In vitro microbial fermentation of tropical grasses at an advanced maturity stage

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Abstract

Four tropical grasses (Cynodon dactylon, Cd, C. plectostachyus, Cp, Brachiaria humidicola, Bh and Pennisetum purpureum, Pp) harvested in Central Brazil during the dry season at 100-day age were compared in terms of chemical composition and in vitro gas production pattern in order to select the most promising species for feeding ruminants during the dry season. Because of its lower proportion of neutral detergent fibre (NDF, 709 g/kg dry matter, DM) and lignin (44 g/kg), and its higher nitrogen (N) content (15.3 g/kg), Pp appears to have the higher nutritive value in such conditions. In contrast, Cp showed a high NDF (843 g/kg) and lignin (115 g/kg) contents, and low N content (8.7 g/kg). Microbial fermentation, estimated by the gas production pattern agreed well with chemical composition of the studied species, and ranked them as follows: Pp > Cd > Bh > Cp. The metabolic energy content of grasses was estimated from the volume of gas produced at 24 h and their crude protein content, and were (in MJ/kg DM): Cd, 5.76; Cp, 5.15; Bh, 5.91; Pp, 9.12. In another experiment, Cp, Bh and Pp were chosen to study the bacterial adhesion (estimated by 15N as microbial marker) and polysaccharidase and β-glucosidase enzymatic activities after in vitro incubation of grasses for 4, 8, 12, 24 and 32 h. The pattern of microbial adhesion and total enzymatic activities agreed well with the chemical composition and gas production results. Although P. purpureum promoted a higher adhesion and total cellulolytic activity, bacterial enzymatic activity (per unit of adhered bacteria) showed that populations associated with the grasses do not differ qualitatively in their cellulolytic capability. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tropical grasses; Gas production; Bacterial adhesion; Enzymatic activity
1. Introduction

Central Brazil is a region of 185 million ha that supports 152 million bovine head, about 80% of the total Brazilian herd (Maehl, 1997). Climate is typically tropical or subtropical, with annual rainfall varying among regions between 600 and 2000 mm and with a marked seasonality: from October to March (‘summer season’), rain is copious and temperatures are high; however, from April to September (‘winter season’) precipitation is scarce. Soils in this area are generally poor, slightly acid, lacking in phosphorus and with an average index of aluminium saturation.

Typical beef production systems in Central Brazil rely on the continuous use of pastures, mostly native or introduced grasses, as only feed during the whole year. In this condition, slaughter weight (450–480 kg) is commonly achieved at about 4 years of age. Pasture availability and quality are high in the summer, when livestock gain weight. However, the lack of rain in winter leads to the flowering and senescence of pastures, with a drop in pasture quality and, thus, digestibility and voluntary intake (Fischer et al., 1996); consequently, growth is at a minimum and animals can even lose part of the weight gained in the previous season.

Quality of pastures strongly affects fibrolytic activity of rumen microorganisms, which may be restricted when poor-quality forages are fed (Jung and Varel, 1988; Fondevila et al., 1997), affecting both bacterial adhesion (Varvikko and Lindberg, 1985; Akin and Rigsby, 1985) and enzymatic activity (Jung et al., 1983; Silva et al., 1987), but the extent of this effect depends on the different anatomical and chemical characteristics of the forage used as substrate (Akin, 1989; Wilson, 1994).

The gas production technique (Menke and Steingass, 1988) has been proved to be a good test to evaluate and classify tropical feeds (Sileshi et al., 1996; Krishnamoorthy et al., 1995; González Ronquillo et al., 1998). However, an additional effort has to be made for studying the microbial activity over the different substrates in order to explain the variations in their fermentation pattern.

As the stage of maturity of plants varies with time according to species and growing conditions (Wilson, 1994), this work was planned with the objective of comparing several widely used grass species from Central Brazil when the dry season is advanced and the quality of available feed resources is declining because of both, plant age (100 days) and water scarcity, the purpose being to find the most suitable grass species for feeding Brazilian bovine herds during this period. Comparison was performed in terms of chemical composition, microbial fermentation pattern and the in vitro microbial activity (bacterial adhesion and enzymatic activity) for those forage species.

