The Role of Bacteria and Fungi on Forage Degradation *in Vitro*

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The role of bacteria and fungi on forage degradation *in vitro*

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Abstract

The study was conducted to evaluate the interactive role of bacteria and fungi on forage degradation *in vitro*. Samples of *Cynodon spp.* were incubated in a 48-h *in vitro* gas assay with incubation medium containing or not antimicrobial substances. Treatments were: antibiotic (Ab), antifungal (Af), negative control (i.e. without antimicrobials) or positive control (i.e. with both Ab and Af). Three replicate assays were conducted and, in each assay the gas volume was measured at 3, 6, 9, 12, 24, 36 and 48 h of incubation. Data of cumulative gas production in each flask in each assay was fitted to a one-pool logistic model which generated three kinetic parameters: total gas production, rate of gas production and lag time. For statistical analysis, data of triplicates in each run were averaged and each run was considered a replicate. All variables were significantly affected by treatments ($P < 0.05$). Compared to negative control treatment, Ab decreased total gas production and the rate of gas production by 26 and 13 %, respectively, and increased the lag time by 5.5 hours. The inclusion of Af also decreased total gas production and the rate of gas production by 5 and 29%, respectively, whereas decreased the lag time by 1 hour. When both Ab and Af were included in the incubation medium, gas production was almost completely inhibited and no convergent data of fermentation parameters was generated. In conclusion, bacteria had a major role on forage degradation what, however, was increased by fungi activity. The mechanisms by which fungi interact with bacteria for degrading forage into the rumen needs to be elucidated.

Introduction

Ruminants have the ability to utilize fibrous carbohydrates from forages as cellulose, hemicellulose and pectin due a symbiotic relationship with microorganisms inhabiting the rumen. These microorganisms, mainly bacteria and fungi, produce enzymes which catalyse the hydrolysis of these carbohydrates releasing free sugars. These compounds are then metabolized by rumen microorganisms producing volatile fatty acids, methane and carbon dioxide, among other products (Van Soest 1994). The species and function of rumen bacteria have been longer studied (Hungate 1966) whereas not consistent information is available on the role of fungi on rumen fermentation. The presence of rumen fungi species was first reported by Orpin (1975), which observed that part of the fungi life cycle was strongly associated with the fibre. Moreover, Mountfort et al. (1982) observed that the rumen fungi species show high cellulolytic activity and, in addition, they are capable to degrade lignified tissues of forage samples (Bauchop 1979; Trinci et al. 1994). Also, Lee et al. (2000) and Zhang et al. (2007) reported that forage degradation was increased by fungi presence. However, other studies have reported that soluble proteins produced by some species of cellulolytic bacteria have the potential to inhibit the cellulase activity of rumen fungi *in vitro* (Wolin and Stewart 1997; Dehority and Tirabasso 2000), indicating a negative interaction between bacteria and fungi. The present study was conducted to evaluate whether the forage degradation *in vitro* is consequence of a summative or interactive activity of bacteria and fungi.

Methods and Study Site

The study was conducted in 2019 at the Universidade Federal de Santa Maria, Santa Maria, RS, Brazil. The substrate used in this experiment was *Cynodon spp*. which was cut from a local pasture in its vegetative stage. Forage sample was dried at 55°C for at least 72 h and ground through a 1-mm screen. Total dry matter was determined by drying at 105°C for at least 16h. Ash was determined by combustion at 600°C during 3h and organic matter (OM) by mass difference. Total N was assayed by the Kjeldahl method (AOAC 1997) and crude protein (CP) calculated as N × 6.25. Neutral detergent fibre (NDF) analysis included ash but did not include either alpha amylase or sodium sulphite. This analysis was performed according to Mertens (2002) except that samples were weighed into polyester filter bags and treated with neutral detergent in autoclave at 110°C for 1 h (Senger et al. 2008). Acid detergent fibre (ADF) and sulfuric acid lignin were analysed according to AOAC (AOAC 1997). The *Cynodon spp*. contained 896, 710, 370, 65 and 154 g/kg (dry matter basis) of OM, NDF, ADF, lignin and CP, respectively. Three replicate *in vitro* assays were conducted to evaluate the impact of antimicrobial substances on *Cynodon spp*. degradation. In each assay, forage samples were weighed

