Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes

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Received 13 July 1999; received in revised form 20 March 2000; accepted 6 July 2000

Abstract

Thirteen plant extracts, selected for their high flavonoid content, were screened on their action on fermentation and protozoa numbers in 1 l dual outflow fermenters supplied with a 50:50 orchard grass hay + barley diet. Using a \(2^{14-10}\) fractional factorial design, we tested each extract at the rate of 0.5 g per day. The corresponding organic matter inflow was equal to 3.4\% of the basal hexoses fermentation rate. \textit{Lavandula officinalis} enhanced acetate (C2), propionate (C3) and butyrate (C4) outflows (+9.2, +11.9 and +11.4\%, respectively), while \textit{Solidago virgaurea} acted mainly on C2 (+11.3\%) and C3 (+15.8\%). \textit{Achillea millefolium} increased C2 relative outflow (+5.7\%) to the detriment of C4 (−7.9\%). Propolis increased C3 production by 10.3\%. Branched chain volatile fatty acids productions were favoured by \textit{A. millefolium} and lowered both by \textit{Arnica chamissonis} and \textit{Fagopyrum esculentum}. Gas production was increased by \textit{L. officinalis} (CO\(_2\): +11.9\%; CH\(_4\): +13.7\%) and \textit{S. virgaurea} (CO\(_2\): +7.8\%; CH\(_4\): +7.7\%), whereas it was lowered by \textit{Equisetum arvense} (CO\(_2\): −10.6\%; CH\(_4\): −14.2\%). Methanogenesis also decreased with \textit{Salvia officinalis} (−8.2\%). Protozoa numbers were little affected by plant extracts. In conclusion, the extracts which could be selected for further evaluation were \textit{L. officinalis} and \textit{S. virgaurea} for promoting the extent of fermentation, and \textit{E. arvense} and \textit{S. officinalis} for their possible inhibitory action on methane production. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Rumen; Plant extract; Fermentation; Methanogenesis; Micro-organism; Flavonoid

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PII: S0377-8401(00)00193-0
1. Introduction

For the past decades, a number of additives, such as ionophores and probiotics, have been introduced into ruminant nutrition, mostly to improve feed utilisation with, ultimately, an increased animal production (Nagaraja, 1995). However, recently in Europe, the use of most of these supplements has become an issue hotly debated by consumer organisations, on grounds of product quality and safety. Moreover, the routine feeding of four antibiotics to livestock, i.e. bacitracin zinc, spiramycin, virginiamycin and tylosin phosphate, has been banned in the European Union since July 1999, because this practice has been suspected to favour bacterial pathogens resistance to these drugs (Coghlan, 1996; European Union, 1998). As managing the microbial activity in the rumen, enhancing feed digestibility to improve weight gain and milk production, or reducing methane emission by ruminants are still pertinent goals for nutritionists, there is a strong need for feed additives acceptable for consumers and showing potential for manipulation of rumen fermentation. Plant extracts with high concentrations of secondary compounds are good candidates for these requirements. They are available in large numbers on an industrial scale, while considered as natural products and authorised for human consumption. Phenolic compounds, such as condensed tannins, are known to have an impact on the rumen microbial metabolism (McAllister et al., 1995). As a matter of fact, a number of commercial supplements based on plant extracts are already available. Pertinent information, though, has to be obtained to assess the nutritional interest of products of this type.

The present essay aimed to screen 13 plant extracts, selected for their high flavonoid content, on their action on fermentation end-product outflows, methanogenesis, fermentation pattern and protozoa numbers in dual outflow fermenters. This work has been preliminarily published in abstract form (Broudiscou et al., 1998).

2. Materials and methods

2.1. Experimental scheme

The primary objective of this work was to assess the nutritional interest of plant extracts of known botanical origin and of guaranteed availability on an industrial scale. The biochemical compositions of these products were too complex to be precisely characterised. For instance, 80 organic substances other than sugars, fatty acids or amino acids have been identified in Achillea millefolium (Duke, 1992). We first chose a batch of 13 extracts for their flavonoid contents and price, but we did not pretend to investigate the effect of specific flavonoids on rumen function, which would belong to the domain of academic research rather than to applied nutrition.

The screening procedure was actually applied to the 14 extracts listed in Table 1. The Dactylis glomerata extract, that is the water-soluble fraction of our dietary forage, was used as a reference for comparison with exogenous extracts. All the extracts, except D. glomerata, were purchased from AMI S.A. (France). The D. glomerata dry extract was prepared in our laboratory as follows. An amount of 500 g of orchard grass pelleted hay
was soaked in 3 l of water adjusted to pH 7.0, and agitated at 39°C for 1 h. Then, the mixture was filtered through a nylon gauze of 150 μM aperture. The filtrate was centrifuged at 13,000 × g for 30 min. The supernatant was freeze-dried and then grounded.

In order to get an independent estimate of all the main effects, we selected a two-level fractional factorial design (Box et al., 1978; Haaland, 1989), to which were added two runs located at the centre of the experimental domain. It must be recalled that the main effects in screening designs are identified with two-factors interactions. The fundamental identity equation summarising all confounding in our study is

\[ I = 125 = 136 = 147 = 238 = 249 = 34.10 = 123.11 = 124.12 = 134.13 = 234.14, \]

each extract being denoted by the factor number given in Table 1. Such an extended confounding was admitted because, in our case, an experiment of 106 runs at least was required to get independent estimates of main effects and two-factor interactions, while most of these were likely to be negligible, in accordance with Pareto’s principle of effect sparsity. Since, 25 to 30 runs should have been added to estimate also the quadratic effects and experimental error, such an experimental design would have been at best unnecessarily expensive, at worst unrealistic. The only acceptable strategy was a sequential approach, beginning with the selection of the most active factors for further testing. However, such an almost unreplicated factorial does not supply enough degrees of freedom to perform powerful F- and t-tests, as the numbers of runs and of coefficients to be estimated are close. Consequently, we implemented half normal and normal plots and Bayesian approaches to detect active experimental factors. These techniques are described below.

