ABSTRACT

The effect of contrasting concentrations of water-soluble carbohydrates of herbage on silage fermentation and composition was examined using grass with high [250 g/kg of dry matter (DM)] concentrations of water-soluble carbohydrates and grass and clover with low (66 g/kg of DM) concentrations of water-soluble carbohydrates. Herbages were ensiled untreated, after inoculation with lactic acid bacteria, or after treatment with formic acid. Good quality silages were produced from herbage with high concentrations of water-soluble carbohydrates, regardless of treatment, and all pH values were below 3.7 after 90 d of ensilage. However, the silage formed from inoculated herbage had a significantly lower concentration of ammonia N and a significantly higher proportion of residual ribulose-1,5-bisphosphate carboxylase compared with the other two silages. Fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) was used to measure ribulose-1,5-bisphosphate carboxylase, and measurement of true plant protein fractions in herbage and silage showed benefits over traditional measurements such as the measurement of N and ammonia N. Herbages with low concentrations of water-soluble carbohydrates produced inferior quality silages that had lower ribulose-1,5-bisphosphate carboxylase contents and higher ammonia N contents, regardless of treatment; few significant differences were observed among treatments. Under good ensiling conditions, when available water-soluble carbohydrate is adequate, the use of inoculants can improve fermentation characteristics and increase the ribulose-1,5-bisphosphate carboxylase content of silages. However, when the herbage has low concentrations of water-soluble carbohydrates, even in inoculated herbages, lactic acid bacteria may follow a heterofermentative pathway instead of a homofermentative pathway, which can result in a decrease in silage quality and a reduction in intact ribulose-1,5-bisphosphate carboxylase.

INTRODUCTION

Silage is produced as a result of the fermentation of water-soluble carbohydrates (WSC) in herbage by the epiphytic microflora, in particular lactic acid bacteria. This fermentation produces mainly lactic acid but also a range of other products (19). The production of silage is thus susceptible to a great number of uncertainties, which include the concentration (37) and availability (23) of WSC in the herbage; the number and types of epiphytic lactic acid bacteria (22, 25); and the numbers and activity of undesirable organisms, such as enterobacteria and clostridia (27, 29), that are present on the herbage to be ensiled. The application of inoculants or acid additives before ensiling can influence the fermentation so that the quality of the silage is more predictable, but, under difficult conditions, such as that when the content of WSC in the herbage is low, the use of these additives may not always guarantee success (19).

In the United Kingdom, grass is the major forage preserved by ensilage for winter feeding. Grass forms 80% of the total silage produced; smaller inputs are contributed by forage maize and other minority crops. In North America, alfalfa, forage maize, and barley (34) are more widely used to produce silage. Climatic conditions are also quite different than those encountered in the United Kingdom. In North America and also in some parts of mainland continental Europe, wilting to a high dry matter content can be achieved easily; however, in the wetter western areas of the United Kingdom, grass and sometimes grass and clover mixtures are often ensiled at a DM content of 20% or less. Grass harvested for ensilage can also vary considerably in WSC content depending on climatic conditions, maturity, fertilizer application, and other factors (19). However, a common factor to both

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North America and Europe is that silage additives are used to improve silage quality.

The goal of ensiling is to reduce the pH of the herbage as rapidly as possible to <4.2, but preferably to <4, so that a stable silage is produced. One of the main factors that influences silage quality is the rate of pH decline in the early stages of fermentation. This pH decline is related to the rate of lactic acid production, which, in turn, is determined by the activity of the natural lactic acid bacterial population or inoculant bacteria applied, and the content and composition of WSC in the herbage. The faster the rate of lactic acid production, the less proteolysis occurs, particularly in crops with high concentrations of WSC for which inoculants have been shown to confer a distinct advantage.

