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LIST OF ABBREVIATIONS

LAB	Lactic acid bacteria
DM	Dry matter
BC	Buffering capacity
WSC	Water soluble carbohydrates
USDA	U.S. department of agriculture
USDA-NASS	U.S. department of agriculture-national agricultural statistics service
USDA-FAS	U.S. department of agriculture-foreign agricultural service
OCPA	Ontario corn producers' association (Canada)
CFUs	Colony forming units
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ARDRA	Amplified ribosomal DNA restriction analysis
ARISA	Automated ribosomal intergenic spacer analysis
DGGE/TGGE	Denaturing gradient/temperature gel electrophoresis
SSCP	Single strand conformation polymorphism
T-RFLP	Terminal restriction fragment length polymorphism
ITS	Internal transcribed spacer
PCR	Polymerase chain reaction
OTUs	Operational taxonomic units
CTAB	Hexadecyltrimethylammonium bromide
rRNA	Ribosomal RNA
NCBI	National center for biotechnology information (US)
BLAST	Basic local alignment search tool
PCA	Principal component analysis
ANOVA	Analysis of variance
pHw	pH of anaerobic stability
VFAs	Volatile fatty acids

sd	Standard deviation
lag	Time prior to the rapid decline of pH, day
k	The rate of pH decline, per day
FM	Fresh material
se	Standard error
SEM	Standard error of the mean
LA	Lactic acid
AA	Acetic acid
LA-ratio	Lactic acid / acetic acid
ND	Not detected
NS	Not significant
H'	Shannon diversity index
NCYC	National collection of yeast cultures (UK)

RÉSUMÉ

L'ensilage est un aliment produit par la fermentation des fourrages sous l'action des bactéries lactiques. Parmi tous les fourrages, le maïs plante entière est une plante importante pour l'alimentation des ruminants et ce, partout dans le monde. Durant la dernière décennie, l'amélioration génétique du maïs a permis d'étendre cette culture toujours plus au nord avec le développement d'hybrides adaptés aux régions plus froides. Plusieurs facteurs influencent le processus de fermentation lors de l'ensilement, dont la température ambiante. L'effet de la température, lorsque celle-ci est inférieure à 20 °C, a été très peu étudié. Considérant ces faits, il devient nécessaire d'étudier l'effet de la température sur le processus d'ensilement du maïs. L'hypothèse émise est que les températures fraîches (5 à 15 °C) affectent le processus d'ensilement du maïs plante entière en diminuant la croissance microbienne, mais aussi en modifiant les populations microbiennes et en particulier les populations de bactéries lactiques épiphytes (BLACE) lors du processus d'ensilement. Ainsi, l'objectif de la présente étude est de déterminer l'effet de la température variant de 5 à 25 °C sur la fermentation et la dynamique des populations microbiennes durant l'entreposage (phase de stabilité anaérobie) de l'ensilage de maïs. Pour étudier ces effets, deux essais ont été réalisés. Avec le premier essai, en utilisant une technique génomique d'évaluation de la diversité microbienne (Chapitre II) de même que les paramètres biochimiques et microbiologiques (Chapitre III), la diversité de la population des BLACE a été suivie pendant 60 jours à cinq températures différentes, variant de 5 à 25 °C. Avec le deuxième essai, le même intervalle de température a été utilisé, mais en simulant une augmentation de température sur des ensilages ayant subi au préalable une fermentation à 10 °C ou à 20 °C, suivi d'un entreposage à 5 °C durant deux mois. La variation des paramètres biochimiques, la composition des populations microbiologiques et la diversité bactérienne (entérobactéries, BLACE) et la population fongique (levures et moisissures) ont été mesurées avec l'augmentation de la température (Chapitre IV). Les résultats du Chapitre II ont démontré que la température exerce une action sélective sur les espèces de BLACE durant le processus d'ensilement. Aux températures les plus élevées (20 et 25 °C), *Lactobacillus plantatum, Pediococcus pentosaceus, Lactobacillus brevis,* et *Lactobacillus buchneri* ont été observés durant la fermentation. À ces températures, un changement de fermentation homofermentaire vers une fermentation hétérofermentaire a été observé avec une domination complète de la population de BLACE par le *L. buchneri* après 60 jours de fermentation. Pour les ensilages incubés à des températures plus froides (5 et 10 °C), *Lactobacillus sakei* et *Lactobacillus curvatus* ont dominé la population de BLACE. La température de fermentation des ensilages de maïs à 15 °C indique un point tournant en regard de la diversité des BLACE et de leur succession durant le processus d'ensilement. Au Chapitre III, les résultats ont démontré que les températures les plus froides ont entravé la fermentation ce qui a retardé l'acidification, diminué la vitesse d'acidification, donné un pH final plus élevé, diminué la production d'acides organiques et augmenté les concentrations résiduelles des sucres solubles (SS). Toutefois, les profils fermentaires de ces ensilages ont démontré une fermentation hétérolactique, mais les espèces de BLACE observées par ces profils étaient différentes. Au Chapitre IV, les résultats ont démontré que l'augmentation des températures de 5 à 25 °C n'a pas modifié de façon importante la composition biochimique

des ensilages dont le processus d'ensilement a été fait à 20 °C. Toutefois, les dénombrements des levures et des entérobactéries ont démontré des niveaux assez élevés lorsque la température a atteint 15 °C et plus. Par ailleurs, pour les ensilages dont la fermentation a été faite à 10 °C, les résultats ont clairement démontré que la fermentation a démarré de nouveau lorsque la température a dépassé 20 °C. Dans ces ensilages, le *L. buchneri* a commencé à dominer la population de BLACE. Aucun autre développement microbien n'a été observé comme à 20 °C. Globalement, ces résultats démontrent que la température exerce un effet sélectif sur les espèces de BLACE impliquées dans le processus de fermentation, mais aussi lorsque la température remonte durant l'entreposage. Ces résultats soulèvent la question de l'efficacité des inoculants lactiques présentement vendus sur le marché lorsque les fourrages sont ensilés à des températures inférieures à 20 °C.

Mots-clés: Ensilage maïs plante entière, température, bactéries lactiques, diversité microbienne, DGGE

ABSTRACT

Silage is a feed produced following fermentation of a forage crop by lactic acid bacteria. Among forages, whole-plant corn is an important crop to feed ruminants all around the world. In the last decade, genetic improvement has expanded corn cultivation further north by developing hybrids adapted to colder regions. Many factors affect silage fermentation and temperature is one of them. The effects of cool temperatures (< 20 °C) have not been very much studied. Knowing this, it was necessary to question this fact. Do cool temperatures affect fermentation and microbial ecology during storage of whole-plant corn silage? The hypothesis was that cool temperatures (5 to 15 °C) affect whole-plant corn silage not only by lowering microbial growth but also by changing microbial populations, and in particular lactic acid bacteria (LAB) populations involved at ensiling. Therefore, the objective of this study was to determine the effects of temperature, ranging from 5 to 25 °C, on the fermentation and microbial ecology during storage (anaerobic phase) of whole-plant corn silage. To study these effects, two ensiling trials were performed. In the first trial, diversity of LAB populations using a culture-independent molecular method (Chapter II), and biochemical and microbiological parameters (Chapter III) were monitored during 60 days at five different temperatures from 5 to 25 °C. In the second trial, effects of increased temperature, from 5 to 25 °C, after a fermentation period of 60 days at either 10 or 20 °C and a storage period of two months at 5 °C were determined on these corn silages. Changes of biochemical and microbiological compositions, as well as bacterial (enterobacteria and LAB) and fungal populations (yeasts and moulds) diversity were measured while temperature was gradually increased (Chapter IV).

In chapter II, results showed that temperature had a selective effect on LAB species during the ensiling process. At warmer temperatures (20 and 25 °C), *Lactobacillus plantatum, Pediococcus pentosaceus, Lactobacillus brevis,* and *Lactobacillus buchneri* predominated the fermentation. At these temperatures, a shift from homo- to hetero-fermentative LAB species during the ensiling process was observed with a complete domination of *L. buchneri* at 60 days. For silages incubated at lower temperature (5 and 10 °C), *Lactobacillus sakei* and *Lactobacillus curvatus* prevailed. Corn silage at 15 °C acted as a transition between higher and lower temperatures in terms of both LAB diversity and their corresponding succession. In chapter III, results showed that lower temperature hampered silage fermentation resulting in delayed acidification, low rates of pH decline, higher final pH, low production of organic acids, and high concentration of residual WSC. However, all silages showed heterolactic fermentation patterns but LAB species involved were different. In chapter IV, results showed that, as incubation temperature increased from 5 to 25 °C, no major change occurred in silages initially fermented at 20 °C, in terms of biochemical compositions. However, yeasts and enterobacteria counts were detected at a rather high level in these silages as incubation temperature reached 15 °C and above. On the other hand, for silage initially fermented at 10 °C, results clearly showed that fermentation resumed as incubation temperature reached 20 °C and over. In these particular silages, *L. buchneri* started to dominate the bacterial LAB

flora. No other microbial population growth was observed. Globally, these results demonstrated that, temperature has a selective effect on LAB species that are involved not only during the ensiling process but also during warming up of temperature. These results also questioned the efficiency of lactic inoculants that are presently on the market when forage crops are ensiled at temperature lower than 20 °C.

Keywords: whole-plant corn silage, temperature, lactic acid bacteria, microbial diversity, DGGE

CHAPTER I

GENERAL INTRODUCTION

1.1 Background of the study

Silage is the feedstuff produced by the fermentation of green forages and crops, based on lactic acid bacteria (LAB) fermentation under anaerobic conditions (Ashbell, *et al.*, 2002; Rossi and Dellaglio, 2007; Weinberg, *et al.*, 2001). Silage can be made from a large variety of forages and crops, such as grasses, corn (*Zea mays*), sorghum (*Sorghum bicolor*), legumes, whole-crop cereals and small grains. It is usually stored in a silo, i.e., in a bunker, a tower, a covered heap or in wrapped bales, to allow the product to ferment.

Compared with haymaking, silage production is less dependent on weather and thus better adapted to harvesting crops at the optimum stage of maturity in order to ensure optimal nutritional value (Allen, *et al.*, 2003). In recent years, silage production has gained popularity in many countries. For instance, in European countries such as the Netherlands, Germany and Denmark, more than 90% of the locally produced forages are stored as silage. Even in countries with generally good weather conditions for haymaking, such as France and Italy, about half of the forages are ensiled (Te Giffel, *et al.*, 2002). In North America, silage has formed a major part of the feed ration of cattle and sheep (Wilkinson, *et al.*, 2003).

Ensiling is a process of complex microbial and enzymatic activities (Weinberg, *et al.*, 2001). Many pre- and post-harvest factors affect it, including forage/crop biochemical characteristics (e.g., dry matter content (DM), buffering capacity (BC), water soluble carbohydrate (WSC) concentration), plant management (e.g., manure and fertilizer application, cutting time, cultivars, maturity), harvesting techniques, weather conditions during harvest period (e.g., rainfall, frost, warm temperature), harvest and silo management (e.g., mowing, chopping, wilting, filling or delayed sealing), and application of biological or biochemical additives (Donald, *et al.*, 1993; Fenlon, 1986; Filya, *et al.*, 2006; Fraser, *et al.*, 2001; Fychan, *et al.*,

2002; Haigh and Parker, 1985; Hassanat, *et al.*, 2006; Hassanat, *et al.*, 2007; Manda, *et al.*, 1985; Marsh, 1979; Muck, 1990; Nishino, *et al.*, 2004; Pauly, *et al.*, 1999; Pedroso, *et al.*, 2005; Ranjit and Kung Jr, 2000; Xing, *et al.*, 2009).

Among all of the above factors, temperature has long been considered a major factor affecting silage fermentation (McDonald, *et al.*, 1966; Muck, *et al.*, 2003). Generally speaking, a moderate temperature between 20 and 30 °C is preferred for silage fermentation (Kim and Adesogan, 2006; McDonald, *et al.*, 1966; Weinberg, *et al.*, 1998; 2001). Probably for this reason, the majority of ensiling experiments in the literature were conducted within this temperature range. Additionally, the impacts of high temperature (> 37 °C) have been well studied due to the detrimental effects on forage preservation (Garcia, *et al.*, 1989; Goering, *et al.*, 1973; Kim and Adesogan, 2006; McDonald, *et al.*, 1966; Weinberg, *et al.*, 1998; 2001). However, the effects of cool temperature (< 20 °C) on silage fermentation have been much less studied. However, in cold climates, cool temperatures can hinder silage fermentation, usually resulting in less intensively fermented silage. In practice, these silages are often found to be associated with high pH and low acid production (Kung, 2010b), as well as high residual WSC and yeast counts which make silages more vulnerable to aerobic deterioration during the feed-out period (Ward and de Ondarza, 2008). Moreover, in all the above studies, the evaluation often has been performed only after moderately short lengths of storage (1 to 82 days). There are no long-term storage studies on the effects of temperature on silage fermentation even though silages may remain stored for longer periods of time on many farms.

Whole-plant corn silage is a popular forage for ruminant animals because of its relatively consistent quality, its high yields and energy content, and its good digestibility. It is one of the most important forages for ruminants in climates where corn can be grown, particularly in Central Europe and North America. In the last decade, cultivation of this crop further north has become possible following the development of hybrids adaptable to colder regions. In Eastern Canada, corn silages are made in autumn when daily mean temperatures are between 0 and 10 °C. Part of the ensiled corn will be stored and consumed during the following winter

and spring season (< 10 °C). The low temperature could impact greatly the ensiling process as well as affect the storage of whole-plant corn silage.

1.2 Whole-plant corn silage

Corn originates from Mesoamerica and was domesticated about 7,000 years ago. When the first European explorers came to the New World, they found corn growing throughout the Americas. Spanish and Portuguese explorers later brought corn back to Europe. Thereafter, corn was introduced throughout the world. Corn can now be found across the world, and it is an essential resource for ensiling to feed ruminant livestock, especially in the Mediterranean region and North America (Wilkinson and Toivonen, 2003). In the United States, corn is planted mainly in the Corn Belt Region (Illinois, Indiana, Iowa, Nebraska, and Minnesota), and it has been the main crop harvested for silage for decades. For instance, in 2000 about 2.46 million ha of corn were harvested for silage, compared with grass (0.39 million ha), legumes (1.32 million ha) and whole-crop cereals (0.11 million ha) (Wilkinson and Toivonen, 2003). According to the latest survey by U.S. Department of Agriculture National Agricultural Statistics Service (NASS), the area planted with corn in the United States was estimated at 39.32 million ha in 2012, and about 2.99 million ha of corn was harvested for silage, an increase of 24.31% from the 2011 census (USDANASS, 2013).

In Canada, corn constitutes the third largest crop (after wheat and barley) and is the most important one in Eastern Canada. It is grown primarily in Ontario and Québec, accounting for more than 90% of Canadian corn. In Eastern Canada, more than 200,000 ha of corn (out of 1,200,000) are harvested as whole-plant corn silage every year (OCPA, 2010). Corn is planted from May to June, and it is harvested mainly from late September to early November when daily mean temperatures are between 0 and 10 °C. Ensiled corn then will be stored and consumed during the winter and spring at even cooler temperature (< 10 °C) (Environment-Canada, 2008). Similarly, many other countries or regions with cool climates, such as the northern USA, northeastern China, Japan, and Korea, are also confronted with low temperatures at harvest as well as during the storage period of whole-plant corn silage (USDA).

In terms of ensiling characteristics, corn can be regarded as an ideal crop because of its relatively high DM content, its low BC, and its adequate level of WSC. Another major advantage of corn is that it offers producers the flexibility to harvest it for forages or grains. Often when corn yields are low, the majority of the crop may be harvested for silage. However, whole-plant corn silage is found to be prone to aerobic spoilage when exposed to air (Ashbell and Weinberg, 1992; Filya and Sucu, 2010; Kung, *et al.*, 1998; Li and Nishino, 2011b). Some researchers have imputed this to the high concentration of residual WSC in corn silage which serve as ideal substrates for aerobic spoilage microbes during feed-out periods (Charmley, 2004).

1.3 The ensiling process

Ensiling is a natural fermentation process under anaerobic conditions, where LAB convert WSC into lactic acid and other organic acids. During the fermentation, pH is reduced and as a consequence, spoilage microbes' activity is inhibited while nutritional value is preserved (McDonald, *et al.*, 1991). The ensiling process can be divided into four principle phases of differing length and intensity: initial aerobic phase, main fermentation phase, stable phase, and feed-out period (Weinberg and Muck, 1996). It is important to control and to optimize each phase of the ensiling process in order to avoid failures and to maintain silage quality.

Phase 1-Initial aerobic phase: This phase normally is confined to a few hours. During this stage, oxygen that is trapped within the forage mass maintains the respiration of plants and microorganisms. Some proteases and carbohydrases remain active. Growth of obligate and facultative aerobic microorganisms is possible (e.g., moulds, yeasts and enterobacteria, etc) which is accompanied by the heat generation from aerobic respiration (McDonald, *et al.*, 1991; Pahlow, *et al.*, 2003). Therefore a long aerobic phase reduces the energy content in silage as WSC are used for respiration and may cause heat damage to proteins. In this phase, the important management practice is to minimize the amount of oxygen present between the plant particles within the silo. Shortening the filling period, packing forage tightly, covering and sealing the silo as soon as possible, will minimize excess air and, in turn, will minimize losses through aerobic respiration. Chopping the forage at proper particle length also helps to pack the forage tightly. Chopping will also favour the growth of epiphytic LAB following the

release of plant sap, which could induce specific physiological responses related to antioxidation and to cell activity of this important bacterial population for the fermentation phase (Pahlow, *et al.*, 2003).

Phase 2-Main fermentation phase: This is the stage where LAB ferment WSC down to mainly lactic and acetic acids. This phase starts once oxygen is depleted. It usually takes one week but can last for more than one month depending on the properties of the ensiled forage crop and the ensiling conditions. In the early stage, pH is still relatively high, allowing spoilage microorganisms to grow. Thus, facultative and obligate anaerobic microorganisms, such as enterobacteria, clostridia, certain bacilli, propionibacteria, and yeasts, theoretically can compete with the LAB flora for the nutrients. Provided that silage fermentation proceeds successfully, LAB quickly develops to be the predominant population, along with the production of organic acids, and a relatively low pH is achieved to inhibit spoilage microbes' activity (Merry, *et al.*, 1997). A rapid and extensive pH decline will minimize growth of spoilage microbes and accordingly will reduce the losses of nutrients during this phase. Some spoilage microbes, such as *Clostridium*, are more adapted to higher moisture environment (McDonald, *et al.*, 1966). The target pH to inhibit the development of *Clostridium* in silage (pHw) is related to forage DM content. The lower the DM content is, the lower the pH should drop downwards to inhibit the development of *Clostridium* (Wieringa, 1969). Therefore harvesting forage at adequate DM content will prevent the undesirable clostridial fermentation (Wieringa, 1969). Applying various additives, such as LAB inoculants, at ensiling can contribute to a rapid decrease of pH in the forage mass.

Phase 3-Anaerobic stable phase: As long as silos remain sealed to maintain anaerobic conditions, rather little activity occurs during this stage, which theoretically can be of any length (Pahlow, *et al.*, 2003). LAB population dominates the microbial flora and typically undergoes a reduction in number in the course of this stage. Only some acid-tolerant proteases and carbohydrases continue to be active. Several acid-tolerant microorganisms, e.g., some yeast species, survive this period in a nearly inactive state, along with others such as clostridia and bacilli which turn to dormancy, as spores. However, if air penetrates into silos due to damage to the silage covering, spoilage microorganisms will grow. Covering the

silage properly and adequately weighting covers prevent oxygen exposure of the forage mass. It is also important to periodically evaluate the condition of the silo's covering and repair any damages (such as holes or tares) as soon as possible. In addition, everything that has been done in previous phases with the aim to inhibit microbial growth and to favour lactic acid fermentation will aid to control further development of spoilage microbes.

