal bacterial genus is *Prevotella* representing approximately 20 % of the bacterial community[14,22,23].

Protozoa

More than 20 species of protozoa had been identified[20], their concentration in the rumen is approximately 10^6 cells/mL. Although the number of protozoal genera is less than that of bacteria, protozoa are physically more massive than bacteria and they may account for approximately half of the total ruminal microbial biomass[19]. Protozoal nitrogen ranges from 4.8 to 12.7 % in the rumen and from 5.9 to 11.9 % in the duodenum[3,7]. Novel reports using high-throughput DNA sequencing have showed that predominant protozoal genera are *Entodinium*, *Epidinium*, *Metadinium*, *Diploplastron*, *Polyplastron* and *Diplodinium*[24]. Over 90 % of the protozoal population in the rumen belong to the class *Litostomatea* which include two groups, *Haptoria* and *Trichostomatia*. The *Trichostomatia* subclass contains *Entodinium* one of the most studied genera, and which accounts around 89 to 91 % of the protozoal population[24].

Fungi

Fungi have been found associated with the more slowly digested fractions of plants, and act as initial colonizers of lignocellulose and increase the bacterial digestion rate of dietary fiber by disrupting lignified plant cell walls[19]. They are small flagellated organisms and they were first misclassified as flagellated protozoa. However, later, it was observed that those flagellates had a cell wall that contained chitin and a reproductive life cycle typical of fungi. The flagellates are fungal zoospores that eventually colonized plant surfaces to produce a mycelium. The mycelium gives rise to sporangia that release more zoospores, and the cycle continues. The fungi increase their residence time by attaching to feed particles. For this reason, it has been difficult to estimate their biomass in the ruminal content[20]. DNA sequencing has recently revealed the presence of 5 major fungal phyla, which include 55 fungal genera. Predominant genera are *Ascomycota* (27 %), *Basidiomycota* (3 %), and *Neocallimastigomycota* (1 %)[25].

Archaea

The archaeal population includes microorganisms that were thought to be bacteria. However, molecular analysis of their DNA has revealed that they belong to a different domain[26]. The density of archaea in the rumen has not precisely been determined. These microorganisms play a special role on feed efficiency given their participation in methane formation, which takes place using carbon dioxide and hydrogen[27]. Because methane emitted into the environment contributes to global warming, abatement of the production of this gas in ruminants is one of the main targets of greenhouse gas mitigation practices for the livestock industry[28]. Recent findings from high-throughput DNA sequencing has revealed that the most abundant archaeal phylum in the rumen is *Euryarchaeota*, which accounts for around 99 % of total ruminal archaeal population. Ten archaeal genera have been detected in the rumen, and the most abundant genus is *Methanobrevibacter*, representing approximately 91 %[26].

The importance of microbial protein as a major source of metabolizable protein with regard to the nutritional state of ruminants and has been recognized a longtime ago[19]. Ruminal bacteria and protozoa contribute to the majority of the metabolizable protein reaching the duodenum. The microbial protein synthesized in the rumen meets at least 50 % of the amino acid requirements of ruminants in various states of production[1,29,30]. Under most dietary conditions, microbial protein accounts for 50 to 85 % of the total amino acid nitrogen entering the small intestine[5]. Other studies suggest that microbial protein synthesized in the rumen contributes from 40 to 90 % of the protein reaching the small intestine, despite the fact that up to 50 % of the microbial protein synthesized could be degraded to ammonia nitrogen in the rumen[30]. Furthermore, the relative contribution of microbial protein reaching the small intestine depends mainly on the quality and solubility of nitrogen intake[30]. This contribution may range from 1,262 to 2,137 g/d in the adult dairy cow, and from 473 to 1,300 g/d in beef cattle; in addition, the concentration of microbial protein in duodenal digesta of sheep has been found to range from 130 to 162 mg/g DM (Table 1).