The measurement of end products, such as ammonia, has traditionally been used to determine the extent of proteolysis that occurs in silage. However, this measurement will only yield an indication of the deamination of amino acids and not the extent of true protein hydrolysis in silage. Cussen et al. (4) suggested that the relationship between ammonia N and residual true protein content is poor; however, other authors (19) suggest otherwise. Thus, the true protein status of silages could be a better indication of silage quality. A useful approach would be to study the fate of the individual plant proteins because they may vary considerably in their susceptibility to degradation. Leaf proteins are complex mixtures of which ribulose-1,5-bisphosphate carboxylase (RUBISCO) is the most abundant component and constitutes up to 39% of the total leaf protein (18). Thus, RUBISCO may form a major part of the protein supply to ruminants, and its fate during ensiling is of considerable interest. Several methods are available to analyze RUBISCO including PAGE (33), gel filtration (13), enzyme activity (28), immunoelectrophoresis (11), and ultracentrifugation (14); PAGE has been used by some researchers to examine the proteins in fresh and conserved grasses and legumes. These methods are all relatively time-consuming, and developments in high speed protein analyses using column chromatography appear to have the potential to measure losses of RUBISCO.

In the present study, the effect of the WSC content of herbage on the protein composition of silage produced by the fermentation of untreated herbage or silage produced by the fermentation of herbage treated with either an acid or inoculant additive was examined; Fast protein liquid chromatography® (FPLC®, Pharmacia, Uppsala, Sweden) was used to assess the influence of additive type on leaf protein hydrolysis. A brief account of these experiments has been published elsewhere (5).

MATERIALS AND METHODS

The study was divided into two separate time-course experiments that used laboratory silos. The first experiment used herbage with adequate WSC for a satisfactory fermentation. The second experiment used herbage with low WSC to create more difficult ensiling conditions. These experiments will subsequently be referred to as high and low WSC experiments and high and low WSC herbages and silages.

Herbage

For the experiment that examined the effect of a high WSC content, the herbage was the first cutting of a perennial ryegrass at the head emergence growth stage that had been mown at 1000 h on May 20, 1993 and chopped using a precision chop forage harvester. For the experiment that examined the effect of a low WSC content, a second regrowth (6 wk after the second cut) of a mixed sward of perennial ryegrass and white clover [ca. 30% clover on a fresh matter (FM) basis] was used. The latter herbage was chosen because, 2 d prior to the experiment, the WSC content was shown to be low (approximately 80 g/kg of DM). In an attempt to reduce WSC concentration even further, the harvest area was shaded with black polythene sheeting for 24 h prior to harvesting. The herbage was cut at 1000 h using a reciprocating mower (Agria 3000; Verkaufs gesellschaft GmbH, Moekmuehl, Germany) on October 7, 1993 and chopped by an electrically operated precision chop forage harvester.

Treatment of Herbage

The experimental treatments were an untreated control (distilled water applied at 6 L/1000 kg); freshly cultured Lactobacillus plantarum (Live System®, Genus, Worcester, United Kingdom; applied at 10⁶ cfu/g of fresh herbage), and formic acid (Add Safe®, BP Nutrition, Northwich, United Kingdom; applied at 3 L/1000 kg of fresh herbage). To ensure even distribution of inoculant throughout the herbage, an application rate of 6 L/1000 kg was used throughout. The inoculant was suspended in 180 ml of distilled water, mixed well, and distributed evenly using a pressure spray system over 30 kg of herbage that had been spread thinly on a polythene sheet. An equivalent volume of distilled water was applied for the untreated control treatment. Formic acid was ap-
plied at 3 L/1000 kg, and distilled water (3 L/1000 kg) was also added to ensure the same total liquid application rate.

**Ensilage**

Three glass laboratory silos (Weck GmbH & Co., Wehr Öffingen, Germany) were filled with herbage (1.1 kg of FM) for each treatment and opening time. Jars were filled within 3 h of cutting and then stored at 21°C for up to 90 d. At the appropriate opening times (high WSC experiment: 0.7, 1.1, 2, 4, 14, 60, and 90 d; low WSC experiment: 1, 4, and 90 d) silage was removed, mixed thoroughly, and sampled for subsequent microbiological and chemical analyses.

**Analysis of Grass and Silages**

Chemical analyses were carried out on silage from each treatment replicate, and an equal portion from each replicate was pooled and mixed to provide one sample for microbiological analysis.