Phase 4-Feed-out phase: During feed-out periods, O_2 has free access to the silage mass. Thus, aerobic microorganisms start to multiply, which finally leads to aerobic deterioration (McDonald, *et al.*, 1991). Aerobic spoilage is usually initiated by lactate-assimilating yeasts which can utilise lactic acid for their metabolism (Wilkinson and Davies, 2013), but occasionally it can also be triggered by acetic acid bacteria (Spoelstra, *et al.*, 1988). Once begun, anaerobic conditions and low pH environment are lost rapidly, and temperature increases due to the increased microbial activities. As a consequence, various groups of aerobic microorganisms begin to proliferate, such as bacilli, some listeria and enterobacteria, and finally, at a later stage, filamentous fungi (Woolford, 1990). Aerobic spoilage of silage is undesirable, because the associated organisms compete for available nutrients and may produce toxins (such as mycotoxins) which are detrimental to the health of humans and animals (Pahlow, *et al.*, 2003). During feed-out, spoilage due to air ingress can be minimized by good management practices, such as sizing the silage structure according to feed-out needs, minimizing the time between removals of silage from the structure and feeding to animals, and maintaining a straight, smooth face to reduce the surface area that is exposed to oxygen. In addition, silage additives capable of decreasing aerobic spoilage losses can be applied at the time of filling.

1.4 Lactic acid bacteria associated with silage

Lactic acid bacteria could be characterized as Gram positive, low-GC, acid-tolerant, generally non-sporulating, non-respiring rod or cocci, which produce lactic acid as the major metabolic end-product of carbohydrate fermentation (Lahtinen, *et al.*, 2011). Their growth temperature ranges from 5 to 50 °C and pH from 4.0 to 6.8 (McDonald, *et al.*, 1991; Pahlow, *et al.*, 2003). These organisms are aerotolerant anaerobes (Stieglmeier, *et al.*, 2009). The principal genera

of LAB are *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus.* The other genera classified as LAB are *Aerococcus*, *Carnobacterium*, *Enterococcus, Oenococcus, Tetragenococcus*, *Vagococcus*, *Weisella* (Axelsson, 2011; Stiles and Holzapfel, 1997) and *Paralactobacillus* (Leisner, *et al.*, 2000). Several LAB genera such as *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Weissella*, *Streptococcus* and *Enterococcus* have been often reported to be associated with silages (McDonald, *et al.*, 1991; Pahlow, *et al.*, 2003).

Based on hexose fermentation metabolism, LAB are divided into two groups: homofermentative and heterofermentative bacteria (Figure 1.1). Homofermentative LAB such as *Pediococcus, Streptococcus, Lactococcus* and some lactobacilli (such as *Lactibacillus ruminis, Lactibacillus plantarum* and *Lactobacillus pentosus*) assimilate hexoses by glycolysis (Embden-Meyerhod-Parnas pathway), producing almost exclusively lactic acid as end-product. Heterofermentative LAB such as *Weissella*, *Leuconostoc,* and some lactobacilli (such as *Lactobacillus brevis* and *Lactobacillus buchneri*) produce not only lactic acid but also ethanol/acetic acid and CO_2 via the pentose phosphate pathway (Axelsson, 2011; Kandler, 1983). Most LAB readily ferment pentoses into lactic and acetic acids without the production of CO_2 (Rooke and Hatfield, 2003). In addition, for species of the genus *Lactobacillus*, three groups have been further defined on the basis of the presence or absence of the enzymes aldolase and phosphoketolase: obligate homofermentative LAB, facultative heterofermentative LAB, and obligate heterofermentative LAB. Obligate homofermentative and facultative heterofermentative lactobacilli belong as former homofermenters, and the difference between these two is that the obligate homofermentative lactobacilli are unable to ferment pentoses as they lack phosphoketolase. Obligate heterofermentative lactobacilli belong as former heterofermenters. This new classification system would allow to include several important formerly recognised homofermenters, such as *L. plantarum* and *L. pentosus* within the group of facultative heterofermentative lactobacilli.

Epiphytic LAB populations vary extensively on different forage crops (Lin, *et al.*, 1992b), and the LAB population on standing or freshly harvested forage crops differs considerably from that found during the ensiling process (Pahlow, *et al.*, 2003). Some genera or species are

often detected on standing crops or only in the early stages of the ensiling process. They contribute very little to silage fermentation and their growth is inhibited soon after anaerobic conditions are achieved. For instance, Ruser found that lactobacilli play an important role in promoting lactic acid fermentation for a longer period, whereas the cocci, e.g., *Streptococci, Leuconostocs*, *Lactococci*, and *Enterococci*, grow vigorously only in the early stage of the ensiling process (McDonald, *et al.*, 1991). Lin *et al.* (1992b) observed that *Enterococcus* species decreased sharply or disappeared entirely after the early stages of the ensiling period, and *P. pentosaceus* and several lactobacilli species, such as *L. plantarum, L. brevis, Lactobacillus homohiochii* and *Lactobacillus gasseri*, were the most frequently identified species during the ensiling process depending of the crops. The epiphytic LAB that predominates during the ensiling process is essential microflora responsible for lactic fermentation.

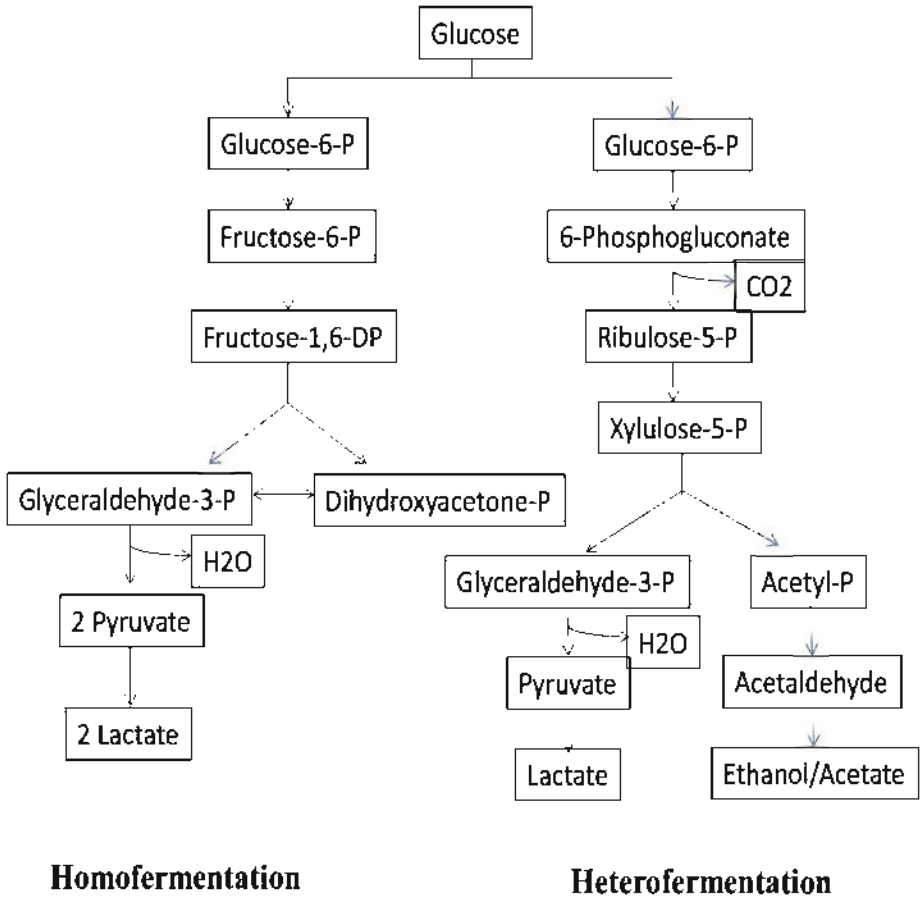

Figure 1.1 Generalized scheme of the pathways of glucose fermentation metabolism in lactic acid bacteria. Modified from (Axelsson, 2011; Caplice and Fitzgerald, 1999; Kandler, 1983)

In addition, a succession of LAB species also occurs during the ensiling process (McDonald, *et al.*, 1991; Muck, *et al.*, 2003). It is generally known that an adequate ensiling process usually starts with a group of homofermentative LAB species quickly after sealing the silo. Later on, when substrate availability becomes limited, more acid-tolerant heterofermentative LAB species would replace them and eventually predominate the fermentation (McDonald, *et al.*, 1991; Seale, *et al.*, 1986; Yang, *et al.*, 2006). Many early studies have confirmed this general consensus about LAB succession during ensiling (Beck, 1972; Brusetti, *et al.*, 2008; Langston and Bouma, 1960; Lin, *et al.*, 1992b; McEniry, *et al.*, 2008; Stevenson, *et al.*, 2006). For instance, Langston & Bouma (1960) observed that *L. plantarum*, *L. brevis* and

Pediococci sp. were three dominant populations in good quality silages, with *L. brevis* usually observed in the later stages of fermentation. Lin *et al.* (1992b) reported predominance of homofermentative LAB, e.g., *L. plantarum* and *P. pentosaceus,* in maize and alfalfa silage throughout the first seven days of ensiling. Whereas, after seven days, heterofermentative *Lactobacillus homohiochii*, *L. brevis* and *L. gasseri* joined the succession and came to prevail. Stevenson *et al.* (2006) observed a shift in dominant LAB species from *L. plantarum* to *L. brevis* in both inoculated and control alfalfa silage. On the other hand, Wang *et al.* (2006) did not observe this shift in alfalfa silage. Instead, during an ensiling period of 30 days, *P. pentosaceaus* was the dominant species in control alfalfa silage, and *L. plantarum* in the alfalfa silage which was inoculated with a previously selected LAB community the predominant strain of which belongs to *L. plantarum.*

1.5 Silage Temperature

The temperature of the silage mass is highly influenced by ambient temperature, in particular the ambient air temperature at harvest and filling time. During the initial aerobic phase, in well-managed silages, the average temperature of the forage mass will rise by 6-11 °C above ambient temperature (White, 2013). However, silage may reach much higher temperatures (> 37 °C) in poorly packed silos where excessive amounts of oxygen are trapped (Kung, 2011). When the fermentation phase is completed, silage temperature in small silos (such as big bales and bag silos) and in the outer layer of big silos drops gradually close to the ambient temperature (Van Saun and Heinrichs, 2008). On the other hand, core temperature of large silos remains almost constantly higher (usually range from 24 to 32 °C), mainly because of the insulation given by the large forage mass (Borreani and Tabacco, 2010; Kung, 2011). Core temperature is usually related to temperature that was prevailing at ensiling (Berthiaume, *et al.*, 2007). In addition, the ingress of oxygen into silos during storage may also result in an increase of silage temperature. Lastly, when opening silos for feeding, aerobic microorganisms such as yeasts, moulds and bacilli start to multiply by oxidizing WSC or organic acids, which generates heat. Silage temperature also rises in spoilage area such as moulded spots or peripheral areas (Borreani and Tabacco, 2010). Although some studies conducted on farm scale level have monitored silage temperature (Williams, *et al.*, 1997), it

is difficult to study the effect of different temperatures on a large scale level because of the difficulty to control ambient temperature. That is the reason why the mainstream way to study the effects of temperature on ensilage is through incubating laboratory-scale silos (e.g., plastic pouches, glass jars or metal containers). Among these laboratory silos, plastic pouches in combination with a vacuum sealer have been reported to be able to assess silage fermentation characteristics. They reduce the amount of work and waste associated with laboratory-scale ensiling (Johnson, *et al.*, 2005; Naoki and Yuji, 2008; Tanaka and Ohmomo, 1995). Recently, these handy silos have become popular as laboratory-scale preparations in Japan (Parvin and Nishino, 2009; Wang and Nishino, 2009), Thailand (Ohmomo, *et al.*, 2002), Canada (Boukhors, 2006; Sylvestre, 2011) as well as European countries, such as the UK (Johnson, *et al.*, 2005), Belgium (Van Ranst, *et al.*, 2009) and Turkey (Kizllsimsek, *et al.*, 2007). This thesis study used experimental plastic pouches to evaluate the effects of temperature on whole-plant corn silage.

1.6 Temperature and lactic acid bacteria in corn silage

Range of the growth temperature differs among LAB species. For instance, most lactobacilli grow at 15 °C but not at 45 °C. *Lactobacillus fermentum* is an exception, as it grows at 45 °C but not at 15 °C. The two species of enterococci commonly found in silage (*Enterococcus faecalis* and *Enterococcus faecium*) both grow within temperatures ranging from 10 to 45 °C. *Pediococcus damnosus*, *P. pentosaceus* and *Pediococcus acidilactici* have temperature optima of 25, 30, and 40 °C respectively (McDonald, *et al.*, 1991). Temperature therefore has a selective effect on the survival and proliferation of different LAB species in silage. Variation in the efficacy of some added LAB inoculants at different temperatures has also been observed. For instance, Weinberg *et al.* (1998) reported, in an ensiling assay of wheat at different temperatures, that treatments inoculated with *L. plantarum* fermented faster at 25 °C, whereas silages inoculated with *Lactobacillus amylovorus* fermented faster at 41 °C. In addition, temperature could also affect the overall LAB population in various fermentation systems, such as Kimchi (Mheen and Kwon, 1984) and sauerkraut (Pederson and Albury, 1953; 1954). Even though many studies have investigated the LAB population associated in silage (Brusetti, *et al.*, 2006; Ennahar, *et al.*, 2003; Gao, *et al.*, 2008; Stevenson, *et al.*, 2006),

unfortunately, very few of them have considered temperature as a factor affecting the LAB population.

For example, with whole-plant corn silage, many studies have investigated the diversity of the associated epiphytic LAB population. These studies include LAB on standing crops (Andrieu and Gouet, 1991; 1992b; Lin, *et al.*, 1992a), LAB on chopped corn prior to ensiling (Andrieu and Gouet, 1991; 1992b; Lin, *et al.*, 1992a; 2012; Pang, *et al.*, 2011). Moreover, Stevenson *et al.* (2006) and Parvin *et al.* (2010) determined the LAB population in corn silage after certain periods of fermentation; Lin *et al.* (1992b) and Brusetti *et al.* (2006) monitored the epiphytic LAB population during the ensiling process. A variety of LAB species were found to dominate in corn silages, such as *L. plantarum, P. pentosaceus, L. brevis, L. buchneri* and *Lactococcus lactis* (Brusetti, *et al.*, 2006; Lin, *et al.*, 1992b; Parvin, *et al.*, 2010; Stevenson, *et al.*, 2006). However, none of the above studies, considered temperature as a factor affecting the population diversity of LAB.

In addition, almost all earlier studies about epiphytic LAB populations during the corn ensiling process were conducted at constant temperatures, varying between 25 and 30 °C depending of the study. No relevant study, to date, has worked with temperatures below 20 °C. However, cool temperature not only could reduce fermentation efficiency by lowering the growth rate and enzymatic activity of microorganisms, but it also may contribute to the change of the composition of the LAB flora in different ecosystems. As the temperature gets colder, species showing better adaptation will dominate the environment, and eventually they will affect overall metabolites. For instance, two non-silage studies observed that low temperatures affected the LAB diversity in the intestinal tract of cultured freshwater fish (Hagi, *et al.*, 2004) (4-10 vs. 13-17 vs. above 20 °C) and in fermented pig feed (Olstorpe, *et al.*, 2008) (10 vs. 15 vs. 20 °C). Their results confirmed that temperature has a selective effect on the LAB population. From the standpoint of inoculants developers, it would be very interesting to learn which groups of LAB species are more competitive at low temperatures, in order to develop suitable inoculants for making silage in cool climates. In addition, considering the expansion of corn cultivation further north to colder regions and noticing the unpredictability of the weather due to climate change, it has become necessary to look into

the effects of low temperatures on LAB population during the ensiling process to better understand its impacts upon silage fermentation and conservation.

1.7 Temperature and silage fermentation

As previously described, ensiling is a process of complex microbial and enzymatic activities (Weinberg, *et al.*, 2001), and temperature has long been considered to be a major factor affecting silage fermentation (McDonald, *et al.*, 1966; Muck, *et al.*, 2003). For instance, Garcia *et al.* (1989) (65 vs. 38 °C), Weinberg *et al.* (2001) (41 vs. 24 °C) and Kim & Adesogan (2006) (40 vs. 20 °C) found that higher temperatures resulted not only in less organic acids production, higher pH values, more residual WSC and nutrients losses, but also in higher susceptibility to aerobic spoilage. Prolonged high temperature, above 45 °C, could result in heat damage through the Maillard reaction (Adesogan and Newman, 2010; Kobayashi, 2003). The Maillard reaction, also known as the nonenzymatic browning, is a chemical reaction between protein and carbohydrates. It is known that this reaction can adversely affect protein digestibility, and, moreover, that the reaction rate is highly temperature dependent (Martins, *et al.*, 2000). The higher the temperature, the higher is the reaction rate and greater is the amount of protein damage.

Temperature also has an effect on plant enzymes. Plant proteases activities were reported to increase progressively at high ensiling temperatures, causing greater protein breakdown (Jones, *et al.*, 1995; McKersie, 1981; Muck and Dickerson, 1988; Weinberg, *et al.*, 2001). Additionally, McDonald *et al.* (1966) observed a shift from a lactic to a clostridial type of fermentation which had resulted from an increase of ensiling temperature from 20 to 42 °C. On the other hand, high ensiling temperatures can benefit cell wall hydrolysis, due to the higher activity of cellulase (Colombatto, *et al.*, 2004; Garcia, *et al.*, 1989).

Cool temperature could be an important factor affecting silage fermentation, particularly in some northern countries or regions. However, to date, very few experiments have been done at temperatures lower than 20 °C. Theoretically, low temperature restricts silage fermentation by reducing enzymatic and microbial metabolisms. The lower the temperature, the slower will be the corresponding chemical reactions, enzymatic activity, and microbial growth rate.

In practice, low temperature could hinder silage production (Schroeder, 2009). Such silages are often found to have high pH value and low acids production (Kung, 2010b; Kung and Shaver, 2001), as well as more residual WSC and high yeast counts (Schroeder, 2009; Ward and de Ondarza, 2008). Even, added silage inoculants may also be impaired and therefore may not be very effective at cold temperature (Schroeder, 2009; Weinberg and Muck, 1996). Recently, Wang *et al.* (2011) studied the effects of a LAB inoculant on the fermentation quality of reed grass (*Phragmites australis* Cav.Trin.ex Sterd.) at two low temperatures: 0 and 4 °C. The LAB inoculants consisted of several strains which had previously been isolated from plant surfaces, including *L. plantarum* (55%), *Leuconostoc mesenteroides* (25%), *Streptococcus faecalis* (15%) and *L. brevis* (5%). It was found that, at such low temperature levels, reed grass inoculated with 6.63 $\log_{10}$ CFU g^{-1} DM started the fermentation process but it would take at least six weeks to get fairly good silage quality. Also, reed grass fermented at 4 °C produced more lactic acid and reached lower pH than at 0 °C over eight weeks of fermentation.

In the few studies which have investigated the effects of temperature, silage storage period was short, generally less than three months (Kim and Adesogan, 2006; Koc, *et al.*, 2009; Weinberg, *et al.*, 2001). On commercial farms, silages may remain stored for longer periods of time. However, there are no long term storage studies on the effects of temperature on silage fermentation and storage which are associated with the anaerobic stability phase.

1.8 Molecular diversity techniques in silage microbiology

With the development of molecular biology tools, many independent-cultural fingerprinting approaches based on DNA or RNA genes have generated the interest to investigate microbial population in complex ecosystems. Several independent-cultural molecular diversity techniques have been developed. They include Amplified Ribosomal DNA Restriction Analysis (ARDRA), Automated Ribosomal Intergenic Spacer Analysis (ARISA), Denaturing Gradient/Temperature Gel Electrophoresis (DGGE/TGGE), 16S library, Single Strand Conformation Polymorphism (SSCP), and Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Nout, *et al.*, 1993). Overall, these techniques contribute to detect

more microorganisms, including less represented species, at lower levels, and uncultivable organisms (Endo and Okada, 2005; Mansfield and Kuldau, 2007).