2. Materials and methods

The four grasses that were evaluated came from the Campus of the Facultade de Zootecnia e Engenheria de Alimentos of the University of Sao Paulo (Pirassununga, Sao Paulo, Brazil; 21˚S, 47˚25’W), located at 634 m above the sea level. The climate is subtropical, with a well-defined dry winter season (6–27˚C) and a humid summer season (27–35˚C, 1500 mm average annual rainfall). Soil, type Dark-red Latosol, was fertilised...
in January 1996 with (kg/ha) N : P : K = 100 : 50 : 50. Forages were harvested in September 1996, after 100 days of regrowth, and total rainfall during the growing period was 58 mm. Species tested were chosen from those most commonly utilised by the farmers in the region, and were: *Cynodon dactylon* (Tifton 85; Cd); *C. plectostachyus* (Cp); *Brachiaria humidicola* (Bh) and *Pennisetum purpureum* cv. Napier (Pp). All forage samples were harvested at 10 cm above the ground level and dried in an air-forced oven and ground through a 1 mm screen before experimental analyses.

The microbial fermentation pattern of forages was studied by measuring the volume of gas produced along the in vitro incubation of samples, according to the technique of Menke and Steingass (1988) as indicated by González Ronquillo et al. (1998). The volume of gas produced by the fermentation of 200 mg dry matter (DM) of duplicate samples of each grass was recorded after 2, 4, 6, 8, 12, 14, 21, 24, 30, 36, 48, 60, 72 and 96 h, in three series of incubation. After each incubation period, the proportion of dry matter disappeared (DMd) was estimated. The final (96 h) gas production (ml) was related to the DMd proportion to calculate the relative gas yielded (RGY, ml of gas per gram of DMd). The apparent organic matter digestibility (OMD, %) and metabolisable energy content (ME, MJ/kg DM) of forages was estimated from the volume of gas produced after 24 h of incubation (GP, ml/200 mg initial DM) and the proportion of crude protein (CP, g/kg DM), according to the following equations, proposed by Menke and Steingass (1988):

\[
\text{OMD} = 24.91 + 0.7222GP + 0.0815CP; \quad n = 185; \quad R^2 = 0.78
\]

\[
\text{ME} = 2.2 + 0.1357GP + 0.0057CP + 0.0002859CP^2; \quad n = 200; \quad R^2 = 0.94.
\]

To estimate the evolution of microbial fermentation, the cumulative gas production was fitted iteratively to the model proposed by France et al. (1993):

\[
y = A\left\{1 - \exp\left[ -b(t - T) - c\left( \sqrt{t} - \sqrt{T} \right) \right] \right\},
\]

where \(y\) denotes cumulative gas production (ml), \(t\) the incubation time (h), \(A\) the asymptote (total gas; ml), \(b\) a rate constant \((h^{-1})\), \(T\) the lag time (h) and \(c\) a rate constant \((h^{-1/2})\). According to these authors, the fractional degradation rate \(\mu\) \((h^{-1})\) may be variable along the fermentation period, and can be estimated from both the rate constants as follows:

\[
\mu = b + \frac{c}{2\sqrt{t}}; \quad t \geq T
\]

Another experiment was performed in two series of incubation to measure microbial activity (bacterial adhesion, using \(^{15}\text{N}\) as a marker, and polysaccharidase and \(\beta\)-glycosidase enzymatic activity) on three forages (Cp, Bh and Pp), that were chosen according to their gas production results. Samples (500 mg DM) of each forage were incubated with 50 ml of the same mixed inoculum described above in duplicated test tubes provided with a Bunsen-type valve. The bacteria were labelled with 52 \(\mu\)g \(^{15}\text{N}\) per test tube, by dissolving 35 mg of \((^{15}\text{NH}_4)_2\text{SO}_4\) (14 + atom% \(^{15}\text{N}\), ISOTEC, Ohio) per litre of inoculum mixture. After incubation of the tubes at 39°C for 4, 8, 12, 24 or 32 h, their content was centrifuged at 1000 \(\times\) g for 5 min and their supernatant discarded. The
residues from duplicated tubes were washed with 25 ml of 0.05 M Na-phosphate buffer (pH 6.7), pooled and centrifuged again under the same conditions. It was assumed that after washing this residue included the undigested forage plus the tightly adherent microorganisms.

