The effect of cultivar, wilting and storage period on fermentation and the clostridial community of alfalfa silage

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ABSTRACT
The objective of this study was to gain deeper insights into chemical transformations and the clostridial community dynamics during the ensiling of alfalfa. A factorial experiment was conducted with four alfalfa cultivars (Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4)) × three wilting durations (0 h (direct-cut), 2 h and 4 h) × three storage periods (14, 28 and 56 days). The clostridial community was examined using denaturing gradient gel electrophoresis. High butyric acid and ammonia nitrogen (NH₃–N) contents and clostridia numbers were observed in direct-cut silage, with higher values observed in A4 than in the other three cultivars. However, butyric acid and NH₃–N contents and clostridia numbers decreased with wilting regardless of the cultivar. Although Clostridium ghonii and Clostridium sartagoforme were common to all direct-cut and wilted silages, bands for these species were faint. Differences in the appearing time of these species were observed, bands for C. ghonii were found after 14 and 28 days of ensiling, while those for C. sartagoforme were only found after 56 days. In addition, in direct-cut silage, distinct bands for Clostridium perfringens were detected in A1, A2 and A3, while those for Clostridium sporogenes were detected in A4. The inactive spores of clostridia could be observed in 4-h wilted silage as no butyric acid was detected. It was concluded that the enhanced clostridial fermentation of direct-cut silage was attributed mainly to C. perfringens and C. sporogenes, while C. ghonii and C. sartagoforme were involved in the restricted clostridial fermentation of 2-h wilted silage.

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Introduction
In recent decades, rapid increase in population and accompanying food demand, particularly that of animal protein, has raised the importance of legume forage in livestock. Alfalfa (Medicago sativa L.) is one of the most common perennial legume forage occupying approximately 324.53 million tonnes worldwide in 2013 (FAO 2015), and it is harvested as both hay and silage for subsequent use in dairy and other livestock diets. However, alfalfa often is viewed as a difficult crop to ensile, primarily because of its high buffering capacity, low water soluble carbohydrates (WSC) content and vulnerability to undesirable secondary clostridial fermentations, especially when ensiled at dry matter (DM) content of less than 300 g/kg (Coblentz and Muck 2012).

Ensilage under farm conditions is not a completely controlled process, because various factors can influence the ensiling process, such as plant species, method of ensiling, climate and DM content (Monteiro et al. 1998; Namihira et al. 2010; Nishino et al. 2012). For the preparation of well-preserved silage, rapid development of the lactic acid fermentation is necessary to reduce the pH and inhibit the growth of inefficient and spoilage microorganisms such as clostridia, enterobacteria, yeast and moulds, which can cause degradation of plant material and/or production of undesirable metabolites (McDonald et al. 1991). However, the epiphytic lactic acid bacteria (LAB) of forages do not always reduce pH rapidly because the initial load can be too low, fast acidifying homofermentative species may be absent, inadequate WSC content of forage, stage of maturation or wilting and conservation procedures may delay or hinder development (Rossi and Dellaglio 2007). Accordingly, moist poorly preserved silage is obtained with high contents of butyric acid and ammonia nitrogen (NH₃–N), which is associated with clostridial activity (Pahlow et al. 2003). In addition to causing reductions in nutritional...
value, the clostridial fermentation affects the hygienic quality of silage by creating conditions that permit an increase in the numbers of pathogenic organisms and their toxins (Flythe and Russell 2004). Moreover, silage of poor microbiological quality has been identified as the main source of clostridia spore contamination in raw milk (Colombari et al. 2001). Therefore, monitoring of the ensiling process with respect to changes in fermentation characteristics and the clostridial community would be helpful for thoroughly understanding and improving alfalfa silage fermentation.

The clostridial community involved in ensiling has been described previously. Of the 84 species described in Bergey’s Manual (Cato et al. 1986), seven have been isolated from silage with more frequent inhabitation of Clostridium tyrobutyricum (Gibson 1965). Rossi and Dellaglio (2007) reported that the occurrence of six clostridia species, and particularly Clostridium perfringens, was associated with the undesirable fermentation of maize and alfalfa silages. However, the vast majority of these investigations utilised culture-based techniques, that while informative, are notorious for underestimating the level of the clostridial diversity (McGarvey et al. 2013). Recent advancements in molecular tools have enabled us to elucidate the entire structure of the complex microbial community found in nature. One frequently used method is denaturing gradient gel electrophoresis (DGGE) analysis, which can demonstrate the dynamics of each microbial constituent in microcosms (Namihira et al. 2010). DGGE analysis has been shown to help understand how LAB and non-LAB species function in the ensiling process for various crop species (Nishino et al. 2012). However, sufficient data are not yet available on the clostridial community involved in alfalfa silage.

In this study, four alfalfa cultivars [Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4)] were ensiled for 14, 28 and 56 days immediately after harvest (wilting for 0 h) or after wilting (2 h and 4 h), and changes in the clostridial community were assessed by DGGE analysis. Data on fermentation characteristics and microbial counts were also provided.

**Materials and methods**

**Plant material and laboratory silage preparation**

This study was organised as a four (alfalfa cultivars) × three (wilting durations) × three (storage periods) factorial arrangement. Four alfalfa cultivars [Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4)] were grown in experimental plots of the Beijing Academy of Agriculture and Forestry Sciences (N39°34’, E116°28’), Beijing, China. Triplicate experimental plots were used, each plot with an area of 3 × 10 m. Approximately 60 kg per hectare of chicken manure was applied prior to sowing, with no additional fertiliser being applied during the crop growing. The average soil organic matter in the experimental plots was 11.1 g/kg; effective N, P and K in the soil were 82.9, 19.0 and 115.0 mg/kg, respectively. First-cut alfalfa was randomly harvested artificially at full-bloom stage on 21 May 2014, from each of the three experimental plots, leaving a stubble of 10 cm. The direct-cut alfalfa was then immediately taken into the laboratory for processing. The alfalfa was divided into three wilting duration groups and wilted in the sun for 0 h (direct-cut alfalfa), 2 h or 4 h. Silage was prepared using a small-scale system for silage fermentation as described by Xu et al. (2008). For each alfalfa cultivar × wilting duration combination, approximately 200 g of alfalfa, chopped into about 20 mm lengths, was packed into plastic film bag silos (Hiroyu KN type, 180 × 260 mm, Asahikasei, Tokyo, Japan) and the bag silos were sealed with a vacuum sealer (BH 950, Matsushita, Tokyo, Japan). Silos were prepared in triplicate and stored at ambient temperature for 14, 28 and 56 days.