Compared to the large number of experiments using molecular methods to study microbial diversity in soil and water, only a few studies were undertaken to detect microbial population dynamics in silage. Mansfield and Kuldau (2007) and May *et al.* (2001) reported the diversity of fungal community associated with corn silage using ITS-PCR-sequencing and PCR-DGGE-sequencing, respectively. Meanwhile, T-RFLP (McEniry, *et al.*, 2008), DGGE (Naoki and Yuji, 2008), and other DNA-based approaches have proven to be potentially viable tools to investigate the dynamics of the bacterial community over the course of the ensiling process. Using DGGE, Li and Nishino (2011a) determined the bacterial and fungal communities of wilted Italian ryegrass silage with and without *Lactobacillus rhamnosus* or *L. buchneri*. Furthermore, developing some groups-, genus- or species-specific primers, have led to providing several specific identification approaches. For instance, lactic acid bacteria or some particular species, such as *L. plantarum*, *Lactobacillus rhamnosus*, and *L. buchneri* have been monitored during the ensiling process (Brusetti, *et al.*, 2006; Chan, *et al.*, 2003; Dellaglio and Torriani, 1986; Ennahar, *et al.*, 2003; Klocke and Mundt, 2004; Klocke, *et al.*, 2006; Schmidt, *et al.*, 2008; Stevenson, *et al.*, 2006; Tannock, *et al.*, 1999). Meanwhile, some fungi like *Penicillium roqueforti* and *Penicillium paneum* also were identified in silage (Boysen, *et al.*, 2000; O'Brien, *et al.*, 2008). In addition, using PCR-DGGE, Drouin and Lafrenière did extensive studies on the *Clostridium* on silages (Drouin and Lafrenière, 2012; Julien, *et al.*, 2008). Unfortunately, many other important microbes such as enterobacteria, acetic acid bacteria, bacillus, listeria, etc., have yet to be examined.

DGGE/TGGE was developed to determine the genetic diversity of complex microbial populations. Its basis is electrophoresis mobility of PCR-amplified 16S rDNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants/temperatures (Muyzer, *et al.*, 1993). Compared to other fingerprinting approaches, DGGE /TGGE is probably a more successfully and frequently used method to provide information on the community structures of bacteria (Bauer, *et al.*, 2000; Endo and Okada, 2005; Gao, *et al.*, 2008; Wang, *et al.*, 2006), fungi (May, *et al.*, 2001) and general microbes (Ercolini, *et al.*,

2006; Kim, *et al.*, 2009; Muyzer and Smalla, 1998). It has several advantages. (1) It can separate same length DNA sequences with as little as one single base pair difference. (2) It is possible to identify constituents that represent as little as only 1% of the total community. (3) By using universal primers, it does not require prior knowledge of the microbial communities; also, it allows a more accurate identification of specific microbial genera or species through using genera- or species-specific probes. (4) It can profile visually and can monitor changes occurring in various microbial communities which are undergoing various treatments, or it can simply monitor microbial shifts in community structure over time. (5) One can assess multiple environmental samples simultaneously. (6) A great advantage of DGGE/TGGE is the ability to excise, re-amplify and sequence selected bands, for identification, and thus to monitor a particular phylotype (Duarte, *et al.*, 2012; Ercolini, 2004; Muyzer, 1999). This thesis study used DGGE to monitor the microbial changes of LAB, of universal bacteria, and of fungi in whole-plant corn silage during the ensiling period.

1.9 Objectives of the study

Whole-plant corn silage is one of the most important forages for ruminant animals. In the last decade, genetic improvement has expanded corn cultivation further north by developing hybrids adapted to colder regions such as Eastern Canada. Observation on the farm has shown that low temperature could result in less efficient fermentation as shown by fermentation patterns. However, very little experimental work has been undertaken to determine the effects of temperatures lower than 20 °C on ensilage. Moreover, in the literature, most evaluations have been done after a short period of storage (≤ three months) at constant temperature. However, on farms, silage may be stored for longer time and silage temperature may change because of ambient temperature during the storage period in the cold of winter and in the following warming-up in the spring.

Therefore, the general purpose of this thesis was to determine the effects of low temperatures, ranging from 5 to 25 °C, on the fermentation of whole-plant corn silage. There are two specific objectives: (a) to study the effects of temperature, ranging from 5 to 25 °C, on the fermentation of whole-plant corn silage; (b) to evaluate the effects of gradually increased

temperatures, from 5 to 25 °C, on the storage of whole-plant corn silage. The first objective was addressed by investigating the effects of temperatures, ranging from 5 to 25 °C, on epiphytic LAB populations during 60 days of fermentation of whole-plant corn silage **(Chapter II)**, and by studying the effects of temperatures, ranging from 5 to 25 °C, on the biochemical and microbiological characters of whole-plant corn silage after 60 days of fermentation (**Chapter III**). The second objective was achieved by determining biochemical and microbiological changes in corn silage, while incubation temperatures were gradually increased from 5 to 25 °C with a previous ensiling process of 60 days followed by two months of storage at 5°C (**Chapter IV**). By understanding the microbial populations and their activities during the ensiling process and coming to discern how temperatures, ranging from 5 to 25 °C, affect the ensiling process and the storage of whole-plant corn silage, we hope to develop a knowledge that would help to produce high quality corn silage in cool climates and to provide some useful information for inoculant developers.

In Chapter II entitled ‘‘Effect of temperatures (5 to 25 °C) on epiphytic lactic acid bacteria populations during the ensiling process of whole-plant corn silage’’, we performed a factorial experiment at five ensiling temperatures (5, 10, 15, 20 and 25 °C) × seven periods of ensiling (0, 1, 2, 3, 7, 28 and 60 d), according to a completely randomised design. Silage samples were subjected to the measurement of pH and to the determination of LAB diversity using PCR-DGGE. The aim was to investigate the effects of temperatures on epiphytic LAB population during the ensiling process of whole-plant corn during a period of 60 days. The hypothesis was that lowering ensiling temperatures could affect greatly epiphytic LAB populations.

Chapter III, entitled ‘‘Effects of temperatures (5 °C to 25 °C) on the fermentation patterns of whole-plant corn silage’’, was set up to extend the study of Chapter II, as well as to further understand the fermentation patterns in silages, dealt with in Chapter II, which were ensiled at 5 to 25 °C for 60 days. The study determined the biochemical and microbiological parameters of silage. Our hypotheses were that low temperatures restrict silage fermentation, and that the fermentation patterns of whole-plant corn silage can be well explained by the epiphytic LAB populations involved at ensiling at different target temperatures.

In Chapter IV, entitled ‘‘Effects of temperature on the long term storage of whole-plant corn silage’’, the study has simulated the fermentation and storage conditions (four to six months) of corn silage in Eastern Canada. A factorial experiment was carried out at two fermentation temperatures (10 and 20 °C) × five storage temperatures (5, 10, 15, 20 and 25 °C), according to a completely randomised design. The purpose was to determine the effect of temperatures, from 5 to 25 °C, on corn silages which was previously fermented at either 10 or 20 °C for 60 days and then stored at 5 °C for an additional period of two months. Biochemical properties and microbiological populations were measured as well as universal bacterial and fungal diversity during the storage period using PCR-DGGE. Our hypothesis was that the initial fermentation temperature at ensiling, greatly affects the fermentation and microbiological characteristics of whole-plant corn silage and affect the anaerobic phase (storage period).

CHAPTER II

EFFECT OF TEMPERATURES (5 °C to 25 °C) ON EPIPHYTIC LACTIC ACID BACTERIA POPULATIONS DURING THE ENSILING PROCESS OF WHOLE-PLANT CORN SILAGE

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2.1 Résumé

PROBLÉMATIQUE : La fermentation du maïs plante-entière n'est pas toujours efficace dans les régions où la température à l'automne est fraîche. Il en résulte souvent des ensilages avec une conservation inadéquate. L'objectif de cette étude était de comprendre de quelle façon ces conditions climatiques influencent la dynamique des populations de bactéries lactiques épiphytes (BLACE) durant les 60 premiers jours de fermentation de l'ensilage de maïs.

RÉSULTATS : Pour étudier les populations de BLACE durant la fermentation du maïs, 145 silos ont été fabriqués et incubés à différentes températures (25, 20, 15, 10 et 5 °C). Ces silos ont ensuite été ouverts à différents temps durant la fermentation (0, 1, 2, 3, 7, 28 et 60 jours). Il a été possible d'observer qu'aux températures d'incubation les plus élevées (20 et 25 °C), les unités taxonomiques opérationnelles (UTO) correspondant aux espèces *Lactobacillus plantarum* et *Pediococcus pentosaceus* ont dominé la population des BLACE à partir de la première journée de fermentation et ce, durant sept jours. Par la suite, l'UTO, correspondant à l'espèce de bactérie lactique hétérofermentaire *Lactobacillus buchneri,* a été observée et elle est demeurée dominante jusqu'à la fin de la période d'incubation. La dynamique des populations de BLACE des ensilages incubés aux températures plus fraîches (5 et 10 °C) a été différente. À ces températures, les BLACE identifiées en début de fermentation ont été associés à l'UTO *Leuconostoc citreum.* Après 60 jours d'incubation, ce sont les UTO correspondant aux espèces *Lactobacillus sakei* et *Lactobacillus curvatus* qui ont dominé les populations de BLACE. Les profils de diversité observés pour les silos incubés à 15 °C correspondaient à une transition entre les deux intervalles de température.

CONCLUSION : Les températures d'incubation lors de la fermentation du maïs-ensilage influencent la diversité des espèces de bactéries lactiques avec une différence marquée entre les températures plus élevées et les températures plus froides de cet essai. Les basses températures (5 et 10 °C) ont permis d'observer des espèces qui n'avaient jamais été identifiés auparavant dans les ensilages de maïs.

2.2 Abstract

BACKGROUND: Fermentation of whole-plant corn silage is not always very efficient where climate is cool during harvest. It often results in poor quality silage. The objective of the present study was to investigate the effect of temperature (5 to 25 °C) on epiphytic lactic acid bacteria (LAB) populations during 60 days of fermentation of whole-plant corn silage.

RESULTS: A total of 145 experimental silos of chopped whole-plant corn were incubated at five different temperatures (5, 10, 15, 20 and 25 °C), and 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 (OTUs) dominated the fermentation within one day. After seven days, one heterofermentative species *Lactobacillus buchneri*-related OTU 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 OTUs 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 between high and low temperature. Colder temperature conditions (5 and 10 °C) have led to the identification of LAB species never observed in corn silage.

2.3 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 wet forages and other crops. Lactic acid bacteria (LAB), which convert water soluble carbohydrates (WSC) into lactic acid and other organic acids, are the main organisms responsible for 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 which dominate the ensiling process, together with other microflora, often dictate the course of fermentation and, accordingly, determine silage quality (Cai, *et al.*, 1998; Lin, *et al.*, 1992b).

Temperature is a major factor affecting ensilage. In the literature, the majority of ensiling experiments were conducted at a moderate temperature between 20 and 30 °C (Kim and Adesogan, 2006; McDonald, *et al.*, 1966; Weinberg, *et al.*, 1998; 2001). Additionally, the impacts of high temperatures (> 37 °C) have been well studied due to their well-known detrimental effects on forage preservation, e.g., resulting in poor quality silage and low aerobic stability (Kim and Adesogan, 2006; Weinberg, *et al.*, 1998; 2001), inducing clostridial fermentation (McDonald, *et al.*, 1966), heat damage (Garcia, *et al.*, 1989; Goering, *et al.*, 1973), etc. However, the effects of low temperatures (< 20 °C) on silage fermentation have been much less studied. But, in cold climates, such as Canada's, low temperature may be an important factor affecting silage fermentation.

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 corn ensiling process (Brusetti, *et al.*, 2006; Lin, *et al.*, 1992b; Parvin, *et al.*, 2010; Stevenson, *et al.*, 2006). 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 (Brusetti, *et al.*, 2006; Lin, *et al.*, 1992b; Parvin, *et al.*, 2010; Stevenson, *et al.*, 2006). However, low temperature not only could reduce fermentation efficiency by lowering the growth rate and enzymatic activity of

microorganisms (Atlas, 1995; Frazier and Westhoff, 1978), 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 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 during the ensiling process of whole-plant corn.

2.4 Materials and methods

2.4.1 Silage preparation

Corn hybrid (Dekalb D26-78) was seeded in Témiscamingue, Québec, Canada, 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. After 140 days of growth, corn plants were harvested and chopped (average particle size of 1.0 cm) with a forage harvester (New Holland 900, USA) on 25 September 2009. No inoculant was added. For each experimental silo, about 400 g of fresh chopped corn was filled into plastic bags (10 x 16 x 6 mil). Air was removed using a commercial vacuum sealer (Nel 216/219M, Hi-Tech Vacuum, Canada) as described by Johnson *et al.* (2005). A total of 145 experimental silos were immediately incubated at 5, 10, 15, 20 and 25 °C, respectively (29 silos at each temperature). Four silos (repetitions) of each temperature treatment were sampled on day 0, 1, 2, 3, 7, 28 and 60 for the determination of pH and LAB diversity during the ensiling process. One extra silo of each treatment was used for the LAB isolation, in order to develop a reference ladder for further DGGE analysis.

2.4.2 Silage analysis

The pH was measured with a pH meter (Accumet® AB15, Fisher Scientific, Canada), where duplicate samples (10.0 g) of silage were macerated in 100 mL distilled water for 60 min at 200 rpm. Dry matter (DM) content of fresh corn forage (day 0) was determined by oven-drying for 72 h at 50 ± 2 °C. WSC content in fresh corn forage and final corn silage were measured on a water extract (100 mg : 2mL distilled water) using the phenol sulphuric acid colorimetric method according to Dubois *et al.* (1956).

2.4.3 Diversity analysis of LAB

Isolation and identification of epiphytic LAB strains used as external reference strains for DGGE

One extra silo of each temperature treatment (5, 10, 15, 20 or 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, USA) was used and the Petri dishes were incubated at 30 °C for 3 days. Approximately 20 colonies were 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 strains 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 to 7 strains) of each group having the same migration distance in 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 (Edwars, *et al.*, 1989). PCR was carried out in a volume of 25 µL containing 1 µL of DNA template (50 ng), 1 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.2 µM of each primer

and 0.025 U μL^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit, New England BioLabs, 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 analyzer 3130XL (Applied Biosystems, Foster City, USA) 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 version 7.1.3.0. 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).

Diversity analysis of LAB

Diversity analysis of LAB was conducted using PCR-DGGE fingerprinting. Total DNA was extracted from silages using the PowerFood™ Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). 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 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.5 µM 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, USA) 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% to 50% in 1X 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, USA) and visualized under UV illumination using a Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules, USA). 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 analyzed using GelCompar II version 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 & 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% to 70%, at 75 V, 60 °C for 20 h.

2.4.4 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 re-PCR using primers L1GC/HAD2 as described above. Confirmation of the positions of these re-PCR products was accomplished 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, USA) and cloned using the pGEM®-T Easy Vector System II (Promega) according to the manufacturer's instruction. Positive clones 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.

2.4.5 Statistical analyses

Species richness was calculated based on the matrix of band presence/absence. Principal component analysis (PCA) was performed with the matrix of band-relative intensity. Indicator species analysis by combining groups of sites was carried out with the "multipatt" function of the "indicespecies" package (version 1.6.2) (De Cáceres, *et al.*, 2010). Pearson's correlation between pH and band-relative intensity was calculated. Mean value ± standard deviation was presented for biochemical results and band-relative intensity. WSC content on day 60 was analyzed using one-way ANOVA with two assumptions verified (i.e., homogeneity of variance was verified with the Fligner-Killeen test and normality of residual error was assessed with the Shapiro-Wilk normality test), followed by a Tukey HSD multiple comparison ($\alpha = 0.05$). All statistical analyses were performed using R (version 2.15.0, http://www.r-project.org).

2.5 RESULTS

2.5.1 Silage conservation

DM content of fresh corn forage was 304.8 ± 8.2 g kg^{-1} fresh material. The pH of final corn silage (day 60) were 4.0 ± 0.0, 3.8 ± 0.0, 3.9 ± 0.1, 4.0 ± 0.1 and 4.3 ± 0.0 for 25, 20, 15, 10 and 5 °C, respectively, which were all lower than the pH of anaerobic stability (i.e., pHw = 4.4). Therefore all silages can be considered well conserved (Wieringa, 1969). WSC content of fresh forage was 86.8 g kg^{-1} DM , and concentration of residual WSC in final silage (day

60) were 11.7 ± 0.9, 13.0 ± 0.5, 24.2 ± 3.2, 36.5 ± 3.1 and 72.7 ± 9.8 g kg^{-1} DM at 25, 20, 15, 10 and 5 °C, respectively.

2.5.2 Isolation and identification of LAB strains

A total of 73 Gram-positive, catalase-negative, rod- or coccus-shaped LAB strains were isolated. Based on PCR-DGGE analysis, they were classified into eleven putative representative operational taxonomic units (OTUs) (Figure 2.1). DNAs of 27 out of the 73 LAB strains were further amplified with primer pA/pH and sequenced. Table 2.1 gives the results of sequence comparison using BLASTn. The eleven putative OTUs were related to species: *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus sakei*, *Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Pediococcus pentosaceus*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Leuconostoc citreum*1 and *Leuconostoc citreum*2 (with single-base difference from *Leuconostoc citreum*1). It was observed that *L. buchneri* produced multiple bands on DGGE gel. In addition, the PCR products, amplified using nine strains consisting of *L. plantarum*, *Leuconostoc citreum*1, *Leuconostoc citreum*2, *L. brevis*, *Leuconostoc lactis*, *L. sakei*, *L. coryniformis* subsp. *torquens*, *P. pentosaceus* and *L. buchneri*, were combined into one sample which was thereafter used as an external reference pattern on each DGGE gel (Figure 2.1).

Table 2.1 Sequence analysis of silage LAB isolates which were thereafter used to develop DGGE reference ladder

Number of isolates	Number of isolates sequenced	GenBank closest relative	GenBank Accession No.	Similarity (%)	Fragment length (bp)	Corresponding to the OTUs in Figure 2
7	2	*Lactobacillus coryniformis* subsp. *torquens*	KC753453	100	1422	C8
2	1	*Lactobacillus brevis*	KC753454	100	1345	C5
8	2	*Lactobacillus curvatus*	KC753455	100	1352	C9
9	2	*Lactobacillus sakei*	KC753456	100	1444	C7
1	1	*Leuconostoc mesenteroides*	KC753457	99	1282	C3
5	2	*Leuconostoc lactis*	KC753458	100	1429	C6
9	2	*Pediococcus pentosaceus*	KC753459	100	1357	C10
12	7	*Lactobacillus buchneri*	KC753460	100	1357	C13
9	4	*Lactobacillus plantarum*	KC753461	100	1434	C1
10	3	*Leuconostoc citreum1*	KC753462	100	1333	C2
1	1	*Leuconostoc citreum2*†	KC753463	99	1332	-

†single-base difference from *Leuconostoc citreum1*

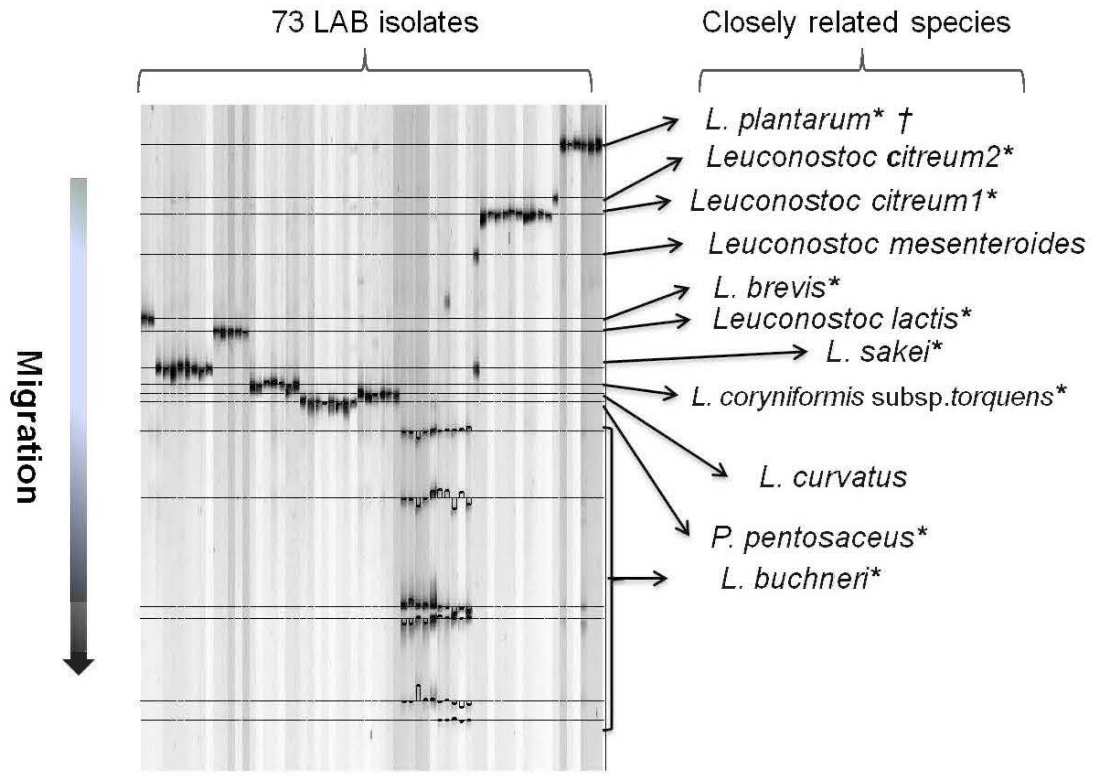


Figure 2.1 73 LAB isolates from corn silage were classified into eleven groups (OTUs) according to their migration distances on DGGE profiles (processed with GelCompar II software). PCR products of nine LAB strains corresponding to nine OTUs labeled with "*" were combined and further served as an external reference pattern on each DGGE gel.