The residue was then sampled (1 g air-dry weight, ca. 250–300 mg DM), and approximately half of it was frozen (−20°C) until analysis for enzymatic activity. The enzymes were extracted from the thawed residue by hydrolysis with lysozyme (Silva et al., 1987), harvested from the supernatant of centrifugation at 23 000 × g for 15 min, and kept frozen (−20°C) until their analysis. Another sample (350 mg air-dry weight, ca. 90–110 mg DM) was kept at 60°C in 3 ml 1 N KOH for 30 min, and then freeze-dried for further determination of 15N in the residue, as an index of bacterial adhesion. The remaining fermentation residue was used to determine DM concentration (60°C, 48 h).

An extra tube with Pp as substrate was also added to each series of incubation, for determining the 15N enrichment in pure bacteria. This tube was incubated for 24 h and processed as above, and then the washed residue was suspended in 70 ml of a 0.1% methylcellulose solution, kept at 4°C for 10 min and centrifuged (1000 × g, 5 min). The supernatant was sampled (20 ml), centrifuged at 23 000 × g for 15 min and the residue processed for 15N analysis as described previously.

Plant samples were analysed for their DM, organic matter (OM) and Kjeldahl nitrogen (N) concentration, according to the Association of Official Analytical Chemists (1980) methods. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined sequentially by the procedures proposed by Van Soest et al. (1991). The acid detergent insoluble N (ADIN) was also measured according to Van Soest et al. (1991). The concentration of total and neutral-detergent (ND) soluble phenolic compounds were determined colorimetrically, according to the procedure of Lau and Van Soest (1981) as specified in Fondevila et al. (1997), and referred to as p-coumaric acid equivalents. All chemical analysis were performed in duplicate.

Polysaccharidase activity of the enzyme extract against carboxymethylcellulose (Sigma; CMCase activity) or xylan from oat spelts (Sigma; Xylanase activity) as substrates was determined by measuring released reducing sugars, according to the Nelson–Somogyi method (Ashwell, 1957). β-Glycosidase (β-D-glucosidase and β-D-xylosidase) activities were determined by measuring colorimetrically (400 nm) the p-nitrophenol released from the p-nitrophenyl compound (Sigma) diluted (5 mmol/l) in 0.05 M phosphate buffer (pH 6.7), after incubation of 1.35 ml substrate with 0.15 ml enzyme at 40°C for 15 min. The reaction was stopped by the addition of 0.5 ml of glycine-NaOH buffer (pH 10.5). The activity of the enzyme extract was expressed as μmol of glucose or xylose (polysaccharidases) or nmol of p-nitrophenol (β-glycosidases) released per ml of extract per min, and referred to the dry weight of the residue from which it was obtained (total enzymatic activity), to the protein content of the extract (specific enzymatic activity), or to the 15N concentration in the fermentation residue (bacterial enzymatic activity). Protein concentration of the enzyme extract was determined by the Bradford (1976) procedure, using bovine serum albumin as a standard. The isotope enrichment of 15N was determined using a mass spectrophotometer (VG PRISM II, IRMS, hooked in series to a DUMAS-style N analyser EA 1108, Carlo Erba, Milan, Italy). The bacterial adhesion was estimated from isotopic proportions of residues
(\textsuperscript{15}N res), bacteria (\textsuperscript{15}N bac) and evaluated forages (\textsuperscript{15}N for; zero time incubation) as follows:

$$\text{Bacterial adhesion} = \frac{[(\text{\textsuperscript{15}N res}) - (\text{\textsuperscript{15}N for})]}{[(\text{\textsuperscript{15}N bac}) - (\text{\textsuperscript{15}N for})]} \times \text{total N res}$$

and was expressed per gram of DM residue.

In the statistical analysis of gas production results, the mean of the two syringes of each sample on each series of incubation was used as the experimental unit for the study of differences, and differences among species and series of incubation were contrasted by analysis of variance per time of incubation, with the interaction series of incubation \times species as the error term (8 degrees of freedom, d.f.). When bacterial activity was studied, the main effects substrate, time of incubation and series were considered, and these and the interaction substrate \times time were contrasted with the residual error (14 d.f.). For all cases, differences between treatment means were identified by the Tukey test at \( p < 0.05 \).