(1.5 g) in triplicate in 160 mL flasks and incubated *in vitro* during 48 h in medium containing 50 mL buffer (Mould et al., 2005) plus 50 mL rumen inoculum, added or not with antimicrobial substances. The inoculum was collected under continuous CO_2 flushing from the rumen cannula of a steer grazing a pasture of *Cynodon spp*. and supplemented with concentrate feedstuffs. A mixture of penicillin, chloramphenicol and streptomycin (500 mg/L of each) was used as antibiotic whereas cycloheximide (50 mg/L) was used as antifungal. *In vitro* fermentations were conducted anaerobically in a slow-stir water-bath system at 39°C. Treatments were: antibiotic (Ab), antifungal (Af), negative control (i.e. without antimicrobials) or positive control (i.e. with both Ab and Af). Bottles without substrate were included as blanks. Gas volume was manually recorded at 3, 6, 9, 12, 24, 36 and 48 h of incubation using a three-outlet valve. The first outlet was connected to a needle (0.6 mm), which was inserted in the bottle across the rubber cap. The second outlet was connected to a graduate column filled with distilled water and the third remained free to remove the gases from inside the bottle after each reading. The volume of gas was measured in mL, corresponding to the displacement of the water in the graduate column. Data of cumulative gas production in each flask in each assay was corrected for blanks and fitted to the unicompartimental logistic model of Schofield et al. (1994) which generated three kinetic parameters: total gas production (mL), rate of gas production (%/h) and lag time (h). Final pH of incubation medium was also recorded at 48 h of incubation. For analysis, data of triplicates in each assay were averaged and each assay was considered a replicate. Statistical analysis was carried out using a general linear model and treatment means were compared through the Student *t* test.

Results

The curves of gas accumulation through the 48 hours of incubation in the different treatments are shown in Figure 1. All kinetic parameters of gas production were significantly affected by treatments ($P < 0.05$, Table 1). Compared to negative control treatment, Ab decreased total gas production and the rate of gas production by 26 and 13 %, respectively, and increased the lag time by 5.5 hours. The inclusion of Af also decreased total gas production and the rate of gas production by 5 and 29%, respectively whereas, however, decreased the lag time by 1 hour. When both Ab and Af were included in the incubation medium, gas production was almost completely inhibited and no convergent data of fermentation parameters were generated. The initial pH of the incubation medium was similar for all treatments and was on average 7.1 whereas the final pH decreased to on average 5.6 in most treatments excepting in C+, which final pH remained near the initial pH.

Table 1. Effect of antimicrobial substances on gas production parameters of *Cynodon spp*. samples incubated *in vitro* during 48 hours.

Variables	Treatments†				s.e.m. ‡
	C-	Ab	Af	C+	
Gas production, mL	148[a]	106[c]	137[b]	NC	1.2
Rate of gas production, %/h	5.6[a]	4.8[b]	4.0[c]	NC	0.07
Lag time, hours	1.8[b]	7.3[a]	0.8[c]	NC	0.13
Initial pH	7.1[a]	7.1[a]	7.1[a]	7.1[a]	0.20
Final pH	5.5[b]	5.7[b]	5.5[b]	6.6[a]	0.28

[a, b, c] Means with different superscripts within a row are different by Student t test ($P \leq 0.05$); NC, curve of gas production did not converge to the logistic model and parameters were not generated.

† C-, negative control (without antimicrobials); Ab, antibiotic (penicillin + chloramphenicol + streptomycin, 500 mg/L of each); Af, antifungal (cycloheximide, 50 mg/L) and C+, positive control (with Ab and Af).

‡ Standard error of means where n = 3 per treatment.

Discussion

The isolated addition of either Ab or Af impacted the kinetic of gas production *in vitro* in different ways. For example, total gas production was partially and more negatively affected by Ab than by Af whereas an inverse effect was observed on the rate of gas production, i.e., the negative impact of Af on this variable was more evident than that observed with Ab addition. The lag time, in turn, was negatively affected by Ab and positively affected by Af. However, when both antimicrobials substances were added to the incubation medium, the fermentation was completely supressed. This result strongly indicates that both bacteria and fungi had a synergistic activity on forage degradation as reported by Lee et al. (2000), even though bacteria had a major role on this process. The increased contribution of rumen bacteria species on forage degradation compared to fungi is coherent with the differences on their microbial mass and metabolic activity into the rumen (Raskin

at al. 1997; Arcuri et al. 2011). Moreover, the fibrolytic bacteria species seems to contain multienzyme complexes for plant cell degradation with higher affinity/specificity for cell-binding with substrate than those found in rumen fungi species (Chesson and Forsberg 1997), what would be coherent with the impact of antimicrobials on lag time. The addition of Ab, a condition by which it would be expected lower bacteria competition and, consequently, higher fungi growth and activity, increased the lag time from average 1.8 to 7.3 hours whereas, in an opposite way, the inclusion of Af, a condition by which only bacteria would be acting on forage colonization and degradation, decreased the lag time of fermentation to only 0.8 hours. These results indicate that fungi species needs more time for reaching high levels of colonisation and multiplication on plant tissues into the rumen than bacteria species (Heat et al. 1986; Dijkstra et al. 2002; Edwards et al. 2008). In addition, it has been reported that rumen fungi species use to colonize and degrade preferentially more lignified plant tissues with lower digestibility (Bauchop 1979; Joblin et al. 2002), what would be coherent with the higher negative impact of Ab on total gas production than that observed with Af addition. However, despite the increased lag time, the inclusion of Ab resulted in lower negative impact on the rate of forage degradation than the inclusion of Af. This result indicate that fungi species needs more time than bacteria for colonizing the forage particles but their fibrolytic activity is similar or even higher than that of rumen bacteria species.