**Chemical and microbiological analyses**

The DM content was determined by drying the material in an oven at 65 °C for 48 h and grinding the dried material to pass through a 1 mm screen. Crude protein (CP) content was analysed according to the method of the AOAC (1990). The WSC content was determined using the method of Owens et al. (1999). Buffer capacity (BC) value was measured by the method of Layne and McDonald (1966). Fermentation products of silage were determined from cold-water extracts. Wet silage (10 g) was homogenised with 90 mL of sterilised distilled water, and then filtered through four layers of medical gauze and a qualitative filter paper. The pH was measured with a glass electrode pH metre (S20K, Mettler Toledo, Greifensee, Switzerland), and NH3–N content was determined by the method of Broderick and Kang (1980). The filtrate was further processed with a dialyser of 0.22 μm to determine organic acid contents, including lactic, acetic and butyric acids, by high performance liquid chromatography (HPLC) (LC-10A; Shimadzu, Tokyo, Japan). The analytical conditions were as follows: column, Shodex RSpak KC-8115-DVB gel C (8.0 mm × 30 cm, Shimadzu, Tokyo, Japan); oven temperature, 50 °C; mobile phase, 3 mmol/L HClO4; flow rate, 1.0 mL/min; injection volume 5 μL; detector, SPD-M10AVP (Shimadzu, Tokyo, Japan).
Wet silage (10 g) was blended with 90 mL of sterilised water and serially diluted (10⁻¹–10⁻⁶) in sterilised water before microbial enumeration. The numbers of LAB were measured by plate count on deMan, Rogosa, Sharpe agar (Difco Laboratories, Detroit, MI) incubated at 37°C for 48 h under anaerobic conditions (Anaerobic box, TE-HER Hard Anaerobox, ANX-1, Hirosawa Ltd., Tokyo, Japan). Yeast was counted on Potato Dextrose agar (Nissui Ltd., Tokyo, Japan) incubated for 48 h at 30°C. The numbers of enterobacteria were determined on Blue Light Broth agar (Nissui Ltd., Tokyo, Japan) incubated at 37°C for 48 h. Clostridia was counted on Clostridia Count agar (Nissui Ltd., Tokyo, Japan) using the Hungate technique. Cold-water extracts were heated to 80°C for 10 min to inactivate the vegetative cells and to trigger the germination of spores, and 200 μL aliquots of the diluted extracts were inoculated into Hungate tubes. The tubes were rolled on ice and incubated at 37°C for 7 days. Black colonies were identified as clostridia.

**Denaturing gradient gel electrophoresis (DGGE)**

The Hungate technique was used for clostridia enumeration, while DGGE was used for the clostridial community analysis and to distinguish between species. Cold-water extracts were heated to 80°C for 10 min to inactivate the vegetative cells and to trigger the germination of clostridia spores, and were incubated with Reinforced Clostridia Medium (Difco Laboratories, Detroit, MI) at 37°C for 7 days. After the enrichment, 5 mL of the cultures were transferred into conical tubes and centrifuged at 14,000 g at 4°C for 5 min. The settled bacterial cells were collected and subjected to DNA extraction by the method of Cocolin et al. (2004).

PCR was used to amplify a variable region (V3 region) of the bacterial 16S rRNA gene, with the GC-clamp attached to the forward primer 341F-GC (5'-CGCCGCGCCGCCTACGGGAGGCAGCAG-3') and the reverse primer 534R (5'-ATTACCGCGGCTGTGCTGG-3') (Muyzer et al. 1993). Amplification was performed in 50 μL of the following reaction mixture: 25 μL of 2× Taq PCR Master Mix, 0.5 μL of each primer (10 μmol/L), 2 μL of template DNA and double-distilled water was added to obtain a final volume of 50 μL. Touchdown PCR was performed in a TP600 Thermo cycler device (Takara Bio Inc., Shiga, Japan) via the following temperature cycling programme: (i) denaturation at 95°C for 4 min, (ii) denaturation at 95°C for 30 s, (iii) annealing at 65°C for 1 min, −0.5°C/cycle (touchdown), (iv) extension at 72°C for 2 min (v) repeat steps (vi)–(vii) for 20 cycles; (vii) denaturation at 95°C for 30 s; (viii) annealing at 55°C for 1 min; (viii) extension at 72°C for 2 min; (ix) repeat steps (vi)–(viii) for 15 cycles; (x) extension at 72°C for 7 min and (xi) hold at 4°C.

The GC-clamp PCR products were separated according to their sequences using a DCode Universal Mutation Detection System (Bio-Rad Ltd., San Diego, CA). The samples were applied directly onto 8% (w/v) polyacrylamide gel (40% acrylamide–bisacrylamide stock solution 37:5:1, Amresco Ltd., Radnor, PA) in a running buffer containing 0.5 × TAE (20 mmol/L Tris-acetate, pH 7.4; 10 mmol/L sodium acetate; 0.5 mmol/L Na₂-EDTA). The gels were prepared with a denaturing gradient from 40% to 70% of urea and formamide (7 mol/L urea and 40% (v/v) deionised formamide as the 100% denaturant). Electrophoresis was conducted at a constant voltage of 150 V for 10 h at 65°C. After electrophoresis, the gels were stained using GelGreen nucleic acid gel stain (Biotium Inc., Hayward, CA) for 30 min, and photographed under UV illumination.