### Table 1
Characteristics of plant extracts

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Plant Part</th>
<th>Solvent</th>
<th>DM&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Phenolic compounds&lt;sup&gt;a&lt;/sup&gt;</th>
<th>OM (%DM)</th>
<th>N (%OM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Achillea millefolium</td>
<td>Aerial</td>
<td>W&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.4</td>
<td>Luteolin-7-glucoside</td>
<td>3.9</td>
</tr>
<tr>
<td>2</td>
<td>Arnica chamissonis</td>
<td>Flower</td>
<td>W</td>
<td>95.1</td>
<td>Hyperoside</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>Betula alba</td>
<td>Leaf</td>
<td>W</td>
<td>96.2</td>
<td>Hyperoside</td>
<td>2.8</td>
</tr>
<tr>
<td>4</td>
<td>Dactylis glomerata</td>
<td>Aerial</td>
<td>W</td>
<td>92.0</td>
<td>Hyperoside</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>Eucalyptus globulus</td>
<td>Leaf</td>
<td>W</td>
<td>96.8</td>
<td>Rutin</td>
<td>0.52</td>
</tr>
<tr>
<td>6</td>
<td>Ginkgo biloba</td>
<td>Leaf</td>
<td>Et 25%</td>
<td>96.5</td>
<td>Quercetin</td>
<td>4.2</td>
</tr>
<tr>
<td>7</td>
<td>Lavandula officinalis</td>
<td>Flower</td>
<td>Et 30%</td>
<td>97.7</td>
<td>Luteolin-7-glucoside</td>
<td>9.8</td>
</tr>
<tr>
<td>8</td>
<td>Lespeziea capitata</td>
<td>Branch</td>
<td>W</td>
<td>97.4</td>
<td>Rutin</td>
<td>36.5</td>
</tr>
<tr>
<td>9</td>
<td>Hypericum perforatum</td>
<td>Flower</td>
<td>Et 25%</td>
<td>97.5</td>
<td>Hyperoside</td>
<td>1.1</td>
</tr>
<tr>
<td>10</td>
<td>Equisetum arvense</td>
<td>Aerial</td>
<td>W</td>
<td>95.0</td>
<td>Rutin</td>
<td>3.65</td>
</tr>
<tr>
<td>11</td>
<td>Propolis</td>
<td>Et 25%</td>
<td>99.0</td>
<td>Phenolic acids&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.4</td>
<td>98.2</td>
</tr>
<tr>
<td>12</td>
<td>Fagopyrum esculentum</td>
<td>Aerial</td>
<td>W</td>
<td>96.2</td>
<td>Rutin</td>
<td>5.5</td>
</tr>
<tr>
<td>13</td>
<td>Salvia officinalis</td>
<td>Leaf</td>
<td>W</td>
<td>95.3</td>
<td>Luteolin-7-glucoside</td>
<td>12.5</td>
</tr>
<tr>
<td>14</td>
<td>Solidago virgaurea</td>
<td>Flower</td>
<td>W</td>
<td>95.3</td>
<td>Rutin</td>
<td>16.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data provided by the manufacturer.

<sup>b</sup> Nature of solvent used for extraction, W: water; Et x%: ethanol x%.

<sup>c</sup> Not determined.

<sup>d</sup> Expressed in caffee acid concentration.
Table 2
Experimental worksheet

<table>
<thead>
<tr>
<th>Plant extract (g per day)</th>
<th>Run numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18</td>
</tr>
<tr>
<td><strong>A. millefolium</strong></td>
<td>0.5 0 0.5 0 0.5 0 0.5 0 0.5 0 0.5 0 0.5 0 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>A. chamissonis</strong></td>
<td>0.5 0.5 0 0 0.5 0.5 0 0.5 0.5 0 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>B. alba</strong></td>
<td>0.5 0.5 0.5 0.5 0 0 0 0.5 0.5 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>D. glomerata</strong></td>
<td>0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0 0 0 0 0 0 0 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>E. globulus</strong></td>
<td>0 0.5 0.5 0 0 0.5 0.5 0 0 0.5 0.5 0 0.5 0.5 0 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>G. biloba</strong></td>
<td>0 0.5 0 0.5 0.5 0 0 0.5 0.5 0 0.5 0 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>L. officinalis</strong></td>
<td>0 0.5 0 0.5 0 0.5 0.5 0 0.5 0.5 0.5 0 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>L. capitata</strong></td>
<td>0 0 0.5 0.5 0.5 0.5 0 0 0 0 0 0.5 0.5 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>H. perforatum</strong></td>
<td>0 0 0.5 0.5 0 0 0.5 0.5 0.5 0.5 0 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>E. arvense</strong></td>
<td>0 0 0 0 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0.5 0 0 0 0 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>Propolis</strong></td>
<td>0.5 0 0 0.5 0 0.5 0.5 0 0.5 0 0 0.5 0.5 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>F. esculentum</strong></td>
<td>0.5 0 0 0.5 0.5 0 0 0.5 0.5 0 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>S. officinalis</strong></td>
<td>0.5 0 0.5 0 0 0.5 0.5 0.5 0.5 0 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
<tr>
<td><strong>S. virgaurea</strong></td>
<td>0.5 0.5 0 0 0 0.5 0.5 0 0.5 0.5 0.5 0.5 0 0.5 0.5 0 0.25 0.25</td>
</tr>
</tbody>
</table>
Table 2 shows the corresponding experimental worksheet made of 18 combinations of experimental treatments. For run numbers 17 and 18, all factors were set at their midpoints, that is the supply of all extracts at the intermediate rate of 0.25 g per day. These two central points participated to the estimation of experimental error. For all other runs, each plant extract planned to be in the diet was supplied at the rate of 0.5 g per day. This rate has been set to limit the total amount of extracts supplied to fermenters to a maximal value of 4 g per day and to simulate a feeding level of approximately 5 g per day for sheep. The 18 runs were randomly assigned to six independent dual outflow fermenters (Hoover et al., 1976; Broudiscou et al., 1997), identically assembled, which were operated for three 7-day contiguous experimental periods.

