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COMMUNICATION

# Aerobe and anaerobe facultative Gram-negative bacteria rod-shaped in the ruminal fluid of dairy cattle fed with different diets containing tropical forages

## Bastoncillos Gram-negativos aerobios o anaerobios facultativos en el líquido ruminal de ganado lechero alimentado con diferentes dietas de forrajes tropicales

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**RESUMEN**

Las bacterias Enterobacteriaceae se encuentran naturalmente en los intestinos y ecosistema ruminal y están ampliamente distribuidas. El objetivo de este estudio fue cuantificar Enterobacteriaceae en el líquido ruminal de vacas lecheras y terneras alimentadas con diferentes dietas de forrajes tropicales. Se recolectaron muestras de fluido ruminal de 30 vacas alimentadas con ensilaje de sorgo y concentrado, 32 vacas alimentadas con *Brachiaria brizantha*, 12 terneras alimentadas con ensilaje de sorgo con concentrado y 11 terneras alimentadas con caña de azúcar. Se obtuvieron 15 ml de fluido ruminal de cada animal con la punción del rumen con catéteres y jeringas estériles, que después de diluciones decimales fueron inoculados en placas con agar MacConkey a 37 °C durante 72 h. Las terneras que recibían el sorgo ensilado tenía un mayor índice de detección y una mayor concentración en el rumen (8,4 x $10^6$ unidades formadoras de colonias UFC / ml) en comparación con las vacas alimentadas con la misma dieta (1,4 x $10^5$ UFC/ml). En las vacas evaluadas, *Enterobacter* spp. fue el género más frecuente para los animales criados en pastos, mientras *Klebsiella* spp. era más común en vacas alimentadas con sorgo ensilado. *Enterobacter* spp. y *Proteus* spp. fueron observados en la mayoría de los aislados de terneras ($P < 0,01$). Estudios a futuro deben dilucidar las diferencias en la población de estos microorganismos para maximizar la productividad y la salud de ganado alimentado con estas dietas.

*Palabras clave*: Enterobacteriaceae, ganado lechero, microbiota ruminal, forrajes tropicales.

**SUMMARY**

The aim of this work was to analyse the population of aerobe and anaerobe facultative Gram-negative rod-shaped in the ruminal fluid of dairy cattle and calves fed with different sources of tropical forage. Samples of ruminal fluid were collected from 30 cows fed with sorghum silage, 32 cows fed with *Brachiaria brizantha* pasture, 12 calves fed with sorghum silage, and 11 calves fed with sugarcane. Fifteen ml of ruminal fluid were collected by sterile catheter and syringe puncture to the rumen. After serial decimal dilutions, samples were inoculated in plates containing MacConkey agar and incubated at 37 °C for 72 h. Calves fed with sorghum silage showed higher detection rate and larger population of these bacteria ($8.4 \times 10^6$ colony forming units CFU/ml) when compared with adult cows fed with the same forage ($1.4 \times 10^5$ CFU/ml). The most frequent genera identified in all groups were *Enterobacter*, *Klebsiella*, and *Proteus*. The most frequently identified bacteria in pasture-fed cows was *Enterobacter* spp., while *Klebsiella* spp. was the most frequently identified bacteria in cows fed with sorghum silage. *Enterobacter* spp. and *Proteus* spp. were more frequently observed in isolates from calves ($P < 0.01$). Future studies should clarify the differences between these populations.

*Key words*: Enterobacteriaceae, dairy cattle, rumen microbiota, tropical forages.

## INTRODUCTION

Facultative anaerobe Gram-negative rod bacteria belonging to the Enterobacteriaceae family are naturally found in the intestines of humans and animals, widely distributed in the environment and frequently found in the ruminal ecosystem. *Escherichia coli* species and the *Salmonella* genus are important groups of enteric and zoonotic pathogens. Ruminants are reservoirs for pathogenic strains, and infections in humans have been related to direct or indirect contact with faeces, ruminal fluid, or animal carcasses (Stevens *et al* 2002, McEvoy *et al* 2003).