**Cloning and sequencing of the DGGE bands**

DGGE bands of interest were excised from the gel with sterile scalpels, and soaked in 100 μL of gel lysis buffer [0.05 mol/L KCl; 1% (v/v) Tris-HCl, 1 mol/L, pH 8.0; and 0.1% (v/v) Triton-X-100] at 95°C for 15 min to allow the DNA to diffuse. Extracted DNA was amplified by PCR using the 341F (without GC-clamp, 5'-CCTACGGGAGGCAGCAG-3') and 534R primers, and the PCR products were purified using a Universal DNA Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China). The purified PCR products were cloned into Escherichia coli TOP10 competent cells (Tiangen Biotech Co., Ltd., Beijing, China) with the pMD18-T Simple Vector (Takara Bio Inc., Shiga, Japan). Plasmid DNA was isolated from the E. coli cells using the TIANprep Mini Plasmid Kit (Tiangen Biotech Co., Ltd., Beijing, China), and subjected to PCR (341F-GC and 534R primers) as described above. The PCR products were checked by DGGE to confirm the purity and the migrating position of the excised band. The plasmids with desirable inserts were sequenced using an ABI 3730XL sequencer (Applied Biosystems Inc., Foster City, CA), using M13F and M13R primers. The GenBank database was searched using the BLAST (Basic Local Alignment Search Tool) programme to determine the closest relatives of the partial 16S rRNA gene sequences. The nucleotide sequences of the V3 region of the 16S rRNA gene in this study were deposited in GenBank (accession numbers from KP895144 to KP895168 for DGGE bands from 1 to 25).
**Statistical analysis**

All microbial counts were log_{10} transformed to obtain log-normal distributed data. To calculate averages, the values below the detection level (detection levels: 50 cfu/g for clostridia and 100 cfu/g for LAB, enterobacteria and yeast, respectively) were assigned a value corresponding to half of the detection level (i.e. 25 cfu/g for clostridia and 50 cfu/g for LAB, enterobacteria and yeast, respectively). Silage chemical compositions and microbial counts data were analysed by a three-way ANOVA for a 4 × 3 × 3 (alfalfa cultivars × wilting durations × storage periods) factorial arrangement of treatments using the GLM procedures of SAS 9.1 (SAS Institute, Cary, NC, 2002), and the Tukey’s test was used for comparisons at 5% significance level.

**Results**

The DM content of direct-cut alfalfa ranged from 216 to 244 g/kg (Table 1), the WSC content was below 45 g/kg DM while the CP content was as high as 200 g/kg DM. The BC value was higher in direct-cut A4 alfalfa than in the other three cultivars. Enterobacteria, which was found as high as 10^7 cfu/g fresh matter (FM), dominated the microbial community of direct-cut alfalfa. Approximate 10^7 cfu/g FM of both LAB and yeast were found, while the clostridia counts were below the detection limit. Wilting of 2 h and 4 h increased the DM content to 332–354 and 416–438 g/kg, respectively. The WSC content, CP content and BC value were numerically lower in both wilted materials than in direct-cut alfalfa. The LAB and enterobacteria counts increased with wilting, especially for the LAB counts which were found as high as 10^6 cfu/g FM in both wilted alfalfa.

Intensive clostridial fermentation occurred in direct-cut alfalfa silage. After 14 days of ensiling, the lactic acid content was only 16.6–28.2 g/kg DM, but the contents of acetic acid, butyric acid and NH_3–N were as high as 12.4–26.5 g/kg DM, 3.4–5.6 g/kg DM and 59.9–96.4 g/kg total nitrogen (TN) for all alfalfa cultivars, respectively. The acetic acid content and pH remained high during ensiling. Prolonged ensiling led to a marked (p < 0.05) increase in the butyric acid and NH_3–N contents. The LAB and clostridia counts were approximate 10^8 and 10^3 cfu/g FM, respectively, during ensiling.

Pre-ensiled alfalfa had a mean pH of about 6.46. After 14 days of ensiling, a gradual decline in pH was observed, in particular, pH in direct-cut A4 silage was as high as 6.06 (Table 2). The pH was lower (p < 0.05) in both wilted silages than in direct-cut silage with an exception for A1, however the effect of the wilting time on pH among different cultivars was distinct after 14 days. For A1 and A4, pH in 4-h wilted silage was lower (p < 0.05) than in 2-h wilted silage, in contrast this was not observed for A2 and A3. Prolonged ensiling decreased (p < 0.05) pH of all silages except for direct-cut A4 (Tables 3 and 4). 4-h wilted silage had lower (p < 0.05) pH than direct-cut and 2-h wilted silages regardless of the cultivar after 56 days.

Irrespective of the wilting time, the lactic acid content was similar in A3 and A4 silages but lower (p < 0.05) than in A1 and A2 silages after 14 days. In 4-h wilted silage, the lactic acid content increased to a greater extent with prolonged ensiling when compared to the increase in the lactic acid content in direct-cut and 2-h wilted silages. After 56 days of ensiling, the lactic acid content in direct-cut A4 silage