2.5.3 LAB population during the ensiling process of corn silage

Our results demonstrated very different profiles of the LAB succession during corn ensiling process at different temperatures (Figure 2.2, see ANNEXE A.1). Ten of 13 OTUs indicative

of different LAB species were successfully identified, which included *L. plantarum*, *Leuconostoc citreum*, *Leuconostoc mesenteroides*, *L. brevis*, *Leuconostoc lactis*, *L. sakei*, *L. coryniformis* subsp. *torquens*, *L. curvatus*, *P. pentosaceus* and *L. buchneri*. As for band C12, it was successfully excised from DGGE gel, cloned and sequenced, and further identified as one uncultured bacterium (GenBank accession no: JX183831.1, similarity: 99%). Excision or re-amplification of bands C4 and C11 from DGGE gel failed. Regarding species richness, the number of LAB species detected from all temperature treatments rose to a rapid peak during the first three days of ensiling, whereupon it slowed down and eventually declined. The mean highest values of LAB species richness were 7.0, 8.5, 8.0, 8.8 and 4.0 at 25, 20, 15, 10 and 5 °C, respectively (see ANNEXE A.2). As for the *Lactococcus*-group of LAB, one sole band was constantly observed on DGGE gel for most silage samples (see ANNEXE A.3). 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.

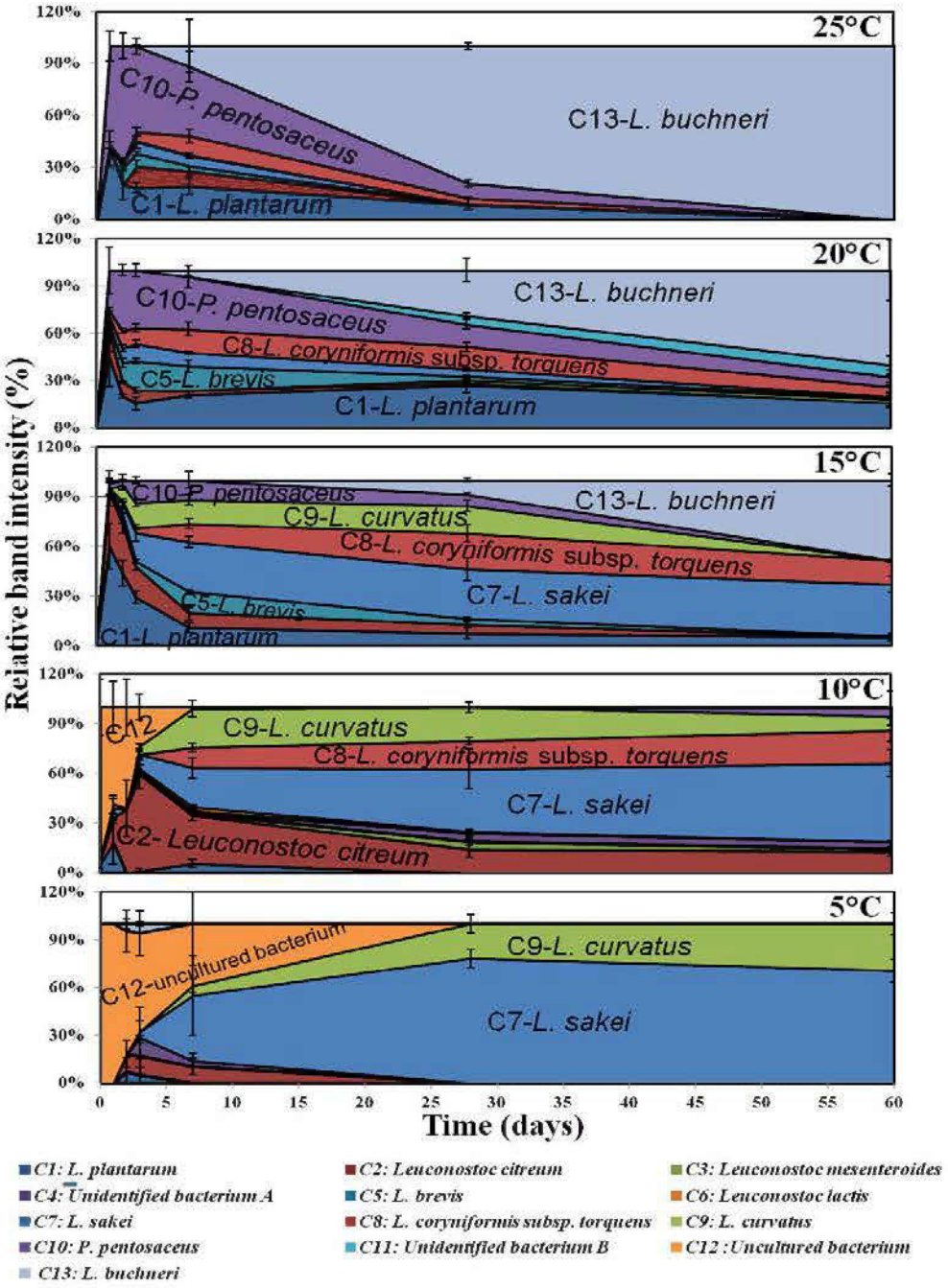


Figure 2.2 Succession of LAB population (OTUs) during the ensiling process of corn silage stored at different temperatures (Band-Relative Intensity (%) ~ Time (days)). Band-relative intensity was described as mean value ± standard deviation. †C1 ~ C13 represents the same LAB-associated OTUs throughout the present study.

Principal component analysis (PCA) was performed in order to obtain an overview of the LAB community at different temperatures. As shown in Figure 2.3a and Figure 2.3b, the contribution percentages of the 1st, 2nd and 3rd principal components were 40.38%, 21.01% and 19.15%, respectively. Using the PC1–PC2 axis, we were able to separate the silage that had been incubated at 10 and 5 °C from those incubated at higher temperatures (20 and 25 °C). Silages incubated at 15 °C are located between the low and warmer temperature groups, but tend to be affiliated with the warmer temperature (PC1–PC3 axis). In addition, LAB-associated OTUs C3 (*Leuconostoc mesenteroides*), C4 (unidentified bacterium A), C6 (*Leuconostoc lactis*) and C11 (unidentified bacterium B) were considered as minor ones due to their low rotation values (eigenvector loadings < 0.01). Referring to Figure 2.3a and 2.3b, OTUs C12 (uncultured bacterium), C2 (*Leuconostoc citreum*), C7 (*L. sakei*) and C9 (*L. curvatus*) prevailed at 5 and 10 °C; whereas C1 (*L. plantarum*), C10 (*P. pentosaceus*) and C13 (*L. buchneri*) dominated at 15, 20 and 25 °C.

Based on the PCA results, we performed indicator species analysis (with a threshold of IndVal = 0.8) by combining groups of ensiling times on silages at lower (5 and 10 °C) and higher temperatures (15, 20 and 25 °C), respectively. Table 2.2 shows that specific LAB-associated OTUs predominated at different stages of the ensiling process. At 5 and 10 °C, C12 (uncultured bacterium) was the only one observed at the beginning of ensilage, and C2 (*Leuconostoc citreum*) appeared on day 2 to day 7 (mainly at 10 °C), and then C7 (*L. sakei*) and C9 (*L. curvatus*) joined the succession and finally dominated. At 15, 20 and 25 °C, C1 (*L. plantarum*), C10 (*P. pentosaceus*), C5 (*L. brevis*) and C2 (*Leuconostoc citreum*) appeared on day 1 and was present there until day 28. C7 (*L. sakei*) and C8 (*L. coryniformis* subsp. *torquens*) appeared on day 2, and C13 (*L. buchneri*) appeared on day 28 and was detected until the end of fermentation.

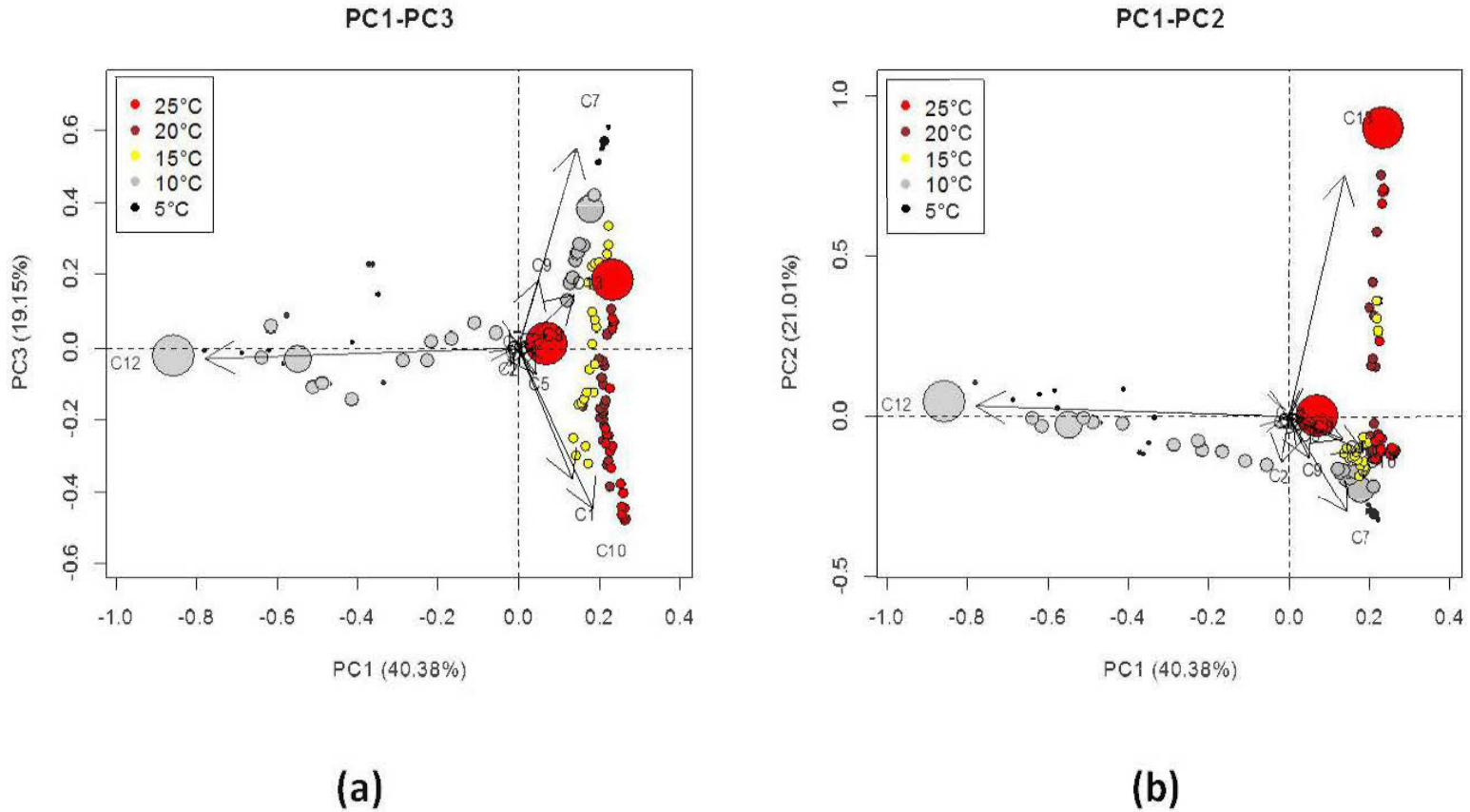
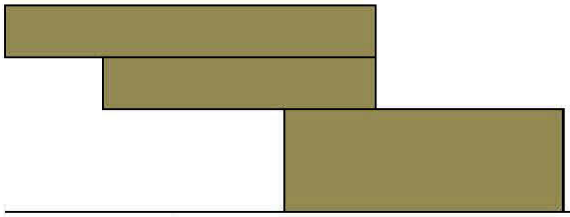
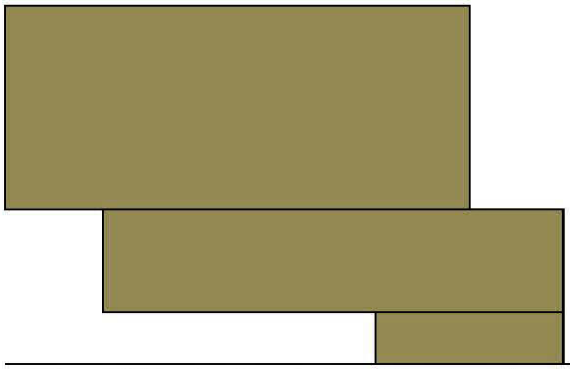


Figure 2.3 PCA analysis of band-relative intensity as a function of OTUs indicative LAB species. PC3 explained 19.15% (> 12.9%) of the variance, higher than what would be predicted under the broken stick model. The size of the circles indicates the frequency of the occurrence of same point.

Table 2.2 Indicator species analysis by combining groups of ensiling times

Days of fermentation						Species	IndVal	*P*	Significance†
1	2	3	7	28	60				(α)
At 5 and 10 °C									
						C12	0.975	0.001	***
						C2	0.847	0.002	**
						C7	0.972	0.001	***
						C9	0.925	0.001	***
At 15, 20 and 25 °C									
						C1	0.974	0.001	***
						C10	0.950	0.001	***
						C5	0.915	0.001	***
						C2	0.894	0.001	***
						C7	0.919	0.001	***
						C8	0.890	0.001	***
						C13	0.976	0.001	***

†** and ***: significant at $P < 0.01$ and 0.001.

2.6 DISCUSSION AND CONCLUSIONS

2.6.1 LAB species associated with corn silage during the ensiling process

A variety of epiphytic LAB species have been reported in corn silage (Brusetti, *et al.*, 2008; Lin, *et al.*, 1992b; Parvin and Nishino, 2010; Stevenson, *et al.*, 2006). Through examination of isolates, Lin *et al.* (1992b) and Brusetti *et al.* (2006) found that *L. plantarum,*

P. pentosaceus and *L. brevis* were three important LAB species. Parvin *et al.* (2010) observed distinctive DGGE bands indicative of *L. plantarum*, *P. pentosaceus* and *Lactococcus lactis* subsp. *lactis*. Using RT-PCR, Stevenson *et al.* (2006) reported that ensiled corn stover developed a predominant population consisting of *L. plantarum* and *L. brevis*, while *L. buchneri* and *P. pentosaceus* were also widely detected. All these studies were carried out at temperatures ranging between 25 and 30 °C, and their results were highly consistent with our findings for corn silage at 20 and 25 °C. In addition, many other LAB species were detected during corn ensiling process. This could be attributed to many factors, e.g., corn hybrid/cultivar (Brusetti, *et al.*, 2008; Lin, *et al.*, 1992b), environmental conditions, sampling time, silage preparation (Parvin and Nishino, 2009), and analysis technique.

For the first time, in corn silage, *L. sakei* and *L. curvatus* were identified at 5 and 10 °C (Figure 2.2). At these low temperatures, one uncultured bacterium (C12) predominated the initial phase of ensilage. Once acidification started, C12 disappeared. Therefore, this species played a very small role during forage acidification, probably due to its high sensitivity to acidity (R = 0.72). Along with acidification, *L. sakei* and *L. 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 which would ensure good conservation. *L. sakei* and *L. curvatus* are phylogenetically close relatives (Pang, *et al.*, 2012). In our assay, both of them were identified as indicator species for corn ensiled at 5 and 10 °C (Table 2.2). Similar strains were found to predominate the microflora in vacuum-packed and processed meat products stored at cold temperatures (Dykes, *et al.*, 1995). Indeed, *L. sakei* proved to contain several transporters for cryo-protective substances and to have more cold stress proteins than other *lactobacilli* (Eijsink and Axelsson, 2005). One strain of *L. curvatus* was isolated from sorghum silage stored at 4 °C, and ensiling trials suggested that it could be used as a silage inoculant at low temperature (Tanaka, *et al.*, 2000). Accordingly, *L. sakei* might be an interesting species for the development of LAB inoculants for cold climates. Moreover, many *L. sakei* strains produce potent bacteriocins, which inhibit growth of pathogenic and spoilage bacteria such as *Listeria* (Eijsink and Axelsson, 2005; Eijsink, *et al.*, 1996). In the future, such strains may be included in silage inoculants in order to improve silage quality and safety.

In this experiment, 15 °C can be viewed as a transition temperature regulating LAB population profiles between two sets of temperature, 20 –25 °C and 5 –10 °C. Characteristic LAB species at both 20 –25 °C (e.g., *L. plantarum*, *P. pentosaceaus* and *L. buchneri*) and 5 – 10 °C (e.g., *L. sakei* and *L. curvatus*) were found in silage at 15 °C (Figure 2.2).

2.6.2 The shift of LAB species during the ensiling process

Results of our study clearly demonstrated that changes in the LAB population diversity occurred during the ensiling process (Figure 2.2). It is generally held that an adequate ensilage usually starts with homofermentative LAB species quickly after sealing; later on, when substrate availability becomes limited, heterofermentative LAB species would replace them and eventually predominate (McDonald, *et al.*, 1991; Seale, *et al.*, 1986; Yang, *et al.*, 2006). Numerous studies have confirmed this shift of LAB flora from homo- to hetero-fermentative species (Beck, 1972; Brusetti, *et al.*, 2006; Langston and Bouma, 1960; Lin, *et al.*, 1992b; McEniry, *et al.*, 2008; Stevenson, *et al.*, 2006). For instance, Langston & Bouma (1960) observed that *L. plantarum*, *L. brevis* and *Pediococcus* spp. were three dominant populations in good quality silage, with heterofermentative *L. brevis* usually observed in later stages. Beck (1972) reported that in ultimately well preserved silages, heterofermentative *L. brevis* and *L. buchneri* replaced homofermentative *L. plantarum* and *L. curvatus* after four days of fermentation. Stevenson *et al.* (2006) also observed the shift from *L. plantarum* to *L. 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 shift 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; Kandler, 1983). Moreover, a similar metabolic change from homolactic to heterolactic fermentation has been observed, and well studied, in LAB species often used in the dairy industries, such as *Lactobacillus casei* (de Vries, *et al.*, 1970), *Streptococcus lactis* (Thomas, *et al.*, 1979), *Lactobacillus bulgaricus* (Rhee and Pack, 1980) and *Lactococcus lactis* (Kowalczyk and Bardowski, 2007; Neves, *et al.*, 2005). 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 grown with excessive substrate, catalysed by lactate dehydrogenase (EC 1.1.1.28) in LAB. While 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 (Liu, 2003; McDonald, *et al.*, 1991; Rhee and Pack, 1980). This is attributed to the low sugar consumption rate which results in 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, i.e., pH, oxygen, type of substrates, substrate limitation, water activity, temperature and salt, were identified to affect the metabolism of LAB and their fermentative patterns (Liu, 2003; Rhee and Pack, 1980; Thomas, *et al.*, 1979). As for the ensiling process, curiously, similar environmental disturbances, e.g., acidity, substrate availability, diffusion of oxygen or moisture, could affect the fermentation and LAB succession during the ensiling process (Brusetti, *et al.*, 2006; Langston and Bouma, 1960; Parvin and Nishino, 2009; 2010; Wang, *et al.*, 2006). In the present study, we assume that 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 ensiled at 20 and 25 °C support the broad consensus concerning the shift from homo- to hetero-fermentative LAB species during ensiling. In these silages, *L. plantarum* and *P. pentosaceus* were gradually replaced by *L. buchneri*. No correlation was found between pH and the band relative intensity of any LAB-associated OTUs ($R_{max}^2 < 0.3$), which is consistent with findings from Lin *et al.* (1992b). 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, $P > 0.05$) suggests that substrate availability could be a factor that regulates the shift of LAB species. The ability of *L. buchneri* to utilize lactic acid could be another factor (Driehuis, *et al.*, 1999). On the other hand, this common assumption of the shift from homo- to hetero-fermentative 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 ($P < 0.05$). 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 C12 (R = 0.72) and acid-tolerance of *L. sakei* (R = − 0.74) and *L. curvatus* (R = − 0.69). Therefore, change in acidity could be the driver of the shift of 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 *L. plantarum* to L. *buchneri* was observed, meanwhile, *L. curvatus* and *L. sakei* accounted for a large proportion of the LAB population in later stages.