*Escherichia coli* and *Salmonella* spp. are important agents in public health and can cause bovine diarrhea and mastitis, leading to major economic losses in dairy farming (Anderson *et al* 2005). Shedding of these pathogens during milking and slaughter can increase the risk of food contamination for human consumption. Strategies to prevent or to reduce the carriage of these pathogens are being sought in order to promote higher food safety and efficiency of livestock (Anderson *et al* 2005, Callaway *et al* 2002).

In diet-induced inflammation in dairy cattle, high concentrate in diets can favour disturbances in the rumen metabolism, which culminate with development of sub-acute rumen acidosis (SARA). Rumen digestive disorder is

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often linked to greater metabolic stress of gastrointestinal (GI) microbiota and lowered fiber digestion, with negative consequences for the host (Zebeli and Metzler-Zebeli 2012). One of the most important factors for the growth of *E. coli* is adequate feed management, since well-fed animals appear less likely to become reservoirs for pathogenic strains (Rasmussen *et al* 1993).

The ruminal lipopolysaccharide (LPS) can be derived from Gram negative bacteria such as Enterobacteriaceae. The gradual increase in dietary concentrate, with low forage-to-concentrate (F:C) ratio, can increase free ruminal LPS concentration and to activate inflammatory response (Gozho *et al* 2006). Permeability of rumen and colon mucosa can increase in the presence high concentration of LPS of *Escherichia coli* at acidic pH values, common in SARA to cattle feed with low F:C ratio (Emmanuel *et al* 2007). It was suggested that under low rumen pH conditions induced by a grain diet, there is a burst in the number of *E. coli* with virulence genes that can take advantage of these rumen conditions to trigger an inflammatory response (Khafipour *et al* 2011).

Little is known about the colonization of the rumen environment by these microorganisms, the role played in the rumen, or their impact on the health of farm animals. Few studies report the influence of diet on the prevalence of these bacteria in the ruminal environment of animals fed with tropical forages and of different ages (Callaway *et al* 2009). Thus, the objective of this study was to characterise the population of aerobes and anaerobe facultative Gram-negative rod-shaped in the ruminal environment of dairy cows and calves fed with diets containing different sources of tropical forages.

## MATERIAL AND METHODS

### LOCALIZATION

Collection of ruminal fluid was performed at the Experimental Farm of the Instituto de Ciências Agrárias (ICA), Universidade Federal de Minas Gerais (UFMG), and in other farms in the municipality of Montes Claros, located in the northern part of the state of Minas Gerais, Brazil. The geographical coordinates of that region correspond to 16°50′52″ latitude south, 43°55′29″ longitude west, and 646 m altitude. The climate, according to the Köppen-Geiger classification, is of an Aw type, considered tropical savanna with a long dry period (October to April) and a short rainy season (November to March). The average temperature during the experimental period of November 2008 to August 2009 was 22.9 °C, and rainfall was 960.0 mm, according to the records of the fifth district of the National Institute of Meteorology, Montes Claros.

### ANIMALS

In a completely randomized design, four different forages in different groups of animals were evaluated. The ruminal fluid of Holstein cows and calves were collected. The mean age of the cows was 5.91 years old, and the mean weight was 546 kg. Calves were on average 12.9 months old and weighed 196.8 kg; all were semi-confined and received forage supplements in stone troughs.

### TREATMENTS

For all treatments, except for grazing animals, feed was supplied at 8 and 16 h. Treatments were compared two by two, with the goal of evaluating the effects of feed and age of the animal on the Enterobacteriaceae population in the rumen.

*Cows fed with sorghum silage (T1)*. Thirty samples were collected from cows fed with sorghum silage. On average, these animals received 35 kg of sorghum silage per animal per day as the only source of forage and 5 kg per animal per day of concentrate feed. This concentrate contained 70% ground corn grains, 25% ground soya grains, 3% mineral premix, and 2% urea. The bromatological composition of sorghum silage and of concentrate feed is described in table 1. All animals had access to a mineral premix and common salt at a proportion of 1:1 in stone troughs, for their consumption *ad libitum*.

*Cows fed on Brachiaria brizantha pasture (T2)*. The second group of 30 cows was fed exclusively on *B. brizantha* pastures in the summer period, from January to April 2009. Results of the bromatological composition of grass samples are found in table 1. The animals also had at their disposal the same mixture of mineral premix and salt supplied to the former group.

