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ORIGINAL ARTICLE

Effect of temperature (5–25°C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage

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Abstract

Aims: The objective of this study was to investigate the effect of temperature (5–25°C) on epiphytic lactic acid bacteria (LAB) populations during 60 days of fermentation of whole-plant corn silage.

Methods and Results: Vacuum bag mini-silos of chopped whole-plant corn were incubated at five different temperatures (5, 10, 15, 20 and 25°C), according to a completely randomized design with four repetitions. The silos were opened and sampled on day 0, 1, 2, 3, 7, 28 and 60. At 20 and 25°C, *Lactobacillus plantarum*- and *Pediococcus pentosaceus*-related operational taxonomic units (OTU) dominated the fermentation within 1 day. After 7 days, the OTU related to the heterofermentative species *Lactobacillus buchneri* began to appear and it eventually dominated silages incubated at these temperatures. Population dynamic of LAB at 5 and 10°C was different. At these temperatures, *Leuconostoc citreum* OTU was identified at the beginning of the fermentation. Thereafter, *Lactobacillus sakei*- and *Lactobacillus curvatus*-related OTU appeared and quickly prevailed. Corn silage at 15°C acted as a transition between 20–25°C and 5–10°C, in terms of LAB diversity and succession.

Conclusion: The conditions of silage incubation temperature affect species diversity of LAB population with notable difference along the temperature gradient. Colder temperature conditions (5 and 10°C) have led to the identification of LAB species never observed in corn silage.

Significance and Impact of the Study: This study demonstrated the impact of temperature gradient on the diversity and some important population shift of lactic acid bacteria communities during fermentation of corn silage.

Introduction

Silage is a very important source of forage for ruminant animals, in particular in areas where animals need to be wintered for long periods of time. It is produced by the anaerobic fermentation of freshly harvested forages and other crops, generally at a dry matter (DM) level varying between 280 and 450 g kg^{-1}. Lactic acid bacteria (LAB), which convert water-soluble carbohydrates (WSC) into lactic acid and other organic acids, are the main organisms responsible for optimal silage fermentation. As a consequence of their activity, pH is reduced and the activity of spoilage microbes is inhibited, allowing the conservation of forage nutrients (McDonald *et al.* 1991). Epiphytic LAB species, particularly those that dominate the ensiling process, together with other microflora, often dictate the course of fermentation and, accordingly, determine silage quality (Cai *et al.* 1998). Optimal ensiling will prevent growth of undesirable micro-organisms like *Clostridium*, reduce proteolysis to lower soluble nitrogen and prevent protein degradation to ammonia.

Temperature is a factor affecting ensilage. Generally speaking, a moderate temperature from 20 to 30°C is preferred for silage fermentation. The impacts of high temperatures (>37°C) have been studied due to their well-known detrimental effects on forage preservation, for example, resulting in poor quality silage and low aerobic stability (Weinberg *et al.* 2001; Kim and Adesogan 2006), inducing clostridial fermentation (McDonald *et al.* 1966), heat damage (Goering *et al.* 1973; Garcia *et al.* 1989), etc. Heat damages of silage directly reduces the feed quality by increasing carbohydrates degradation, from increasing Maillard reactions between carbohydrates and proteins, and from degradation of proteins. However, the effects of low temperature (<20°C) on silage have hardly been studied. But, in cold climates, such as Canada's, low temperature may be an important environmental factor during ensiling. In practice, low temperature could hinder silage production. Such silages are often found to have high pH value, low rate of pH decline (Ali *et al.* 2015), low acids production (Kung 2010b), as well as more residual WSC and high yeast counts. Even, added silage inoculants may be impaired and, therefore, may not be very effective at low ambient temperatures (Weinberg and Muck 1996). Recently, Wang *et al.* (2011) studied the effects of a lab-made LAB inoculant on a reed grass silage (*Phragmites australis*) at 0 and 4°C. It was found that it would take at least 6 weeks to get fairly good silage quality at an inoculation level of 6·63 $\log_{10}$ CFU g^{-1} DM.

Whole-plant corn silage is one of the most important forages for ruminants in Central Europe and North America. Several studies have investigated the diversity of epiphytic LAB during the corn ensiling process (Lin and Bolsen 1992; Brusetti *et al.* 2006; Stevenson *et al.* 2006; Parvin *et al.* 2010). However, all these studies were conducted at constant temperatures set between 20 and 30°C. These studies did not consider temperature as a factor which could affect the epiphytic LAB population diversity, and none of them examined the epiphytic LAB population at a temperature below 20°C (Lin and Bolsen 1992; Brusetti *et al.* 2006; Stevenson *et al.* 2006; Parvin and Nishino 2010). However, low temperature not only could reduce fermentation efficiency by lowering the growth rate and enzymatic activity of micro-organisms, but it also may change the composition of LAB flora in different ecosystems, by selecting certain species adapted to low growth temperatures. For instance, Hagi *et al.* (2004) reported that the changes in water temperature (4°C to above 20°C) resulted in the seasonal changes in LAB composition in the intestinal tract of freshwater fish. Olstorpe *et al.* (2008) found that the population diversity of LAB in a fermented pig feed differed at different low temperatures (10, 15 and 20°C). Unfortunately, to date, no relevant research studies were conducted on silages.

In the last decades, genetic improvement has expanded corn cultivation further north by developing hybrids adapted to colder regions. In Eastern Canada, whole-plant corn is often harvested and directly ensiled mainly between late September and early November, when daily mean temperatures are between 0 and 10°C. This low-temperature condition could greatly affect epiphytic LAB population and accordingly the fermentation in corn silage. Therefore, the aim of this study was to investigate the effects of temperatures ranging between 5 and 25°C upon epiphytic LAB population and the fermentation pattern during the ensiling process of whole-plant corn.

Materials and methods

Silage preparation

Corn hybrid (Dekalb D26-78) was seeded in Témiscamingue, Québec, Canada (the average Corn Heat Unit in this region is around 2300), at a density of 74 000 plants ha^{-1}. A total of 150 kg N ha^{-1}, 70 kg P ha^{-1} and 40 kg K ha^{-1} were applied during the growing season. Corn was harvested after 140 days of growth and chopped (average particle size of 1·0 cm) with a forage harvester (New Holland 900, New Holland Inc., New Holland, PA, USA) not equipped with a kernel processor. The mean DM level of the fresh chopped material was 308·3 g kg^{-1} ($P = 0{\cdot}381$) and the kernels were at half milk line. No inoculant was added on the forage. For each experimental silo, about 400 g of fresh chopped corn was filled into polyvinyl fluoride film bags (25·4 × 40·6 cm; 0·0508 mm of thickness; Tedlar, SKC Inc. Eighty Four, PA, USA; oxygen permeability of 50 cm^3 m^{-2} day^{-1} bar^{-1}). Air was removed using a commercial vacuum sealer (Nel 216/219M; Hi-Tech Vacuum, Saint-Cyrille-de-Wendover, QC, Canada) as described by Johnson *et al.* (2005). The vacuum bags were not purged during the incubation. Eight bags were resealed in the first 5 h of incubation due to presence of forage along the seams. The trial was a complete randomized design with a five ensiling temperatures (5, 10, 15, 20 and 25°C) x seven openings (0, 1, 2, 3, 7, 28 and 60 days) factorial arrangement of treatments. In order to minimize field-related variation of corn physiological status, the forage was collected from the same trailer of chopped corn, immediately after unloading at the bunker. The forage was immediately transported to the laboratory in 50-l containers and the silos were prepared within 1 h after the harvest. Four repetitions of the treatments were performed. The repetitions were distributed along treatments in relation with ensiling time to potentially block this factor of variation. Incubations at 5, 10 and 15°C were performed in refrigerated incubators (Model New Brunswick Innova 44R; Eppendorf, Hauppauge, NY, USA) while incubation

at 20 and 25°C were performed in growth chamber (BDR16, Conviron, Winnipeg, MB, Canada) and a convection laboratory incubator (BD, Binder, Tuttlingen, Germany) respectively. In addition, one extra silo of each temperature treatment was prepared for the LAB isolation, in order to develop a LAB reference ladder for denaturing gradient gel electrophoresis (DGGE) profiling. Extra silos were incubated for 14 days.