2.2. Incubation procedure

Three wethers, fed on 900 g per day chopped timothy hay and 300 g per day ground and pelleted barley twice daily, were used as donor animals. The rumen contents used as inoculum were taken after a 24 h fasting, with free access to water, and processed as in Broudiscou et al. (1997).

A pelleted diet, made of 500 g kg\(^{-1}\) orchard grass hay and 500 g kg\(^{-1}\) ground barley, was supplied to the fermenters at the rate of 18 g at 11.00 h and 18 g at 23.00 h. The compositions of the feeds are given in Table 3. The plant extracts were introduced simultaneously to the diet, in amounts adequate to comply with the experimental worksheet. All the diets were kept isonitrogenous by adding from 2.48 to 5 ml per meal of a 47.19 g l\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\) solution. The fermentation broths were separately supplemented with 31.7 mg per day CaCl\(_2\), 47.5 mg per day MgCl\(_2\). The dilution rates of particle and liquid phases were, respectively, set at 0.055 and 0.027 h\(^{-1}\). The fermenters were operated in a variable working volume mode. While the filtered effluents were continuously pumped, the displaced effluents were collected once a day at 9.45 h under N\(_2\) atmosphere. Between two daily withdrawals, the working volume in the vessels was allowed to increase up to 1200 ml. The procedures followed to control both turnover rates have been described in Broudiscou et al. (1997).

Each fermenter, maintained at 39\(^\circ\)C, was continuously infused with artificial saliva, alternatively acidic or alkaline, in order to maintain the pH of the fermentation medium at a set point of 6.0 ± 0.05. The feedback control loop included a gel-filled combination pH

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Characteristics of the feeds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hay</td>
</tr>
<tr>
<td>DM (g kg(^{-1}) wet sample)</td>
<td>921</td>
</tr>
<tr>
<td>Composition of DM (g kg(^{-1}) wet sample)</td>
<td></td>
</tr>
<tr>
<td>Organic matter</td>
<td>913</td>
</tr>
<tr>
<td>Crude protein (N × 6.25)</td>
<td>148</td>
</tr>
<tr>
<td>Starch</td>
<td>Traces</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>634</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>342</td>
</tr>
</tbody>
</table>
probe (Broadley-James Corp., Santa Ana, CA), a transmitter (Omega engineering, Stamford, Connecticut) and a data acquisition system (SAM 80, AOIP Mesures, Evry, France) transferring data to a PC and transmitting actions to a three-way pinch valve. The software used for data analysis and process control was provided by Dr. A. Lecomte (laboratoire de Biométrie, Avignon). The pH was measured every minute, and the comparison of the measured value to the setpoint, switched the pinch valve on the appropriate saliva, upstream from the peristaltic pump. The average mineral composition of the resulting buffer was close to the optimum determined in Broudiscou et al. (1999a).

The pH values in vessels were checked by having them continuously plotted against time on the PC monitor and by daily comparison to a standard determination in a filtered sample, taken at 9.45 h, using a pH meter calibrated with certified buffers (Hanna Instruments, Italy). The on/off control, compared to a PID control, proved to be satisfactory. The pH was maintained within a range of ±0.05 pH unit around the set value.

2.3. Analytical methods

The amount of collected effluents, delivered saliva and the fermentation gas volumes were measured every day. After a 5-day adaptation term, 10 ml of filtered (pore size of the filters = 200 µm) fermentation broth was taken at 9.45 h for redox potential and subsampled for volatile fatty acids (VFA) and protozoa population density determination. Protozoa were counted according to Broudiscou et al. (1997). The displaced and filtered effluents collected in a container at +4°C on days 6 and 7 were pooled and subsampled for VFA determination. All the samples for VFA analysis were mixed with 0.1 volume of H₃PO₄ 8.2% (w/w) and stored at −20°C until processed as described by Jouany (1982). The fermentation gas composition was determined on days 6 and 7 by gas chromatography as described by Broudiscou et al. (1999b).

The daily amount of hexoses fermented (HF) was estimated by Eq. (1) from outflows of individual VFA (Demeyer and Van Nevel, 1975).

\[
HF \text{ (mol per day)} = \frac{(C2 + C3)}{2} + C4 + C5
\]  

in which C2, C3, C4, and C5 are the daily outflows (mol per day) of acetate, propionate, butyrate and valerate, respectively.

2.4. Statistical analyses

The results were submitted to linear regression by a Minitab procedure (Minitab, 1998). Data were fitted to the following model:

\[
Y = b_0 + \sum_{i=1}^{14} b_i X_i
\]  

where \( Y \) was the response, \( X_i \) were the 14 coded variables related to extracts, \( b_0 \) and \( b_i \), the 15 regression weights. The coded variable of a given extract \( X_i \) was related to its daily
input rate $N_i$, expressed in g per day, by the Eq. (3).

$$X_i = \frac{(N_i - 0.25)}{0.25} \tag{3}$$

Hence, $X_i$ varied from $-1$, when the extract $i$ was absent, to $+1$, when it was supplied at the maximal rate of 0.5 g per day. The predicted response on basal diet $Y_b$ equalled

$$Y_b = b_0 - \sum_{i=1}^{14} b_i \tag{4}$$

Besides, the supply of plant extract $i$ at the rate of 0.5 g per day had an estimated effect on $Y$ twice as large as $b_i$. As an illustration, the addition of *Solidago virgaurea* extract to the basal diet increased the predicted VFA concentration from 86.5 to 96.1 mM, that is an 11% increase.