*Calves fed with sorghum silage (Sorghum bicolor, L.; T3)*. Following a period of 60 days feeding on sorghum silage, 12 female calves were submitted to collection of ruminal fluid. These animals consumed on average 15 kg per animal per day of the same silage supplied to the cows and 2 kg per animal per day of concentrate feed (table 1). The ingredients used in the preparation of this concentrate were crushed sorghum grain (70%), soya bran (25%), urea (2%), and mineral premix (3%). The calves had access *ad libitum* to a mix of minerals with common salt in proportions of 1.5:1.

*Calves fed with sugarcane fodder (Saccharum officinarum, L.; T4)*. The female calves used in this treatment were adapted to the diet of chopped sugarcane fodder (table 1) for a period of 43 days, reaching an average consumption of 9.2 kg per animal per day. Before feeding, urea and ammonium sulphate were added at a proportion of 9:1, both diluted in water. Forty grams of the urea and ammonium sulphate mixture was supplied for each 100 kg animal body weight. The mineral premix was also supplied as described.

**Table 1**. Composition of sorghum silage, *Brachiaria brizantha* grass, chopped cane, and concentrate feed supplied to the cows and calves.

Composición del ensilaje de sorgo, pasto de *Brachiaria brizantha*, caña de azúcar picada, y concentrado administrado para las vacas y terneras.

| Parameters | Sorghum silage | *B. brizantha* | Chopped sugarcane* | Concentrate cows | Concentrate calves |
|---|---|---|---|---|---|
| DM | 31.04% | 32.76% | 24.84% | 85.36% | 86.50% |
| FND | 46.28% | 37.49% | 36.68% | 10.56% | 18.12% |
| FAD | 38.21% | 31.18% | 30.55% | 8.02% | 13.86% |
| TP | 5.45% | 6.02% | 5.16% | 18.65% | 11.61% |
| EE | 2.93% | 2.02% | 0.90% | 5.15% | 4.71% |
| MC | 7.44% | 6.61% | 7.37% | 8.27% | 4.39% |
| TC | 84.18% | 85.35% | 86.57% | 67.58% | 79.29% |
| NFC | 37.90% | 47.86% | 49.89% | 57.02% | 61.17% |

Notes: *, chopped sugarcane without added urea or ammonium sulfate.
DM: dried matter; FND: fiber in neutral detergent; FAD: fiber in acid detergent; TP: total protein; EE: ether extract; MC: mineral content; TC: total carbohydrate; NFC: non-fibrous carbohydrate.
According to Van Soest (24), TC = 100 – (TP + EE + MC) and NFC = TC – FND.

### SAMPLE COLLECTION

Animals were immobilized in a containment chute in the morning before their first meal. To collect ruminal fluid, aseptic trichotomy was performed with a 1% povidone-iodine (PVP-I) solution on an area of approximately 5 $cm^2$ on the ventral part of the left abdomen, below the paralumbar fossae and cranial to the knee joint (Dirksen 1993).

### CULTIVATION, QUANTIFICATION, ISOLATION, AND IDENTIFICATION

Decimal dilutions of the ruminal fluid were prepared in tubes containing 9 ml of sterile saline solution. Afterwards, each dilution solution was homogenised with a vortex for 1 min. Aliquots of 100 µL were inoculated onto 90 × 90 mm sterile plates containing Mac Conkey agar (Acumedia® Manufacturers, Lansing, MI, USA). Inoculations were spread with sterile Drigalski spatulas, incubated in a BOD incubator at 39 °C, and monitored for bacterial growth for up to 7 days.

To identify the most prevalent genera, bacteria were re-isolated, grown in plates with Mac Conkey agar, and incubated at 37 °C for 24 h. After exponential growth, each isolate was inoculated in tube containing Rugai and Araújo medium, modified by Pessoa and Silva (MBiolog Diagnósticos, Brazil). The tubes were incubated in a BOD incubator at 39°C, results were evaluated after 24 h, and presumptive identification of the genera was conducted, according to methods described by Pessoa and Silva (1972). This genus classification takes also into account the production of indoles, sulfides, and gasses; the use of tryptophan, lactose, citrate, lysine, glucose, saccharose, and urea; and motility (Mc Faddin 2000, Murray *et al* 2007).