**Table 1. Chemical composition and microbial count of pre-ensiled alfalfa.**

| Cultivar<sup>a</sup> | Wilting<sup>b</sup> | Chemical compositions<sup>c</sup>, g/kg DM | Microbial counts<sup>d</sup>, log_{10} cfu/g FM |
|---|---|---|---|
| | | DM<sup>e</sup> | WSC<sup>f</sup> | CP<sup>e</sup> | BC<sup>a</sup> | LAB<sup>b</sup> | ENT<sup>b</sup> | Clostridia<sup>b</sup> | Yeast<sup>b</sup> |
| A1 | 0-h | 6.50 | 243 | 45 | 204 | 551.5 | 4.39 | 6.89 | <1.70 | 3.85 |
| | 2-h | 6.44 | 354 | 42 | 203 | 528.5 | 6.34 | 7.30 | <1.70 | 4.03 |
| | 4-h | 6.35 | 438 | 38 | 199 | 532.2 | 6.49 | 8.58 | <1.70 | 3.50 |
| A2 | 0-h | 6.52 | 227 | 39 | 210 | 570.2 | 4.42 | 7.17 | <1.70 | 4.52 |
| | 2-h | 6.46 | 332 | 33 | 204 | 551.3 | 5.07 | 7.42 | <1.70 | 4.40 |
| | 4-h | 6.40 | 417 | 30 | 200 | 530.1 | 6.25 | 8.34 | <1.70 | 4.42 |
| A3 | 0-h | 6.59 | 216 | 41 | 193 | 540.2 | 4.44 | 7.28 | <1.70 | 3.95 |
| | 2-h | 6.35 | 339 | 39 | 191 | 522.0 | 6.09 | 7.40 | <1.70 | 4.34 |
| | 4-h | 6.31 | 416 | 36 | 188 | 518.3 | 6.60 | 8.24 | <1.70 | 3.65 |
| A4 | 0-h | 6.61 | 244 | 43 | 213 | 589.1 | 4.76 | 7.96 | <1.70 | 4.08 |
| | 2-h | 6.46 | 338 | 40 | 209 | 532.7 | 6.36 | 8.03 | <1.70 | 4.29 |
| | 4-h | 6.49 | 438 | 36 | 204 | 524.9 | 6.62 | 8.57 | <1.70 | 4.28 |

<sup>a</sup>Four alfalfa cultivars were Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4).

<sup>b</sup>Three wilting durations were wilting for 0 h (0-h), 2 h (2-h) and 4 h (4-h).

<sup>c</sup>DM: dry matter; WSC: water soluble carbohydrates; CP: crude protein; BC: buffering capacity, mE/kg DM.

<sup>d</sup>FM: fresh matter; LAB: lactic acid bacteria; ENT: enterobacteria.

<sup>e</sup>Means of three samples.
(generally undetectable) was lower \((p < .05)\) than in the other three cultivars (22.5–32.0 g/kg DM); in contrast there were no significant differences \((p > .05)\) among cultivars in the lactic acid content within either 2-h or 4-h wilted silages with an exception for 2-h wilted A2 silage.

At any storage period, both wilted silages had lower \((p < .05)\) acetic acid content compared to direct-cut silage with no effects due to the cultivar. While there were no significant differences \((p > .05)\) between 2-h and 4-h wilted silages in the acetic acid content. The acetic acid content in both wilted silages was consistent throughout the ensiling period, with it averaging 12.1 and 10.0 g/kg DM for 2-h and 4-h wilted silages, respectively. In contrast, the acetic acid content in direct-cut silage increased \((p < .05)\) because

### Table 2. Fermentation characteristics of alfalfa silage ensiled for 14 days.

| Cultivar | Wilting | pH | LA  | AA  | LA/AA | BA  | NH3-N | LAB | Clostridia |
|----------|---------|----|-----|-----|-------|-----|-------|-----|------------|
| A1       | 0-h     | 5.17<sup>a</sup> | 22.93<sup>b</sup> | 15.33<sup>bc</sup> | 1.54<sup>bc</sup> | 3.49<sup>b</sup> | 60.80<sup>b</sup> | 8.83<sup>c</sup> | 2.21<sup>bc</sup> |
|          | 2-h     | 5.35<sup>a</sup>  | 9.00<sup>e</sup>  | 9.61<sup>de</sup>  | 1.02<sup>de</sup> | 2.44<sup>c</sup> | 44.71<sup>c</sup> | 9.11<sup>bc</sup> | 1.40<sup>c</sup> |
|          | 4-h     | 5.23<sup>d</sup>  | 6.63<sup>*</sup>  | 10.89<sup>cde</sup> | 0.62<sup>ef</sup>  | 0.00<sup>d</sup> | 16.60<sup>d</sup> | 9.16<sup>bc</sup> | 1.60<sup>c</sup> |
| A2       | 0-h     | 5.55<sup>c</sup>  | 28.19<sup>a</sup> | 18.40<sup>b</sup>  | 1.56<sup>bc</sup> | 3.60<sup>b</sup> | 59.87<sup>b</sup> | 8.91<sup>de</sup> | 2.23<sup>bc</sup> |
|          | 2-h     | 4.96<sup>d</sup>  | 22.51<sup>b</sup> | 10.99<sup>cd</sup> | 2.05<sup>a</sup>  | 2.51<sup>c</sup> | 41.40<sup>c</sup> | 9.26<sup>d</sup> | 1.60<sup>c</sup> |
|          | 4-h     | 5.24<sup>c</sup>  | 7.33<sup>de</sup> | 6.80<sup>e</sup>  | 1.21<sup>bc</sup> | 0.00<sup>d</sup> | 10.64<sup>e</sup> | 9.39<sup>c</sup> | 1.40<sup>c</sup> |
| A3       | 0-h     | 5.63<sup>b</sup>  | 18.65<sup>c</sup> | 12.36<sup>cd</sup> | 1.52<sup>bc</sup> | 3.40<sup>bc</sup> | 61.10<sup>b</sup> | 8.91<sup>de</sup> | 2.23<sup>bc</sup> |
|          | 2-h     | 5.14<sup>b</sup>  | 18.05<sup>d</sup> | 10.50<sup>cde</sup> | 1.83<sup>d</sup>  | 2.31<sup>e</sup> | 39.68<sup>d</sup> | 9.25<sup>de</sup> | 2.00<sup>c</sup> |
|          | 4-h     | 5.27<sup>d</sup>  | 3.53<sup>f</sup>  | 9.04<sup>de</sup>  | 0.39<sup>f</sup>  | 0.00<sup>d</sup> | 13.94<sup>d</sup> | 9.00<sup>d</sup> | 1.93<sup>bc</sup> |
| A4       | 0-h     | 6.06<sup>a</sup>  | 16.62<sup>d</sup> | 26.16<sup>b</sup>  | 18.34<sup>bc</sup> | 1.43<sup>bc</sup> | 4.74<sup>b</sup>  | 79.57<sup>c</sup> | 8.62<sup>de</sup> |
|          | 2-h     | 4.96<sup>ef</sup> | 19.72<sup>cde</sup> | 9.61<sup>d</sup> | 2.06<sup>ab</sup> | 3.30<sup>c</sup> | 56.63<sup>d</sup> | 8.91<sup>de</sup> | 4.01<sup>a</sup> |
|          | 4-h     | 4.99<sup>gh</sup> | 19.98<sup>b</sup>  | 10.27<sup>de</sup> | 2.13<sup>ab</sup> | 0.00<sup>d</sup> | 19.70<sup>b</sup> | 8.70<sup>b</sup> | 1.60<sup>c</sup> |
|          | 0-h     | 4.95<sup>d</sup>  | 22.97<sup>bcd</sup> | 19.03<sup>b</sup>  | 1.23<sup>bc</sup> | 4.32<sup>d</sup> | 87.20<sup>a</sup> | 8.51<sup>c</sup> | 1.90<sup>b</sup> |
|          | 2-h     | 4.95<sup>de</sup> | 31.58<sup>a</sup> | 10.52<sup>d</sup> | 3.04<sup>d</sup>  | 3.19<sup>c</sup> | 46.50<sup>e</sup> | 8.91<sup>de</sup> | 1.40<sup>c</sup> |
|          | 4-h     | 4.95<sup>de</sup> | 22.59<sup>bcde</sup> | 12.02<sup>d</sup> | 2.13<sup>ab</sup> | 0.00<sup>d</sup> | 28.00<sup>f</sup> | 8.92<sup>de</sup> | 1.50<sup>c</sup> |
|          | 0-h     | 5.21<sup>de</sup> | 18.91<sup>b</sup>  | 15.91<sup>cd</sup> | 1.21<sup>bc</sup> | 4.32<sup>d</sup> | 87.20<sup>a</sup> | 8.91<sup>de</sup> | 1.40<sup>c</sup> |
|          | 2-h     | 5.40<sup>de</sup> | 21.07<sup>cde</sup> | 13.12<sup>cd</sup> | 1.21<sup>bc</sup> | 0.00<sup>d</sup> | 28.00<sup>f</sup> | 8.92<sup>de</sup> | 1.50<sup>c</sup> |
|          | 4-h     | 5.43<sup>de</sup> | 18.22<sup>de</sup> | 10.57<sup>d</sup> | 1.80<sup>bc</sup> | 0.00<sup>d</sup> | 31.81<sup>f</sup> | 8.80<sup>a</sup> | 1.80<sup>bc</sup> |
| SEM<sup>5</sup> | 0.032 | 0.452 | 0.937 | 0.077 | 0.202 | 5.978 | 0.031 | 0.081 |