PCR-DGGE analysis

Diversity analysis of LAB was conducted using PCR-DGGE fingerprinting. Total DNA was extracted from 20 g of silages suspended in 180 ml of peptone buffer and shaken for 120 s in a paddle shaker (Seward, Inc., Worthing, UK). A 1 ml aliquot of the corn silage suspension was centrifuged and the pellet was transferred to the beads-containing tubes of the PowerFood™ Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, NM) according to the manufacturer's instructions (Marsh *et al.* 2013). Microbial cells lysis was optimized by a 2 min' mechanical lysis in a MixerMill 400 (Retsch, Inc., Haan, Germany) at a speed of 15 revolutions per second. Primers L1 (5′-CAG CAG TAG GGA ATC TTC C-3′) (Meroth *et al.* 2003) and HAD2 (5′-GTA TTA CCG CGG CTG CTG GCA-3′) (Tannock *et al.* 1999), specific to *Lactobacillus*-group including genus *Lactobacillus*, *Weissella*, *Pediococcus* and *Leuconostoc*, were used to amplify a 185-bp fragment of the V3 region of the 16S rRNA genes. A 40-bp GC clamp (5′-CGC CCG GGG CGC GCC CCG GGC GGC CCG GGG GCA CCG GGG G-3′) was attached to the 5′ end of L1 for DGGE analysis (Walter *et al.* 2001). PCR was performed according to Meroth *et al.* (2003) using same *Taq* PCR Kit. PCR reactions (25 μl) contained 1 μl of DNA template (50 ng), 1× standard *Taq* reaction buffer, 200 μmol l^{-1} of each deoxynucleotide, 0·5 μmol l^{-1} of each primer and 0·05 U μl^{-1} of *Taq* DNA polymerase. PCR cycles comprised an initial DNA denaturation at 95°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 66°C for 30 s, extension at 68°C for 1 min and a final elongation step at 68°C for 7 min.

DGGE was carried out using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) according to Kebli *et al.* (2011) with a modification of the range of denaturing gradient. PCR products (10 μl) were applied on 8% polyacrylamide gels (acrylamide: bis-acrylamide, 37·5 : 1) with a linear denaturing gradient range of 41–50% in 1× TAE electrophoresis buffer. Electrophoresis was performed at 75 V and 60°C for 16 h. Then the gels were stained with SYBR Gold (Invitrogen, Carlsbad, NM) and visualized under UV illumination using a Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories). In addition, PCR products of nine LAB strains previously isolated from the same silages were combined and served as an external reference pattern to which all gels were aligned. DGGE profiles were analysed using GelCompar II ver. 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Band matching analysis was performed by excluding rare bands and by assigning multiple bands deriving from same LAB species into single band class. One matrix of band-relative intensity and one of band presence/absence were obtained for further diversity analyses. The relative intensity of each band was calculated by dividing the intensity of the band by the sum of the intensity of all the bands within the lane.

Diversity analysis of *Lactococcus*-group was carried out using a primer set consisting of Lac3 (5′-AGC AGT AGG GAA TCT TCG G-3′) and Lac2GC (5′-CGC CCG GGG CGC GCC CCG GGC GGC CCG GGG GCA C CG GGG GAT TTC ACC GCT ACA CAT G-3′), which amplified a 380-bp fragment of the V3 region of the 16S rDNA genes. This primer set is specific to the following genera: *Lactococcus*, *Streptococcus*, *Enterococcus*, *Tetragenococcus* and *Vagococcus* (Endo and Okada 2005). The PCR amplification was performed as described by Endo and Okada (2005) but with an optimized annealing temperature of 59°C. DGGE was conducted using a 8% polyacrylamide gel with a denaturing gradient range of 30–70%, at 75 V, 60°C for 20 h.

Denaturing gradient gel electrophoresis reference ladder using epiphytic lactic acid bacteria strains isolated from silages

One extra silo of each temperature treatment (5, 10, 15, 20 and 25°C) was taken out for LAB isolation after 14 days of fermentation. Each silage sample (20·0 g) was homogenized with 180 ml of peptone water (0·2% Bacto peptone (w/v) with 0·01% Tween 80 (w/v)), and serial dilutions were prepared with the same peptone water. A MRS medium (Difco) was used and the Petri dishes were incubated at 30°C for 3 days. A mean of 20 colonies was randomly picked up from each temperature treatment. All purified isolates were characterized by their Gram reaction, catalase activity and cell morphology.

In order to design the molecular ladder that would be used for the DGGE, total DNA of LAB isolates was extracted using a hexadecyltrimethylammonium bromide (CTAB) method (Griffiths *et al.* 2004). The 16S rRNA gene of LAB isolates was screened and grouped by PCR-DGGE based on the mobility differences of their responding bands, where a primer set of L1GC/HAD1 was used as described below. Representatives (1–7 isolates) of each group having the same migration distance on the DGGE gel were selected. Their DNA was then amplified using a

universal eubacterial primer set consisting of pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′), which can amplify a contiguous sequence of a nearly complete 16S rRNA gene spanning 1·5 kb (Edwards *et al.* 1989). PCR was carried out in a volume of 25 μl containing 1 μl of DNA template (50 ng), 1× standard *Taq* reaction buffer, 200 μmol l^{-1} of each deoxynucleotide, 0·2 μmol l^{-1} of each primer and 0·025 U μl^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit; New England BioLabs, Whitby, ON, Canada). PCR cycles comprised an initial DNA denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, extension at 68°C for 1 min and a final elongation step at 68°C for 5 min. Finally, sequence analysis was conducted with a BigDye® Terminator v3.1 Cycle Sequencing Kit on the genetic analyser 3130XL (Applied Biosystems, Foster City, CA) at the Plate-forme d'Analyses Biomoléculaires (Université Laval, Canada). PCR amplicons were sequenced in both directions and nucleotide sequences were aligned using software BioEdit ver. 7.1.3.0. (Ibis Biosciences, Carlsbad, CA, USA) DNA sequence similarity searches were run via blastn against the GenBank database of the United States National Center for Biotechnology Information (NCBI). The sequences generated by this study were deposited in GenBank (accession numbers: KC753453 to KC753463).