Table 1: Comparative data of microbial protein reaching the small intestine measured by different methods

Source	Marker used[1]	Type of animal	Microbial protein reaching the duodenum
Glenn *et al*.[86]	PB	Holstein steers	1,093 g/d
Ipharraguerre *et al*.[89]	PB	Dairy cattle	1,825 g/d
Cooper *et al*.[85]	PB	Beef cattle	1,300 g/d
Sylvester *et al*.[83]	rDNA	Dairy cattle	1,693 g/d
Schwab *et al*.[90]	LN	Dairy cattle	2,137 g/d
Moorby *et al*.[88]	Cytosine	Dairy cattle	944 g/d
Hristov *et al*.[84]	LN	Dairy cattle	1,906 g/d
Leupp *et al*.[87]	PB	Beef cattle	545 g/d
Belanche *et al*.[8]	PB	Sheep	162 mg/g DM
Belanche *et al*.[8]	rDNA	Sheep	130 mg/g DM
Castillo-Lopez *et al*.[5]	DAPA	Beef cattle	473 g/d
Castillo-Lopez *et al*.[5]	rDNA	Beef cattle	561 g/d
Castillo-Lopez *et al*.[3]	PB	Dairy cattle	1,881 g/d
Castillo-Lopez *et al*.[3]	rDNA	Dairy cattle	1,262 g/d

PB= Purine bases; DAPA= Diaminopimelic acid; LN= Labelled nitrogen (^{15}N).

Moreover, ruminal microbes are a major source of other nutrients for the ruminant[31]. Major chemical components of ruminal microorganisms are nitrogen, carbohydrates, lipids and ash[32] (Table 2). The content of organic matter, nitrogen and amino acids in mixed rumen bacteria increase by decreasing the level of forage in the diet[19]. These variations could be due to difference in the species of bacteria resulting from different diets[33]. High-throughput DNA sequencing has recently confirmed this suggestion[21]. An increase in the organic matter and nitrogen concentrations of the protozoal population observed in response to an increase in the amount of starch in the diet has been observed[19], which may be due to changes in the protozoal community, recently confirmed using molecular methods[34].

Table 2: Nutrient composition of ruminal microorganisms[30] isolated through centrifugation

	Centrifugal fraction		
Nutrient content (g/kg DM)	**A[1]**	**B[2]**	**SEM[3]**
Moisture	62	50	2.3
Nitrogen	100	103	1.6
Carbohydrate	91	93	7.1
Lipid	92	94	6.7
Ash	116	98	4.0

[1]Supernatant centrifuged at 19,000 ×g for 8 min considered to contain the bulk of microorganisms.

[2]Supernatant re-centrifuged at 19,500 ×g for 15 min considered to harvest virtually all remaining microorganisms.

[3]Standard error of the mean.

Factors influencing the synthesis of ruminal microbial protein

Ruminal microbial growth depends on their capability to degrade and ferment feed ingredients. Bacterial cells have diverse transport systems for taking up low-molecular weight and soluble nutrients such as sugars[35]. Because feed ingredients are primarily composed of complex polymers such as starch, protein and cellulose, these polymers are first degraded by extracellular enzymes to low molecular weight substances, which are then utilized by bacteria. The amount of *in vivo* bacterial yield ranges from 1.9 to 3.0 mg per 100 mg of organic matter truly digested[36]. Some of the main factors that influence ruminal microbial protein synthesis include availability of dietary carbohydrate, ruminally degradable protein, dietary fat, ruminal pH and feed intake[37]. The model used to predict microbial protein (g/d) flow to small intestine is related to total digestible nutrientes, MN= 0.0166TNDkg[38]; or MCP= 0.087TDNintake+42.73[18].

Effect of dietary carbohydrates

Efficient utilization of degraded dietary nutrient requires that the energy from the fermentation of dietary organic matter be supplied at a rate which matches the synthetic abilities of the ruminal microbes. Readily available carbohydrates such as starch are effective for increasing utilization of degraded nutrients and increasing microbial growth[39]. In addition, there is an increase in microbial growth in continuous cultures (15.0 to 19.5 g microbial protein/100 g DM digested) in response to increased dietary nonstructural carbohydrate levels (32 to 49 % of DM)[39]. Thus, the type of dietary carbohydrates may influence bacterial metabolism. Feedlot cattle fed high-carbohydrate diets are virtually free of protozoa. However, other investigations carried out utilizing wheat[40] and corn- and sorghum-based diets[41] have showed high concentrations of protozoa in the rumen with high-grain diets. The shifts in the bacterial and protozoal population due to dramatic increase in dietary starch[21] is presumably due to the decrease in ruminal pH[38].