To conclude, during the ensiling process, the adaptation of epiphytic LAB population to differing temperature conditions is achieved through the selection of LAB species by temperature. Also, it has been clearly indicated that a change in LAB composition during ensiling occurred at all temperature treatments; however, more research is needed to gain comprehensive knowledge of the underlying mechanisms. 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 to compete, and also to screen appropriate LAB strain(s) not only from final silage products but from different ensiling stages.

2.7 ACKNOWLEDGEMENTS

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CHAPTER III

EFFECTS OF TEMPERATURE (5 °C-25 °C) ON THE FERMENTATION PATTERNS OF WHOLE-PLANT CORN SILAGE

Yiqin Zhou, Pascal Drouin, Carole Lafrenière

Article in preparation for Grass and Forage Science

3.1 Résumé

Cet essai a été réalisé pour étudier l'effet de cinq températures différentes (5, 10, 15, 20 and 25 °C) sur les profils fermentaires du maïs ensilage. Les résultats des analyses biochimiques et microbiologiques ont démontré que les températures les plus froides ont réduit l'intensité de la fermentation du maïs, ce qui a résulté en un pH plus élevé, une baisse de la synthèse des acides organiques et une teneur plus élevée en sucres solubles résiduels. Tous les profils fermentaires des ensilages ont démontré une fermentation hétérolactique après 60 jours de fermentation. Toutefois, avec une température d'incubation de 20 et 25 °C, la fermentation hétérolactique a été reliée à la dégradation de l'acide lactique par *Lactobacillus buchneri* alors qu'aux températures plus froides de 5 et de 10 °C, il s'agissait d'une fermentation hétérolactique. Le profil de fermentation, avec une température d'incubation de 15 °C, a démontré une zone de transition pour les caractéristiques biochimiques et microbiologiques. L'observation de levures avec des températures de 15 °C et moins, pourrait expliquer les problèmes de stabilité aérobie des ensilages souvent observées sur les fermes commerciales. Les profils fermentaires des ensilages de maïs sont expliqués par les bactéries lactiques d'origine épiphyte lesquelles varient avec la température. De plus, la survie des levures a été favorisée lorsque la fermentation des ensilages a été faite à des températures de 15 °C et moins. L'effet des levures sur la diminution de la stabilité aérobie des ensilages lors de l'ouverture des silos est bien connu.

3.2 Abstract

This experiment was carried out to study effects of temperature on the fermentation patterns of whole-plant corn silage at five different temperatures (5, 10, 15, 20 and 25 °C). Biochemical and microbial results showed that lower temperature restricted silage fermentation resulting in higher pH value, less organic acids production and more residual water soluble carbohydrates. All silages showed heterolactic fermentation pattern after 60 days. However, at 20 and 25 °C heterolactic fermentation pattern was linked to lactic acid degradation by *Lactobacillus buchneri* but at 5 – 10 °C it was associated to a heterolactic fermentation. The 15 °C treatment acted as a transition temperature treatment between 20 -25 °C and 5 -10 °C, in terms of both biochemical and microbiological characteristics. Yeasts persisted in silages at temperature equal and below 15 °C. This might explain aerobic instability problems often encountered with whole-plant corn silage on commercial farms. These results showed that fermentation patterns of whole-plant corn silage can be explained by epiphytic LAB populations at ensiling which varied with temperature. Low temperature (15 °C and lower) favoured the survival of yeasts. This population is well known to affect aerobic stability of silage at opening of silos.

3.3 Introduction

Silage is produced by the fermentation of wet forages and other crops. Ensiling is a process of complex succession of microbial and enzymatic activities, which are strongly influenced by temperature (Muck, *et al.*, 2003; Weinberg, *et al.*, 2001). Indeed, temperature has long been regarded as a major factor affecting silage fermentation (McDonald, *et al.*, 1966).

Generally speaking, a moderate temperature from 20 to 30 °C is preferred for silage fermentation, principally because the optimal growth temperature of the lactic acid bacteria (LAB) species involved lies within this range (Pahlow, *et al.*, 2003). In addition, earlier studies have concluded that high ensiling temperatures (> 37 °C) are detrimental to silage preservation. For instance, Garcia *et al.* (1989) (65 vs. 38 °C), Weinberg *et al.* (2001) (41 vs. 24 °C) and Kim and Adesogan (2006) (40 vs. 20 °C) found that high temperatures resulted, not only in less organic acid production, higher pH, more residual WSC and greater nutrient losses, but also higher susceptibility to aerobic spoilage. The activity of plant proteases was reported to increase progressively with an increment of the temperature, leading to greater protein breakdown and ammonia accumulation in silages (Kim and Adesogan, 2006; Muck and Dickerson, 1988). Prolonged temperatures above 45 °C can lead to heat damage to the proteins (Van Saun and Jud Heinrichs, 2008; White, 2013). Moreover, McDonald *et al.* (1966) observed that ensiling grass silage at 42 °C resulted in a clostridial type of fermentation, instead of the desirable lactic acid fermentation. Thus, special care should be taken while making silage in warm climates in order to keep silage temperature in the cool range.

On the other hand, low temperature (< 20 °C) is also not favourable, because it tends to restrict fermentation which usually results in poor quality silage. These silages are often associated with higher pH and low organic acid production (Kung, 2010b; Kung and Shaver, 2001), as well as high residual WSC and yeasts counts, making silages more vulnerable to aerobic deterioration during feed-out (Schroeder, 2009; Ward and de Ondarza, 2008). Besides, the growth of inoculated LAB may also be impaired (Schroeder, 2009; Weinberg and Muck, 1996), since these strains may not be adapted to grow at low temperatures. In northern countries, low temperatures at harvest challenge silage production (Schroeder,

2009). For this reason, some researchers have attempted to look for suitable LAB strains for making silages in cool climates such as the Tibetan Plateau (Pang, *et al.*, 2012) and Japan (Tanaka, *et al.*, 2000).

Extensive work has been done on the effects of moderate and high temperature on silage fermentation. Despite field observations as those described above, however, the effects of low temperature (< 20 °C) on ensilage have hardly been studied. In a previous study, using molecular techniques, we found that temperature, ranging from 5 to 25 °C, affected the composition of epiphytic LAB populations during whole-plant corn ensiling process (Chapter II). Therefore, silages previously done to study the LAB population dynamic were characterized for their fermentation pattern. The objective was to link LAB population to the fermentation pattern in order to better understand temperature issues to ameliorate silage conservation in cool climate. Other microorganism populations were also studied.

3.4 Materials and Methods

3.4.1 Silage preparation

Corn hybrid (Dekalb D26-78) was grown in the Témiscamingue region, Québec (Canada) (the average CHU in this region is about 2300), at a density of 74074 plants ha^{-1}. 150 kg N ha^{-1}, 70 kg P ha^{-1} and 40 kg K ha^{-1} were applied during the growing season. On 25th September 2009, corn plants were chopped (average particle size of 1.0 cm) with a forage harvester (New Holland 900, USA) after 140 days of growth. The mean low and high temperatures on the week of sampling were 8 and 19 °C, respectively (source from www.weatherspark.com). No inoculant was added. For each experimental silo, about 400 g of freshly chopped corn was filled into a plastic bag (10 x 16 x 6 mil). Air was removed using a commercial vacuum sealer (Nel 216/219M, Hi-Tech Vacuum, Canada) according to the protocol described by Johnson *et al.* (2005). A factorial experiment with five ensiling temperatures of 5, 10, 15, 20 and 25 °C and seven opening periods on day 0, 1, 2, 3, 7, 28 and 60 was conducted according to a completely randomized design with four repetitions.

3.4.2 Biochemical analyses

A total of 20 g silage from each treatment was sampled for the pH measurement on day 0, 1, 2, 3, 7, 28 and 60. Silage pH was measured with a pH meter (Accumet® AB15, Fisher Scientific, Canada), where duplicate samples (10.0 g) were macerated with 100 mL distilled water for 24 h at spun at 200 rpm for 60 min. Corn forage (100 g) was collected prior to ensiling (day 0) for the determination of dry matter (DM) content, total N, water soluble carbohydrates (WSC) and buffering capacity (BC). 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 (1990). WSC was measured following water extraction (100 mg : 25 mL distilled water) and determined by the phenol sulphuric acid colorimetric method according to Dubois *et al.* (1956). BC 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 (VFAs, i.e., acetic acid, propionic acid, n-butyric acid and iso-butyric acid). A silage sample of 20.0 g was macerated in 200 mL of 0.1N HCl for 60 min at 200 rpm and then filtered through a Whatman #541 paper. This filtrate was used for the analyses of lactic acid and ammonia. Lactic acid was determined by a spectrophotometric method according to Taylor (1996). Ammonia was determined as described by Flipot *et al.* (1976) on an automated Kjeltec 1030 (Foss, Eden Prairie, USA). Ethanol and VFAs 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, Ont. Canada) equipped with a 25 m capillary column (i.d. 0.319 mm ; film tickness, 0.50 µm ; DB-FFAP, J & W 123-3223) and a flame ionization detector. At the time of sample injection of 0.5 µL, the column temperature was 60 °C for 1 min, then ramped at 20 °C min^{-1} to 120 °C, and then at 15 °C min^{-1} to 150 °C, and then at 35 °C min^{-1} to 220 °C and maintained for 5 min. Inlet and detector temperature were 220 and 300 °C respectively. The split ratio was 25:1. The flow rate for 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 quantify using pure standard of acetic acid (Fisher A38), propionic acid (Anachemia 75992-320), iso-butyric acid (Sigma 1754), n-butyric acid (Aldrich 109959) and ethanol (Alcools de Commerce Ltd.).

3.4.3 Microbiological analyses

Microbial enumerations were performed on day 0, 28 and 60. Each silage sample (20.0 g) was blended in a Stomacher (Seward, UK) for 2 minutes 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. Total colony forming units (CFUs) of LAB, enterobacteria and fungi (i.e., yeasts and moulds) were measured 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). Triplicates of each dilution series were made.

3.4.4 Statistical analyses

For biochemical and microbiological results, normality of residual error was assessed using Shapiro-Wilk normality test; homogeneity of variance was verified with Fligner-Killeen test. Besides, above assumptions were also checked using a graphic method, i.e., plot. If these two criteria were met, the data were analysed using one-way ANOVA, and Tukey HSD multiple comparison was further performed if a significant response was found. Otherwise, mean values and standard deviations (sd) were presented in cases where either or both of the two assumptions were violated. A non-linear regression model was used to fit the pH data, and the lag time (lag, time prior to the rapid decline, in days) and the rate of pH decline (k, per day) were calculated (Jones, *et al.*, 1992). One-way ANOVA followed by Tukey's HSD test were then performed to check the effects of temperature treatments on lag and k. An alpha level of 0.05 of statistical significance was used. In addition, a principal component analysis (PCA) was performed with the chemical and microbiological results. The pH change over time was analysed with SAS (version 9.2), and all other statistical analyses were performed using R (version 2.15.0, http://www.r-project.org).

3.5 Results

3.5.1 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. BC was 24.32 g lactic acid kg^{-1} DM and pH was 5.8. Taking account of DM content, WSC content and BC, the corn forage used in our experiment had a good ensilability. The numbers of LAB, yeasts, enterobacteria and moulds in 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 ($P > 0.05$), which ensured similar initial conditions for different temperature treatments.

3.5.2 pH Change

Figure 3.1 shows the kinetics of pH change for corn silage incubated at the five tested temperatures. A non-linear regression model developed by Jones *et al.* (1992) was able to fit to the data at 10, 15, 20 and 25 °C, but not at 5 °C (Table 3.1). Forage acidification started so rapidly that no lag times were observed at higher incubation temperatures (i.e., 20 and 25 °C), whereas the acidification was delayed by 1.890 days at 15 °C, and by 3.211 days at 10 °C. The rates of pH decline (per day) were 0.095, 0.530, 0.358 and 0.668 at 10, 15, 20 and 25 °C, respectively (see ANNEXE B.1). It was observed that temperature significantly affected the rates of pH decline ($P < 0.0001$) as well as the lag time ($P < 0.0001$). Notably, a slight increase of pH was observed at 25 °C from day 28 to day 60 (3.85 vs. 3.98, $P < 0.05$). In addition, at 5 °C, forage acidification was greatly delayed (≥ seven days), and the decline of pH was the slowest among all five temperature treatments.

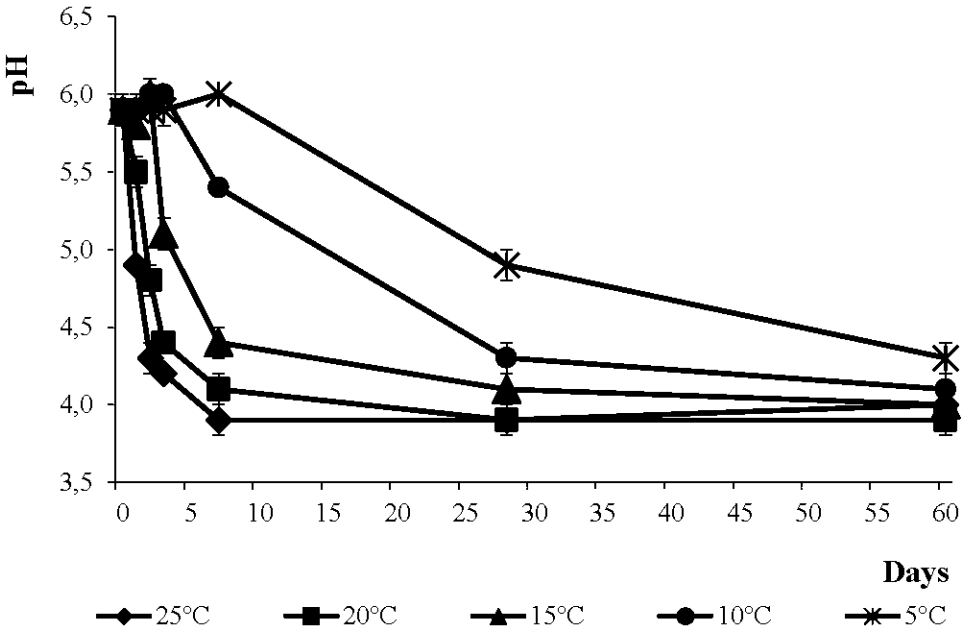

Figure 3.1 Kinetics of pH for whole-plant corn ensiled at different temperatures. pH data is represented as a mean value ± standard deviation.

Table 3.1 Effect of temperature on the pH decline of whole-plant corn silage

Parameter	Temperatures				SEM_{max}
	10 °C	15 °C	20 °C	25 °C	
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 unlike superscripts ($P \leq 0.05$); SEM_{max}: the maximum value of the standard error of the mean within the same row.

3.5.3 Chemical composition of corn silage

As shown in Table 3.2, all silages were well preserved after 60 days of fermentation. Their pH values were lower than the calculated pH of anaerobic stability (computed pH_w was 4.4) (Wieringa, 1969). It was observed that final pH of the silage was significantly ($P < 0.05$) higher at lower incubation temperature. Silages fermented at lower temperatures contained less lactic acid and ammonia, and higher contents of residual WSC. No significant difference was found for the ethanol concentration between the different treatments. Silages incubated at higher temperatures (from 15 to 25 °C) tended to produce more VFAs. For instance, significant higher levels of acetic acid were produced at higher incubation temperatures than at 5 or 10 °C ($P < 0.05$). Higher concentration of propionic acid seemed to be present at higher incubation temperatures. Other VFAs were also detected but the very high variation between temperature treatments made results difficult to interpret.

Table 3.2 Effect of temperature on chemical composition (g kg^{-1} DM) after 60 days of fermentation

	Incubation temperature (day 60)				
	25 °C	20 °C	15 °C	10 °C	5 °C
pH	3.98^{bc}†	3.85^{d}	3.95^{c}	4.05^{b}	4.32^{a}
DM, (g kg^{-1} FM)	315.7^{a}	301.8^{bc}	295.2^{c}	304.0^{bc}	309.9^{ab}
WSC	11.7^{d}	13.0^{d}	24.2^{c}	36.5^{b}	72.7^{a}
Total N	12.18	12.57	12.19	11.88	11.93
Ammonia-N/ Total N, (%)	6.17^{a}	4.62^{b}	4.65^{b}	3.63^{c}	3.80^{c}
Lactic acid	42.76^{c}	63.34^{a}	50.98^{b}	32.35^{d}	30.59^{d}
Acetic acid	75.24^{a}	80.03^{a}	75.92^{a}	40.97^{b}	25.38^{c}
LA-ratio	0.57	0.79	0.67	0.79	1.20
Ethanol	2.73	3.16	4.05	3.96	1.99
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	ND	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

† One-way ANOVA followed by Tukey's HSD test were performed. Within a row, means with unlike superscripts differ significantly ($P < 0.05$). "LA-ratio", the ratio of Lactic acid/ Acetic acid. "ND", not detected. Mean ± sd was presented for Priopionic, n-Butyric and Isobutyric acid.

3.5.4 Microbial counts of corn silage

Microbial counts were made on corn silage after 28 and 60 days of fermentation (Table 3.3). This allowed us to observe the microbiological changes during fermentation, in particular for the two undesirable bacteria groups, i.e., enterobacteria and yeasts. Silages incubated at 20

and 25 °C contained slightly higher numbers of LAB, and the number of LAB stayed stable from day 28 to day 60 for all temperature treatments. 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.

Table 3.3 Effect of temperature on microbial counts ($\log_{10}$ CFU g^{-1} FM) after 28 and 60 days of fermentation

Temperature	LAB		Yeast		Enterobacteria	
	d28	d60	d28	d60	d28	d60
25 °C	9.39[a]	9.09[a]	ND†	ND	ND	ND
20 °C	8.93[b]	9.12[a]	3.58	ND	ND	ND
15 °C	8.27[c]	8.81[b]	3.55	3.37	ND	ND
10 °C	8.16[c]	8.15[d]	3.76	4.43	ND	ND
5 °C	8.38[c]	8.48[c]	3.24	3.99	4.23	ND

† "ND", under the level of detection (< 2 $\log_{10}$ CFU g^{-1} FM); clostridial spores in all silage samples were under the level of detection. One-way ANOVA followed by Tukey's HSD test were performed, and means in a column with unlike superscripts differ ($P < 0.05$).

3.6 Discussion

3.6.1 Low temperature restricts silage fermentation

The effects of low temperatures (< 20 °C) on ensilage have hardly been investigated. 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 temperatures restrict fermentation. During 60 days of ensilage, warmer temperatures (20 and 25 °C) resulted in a more rapid start of forage acidification and decline, whereas lower temperatures generally led to delayed

acidification and lower rates of pH decline especially at 10 and 5 °C (Figure 3.1, Table 3.1). Nevertheless, sufficiently low pH values were eventually reached in all silage samples to ensure their anaerobic stability ($pH < pH_w$) (Wieringa, 1969). This is mainly attributed to the good ensilability of whole-plant corn.