3. Results

Chemical composition of the tropical grasses, including their proportions of total and soluble phenolic compounds expressed as \( p \)-coumaric acid equivalents, is presented in Table 1. Nitrogen concentration was lower, and NDF, ADF and ADL higher, in \( C_p \) and \( C_d \) than in \( P_p \), recording intermediate values in \( B_h \). However, differences were minor in the ADIN content, except for \( P_p \). Content of both, the total and soluble phenolics were not related to the ADL proportion. Differences in total phenolics were not important, and soluble phenolics were higher in \( B_h \) and \( C_d \).

Fig. 1 shows the average pattern of microbial fermentation of forages, measured in terms of the volume of gas produced in their in vitro incubation, and Table 2 presents the

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Chemical composition (g/kg dry matter) of the studied tropical grasses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( C. \ dactylon )</td>
</tr>
<tr>
<td>OM\textsuperscript{a}</td>
<td>945</td>
</tr>
<tr>
<td>N\textsuperscript{b}</td>
<td>10.3</td>
</tr>
<tr>
<td>NDF\textsuperscript{c}</td>
<td>836</td>
</tr>
<tr>
<td>ADF\textsuperscript{d}</td>
<td>447</td>
</tr>
<tr>
<td>ADL\textsuperscript{e}</td>
<td>75</td>
</tr>
<tr>
<td>ADIN\textsuperscript{f}</td>
<td>1.7</td>
</tr>
<tr>
<td>Total phenolics\textsuperscript{g}</td>
<td>28.9</td>
</tr>
<tr>
<td>Soluble phenolics\textsuperscript{g}</td>
<td>15.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Organic matter.  
\textsuperscript{b} Total nitrogen.  
\textsuperscript{c} Neutral detergent fibre.  
\textsuperscript{d} Acid detergent fibre.  
\textsuperscript{e} Acid detergent lignin.  
\textsuperscript{f} Acid detergent-insoluble nitrogen.  
\textsuperscript{g} mg \( p \)-coumaric acid equivalents/g DM.
coefficients of gas production evolution, obtained from the adjustment of recorded data to
the curve model, together with average DMd and RGY values and the estimated OMD
and ME content of grasses. Fermentation of \textit{P. purpureum} presented a higher ($p < 0.001$)
gas production from 6-h incubation onwards, and differences between the volume of gas

\textbf{Fig. 1.} Pattern of the volume of gas produced by in vitro microbial fermentation of \textit{C. dactylon} (○), \textit{C. plectostachyus} (●), \textit{B. humidicola} (■) and \textit{P. purpureum} (▲). Upper vertical bars show standard errors of means.

\textbf{Table 2}

Parameters of curves fitted with in vitro gas production results, dry matter disappeared (DMd, mg/g), relative gas yielded (RGY, ml gas/g DM disappeared) and estimated organic matter digestibility (OMD, g/kg) and metabolisable energy content (ME, MJ/kg DM) of grasses

<table>
<thead>
<tr>
<th></th>
<th>\textit{C. dactylon} &amp;</th>
<th>\textit{C. plectostachyus} &amp;</th>
<th>\textit{B. humidicola} &amp;</th>
<th>\textit{P. purpureum} &amp;</th>
<th>s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{A} &amp; 174 b &amp; 122 c &amp; 195 b &amp; 255 a &amp; 7.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{b} &amp; 0.032 &amp; 0.028 &amp; 0.022 &amp; 0.036 &amp; 0.0038</td>
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<tr>
<td>\textit{c} &amp; -0.046 b &amp; 0.011 a &amp; -0.031 b &amp; -0.080 b &amp; 0.0164</td>
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</tr>
<tr>
<td>\textit{T} &amp; 0.36 &amp; 3.19 &amp; 0.59 &amp; 3.10 &amp; 1.475</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>\textit{μ6} &amp; 0.022 &amp; 0.031 &amp; 0.016 &amp; 0.019 &amp; 0.0049</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>\textit{μ12} &amp; 0.025 &amp; 0.030 &amp; 0.017 &amp; 0.024 &amp; 0.0044</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>\textit{μ24} &amp; 0.027 &amp; 0.030 &amp; 0.019 &amp; 0.027 &amp; 0.0041</td>
<td></td>
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<td></td>
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<tr>
<td>\textit{μ48} &amp; 0.028 &amp; 0.029 &amp; 0.020 &amp; 0.030 &amp; 0.0039</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMd &amp; 402 b &amp; 258 c &amp; 428 b &amp; 627 a &amp; 7.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGY &amp; 411 ab &amp; 450 a &amp; 371 b &amp; 416 ab &amp; 12.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMD &amp; 409 &amp; 389 &amp; 401 &amp; 527 &amp; –</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME &amp; 5.76 &amp; 5.15 &amp; 5.91 &amp; 9.12 &amp; 9.12</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