Effect of ruminally degradable protein

High producing ruminants are generally unable to meet their requirements for amino acids from rumen microbes alone[42]. Therefore, inclusion of ruminally undegraded protein in the diet may increase the total amino acid supply to the small intestine and modify the duodenal amino acid profile. However, feeding low-degradable protein sources may also limit microbial fermentation, resulting in reduced supply of energy and microbial amino acid to the host animal. Ruminal degradation of dietary protein is a time-dependent process, and rate of degradation relative to rate of passage is a critical dynamic property affecting the amount of ruminally undegraded protein escaping the rumen[43]. A diet with 5.3 % ruminally degradable protein results in a higher bacterial nitrogen flow (415 g/d as opposed to 365 g/d when 4.8 % ruminally degradable protein is fed)[42]. More ruminally available nitrogen likely improves the efficiency of energy utilization stimulating the growth of the bacterial population[43]. This indicates that if energy from carbohydrate for microbial growth is not limiting, the resulting peptides and amino acids are used for microbial protein synthesis more efficiently. However, if carbohydrates are limiting, a considerable fraction of the protein is broken down to ammonia, which can be partially wasted through urine. Thus, there should

be coordination between the availability of energy and nitrogen in the rumen[15]. More recently, equations have predicted microbial protein production as a linear function of ruminally degradable protein intake in dairy cattle[17]. In addition, in feedlot cattle, a minimum of 100 g of soluble nitrogen/kg of organic matter digested in total tract is required to maximize microbial nitrogen flow[44,45].

Effect of dietary fat

Although negative effects of dietary fat has been acknowledge several decades ago, novel advances in molecular methods have revealed that ruminal microorganisms belonging to the genera *Fibrobacter*, *Ruminocuccus*, *Butyrivibrio* and *Prevotella* can be very sensitive to fat[46,47]. It is important to note that unsaturated fatty acids have been shown to be toxic to ruminal bacteria, especially fiber digesting bacteria. This toxicity could be due to an impediment in the nutrient digestion due to fatty acids adhering to the cell wall[46]. Thus, it is not surprising that one of the major actions of some ruminal bacterial genera is fatty acid biohydrogenation to minimize the negative impacts of unsaturated fatty acids on microbial growth. Detrimental effects of dietary fat on ruminal protozoa, fungi and archaea have also been reported when feeding linseed or soybean oil[46].

Effect of ruminal pH

The effects of pH on growth of some ruminal bacteria have been recognized[48]. Species like *Fibrobacter succinogenes* and *Ruminoccosus albus* are very sensitive to acidic ruminal pH[49]. This sensitivity can be explained by negative effects of pH on glucose uptake. Other bacterial species such as *Prevotella ruminicola* and *Selenomonas ruminantium* are fairly resistant to decline in extracellular pH[50]. The type of transport mechanisms used by bacteria influences their sensitivity to pH. For example, transport of arabinose and xylose by *Prevotella ruminicola* is more sensitive to declines in extracellular pH than is glucose transport[51]. Low extracellular pH also decreases the transport of arginine, glutamate, and

leucine in *Streptococcus* sp.[49]. Development of high-throughput DNA sequencing have described the effect of ruminal pH on bacterial taxa of the entire bacterial population. For example, fiber digesting bacteria have been shown to be more sensitive to low ruminal pH compared to starch digesting bacteria[37,21]. In addition, researchers have found that mild or severe ruminal acidosis can induce drastic shifts in the bacterial population of the rumen. Rapid proliferation of some bacteria such as *Streptoccosus bovis* and *Lactobacillus* sp. has been reported in cattle in situations where the rumen contains high proportions of rapidly fermented carbohydrates and low ruminal pH[52,53]. Furthermore, the decrease in ruminal pH due to feeding high-grain diets negatively affects protozoal growth[54]; consequently, the model to predict microbial protein includes neutral detergent fiber as an adjustment factor[29].

Effect of feed intake

Decreased feed intake may affect bacterial activity and decrease microbial efficiency due to insuficiency in soluble nitrogen and fermentable organic matter[45]. However, if feed intake restriction is not severe, then microbial efficiency is increased (grams microbial nitrogen/kilograms organic matter fermented), but microbial yield (grams of microbial nitrogen reaching duodenum) is decreased as a consequence of less organic matter fermented in rumen. Other studies that have reported a positive relationship between increased feed intake and microbial yield; this is because higher feed intake increases passage rate which prevents protozoa predation[44,53]. In addition, the increased quantity of bacterial nitrogen reaching the duodenum with increased feed intake is expected because bacterial nitrogen production is positively correlated with the intake of digestible organic matter[53].