We assessed the goodness or the lack of fit of the model by visual analysis of the response residuals. The coefficient estimates were compared to zero by a Student $t$-test. The relative importance of the estimated effects was compared using Pareto charts (Ishikawa, 1976), which consisted of sorting and plotting the square effects expressed as percentages of the sum of all square effects. A number of statistical tools based on different properties of the normal distribution were combined to identify active factors, using NEMROD procedures (NEMROD, 1999). Both normal probability and half normal probability plots (Daniel, 1959) gave graphical outputs of normal probabilities versus coefficient estimates. The active estimates depart from the fitted line, as they do not follow the normal distribution of error. The Bayes plot (Box and Meyer, 1986), also called active contrast plot, is based on the assumption of effect sparcity. It gives the range of posterior probabilities that each effect is active, for different combinations of two parameters supplied by the analyst: the prior probability that any given effect is active, and the scale factor, which is the difference in scale between active and inactive effects and defines the increase of residual variance due to active effects. Important factors exhibit high posterior probabilities. In our approach, the prior probability and the scale factor varied from 0.1 to 0.4, and from 5 to 20, respectively. These tools have been extensively described by Haaland (1989, pp. 65–68) and Kobilinsky (1997, pp. 87–91).

### 3. Results

None of our dependent variables exhibited an anomalous distribution of residuals. The main characteristics of the fermentation broths sampled at 9.40 h are shown in Table 4. The redox potential, averaging $-309.1$ mV, was satisfactory in all runs. It was slightly enhanced by *Arnica chamissonis* and lowered by *Salvia officinalis*. The protozoa population density ranged from 41.6 to 87.6 $\mu l^{-1}$. It tended to increase with *Lespedeza capitata* and *Hypericum perforatum*, and decrease with propolis. The numbers of large protozoa were increased by *A. millefolium* and depressed by *A. chamissonis*. The predicted fermentation pattern with the basal diet was characterised by a VFA
concentration of 86.5 mM and C2, C3 and C4 molar proportions of 48.6, 20.6 and 25.6%, respectively. It was little affected by the supply of plant extracts. One can only notice that *S. virgaurea* enhanced VFA concentration by approximately 11%, along with a number of minor shifts between molar proportions induced by *A. millefolium* and *A. chamissonis*. *D. glomerata* extract had negligible effects on the fermentation pattern, merely a slight decrease of C4 proportion in favour of C2 and C3. The daily productions of individual VFAs are given in Table 5. In the absence of any plant extract, the outflows of VFA predicted by the models were, C2: 58.4 mmol per day; C3: 25.3 mmol per day; IC4: 0.95 mmol per day; C4: 31.7 mmol per day; IC5: 1.41 mmol per day, and C5: 2.51 mmol per day. These fermentation rates were influenced by several extracts. *Lavandula officinalis* enhanced C2, C3 and C4 outflows (‡9.2%, ‡11.9% and ‡11.4%, respectively), while *S. virgaurea* acted mainly on C2 (‡11.3%) and C3 (‡15.8%). *A. chamissonis* increased C4 outflow by 18.3% to the detriment of C2 (ÿ4.8%) and C3 (ÿ12.6%). Propolis increased C3 production by 10.3%. Branched chain VFA productions were favoured by *A. millefolium* (IC4: +6.3%; IC5: +11.3%), and lowered both by *A. chamissonis* (IC4: −6.3%; IC5: −14.2%) and *Fagopyrum esculentum* (IC4: −8.4%; IC5: −7.1%). As shown in Table 6, the rate of fermentation of hexoses equalled 77.2 mmol per day on the basal diet, and was strongly enhanced by *L. officinalis* (+10.1%) and *S. virgaurea* (+6.7%). As a matter of comparison, when a plant extract (except

<table>
<thead>
<tr>
<th>Responses</th>
<th>Eh (mV)</th>
<th>Protozoa</th>
<th>VFA (mM)</th>
<th>Molar proportion (%VFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;70 µm (µl⁻¹)</td>
<td>Total (µl⁻¹)</td>
<td>C2</td>
<td>C3</td>
</tr>
<tr>
<td>R²</td>
<td>0.80</td>
<td>0.84</td>
<td>0.78</td>
<td>0.99</td>
</tr>
<tr>
<td>RSD</td>
<td>27.7</td>
<td>1.75</td>
<td>14.6</td>
<td>5.95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Terms</th>
<th>Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>−309.1 2.2 59.0 99.6 50.59 22.27 22.14</td>
</tr>
<tr>
<td><em>A. millefolium</em></td>
<td>−4.0 1.1b,* 3.8 0.3 1.01b,* 0.18 −1.43</td>
</tr>
<tr>
<td><em>A. chamissonis</em></td>
<td>12.4b −0.9a 0.9 −0.9 −0.71 −1.18a,* 1.97b</td>
</tr>
<tr>
<td><em>B. alba</em></td>
<td>6.4 0.3 4.1 0.9 0.9 −0.03 −0.68 0.80</td>
</tr>
<tr>
<td><em>D. glomerata</em></td>
<td>−3.4 −0.3 −0.6 1.5 0.43 0.56 −1.28</td>
</tr>
<tr>
<td><em>E. globulus</em></td>
<td>−2.5 0.5 1.6 2.6 0.31 −0.05 0.17</td>
</tr>
<tr>
<td><em>G. biloba</em></td>
<td>−9.3 0.4 −0.2 1.3 0.89b,* 0.18 −1.17</td>
</tr>
<tr>
<td><em>L. officinalis</em></td>
<td>10.0 −0.3 2.1 2.0 −0.61 0.21 0.63</td>
</tr>
<tr>
<td><em>L. capitata</em></td>
<td>0.7 0.4 5.3 −0.6 0.50 −0.39 −0.25</td>
</tr>
<tr>
<td><em>H. perforatum</em></td>
<td>3.3 −0.2 5.3 −1.6 −0.36 0.42 0.04</td>
</tr>
<tr>
<td><em>E. arvense</em></td>
<td>1.3 0.1 −3.3 1.0 0.35 0.58 −1.08</td>
</tr>
<tr>
<td>Propolis</td>
<td>−2.2 −0.1 −4.8 1.2 −0.24 0.59 −0.20</td>
</tr>
<tr>
<td><em>F. esculentum</em></td>
<td>−1.4 0.1 −1.6 −0.6 −0.13 0.34 −0.13</td>
</tr>
<tr>
<td><em>S. officinalis</em></td>
<td>−11.4b 0.7 3.0 1.2 0.44 0.20 −0.82</td>
</tr>
<tr>
<td><em>S. virgaurea</em></td>
<td>−0.7 −0.1 −0.6 4.8a,* 0.18 0.74 −0.69</td>
</tr>
</tbody>
</table>

*a* The factor is identified as active using Bayesian approach, normal and half-normal probability plots.