\textsuperscript{a} Among rows, different letters show significant differences ($p < 0.05$).

\textsuperscript{b} A. total gas production (ml/g incubated DM); \textit{b}, fermentation rate (h\textsuperscript{-1}); \textit{c}, fermentation rate (h\textsuperscript{-1/2}); \textit{T}, lag time (h); \textit{μ6}, \textit{μ12}, \textit{μ24}, \textit{μ48}: fractional fermentation rates at different incubation times (h\textsuperscript{-1}), estimated from the rate constants \textit{b} and \textit{c}.

\textsuperscript{c} $p < 0.001$.

\textsuperscript{d} $p < 0.05$. 
produced from Bh and Cd incubation and that from Cp become significant from 36 and 48 h incubation, respectively. Total gas production, as shown from the adjusted curves coefficient A, presented significant differences among species \( p < 0.001 \), it being higher for Pp incubation than for Bh and Cd and giving the lowest value for Cp. The fermentation rate c also showed differences \( p < 0.05 \), being higher (positive) for Cp, and negative with the other grasses. This means that the fractional fermentation rate \( (\mu) \) for Cp diminished as the incubation proceeded, whereas the opposite was observed for the others. The DMD \( p < 0.001 \) and both the estimates of OMD and ME content showed the same pattern of differences as A.

The development of microbial adhesion to the substrate, measured as the increased amount of \( ^{15} \)N in the dry residue of in vitro fermentation of Cp, Bh and Pp, is presented in Fig. 2. Overall means showed a higher \( p < 0.001 \) bacterial adhesion to substrate for Pp than for Bh and Cp, but no significant differences were observed between Bh and Cp. Throughout all the period of study (32 h), adhesion does not seem to reach a plateau for both, Pp and Bh, whereas plateau became apparent for Cp from 12 h incubation.

Overall means for total, bacterial and specific enzymatic activities are presented in Table 3, and the patterns of both, total and bacterial polysaccharidases (CNCCase and xylanase) and ɣ-glycosidase (ɣ-glucosidase and ɣ-xyllosidase) activities, are shown in Figs. 3 and 4, respectively. Total CNCCase and γ-xyllosidase activities were higher over Pp than over Cp or Bh \( p < 0.001 \). However, there were no significant differences in xylanase activity between Pp and Bh, nor among the three grasses in γ-glucosidase activity \( p > 0.05 \). Specific activities were higher over Cp and Bh than over Pp. Only differences in bacterial xylanase \( p < 0.001 \) and γ-xyllosidase \( p < 0.01 \) over the studied substrates were observed.

As in both, the gas production and microbial adhesion curves (Figs. 1 and 2), the pattern of total CNCCase activity (Fig. 3) showed a higher response to Pp than to Cp or Bh. However, the other enzymatic activities studied showed a pattern different from that of CNCCase. The drop observed at 32 h for Pp appeared earlier in xylanase activity (12 h),
### Table 3

Total (g\(^{-1}\) dry matter residue per min), specific (per mg extracted protein per min) or bacterial (per mg \(^{15}\)N per min) polysaccharidase (CMCase or Xylanase; \(\mu\)mol sugar released), or \(\beta\)-glycosidase (\(\beta\)-glucosidase or \(\beta\)-xylosidase; nmoles pnp released) overall activities of extracts from the solid fermentation residue of the selected grasses.