Measurement of microbial protein and its contribution to metabolizable protein

Measurement of intestinal flow of microbial protein requires the isolation of ruminal

microbial pellets, which are then used as reference to establish the ratio of microbial marker/nitrogen. Then, the marker in intestinal digesta is quantified. During the last few decades, several methods have been used to estimate microbial protein synthesis and the proportion that leaves the rumen[4](Table 1). One of the critical challenges in this process is obtaining a reference microbial pellet representative of both the fluid and particulate phases[10]. The nitrogen content of liquid-associated bacteria is 8.5 % and that of the particulate-associated bacteria is 7.0 %[19]. Consequently, if only the liquid-associated bacteria are used as reference to establish the ratio of marker/protein, this ratio would lead to underestimated values.

Measuring intestinal digesta flow is also needed to estimate microbial protein flow[53,54]. One of the most commonly used external digesta markers is chromic oxide (Cr_2O_3). For this procedure, Cr_2O_3 is placed in gelatin capsules and dosed into the rumen[55,56] twice daily during 10 d to reach a stable flow of the marker through the gastrointestinal track[5,57,58]. Although, it has also became common to incorporate Cr_2O_3 in the diet at concentrations that range between 0.25 and 0.40 %, on a DM-basis. In addition, indigestible ADF (iADF) is an internal digesta marker routinely used[59,60]. With this approach, the concentration of iADF in samples is determined after a 288-h *in situ* incubation in the rumen. Intestinal digesta flow is then calculated based on the amount of the marker fed (iADF) or dosed (Cr_2O_3) and the concentration of the respective marker in duodenal samples[61]. From these values, the flow of microbial protein; and thus, its contribution to total metabolizable protein is estimated. Other techniques for measuring digesta flow include labeling the particulate and fluid digesta phases with $YbCl_3$ and Cr-EDTA, respectively[6].

This review will focus on conventional microbial markers widely used for the estimation of microbial protein, such as total purines[4], diaminopimelic acid[5], and labeled nitrogen[6]. In addition, the use of DNA[3] through real-time PCR to measure protein originating from bacterial, protozoa and yeast will be discussed. The ideal microbial marker should not be present in the feed, not be absorbed, be biologically stable, occur in a similar percentage between the various types of microbes, be a constant percentage of the microbial cell in all stages of growth, and all forms should flow at a similar rate[62].

The use of total purines as a microbial marker

Purine bases (adenine and guanine) are part of nucleic acids of microbial cells[63]. Briefly, this procedure combines standard methods for the hydrolysis of nucleotides by perchloric acid. The first step is followed by precipitation of free purines with silver nitrate to separate the purines from interfering compounds. In this method, acid resolubilized purines are quantitated with a spectrophotometer at 260 nm. Then, microbial protein is estimated by the ratio of purines/nitrogen of reference bacterial pellets[64] and the concentration of purines in samples.

The use of purines is considered to have some inherent challenges. For example, purines from feed, which escape destruction in the rumen may cause overestimation of microbial protein[63]. Sloughed epithelial gut cells may also contribute purines to the digesta, and therefore cause an overestimation. In addition, greater purine concentration in duodenum than in abomasum in lambs has been reported, which was attributed to sloughed cells and bile secretion[8]. The correction factor 0.195 × BW^0.75 has been utilized to mitigate this overestimation[65]. Other major challenges encountered when using total purines as a microbial marker seems to be whether the purines are present in a similar percentage in the different species and in all stages of microbial growth. It has been reported that values for purines in mixed ruminal bacteria vary widely. For example, a study found a mean purine concentration of 7.28 % with values ranging from 2.40 to 13.02 %[53]. Variations in purine concentrations of mixed ruminal bacteria grown in continuous culture using several different protein sources have been reported[66]. This variation has also been reported among pure cultures[64]. Concentrations as a percentage of DM ranges from 0.69 to 5.57 %, with a mean value of 2.98 %. This situation indicates that if the ratio purine/nitrogen is used to estimate microbial protein at the duodenal level, values would be overestimated. Among the biological factors that may be responsible for these variations are the difference in chemical composition among liquid- and particle-associated bacteria and the stage of bacterial growth[19]. Therefore, the bacterial isolation procedure should gather a bacterial pellet that represents not only different locations of the rumen, but also liquid- and particle-associated bacteria[67].

The use of daminopimelic acid as a microbial marker

This method is based on the estimation of the ratio diaminopimelic acid/nitrogen in ruminal bacteria and the amount of the microbial marker in digesta[5]. From these values the amount of bacterial nitrogen in intestinal digesta is calculated[68]. Briefly, lyophilized samples are hydrolyzed with methasulfonic acid then centrifuged. Then, 20 µL of derivatized sample are injected into the column and subjected to HPLC analysis. During the oxidation process, methionine is converted into methionine-sulfone. In the last step of the process ion-exchange column chromatographic separation is conducted.