Herbage and silage N, pH and DM were determined as described by Merry et al. (20). Ammonia N and lactic acid concentrations were measured as described by Merry et al. (22). Silage VFA were determined using HPLC (2). The ADF content was determined on freeze-dried samples of the herbage using acetyltrimethyl ammonium bromide detergent in 0.5 M sulphuric acid. The CP content of the herbage was calculated by multiplying the herbage N value by 6.25. The NDF content was determined on a freeze-dried sample of the herbage using sodium lauryl sulphate and ethylene diamine tetraacetic acid. Digestible OM (DOM) was predicted from solubility in cellulase by applying the following regression equation of Jones and Hayward (12) to in vivo data: DOM = [percentage of OM digested by cellulase enzyme × 0.639] + 30.638.

Lactic acid bacteria and enterobacteria were enumerated according to the methods described by Merry et al. (22).

**FPLC®**

This technique has been briefly described by Williams et al. (36) and was used to quantify RUBISCO in the original herbage and after ensilage for 90 d. Proteins were extracted from frozen fresh material by homogenizing with 0.05 M phosphate buffer (pH 7.5) at 4°C. Reducing conditions were maintained by adding 4.6 mM sodium-D-isoascorbate, 0.1 mM sodium diethyldithiocarbamate, and 1 mM 2-mercaptoethanol to the buffer. After centrifugation at 10,000 × g at 2°C for 15 min, the supernatant was stored at -19°C prior to analysis. The extraction procedure was reproducible, and the recovery (95%) of pure RUBISCO added to herbage and silages at the outset of the extraction procedure was similar to that found by Grum et al. (8).

The RUBISCO was measured by FPLC® using a monoQ HR5/5 strong anion exchange column (50 × 4.6 mm; Pharmacia). Elution was carried out with a gradient of two buffers at a flow rate of 1.0 ml/min. Buffer A was 20 mM ethanolamine (pH 9.0), and buffer B was 20 mM ethanolamine/1 M NaCl (pH 9.0). The gradient applied was 100% buffer A and 0% buffer B for 3 min; 100 to 65% buffer A and 0 to 35% buffer B for 20 min; 70 to 0% buffer A and 30 to 100% buffer B for 4 min, holding for 5 min at 0% buffer A and 100% buffer B; then 0 to 100% buffer A and 100 to 0% buffer B for 1 min before holding for 5 min at 100% buffer A and 0% buffer B. The total elution required 38 min. The detection wavelength was 280 nm. The retention time for RUBISCO was 27.6 min.

The retention time for RUBISCO was established by chromatography of samples of the highly purified protein under identical elution conditions: 1) isolation from ryegrass according to the method of Jones and Mangan (14) or 2) isolation from lucerne and spinach (kindly provided by J. L. Mangan, Institute of Animal Physiology and Genetics Research, Cambridge, United Kingdom). The identity of RUBISCO was confirmed by PAGE and amino acid analysis after carrying out preparative separations under con-

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**Figure 1.** Changes in pH for silages produced from the fermentation of untreated herbage (◊), inoculated herbage (○), and acidified herbage (□). All herbage in this experiment had a high concentration of water-soluble carbohydrates.
Figure 2. Changes in lactic acid concentrations for silages produced from the fermentation of untreated herbage (◊), inoculated herbage (○), and acidified herbage (○). All herbage in this experiment had a high concentration of water-soluble carbohydrates.

Figure 3. Changes in ammonia N concentrations for silages produced from the fermentation of untreated herbage (◊), inoculated herbage (○), and acidified herbage (○). All herbage in this experiment had a high concentration of water-soluble carbohydrates.

Statistical Analysis

Differences in silages resulting from fermentation time and treatment were determined using two-way analysis of variance (7). The least significant difference test was used to separate means (7).

RESULTS

High WSC Experiment

The herbage had the following composition: 193 g of DM/kg of FM, 17.4 g of N/kg of DM, 224 g of ADF/kg of DM, 412 g of NDF/kg of DM, 656 g of DOM/kg of DM, 250 g of WSC/kg of DM. The pH of the high WSC herbage was 6.18.

Changes in pH and in lactic acid and ammonia N concentrations over the first 14 d of the ensiling period are shown in Figures 1, 2, and 3. The pH of the silage produced from the fermentation of inoculated herbage fell most rapidly to a value of 3.60 by 4 d (Figure 1). The pH of the silage produced from the fermentation of acidified herbage declined most slowly, not falling below 4 until after 4 d, and the pH of the silage produced from the fermentation of untreated herbage showed an intermediate rate of pH decline.