Levels of significance<sup>6</sup>
- Cultivar (C): *** *** *** *** *** *** *** ***
- Wilting (W): *** *** *** *** *** *** *** ***
- C × W: *** *** *** *** *** *** *** ***

<sup>a–h</sup> Values in the same column with different superscript letters are significantly different \((p < .05)\).

<sup>1</sup> Four alfalfa cultivars are Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4).

<sup>2</sup> Three wilting durations are wilting for 0 h (0-h), 2 h (2-h) and 4 h (4-h).

<sup>3</sup> LA: lactic acid; AA: acetic acid; DM: dry matter; LA/AA: lactic to acetic acid ratio; NH<sub>3</sub>-N: ammonia nitrogen, g/kg total nitrogen.

<sup>4</sup> FM: fresh matter; LAB: lactic acid bacteria.

<sup>5</sup> SEM: standard error of the mean.

<sup>6</sup> *** \( p < .001 \); ** \( p < .01 \); * \( p < .05 \); NS = not significant \((p > .05)\).
of prolonged ensiling, and was highest (p < .05) for A4 (26.5–36.2 g/kg DM), intermediate (p < .05) for A2 (18.4–33.4 g/kg DM), lowest (p < .05) for A1 (15.3–21.6 g/kg DM) and A3 (12.4–21.8 g/kg DM).

The lactic to acetic acid ratio was higher (p < .05) in direct-cut and 2-h wilted silages than in 4-h wilted silage after 14 days of ensiling. The effect of the cultivar on the ratio among different wilting time was distinct after 14 days. The ratio in direct-cut silage was similar for A1, A2 and A3 but higher (p < .05) than for A4; the ratio in 2-h wilted silage was similar for A1 and A4 but lower (p < .05) than for A2 and A3; the ratio in 4-h wilted silage was similar for A1, A3 and A4 but lower (p < .05) than for A2. Both wilted silages had higher lactic to acetic acid ratio than direct-cut silage after 28 days of ensiling, but a significant difference (p < .05) was only observed between 2-h wilted and direct-cut silages with an exception for A1. After 56 days of ensiling, the ratio increased to more than 5 g/kg DM in both wilted silages. The NH3-N content increased to more than 100 g/kg TN in both wilted silages. The NH3-N content in both wilted silages was below 100 g/kg TN throughout the ensiling period, with mean values of 63.2 and 25.2 g/kg TN in 2-h and 4-h wilted silages, respectively. In contrast, the NH3-N content increased to more than 100 g/kg TN in direct-cut silage after 56 days of ensiling, and was highest (p < .05) for A4 (34.8 g/kg DM), intermediate (p < .05) for A2 (16.3 g/kg DM), lowest (p < .05) for A1 (5.5 g/kg DM) and A3 (5.6 g/kg DM).

Wilting resulted in lower (p < .05) NH3-N content compared to direct-cut silage among all cultivars and storage periods. The NH3-N content increased (p < .05) with prolonged ensiling. In direct-cut silage, A4 had higher (p < .05) NH3-N content than the other three cultivars at a given storage period. However, this difference because of the cultivar was not observed in both wilted silages. The NH3-N content in both wilted silages was below 100 g/kg TN throughout the ensiling period, with mean values of 63.2 and 25.2 g/kg TN in 2-h and 4-h wilted silages, respectively. In contrast, the NH3-N content increased to more than 100 g/kg TN in direct-cut silage after 56 days of ensiling, and was highest (p < .05) for A4 (200.6 g/kg TN), intermediate (p < .05) for A2 (167.2 g/kg TN), lowest (p < .05) for A1 and A3 (108.8 and 105.7 g/kg TN, respectively).