<table>
<thead>
<tr>
<th>Species</th>
<th>CMCase</th>
<th>Xylanase</th>
<th>(\beta)-Glucosidase</th>
<th>(\beta)-Xylosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. plectostachyus</em></td>
<td>2.31 b</td>
<td>2.35 b</td>
<td>3.78 a</td>
<td>0.183 (***</td>
</tr>
<tr>
<td><em>B. humidicola</em></td>
<td>2.45 b</td>
<td>4.96 a</td>
<td>4.72 a</td>
<td>0.290 (***</td>
</tr>
<tr>
<td><em>P. purpureum</em></td>
<td>3.78 a</td>
<td>4.72 a</td>
<td>121.2</td>
<td>7.01 (NS)</td>
</tr>
<tr>
<td>SEM</td>
<td>0.183</td>
<td>0.290</td>
<td>7.01 (NS)</td>
<td></td>
</tr>
<tr>
<td><strong>Specific</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. plectostachyus</em></td>
<td>1.15 a</td>
<td>1.00 a</td>
<td>0.68 b</td>
<td>0.105 (**)</td>
</tr>
<tr>
<td><em>B. humidicola</em></td>
<td>1.21 b</td>
<td>1.78 a</td>
<td>0.71 c</td>
<td>0.113 (***</td>
</tr>
<tr>
<td><em>P. purpureum</em></td>
<td>56.4 a</td>
<td>43.3 a</td>
<td>18.8 b</td>
<td>3.66 (***</td>
</tr>
<tr>
<td>SEM</td>
<td>0.105</td>
<td>0.113</td>
<td>3.66 (***</td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. plectostachyus</em></td>
<td>0.451</td>
<td>0.520</td>
<td>0.493</td>
<td>0.0266 (NS)</td>
</tr>
<tr>
<td><em>B. humidicola</em></td>
<td>0.474 c</td>
<td>1.052 a</td>
<td>0.712 b</td>
<td>0.0641 (***</td>
</tr>
<tr>
<td><em>P. purpureum</em></td>
<td>22.1</td>
<td>24.6</td>
<td>22.6</td>
<td>2.21 (NS)</td>
</tr>
<tr>
<td>SEM</td>
<td>0.0266</td>
<td>0.0641</td>
<td>2.21 (NS)</td>
<td></td>
</tr>
</tbody>
</table>

* Among rows, different letters show significant differences (\(p < 0.05\)).

**Within brackets, signification of the main effect grass species: *** = \(p < 0.001\); ** = \(p < 0.01\); * = \(p < 0.05\); NS = \(p > 0.05\).*

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**Fig. 3.** Total polysaccharidase (\(\mu\)mol sugar released/g DM residue) activity (CMCase, SEM = 0.409, and xylanase, SEM = 0.648), and \(\beta\)-glycosidase (nmol \(p\)-nitrophenol released/g DM residue) activity (\(\beta\)-glucosidase, SEM = 15.68, and \(\beta\)-xylosidase, SEM = 12.22) of extracts from the solid fermentation residue of *C. plectostachyus* (●), *B. humidicola* (■) and *P. purpureum* (▲) at different incubation times.
whereas this activity over Bh peaked at 12 and 24 h. The pattern of β-glucosidase activity was very irregular, showing this and β-xylosidase a high magnitude over Pp at 4- and 8-h incubation, descending thereafter to levels similar to both, Cp and Bh. However, β-glucosidase for Bh peaked at 12 h, even over the recorded values for Pp. Bacterial CMCase (Fig. 4) showed a stable activity throughout the incubation period over the three grasses, but bacterial xylanase activity presented a maximum at 4–8 h for Pp or at 12 h for Bh, descending thereafter. Pattern of β-glucosidase was again very variable on time within species, and presented a peak at 4 h for Pp and at 12 h for Bh, but only the former response was observed on bacterial β-xylosidase activity.