Diaminopimelic acid is found in the cell membrane of ruminal bacteria and it is absent in feedstuffs commonly fed to ruminants[68]. The accuracy of the technique depends on a constant diaminopimelic acid/nitrogen ratio among various microbial species, or the maintenance of a constant ratio of microbial species in the rumen. However, the latter assumption is not consistent with the sequential nature of rumen fermentation. In addition, the diaminopimelic/nitrogen ratio may vary among ruminal bacterial species[39]. The different bacteria have different peptidoglycan concentrations in the cell wall, therefore, different diaminopimelic acid concentration. For example, gram-positive bacteria contain 30 to 70% peptidoglycan in the cell wall; the gram-negative bacteria contain only 10 %. Furthermore, if cattle are fed with only forage diets, the gram-negative bacteria will be predominant in the rumen, and if cattle consume more concentrate, the proportion of gram-positive bacteria will increase[69,70]. Therefore, variations in the relative abundance of gram-positive and gram-negative bacteria may affect the estimation of bacterial nitrogen synthesis. For example, if gram-positive bacteria predominate in the rumen, this ratio will be greater, which would lead to an underestimation of bacterial protein synthesis if reference pellets are not representative of whole digesta.

The use of labeled nitrogen as a microbial marker

The synthesis of microbial protein has also been estimated by quantifying ^{15}N incorporation into microbes from $(^{l5}NH_4)_2SO_4$[15,6]. The ^{15}N is infused into the rumen via a ruminal cannula at a constant rate of approximately 1 L per day. This method is based on the incorporation of

labeled nitrogen from ammonia and do not account for microbial protein synthesized directly from amino acids or peptides. Besides being costly, the technique of utilizing nitrogen as a marker is quite complicated and as a result has not been extensively used. One of the advantages of this approach as compared to the purine analysis is the fact that ^{15}N-labeled protein leaving the rumen will only be of microbial origin, whereas a portion of the purines leaving the rumen may be of dietary origin[71]. However, the marker/nitrogen ratio has been shown to differ between bacteria associated with fluid and particle phases[30]. Thus, establishing this ratio from a representative bacterial pellet has been challenging.

The use of DNA as a microbial marker

The real-time PCR is a powerful tool used for quantitative nucleic acid analysis. DNA has been recently tested as a microbial marker through this method. The real-time PCR is a refinement of the original PCR developed in the mid 1980's[72,73], which allows rapid detection of microbial DNA, thus indicating the presence of a target microorganism or group of microorganisms. Compared with a conventional PCR method employing two primers, a forward and a reverse, an additional fluorescent probe is required in real-time PCR assays. Therefore, this is highly specific and sensitive because three oligonucleotides complementary to the target DNA marker are employed[74]. One advantage of this approach is the ability to quantify microbial protein originating from bacteria, protozoa and yeast, which could not be achieved using the conventional microbial markers. Therefore, the method is based on quantification of a DNA segment specific to these microbial domains[74].

One of the first studies that employed DNA through real-time PCR for the estimation of microbial protein was conducted in 2005[75] by using the protozoal 18S gene as a microbial marker. This assay was used to quantify the amount of protozoal biomass in ruminal fluid and digesta from the small intestine. These authors also reported that duodenal digesta subjected to two freeze-thaw cycles decreased recovery by almost half, but one freezing (a standard practice) appeared to increase recovery of microbial DNA. The assay includes procedures for isolating protozoal cells from the rumen for use as a standard to convert 18S gene copies to a biomass basis. The protozoal nitrogen has been determined to be 12.7 % of total rumen microbial nitrogen pool and 11.9 % of the duodenal microbial nitrogen for diets containing high forage[75].

Researchers have also reported the use of microbial DNA as a marker to estimate bacterial protein by measuring the 16S gene[5,8], or yeast protein by measuring part of the second chromosome of *Saccharomyces cerevisiae*[9]. One of the advantages of using DNA as a microbial marker is the high specificity for targeting an amplicon that is part of either bacteria, protozoa or yeast excluding any extraneous materials originating from undegraded

feed, which prevents contamination[8,64]. The quantification of yeast protein from the microbial protein pool is particularly important when ruminant rations include feed ingredients containing yeast cells from *Saccharomyces cerevisiae*, like those from the ethanol industry[76]. If the contribution of dietary yeast protein is not quantified separately, then microbial protein originating from bacteria and protozoa would be overestimated.