A total of 73 Gram-positive, catalase-negative, rod- or coccus-shaped LAB strains were purified. Based on PCR-DGGE analysis, they were classified into eleven putative representative operational taxonomic units (OTU). DNA of 27 out of the 73 LAB strains were further amplified with primers pA/pH and sequenced. The sequences were compared to Genbank database using the blastn algorithm. The eleven putative OTU were related to: *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Pediococcus pentosaceus*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Leuconostoc citreum*1 and *Leuc. citreum*2 (with single-base difference from *Leuc. citreum*1). It was observed that *Lact. buchneri* produced multiple bands under the DGGE gel parameters used. The PCR products of the nine strains consisting of *Lact. plantarum*, *Leuc. citreum*1, *Leuc. citreum*2, *Lact. brevis*, *Leuc. lactis*, *Lact. sakei*, *Lact. coryniformis* subsp. *torquens*, *Ped. pentosaceus* and *Lact. buchneri*, were combined into one sample which was thereafter used as an external reference pattern on each DGGE gel (Table S1).

Denaturing gradient gel electrophoresis band excision, cloning and sequencing

Bands on DGGE gels that could not be aligned with our LAB external reference strains were excised from polyacrylamide gel. Their DNA was eluted with 70 μl sterile deionised water at 4°C overnight and subsequently used as a template for a nested PCR using primers L1GC/HAD2 as described above. Confirmation of the positions of these nested PCR products was verified by running DGGE along with the PCR products of original silage samples. Amplicons were then purified using Wizard® SV Gel and the PCR Clean-Up System (Promega, Madison, WI) and cloned using the pGEM®-T Easy Vector System II (Promega) according to the manufacturer's instruction. Positive clones, a minimum of two for each band, were screened and streaked twice, and their plasmids were isolated using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). Sequence analysis was done by the Plate-forme d'Analyses Biomoléculaires as previously described, but with Sp6 and T7 promoter primers. The nucleotide sequences were aligned using BioEdit software and DNA sequence similarity searches were done via blastn against the NCBI.

Biochemical analyses

A total of 20 g silage from each treatment was sampled for pH measurement on day 0, 1, 2, 3, 7, 28 and 60. Silage pH was measured with a pH meter (Accumet® AB15; Fisher Scientific, Ottawa, ON, Canada), where triplicate subsamples (10·0 g) were macerated with 100 ml distilled water for 60 min at 4°C (Parker 1979). Four samples per temperature treatment of the corn forage (100 g) were collected prior to ensiling (day 0) for the determination of DM content, total N, WSC and buffering capacity. DM content was determined by oven drying after 72 h at 50 ± 2°C. Total N was determined according to the method 7·022 of AOAC (2005). WSC was measured on a water extract (100 mg: 25 ml distilled water) using the phenol sulphuric acid colorimetric method according to Dubois *et al.* (1956). Buffering capacity was determined by lactic acid titration (DeMarquilly 1986). In addition, 100 g of final silages (day 60) were sampled for the determination of DM content, total N, WSC, ammonia, lactic acid, ethanol and volatile fatty acids (VFA, that is, acetic acid, propionic acid, n-butyric acid and iso-butyric acid). For lactic acid and ammonia concentration, a 20·0 g subsample of silage was macerated in 200 ml of 0·1N HCl for 60 min with frequent manual shaking, centrifuged 5 min at 200 rev min^{-1} and then filtered through a Whatman #541 paper (Berthiaume *et al.* 2006). This filtrate was used for the analyses of lactic acid and ammonia. Lactic acid was determined by a spectrophotometric method according to Taylor (Taylor 1996). Ammonia was determined as described by Fliplot *et al.* (1976) on an automated Kjeltec 1030 (Foss, Eden Prairie, MN). Ethanol and VFA were determined

on water extract of silage according to Fussell and McCalley (1987). VFA analyses were conducted with a gas chromatograph (Model 6850; Agilent, Mississauga, ON, Canada) equipped with a 25 m capillary column (i.d. 0·319 mm; film thickness, 0·50 μm; DB-FFAP, J & W 123-3223) and a flame ionization detector. At the time of injection, 0·5 μl of water extracts were used. Column temperature was set to 60°C for 1 min, then temperature increased at a rate of 20°C min^{-1} to 120°C, then at 15°C min^{-1} to 150°C, and finally at 35°C min^{-1} to 220°C, which was maintained for 5 min until end of the run. Inlet and detector temperatures were 220 and 300°C respectively. The split ratio was 25 : 1. The flow rate for the hydrogen carrier gas was 30 ml min^{-1}. The detector gases and their flow rate were: 30 ml min^{-1} for hydrogen, 400 ml min^{-1} for air. Each peak was identified and quantified using pure standard of acetic acid (Fisher Scientific), propionic acid (Anachemia Science, Lachine, QC, Canada), iso-butyric acid (Sigma-Aldrich), n-butyric acid (Sigma-Aldrich) and ethanol (Alcools de Commerce Ltd., Boucherville, QC, Canada).

Microbiological analyses

Microbial enumerations were performed on day 0, 28 and 60. Each 20 g silage sample was blended in a Stomacher paddle mixer (Seward) for 2 min with 180 ml of peptone water (0·2% Bacto peptone (w/v), 0·01% Tween 80 (w/v)), and serial dilutions were prepared with the same peptone water. Total colony forming units (CFU) of LAB, enterobacteria and fungi (i.e. yeasts and moulds) were counted after incubation at 28°C on plates of Rogosa Agar (Oxoid), Violet Red Bile Agar (Oxoid) and Malt Extract Agar (Difco), respectively. Clostridial spores were counted on Reinforced Clostridial Agar (Oxoid) according to Jonsson (1990). For each culture media, triplicates from each dilution series were made.

Statistical analyses

The data were analysed according to a complete random block design with four replications distributed in time required for ensiling of the vacuum bags mini-silos. A complete analysis of the biochemical and microbiological results was performed only for the 60-days opening using R (ver. 3.1.2, www.r-project.org). The normality of residual error was assessed using Shapiro–Wilk normality test and graphical inspection method; homogeneity of variance was verified using Fligner–Killeen test. If these criteria were met, the biochemical and microbial results were analysed using one-way ANOVA where the fixed factor was the temperature of incubation. If a significant response was observed ($P \leq 0{\cdot}05$), a Tukey HSD multiple comparisons was performed ('multcomp' package). Otherwise, mean values and standard deviations (SD) were presented in cases where either one or both of the assumptions were violated. Log transformation of microbial enumerations was done. Different transformations were tested for variables that did not comply to ANOVA criteria.

A nonlinear regression model ($pH = pH_a \times \exp^{-k(day-lag)} + pH_z$) was used to fit the pH data (pH_a, initial minus final pH; pH_z, final pH at day 60), the lag time (lag, time prior to the rapid decline, in days) and the rate of pH decline (highest k, per days between sample periods) was calculated (Jones *et al.* 1992). In order to understand how LAB diversity interacts with biochemical and physical parameters, a principal component analysis (PCA) was performed with the matrix of band-relative intensity from the DGGE profiles and the main fermentation parameters, including pH, VFA and microbial counts. An indicator species analysis was also carried out from the PCA results, using the 'multipatt' function of the 'indicespecies' package (ver. 1.6.2) (De Cáceres *et al.* 2010). Pearson's correlation between pH and band-relative intensity was calculated.