*b* The factor is identified as active using Bayesian approach and either normal or half-normal probability plot.

*P* < 0.10 (null hypothesis).
was supplied at the rate of 0.5 g per day to fermenters, the corresponding organic fraction represented an average inflow of 2.72 mmol per day of hexoses, which was equal to 3.5% of the basal hexoses fermentation rate. The VFA production pattern on the basal diet was 76.0, 32.8 and 40.6 mol per 100 mol of HF for C2, C3 and C4, respectively. It was influenced by A. millefolium (C2: −5.7%; C4: −7.9%), A. chamissonis (C2: −1.4; C3: −0.7**; C4: −0.03b), B. alba (−0.0; −0.02; 1.6), D. glomerata (−0.2; 0.4; −0.01), E. globulus (1.8), G. biloba (2.1), L. officinalis (2.7), L. capitata (0.7), H. perforatum (−0.7; 0.00), E. arvense (−0.2; 0.01; −1.1), Propolis (1.4), F. esculentum (−0.6; 0.1), S. officinalis (0.9), S. virgaurea (3.3*). The variables related to gas production are presented in Table 7. The predicted volume of fermentation gases on the basal diet was equal to 5.21 l per day, and was composed of 170.2 mmol per day of carbon dioxide, 36.5 mmol per day of methane and 0.70 mmol per day of hydrogen. Gas production tended to increase with L. officinalis (CO2: +11.9%; CH4: +13.7%) and S. virgaurea (CO2: +7.8%; CH4: +7.7%), whereas it was lowered by Equisetum arvense (CO2: −10.6%; CH4: −14.2%). Methanogenesis also tended to decrease with S. officinalis (−8.2%). Hydrogen net production was increased by E. arvense (+30%) and lowered by L. officinalis (−50%). When individual gas outflows were expressed as fractions of hexoses fermented, E. arvense, followed by S. officinalis, G. biloba and Eucalyptus globulus, appeared to divert fermentation from gas production. Besides, D. glomerata extract did not significantly influence the production of gases.

Table 5
Effects of plant extracts on volatile fatty acid production (mmol per day)*

<table>
<thead>
<tr>
<th>Responses</th>
<th>C2</th>
<th>C3</th>
<th>IC4</th>
<th>C4</th>
<th>IC5</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>R²</td>
<td>0.88</td>
<td>1.00</td>
<td>0.62</td>
<td>0.74</td>
<td>0.78</td>
<td>0.88</td>
</tr>
<tr>
<td>RSD</td>
<td>4.94</td>
<td>0.062</td>
<td>0.118</td>
<td>7.04</td>
<td>0.240</td>
<td>0.253</td>
</tr>
<tr>
<td>Terms</td>
<td>Coefficients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>69.3</td>
<td>30.8</td>
<td>0.92</td>
<td>31.4</td>
<td>1.40</td>
<td>3.15</td>
</tr>
<tr>
<td>A. millefolium</td>
<td>1.1</td>
<td>0.2</td>
<td>0.03b</td>
<td>−1.6</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>A. chamissonis</td>
<td>−1.4</td>
<td>−1.6b**</td>
<td>−0.03b</td>
<td>2.9b</td>
<td>−0.10b</td>
<td>0.06</td>
</tr>
<tr>
<td>B. alba</td>
<td>−0.0</td>
<td>−0.7**</td>
<td>0.02</td>
<td>1.6</td>
<td>−0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>D. glomerata</td>
<td>−0.2</td>
<td>0.4*</td>
<td>−0.01</td>
<td>−2.2</td>
<td>0.06</td>
<td>−0.06</td>
</tr>
<tr>
<td>E. globulus</td>
<td>1.8</td>
<td>0.5*</td>
<td>0.00</td>
<td>0.6</td>
<td>0.01</td>
<td>0.07</td>
</tr>
<tr>
<td>G. biloba</td>
<td>2.1</td>
<td>0.5*</td>
<td>−0.01</td>
<td>−1.2</td>
<td>−0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>L. officinalis</td>
<td>2.7</td>
<td>1.5b**</td>
<td>0.01</td>
<td>1.8</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>L. capitata</td>
<td>0.7</td>
<td>−0.7**</td>
<td>0.00</td>
<td>−0.6</td>
<td>0.06</td>
<td>−0.12</td>
</tr>
<tr>
<td>H. perforatum</td>
<td>−0.7</td>
<td>0.5*</td>
<td>−0.01</td>
<td>0.0</td>
<td>−0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>E. arvense</td>
<td>−0.2</td>
<td>0.7**</td>
<td>0.01</td>
<td>−1.1</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Propolis</td>
<td>1.4</td>
<td>1.3b**</td>
<td>0.01</td>
<td>0.6</td>
<td>−0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>F. esculentum</td>
<td>−0.6</td>
<td>0.4*</td>
<td>−0.04b</td>
<td>−0.2</td>
<td>−0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>0.9</td>
<td>0.5*</td>
<td>0.00</td>
<td>−0.8</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>S. virgaurea</td>
<td>3.3*</td>
<td>2.0b**</td>
<td>−0.01</td>
<td>−0.1</td>
<td>−0.01</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* The factor is identified as active using Bayesian approach, normal and half-normal probability plots.
** The factor is identified as active using Bayesian approach and either the normal or half-normal probability plot.
* P < 0.10 (level of significance for the null hypothesis).
** P < 0.025 (level of significance for the null hypothesis).