Major components of the real-time PCR assay include 1) A forward primer, which is an oligonucleotide of 20 to 24 base pairs, the 5' end of this oligonucleotide anneals to the 3' end of the target DNA marker[73]; 2) A reverse primer is also needed, the length of this oligonucleotide should be of 20 to 24 base pairs, the 3' end of the target microbial DNA marker should be complementary to the 5' end of this primer[73]; 3) A dual labelled *Probe* is also required[9]. The real-time PCR reaction is performed by temperature cycling. High temperature (95 ºC) is applied to separate the strands of the double helical DNA, then temperature is lowered at 60 ºC to let primers anneal to the target DNA marker, and finally the temperature is set around 72 ºC, which is optimum for the polymerase that extends the primers[74].

Recent reports, however, indicate that some bacterial[77] and protozoal[7] species may present varying copy numbers of the DNA markers utilized. For example, the phyla *Firmicutes* and *Gammaproteobacteria* may contain 5-fold more copies of the 16S gene compared to the rest of bacterial phyla[77], which could introduce bias in the method if bacterial pellets used as reference are not representative of samples being analyzed. In addition, it has been suggested that the lower estimates of microbial protein[3] may be attributed to incomplete recovery of DNA copies from samples[8] or because the universal primers used do not bind to 100% of the microbial 16S and 18S genes, when quantifying bacterial or protozoal protein, respectively.

DNA sequencing improves knowledge on factors affecting measurement of microbial protein flow

The use of high-throughput DNA sequencing

Recent advances in molecular techniques applied to high-throughput DNA sequencing of microbial DNA in combination with bioinformatic analysis of the microbial population

inhabiting the rumen have made significant contributions to our knowledge on the ruminal microbiome[12]. This technique has been applied in a variety of research topics related to ruminant nutrition. For example, new insights have been achieved on shifts in the ruminal bacterial population due to change in diet composition[21], change in ruminal pH[37], biohydrogenating bacteria[14,78], the role of bacteria on milk fat composition[79], and archaea involved in methane formation[22,80]. In addition, DNA sequencing can improve our understanding on factors affecting the estimation of microbial protein and its flow to the small intestine. More specifically, detailed phylogenetic differences have been revealed between bacterial pellets used as reference and the bacterial population of the intestinal digesta.

Taxonomic differences of bacteria from ruminal fluid and whole intestinal digesta

One of the main challenges in the estimation of microbial protein synthesis and its flow to the small intestine is obtaining a reference bacterial pellet representative of microbial population of whole ruminal contents. Although differences between bacteria from ruminal fluid and bacteria from whole digesta flowing has been longtime assumed[10], it is not until recent years that the use of high-throughput DNA sequencing has enabled researchers to examined the complete taxonomic profile of the reference pellets and whole intestinal digesta[14]. Those findings have revealed that taxonomic profile of bacterial pellets isolated from ruminal fluid differs drastically compared to that of bacteria in intestinal digesta when analyzed at the taxonomic levels of phylum, order, family and genus (Tables 3,4). For example, greater proportions of the predominant bacterial phyla *Firmicutes*, *TM7* and *Tenericutes* have been found in the reference bacterial pellets compared to whole intestinal digesta. In addition, greater proportions of the bacterial orders *Bacteroidales* and *Clostridiales*, as well as higher levels of the bacterial family *Lachnospiraceae* were found in the reference pellets. On the other hand, the reference bacterial pellets contained lower proportions of the phyla *Fibrobacteres*, *Spyrochaetes*, *Proteobacteria* and *Lentisphare*, and contained lower proportion of the family *Ruminoccocaceae* and the genus *Butyrivibrio*. These findings support the notion that the use of solely fluid-associated bacteria as reference pellets would lead to bias in the estimation of microbial protein synthesis, and that there is a need to develop effective detachment procedures to obtain bacterial pellets that are more representative of whole ruminal contents[10] for accurate estimation of the contribution of microbial protein to total metabolizable protein. These findings show that detachment of the

fibrolytic bacteria from the solid fraction of ruminal contents is particularly important for obtaining representative reference microbial pellets.