Results

Initial characteristics of fresh corn forage

Fresh corn forage contained 304·8, 86·8 and 12·2 g kg^{-1} DM, WSC and total N respectively. Buffering capacity was 24·32 g lactic acid kg^{-1} DM and the pH was 5·8. The numbers of LAB, yeasts, enterobacteria and moulds in fresh corn forage were 3·31, 4·19, 7·37 and 5·03 log_{10} CFU g^{-1} fresh material (FM) respectively; clostridial spores were under the detection level (<2 log_{10} CFU g^{-1} FM). Moreover, no significant difference was found among different temperature treatments for all the parameters measured prior to ensiling ($P > 0{\cdot}05$), which ensured similar initial conditions for different temperature treatments.

Chemical composition of corn silage

As shown in Table 1, all silages were well preserved after 60 days of fermentation as the measured pH values were lowered than the pH of anaerobic stability (pH_w) (Wieringa 1969), that was calculated to be of 4·4. Final pH of the silage was significantly ($P < 0{\cdot}001$) higher at 5°C, compared to the other incubation temperatures. Silages fermented at the lower temperatures, 5 and 10°C, contained less lactic acid and ammonia, and higher contents of residual WSC. No significant difference was found for the ethanol concentration between the different

Table 1 Chemical analysis of the corn silages after 60 d of ensiling (g kg^{-1} DM ± SD) for the different temperature treatments

	Temperature					
	25°C	20°C	15°C	10°C	5°C	SEM
pH	3·95*^{c}	3·83^{d}	3·94^{c}	4·04^{b}	4·30^{a}	0·030
DM (g kg^{-1} FM)	315·6^{a}	301·8^{bc}	295·2^{c}	304·0^{bc}	309·9^{ab}	0·341
WSC	11·7^{d}	13·0^{d}	24·2^{c}	36·5^{b}	72·7^{a}	0·342
Total N	12·2	12·6	12·2	11·9	11·9	0·033
Ammonia-N/Total N, %	6·17^{a}	4·62^{b}	4·65^{b}	3·63^{c}	3·80^{c}	0·086
Lactic acid	42·8^{c}	63·3^{a}	51·0^{b}	32·4^{d}	30·6^{d}	2·546
Acetic acid	75·2^{a}	80·0^{a}	75·9^{a}	41·0^{b}	25·4^{c}	6·272
Ethanol	2·73	3·16	4·05	3·96	1·99	0·715
Lactic/Acetic + Ethanol	0·55^{a}	0·78^{a}	0·64^{a}	0·73^{a}	1·12^{b}	0·082
Propionic acid†	5·98 ± 3·84	2·90 ± 0·38	2·06 ± 1·03	1·09 ± 0·09	1·32 ± 0·69	
n-Butyric acid†	1·96 ± 1·23	0·54 ± 0·39	n.d.	1·10 ± 0·15	0·62 ± 0·17	
Isobutyric acid†	1·06 ± 0·25	0·69 ± 0·22	0·20 ± 0·17	0·22 ± 0·05	0·35 ± 0·28	
n-Valeric acid†	6·13 ± 3·01	2·00 ± 1·90	0·33 ± 0·31	0·32 ± 0·17	0·60 ± 0·65	
Isovaleric acid†	1·51 ± 0·81	6·75 ± 7·32	1·17 ± 1·70	0·68 ± 0·04	0·73 ± 0·20	

DM, dry matter; FM, fresh material; WSC, water-soluble carbohydrates.
*Within a row means followed by the different letters differ significantly ($P \leq 0·05$). 'n.d.': under detection level.
†Lack of normality did not allow ANOVA to be performed on those variables.

treatments ($P = 0·057$), but its concentration tend to be higher at 15°C compared to the other incubation temperatures. Silages incubated at higher temperatures (from 15 to 25°C) produced significantly higher levels of acetic acid than at 5 or 10°C ($P < 0·001$). Higher concentration of propionic acid seemed to be present at higher incubation temperatures, but high variability between samples impaired statistical analysis. Other VFA were also detected, but the high variation within temperature treatments lowered the potential to report statistical difference.

pH Change

Figure 1 shows the kinetics of pH change for corn silage incubated at the five tested temperatures. A nonlinear regression model was able to fit the data at 10, 15, 20 and 25°C, but not at 5°C (Table 2) (Jones *et al.* 1992). Forage acidification started rapidly. No lag times were observed at higher incubation temperatures (i.e. 20 and 25°C), whereas acidification was delayed by 1·890 days at 15°C, and by 3·211 days at 10°C ($P < 0·001$). The rates of pH decline (k) were 0·095, 0·530, 0·358 and 0·668 day^{-1} at 10, 15, 20 and 25°C respectively ($P < 0·001$). Acidification data at 5°C could not be explained using the model. At this temperature, forage acidification was greatly delayed (≥7 days), and the decline of pH was the slowest among the treatments (Fig. 1).

Microbial counts of corn silage

Fresh corn microbial counts were of 3·4, 7·4, 4·1, 4·8 $\log_{10}$ g^{-1} FM of lactic acid bacteria, enterobacteria,

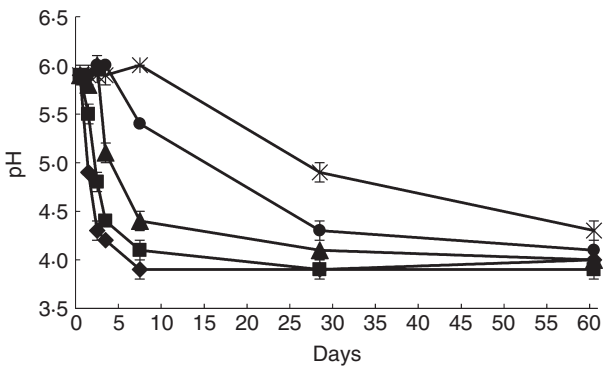

Figure 1 Kinetics of pH for whole-plant corn ensiled at different temperatures. pH data are represented as mean value ± standard deviation. (♦) 25°C; (■) 20°C; (▲) 15°C; (●) 10°C and (★) 5°C.

Table 2 Effect of temperature on the pH decline of whole-plant corn silage

	Temperatures				
Parameter	10°C	15°C	20°C	25°C	SEM_{max}
Lag time (lag, day)	3·211^{a}*	1·892^{b}	0·000^{c}	0·000^{c}	0·086
Rate of pH decline (k, per day)	0·095^{d}	0·530^{b}	0·358^{c}	0·668^{a}	0·033

*According to a one-way ANOVA followed by Tukey's HSD test, statistical differences among the means of lag and k within the same row are indicated by different superscripts letters ($P \leq 0·05$); SEM_{max}: the maximum value of the standard error of the mean within the same row.