After 60 days of fermentation, it was observed that lower temperatures generally resulted in higher pH, less organic acids produced and more residual WSC. Probably due to the delay in acidification and the low rate of pH decline at lower temperatures, undesirable microbes survived for 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. 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 temperatures, the metabolic activity of enterobacteria could be very low, and moreover, the activity of plant proteases which also play an important role in ammonia production may be greatly inhibited (Rooke and Hatfield, 2003). Overall, the restricted silage fermentation at low temperatures has mainly been attributed to the thermodynamic implication that low temperatures inhibit bacterial metabolism and plant enzymatic activities. However, results from our earlier work indicated that distinct epiphytic LAB populations were involved at different ensiling temperatures, and this could be another important factor.

3.6.2 Epiphytic LAB populations and silage fermentation patterns

Our previous work with the same corn silage samples demonstrated that, at 20 and 25 °C, during 60 days of fermentation, *Lactobacillus plantarum* and *Pediococcus pentosaceus* prevailed in the early stages, but the heterofermentative *L. buchneri* started to proliferate by day 28 and thereafter dominated the fermentation (Chapter II). A group of scientists (Driehuis, *et al.*, 1999) isolated one *L. buchneri* strain from whole-plant corn silage. Under anaerobic conditions, this strain was capable of degrading lactic acid to acetic acid and 1,2-propanediol (Oude Elferink, *et al.*, 2001) which could then be converted to propionic acid and 1-propanol (Krooneman, *et al.*, 2002). In this study, at 20 and 25 °C, the low LA-ratios

(lactic acid/acetic acid) (< 1) together with the slightly increased silage 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 ($P < 0.05$), which was also confirmed by the tendency of silage to contain more propionic acid at 25 °C. In addition, our earlier findings that *L. buchneri* accounted for a larger proportion of the LAB flora at 25 °C than at 20 °C (100% vs. 60.3 ± 19.2%) (Chapter II), were consistent with above-mentioned biochemical characteristics. Oude Elferink *et al.* (2001) also showed that the utilization of lactic acid is pH and temperature dependant.

Corn silages were also well conserved at 5 and 10 °C even though the fermentation was restricted, as described above. Our previous work showed that the facultative heterofermentative *Lactobacillus sakei* and *Lactobacillus curvatus* dominated in these silages (Chapter II). Herein, a LA-ratio close to 1:1 suggested heterolactic fermentation, which was in accordance with the predominance of *L. sakei* and *L. curvatus*. Additionally, we performed a principal component analysis (PCA) with the data of silage biochemical and microbiological compositions (Figure 3.2). 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, i.e., the dominant LAB species at both 20 and 25 °C (i.e., *L. plantarum, P. pentosaceus* and *L. buchneri*) and at 5 and 10 °C (i.e., *L. sakei* and *L. curvatus*) were co-present at 15 °C (Chapter II).

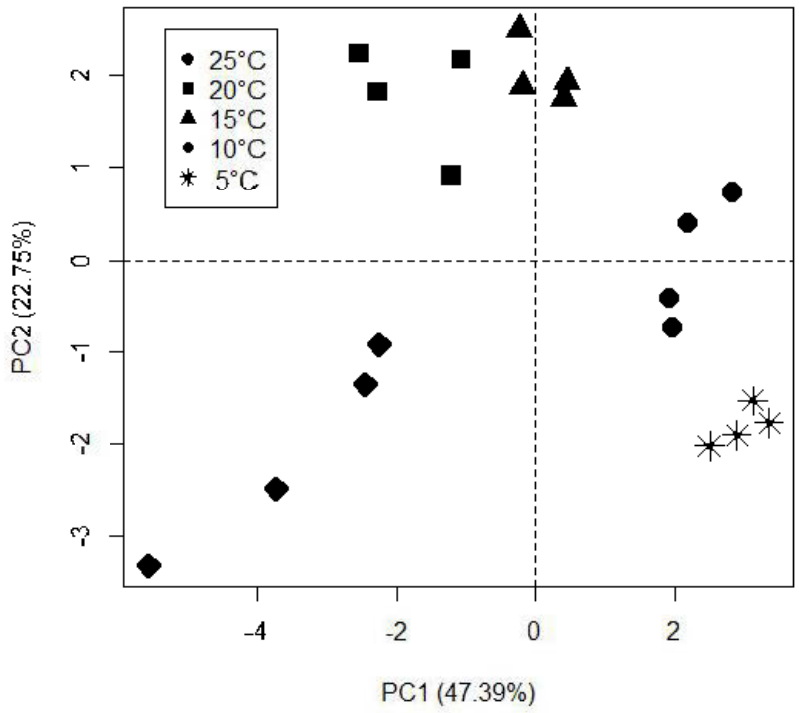

Figure 3.2 PCA analysis of the chemical and microbiological compositions of corn silage stored at different temperatures. Because different types of variables with different units were included, all data were scaled first to have zero unit variance before the analysis took place.

3.6.3 Yeasts

Yeasts are believed to be the primary initiators of silage aerobic spoilage (Woolford, 1990). Silages with high yeast counts are generally more prone to aerobic spoilage when exposed to air (Beck and Gross, 1964; Courtin and Spoelstra, 1990; Pitt, *et al*., 1991). In this study, the log number of yeasts on corn forage was 4.19 prior to ensiling. During 60 days of fermentation, yeasts were under the detection level at 25 °C, and then at 20 °C, whereas similar number of yeasts (about 4 $\log_{10}$ CFU g^{-1} FM) persisted at 15, 10 and 5 °C (Table 3.3).

Undissociated fatty acids, in particular acetic acid, have been recognized as important yeast inhibitors, and this is also the reason why heterolactic fermentation has been advocated in recent years to improve silage aerobic stability (Weinberg and Muck, 1996). In our study, similar levels of acetic acid at 25, 20 and 15 °C ($P > 0.05$) indicated that acetic acid was not the sole inhibitor. Moreover, the considerable differences in acetic acid content at 15, 10 and 5 °C ($P < 0.05$) suggested that acetic acid apparently was not a crucial yeast inhibitor in these silages (Table 3.2). 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 its concentration was 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 natural concentrations of this compound affect populations of yeasts in silages (Kung, *et al.*, 2000). The other possibility is propionic acid which is much more inhibitory than acetic acid (Moon, 1983). Our previous study showed that, after 60 days of fermentation, *L. buchneri,* a propionic acid producer (Krooneman, *et al.*, 2002; 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 (Chapter II). This finding was in accordance with the tendency of silages to contain more propionic acid at warmer temperatures (Table 3.2).

The overall results of the survival of yeasts showed that, the lower was the incubation temperature, the longer it would generally take to inhibit yeasts. However, the survival of yeasts seemed to persist in silage at a temperature equal and below 15 °C within the 60 days of incubation. In practice, many whole-plant corn forages are harvested for silage at around 15 °C or lower temperatures during the fall, particularly in 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, *L. buchneri* has been widely used in silage production, aiming to enhance aerobic stability through inhibiting the growth of yeasts (Combs and Hoffman, 2001; Kung, 2010a), but, our results showed that this bacterium was not adapted to low temperatures (< 15 °C) (Chapter II). It main explain failure that is sometimes observed with corn silage

3.7 Conclusion

Low temperatures restrict silage fermentation, and the fermentation patterns of whole-plant corn silage can be well explained by the epiphytic LAB populations involved at ensiling. In addition, the survival of yeasts at low temperatures (< 15 °C) may explain the problem of aerobic instability of whole-plant corn silage. Moreover, the effects of inoculating *L. buchneri*, aiming to improve silage aerobic stability, need to be further tested at low temperatures.

3.8 Acknowledgements

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CHAPTER IV

EFFECTS OF TEMPERATURE ON THE LONG TERM STORAGE OF WHOLE-PLANT CORN SILAGE

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Article in preparation for Grass and Forage Science

4.1 Résumé

Cette étude a été réalisé pour déterminer l'effet d'une augmentation progressive de la température de 5 à 25 °C durant l'entreposage d'ensilages de maïs plante entière ayant au préalable été fermentés à 10 et 20 °C pendant une période de 60 jours, puis entreposés à 5 °C pendant deux mois additionnels. Les résultats de fermentation réalisés avant l'augmentation de température ont démontré une fois de plus que la température de 10 °C a restreint l'intensité de la fermentation des ensilages comparativement à une température de 20 °C. Pour les ensilages préalablement fermentés à 20 °C suivi d'une augmentation de la température de 5 à 25° C, peu de changement de la composition biochimique et microbiologique a été noté. Toutefois, pour ces ensilages, le nombre d'entérobactéries et de levures a augmenté de façon importante dès que la température d'entreposage a été supérieure à 15 °C (4-5 $\log_{10}$ UFC g MF^{-1}). L'analyse de la diversité moléculaire par PCR-DGGE a indiqué une dominance de la levure *Candida humilis*. Pour les ensilages préalablement fermentés à 10 °C, peu de changement de la composition biochimique a été observé durant l'entreposage jusqu'à ce que la température atteigne 20 °C. Toutefois, entre 20 et 25 °C, la fermentation de ces ensilages a démarré de nouveau. Le nombre de bactéries lactiques a alors augmenté de façon importante, une augmentation de la concentration en acide acétique et une diminution du pH et de la concentration en sucres solubles ont alors été observés. Les patrons de diversité moléculaire par PCR-DGGE ont indiqué une dominance de *Lactobacillus buchneri* entre 20 et 25 °C. Ainsi, la température durant la fermentation de même que la température durant l'entreposage ont un effet sur le développement des populations de microorganismes dans les ensilages ce qui modifient les profils fermentaires. Les ensilages fermentés à 20 °C pourraient être plus à risque lors de leur ouverture en raison d'une meilleure survie des levures et entérobactéries.

4.2 Abstract

This study was carried out to evaluate the effects of gradually increasing temperatures, from 5 to 25 °C on corn silage which was previously fermented at either 10 or 20 °C for 60 days and then stored at 5 °C for two months. After that storage period, results showed that temperature of 10 °C during fermentation restricted silage fermentation compared to fermentation temperature of 20 °C. After two months of storage at 5 °C, as storage temperature was increased from 5 to 25 °C, little changes occurred in silages fermented at 20 °C, in terms of most biochemical parameters as well as bacterial and mould populations. However, in these silages, a fairly high number of enterobacteria and yeasts (4-5 $\log_{10}$ CFU g FM^{-1}) were detected at 15 °C and above. PCR-DGGE profile showed that *Candida humilis* predominated the fungi flora in these silages. For silage where fermentation occurred at 10 °C, no significant changes were observed in most silage characteristics in silages when temperature was increased from 5 to 20 °C. However, above 20 °C, silage fermentation resumed as observed from the significantly increased number of LAB colonies, acetic acid content, and the rapid decline in pH and WSC concentration. In these silages, DGGE results showed that *Lactobacillus buchneri* started to dominate the bacterial flora as temperature increased from 20 to 25 °C. Therefore, temperature during fermentation as well as temperature during storage modulates microorganism population development and fermentation patterns. Silage fermented at 20 °C seems to be more at risk for aerobic stability at opening because of better survival of yeasts and enterobacteria.

4.3 Introduction

Silage is produced by the fermentation of humid forages and crops by lactic acid bacteria (LAB). In term of ensilability, whole-plant corn can be regarded as an ideal crop, because of its relatively high dry matter content (DM), low buffering capacity and adequate level of water soluble carbohydrates (WSC) (Allen, *et al.*, 2003). However, compared to other forage crops, corn silage is found to be more prone to aerobic spoilage when exposed to air (Ashbell and Weinberg, 1992; Filya and Sucu, 2010; Kung, *et al.*, 1998; Li and Nishino, 2011b). Aerobic spoilage of silage is associated with the penetration of oxygen into silos during storage or feed-out periods (McDonald, *et al.*, 1991). Lactate-assimilating yeasts, and occasionally acetic acid bacteria (Spoelstra, *et al.*, 1988), are believed to be the primary spoilage initiators (Wilkinson and Davies, 2013). Silages with yeast counts of more than 100,000 per gram are generally at a higher risk of aerobic spoilage at feed-out (Wilkinson and Davies, 2013). Also, silages with high levels of residual sugars (Ruxton, *et al.*, 1975), high concentrations of lactic acid (Sebastian, *et al.*, 1996), and low levels of volatile fatty acids (VFAs) (e.g., acetic and propionic acid) (Moon, 1983) are more susceptible to aerobic deterioration at feeding. This is because sugars and lactate can be readily utilized by yeasts, and VFAs inhibit yeast growth (Weinberg, *et al.*, 1993).

Temperature affects silage fermentation (Muck, *et al.*, 2003). In most of the studies investigating the effects of temperature on ensilage, the majority of the ensiling experiments were conducted at a moderate temperature (from 20 to 30 °C) (Kim and Adesogan, 2006; McDonald, *et al.*, 1966; Weinberg, *et al.*, 1998; 2001), and / or a fixed elevated temperature (> 37 °C). High temperatures are well-known for their detrimental effects on silage fermentation, resulting in poor quality and low aerobic stability silages (Kim and Adesogan, 2006; Weinberg, *et al.*, 1998; 2001), inducing clostridial fermentation (McDonald, *et al.*, 1966), and heat damage (Garcia, *et al.*, 1989; Goering, *et al.*, 1973). Very little information is available concerning the effects of temperatures lower than 20 °C on silage fermentation. Moreover, in all previous studies, evaluation has been conducted after moderately short lengths of ensiling (≤ 82 days). However, on many farms, silages may remain stored for

longer periods of time. To our knowledge, there are no long-term studies on the effects of temperature on silage fermentation.

In the last decades, breeding has expanded corn cultivation further north by developing hybrids adapted to cool climates. In climates similar to Eastern Canada, corn is harvested for silage in autumn when mean temperature is around 10 °C. Ensiled corn will be stored and consumed during winter and spring seasons when other green forages are still not available. In spring, as temperature warms up, corn silage, particularly the outer layers of the silo will be confronted to the rising temperatures.

In our previous study, it was found that, during 60 days of incubation, different fermentation temperatures induced distinct LAB populations during corn ensiling process (Chapter II), and resulted in very different biochemical and microbiological profiles (Chapter III). We venture the hypothesis that similar changes in silage profiles will also be observed with rising temperatures after an additional two months of storage at 5 °C, which should simulate the temperature increase observed in spring. In this study, we simulated the fermentation and storage conditions of corn silage in Eastern Canada, and our purpose was to determine the effect of an increase of incubation temperature, from 5 to 25 °C, on whole-plant corn silage which was previously ensiled at either 10 or 20 °C for 60 days and then stored at 5 °C for an additional period of two months. Biochemical properties and microbiological populations including the bacterial and fungal population diversity were determined.

4.4 Materials and Methods

4.4.1 Silage preparation

Corn hybrid (Dekalb D26-78) was seeded in the Témiscamingue region of Québec, Canada, at a density of 74074 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. After 129 days of growth, corn plants were harvested at 1/3 milk line and chopped (average particle size of 1.0 cm) with a forage harvester (New Holland 900, USA) on 20 September 2010. No silage inoculants were added. The fresh material was immediately transported to the laboratory where experimental silos were

prepared. About 350 g of chopped corn were filled into plastic pouches (10 x 16 x 6 mil). Air was removed using a commercial vacuum sealer (Nel 216/219M, Hi-Tech Vacuum, Canada) according to the protocol described by Johnson *et al.* (2005).

A factorial experiment with Fermentation Temperature (10 and 20 °C) × Storage Temperature (5, 10, 15, 20 and 25 °C) was carried out. A total of 40 experimental silos were prepared. They were split into two groups with 20 silos each, and incubated at 10 and 20 °C, respectively, for two months, and then stored at 5 °C for another two months. Afterwards, storage temperature was increased progressively from 5 to 25 °C following a weekly increment of 2.5 °C. Four repetitions were made for each treatment. It took two weeks for storage temperature increased from 5 to 10 °C, 10 to 15 °C and etc. Four silos from each storage temperature treatment (5, 10, 15, 20 and 25 °C) were sampled at the end of each incubation period. Thus, silos which were sampled at 5 °C had been stored for four months and silos which were sampled at 25 °C had been stored for six months. This represents what is going on commercial farms.

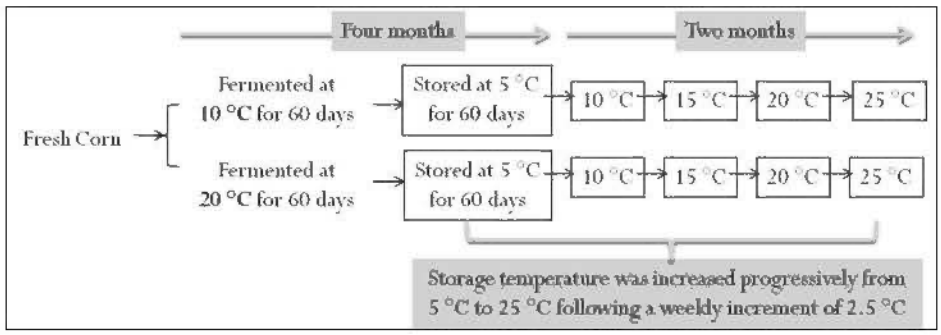

Figure 4.1 The workflow of silage treatments

4.4.2 Sampling

For each experimental silo, 20 g of silage were sampled for pH measurement whereas 100 g of silage were sampled for the determination of DM content, WSC, total N, ammonia, lactic

acid, ethanol and VFAs (i.e., acetic acid, propionic acid, n-butyric acid and iso-butyric acid). Finally, 20 g of silage were used to perform microbial enumeration and another 20 g were used for molecular microbial diversity analyses. Fresh chopped corn samples prior to ensiling were submitted to similar biochemical analyses (i.e., pH, DM, WSC and total N) and microbial enumeration.

4.4.3 Biochemical analyses

The pH was measured with a pH meter (Accumet® AB15, Fisher Scientific, Canada), where duplicate samples (10.0 g) of silage were macerated with 100 ml distilled water for 24 hand spun at 200 rpm for 60 min. The DM content was determined by oven-drying for 72 h at 50 ± 2 °C. Total N was determined according to the method 7.022 of AOAC (1990), and WSC were extracted using water (100 mg : 25 mL distilled water) and measured by the phenol sulphuric acid colorimetric method according to Dubois *et al.* (1956). Ethanol and VFAs were determined on silage water extract according to Fussell and McCalley (1987). Analyses were conducted with a gas chromatograph (Model 6850, Agilent, Mississauga, Ont. 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 moment of sample injection of 0.5 µL, the column temperature was set to 60 °C for 1 min, then oven temperature increased to 120 °C at a rate of 20 °C min^{-1}, to 150 °C at a rate of 15 °C min^{-1}, and then to 220 °C at a rate of 35 °C min^{-1} and maintained for 5 min. Inlet and detector temperature were 220 and 300 °C respectively. The split ratio was 25:1. The flow rate of hydrogen which was used as 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. Peaks were identified and quantified by comparison with pure standards of acetic acid (Fisher A38), propionic acid (Anachemia 75992-320), iso-butyric acid (Sigma 1754), n-butyric acid (Aldrich 109959) and ethanol (Alcools de Commerce Ltd.).

In addition, 20.0 g of silage was macerated in 200 ml of 0.1N HCl for 60 min on a reciprocal shaker set at 200 rpm and then filtered through a Whatman #541 paper. The filtrate was used for the analyses of lactic acid and ammonia. Lactic acid was determined by a

spectrophotometric method according to Taylor (1996). Ammonia was determined as described by Flipot *et al.* (1976) on an automated Kjeltec 1030 (Foss, Eden Prairie, USA).

4.4.4 Microbial enumeration

Each silage samples (20.0 g) were blended in a Stomacher (Seward, UK) for 2 min 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. Total colony forming units (CFUs) of LAB, enterobacteria and fungi (i.e., yeasts and moulds) were measured after incubation at 28 °C for three days 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). Triplicates of each dilution series were made.

4.4.5 Analyses of bacterial and fungal diversity

PCR-DGGE fingerprinting was used to analyze the bacterial and fungal diversity in corn silage. Total DNA was extracted from silage samples using the PowerFood™ Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA).