Table 3: Predominant bacterial phyla and orders found in reference bacterial pellets isolated from ruminal fluid and in whole intestinal digesta of beef steers (%)

Bacterial taxa	Origin of bacteria		SEM[2]	*P*-value
	Reference pellets from ruminal fluid[1]	Whole intestinal digesta		
Phylum, % of total				
Firmicutes	45.95	31.32	2.675	< 0.001
Bacteroidetes	44.22	42.02	2.497	0.53
Fibrobacteres	0.1	10.1	0.06	< 0.01
Chloroflexi	1.69	2.00	0.303	0.41
TM7	1.60	0.29	0.227	< 0.001
Tenericutes	1.45	1.00	0.199	0.027
Spyrochaetes	0.92	1.78	0.138	< 0.001
Proteobacteria	0.63	3.81	0.241	< 0.001
SR1	0.33	0.22	0.150	0.58
Planctomyces	0.26	0.11	0.042	0.014
Lentisphaera	0.13	2.02	0.128	< 0.001
Synergistetes	0.11	0.14	0.031	0.552
Verrucomicrobia	0.12	0.52	0.056	< 0.001
WPS2	0.10	0.30	0.051	< 0.001
Other	2.49	14.46	0.122	0.001
Order, % of total				
Bacteroidales	44.22	41.90	2.498	0.51
Clostridiales	34.99	28.31	1.748	0.004
Coriobacteriales	5.32	1.41	0.881	< 0.001
Anaerolineales	1.69	2.00	0.303	0.41
TM7	1.60	0.30	0.227	< 0.001
Campylobacterales	0.32	0.42	0.039	0.067
Pirellulales	0.26	0.11	0.042	0.014
Erysipelotrichaeles	0.17	0.07	0.020	< 0.001
Victivallales	0.12	1.78	0.125	< 0.001
Sipochaetales	0.12	0.16	0.021	0.17
Sphaerochaetales	0.09	1.79	0.235	< 0.001
Rhizobiales	0.04	0.01	0.010	0.084
Desulfovibrionales	0.04	0.04	0.018	0.97
YS2	0.03	0.57	0.051	< 0.001
Rickettsiales	0.02	0.29	0.046	< 0.001
Other	10.97	20.84	---	---

[1] Bacteria isolated by differential centrifugation. Adapted from Castillo-Lopez *et al*.[14].

[2] The largest standard of the mean.

Table 4: Predominant bacterial families and genera found in reference bacterial pellets isolated from ruminal fluid and in whole intestinal digesta of beef steers (%)

Bacterial taxa	Origin of bacteria		SEM[2]	*P*-value
	Reference pellets from ruminal fluid[1]	Whole intestinal digesta		
Family, % of total				
Unclassified *bacteroidales*	27.93	17.80	2.442	0.006
Lachnospiraceae	17.38	9.27	0.979	< 0.001
Rominococcaceae	11.48	13.66	0.895	0.061
Prevotellaceae	10.99	12.12	1.225	0.52
Unclassified *clostridiales*	7.70	2.47	0.857	< 0.001
Paraprevotellaceae	3.78	4.27	0.857	0.671
F16	2.80	0.47	0.884	0.055
Clostridiaceae	2.11	1.39	0.202	< 0.001
Anaerolinaceae	1.68	2.00	0.303	0.418
Coriobacteriaceae	1.30	0.05	0.261	< 0.001
Anaeroplasmataceae	0.98	0.80	0.185	0.351
Veillonellaceae	0.88	1.44	0.191	0.047
Spirochaetaceae	0.78	1.48	0.130	< 0.01
Catabacteriaceae	0.72	1.24	0.147	0.011
BS11	0.68	0.18	0.293	0.01
Genus, % of total				
Unclassified *bacteroidales*	27.94	17.81	2.442	0.006
Unclassified *lachnospiraceae*	11.73	6.39	0.582	< 0.001
Prevotella	10.94	12.02	1.127	0.53
Unclassified *ruminococcaceae*	8.51	9.71	0.770	0.15
Unclassified *clostridia*	7.71	2.47	0.857	< 0.001
Unclassified *coriobacteriales*	4.02	1.36	0.659	< 0.001
Unclassified *paraprevotellaceae*	3.79	4.27	0.857	0.67
Ruminococcus	2.84	3.51	0.326	0.162
Butyrivibrio	2.59	0.88	0.203	< 0.001
SHD231	1.69	2.00	0.303	0.41
Clostridum	1.48	0.66	0.120	< 0.001
Unclassified *coriobacteriaceae*	1.10	0.05	0.213	< 0.001
Coprococcus	0.97	0.23	0.225	0.011
Succiniclasticum	0.81	1.41	0.189	0.032
Shuttleworthia	0.76	0.10	0.150	< 0.001
Unclassified *catabacteriaceae*	0.73	1.25	0.147	0.011
BS11	0.68	1.83	0.293	0.01
Pseudobutyrivibrio	0.64	0.73	0.103	0.553

[1]Bacteria isolated by differential centrifugation. Adapted from Castillo-Lopez *et al*.[14].