Inverse trends were observed for lactic acid concentrations (Figure 2). The silage produced from the fermentation of inoculated herbage contained considerably higher concentrations of lactic acid up to 4 d; the lowest concentrations were consistently observed in silages produced from the fermentation of herbage treated with formic acid.

Ammonia N concentration was generally highest and increased most rapidly in silage produced from the fermentation of acidified herbage (Figure 3). After 2 d, the values for ammonia N in silages produced from the fermentation of untreated and acidified herbage were very similar. Silage produced from the fermentation of inoculated herbage had a consistently lower ammonia N concentration during the first 14 d of fermentation.

Changes in the numbers of lactic acid bacteria and enterobacteria during fermentation are shown in Table 1. Total numbers of lactic acid bacteria in the chopped herbage entering the silo were approximately 10^4 cfu/g of FM. For the inoculated herbage, numbers...
of lactic acid bacteria were approximately five times higher after 0.7 d of ensilage compared with corresponding values for the untreated herbage. Numbers of lactic acid bacteria in silages produced from the fermentation of acidified herbage were considerably lower after 0.7 d of fermentation. After 1.1 d of ensilage, silages produced from the fermentation of both untreated herbage and inoculated herbage contained greater than $10^9$ cfu of lactic acid bacteria/g of FM, but, in silage produced from the fermentation of acidified herbage, this value was only achieved by d 4. Enterobacteria ($2.83 \times 10^4$ cfu/g of FM) were initially more prominent than lactic acid bacteria and increased to almost $10^8$ cfu/g of FM in silage produced from the fermentation of both the untreated herbage and inoculated herbage by 1.1 d of ensilage. The acidified herbage initially inhibited the growth of enterobacteria, but this trend was later reversed on d 2 and 4 of fermentation. The opposite effect was observed for untreated herbage and inoculated herbage. An initial increase in enterobacteria was followed by a decline to approximately $10^4$ cfu/g of FM by d 4. After d 4, enterobacteria were not detected in any of the silages.

The chemical composition of the mature (90 d) silages is shown in Table 2. The overall mean pH value of silages was 3.54, and, although differences were very small, the pH of silage produced from the fermentation of untreated herbage was greater ($P < 0.05$) than the pH of the silage produced from the fermentation of acidified herbage, which was greater than ($P < 0.05$) that of silage produced from the fermentation of inoculated herbage. Nonsignificant differences were observed in the concentrations of lactic acid, which ranged from 94 g/kg of DM in silage produced from the fermentation of acidified herbage

<table>
<thead>
<tr>
<th>Opening time of herbage</th>
<th>Untreated herbage</th>
<th>Inoculated herbage</th>
<th>Acidified herbage</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EB (log$_{10}$ cfu/g of fresh matter)</td>
<td>EB</td>
<td>LAB</td>
<td>EB</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.5</td>
<td>3.6</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>0.7 d</td>
<td>7.5</td>
<td>8.0</td>
<td>7.5</td>
<td>8.5</td>
</tr>
<tr>
<td>1.1 d</td>
<td>8.0</td>
<td>9.2</td>
<td>7.5</td>
<td>9.4</td>
</tr>
<tr>
<td>2 d</td>
<td>7.3</td>
<td>9.3</td>
<td>5.6</td>
<td>9.5</td>
</tr>
<tr>
<td>4 d</td>
<td>5.2</td>
<td>9.1</td>
<td>4.7</td>
<td>9.6</td>
</tr>
<tr>
<td>14 d</td>
<td>ND$^1$</td>
<td>8.3</td>
<td>ND</td>
<td>8.4</td>
</tr>
<tr>
<td>90 d</td>
<td>ND</td>
<td>&gt;7.0</td>
<td>ND</td>
<td>3.7</td>
</tr>
</tbody>
</table>

$^1$Not detected.
TABLE 3. Changes in chemical composition of perennial ryegrass and clover silage during the initial stages of fermentation. All herbages in this experiment had a low concentration of water-soluble carbohydrates.