For bacterial diversity, we tested four primer sets including 357F (5´-CCT ACG GGA GGC AGC AG-3´) / 517R (5´-ATT ACC GCG GCT GCT GG-3´) (Gao, *et al.*, 2008), F984 (5´-AAC GCG AAG AAC CTT AC-3´) / R1378 (5´-CGG TGT GTA CAA GGC CCG GGA ACG-3´) (Heuer, *et al.*, 1997), 16F945 (5´-GGG CCC GCA CAA GCG GTG G-3´) / 16R1401 (5´-CAC GGG GGG CGG TGT GTA CAA GAC CC-3´) (Thanh, *et al.*, 2008) and W01 (5'-AGA GTT TGA TC[AC] TGG CTC-3') / W012 (5'-TAC GCA TTT CAC C[GT]C TAC A-3') - HDA1 (5'-ACT CCT ACG GGA GGC AGC AGT-3') / HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA-3') (a nested PCR) (Ogier, *et al.*, 2004). 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 primer 357F, F984, 16R1401 and HDA1 for DGGE analyses (Walter, *et al.*, 2001). PCR amplification failed with 16F945/16R1401GC. By comparing their DGGE profiles, primer set 357FGC/517R produced more distinct bands and therefore was selected to amplify the V3 regions of the 16S rDNA of bacteria in silages (see ANNEXE C.1 and

ANNEXE C.2). PCR was carried out in a volume of 15 µL containing 1 µL of DNA template (50 ng), 1 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.3 µM of each primer and 0.025 U μL^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit, New England BioLabs, Canada). PCR cycles consisted of an initial DNA denaturation at 95 °C for 10 min, 30 cycles of denaturation at 93 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. DGGE was carried out using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) according to Kebli *et al.* (2011) with a modification in the range of denaturing gradient. PCR products (15 µL) were applied on 8 % polyacrylamide gels (acrylamide : bis-acrylamide, 37.5:1) with a linear denaturing gradient range of 32 % to 60 % in 1X TAE electrophoresis buffer. Electrophoresis was performed at a constant voltage of 75 V and a temperature of 60 °C for 16 h. Then the gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) and visualized under UV illumination using a Molecular Imager® ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules, CA, USA). DGGE profiles were analyzed using software GelCompar II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). One matrix of band relative intensity was obtained. And 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.

As for the fungal diversity (i.e., yeasts and moulds), we tested five primer sets including NS1 (5'-GTA GTC ATA TGC TTG TCT C-3') / Fung (5'-ATT CCC CGT TAC CCG TTG-3') (May, *et al.*, 2001), NL1 (5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3') / LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Stringini, *et al.*, 2008), NL3A (5'-GAG ACC GAT AGC GAA CAA G -3') / NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') (Thanh, *et al.*, 2008), ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') / ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Bonito, *et al.*, 2010), and ITS1F (5'-TTG GTC ATT TAG AGG AAG TAA-3') / ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') (Kebli, *et al.*, 2011). A same 40-bp GC clamp (Walter, *et al.*, 2001) was attached to the 5' end of primer Fung, NL1, NL4, ITS1 and ITS1F for DGGE analyses. PCR amplification failed with ITS1GC/ITS4. By comparing their DGGE profiles, primer set ITS1FGC / ITS2 produced more distinct bands and therefore was selected to amplify a fragment of 280 bp of the SSR region of fungi in silages (see ANNEXE

C.3 and ANNEXE C.4). PCR was carried out in a volume of 15 µL containing 1 µL of DNA template (50 ng), 1 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.3 µM of each primer and 0.025 U μL^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit, New England BioLabs, Canada). PCR cycles consisted of an initial DNA denaturation at 95 °C for 3 min, 30 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec, extension at 72 °C for 1 min 15 sec, and a final elongation step at 72 °C for 5 min. DGGE was performed according to the same protocol described above, with a linear denaturing gradient range of 20% to 50%. DGGE profiles were analyzed as described above.

A protocol based on band excision-reamplify was initially used to identify DGGE bands. Despite the time-consuming and laborious procedures, results of the following check-up DGGE showed that this protocol failed to extract specific bands from DGGE gels. High bacterial diversity in the silage samples probably explains this failure. Similar difficulties has also been encountered by many other DGGE users (Green, *et al.*, 2009). We thereafter tried to clone the PCR products of representative silage samples, and then identified individual DGGE bands by aligning the PCR amplicons of the clones with the PCR products of silage samples on same DGGE gels. To be detailed, this procedure required that the DNAs of silage samples were amplified with same selected primers as described above, the PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and subsequently cloned using the pGEM®-T Easy Vector System II (Promega) according to the manufacturer's instruction. 80 bacterial and 80 fungal positive clones were screened and streaked twice. Their plasmids were isolated using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). Sequence analysis was conducted with a BigDye® Terminator v3.1 Cycle Sequencing Kit on the genetic analyzer 3130XL (Applied Biosystems, Foster City, CA, USA) at the Plate-forme d'Analyses Biomoléculaires (Université Laval, Québec, Canada), where promoter primers Sp6 and T7 were used. The nucleotide sequences of clones were aligned using BioEdit software (version 7.1.3.0) and DNA sequence similarity searches were done via BLASTn against the GenBank database of the United States National Center for Biotechnology Information (NCBI). Finally, representative clones with different sequences were re-amplified using same primers, and then aligned with the PCR amplicons of silage samples on same DGGE gels.

4.4.6 Statistical analyses

For this factorial experiment [Fermentation Temperature (10 and 20 °C) × Storage Temperature (5, 10, 15, 20 and 25 °C)], firstly, a two-way ANOVA with interaction was done with the biochemical and microbiological results. Interaction was found significant for several parameters (see ANNEXE C.5 and C.6). Thereafter, one-way analysis of variance (one-way ANOVA) was performed on the biochemical and microbiological compositions of corn silages. Normality of residual error was assessed using Shapiro-Wilk normality test and homogeneity of variance was verified with Fligner-Killeen test. If these two criteria were met, the data were analyzed using one-way ANOVA. Differences between treatment means were tested by Tukey HSD test in cases where statistical significance was observed ($\alpha = 0.05$). Otherwise, mean value and standard error of the mean were presented in cases where either or both of the two assumptions were violated. Standard error of the mean (se or SEM) was calculated using the 'std.error' function of the 'plotrix' package (version 3.4-8). Shannon diversity index were calculated based on the matrix of band relative intensity. All statistical analyses were performed using R (version 3.0.0, http://www.r-project.org).

4.5. Results

4.5.1 Initial conditions of fresh corn forage

Fresh corn forage contained 309.90 ± 2.72, 125.55 ± 2.59, 13.06 ± 0.03 g kg^{-1} DM, WSC and total N, respectively. Fresh corn forage pH was 5.85 ± 0.03. The numbers of LAB, yeasts, enterobacteria, and moulds were 4.43 ± 0.10, 4.95 ± 0.04, 7.04 ± 0.19 and 4.38 ± 0.05 $\log_{10}$ CFU g^{-1} fresh materials (FM), respectively. The number of clostridial spores was 3.18 ± 0.20 $\log_{10}$ CFU g^{-1} FM. Overall, the biochemical and microbiological compositions of the fresh corn forage used in the present experiment was normal for whole-plant corn harvested at one-third milk line.

4.5.2 Biochemical composition of corn silage

Table 4.1 gives the results of the effects of fermentation temperature on the biochemical composition of corn silages after two months of fermentation (20 vs. 10 °C) and two months

of storage at 5 °C. All silages could be considered as well conserved (pH < pHw, pH of anaerobic stability) (Wieringa, 1969). Silages incubated at 10 °C were less fermented than those at 20 °C, and had significantly higher pH value (4.04 vs. 3.74) and residual WSC (95.84 vs. 35.94 g kg^{-1} DM) ($P < 0.01$). Silages fermented at 20 °C also contained significantly ($P < 0.01$) higher contents of lactic acid (59.24 vs. 34.99 g kg^{-1} DM), ethanol (10.66 vs. 2.83 g kg^{-1} DM) and NH_3-N/Total-N (5.41% vs. 3.13%). In addition, no significant differences were found on DM and total N content, as well as single VFA concentration.

Table 4.2 and Figure 4.2 present the biochemical changes in corn silages as storage temperature increased from 5 to 25 °C. For corn silages initially fermented at 20 °C, no important changes were observed in pH and WSC content as storage temperature increased from 5 to 25 °C. No significant change ($P > 0.05$) was found in the content of DM, total N, lactic acid, or most VFAs except acetic acid. The concentration of acetic acid, ethanol and ammonia (NH_3-N/Total-N) in these silages tended to increase as storage temperature rose up to 25 °C. On the other hand, the results from corn silages initially fermented at 10 °C differ from those from silages fermented at 20 °C. As storage temperature increased to 25 °C, silage fermentation resumed as demonstrated by a substantial decline in WSC content (from 108.65 to 47.04 g kg^{-1} DM) and pH (from 4.02 to 3.80) ($P < 0.05$). Simultaneously, significant increases were observed in the production of acetic acid and ammonia at 25 °C ($P < 0.001$).

Table 4.1 Effects of fermentation temperature (10 °C vs. 20 °C) on the biochemical (g kg^{-1} DM) and microbiological (Log_{10} CFU g FM^{-1}) compositions of corn silage

Fermentation Temp	pH	DM (g kg^{-1} FM)	WSC	Total N	Lactic acid	Acetic acid	Ethanol	Propionic acid	n-Butyric acid	iso-Butyric acid	NH_3-N/ Total N (%)	LAB	Clostridia spores
20 °C	3.74	299.4	35.94	13.78	59.24	80.01	10.66	1.17	0.53	0.28	5.41	7.85	2.80
10 °C	4.04	308.3	95.84	13.80	34.99	78.41	2.83	0.95	0.45	0.23	3.13	5.80	3.12
SEM	0.056	2.639	11.386	0.102	5.582	0.637	1.590	0.282	0.053	0.088	0.442	0.389	0.154
P	***	NS	***	NS	**	NS	***	NS	NS	NS	***	***	NS

Note: biochemical measurement and microbial enumeration were done after two months of fermentation at each temperature and another two months of storage at 5 °C. SEM: standard error of the mean; NS, not significant; ** and ***: significant at $P \leq 0.01$ and 0.001. Enterobacteria, yeasts and moulds counts were under the detection level (< 2 log_{10} CFU g FM^{-1}) for both fermentation temperatures.

Table 4.2 Effects of the increasing temperature during storage period on the chemical composition (g kg^{-1} DM) of corn silage initially fermented at two different temperatures

Storage temperature	Total N	DM (g kg^{-1} FM)	Lactic acid	Acetic acid	Ethanol	NH_3-N/ Total N (%)	Propionic acid	n-Butyric acid	iso-Butyric acid
Fermentation temperature = 20 °C									
5 °C	13.78	299.4	59.24	80.01ab	10.66	5.41a	1.17	0.53	0.28
10 °C	13.87	307.9	55.92	70.64a	7.50	5.35a	0.94	1.32	0.07
15 °C	13.86	306.9	59.01	93.22bc	8.10	5.42a	0.90	1.64	0.10
20 °C	13.53	303.9	50.98	107.63c	13.45	5.63ab	0.76	1.02	0.13
25 °C	13.59	306.6	61.33	107.86c	15.25	6.16b	1.21	1.85	0.28
SEM	0.080	1.25	1.912	4.081	0.895	0.086	0.144	0.208	0.045
Effects of increasing temperature	NS	NS	NS	***		**	NS	NS	NS
Fermentation temperature = 10 °C									
5 °C	13.80	308.3	34.99	78.41A	2.83	3.13A	0.95	0.45	0.23
10 °C	13.63	314.0	40.49	80.96A	2.79	3.40A	1.10	0.22	0.19
15 °C	13.70	311.3	36.08	80.17A	3.16	3.44A	1.07	0.21	0.17
20 °C	13.68	304.8	44.39	81.16A	2.52	3.55A	1.41	0.54	0.36
25 °C	13.97	305.2	45.41	139.17B	4.08	5.34B	1.27	0.56	0.30
SEM	0.089	1.60	1.531	5.569	0.213	0.182	0.054	0.059	0.039
Effects of increasing temperature	NS	NS	NS	***	NS	***	0.063	NS	NS

Note: For each fermentation temperature (20 and 10 °C), values with different letters within the same column are statistically different ($P \leq 0.05$); SEM: standard error of the mean; NS, not significant; *, ** and ***: significant at $P \leq$ 0.05, 0.01 and 0.001, respectively. The data of Ethanol and WSC were log transformed for the verification of the two assumptions before ANOVA analysis.

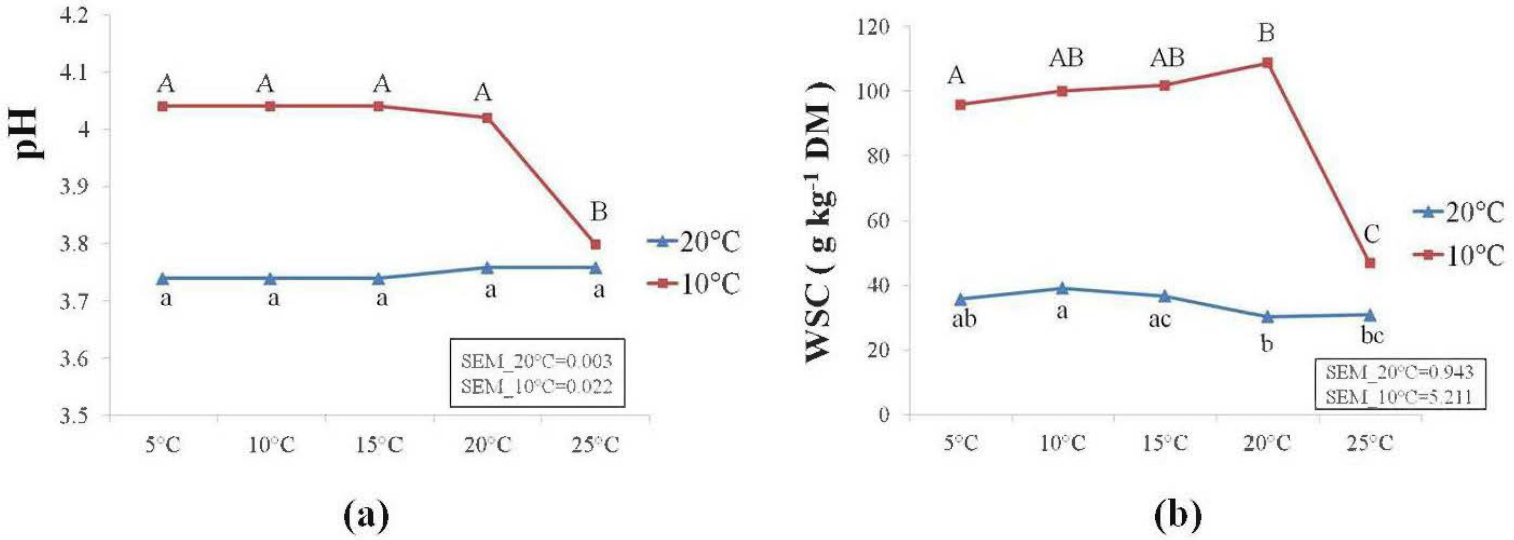

Figure 4.2 Effects of the increasing temperature (from 5 to 25 °C) during storage period on the pH (a) and WSC content (b) in corn silage initially fermented at two temperatures (10 and 20 °C). For each fermentation temperature (20 and 10 °C), values labelled with different letters within same pane are statistically different ($P \leq 0.05$).

4.5.3 Microbial counts of corn silage

Microbial enumeration was made on corn silage (Table 4.3). Regarding the effects of initial fermentation temperature (20 vs. 10 °C), significant higher LAB counts were detected at 20 °C than at 10 °C (7.85 vs. 5.80 log $_{10}$ CFU g FM^{-1}) ($P < 0.001$) (Table 4.1). For silages initially fermented at 20 °C, LAB counts tended to slightly increase as storage temperature increased from 20 to 25 °C. Undesirable microbes (i.e., enterobacteria and yeasts) started to appear at 15 °C and above. On the contrary, in silages initially fermented at 10 °C, LAB counts increased sharply as storage temperature reached 20 and 25 °C ($P < 0.05$), and neither enterobacteria nor yeasts were detected (< 2.00 $\log_{10}$ CFU g^{-1} FM).

No moulds were detected in all silage samples (< 2.00 $\log_{10}$ CFU g^{-1} FM). Number of clostridial spores was not affected by neither initial fermentation temperatures (20 vs. 10 °C) (Table 4.1) nor following temperature increases from 5 to 25 °C during the storage period. Moreover, the numbers of spores in corn silage were similar to those in corn forage prior to ensiling. These findings indicated that the growth of *Clostridium* was inhibited, and no germination of clostridial spores took place during the entire experimental period.

Table 4.3 Effects of the increasing temperature during storage period on the microbiological composition (log_{10} CFU g FM^{-1}) of corn silage initially fermented at two different temperatures

Storage temperature	LAB	Enterobacteria	Yeasts	Moulds	Clostridia spores
Fermentation temperature = 20 °C					
5 °C	7.85 a	ND	ND	ND	2.80
10 °C	7.82 a	ND	ND	ND	3.21
15 °C	7.67 a	4.02 ± 0.07	3.93 ± 0.08	ND	3.52
20 °C	7.81 a	4.71 ± 0.47	4.85 ± 1.21	ND	3.43
25 °C	8.19 b	4.78 ± 0.10	4.44 ± 1.11	ND	3.68
SEM	0.049	0.517	0.504	0	0.108
Effects of increasing temperature	**				NS
Fermentation temperature = 10 °C					
5 °C	5.80 A	ND	ND	ND	3.12
10 °C	5.85 A	ND	ND	ND	3.37
15 °C	5.85 A	ND	ND	ND	3.28
20 °C	7.43 B	ND	ND	ND	3.54
25 °C	8.54 C	ND	ND	ND	3.58
SEM	0.255	0	0	0	0.061
Effects of increasing temperature	***				NS

Note: For each fermentation temperature (20 and 10 °C), values with different letters within the same column are statistically different ($P \leq 0.05$); Mean ± se was presented for enterobacteria and yeast counts; ND, not detected (< 2.00 log_{10} CFU g FM^{-1}); SEM: standard error of the mean; NS, not significant; ** and ***: significant at $P \leq 0.01$ and 0.001, respectively.

4.5.4 Bacterial diversity of corn silage

We identified the main bacterial clones (Table 4.4). LAB species represented the major bacterial population, in particular some heterofermentative species such as *Lactobacillus buchneri*, *Lactobacillus brevis*, *Weissella koreensis* and *Leuconostoc citreum*. Other LAB species such as *Lactobacillus oryzae* and *Pediococcus parvulus* were also found. Enterobacteria such as *Pantoea agglomerans* were detected. Our results indicated that, a clone identified as of the genus *Chryseobacterium,* was observed for the first time in corn silage in rather high occurrence as shown by its high relative intensity in Figure 4.3. Figure 4.3 presents the DGGE profiles of universal bacteria in corn silages and the corresponding Shannon diversity index (H'). Higher diversity was observed in corn silages initially fermented at 10 °C compared to 20 °C. H' of silages initially fermented at 20 °C was not affected by storage temperatures ($P > 0.05$). However, in silages which were fermented at 10 °C, a significant decrease in H' was observed as storage temperature increased to 25 °C ($P < 0.05$). At 25 °C, the H' value of both initial fermentation treatments reached the same value. In addition, six OTUs in the DGGE profiles were aligned to bacteria clones. It was noticed that the relative intensity of *L. buchneri* (OTU E) tended to increase at higher storage temperatures, particularly in silage samples which were initially fermented at 10 °C.

Table 4.4 Sequence analysis of bacterial clones

Number clones sequenced	Most closely related bacterial sequence	GenBank Accession No. of related sequence	Similarity %	Environment from which related sequence was isolated
20 [E]	*Lactobacillus buchneri*	JQ249065.1	100	Fermented cucumber (USA)
12 [C]	*Lactobacillus brevis*	KC713915.1	100	Fermented bamboo shoot (India)
5 [D]	*Chryseobacterium* sp.	AB461706.1	100	Stems of field-grown soybeans (Japan)
4 [A]	*Weissella koreensis*	NR075058.1	100	Kimchi (USA)
1 [B]	*Leuconostoc citreum*	KC417025.1	99	Wheat flours (Italy)
8	Uncultured bacterium isolate	JX183833.1	98	Jejunum, ileum and cecum of weaned piglets (China)
6	*Pantoea agglomerans*	KC355300.1	99	Pepper (South Korea)
2	*Lactobacillus oryzae*	AB731661.1	99	Fermented rice grain (Japan)
2	*Pediococcus parvulus*	AB601176.1	100	Italian ryegrass silage (Japan)
1	*Stenotrophomonas maltophilia*	KC764984.1	100	Tobacco rhizosphere soils (China)

Note: Clones labeled with superscripts "A" to "E" correspond to the marked out OTUs in Figure 4.3. Sequence analysis of 19 universal bacterial clones failed.