[2]The largest standard of the mean.

Implications of taxonomic differences between bacteria from ruminal fluid and whole intestinal digesta on microbial markers

Results obtained from the use of high-throughput DNA sequencing elucidate potential factors that may bias the estimation of microbial protein flow when isolating the representative pellet only from the liquid phase of ruminal contents. For example, studies have suggested that particle-associated bacteria contain lower proportions of purines than liquid-associated bacteria[81]. Data on purine content among different bacterial taxa are limited, and because a large proportion of particle associated bacteria are excluded from the reference pellet isolated during differential centrifugation, taxonomic profile divergence between the isolate and intestinal digesta likely represents a source of potential underestimation of intestinal supply of microbial protein. This may partially explain the lower estimates observed compared to predicted values[3].

In addition, peptidoglycan concentration varies among bacteria, thus they have different diaminopimelic acid concentrations. Gram-positive bacteria contain more peptidoglycan in the cell wall than gram-negative bacteria[68]. Given the greater proportion of *Firmicutes* (phylum), *Clostridiales* and *Coriobacteriales* (orders) and *Lachnospiraceae* (family), largely represented by gram-positive bacteria, in the reference pellets isolated from ruminal fluid compared to bacteria from whole intestinal digesta, when diaminopimelic acid is used as microbial marker, researchers should be aware of potential underestimation of intestinal microbial protein flow.

There is limited information on how the concentration of labeled nitrogen varies among ruminal bacteria. However, reports indicate that the marker/nitrogen ratio differs between fluid- and particle-associated bacteria and that ^{15}N enrichment is higher in liquid-associated bacteria compared to particle-associated bacteria[82]. Therefore, results on taxonomic profile between bacteria from ruminal fluid and bacteria from whole intestinal digesta suggest that when the reference microbial pellets are obtained only from ruminal fluid, potential underestimation of intestinal microbial protein flow may occur.

Lastly, given the variations in copy numbers of the 16S gene across ruminal bacteria, particularly greater copy numbers in *Firmicutes*, and because of the greater proportions of *Firmicutes* in bacteria isolated form ruminal fluid, when DNA is used as microbial marker, underestimated values for bacterial protein would likely be obtained if reference bacterial pellets are not representative of whole digesta flowing to the small intestine.

Conclusions

The ruminal microbiome plays an essential role in ruminant nutrition; major contributors to microbial protein are bacteria and protozoa. Main dietary and animal related factors that influence microbial protein synthesis in the rumen are the availability of carbohydrates, ruminally degradable protein, dietary fat, feed intake and ruminal pH. All microbial markers have advantages and disadvantages; conventional microbial markers that are commonly utilized to estimate microbial protein synthesis include total purines, diaminopimelic acid and labeled nitrogen. Recently, the use of DNA as a microbial marker through real-time PCR allows detection of bacterial, protozoal and yeast protein separately. Therefore, when researchers are interested in evaluating the contribution of protozoa to the metabolizable protein pool or when evaluating the contribution of dietary yeast, the use of DNA marker with real-time PCR represents an alternative method. However, investigators should be aware of potential biase when using any of these methods.

One of the major difficulties in the estimation of microbial protein flow is obtaining a representative microbial pellet from the rumen to establish the ratio of microbial marker/nitrogen. Differences between particulate-associated bacteria and fluid-associated bacteria have been recognized. However, to the authors' knowledge, no studies have compared the taxonomic community composition between fluid associated bacteria and bacteria found in whole intestinal contents and discuss implications on microbial markers as well as potential effects on the estimation of microbial protein flow to the small intestine. Recent advances in high-throughput DNA sequencing in combination with bioinformatics have revealed significant phylogenetic divergence from many bacterial taxa at the phylogenetic levels of phylum, family and genus between the reference microbial pellets isolated solely from ruminal fluid and bacteria found in whole intestinal contents. These findings indicate that further research to develop effective methods for detaching bacteria from feed particles to obtain reference microbial pellets representative of whole ruminal contents is warranted in order to prevent bias when quantifying the microbial protein contribution to the metabolizable protein supply in ruminants.

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