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Short communication

Ruminal bacterial community changes during adaptation of goats to fresh alfalfa forage

Diego Javier Grilli [a,e,*], Jakub Mrázek [b], Kateřina Fliegerová [b], Jan Kopečný [b], Sebastian Paez Lama [c], María Esperanza Cerón Cucchi [d], Miguel Angel Sosa [e], Graciela Nora Arenas [f]

[a] Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan Agustín Maza, Av. Acceso Este Lateral Sur 2245, CP 5519 Mendoza, Argentina
[b] Institute of Animal Physiology and Genetics, Czech Academy of Sciences, v.v.i., Videnska 1083, CP 142 20 Prague, Czech Republic
[c] Instituto Argentino de Investigaciones de las Zonas Áridas, Centro Científico y Tecnológico, M5502BPB Mendoza, Argentina
[d] Instituto de Patobiología. Centro de Investigación en Ciencias Veterinarias y Agronómicas. Instituto Nacional de Tecnología Agropecuaria. De Los Reseros y Dr. Nicolás Repetto S/N, Hurlingham, CP 1686 Buenos Aires, Argentina
[e] Instituto de Histología y Embriología de Mendoza, Universidad Nacional de Cuyo, Casilla de Correo 56, CP 5500 Mendoza, Argentina
[f] Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Casilla de Correo 56, CP 5500 Mendoza, Argentina

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ABSTRACT

Using culture-independent molecular approaches, we studied the bacterial population changes in the rumen of goats abruptly converted from alfalfa hay to fresh alfalfa diet. Administration of fresh forage with significantly increased soluble nitrogen and soluble protein nitrogen resulted in frothy bloat. Changes of the bacterial composition of rumen were monitored using DGGE analysis of 16S rDNA gene amplicons and quantitative PCR method. As the diet changed, the bacterial population of Bacteroidetes and γ-Proteobacteria decreased, even if animals have not shown signs of frothy bloat. The most severely bloated animals showed an increase of Bacteroidetes phylum. *Lactobacillus/Streptococcus* group belonging to Firmicutes phylum decreased in response to transferring the animals from hay to a fresh forage-based diet, and did not achieve the values observed at the beginning of the experiment. In summary, changes in the diet and subsequent frothy bloat occurrence produce long-lasting changes in the structure of the microbial community and may be associated with a specific bacterial population belonging to the Bacteroidetes phylum.

1. Introduction

The alfalfa (*Medicago sativa* L.) is widespread in large areas of goat production system in Argentina. However, this forage is associated with the problem of frothy bloat in ruminants, when the mechanism for the eructation of rumen gas is inhibited or impaired and gas production exceeds the animal's ability to expel it (Majak et al., 2003). Saponins, soluble protein nitrogen, and hemicelluloses of alfalfa are supposed to be the primary foaming agents (Moeller et al., 2012). However, the bloat potential of legumes also depends on their digestibility by rumen bacteria. The changes in bacterial population associated with bloat have been described by the culture independent methods in cattle grazing wheat forage (Min et al., 2013, 2006; Pitta et al., 2014). To our knowledge, no study so far has monitored the influence of bloat-causing legumes on the digestive microbiome of goats. This is the first study dealing with in vivo effect of fresh alfalfa forage on ruminal bacterial population of cannulated Creole goats. We have monitored the changes of the overall bacterial composition of rumen samples using DGGE analysis of 16S rDNA gene amplicons and quantitative PCR method to assess the role of important groups of bacteria during the adaptation to a fresh forage diet.

2. Material and methods

2.1. Forage and feeding experiment

Alfalfa was cultivated under the climatic conditions of north-east Mendoza, Argentina. The first growth in the pre-bloom stage was collected in November 20th, 2013. The re-growths in the

* Corresponding author at: Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan Agustín Maza, Av. Acceso Este Lateral Sur 2245, CP 5519 Mendoza, Argentina.
E-mail address: diegogrilli@yahoo.com.ar (D.J. Grilli).