After 14 days of ensiling, LAB numbers increased to 10^5 cfu/g FM, and increased with the longer wilting time regardless of the cultivar. After 28 days of ensiling, LAB numbers in both wilted silages were not significantly different (p > .05) from the numbers in direct-cut silage, although they were numerically

| Cultivar | Wilting | pH | LA  | AA  | LA/AA | BA  | NH3-N | LAB | Clostridia |
|----------|---------|----|-----|-----|-------|-----|-------|-----|------------|
| A1       | 0-h     | 5.01 | 32.01 | 21.61 | 1.49 | 5.51 | 108.80 | 8.10 | 3.00       |
| A1       | 2-h     | 4.96 | 26.54 | 11.08 | 2.43 | 4.47 | 87.83 | 8.37 | 2.66       |
| A1       | 4-h     | 4.63 | 25.44 | 9.52  | 2.74 | 0.00 | 33.30 | 8.28 | 1.80       |
| A2       | 0-h     | 5.33 | 25.20 | 33.44 | 0.76 | 16.34 | 167.21 | 7.15 | 3.38       |
| A2       | 2-h     | 5.05 | 18.42 | 18.27 | 1.87 | 4.68 | 89.37 | 8.22 | 2.98       |
| A2       | 4-h     | 4.73 | 21.48 | 9.43  | 2.36 | 0.00 | 32.32 | 7.19 | 1.40       |
| A3       | 0-h     | 5.02 | 22.55 | 21.78 | 1.05 | 5.56 | 105.68 | 8.09 | 3.02       |
| A3       | 2-h     | 5.03 | 24.52 | 15.01 | 1.64 | 4.62 | 86.08 | 7.79 | 1.96       |
| A3       | 4-h     | 4.66 | 19.41 | 8.57  | 2.33 | 0.00 | 36.01 | 7.18 | 1.90       |
| A4       | 0-h     | 6.77 | 0.00  | 36.19 | 0.00 | 34.78 | 200.62 | 6.68 | 5.96       |
| A4       | 2-h     | 5.30 | 21.56 | 15.15 | 1.46 | 6.00 | 110.55 | 7.97 | 1.60       |
| A4       | 4-h     | 4.84 | 21.37 | 8.73  | 2.66 | 0.00 | 38.86 | 7.91 | 1.70       |

Levels of significance:
- **p < .001,** **p < .01,** **p < .05,** NS = not significant (p > .05).

| Cultivar | Wilting | pH | LA  | AA  | LA/AA | BA  | NH3-N | LAB | Clostridia |
|----------|---------|----|-----|-----|-------|-----|-------|-----|------------|
| A1       | 0-h     | 5.01 | 32.01 | 21.61 | 1.49 | 5.51 | 108.80 | 8.10 | 3.00       |
| A1       | 2-h     | 4.96 | 26.54 | 11.08 | 2.43 | 4.47 | 87.83 | 8.37 | 2.66       |
| A1       | 4-h     | 4.63 | 25.44 | 9.52  | 2.74 | 0.00 | 33.30 | 8.28 | 1.80       |
| A2       | 0-h     | 5.33 | 25.20 | 33.44 | 0.76 | 16.34 | 167.21 | 7.15 | 3.38       |
| A2       | 2-h     | 5.05 | 18.42 | 18.27 | 1.87 | 4.68 | 89.37 | 8.22 | 2.98       |
| A2       | 4-h     | 4.73 | 21.48 | 9.43  | 2.36 | 0.00 | 32.32 | 7.19 | 1.40       |
| A3       | 0-h     | 5.02 | 22.55 | 21.78 | 1.05 | 5.56 | 105.68 | 8.09 | 3.02       |
| A3       | 2-h     | 5.03 | 24.52 | 15.01 | 1.64 | 4.62 | 86.08 | 7.79 | 1.96       |
| A3       | 4-h     | 4.66 | 19.41 | 8.57  | 2.33 | 0.00 | 36.01 | 7.18 | 1.90       |
| A4       | 0-h     | 6.77 | 0.00  | 36.19 | 0.00 | 34.78 | 200.62 | 6.68 | 5.96       |
| A4       | 2-h     | 5.30 | 21.56 | 15.15 | 1.46 | 6.00 | 110.55 | 7.97 | 1.60       |
| A4       | 4-h     | 4.84 | 21.37 | 8.73  | 2.66 | 0.00 | 38.86 | 7.91 | 1.70       |

Levels of significance:
- **p < .001,** **p < .01,** **p < .05,** NS = not significant (p > .05).
higher in both wilted silages. The effect of the cultivar on LAB numbers among different wilting time was distinct after 56 days. LAB numbers in direct-cut silage were higher ($p < .05$) for A1 and A3 than A2 and A4; the numbers in 2-h wilted silage were similar among all cultivars; A1 and A4 had higher ($p < .05$) LAB numbers in 4-h wilted silage compared to A2 and A3.

Clostridia numbers in direct-cut silage increased to $10^2$ cfu/g FM after 14 days of ensiling, in particular, the numbers in direct-cut A4 silage were as high as $10^6$ cfu/g FM. In contrast, both wilted silages had clostridia numbers of less than $10^6$ cfu/g FM with an exception for 4-h wilted A4. An increase in clostridia numbers was not observed in all direct-cut and wilted silages after 28 days of ensiling, except for direct-cut A4 in which the numbers increased to $10^5$ cfu/g FM. The effect of the cultivar on clostridia numbers among different wilting time was also distinct after 56 days. In direct-cut silage, A4 ($10^6$ cfu/g FM) resulted in a more rapid increase ($p < .05$) in clostridia numbers than the other three cultivars (mean $10^3$ cfu/g FM); in 2-h wilted silage, clostridia numbers were higher ($p < .05$) for A1 and A2 than A3 and A4; there were no significant differences ($p > .05$) in clostridia numbers in 4-h wilted silage among all cultivars.