Table 3 Enumeration of three main bacteria group of corn silages (log_{10} CFU g^{-1} FM) for the different temperature treatments during corn fermentation

	LAB		Yeast		Enterobacteria	
Temperature (°C)	28 days	60 days	28 days	60 days	28 days	60 days
25	9·39 ± 0·17	9·09 ± 0·02	n.d.	n.d.	n.d.	n.d.
20	8·93 ± 0·06	9·12 ± 0·04	3·58 ± 0·11	n.d.	n.d.	n.d.
15	8·27 ± 0·01	8·81 ± 0·08	3·55 ± 0·41	3·37 ± 0·28	n.d.	n.d.
10	8·16 ± 0·04	8·15 ± 0·03	3·76 ± 0·83	4·43 ± 0·83	n.d.	n.d.
5	8·38 ± 0·13	8·48 ± 0·04	3·24 ± 0·13	3·99 ± 0·29	4·23 ± 0·16	n.d.

FM, fresh material; LAB, lactic acid bacteria.
n.d.: under the level of detection (<2 log_{10} CFU g^{-1} FM); moulds and clostridial spores were under the level of detection in all silages after 28 and 60 day of ensiling.

yeasts and moulds respectively. Clostridia counts were under detection level (<2 log_{10} CFU g^{-1} FM). Microbial counts were made on corn silage after 28 and 60 days of fermentation (Table 3). This allowed us to observe microbiological changes during fermentation, in particular for undesirable bacteria groups, that is, clostridia, enterobacteria and yeasts. Acid-tolerant yeasts survived the 60 days of fermentation at lower temperatures, whereas they were under detection level (<2 log_{10} CFU g^{-1} FM) on day 28 and day 60 at 25°C, and on day 60 at 20°C. Enterobacteria were only detected at 5°C on day 28. The numbers of moulds and clostridial spores were below the level of detection (<2 log_{10} CFU g^{-1} FM) in all silage samples (data not shown).

Lactic acid bacteria population during the ensiling process of corn silage

Our results demonstrated very different profiles of the LAB population during corn ensiling process in relation to the different incubation temperatures (Figs 2 and S2). Thirteen dominant OTU were selected for further analysis. Ten OTU were successfully identified following cloning and sequencing of the PCR-DGGE band. These OTU are also related to the isolated strains used for the DGGE ladder. They included *Lact. plantarum*, *Leuc. citreum*, *Leuc. mesenteroides*, *Lact. brevis*, *Leuc. lactis*, *Lact. sakei*, *Lactobacillus coryneformis*, *Lact. coryniformis* subsp. *torquens*, *Lact. curvatus*, *Ped. pentosaceus* and *Lact. buchneri*. Excision or re-amplification of bands C4 from DGGE gel failed. As for the *Lactococcus*-group, one sole band was constantly observed on DGGE gel for all silage samples (data not shown). This band was successfully excised, cloned and sequenced, and further identified as *Lactococcus lactis* subsp. *lactis* (GenBank accession no: AB775185.1, similarity: 99%). Since no variation was found among silage samples regardless of temperature, no further gel or statistical analyses were done for this group.

PCA was performed in order to obtain an overview of the LAB community and biochemical parameters at different temperatures (Fig. 3a,b). The 1st and 2nd principal components explained 47·39 and 22·75% of the variation respectively (Fig. 3a). The first axis was positively correlated with the WSC concentration and yeasts cell counts while negatively correlated with acetic and lactic acid concentration. The second axis explained less than what would be predicted under the broken stick model given 19 variables (13·4%).

Based on the PCA results, we performed an indicator species analysis (with a threshold of IndVal = 0·8) by combining groups of ensiling temperature on silages at lower (5 and 10°C) and higher temperatures (15, 20 and 25°C) respectively. At 5 and 10°C, C12 (*Lact. coryneformis*) was the only one observed at the beginning of ensilage, and C2 (*Leuc. citreum*) appeared on day 2 to day 7 (mainly at 10°C), and then C7 (*Lact. sakei*) and C9 (*Lact. curvatus*) joined the succession and finally dominated. At 15, 20 and 25°C, C1 (*Lact. plantarum*), C10 (*Ped. pentosaceus*), C5 (*Lact. brevis*) and C2 (*Leuc. citreum*) appeared on day 1 and were present until day 28. C7 (*Lact. sakei*) and C8 (*Lact. coryniformis* subsp. *torquens*) appeared on day 2, and C13 (*Lact. buchneri*) appeared on day 28 and was detected until the end of fermentation.

Discussion

Low temperature restricts silage fermentation

Compared to extensive work with moderate and high temperatures, the effects of low temperature (<20°C) on ensilage have hardly been investigated (Muck 2013). Thus, in this study, we looked into the effects of temperature, ranging from 5 to 25°C, on whole-plant corn silage fermentation. Our results confirmed that low temperature restricts fermentation. During 60 days of fermentation,