4. Discussion

Cell wall proportion and composition, and N proportion of studied forages (Table 1), compared with what it may be expected from results of previous studies with less mature forages (data not shown), indicate the reduction in nutritive value caused by stage of maturity. In addition, other plant constituents, such as phenolic compounds, may also reduce the nutritive value of forages by a toxic effect on rumen microorganisms (Jung et al., 1983; Akin et al., 1988). The analysis of phenolic compounds applied here (Lau and Van Soest, 1981), though cannot discriminate among the different compounds, may give an index of total and ND-soluble phenolics which has been related to straw cell walls digestion (Lau and Van Soest, 1981; Fondevila et al., 1997). In this case, there is no
obvious relationship between phenolic concentration and the coefficients of microbial fermentation, but this may happen because of the importance of other restrictions (lignin, scarcity of soluble components) also affecting digestibility.

Despite a strong relationship between chemical composition and microbial degradation is not expected, especially in tropical forages, Vadiveloo and Fadel (1992) stated that ADF, total N and condensed tannins (if present) are the most limiting chemical factors upon digestibility, and Ford and Elliot (1989) proposed the lignin : hemicellulose ratio as the best index of forage degradability. Among the studied forages, considering their cell wall proportions and composition and their N concentrations, *P. purpureum* would appear as the most promising forage at 100 days of age in the growing conditions of the dry season. In addition, soluble phenolics in *P. purpureum* were lower than the other forages. Relative to these parameters, the expected nutritive value of the other forages: *B. humidicola* ≥ *C. dactylon* > *C. plectostachyus* would be in accordance with their gas production results (Fig. 1 and Table 2).

Aumont et al. (1995) reported a higher degradation, either in vivo or in vitro, for *Pp* compared with most of the other 22 tropical grasses tested. Fitting of data for *Pp* to the curve presented a high lag time (*T*, Table 2), but it has been observed sometimes that this parameter does not respond to real delays in the beginning of fermentation (González Ronquillo et al., 1998), as it can be seen when compared with gas production pattern (Fig. 1). At an expected rumen retention time of forages of 48 h, that can be assumed theoretically as the moment of effective comparison of microbial fermentation among forages (AFRC, 1993), the volume of gas produced (ml/g DM) was: *Pp*, 218; *Cd*, 122; *Bh*, 113; *Cp*, 90, indicating that differences between *C. dactylon* and *B. humidicola* were higher than those at the end of the fermentation period, and those between *B. humidicola* and *C. plectostachyus* were not as high as shown by the total gas production coefficient *A* (Table 2). The extent of DM disappearance after 96 h fermentation (Table 2) agrees with coefficient *A*, supporting the high relationship between gas production and rumen degradation (Sileshi et al., 1996; Getachew et al., 1998). Estimates of OMD and ME content of grasses, obtained by using both, the gas production and N content, agree with the above results. Krishnamoorthy et al. (1995), using a similar calculation from gas production results, obtained a ME content for *P. purpureum* (of unknown maturity stage) of 9.4 MJ/kg DM. Converting data of average samples of different ages of *P. purpureum*, *B. humidicola* and *C. dactylon* given by Aumont et al. (1995) to ME content (ME = 0.81 × DE; AFRC, 1993), their ME energy content was 8.5, 8.7 and 8.1 MJ/kg DM, respectively. However, RGY ranked *Cp* ≥ *Pp* ≥ *Cd* ≥ *Bh*, though only significant differences were observed between *Cp* and *Bh*. In this regard, it has been suggested that a higher content of rapidly fermentable carbohydrates, shown here by the lower acetate:propionate ratio, promotes an underestimation of the fermentation by the volume of gas produced (Getachew et al., 1998).

One of the objectives of this work was to study the processes involved in microbial action on the forages for a better understanding of fermentation results. Microbial adhesion is a necessary previous step for digestion of highly-resistant tissues in tropical forages (Akin and Rigsby, 1985), that is restricted by their lignin and phenolic content (Akin et al., 1988). In our work, the advanced age of grasses indicates the lack of cell wall solubles (to a lesser extent for *Pp*, where maturity stage is not so advanced at 100 days;
Vieira et al., 1997), which supports the need for a tight adhesion for microbial degradation, and at the same time suggests that the process may occur slowly. This may explain why adhesion does not achieve the plateau after 32 h (Fig. 2), except for Cp, where low quality justifies the low extent of microbial adhesion. In any case, adhesion curves agree well with gas production pattern, showing a higher extent of bacterial adhesion over Pp after 12 h incubation. Data of Varvikko and Lindberg (1985) reported a microbial colonisation after 24 h incubation of 12.6 and 7.4 mg $^{15}$N/g residual DM for rye grass and barley straw incubated in the rumen, and those for straw at 5 and 12 h incubation (2.9 and 4.0 mg $^{15}$N/g DM) fitted well with our data for Cp and Bh, indicating that high-maturity, low-quality tropical forages behave similar to straw for microbial adhesion.