D. glomerata was supplied at the rate of 0.5 g per day to fermenters, the corresponding organic fraction represented an average inflow of 2.72 mmol per day of hexoses, which was equal to 3.5% of the basal hexoses fermentation rate. The VFA production pattern on the basal diet was 76.0, 32.8 and 40.6 mol per 100 mol of HF for C2, C3 and C4, respectively. It was influenced by A. millefolium (C2: +5.7%; C4: −7.9%), A. chamissonis (C2: −7.6%; C3: −15.0%; C4: +13.3%), Betula alba (C3: −8.1%; C4: +6.5%), D. glomerata (C3: +8.2%; C4: −9.4%), Ginkgo biloba (C2: +5.7%; C4: −7.6%) and S. virgaurea (C3: +7.8%). The variables related to gas production are presented in Table 7. The predicted volume of fermentation gases on the basal diet was equal to 5.21 l per day, and was composed of 170.2 mmol per day of carbon dioxide, 36.5 mmol per day of methane and 0.70 mmol per day of hydrogen. Gas production tended to increase with L. officinalis (CO2: +11.9%; CH4: +13.7%) and S. virgaurea (CO2: +7.8%; CH4: +7.7%), whereas it was lowered by Equisetum arvense (CO2: −10.6%; CH4: −14.2%). Methanogenesis also tended to decrease with S. officinalis (−8.2%). Hydrogen net production was increased by E. arvense (+30%) and lowered by L. officinalis (−50%). When individual gas outflows were expressed as fractions of hexoses fermented, E. arvense, followed by S. officinalis, G. biloba and Eucalyptus globulus, appeared to divert fermentation from gas production. Besides, D. glomerata extract did not significantly influence the production of gases.
4. Discussion

Assessing the impact of a large number of factors on animal nutrition by applying a $2^{(k-p)}$ design is the first step of a sequential research strategy. It allows to select the most important additives for further evaluation within a reasonably small number of experimental runs. However, when analysing the results, one should keep in mind that main effects are identified with interactions, and have to be confirmed by an appropriate trial, a full factorial design for instance.

The in vitro screening conditions chosen by us related to diets rich in nonstructural carbohydrates. The NDF and starch fractions of our basal ration were equal to 41 and 31% dry matter (DM), respectively. In order to test plant extracts in suboptimal culture conditions, we set the pH at the lower limit of physiological interval, without simulating, though, rumen acidosis which is diagnosed only when rumen pH is less than 5.8. In our trial, C4 production rates were higher, and C2 production rates lower than those reported in the literature for similar diets. Høngerholt et al. (1998) observed C2, C3 and C4 concentrations averaging 56.4, 21.2 and 16.3 mmol l$^{-1}$, respectively, in the effluents of dual outflow fermenters maintained on a grain-dried grass pasture ration. The inclusion of $D. \text{glomerata}$ extract in our trial was an attempt to examine how the water-soluble fraction of dietary forage could influence rumen microbial metabolism, and to assess the

### Table 6

Effects of plant extracts on the daily amounts of hexoses theoretically fermented (HF) and on the relative productions of C2, C3 and C4

<table>
<thead>
<tr>
<th>Responses</th>
<th>HF (mmol per day)</th>
<th>C2 (mol 100 mol$^{-1}$ HF)</th>
<th>C3 (mol 100 mol$^{-1}$ HF)</th>
<th>C4 (mol 100 mol$^{-1}$ HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R$^2$</td>
<td>0.72</td>
<td>0.92</td>
<td>0.84</td>
<td>0.83</td>
</tr>
<tr>
<td>RSD</td>
<td>8.88</td>
<td>3.69</td>
<td>3.84</td>
<td>4.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Terms</th>
<th>Coefficients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>86.2</td>
<td>80.61</td>
<td>35.90</td>
<td>36.28</td>
</tr>
<tr>
<td>$A. \text{millefolium}$</td>
<td>$-0.8$</td>
<td>$2.16^{*}$</td>
<td>$0.525$</td>
<td>$-1.60$</td>
</tr>
<tr>
<td>$A. \text{chamissonis}$</td>
<td>$1.4$</td>
<td>$-2.87^{*}$</td>
<td>$-2.45^{*}$</td>
<td>$2.70^{*}$</td>
</tr>
<tr>
<td>$B. \text{alba}$</td>
<td>$1.2$</td>
<td>$-1.08$</td>
<td>$-1.32$</td>
<td>$1.32$</td>
</tr>
<tr>
<td>$D. \text{glomerata}$</td>
<td>$-1.9$</td>
<td>$1.75$</td>
<td>$1.34$</td>
<td>$-1.90$</td>
</tr>
<tr>
<td>$E. \text{globulus}$</td>
<td>$1.8$</td>
<td>$0.35$</td>
<td>$-0.17$</td>
<td>$-0.03$</td>
</tr>
<tr>
<td>$G. \text{biloba}$</td>
<td>$0.3$</td>
<td>$2.16^{b,*}$</td>
<td>$0.47$</td>
<td>$-1.54$</td>
</tr>
<tr>
<td>$L. \text{officinalis}$</td>
<td>$3.9^{a}$</td>
<td>$-0.67$</td>
<td>$0.21$</td>
<td>$0.53$</td>
</tr>
<tr>
<td>$L. \text{capitata}$</td>
<td>$-0.5$</td>
<td>$1.51$</td>
<td>$-0.52$</td>
<td>$-0.58$</td>
</tr>
<tr>
<td>$H. \text{perforatum}$</td>
<td>$-0.1$</td>
<td>$-0.67$</td>
<td>$0.63$</td>
<td>$0.08$</td>
</tr>
<tr>
<td>$E. \text{arvense}$</td>
<td>$-0.7$</td>
<td>$0.49$</td>
<td>$1.14$</td>
<td>$-1.07$</td>
</tr>
<tr>
<td>Propolis</td>
<td>$2.0$</td>
<td>$-0.41$</td>
<td>$0.67$</td>
<td>$-0.07$</td>
</tr>
<tr>
<td>$F. \text{esculentum}$</td>
<td>$-0.3$</td>
<td>$-0.34$</td>
<td>$0.69$</td>
<td>$-0.12$</td>
</tr>
<tr>
<td>$S. \text{officinalis}$</td>
<td>$0.1$</td>
<td>$1.02$</td>
<td>$0.64$</td>
<td>$-1.00$</td>
</tr>
<tr>
<td>$S. \text{virgaurea}$</td>
<td>$2.6^{a}$</td>
<td>$1.25$</td>
<td>$1.28$</td>
<td>$-1.07$</td>
</tr>
</tbody>
</table>

$a$ The factor is identified as active using Bayesian approach, normal and half-normal probability plots.