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budding stage of maturity were collected 14 days after the previous cut. The fresh-cut material was directly offered to animals as fresh alfalfa forage (FAF) from December 4th to 29th. Samples of FAF collected on December 4th, 20th, and 29th and of hay (AH) were dried at 60 °C for 72 h and used for chemical analysis: dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) (Association of Official Analytical Chemists, 2006); hemicellulose (H) (van Soest et al., 1991); total nitrogen (TN), insoluble nitrogen (IN), soluble nitrogen (SN), soluble protein nitrogen (SPN) and soluble non-protein nitrogen (SNPN) (Min et al., 2005). The saponins content was calculated from the foaming index (FI) according to WHO/PHARM/92559 (1998). Samples were analyzed in duplicate. The nutritional components of the diets were compared by ANOVA followed by Tukey's HSD procedure ($P < 0.05$), using *Infostat* statistical program (Di Rienzo et al., 2011). Four goats fitted with a rumen fistula were used in this study. Experiment was permitted by The Committee for the Care and Use of Animals in Research (Approval no. 102/2013) and was in agreement with the Guide of the Federal Animal Science Society (2010). The animals were housed and fed in pens with defined amounts of forages averaging 0.76 kg of DM per day to meet the animals' nutrient requirements (National Research Council, 2007). The goats were fed on AH diet for a period of 30 days (from November 4th to December 3rd). Then, the goats were abruptly shifted to FAF diet for 25 days (from December 4th to December 29th). Rumen samples were collected from each goat on 4 days previous to FAF experimental period (day 1, December 1st) and then on days 4, 17, 20, 21, 22, and 29 of the experimental period according to the incidence and severity of bloat of four goats fed on FAF diet. The scoring system of Paisley and Horn (1998) was employed to characterize the incidence and severity of bloat. Bloat scores were described as follows: BS-0=normal, BS-1=slight distention of left side of animal, BS-2= marked distention of left side of animal, and BS-3=severe distention. Samples of the whole-rumen contents with similar solid/liquid proportions were collected in a sterile container, freeze-dried and transferred to the laboratory.

2.2. DNA isolation and PCR-DGGE analysis

The genomic DNA was isolated using method of Yu and Morrison (2004) combining bead-beating cell disruption with the column filtration steps of the QIAamp DNA Stool Mini Kit (Qiagen, Germany). The PCR reaction with primers 338GC and 534 (Table S1) was performed using PPP Master Mix kit (Top-Bio, Czech Republic) according to Muyzer et al. (1993). DGGE analysis was performed on DCode Mutation Detection System (BioRad Laboratories Ltd, Germany) on a polyacrylamide gel with 35–60% denaturing chemical concentration. The gel was stained with Gel Green Dye and digitized using the BioRad system (BioRad Laboratories Ltd, Germany). Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 6, Applied Maths, Inc., Austin, TX, USA) to create similarity matrices in order to compare 16S rDNA amplicon patterns of four nonbloated and bloated goats grazing alfalfa hay (AH diet, d 1) and fresh alfalfa forage (FAF diet, d 20). Using average Pearson's similarity coefficient index, with an optimization of 10.0%, clustering was carried out using UPGMA. Bands of interest were cut from the gel, the DNA was sequenced and identified by using BLASTn application.

Real-time PCR: The quantification of Firmicutes and *Clostridium leptum* group, Bacteroidetes, Actinobacteria, γ-Proteobacteria, *Bacteroides*/*Prevotella* group, *Lactobacillus*/*Streptococcus* group, and *Butyrivibrio* group were performed on MX3005P QPCR System (Stratagene, U.S.A) according to Table S1. To avoid distorting effect of absolute quantification, the relative quantification approach was used for comparison of all studied samples. ANOVA followed by Tukey's HSD procedure ($P < 0.05$) using *Infostat* statistical program (Di Rienzo et al., 2011) has been applied to determine significant differences among DNA based quantity of bacteria in samples retrieved from bloated and non-bloated animals.