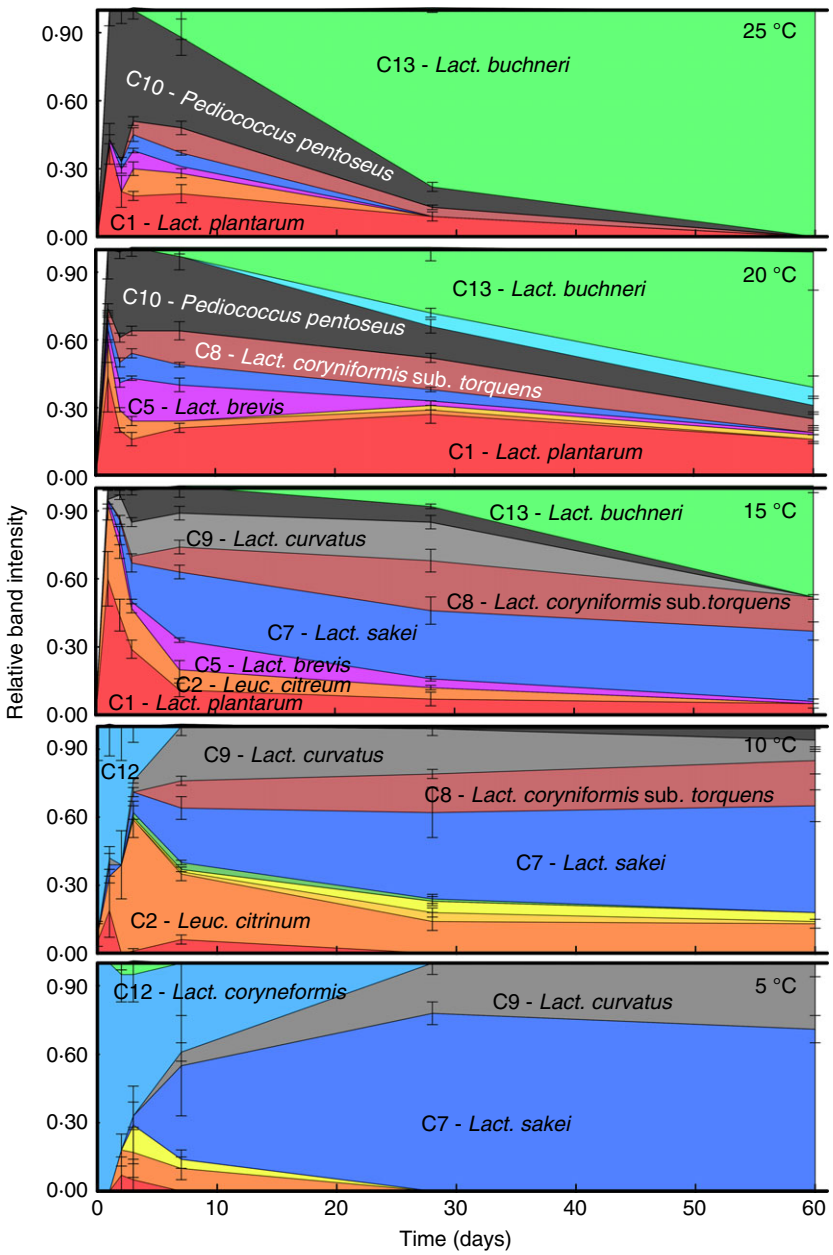

Figure 2 Succession of lactic acid bacteria (LAB) population (OTUs) during the ensiling process of corn silage stored at different temperatures (Band-Relative Intensity (%) ~ Time (days)). Band-relative intensity was described as mean value ± standard deviation. C1–C13 represents the same LAB-associated OTUs described throughout this study: C1 – *Lactobacillus plantarum* ■, C2 – *Leuconostoc citreum* ■, C3 – *Leuconostoc mesenteroides* ■, C4 – unidentified OTU A ■, C5 – *Lactobacillus brevis* ■, C6 – *Leuconostoc lactis* ■, C7 – *Lactobacillus sakei* ■, C8 – *Lactobacillus coryneformis* subs. torquens ■, C9 – *Lactobacillus curvatus* ■, C10 – *Pediococcus pentosaceus* ■, C11 – *Lact. coryneformis* ■, C12 – *Lact. coryneformis* ■, C13 – *Lactobacillus buchneri* ■.

warmer temperatures (20 and 25°C) resulted in quick start of forage acidification and rapid pH decline, whereas lower temperatures generally led to delayed acidification and lower rates of pH decline especially at 10 and 5°C (Fig. 1, Table 2). Nevertheless, sufficiently low pH values were eventually reached in all silage samples to ensure their anaerobic stability (Wieringa 1969). This might be attributed to the good ensilability of whole-plant corn.

After 60 days of fermentation, it was observed that lower temperature generally resulted in higher pH, low acids production and more residual WSC. Probably due to the delay in acidification and the low rate of pH decline at lower temperatures, undesirable microbes survived a longer period in these silages. For instance, after 28 days of fermentation at 5°C, enterobacteria were still present (4·23 ± 0·16 $\log_{10}$ CFU g^{-1} FM) due to the high silage pH of 4·9, which was above the critical value of 4·5 (Pahlow *et al.* 2003). In addition, the lowest ammonia level was found at 5°C, although enterobacteria were detected in those samples. This seems to disagree with the general assumption that the production of ammonia in silage usually results from the activity of enterobacteria if clostridia are not detected (McDonald *et al.* 1991). But, at such low temperature, enterobacteria metabolic activity could be very low, and moreover, the activity of plant proteolysis enzymes, which also plays an

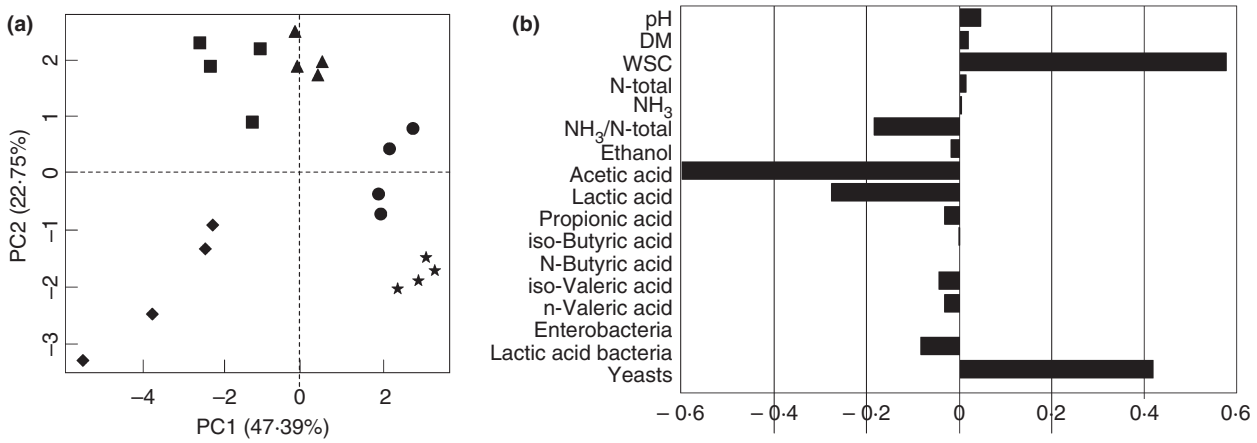

Figure 3 (a) Principal component analysis (PCA) analysis of band-relative intensity as a function of operational taxonomic units indicative lactic acid bacteria species. (b) The eigenvector loading for PC1 of the PCA on the chemical and microbiological data set. (♦) 25°C; (■) 20°C; (▲) 15°C; (●) 10°C and (★) 5°C.

important role in ammonia production, may be greatly reduced (Rooke and Hatfield 2003). Overall, the inefficient silage fermentation at low temperature might be attributed to the thermodynamic implication that low temperature inhibits bacterial metabolism and plant enzymatic activities.

Lactic acid bacteria species associated with corn silage during the ensiling process

A variety of epiphytic LAB species have been reported in corn silage (Stevenson *et al.* 2006; Brusetti *et al.* 2008; Parvin and Nishino 2010; Wu *et al.* 2014). Through examining isolates, Lin *et al.* (1992) and Brusetti *et al.* (2008) found that *Lact. plantarum*, *Ped. pentosaceus* and *Lact. brevis* were three important LAB species. Parvin *et al.* (2010) observed distinctive DGGE bands indicative of *Lact. plantarum*, *Ped. pentosaceus* and *Lc. lactis* subsp. *lactis*. Using RT-PCR, Stevenson *et al.* (2006) reported that ensiled corn stover developed a predominant population consisting of *Lact. plantarum* and *Lact. brevis*, while *Lact. buchneri* and *Ped. pentosaceus* were also widely detected. All these studies were carried out at temperatures ranging between 25 and 30°C. These results are consistent with our findings for corn silage at 20 and 25°C. However, other LAB species were also identified during corn ensiling process for the previously mentioned studies. This difference in diversity could be attributed to many factors, for example, corn ybrid/cultivar (Lin *et al.* 1992; Brusetti *et al.* 2008), environmental conditions, sampling time, silage preparation (Parvin and Nishino 2009) and analysis technique (strain purification to DNA fingerprinting).