<table>
<thead>
<tr>
<th>Opening time and composition</th>
<th>Silage produced from</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated herbage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inoculated herbage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acidified herbage</td>
<td></td>
</tr>
<tr>
<td>pH d1</td>
<td>5.66</td>
<td>4.81</td>
</tr>
<tr>
<td>DM, g/kg of FM</td>
<td>146.8</td>
<td>149.6</td>
</tr>
<tr>
<td>Lactic acid, g/kg of DM</td>
<td>5.68</td>
<td>11.06</td>
</tr>
<tr>
<td>Ammonia N, g/kg of DM</td>
<td>0.00</td>
<td>0.79</td>
</tr>
<tr>
<td>pH d4</td>
<td>4.06</td>
<td>4.42</td>
</tr>
<tr>
<td>DM, g/kg of FM</td>
<td>146.6</td>
<td>148.5</td>
</tr>
<tr>
<td>Lactic acid, g/kg of DM</td>
<td>70.64</td>
<td>14.25</td>
</tr>
<tr>
<td>Ammonia N, g/kg of DM</td>
<td>1.34</td>
<td>1.51</td>
</tr>
</tbody>
</table>

Changes in the numbers of lactic acid bacteria and enterobacteria during ensilage are shown in Table 4. Over the first 4 d, the pH of the silage produced from the fermentation of inoculated herbage fell slightly more rapidly than did the pH of the silage produced from the fermentation of untreated herbage; the pH of silage produced from the fermentation of acidified herbage slowly declined to 4.42 by d 4. Inverse trends were observed over the first 4 d for lactic acid concentrations. Silage produced from the fermentation of inoculated herbage contained the highest concentration of lactic acid followed by silage produced from the fermentation of untreated herbage; the lowest lactic acid concentrations during this time occurred in silage produced from the fermentation of herbage treated with formic acid. Ammonia N concentrations were considerably lower in silage produced from the fermentation of inoculated herbage after 1 and 4 d of ensilage compared with concentrations in either of the other two silages (Table 3).

**Low WSC Experiment**

The herbage contained 143.5 g of DM/kg of FM, 35.4 g of N/kg of DM, 226 g of ADF/kg of DM, 352 g of NDF/kg of DM, 648 g of DOM/kg of DM, and 66 g of WSC/kg of DM and had a pH of 5.75.

The pH values and lactic acid and ammonia N concentrations for d 1 and 4 of the ensiling period are to approximately 136 g/kg of DM in silage produced from the fermentation of inoculated herbage. Concentrations of acetic acid were higher ($P < 0.05$) in silage produced from the fermentation of untreated herbage than in silage produced from the fermentation of inoculated herbage, and none was detected in silage produced from the fermentation of acidified herbage. Corresponding ratios of lactic acid to acetic acid were higher ($P < 0.05$) in silage produced from the fermentation of inoculated herbage than in silage produced from the fermentation of untreated herbage. Butyric and propionic acids were not detected in any of the silages.

Ammonia N concentrations were generally low, but they were lower ($P < 0.05$) in silages produced from the fermentation of inoculated herbage than in the other silages. This trend was reflected in the RUBISCO content. A greater proportion ($P < 0.05$) of RUBISCO remained after fermentation in silage produced from the fermentation of inoculated herbage than in the other two silages. No enterobacteria were detected in the final 90-d silages. Lactic acid bacteria were still in excess of $10^7$ cfu/g of FM in silages produced from the fermentation of untreated herbage. The numbers of these bacteria were similar in silages produced from the fermentation of inoculated and acidified herbages ($5.0 \times 10^3$ and $2.0 \times 10^4$ cfu/g of FM, respectively).
Table 4. Numbers of lactic acid bacteria (LAB) and enterobacteria (EB) in untreated herbage and in silage during fermentation of untreated, acidified, and inoculated herbages. All herbages in this experiment had a low concentration of water-soluble carbohydrates.