3. Results and discussion

3.1. Nutritive value of forage and bloating

AH was characterized by significantly ($P < 0.05$) lower CP ($15 \pm 0.1\%$ DM), TN (25 ± 0.1 mg/g dry weight, DW), SN (11 ± 0.1 mg/g DW), and SPN (7 ± 0.4 mg/g DW). Compared to AH, FAF at the beginning of the feeding period, contained significantly ($P < 0.05$) higher CP ($21 \pm 1\%$ DM) and lower NDF ($35 \pm 0.7\%$ DM), ADF ($33 \pm 0.3\%$ DM) and H ($2 \pm 0.3\%$ DM). After 17 days of FAF feeding, the goats started to show obvious clinical signs of frothy bloat (BS-2), which were even more severe after 3 days (BS-3) (Fig. 1). The FAF consumed in this period (day 20) contained significantly ($P < 0.001$) higher values of SN (26 ± 1 mg/g DW) and SPN (17 ± 0.1 mg/g DW) regarding to the values obtained in FAF at 4 (18 ± 0.8 and 14 ± 0.4 mg/g DW, respectively) and 29 days (19 ± 1 and $13 \pm$ mg/g DW, respectively). This coincides with the results of Howarth et al. (1977) who reported that SN and SP are the most reliable and practical chemical parameters for predicting the bloat potential of alfalfa forage. In the following days the signs of bloat decreased progressively until day 29 when none of the animals suffered from bloating. In the end of the experiment, NDF ($46 \pm 0.2\%$ DM), ADF ($39 \pm 0.4\%$ DM), IN (15 ± 3 mg/g DW) and SNPN (6 ± 0.3 mg/g DW) of FAF was comparable with values of AH. Majak et al. (2003) reported that fragile plants with thin cell walls have a higher probability to cause pasture bloat than plants with thicker cell walls. However, in this study, the observed clinical signs of bloat in goats were not possible to relate either with the concentration of NDF, ADF, H, TN, IN, SNPN or with the saponins value (FI) of FAF diet. In comparison to the results of Min et al. (2006) describing clinical signs (BS-1) of frothy bloat in steers grazing wheat forage with 28% of CP, 44% of NDF and 29% of ADF after 40 days, the goats used in this study showed greater severity of associated signs to frothy bloat (BS-3) with lower values of CP (24%) and NDF (43%), and higher values of ADF (37%) after 20 days. However, Pitta et al. (2014) demonstrated that cattle requires at least 14 days to adapt to vegetative wheat pasture, similarly to FAF-diet fed goats used in this study.

3.2. DGGE profile of bacterial community

The comparison of 16S rDNA amplicon patterns of four nonbloated and bloated goats grazing AH (d 1) and FAF (d 20) shows the individual responses of animals to dietary change (Fig. 2). However the different intensity of dominant bands can indicate the quantitative changes in the bacterial community composition. Based on the positions of each band from the PCR-DGGE band patterns, 4 dominant bands were excised and sequenced (Fig. 2). Sequences of the bands 1, 2, 3 and 4 have high similarity with Bacteroidales, *Lachnospira multipara*, Clostridiaceae and Clostridiales, respectively. Min et al. (2006), using DGGE analysis, reported two different bacterial populations between bloated and nonbloated steers grazing wheat forage with greater proportions of high-G+C-containing bacterial strains and a few low-G+C-containing strains in nonbloated animals. Such differences have not been observed in this study, even if considerable shift in DGGE profiles of two goats (1 and 2) was apparent (Fig. 2). DGGE analysis comparing bacterial profile of the nonbloated and bloated goats was unable to elucidate the effect of diet on rumen bacterial composition.