For the first time, *Lact. sakei* and *Lact. curvatus* were identified in corn ensiled at 5 and 10°C (Fig. 2). Also, at these low temperatures, one *Lact. coryneformis* (C12) predominated the initial phase of ensilage. Once acidification started, OTU C12 disappeared. Therefore, this species played a very small role during forage acidification, probably due to its high sensitivity to acidity ($R = 0{\cdot}72$). Along with acidification, *Lact. sakei* and *Lact. curvatus* quickly prevailed, and a low pH of 4·0 at 10°C and of 4·3 at 5°C was reached after 60 days of fermentation. *Lact. sakei* and *Lact. curvatus* are phylogenetically close relatives (Pang *et al.* 2012). In our assay, both the *Lact. sakei* and the *Lact. curvatus* OTU were identified as indicator species for corn ensiled at 5 and 10°C. Similar strains were found to predominate the microflora in vacuum-packed and processed meat products stored at cold temperatures (Dykes *et al.* 1995). Indeed, *Lact. sakei* proved to contain several transporters for cryo- and osmo-protective substances and to have more cold stress proteins than other *lactobacilli* (Eijsink and Axelsson 2005). Both species might be candidates as a LAB inoculant of forage. In fact, it has been suggested that one strain of *Lact. curvatus* isolated from sorghum silage stored at 4°C could be used as a silage inoculant at low temperature (Tanaka *et al.* 2000). Accordingly, *Lact. sakei* might be an interesting species for the development of LAB inoculants for cold climates. Strains of *Lact. sakei* are often used in meat processing technologies and it was demonstrated that they produce potent bacteriocins during the fermentation of meat, like sausage. These bacteriocins inhibit the growth of pathogenic and spoilage bacteria such as *Listeria* (Eijsink *et al.* 1996; Eijsink and Axelsson 2005).

In this experiment, corn silage ensiled at 15°C can be viewed as a transition temperature regulating LAB population profiles between two set of temperatures, 20–25°C and 5–10°C. Characteristic LAB species at both 20–25°C

(e.g. *Lact. plantarum*, *Ped. pentosaceaus* and *Lact. buchneri*) and 5–10°C (e.g. *Lact. sakei* and *Lact. curvatus*) were found in silage at 15°C (Fig. 2).

The shift of lactic acid bacteria species during the ensiling process

Results of our study clearly demonstrated that changes in the LAB population diversity occurred during the ensiling process (Fig. 2). It is generally recognize that an adequate ensilage usually starts with homofermentative LAB species quickly growing after sealing; later on, when substrate availability becomes limited, heterofermentative LAB species would replace them and eventually predominate (Seale *et al.* 1986; McDonald *et al.* 1991; Yang *et al.* 2006). Numerous studies have confirmed this shift of LAB flora from homo- to hetero-fermentative species (Langston and Bouma 1960; Beck 1972; Lin and Bolsen 1992; Brusetti *et al.* 2006; Stevenson *et al.* 2006; McEniry *et al.* 2008). For instance, Langston and Bouma (1960) observed that *Lact. plantarum*, *Lact. brevis* and *Pediococcus* spp. were three dominant species in good quality silage, with heterofermentative *Lact. brevis* usually observed in later stages. Beck (1972) reported that in ultimately well-preserved silages, heterofermentative *Lact. brevis* and *Lact. buchneri* replaced homofermentative *Lact. plantarum* and *Lact. curvatus* after 4 days of fermentation. Stevenson *et al.* (2006) also observed the shift from *Lact. plantarum* to *Lact. brevis* in alfalfa silage.

One interesting question thereby arises: What causes this phenotypic shift? Surprisingly, to date, little if any work has been reported on the regulatory mechanism of the succession of LAB species during the ensiling process. Instead, much attention has been focused on the sugar metabolism of individual LAB species, partially because of their biotechnological relevance (Axelsson 2011). Moreover, a similar metabolic change from homolactic to heterolactic fermentation has been observed and well-studied in LAB species often used by the dairy industries, such as *Lactobacillus casei*, *Streptococcus lactis*, *Lactobacillus bulgaricus* and *Lc. lactis* (Zaunmüller *et al.* 2006; Kowalczyk and Bardowski 2007). Briefly, hexoses are firstly degraded to glyceraldehyde-3-phosphate, followed by oxidization to pyruvate via the glycolytic pathway. The pyruvate is then reduced to lactate when growth occur with excessive substrate, catalysed by lactate dehydrogenase (EC 1.1.1.28) in LAB. Under the condition of low substrate availability, some LAB adapt themselves to alternative pathways of pyruvate conversion (i.e. phosphoroclastic split) with acetate, formate and ethanol as major end-products (Rhee and Pack 1980; McDonald *et al.* 1991; Lin and Bolsen 1992; Liu 2003). This contributes to the low sugar consumption rate which results in a low intracellular concentration of fructose-1,6-bisphosphate, an essential activator of lactate dehydrogenase (de Vries *et al.* 1970; Thomas *et al.* 1979). A variety of environmental factors, that is, pH, oxygen, type of substrates, substrate limitation, water activity, temperature and salt, were identified to affect the metabolism of LAB and their fermentative patterns (Thomas *et al.* 1979; Rhee and Pack 1980; Liu 2003). As for the ensiling process, curiously, similar environmental disturbances, for example, acidity, substrate availability, aerobiosis or moisture, could affect the fermentation and LAB succession during the ensiling process (Langston and Bouma 1960; Brusetti *et al.* 2006; Wang *et al.* 2006; Parvin and Nishino 2009, 2010). In the present study, we assume that the LAB community associated with ensilage behaves similarly to single LAB species, albeit with differences due to the complex fermentation environments and microbial interactions.

Herein, our observations on whole corn silage at 20 and 25°C support the broad consensus concerning the shift from homo- to heterofermentative LAB species during ensiling. In these silages, the growth of *Lact. plantarum* and *Ped. pentosaceus* was gradually replaced by *Lact. buchneri*. No correlation was found between pH and the band-relative intensity of any LAB-associated OTU ($R^2_{max} < 0{\cdot}3$), which is consistent with findings from Lin *et al.* (1992). In the present trial, substrates were not monitored as ensiling proceeded. Although, low level of residual WSC in final silages at 25 and 20°C (11·7 *vs* 13·0 g kg^{-1} DM, $P > 0{\cdot}001$) suggests that substrate availability could be a factor that regulates the shift of LAB species. The ability of *Lact. buchneri* to utilize lactic acid could be another factor (Oude Elferink *et al.* 2001).

On the other hand, this common assumption of the shift from homo- to heterofermentative LAB species does not explain what was observed at 5 and 10°C. After 60 days of fermentation, these silages still contained very high residual WSC, which were 83·8 and 42·0% of the initial content, respectively. This observation suggested that substrate was not limited for the microbiota throughout the whole ensiling process and storage period. Hence, nutrient availability was probably not a factor that regulated the shift of LAB species in these silages. On the other hand, a correlation was observed between pH change and the relative intensity of indicator species, which suggests high acid-sensitivity of *Lact. coryneformis* (OTU C12) ($R = +0{\cdot}72$) and acid-tolerance of *Lact. sakei* ($R = -0{\cdot}74$) and *Lact. curvatus* ($R = -0{\cdot}69$). Therefore, change in acidity could be the driving mechanism of the shift in LAB species during corn ensiling at 5 and 10°C. Finally, corn silage stored at 15°C acted as a transition between 20–25°C and 5–10°C. A shift from *Lact. plantarum* to *Lact. buchneri* was observed at higher

temperature, meanwhile, *Lact. curvatus* and *Lact. sakei* accounted for a large proportion of the LAB population in a later stage at lower temperature.