### STATISTICAL ANALYSIS

After an exploratory analysis of the quantification data using the Lilliefors and Bartlett's assays, $\log_{10}(x + 10)$ transformation was performed. Average values were compared using the Student's t-test, with the following comparisons (T1 with T2; T1 with T3; and T3 with T4. Positive rates and distributions of bacterial genera for the different animal groups were evaluated with the chi-squared test (Sampaio 1998). All analyses were performed with the statistical software package Sistema para Análises Estatísticas e Genéticas (SAEG) with significance set at $P < 0.05$.

## RESULTS AND DISCUSSION

After cultivation, all evaluated groups showed growth of aerobes and anaerobe facultative Gram-negative rod-shaped in percentages indicated in table 2. Considering the two treatments involving calves, the percentage of cultures positive for non-lactose-fermenting (Lac-) was lower for samples from calves fed sugarcane ($P < 0.05$). Although there have been no reports of this in the literature, this result may suggest the existence of inhibitory factors on the population of non-fermenting lactose bacteria present in sugar cane or produced by the population of microorganisms using sucrose.

The diet influenced significantly the population of these bacteria. When comparing the groups of female calves evaluated, the large population in samples from animals fed with sugarcane and urea could have been related to the type and concentration of carbohydrates available in this diet (table 2, $P < 0.05$). These bacteria easily degrade simple carbohydrates (Ostling and Lindgren 2006). Therefore, diets rich in soluble carbohydrates, such as the saccharose present in cane fodder, could have favoured the growth of Enterobacteriaceae. Theses bacteria could be favouring to the digestion of nutrients of this diet, contributing to the formation of microbial protein in the rumen.

Cows (T1) and calves (T3) feed with sorghum silage and showed lest development of these microorganisms in the rumen (table 2). This reduction could be justified by the fermentation that occurs in forages containing high

**Table 2**. Comparison of populations of aerobes and anaerobe facultative Gram-negative rod-shaped in samples of ruminal fluid from cows fed with sorghum silage (n = 32), cows fed with *Brachiaria brizantha* pasture (n = 30), calves fed with sorghum silage (n = 12), and calves fed with sugarcane (n = 11).

Comparación de las poblaciones de los bastoncillos Gram negativos aeróbicos y anaeróbicos en muestras de fluido ruminal de vacas alimentadas con ensilaje de sorgo (n = 32), las vacas alimentadas con pastos de *Brachiarai brizantha* (n = 30), las terneras alimentadas con ensilaje de sorgo (n = 12), y las terneras alimentadas con caña de azúcar (n = 11).

| Group (n) | Quantification | Positive cultures | | |
|---|---|---|---|---|
| | CFU/ml | Total % | Lac – % | Lac + % |
| Cows silage (T1) | $1.4 \times 10^5$ A | 83.3% | 50.0% B | 76.7% |
| Cows grass (T2) | $6.0 \times 10^5$ A | 96.7% | 93.3% A | 96.7% |
| Calves silage (T3) | $8.4 \times 10^6$ B | 100% | 100.0% B | 100% |
| Calves sugarcane (T4) | $2.1 \times 10^8$ A | 100% | 36.7% A | 100% |
| Cows silage (T1) | $1.4 \times 10^5$B | 83.3% | 50.0% A | 76.7% |
| Calves silage (T3) | $8.4 \times 10^6$ A | 100% | 100.0% B | 100% |

Notes: Lac-, lactose non-fermentative; Lac+, lactose fermentative.
CFU/ml averages followed by different letters were significantly different from each other (Student's t-test, 5% significance). Coefficient of variation = 30.5%.

levels of lactic acid, which is inhibitory to coliforms (Ostling and Lindgren 2006). During the normal and adequate silaging process, Enterobacteriaceae initially grow, but the population decreases rapidly when the medium is acidified, since acidic conditions are unfavourable to these bacteria (Ostling and Lindgren 2006). Further researches should clarify if there are inhibitors of these bacteria in sorghum silage, allowing for the achievement of equilibrium and control of these microorganisms in the ruminal environment.