$b$ The factor is identified as active using Bayesian approach and either normal or half-normal probability plot.

$* P < 0.10$ (null hypothesis).
Table 7
Effects of plant extracts on gas production

<table>
<thead>
<tr>
<th>Responses</th>
<th>Volume (l per day)</th>
<th>CO₂ (mmol per day)</th>
<th>CH₄ (mol 100 mol⁻¹ HF)</th>
<th>H₂ (mol 100 mol⁻¹ HF)</th>
<th>CO₂ (mol 100 mol⁻¹ HF)</th>
<th>CH₄ (mol 100 mol⁻¹ HF)</th>
<th>H₂ (mol 100 mol⁻¹ HF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R²</td>
<td>0.63</td>
<td>0.63</td>
<td>0.68</td>
<td>0.85</td>
<td>0.78</td>
<td>0.69</td>
<td>0.82</td>
</tr>
<tr>
<td>RSD</td>
<td>0.88</td>
<td>36.2</td>
<td>10.6</td>
<td>0.202</td>
<td>22.5</td>
<td>8.05</td>
<td>0.343</td>
</tr>
<tr>
<td>Terms</td>
<td>Coefficients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>5.43</td>
<td>176.4</td>
<td>37.1</td>
<td>0.70</td>
<td>204.4</td>
<td>42.9</td>
<td>0.83</td>
</tr>
<tr>
<td>A. millefolium</td>
<td>-0.07</td>
<td>-4.9</td>
<td>-0.5</td>
<td>0.02</td>
<td>-3.7</td>
<td>-0.2</td>
<td>0.04</td>
</tr>
<tr>
<td>A. chamissonis</td>
<td>0.14</td>
<td>7.9</td>
<td>2.1</td>
<td>0.02</td>
<td>5.5</td>
<td>1.6</td>
<td>0.02</td>
</tr>
<tr>
<td>B. alba</td>
<td>0.06</td>
<td>3.6</td>
<td>1.3</td>
<td>-0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3</td>
<td>1.0</td>
<td>-0.08</td>
</tr>
<tr>
<td>D. glomerata</td>
<td>0.10</td>
<td>1.8</td>
<td>0.3</td>
<td>0.05</td>
<td>6.2</td>
<td>1.2</td>
<td>0.10</td>
</tr>
<tr>
<td>E. globulus</td>
<td>-0.07</td>
<td>-2.3</td>
<td>-0.8</td>
<td>0.01</td>
<td>-6.8</td>
<td>-1.7</td>
<td>-0.04</td>
</tr>
<tr>
<td>G. biloba</td>
<td>-0.07</td>
<td>-4.6</td>
<td>-1.4</td>
<td>0.03</td>
<td>-6.3</td>
<td>-1.8</td>
<td>0.05</td>
</tr>
<tr>
<td>L. officinalis</td>
<td>0.25</td>
<td>10.1</td>
<td>2.5</td>
<td>-0.15&lt;sup&gt;b,*&lt;/sup&gt;</td>
<td>2.4</td>
<td>0.9</td>
<td>-0.22&lt;sup&gt;b,*&lt;/sup&gt;</td>
</tr>
<tr>
<td>L. capitata</td>
<td>0.11</td>
<td>3.3</td>
<td>1.0</td>
<td>0.03</td>
<td>4.8</td>
<td>1.3</td>
<td>0.06</td>
</tr>
<tr>
<td>H. perforatum</td>
<td>-0.07</td>
<td>-1.2</td>
<td>-0.6</td>
<td>0.00</td>
<td>-1.1</td>
<td>-0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>E. arvense</td>
<td>-0.23</td>
<td>-9.0</td>
<td>-2.6</td>
<td>0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12</td>
</tr>
<tr>
<td>Propolis</td>
<td>0.11</td>
<td>3.7</td>
<td>0.3</td>
<td>-0.02</td>
<td>-0.5</td>
<td>-0.6</td>
<td>-0.06</td>
</tr>
<tr>
<td>F. esculentum</td>
<td>-0.11</td>
<td>-3.8</td>
<td>-0.9</td>
<td>0.06</td>
<td>-3.5</td>
<td>-1.0</td>
<td>0.08</td>
</tr>
<tr>
<td>S. officinalis</td>
<td>-0.11</td>
<td>-5.0</td>
<td>-1.5</td>
<td>0.02</td>
<td>-6.2</td>
<td>-1.8</td>
<td>0.01</td>
</tr>
<tr>
<td>S. virgaurea</td>
<td>0.18</td>
<td>6.6</td>
<td>1.4</td>
<td>0.01</td>
<td>1.4</td>
<td>0.3</td>
<td>-0.04</td>
</tr>
</tbody>
</table>

<sup>a</sup> The factor is identified as active using Bayesian approach, normal and half-normal probability plots.
<sup>b</sup> The factor is identified as active using Bayesian approach and either normal or half-normal probability plot.
<sup>*</sup> <i>P < 0.10</i> (null hypothesis).
possible effects on fermentation of a low supply of soluble nutrients of forage origin, in relatively high grain rations. This extract appeared to be quite a minor factor, which merely shifted the VFA production rates to greater relative amounts of C2 and C3, without any favourable effect on the extent of fermentation.

Among commercial extracts, *L. officinalis* and, to a lesser extent, *S. virgaurea* strongly favoured microbial fermentation of dietary OM. Both extracts appeared to promote overall fermentation processes. *L. officinalis* did not affect the partitioning of end-products into individual VFA and gases. On the contrary, several other plant extracts enhanced the relative production of particular VFAs: C2 with *A. millefolium*, *D. glomerata*, *G. biloba*, *L. capitata* and *S. virgaurea* extracts, C3 with *E. arvense*, and C4 with *A. chamissonis* and *B. alba*. Besides, several extracts slightly inhibited methanogenesis, *E. arvense* being the most effective, along with increased C3 and H2 net productions. The accumulation of H2 following *E. arvense* supplementation was not high enough to affect negatively the extent of microbial feed degradation and fermentation or biomass net production (Broudiscou et al., 1999c). *S. officinalis* was a less active methane inhibitor than *E. arvense*, and induced a smaller shift of fermentation pattern towards propionate.