Epiphytic lactic acid bacteria populations and silage fermentation patterns

In 1999, a group of forage scientists isolated one *Lact. buchneri* strain from whole-plant corn silage (Driehuis *et al.* 1999). Under anaerobic conditions, this strain was capable of degrading lactic acid to acetic acid and 1,2-propanediol, which could then be converted to 1-propanol and propionic acid by *Lactobacillus diolivorans* (Oude Elferink *et al.* 2001; Krooneman *et al.* 2002). In this study, at 20 and 25°C, the low LA-ratios (lactic acid/acetic acid) (<1) together with a slight increase in pH at 25°C indicated lactate degradation in these silages. The significantly lower LA-ratio (0·57 *vs* 0·79), higher pH value (4·0 *vs* 3·8) and lower lactic acid concentration (4·28% *vs* 6·33%) suggested a greater extent of lactate degradation at 25°C than at 20°C ($P < 0{\cdot}001$), which was also confirmed by the tendency of samples from the 25°C treatment to contain higher concentration of propionic acid. In addition, our observations that *Lact. buchneri* accounted for a larger proportion of the LAB flora at 25°C than at 20°C (100% *vs* 60·3 ± 19·2%), also kept consistency with above-mentioned biochemical characteristics.

Corn silages were also well conserved at 5 and 10°C although the fermentation was restricted, as described above. A LA-ratio close to 1 : 1 suggested heterolactic fermentation, which was in accordance with the predominance of *Lact. sakei* and *Lact. curvatus*. Additionally, we performed a PCA from the biochemical and microbiological data (Fig. 3). It was observed that whole-plant corn ensiled at 15°C lied close to 0 on the first axis, along with warmer and cooler temperatures on both sides. This indicated that 15°C was a transition temperature treatment between 20–25°C and 5–10°C. This finding could also be explained by the epiphytic LAB population involved, that is, the dominant LAB species at both 20 and 25°C (i.e. *Lact. plantarum*, *Ped. pentosaceus* and *Lact. buchneri*) and at 5 and 10°C (i.e. *Lact. sakei* and *Lact. curvatus*) were co-present at 15°C.

Yeast

Yeasts have often been identified as primary initiators of silage aerobic spoilage (Woolford 1990). Silages with high yeast counts are generally prone to aerobic spoilage when exposed to air (Pahlow *et al.* 2003). In this study, the log number of yeasts on corn forage was 4·19 prior to ensiling. At 25°C, yeast number was under detection level in the samples from 28 and 60 days of incubation. For the 20°C treatment, yeasts were present at 28 days (3·58 $\log_{10}$ CFU g^{-1} FM), but under detection level after 60 days of fermentation, whereas similar number of yeasts, about 4 $\log_{10}$ CFU g^{-1} FM, persisted at 15, 10 and 5°C (Table 3).

Undissociated VFA, in particular acetic acid, have been recognized as important yeast inhibitors, and this is also the reason why heterolactic fermentation has been advocated to improve silage aerobic stability (Weinberg and Muck 1996). In our study, similar levels of acetic acid at 25, 20 and 15°C ($P > 0{\cdot}001$) indicated that acetic acid was not the sole inhibitor of yeast since yeasts counts of 3·37 $\log_{10}$ CFU g^{-1} FM was measured after 28 days of fermentation at 15°C. Moreover, the considerable differences in acetic acid content at 15, 10 and 5°C compared to higher incubation temperatures ($P < 0{\cdot}001$) suggested that acetic acid apparently was not a crucial yeast inhibitor in these silages (Table 1). Butyric acid also inhibits the growth of yeasts (Driehuis *et al.* 1999; Kung 2010a). But, in this study, it was not likely the issue because the concentration of butyric acid was low and biologically negligible, and clostridia spores were under the detection level (<2 $\log_{10}$ CFU g^{-1} FM) throughout the assay. In addition, ammonia also has good antifungal activity, but it is doubtful that the observed concentrations of this compound affect populations of yeasts in silages (Kung *et al.* 2000). The other potential inhibitor is propionic acid, which is much more inhibitory than acetic acid (Moon 1983). Our results showed that, after 60 days of fermentation, *Lact. buchneri*, a 1,2-propanediol producer (Oude Elferink *et al.* 2001), accounted for 100%, 60·3 ± 19·2%, 48·5 ± 3·6%, 0·3 ± 0·2% and 0% of the LAB flora at 25, 20, 15, 10 and 5°C respectively. Although *Lact. diolivorans* was not identified from our DGGE bands, this finding was in agreement with the tendency of our silage samples to have a higher concentration of propionic acid at warmer temperatures (Table 1).

Lower environmental temperature could indirectly favour the yeasts survival by allowing a slower metabolism, which will allow optimized pumping-out of the protons released by the organic acid in the cytoplasm. Yeasts could benefit from the higher concentration of WSC observed at the lower incubation temperatures to fuel this survival strategy. This hypothesis could explain the correlation between yeast counts and higher concentration in WSC observed from the PCA (Fig. 3b). At the lower incubation temperature, other metabolisms associated to low-temperature stresses can also reduce the efficiency of the organic acids to kill the yeasts, that is, less permeability of the membrane to organic acids (Beales 2004).

The overall results of the survival of yeasts showed that, as incubation temperature get lower, the longer it would generally take to inhibit yeasts. However, survival of yeasts seemed to persist in silage at a temperature equal and below 15°C within the 60 days of incubation. In practice, on many farms, whole-plant corn forages are harvested for silage at around 15°C or lower mean daily temperatures during the fall, particularly in some cool climates such as Eastern Canada. This may explain why whole-plant corn silage is particularly susceptible to heating and spoilage during feed-out. On the other hand, *Lact. buchneri* inoculation has been widely used in silage production, aiming to enhance aerobic stability through inhibiting the growth of yeasts. But, our results showed that this bacterium was not adapted to low temperatures (<15°C).

In summary, during the ensiling process, the adaptation of epiphytic LAB population to differing temperature conditions was achieved through the distinct succession of LAB population. Also, it has been clearly indicated that a change in LAB composition during ensiling occurred for all temperature treatments. However, more research is needed to gain comprehensive knowledge of the underlying mechanisms of this succession. Therefore, for the development of new LAB inoculants, it may be important to test for the temperature range at which the targeted LAB strain(s) are capable of competing, and also to screen appropriate LAB strain(s) not only from final silage products but from different ensiling stages.

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Conflict of Interest

Y. Zhou, P. Drouin and C. Lafrenière declare that they have no conflict of interest. As no microbial additive was used for this project, no possible conflict of interest exists with current position at Lallemand Animal Nutrition of P Drouin.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Main characteristics of the fresh corn prior to the fermentation.

Figure S2. Seventy-three lactic acid bacteria isolates following plate counts from corn silage samples of all five incubation temperatures were classified into 11 groups (OTU) according to their migration distances on DGGE profiles (processed with GelCompar II software, Applied Math, Austin TX, USA).

Figure S3. Global diversity patterns of lactic acid bacteria measured by PCR-DGGE in corn silages stored at 5, 10, 15, 20 and 25°C for 60 days.