The clostralidial community determined from the 16S rRNA gene sequences is shown in Figure 1. A total of eight clostridia species were detected in pre-ensiled alfalfa and alfalfa silage: *C. perfringens*, *Clostridium sporogenes*, *Clostridium ghonii*, *Clostridium sartagoforme*, *Clostridium proteolyticum*, *Clostridium baratii*, uncultured *Clostridium* sp. and *C. tyrobutyricum*. The most distinct bands corresponded to *C. perfringens* and were observed on the DGGE gel in the case of pre-ensiled alfalfa. *C. proteolyticum* was also present in pre-ensiled alfalfa, but bands for *C. proteolyticum* was faint when compared to those for *C. perfringens*. In addition, *C. sporogenes*, *C. baratii* and *C. ghonii* were also observed in a limited number of pre-ensiled alfalfa samples. However, bands for *C. proteolyticum* and *C. baratii* were not detectable after ensiling. In contrast, bands for *C. sartagoforme*, *C. tyrobutyricum* and uncultured *Clostridium* sp., which were undetectable in pre-ensiled alfalfa, were found in several silages. After 14 days of ensiling, distinct bands for *C. perfringens* were observed in the case of direct-cut A1, A2 and A3 silages, while a distinct band was observed for *C. sporogenes* in the case of direct-cut A4 silage. These bands were also clearly seen in the corresponding silages after 56 days of ensiling, puzzlingly, they were not observed after 28 days. Bands for *C. ghonii* were common to all direct-cut and wilted silages after 14 and 28 days of ensiling, although they were faint when compared to those for *C. perfringens* and *C. sporogenes*. Faint bands were observed for *C. sartagoforme* in the case of all 56-day silages. Besides *C. ghonii* and *C. sartagoforme*, bands for *C. perfringens*, *C. sporogenes*, *C. tyrobutyricum* and uncultured *Clostridium* sp. were observed in a limited number of wilted silages. The presence of these clostridia species, however, did not show a marked association with butyric acid production in 4-h wilted silage. In addition, two LAB, *Lactobacillus plantarum* and *Pediococcus acidilactici*, were uniformly distributed in all silages.

**Discussion**

In this study, intensive clostralidial fermentation was seen in the case of direct-cut alfalfa silage using the thresholds described by Pahlow et al. (2003), likely due to the low contents of DM, WSC and LAB numbers and the high BC value. For the forages studied, initial WSC content was lower than 60–70 g/kg DM which was recommended as theoretical requirement to achieve well-preserved fermentation (Smith 1962). Heinritz et al. (2012) reported that the fermentability coefficient which was based on chemical pre-condition of pre-ensiled materials (e.g. DM, WSC, BC, nitrate and tannin) predicted quite well the silage quality for a couple of materials. In this study, higher BC value in direct-cut A4 alfalfa could partly contribute to the higher pH in direct-cut A4 silage than in the other three cultivars.

Nishino et al. (2012) suggested that *Lactobacillus plantarum* was involved in the increase in acetic acid content with prolonged ensiling, because *L. plantarum* can metabolise lactic acid to acetic acid under sugar-deficient conditions. The same metabolic process could have occurred in the case of direct-cut silage because of prolonged ensiling. Homofermentative LAB, *P. acidilactici*, was likely to be associated with the increase in lactic acid content. In this study, we found that the lactic to acetic acid ratio decreased with prolonged ensiling in direct-cut silage. This effect was ascribed to a large increase in acetic acid rather than changes in lactic acid contents (Parvin and Nishino 2009). In addition, it seemed that heterofermentative LAB metabolism persisted in direct-cut A4 silage as the lactic to acetic acid ratio remained lower than 1.0. Therefore, it suggested that a decline in the lactic to acetic acid ratio of direct-cut silage could be because of the production of acetic acid from lactic acid and the enhanced activity of heterofermentative LAB. As a result of the enhanced acetic acid production, pH decreased slowly, thereby allowing the clostralidial fermentation of direct-cut silage (Pahlow et al. 2003).
One of the critical features of a forage to be ensiled is the proper DM content. Undesirable fermentation of direct-cut alfalfa silage without any additives was observed in previous studies (Wang et al. 2009; Bai et al. 2011; Coblentz and Muck 2012). Therefore, alfalfa is often wilted to contents above 300 g DM/kg before ensiling to reduce the chances of the clostridial fermentation (Leibensperger and Pitt 1987). Conditions in some geographical areas for wilting silage are particularly good, and it is common to find silages containing more than 450 g DM/kg (Whiter and Kung 2001). In this study, DM content of alfalfa was about 332–354 and 416–438 g/kg after wilting for 2-h and 4-h, respectively. Results showed that acetic and butyric acids contents were lower in silage with higher DM content. Therefore, microorganisms responsible for acetic and butyric acids production in silages were limited to growth because of the increased DM content (Whiter and Kung 2001; Pahlow et al. 2003).