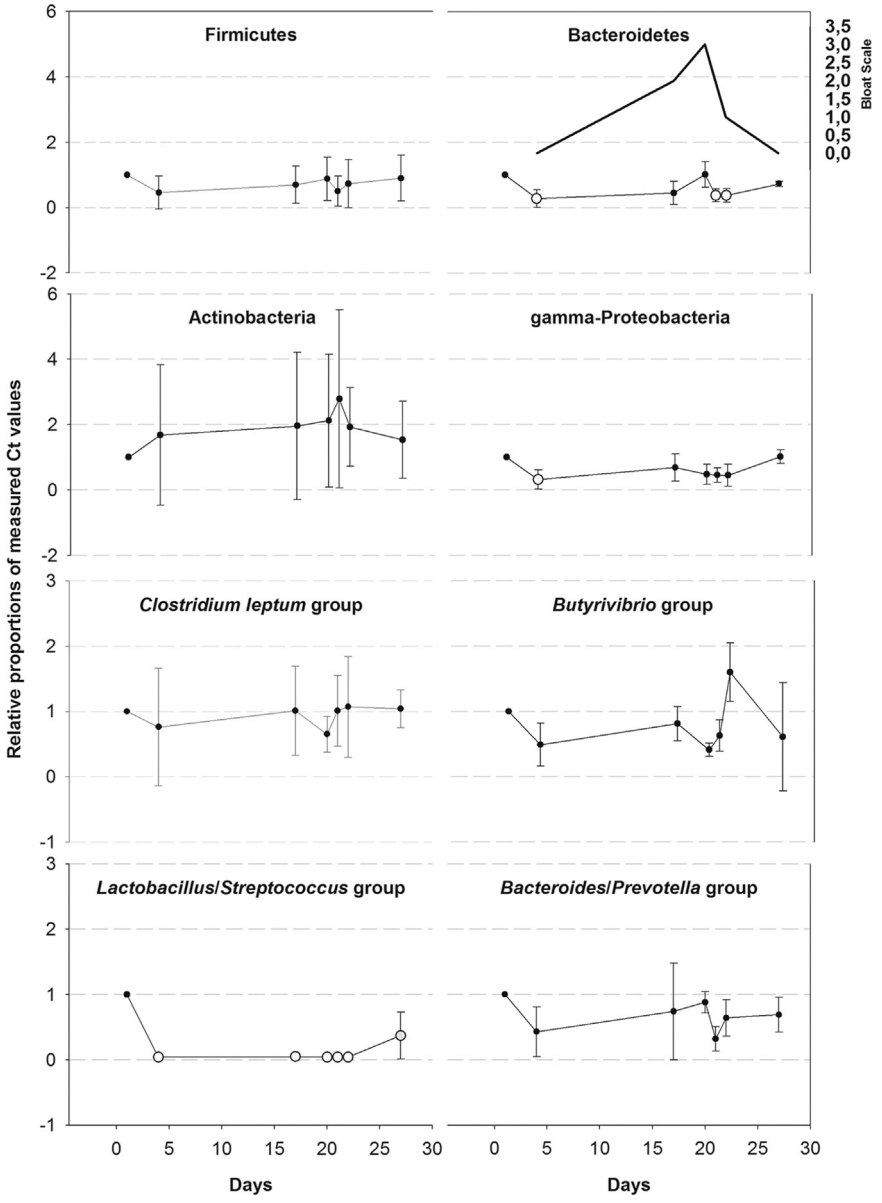

Fig. 1. Bloat scale and results of relative qPCR quantification of Firmicutes, Bacteroidetes, γ-Proteobacteria, Actinobacteria, *Clostridium leptum* group, *Butyrivibrio* group, *Lactobacillus*/*Streptococcus* group, and *Bacteroides*/*Prevotella* group in the rumen samples of AH- and FAF-diet fed goats. Data are expressed as relative proportions of measured Ct values ± SE (n=4). Sample of day 1 (AH diet) was used as the calibrator. Open symbols indicate significant differences ($P < 0.01$) compared to the day 1.

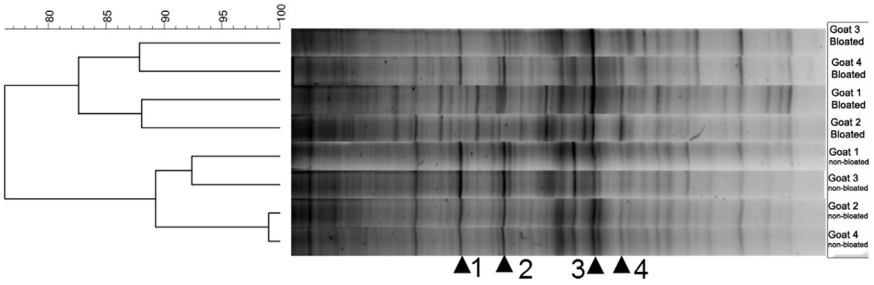

Fig. 2. PCR-DGGE profiles of the rumen bacterial 16S rDNA gene (V3 region). Cluster analysis using average Pearson's similarity coefficient index (Optimization: 10%) and unweighted pair group method with arithmetic means (UPGMA) was performed from four nonbloated and bloated goats grazing alfalfa hay and fresh alfalfa forage, respectively. The comparison of the PCR-DGGE profiles was generated with the BioNumerics software package. Band 1 Bacteroidales, band 2 *Lachnospira multipara*, band 3 Clostridiaceae and band 4 Clostridiales.

3.3. Quantitative bacterial analysis

Real-time qPCR analysis was carried out in each sampling time from rumen of FAF- and AH-diet fed goats. The four most important bacterial phyla (Kim et al., 2011) exhibited different sensitivity to dietary changes (Fig. 1). The Firmicutes and Actinobacteria levels did not change significantly during the feeding of the animals with the FAF diet. However, Actinobacteria showed