Krause *et al* (2003) evaluated the effects of diet on populations of *E. coli* and reported a negative relationship between total coliforms and *Lactobacillus* spp. in the digestive tract of cattle. Diet was the major factor regulating bacterial composition, since cattle receiving a high-grain diet had higher populations of these coliforms. Similarly and Callaway *et al* (2009) reported that the *E. coli* populations were abundant in the rumen of cattle fed with higher levels of grain than those fed with forage.

On the other hand, calves (T3) exhibited significant increased growth of bacteria within this group compared to cows fed with the same diet (T1). This could be due to the immaturity of the ruminal environment of young animals, which are characterised by an unstable and continually adapting microbial population permitting a higher growth of Enterobacteriaceae (Ruiz-Lacaz 1992, Callaway *et al* 2009).

Tkalcic *et al* (2000) evaluated the influence of diet on the *E. coli* O157:H7 population in the rumen of calves and found that the Enterobacteriaceae population was similar among animals who received diets with high levels of concentrates and those that received diets with high levels of forage. The immaturity of the digestive tract of these young ruminants have favoured the high concentration of Enterobacteriacea in both diets.

GENUS DISTRIBUTION

The distribution of genera of the bacterial isolates from the four groups of animals under study is described in table 3, according to the presumptive identification by biochemical and micro-morphological assays. The genera most frequently identified were *Enterobacter*, *Klebsiella*, and *Proteus*.

When comparing the occurrences of these genera in the calves sampled, *Enterobacter* spp. and *Klebsiella* spp. were more frequently identified in animals fed sugarcane and urea, while *Proteus* spp., together with these two genera, were more frequent in calves fed with sorghum silage ($P < 0.05$). In the cows evaluated, *Klebsiella* spp. was more frequent in animals fed with silage, while *Enterobacter* was predominant among the isolates from cows fed with pasture forage ($P < 0.01$).

Preliminary studies have reported that *Klebsiella* spp. may have the ability to degrade mimosine, a toxic compound present in the forage legume *Leucaena leucocephala*[1]. Therefore, in the present study, diet containing sorghum silage could be considered a favourable factor for this genus, which could reduce the risk of contamination for animals fed with that legume.

*Proteus* spp. is a producer of urease and could contribute to the use of the urea added to the group fed with sugarcane. Most published reports have suggested that there is an association between the presence of this genus and urinary infections in humans (Rugai and Araújo 1968,

[1] Aung A, T Ngwe, U Meulen, F Gessler, H Böhnel. 2006. Control of Leucaena toxicosis in Myanmar using IBT-Goettinger Bioreactor grow mimosine degrading ruminal Klebsiella spp. In: TROPENTAG 2006: Conference on International Agricultural Research for Development.: University of Bonn, Germany; www.tropentagde/2006/abstracts/full/202pdf

**Table 3**. Genus distribution of Gram-negative, rod-shaped, facultative anaerobes in the ruminal fluid of dairy cattle fed with different tropical forages

Distribución de los géneros de bacterias Gram-negativas, bastoncillos y aerobios o anaerobios facultativos, en el fluido ruminal de ganado lechero alimentado con diferentes forrajes tropicales

| Genera | Total | T1 cows-silage | | T2 cows-grass | | T3 calves-silage | | T4 calves-cane | |
|---|---|---|---|---|---|---|---|---|---|
| | n | n | % | n | % | n | % | n | % |
| *Alcaligenes* | 8 | 2 | 7.1 | 2 | 7.7 | 4 | 13.8 | 0 | 0.0 |
| *Citrobacter* | 1 | 0 | 0.0 | 1 | 3.8 | 0 | 0.0 | 0 | 0.0 |
| *Escherichia* | 9 | 2 | 7.1 | 2 | 7.7 | 4 | 13.8 | 1 | 11.1 |
| *Edwardsiella* | 1 | 1 | 3.6 | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| *Enterobacter* | 32* | 5 | 17.9 | 13* | 50.0 | 8* | 27.6 | 6* | 33.3 |
| *Klebsiella* | 27* | 12* | 42.9 | 6 | 23.1 | 4 | 13.8 | 5* | 27.8 |
| *Proteus* | 17* | 3 | 10.7 | 1 | 3.8 | 8* | 27.6 | 5* | 27.8 |
| *Providencia* | 1 | 0 | 0.0 | 1 | 3.8 | 0 | 0.0 | 0 | 0.0 |
| *Pseudomonas* | 4 | 3 | 10.7 | 0 | 0.0 | 1 | 3.4 | 0 | 0.0 |
| Total | 100 | 28 | 100.0 | 26 | 100.0 | 29 | 100.0 | 17 | 100.0 |