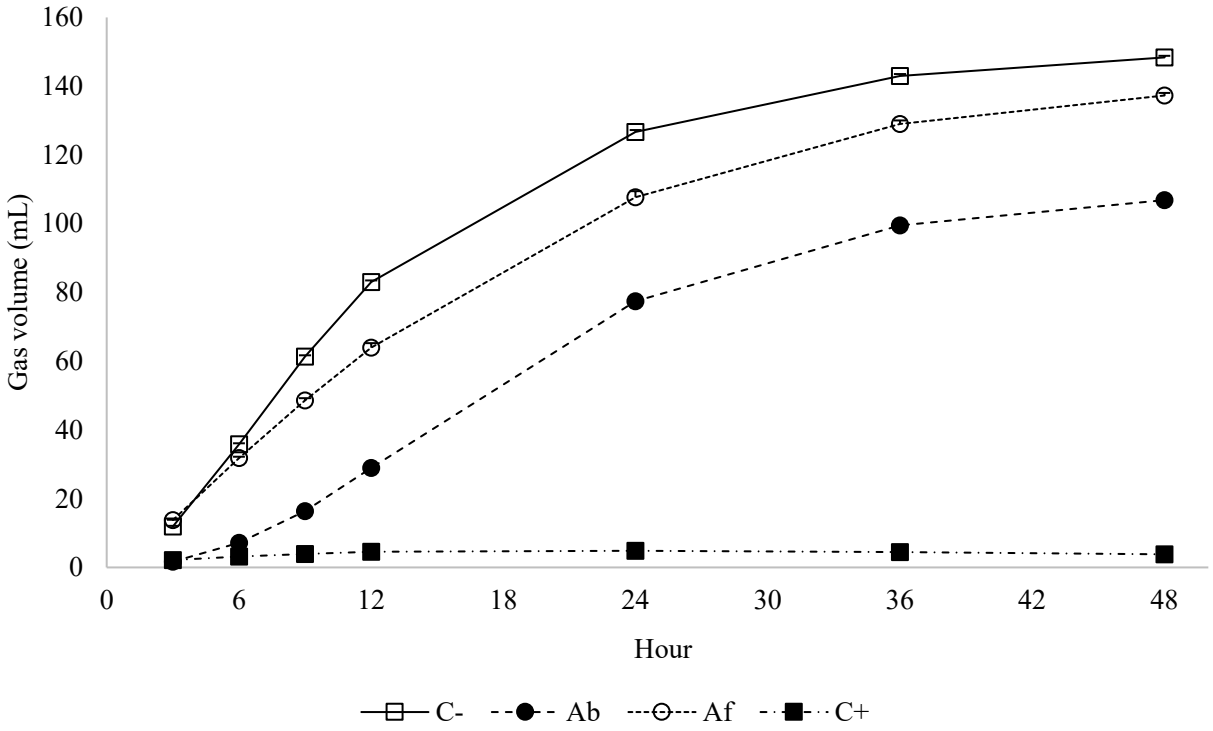

Figure 1. Effect of antimicrobial substances on kinetics of cumulative gas production of *Cynodon spp.* in a 48-h *in vitro* gas production assay. C-, negative control (without antimicrobials); Ab, antibiotic (penicillin, chloramphenicol and streptomycin 500 mg/L of each); Af, antifungal (cycloheximide 50 mg/L) and C+, positive control (with Ab and Af).

Excepting for the positive control treatment, where the fermentation was completely supressed and the pH of the incubation medium remained near the initial pH, the final pH of other treatments decreased to values below 6.0 after 48 hours of fermentation, a condition by which the activity and/or growth of fibrolytic rumen bacteria species are greatly and negatively affected (Mould and Ørskov 1983; Martin and Michalet-Doreau 1994; Zhang et al. 2018). However, it is not known which would be the impact of low pH on rumen fungi activity or even on the interaction between rumen fungi and bacteria on forage degradation. In conclusion, bacteria had a major role on forage degradation what, however, was increased by fungi activity. However, the mechanisms by which fungi interact with bacteria for degrading forage into the rumen needs to be elucidated. Data of biomolecular (i.e. qPCR and DNA sequencing) and microscopy techniques may contribute for this task.

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