Not all vegetal tissues are colonised equally, or by the same populations (Akin, 1989), so differences in enzymatic capability of the adhered population to degrade different carbohydrates may also be expected and considered. Total enzymatic activity is associated with the carbohydrate digestive capability of the adhered population, and sometimes it has been used as an index of microbial colonisation (Silva et al., 1987). Specific enzymatic activity is related to the hydrolytic potential of produced enzymes and mainly serves as a comparison among trials, whereas bacterial activity gives a more approximate idea of the ability of adhered population to degrade structural polysaccharides.

In this work, total CMCase pattern agrees with those of both, microbial adhesion and gas production, indicating that both, cellulolytic and non-cellulolytic adhered bacteria were increased when incubating Pp. Kabré et al. (1994) suggest that poor availability of an easily degradable substrate would result in a greater expression of the degradative potential of the adhering microorganisms, and, moreover, it minimises the possibility of a negative feedback on cellulase production and activity of cellulolytic bacteria. This may explain the lower specific CMCase activity over Pp than over Bh and Cp, and the lack of differences in this bacterial activity. In the same way, the weakness of cellulase hydrolytic potential (CMCase and β-glucosidase specific activities) on Pp compared with Bh and Cp (Table 3) may be related with the expression of feedback mechanisms of cellobiose upon cellulose and glucose upon both, cellulose or soluble carbohydrates.

It is noteworthy that there is a delayed increase of xylanase activity in B. humidicola up to 8 h incubation, compared with Pp. This might be explained by the higher content of ND-soluble phenolics (Table 1) that are released at a faster rate from forage particles, affecting in an initial stage the population involved in fibre breakdown (Fondevila et al., 1997). However, no differences in overall means for total xylanase activity were observed between Bh and Pp (Table 3). It can be inferred from Fig. 3 that cellulose digestion for Pp is the main responsible factor for the higher gas production (Fig. 1), whereas hemicellulose (xylan) is the most utilised structural polysaccharide in Bh, though to a similar extent as in Pp. It is worth noting that the scarce differences in adhesion and total enzymatic activities (except for xylanase) between Cp and Bh up to 32 h are in accordance with gas production results (Fig. 1), indicating that the limited potential for microbial fermentation for Cp is caused by its low nutritive value, and not by a restricting access to degradable nutrients for bacteria or their enzymes because of its higher lignin or phenolic contents. Adhered bacteria are similar in their enzymatic activity among forages...
(Fig. 4), indicating that no major qualitative differences induced by substrate occurred in adhered population. Whereas both, cellulosytic and non-cellulosytic bacteria are responsible in a similar extent for the higher adhesion over *P. purpureum*, the higher specific xylanolytic activity on *B. humidicola* indicates that the adhering bacteria are more prone to use xylan than cellulose as energy source.

5. Conclusions

Our results and those previously published by others indicate that the nutritive value of *P. purpureum* is less affected by age, and it is the most promising grass species for ruminant feeding after 100 days of age in the dry season conditions. On the other hand, under these conditions *C. plectostachyus* appears to be a low quality forage, although differences at the expected rumen retention times were not so conclusive. Bacterial adhesion and their enzymatic activity upon structural carbohydrates differ widely among forages, but generally agree with these fermentation results. Although *P. purpureum* promoted a higher adhesion, solid-associated populations on the different grasses do not differ qualitatively in their cellulosolytic abilities.

This work was carried out in vitro under controlled fermentation conditions, and differences among forages in a practical situation must have been affected by dietary characteristics, specially regarding their N contents because of nitrogen’s effect on rumen ammonia concentration. Independent of the ranking established by their estimated energy contents, all these grasses need nitrogen supplementation for ensuring an optimal forage intake and satisfying protein requirements of ruminants.

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