<table>
<thead>
<tr>
<th>Opening time of herbage</th>
<th>Untreated herbage</th>
<th>Inoculated herbage</th>
<th>Acidified herbage</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EB (log₁₀ cfu/g fresh matter)</td>
<td>EB</td>
<td>EB</td>
<td>EB</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.7</td>
<td>4.0</td>
<td>. . .</td>
<td>. . .</td>
</tr>
<tr>
<td>1 d</td>
<td>6.5</td>
<td>7.3</td>
<td>6.2</td>
<td>8.2</td>
</tr>
<tr>
<td>4 d</td>
<td>4.3</td>
<td>8.9</td>
<td>1.8</td>
<td>9.3</td>
</tr>
<tr>
<td>90 d</td>
<td>3.1</td>
<td>&gt;7.0</td>
<td>2.8</td>
<td>&gt;7.0</td>
</tr>
</tbody>
</table>

¹Not detected.
²Not applicable.

Greater than 10⁶ cfu/g of FM in the silages produced from the fermentation of untreated and inoculated herbages; formic acid treatment of herbage initially depressed numbers of enterobacteria in that silage. The opposite was observed in silages produced from the fermentation of untreated and inoculated herbages; by d 4, the initial increase in enterobacteria was followed by a decline to 2 × 10⁴ and 6 × 10¹ cfu/g of FM, respectively, for silages produced from the fermentation of untreated and inoculated herbages. After 90 d of ensiling, enterobacteria populations greater than 10² cfu/g of FM were still present in all silages except silage produced from the fermentation of acidified herbage.

The chemical composition of the 90-d silages is shown in Table 5. The overall mean pH of silages was 4.89 (SEM = 0.261), and pH did not differ among treatments. Lactic acid concentrations ranged from 54 to 81 g/kg of DM, but, again, no significant differences among any of the treatments were observed. Acetic acid concentrations were high and similar for all silages, ranging from 63 to 71 g/kg of DM. Corresponding ratios of lactic acid to acetic acid were highest in the silage produced from the fermentation of acidified herbage. Butyric acid was only detected in the silage produced from the fermentation of untreated herbage, and propionic acid was detected at low concentrations in the silages produced from the fermentation of untreated and inoculated herbages.

Ammonia N concentration ranged from 72 to 305 g/kg of N. However, no significant differences were found among any of the treatments. The residual RUBISCO content of all silages was similar, and no significant differences were detected.

**DISCUSSION**

Two crops of differing chemical composition, in particular WSC content, were chosen to provide contrast-
ing challenges to different silage additives. The crops were a first cut of perennial ryegrass with a particularly high WSC content (250 g/kg of DM) and a third cut of a perennial ryegrass and white clover mixed sward in which several factors (i.e., late season, low DM herbage containing clover, and shading) were used to achieve a low WSC content. A reduction in WSC content (to less than 70 g/kg of DM) was achieved by shading, similar to the results of a study by Lunden-Pettersson and Lindgren (16) using the same approach. The crops also differed in their N content; the low WSC herbage had a higher value than did the high WSC herbage. This difference can be explained by the presence of clover in the low WSC herbage, giving it a greater N content than the pure grass sward that was used for the high WSC herbage.

A second major factor that determines the type of fermentation achieved, the population density of epiphytic lactic acid bacteria, was moderate on both crops after chopping when compared with values reported in the literature (20). Only one order of magnitude of difference existed between numbers of lactic acid bacteria on the two crops, although the species diversity on the low and high WSC crops was not determined and might have been quite different.

A more rapid initial (d 1 to 4) pH decline was observed in both experiments in the silage produced from the fermentation of inoculated herbage compared with that for silage produced from the fermentation of untreated herbage. In the terminal (90 d) silages prepared from the high WSC herbage, the ratios of lactic to acetic acid were high in the silage produced from the fermentation of inoculated herbage, indicative of a homolactic fermentation (4, 22). Although the inoculant initially accelerated the fermentation by rapid lactic acid production in the low WSC silages, this was not reflected in the ratios of lactic to acetic acid after 90 d. Although enterobacteria numbers (generally recognized as good indicators of the rate of acidification) initially declined more rapidly in silages produced from the fermentation of inoculated herbage, the final silage prepared from herbage with a low WSC content and treated with formic acid was the only one in which no enterobacteria were detected. This result is contrary to previous observations made by Merry et al. (22), despite the fact that the rate of pH decline in the present experiment appeared to be slower in the silages produced from the fermentation of acidified herbage.