The changes in methane production did not always combine with similar variations in protozoa numbers, e.g. following supplementation with *S. officinalis*, although several rumen ciliates and methanogens are known to be symbionts (Vogels et al., 1980; Stumm et al., 1982). As a matter of fact, the Pearson correlation coefficient between the effects of plant extracts on total protozoa number and methane outflow equalled 0.24, and was not significantly different from null (*P* = 0.42). In other experimental conditions, the symbiotic relation between rumen protozoa and methanogenic bacteria has appeared to be of minor importance as well, e.g. in the in vitro reduction of methanogenesis following sheep supplementation with soya oil hydrolysat (Broudiscou et al., 1990). It appears that Sharp et al. (1998) have reported an experimental study which could illuminate this issue. Using group-specific rRNA probes, they have observed that the most abundant family of methanogens in the rumen, belonging to Methanobacteriacea, were either free living organisms or associated with protozoa, and that they were partially replaced by the free living Methanomicrobiales, during the loss of protozoa in dual outflow fermenters.

The larger protozoa, mainly identified as *Polyplastron multivesiculatum* and *Eudiplodinium* sp., were specifically influenced by *A. millefolium* and *A. chamissonis*. The data on biomass synthesis suggest that both extracts acted indirectly by modifying the extent of bacterial growth (Broudiscou et al., 1999c). The microbial N outflow, estimated with nucleobases as the marker, was positively correlated with large protozoa numbers (*r*² of 0.64, *P* = 0.015), but its variations could not be explained by changes in protozoa biomass. For both plant extracts, the variations in N output originating from large ciliates accounted for 25% at most of the changes in microbial N outflow. The increase in bacterial biomass induced by *A. millefolium* supplementation may have favoured the development of larger protozoa through an interspecies (possibly prey– predator) interaction, this influence being reversed in the case of *A. chamissonis*.

As far as we know, data on long-term effects of plant extracts on rumen microbial metabolism, in continuous cultures as well as in vivo, are lacking. Scehovic (1999) has extracted the buffer-soluble contents of about a 100 plant species harvested in natural
grasslands, and individually quantified their action on rumen bacteria during 4 h batch incubations by measuring pH decrease. The incubation method and the type of measurements, rendered any comparison with our data arduous. Furthermore, this stimulating work was designed to assess the quality of natural grassland herbage rather than explore new means for manipulating rumen fermentation. However, this study revealed strong interspecific differences and stressed how difficult it was to relate stimulating or inhibiting actions of plants to the presence of particular phytochemicals.

In the present essay, we have selected flavonoid-rich plant extracts, because these compounds have been reported to interact with micro-organisms in other ecosystems, in a positive as well as in a negative way. On the one hand, it is now well-established that legume plants flavonoids are engaged in the transcription of nodulation genes in symbiotic bacteria, in a highly specific interaction (Cleyel-Marel et al., 1996; Gough et al., 1997). On the other hand, propolis has been reported to have antibacterial action (Bevilacqua et al., 1997). It was found bactericidal against gram-positive and some gram-negative bacteria, apparently by modifying the bioenergetic status of the bacterial membrane and inhibiting bacterial motility (Mirzoeva et al., 1997). Moreover, Naringenin was reported to inhibit the growth of Xanthomonas strains, a pathogen of rice (Padmavati et al., 1997). Nevertheless, our extracts are complex mixtures of biochemical compounds, and one must keep in mind that the phytochemical origin of the effects observed may not be limited to the flavonoid fraction. As a matter of fact, E. globulus, F. esculentum, S. virgaurea and L. capitata extracts presented a rutin content ranging from 0.7 to 36.5% DM, but this hierarchy did not emerge from our experimental observations. Therefore, flavonoids may not be the active fraction or may work in interaction with other plant components. We surveyed the biological activity of phytochemicals identified in A. millefolium, A. Montana, L. officinalis, E. arvense, F. esculatum, S. officinalis and S. virgaurea (Duke, 1992). The group of water-soluble compounds included 19 molecules with antibacterial activity, such as caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, thymol and vanillic acid (Pizzorno and Murray, 1985; Osawa et al., 1990; Didry et al., 1993; Aziz et al., 1998; Scehovic, 1998), five with antiviral activity, and one with prebiotic activity, inulin present in A. millefolium and A. Montana (Gibson, 1999). A second group, characterised by a much lower solubility in water, contained 16 compounds with antibacterial activity, one with antifungal activity, and one with antiviral activity. In fact, the biochemical complexity of plant extracts may prove to be most awkward in attempts at explaining their mode of action.

Even if plant extracts are more likely than chemicals to find social acceptance and although all our extracts, except D. glomerata, have been chosen within a list of products used in human nutrition, they should be carefully investigated for possible toxicity to livestock, as any other feed additive. In fact, H. perforatum, which contains a photodynamic toxin, hypericin, may cause sunburn in livestock, as well as some types of F. esculentum. E. arvense is known to contain thiaminase in amounts large enough to induce a vitamin deficiency and eventually cerebrocortical necrosis (Edwin and Jackman, 1973, 1982). Raw E. arvense extract could possess a high thiaminase activity, that would make its long-term consumption by ruminants inappropriate. Thus, the role of S. officinalis and propolis in decreasing methane production is also worth investigating, as part of a mitigation strategy (Van Nevel and Demeyer, 1996; Minami and Takata, 1997).
In conclusion, the extracts which could be selected for further evaluation were *L. officinalis* and *S. virgaurea* for promoting the extent of fermentation, and *E. arvense* and *S. officinalis* for their possible inhibitory action on methane production. These favourable effects have now to be confirmed by using a full factorial design.

**Acknowledgements**

The authors would like to thank Mrs. B. Lassalas for laboratory analyses, and L. L’Hotelier for taking care of experimental animals.

**References**