great variation in the relative proportions evidenced for each individual animal. The significantly decreased levels ($P < 0.01$) of Bacteroidetes and γ-Proteobacteria were detected in response to the change from AH to FAF-based diet. The raised levels of γ-Proteobacteria occurred on day 17 (BS-2) and remained similar to day 1 until the end of the experiment. This is the first report about the suppressive effect of fresh forage on γ-Proteobacteria and evaluation of this result is difficult due to the lack of this type of data. However, Petri et al. (2013) did not detect this proteobacterial class in forage fed heifers. Kocherginskaya et al. (2001) detected much lower percentage of Proteobacteria phylum in the rumen of steers on the hay diet compared to corn diet. On the day 20 when the most severe signs of bloat (BS-3) were observed, numbers of Bacteroidetes was increased again, achieving the levels of day 1 (Fig. 1). The following days (day 21 and 22), the animals decreased the intensity of the signs of bloat (BS-2 and BS-1, respectively) and showed significant decrease ($P < 0.01$) in Bacteroidetes, similar to observed values after the change of diet. The number of Bacteroidetes was recovered in the end of experiment. Therefore, compared to AH diet, the bloat of goat induced by FAF diet can be statistically correlated only with decreased levels of Bacteroidetes and γ-Proteobacteria, but no correlation with intensity of bloating was observed. Group specific qPCR was further performed to elucidate the influence of bloating on rumen bacterial composition. The graph of quantification of the *Bacteroides/Prevotella* group exhibited the same development in time as the Bacteroidetes phylum (Fig. 1). The decrease after dietary shift and the changes during the FAF diet experimental period have not been significantly probative and therefore it was not possible to relate the decrease in the concentration of bacteria belong *Bacteroides/Prevotella* group with the intensity of changes of Bacteroidetes phylum. These findings indicate that frothy bloat in goats is associated with a specific bacterial population belonging to the Bacteroidetes phylum and bloat generated changes in the microbial community structure persist after cessation of clinical manifestations. Pitta et al. (2014) observed a large percentage of unclassified genus of the *Bacteroidaceae* family in cattle fed vegetative wheat pasture with high crude protein content. The same study also reported the significantly increased number of *Prevotella* and significantly lower number of *Bacteroides* in liquid rumen fluid fraction associated with mild frothy bloat of cattle. This indicates the different sensitivity of these two genera to the chemical composition of rumen fluid during the bloating. The specific primers of Bartosch et al. (2004) used in this study however amplify the 16S rDNA gene of both *Bacteroides* and *Prevotella* sp., and thus cannot disclose the possible different response to bloat conditions. Moreover, the different phylotypes of *Prevotella* known as the predominant genus in goats (Sun et al., 2010) and cows (Min et al., 2013) on a hay based diet and abundant genus in the rumen of goats during transition from forage to concentrate as well as during ruminal acidosis (Sun et al., 2010), probably can have diverse functions, even if their role in the rumen has been widely described (Stevenson and Weimer, 2007). The quantification of two groups of Firmicutes phylum including *Butyrivibrio* group and *C. leptum* group (Fig. 1) indicated their suppression after dietary change and at day 20, but bacterial levels did not differ statistically at sampling times. Pitta et al. (2014) described in the rumen of steers grazing vegetative wheat forage that greater accumulation of mucopolysaccharide biofilm during bloating was associated with the abundance of Firmicutes lineage such as *Clostridium*, *Ruminococcus*, *Oscillospira* and *Moryella*, however only increased *Ruminococcus* level significantly correlated with bloating. Higher DNA density signal for *Ruminococcus flavefaciens* in bloated steers was described by Min et al. (2013). Despite these results, in our study the *C. leptum* group (covering also the *Ruminococcus* sp.) and *Butyrivibrio* group were non-significantly reduced at day of bloat (d 20). The significantly decreased level ($P < 0.001$) of *Lactobacillus/Streptococcus* group was detected as response to transfer form hay to fresh forage (d 4, BS-0). The levels of this group remained diminished until day 22. On the day 29, numbers of *Lactobacillus* and *Streptococcus* sp. were significantly increased ($P < 0.001$) again, but not achieving the levels of day 1. The rapid growth of *Streptococcus bovis* had not been however reported in animals on a forage diet (Petri et al., 2013; Sun et al., 2010).

4. Conclusion

This study is the first report on the in vivo effect of fresh alfalfa forage on the ruminal bacterial population of goats. Increased content of soluble nitrogen and soluble protein nitrogen of fresh forage was determined as the inducing factor of bloating. In the most seriously bloated animals the culture-independent molecular methods revealed a significant increase of Bacteroidetes not supported by increased levels of the *Bacteroides/Prevotella* group. The severe form of frothy bloat thus may be associated with a specific bacterial population belonging to the Bacteroidetes phylum, which is still uncultured and therefore unknown. Our results also indicated that the frothy bloat in goats produce long-lasting changes in the structure of the rumen microbial community, which persist even after the cessation of clinical manifestations of animal bloat.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.livsci.2016.08.001.

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