When a forage crop is ensiled, an extensive hydrolysis of protein occurs, which affects fermentation by counteracting the desired rapid fall in silage pH and results in low efficiency of nitrogen utilisation by ruminants (Wang et al. 2009). In this study, an extensive

![Denaturing gradient gel electrophoresis (DGGE) analysis of the clostridial community as influenced by alfalfa cultivars, wilting durations, and storage periods.](image)

**Figure 1.** Denaturing gradient gel electrophoresis (DGGE) analysis of the clostridial community as influenced by alfalfa cultivars (Sanditi (A1), AC Caribou (A2), WL319HQ (A3) and 4030 (A4)), wilting durations (0-h, 2-h and 4-h) and storage periods (14, 28 and 56 days). M, DNA marker; DGGE bands: 1, *Lactobacillus plantarum*; 2, *Enterococcus faecium*; 3, *Pediococcus pentosaceus*; 4, *Enterococcus faecalis*; 5, uncultured *Clostridium* sp.; 6, *Pediococcus acidilactici*; 7, *Bacillus* sp.; 8, *Clostridium perfringens*; 9, *Clostridium sporogenes*; 10, *Clostridium perfringens*; 11, *Clostridium perfringens*; 12, *Clostridium perfringens*; 13a and 13b, *Clostridium perfringens*; 14, *Clostridium baratii*; 15, *Clostridium ghonii*; 16, *Clostridium proteolyticum*; 17, *Weissella hellenica*; 18, *Pediococcus loli*; 19, *Escherichia fergusonii*; 20, *Clostridium ghonii*; 21, *Lactobacillus helsingborgensis*; 22, *Lactobacillus gasseri*; 23, *Lactobacillus oris*; 24, *Clostridium sartagoforme*; 25, *Clostridium tyrobutyricum*.
proteolysis was observed in direct-cut silage as indicated by high NH$_3$–N content. Wilting is beneficial because a more rapid fall in silage pH can result in less proteolysis and deamination, and therefore lower NH$_3$–N content (Moselhy et al. 2015). Whiter and Kung (2001) reported similar findings in alfalfa silages with DM content of 300 and 540 g/kg. The formation of NH$_3$–N reflects part of the proteolysis occurring during ensiling and is explained by plant enzymatic, clostridial (Heron et al. 1989), proteolytic activity of microorganisms might have contributed to the production of NH$_3$–N after a longer storage period. When relating the butyric acid content, as an indicator of the clostridial fermentation, to NH$_3$–N, NH$_3$–N content in direct-cut and 2-h wilted silages could be most likely explained by clostridial activity. However, the bacteria responsible for the increased NH$_3$–N content in 4-h wilted silage were difficult to identify. Small NH$_3$–N increases in non-clostridial silages can be caused by plant enzymes, lactic acid bacteria, other bacteria and nitrate reduction (Tremblay et al. 2001).

Both *C. perfringens* and *C. sporogenes* are clostridia known to be associated with anaerobic deterioration of silage (Flythe and Russell 2004; Rossi and Dellaglio 2007). It was reported that *C. perfringens* and *C. sporogenes* could ferment proteins, protein hydrolysates or amino acids and produce large amounts of acetic and butyric acids (Cato et al. 1986; Allison and Macfarlane 1990; Myers et al. 2006). In this study, *C. perfringens* and *C. sporogenes* have mainly contributed to the clostridial fermentation in direct-cut alfalfa silage. *C. sporogenes*, which is classified as a proteolytic member of the genus *Clostridium*, has high specific activity of protein degradation than the saccharolytic species *C. perfringens*. In addition, protease production by *C. sporogenes* is strongly enhanced under carbohydrate-limiting conditions (Allison and Macfarlane 1990). Therefore, higher contents of butyric acid and NH$_3$–N in direct-cut A4 silage than in the other three cultivars could partly attribute to *C. sporogenes*. The disappearance of bands for *C. perfringens* and *C. sporogenes* in direct-cut silage after 28 days of ensiling is puzzling. Because we did not examine some faint bands, the entire clostridial community was not revealed in this study.

The effect of wilting on alfalfa silage fermentation can be partly accounted for by changes in the clostridial community, with a particular inhibition of *C. perfringens* and *C. sporogenes*. In contrast, *C. ghonii* and *C. sartagoforme* were still found in 2-h wilted silage. *C. ghonii* and *C. sartagoforme* have been found in soil, marine sediments, rumens, soft-tissue infections in humans and human faeces (Cano et al. 2000; Nathani et al. 2016), but have never been detected in silage. From this study, however, alfalfa silage can be regarded as a habitat of *C. ghonii* and *C. Sartagoforme*. The appearance of *C. ghonii* and *C. sartagoforme* in 2-h wilted silages might suggest that they are low water activity-tolerant. Protein degradation and butyric acid production characterisations were also observed in *C. ghonii* and *C. sartagoforme* (Hashimi et al. 2013; Zhang et al. 2015; Nathani et al. 2016). The findings reported here might possibly point to a more important role for *C. ghonii* and *C. sartagoforme*, their uniform distribution indicated that *C. ghonii* and *C. sartagoforme* could be attributed mainly to the clostridial fermentation in 2-h wilted silage. When the environment became more unfavourable in 4-h wilted silage, the clostridial community existed as inactive spores as no butyric acid was detected.

Further experiments, including isolation and characterisation of *C. perfringens*, *C. sporogenes*, *C. ghonii* and *C. sartagoforme* and examination of the effect of inoculating alfalfa silage with these clostridia species, are now in progress to complement the results of this study. Most studies in which clostridia were isolated from silage were directed at problems with late blowing of cheese (Garde et al. 2011). Bacteriocin-producing LAB strains, such as *Lactobacillus paracasei* complex and *Lactococcus lactis* IFPL 3593, have been screened for control of late blowing of cheese by their ability to inhibit the growth of clostridia (Christiansen et al. 2005; Carmen Martinez-Cuesta et al. 2010). Similarly, in future research, we hope that LAB strains with antimicrobial activity against *C. perfringens*, *C. sporogenes*, *C. ghonii* and *C. sartagoforme* can be screened and used as inoculants to improve alfalfa silage quality by suppressing the clostridial fermentation.

**Conclusions**

For direct-cut alfalfa silage, *C. sporogenes* resulted in the enhanced clostridial fermentation of A4, while *C. perfringens* was associated with the clostridial fermentation of the other three cultivars. Wilting could be regarded as an efficient way to depress the clostridial fermentation. *C. ghonii* and *C. sartagoforme* were involved in the restricted clostridial fermentation of 2-h wilted silage. The clostridial fermentation was completely inhibited in 4-h wilted silage as no butyric acid was detected. We also found that high acetic acid...
production resulted in a slow drop in pH, thereby allowing the clostridial fermentation.

**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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