Notes*, Values significantly higher when compared within the same treatment ($P < 0.05$).

Chromek *et al* 2005) and carnivores (Gaastra *et al* 1996), since urine is rich in urea. In a recent study, Silveira *et al* (2008) added urea to a diet containing high levels of corn and observed a lower excretion of faecal coliforms, suggesting the presence of favourable fermentative conditions in the rumen.

The implications and interactions of these microorganisms in the ruminal environment are not fully known. Enterobacteriaceae have a significant and beneficial effect on the decrease of oxygen in the rumen (Ruiz-Lacaz *et al* 1992). On the other hand, the potential of these bacteria in industrial microbiology is promising. Strains of *Klebsiella oxytoca* from the lignocellulose-degrading microbiota have been isolated from feces of sheep and used in the direct bioconversion of Napier grass (*Pennisetum alopecoider*) to ethanol, supporting the biotechnological potential of these isolates (Lin *et al* 2010). Another study reported the participation of Enterobacteriaceae in the digestion of cellulose and xylan in bat diets. Among 14 isolates belonging to the species *Proteus vulgaris*, *P. mirabilis*, *Citrobacter freundii*, *Serratia liquefaciens*, and *K. oxytoca*, eight presented cellulolytic and xylanolytic activity (Anand and Sripathi 2004).

Other positive interactios have been reported. Methanogenesis was markedly decreased by addition of nitrate and nitrite, and inoculation of *E. coli* W3110-reduced methanogenesis by 27%. Inoculation of *E. coli* W3110 to nitrite containing cultures decreased nitrite accumulation, while keeping methanogenesis low. The culture of wild-type *E. coli* W3110 may decrease methanogenesis in cultures of mixed rumen microbes, suggesting that inoculation of wild-type *E. coli* W3110 may be a practical way to suppress nitrite toxicity when nitrite is used to abate methanogenesis in the rumen (Sar *et al* 2005).

The isolates found in this work may also possess biotechnological potential, which should be investigated. Meanwhile, studies investigating the influence of these bacteria on the nutrition and health of the animals, not just on public health should be performed, bearing in mind that these microorganisms are important agents of food poisoning and possible carriers of antimicrobial multi-resistance genes (Gaggìa *et al* 2010).

This study evaluated the population of coliforms in the ruminal environment of animals fed with diets containing high proportions of tropical forage, and the results indicated that there was a lower occurrence of *E. coli* and absence of *Salmonella* spp., which suggests that the ruminal environment has a lower risk for contamination by these zoonotic agents. As such, these results corroborate recent research indicating that proper dietary management, with higher levels of forages, is a major factor in the control of pathogenic strains of Enterobacteriaceae (Rasmussen *et al* 1993).

In this work, a higher detection rate and a larger population of Enterobacteriaceae in the ruminal environment of female calves fed with sorghum silage compared with cows fed with the same forage were observed, taking into account the effect of the age of the animal. For the group of calves, sugarcane and urea favoured the growth of these bacterial populations, compared to sorghum silage supplementation. For the group of cows, the type of forage that the animal consumed did not influence the ruminal concentration of this bacterial family.

*Enterobacter* and *Klebsiella* genera were more prevalent in the rumen of cows fed with pasture and sorghum silage, respectively. On the other hand, *Enterobacter* spp. and *Proteus* spp. were more frequent in calves fed with sorghum silage. Future studies should clarify the differences

found in the Enterobacteriaceae populations, with goals of enhancing the productivity of cattle fed the forages evaluated and reducing the population of pathogenic zoonotic strains in farm animals.

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