The final silages prepared from high WSC herbage were all well preserved in terms of traditional indicators of silage quality (high lactic acid concentrations, low pH values, and low ammonia N contents). Ammonia N concentration was significantly lower, and residual RUBISCO content was higher ($P < 0.05$), in silage produced from the fermentation of inoculated herbage than in the silage produced from the fermentation of untreated herbage. Concentrations of RUBISCO were reported as proportions of the concentration in the original herbage rather than preparing fresh RUBISCO each time and providing an absolute value for the RUBISCO content. Similar approaches were adopted in studies (6, 8, 24) using electrophoretic measurement of RUBISCO in alfalfa and other forage crops. The observations on residual RUBISCO content support the recent findings of Cussen et al. (4) and those of Williams et al. (36) who also ensiled herbage with a relatively high WSC content. Changes during the initial stages of fermentation have been highlighted as crucial factors to determine the effect of additives on silage quality (32). Thus, these changes are also likely to be critical in the modulation of the extent of proteolysis that occurs in the silo, particularly as the pH optima of some of the plant enzymes that play a major role in proteolysis (18) are close to those of herbages at the start of the ensiling period (9). Merry et al. (21) suggested that one of the major benefits of inoculants that contain lactic acid bacteria is their ability to increase the efficiency and rate of lactic acid production and thereby reduce proteolysis in the silo, effecting potential benefits in terms of animal production response. Nevertheless, other work has suggested that the concentration (19, 31) and availability (23) of WSC, in particular fructan, may compromise the efficacy of silage inoculants in terms of rate and extent of pH decline. This possibility was indicated in the present experiments when the WSC content of herbage was very low. When the inoculant was used in low WSC conditions, the initial rate of pH decline appeared to be slower, and the final pH value appeared to be higher compared with the silages produced from the fermentation of high WSC herbage. In addition, the ammonia N values suggest that proteolytic clostridia were involved, although the lack of butyric acid indicates that clostridia that ferment lactic acid were not active. Lactic acid bacteria in the inoculant that possess the ability to degrade grass...
fructans may have an advantage when low sugar herbages are ensiled (23, 26).

After 90 d, the pH values of all of the silages produced from the fermentation of low WSC herbages had risen, presumably because of a secondary fermentation, but, because butyric acid was only detected in low concentrations in some silages, clostridia that ferment lactic acid cannot be implicated. However, increased production of acetate at the expense of lactate is a likely explanation for which there are two possible reasons. First, fermentation of lactic acid to acetic acid by lactic acid bacteria has been shown to occur under sugar-limiting conditions (3, 14, 30). In indirect support of the shift from lactic acid to acetic acid, a much smaller rise in pH took place in the silage produced from the fermentation of acidified herbage in which microbial activity had been inhibited. Second, heterofermentative lactic acid bacteria, which are often present during the latter stages of ensilage, are more tolerant to acidic conditions (17) and produce acetic acid in addition to lactic acid as one of their fermentation end products.

When adequate WSC is present in an inoculated herbage, high quality, anaerobically stable silages are produced with relative ease. The absolute requirement for available WSC to prepare good quality silage can be lessened when using an inoculant than when silages are prepared without an additive treatment (16). Under such conditions freshly cultured inoculants, such as those used in the present studies, may increase the residual intact protein content of the resultant silage. However, when WSC is limiting or not readily available, a different scenario emerges, and treatment with formic acid offers some distinct advantages (16) that must be offset against disadvantages such as adverse effects of acids on human health and safety. Nevertheless, even direct acidification with formic acid did not markedly influence protein hydrolysis. In the present study RUBISCO, the major soluble leaf protein, was satisfactorily resolved by FPLC® with the monoQ column. This satisfactory result suggests the FPLC® is a rapid method for the determination of RUBISCO, which is ideally suited to time-course studies. Use of FPLC® has advantages over PAGE analysis because peak area can be used more easily to compare silage samples relative to the original herbage rather than relying on the more subjective approach of either scanning or visual comparison of the density of bands separated on polyacrylamide gels. However, no single method can provide all of the information on such a complex mixture and development of further methods to characterize these nitrogenous components is in progress. This information combined with a greater understanding of substrate supply in the herbage will help to develop silage inoculants that improve the protein quality of conserved forages.

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