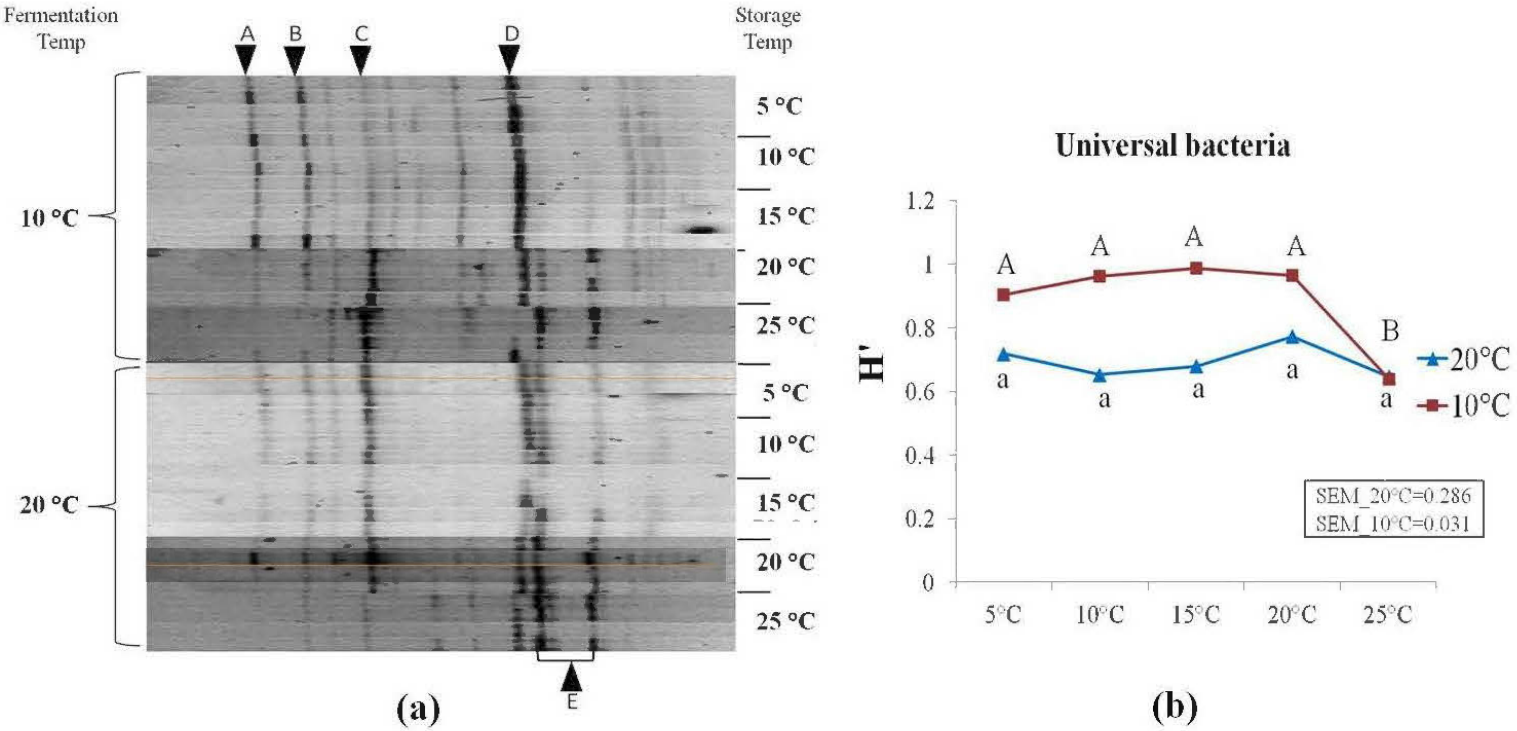

Figure 4.3 DGGE profiles of universal bacteria in corn silage (a) and their corresponding Shannon diversity index (H') (b). Labeled bands with letter "A" to "E" were allotted to the following species: A: *Weissella koreensis*; B: *Leuconostoc citreum*; C: *Lactobacillus brevis*; D: *Chryseobacterium* sp.; E: *Lactobacillus buchneri*. Four repetitions of every experimental treatment are included.

4.5.5 Fungal diversity of corn silage

As shown in Table 4.5, several species of mould flora were detected in corn silage samples using the cultural-independent approach. However, the microbial counts showed that moulds were under the detection level on the MEA medium (Table 4.3). In addition, some mould spores may also be present in silages, but cannot germinate and grow on the MEA plates due to the unsuitable lab conditions. This suggests that culturable fungi species on the MEA medium accounts for only a small portion of whole fungal community in silages, and the method of enumeration of microbial colonies on this medium underestimates the true number of moulds present in silage. Following sequencing, several mould genera were detected, including *Oidiodendron*, *Cladosporium*, *Fusarium*, *Davidiella*, *Basidiomycota, Tremellales, Alternaria*. It was also observed that the yeast species, *Candida humilis,* initially named as *Candida milleri* (NCYC, 2013) were frequently detected in our samples. The DGGE profiles of silages fermented at 10 °C differed from those fermented at 20 °C (Figure 4.4). Higher diversity level of fungi (i.e., yeasts and moulds) was observed in silages fermented at 10 °C compared to 20 °C. As storage temperature increased from 5 to 25 °C, no important changes were observed within neither of the two fermentation temperatures. This can also be seen with the trend of H' (Figure 4.4).

Table 4.5 Sequence analysis of fungal clones

Number of clones sequenced	Most closely related fungal sequence	GenBank Accession No. of related sequence	Similarity %	Environment from whichrelated sequence was isolated
23[A]	*Candida humilis*	AY493349.1	98	Natural tequila fermentation
8	Uncultured *Oidiodendron*	JF796748.1	99	Oil pumpkin flower (Austria)
7	Uncultured fungus clone	FJ757776.1	100	*Quercus macrocarpa* phyllosphere (USA)
6	Uncultured *Cladosporium*	KC143740.1	100	Human stool (France)
6	Uncultured *Fusarium*	HE977545.1	99	Soil (UK)
5[B]	Uncultured *Davidiella*	JX448366.1	100	Agarwood (India)
4	Uncultured fungus clone	FJ757067.1	100	*Quercus macrocarpa* phyllosphere (USA)
3[C]	Uncultured *Basidiomycota*	HE977542.1	100	Soil (UK)
2	Uncultured fungus clone	JN906946.1	100	European beech (*Fagus sylvatica*) phyllosphere (France)
2	Uncultured fungus clone	FJ758346.1	100	*Quercus macrocarpa* phyllosphere (USA)
2	Uncultured *Alternaria*	JQ346916.1	100	Roots of herbs (endophyte) (China)

Note: clones labeled with superscripts "A" to "C" correspond to the marked out OTUs in Figure 4.4. Five other single uncultured fungi were also detected, and cloning and/or sequence analysis of 7 fungus clones failed.

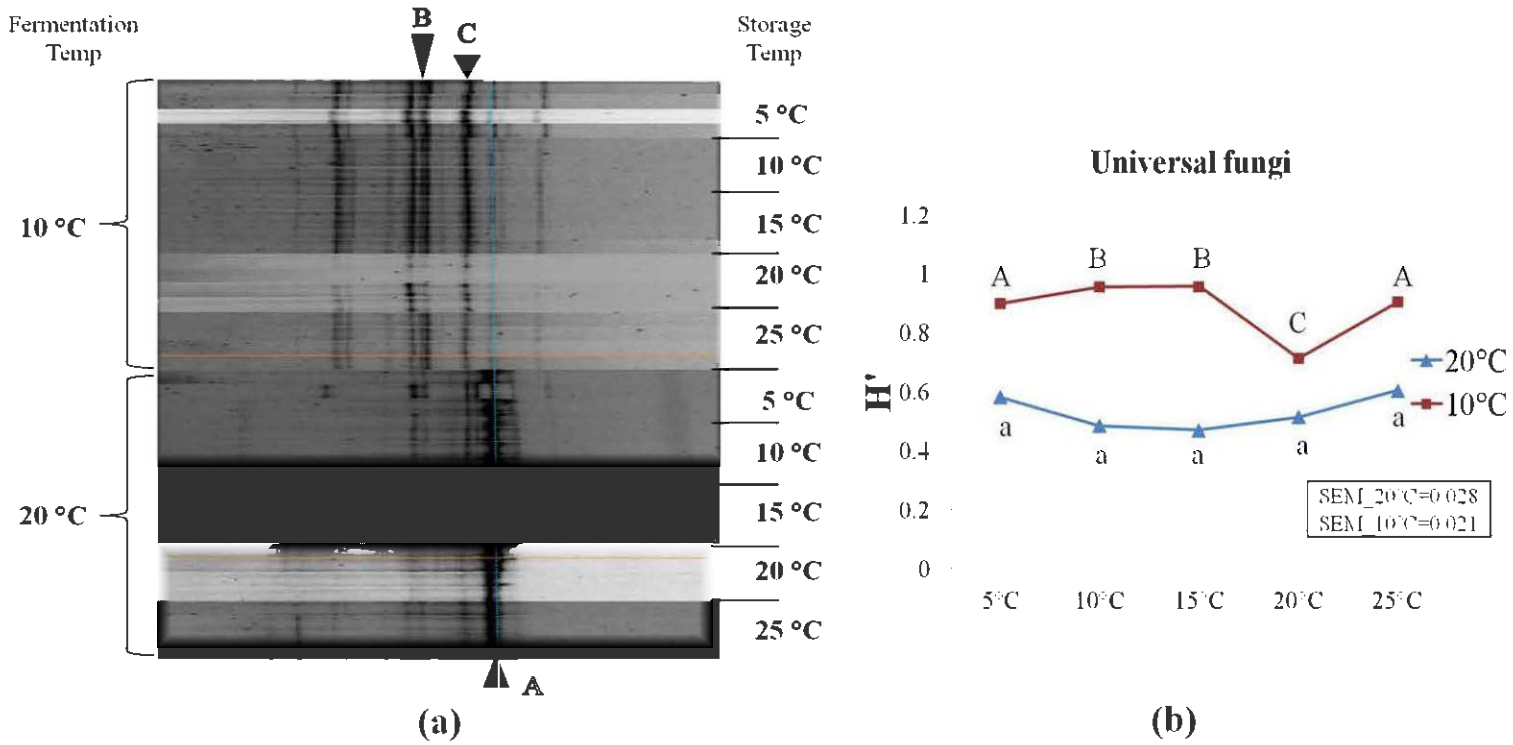

Figure 4.4: DGGE profiles of universal fungi in corn silage (a) and their corresponding Shannon diversity index (H') (b). Labeled bands with letter "A" to "C" were allotted to the following species: A: *Candida humilis*; B: *Uncultured Davidiella*; C: *Uncultured Basidiomycota*. Four repetitions of every experimental treatment are included.

4.6 Discussion

4.6.1 Effects of fermentation temperature (10 vs. 20 °C) on the fermentation of whole-plant corn silage

The results from the current study are generally consistent with our earlier findings in chapter III that low fermentation temperature (10 vs. 20 °C) restricts silage fermentation, resulting in higher pH, more residual WSC, less lactic acid production, lower levels of ethanol and ammonia, as well as lower numbers of LAB ($P < 0.05$) (Table 4.1).

4.6.2 Corn silages initially fermented at 20 °C did not stabilize as storage temperature increased from 5 to 25 °C

After an additional two months of storage at 5 °C, a highly acidic environment was reached (pH = 3.74) in corn silage initially fermented at 20 °C. Little changes occurred as storage temperature increased from 5 to 25 °C. Basically, the increasing storage temperature from 5 to 25 °C did not affect much the fermentation profiles, in terms of most biochemical parameters (i.e., pH, WSC, DM, total-N, lactic acid and most VFAs) (Figure 4.2 and Table 4.2), as well as for the bacterial and fungal populations (Figure 4.3 and Figure 4.4). The number of LAB colonies as determined from plate counts stayed rather stable, although a statistically significant increase was observed at 25 °C (Table 4.3). In addition, DGGE analysis results showed that the LAB flora in corn silage consisted of mainly heterofermentative species, such as *L. buchneri*, *L. brevis*, *Leuconostoc citreum* and *Weissella koreensis* (Figure 4.3). This is in agreement with our results from Chapter II as well as many previous studies that heterofermentative LAB species usually predominate in the latter stages of ensilage (McDonald, *et al.*, 1991; Seale, *et al.*, 1986; Yang, *et al.*, 2006).

It should be noticed that, as incubation temperatures rose up to 15 °C, a fairly high number of enterobacteria and yeasts (4-5 $\log_{10}$ CFU g FM^{-1}) were detected. Enterobacteria and yeasts are facultative anaerobes. Under anaerobic conditions, enterobacteria ferment available sugars producing mainly acetic acid, with lesser quantities of ethanol, formic acid, 2,3-butanediol and CO_2. Some enterobacteria can also deaminate amino acids to ammonia. Yeasts ferment sugars to mainly ethanol and CO_2 along with small amounts of other alcohols

and VFAs (Pahlow, *et al.*, 2003). In addition, the activity of plant proteases was reported to increase as temperature increases, which results in greater protein breakdown and ammonia accumulation in silages (Kim and Adesogan, 2006). This explained the significantly higher contents of acetic acid and ammonia ($P < 0.05$) at higher storage temperatures, as well as the trend for silages to contain more ethanol (Table 4.2). Furthermore, our DGGE results showed that the yeast *C. humilis* predominated the fungi flora in these silages (Figure 4.4). *Candida* spp. are commonly detected at ensiling, and *C. humilis* has been reported to predominate in whole-plant corn silage (Middelhoven and Franzen, 1986; Middelhoven and van Baalen, 1988). Most *Candida* spp. can readily assimilate lactate for their metabolism under aerobic conditions (Pahlow, *et al.*, 2003). Therefore, these corn silages which were initially ensiled at 20 °C might be prone to aerobic deterioration, as it was reported that yeasts numbers higher than 10^5 CFUs per gram are considered a threshold level to induce aerobic deterioration at feed-out (Pitt, *et al.*, 1991).

4.6.3 Corn silage initially fermented at 10 °C resumed fermentation as storage temperature increased from 5 to 25 °C

As storage temperature increased from 5 to 20 °C, no significant changes were observed in most silage characteristics, including pH, WSC content, lactic and acetic acid production, ethanol and most microbial counts (Table 4.2, Table 4.3 and Figure 4.2). But, a significant increase in the number of LAB colonies was observed at 20 and 25 °C (Table 4.3). Results of the bacterial diversity measurements further showed that, as temperatures reached 20 and 25 °C, two heterofermentative species, *L. buchneri* and *L. brevis*, became the dominant species of the LAB flora in these silages (Figure 4.3). As a consequence, the content of acetic acid in these silages increased considerably (by 71.5%) at 25 °C, and this was in accordance with the rapid decline in pH and WSC concentration (Table 4.2 and Figure 4.2). In addition, ammonia concentration also increased as temperature rose up to 25 °C probably due to the increased plant proteolysis. All above changes clearly indicated that silage fermentation resumed as temperatures increased. Indeed, it has been observed in cool climates that silage fermentation could restart as weather warms up, in particular for the less fermented silages due to low temperature at ensiling (Pereira, *et al.*, 2009; Schroeder, 2009).

Yeasts were under the detection level in these silages (< 2.00 $\log_{10}$ CFU g^{-1} FM) (Table 4.3), and DGGE results showed that the lactate-assimilating species *C. humilis* represented only a small fraction of the fungi flora (Figure 4.4). As storage temperature increased to 20 and 25 °C, *L. buchneri*, started to prevail (Figure 4.3). The metabolism of *L. buchneri* (Driehuis, *et al.*, 1999; Krooneman, *et al.*, 2002; Oude Elferink, *et al.*, 2001) and the merits (Kung, *et al.*, 2007; Mari, *et al.*, 2009; Nkosi, *et al.*, 2009; Reich and Kung, 2010; Schmidt, *et al.*, 2009; Taylor, *et al.*, 2002) of inoculating with *L. buchneri* to improve silage aerobic stability have been well studied. Hence, thanks to *L. buchneri*, more acetic acid was produced ($P < 0.001$) along with slightly increased contents of propionic acid ($P = 0.063$), ensuring the absence of undesirable yeasts in silage.

4.6.4 *Chryseobacterium* sp.

The occurrence of DGGE OTU (Figure 4.3) and clones (Table 4.4) belonging to *Chryseobacterium* sp. is puzzling since this bacterium is rarely mentioned in silage, neither do other members of this family. The only occurrence was very recent in a metagenome analysis of grass silage (Eikmeyer, *et al.*, 2013). They reported that 3% of the diversity was related to *Flavobacterium* and *Chryseobacterium*, and nearly 10% for the Flavobacteria. *Chryseobacterium* is a ubiquitous bacterium belonging to the Flavobacteriaceae family. Some bacteria from this family are often used to produce extracellular enzymes as silage additives, but this does not explain their presence. Although they are frequently observed in soils, they were also be isolated from decayed plants (Behrendt, *et al.*, 2008). Since some of the corn tissues are in senescence, this bacterium could actively colonize leaves or flowers. By doing so, they would become part of the epiphytic microflora of corn. Residual DNA would then have been amplified by PCR. Presence of the corresponding OTU on fresh material (see ANNEXE C.7) confirmed this hypothesis. It was also observed that the intensity of this OTU was higher following ensiling at 10 °C than at 20 °C, and it diminished as storage temperature increased. Degradation of ghost cells and residual DNA from this organism could possibly be more important as the temperature increased. The occurrence of this organism on corn plants prior to ensiling could possibly affect silage quality in relation with suboptimal fermentation parameters in early stages of ensilage.

To conclude, this experiment simulated what happens to commercial silages as storage temperature gradually increases in spring time. Temperature at ensiling greatly affects the fermentation characteristics of whole-plant corn silage during long-term storage if temperature variations occur. Previously, it has been concluded in many short-term silage studies that moderate temperatures (between 20 and 30 °C) are more favourable for ensilage and result in better quality silage. However, in this long-term study, we showed that it was true with many fermentation parameters, but might not be so in terms of the survival of spoilage microbes in silage (such as yeasts and enterobacteria). This partially explains why well-fermented silages are sometimes observed to be prone to aerobic deterioration. On the contrary, although low temperatures at ensiling restrict silage fermentation, fermentation could resume following warm-up weather. Moreover, the survival of yeasts in these silages is lower than in silages made at warmer temperature at ensiling. In practice, these observations allowed us to recommend that producers feed silages which were initially ensiled at warmer temperature earlier and quickly during winter or early spring time, in order to reduce aerobic spoilage. Moreover, for silages which were ensiled at low temperatures, it is suggested to add inoculants, such as *L. buchneri*, to ensure silage aerobic stability as ambient temperature warms up.

4.7 Acknowledgements

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CHAPTER V

GENERAL CONCLUSION

5.1 Conclusions

Whole-plant corn silage is a very important feed for ruminant animals. In the last decade, the cultivation of corn has expanded further north. Thus low temperature becomes a potential factor that may affect silage production. In Eastern Canada, whole-plant corn is usually harvested for silage from late September to early November when daily mean temperatures are between 0 and 10 °C. Ensiled corn will then be stored and consumed during the following winter and spring. Other countries or regions where cool temperatures prevail during ensiling might encounter similar issues. Thus, the purpose of this thesis was to study the effects of cool temperature on the fermentation and storage of whole-plant corn silage.

Ensiling is a process of complex microbial and enzymatic activities. Temperature is one of the factors affecting silage fermentation. Many earlier studies have investigated the effects of moderate (between 20 and 30 °C) or elevated temperatures (> 37 °C) on ensilage. However, very little work has been done to understand the implications of fermentation at temperatures lower than 20 °C for silages prepared from any types of forages. Moreover, most previous studies were done after a moderately short period of time, usually less than three months.

In this study, we simulated the fermentation and storage conditions of whole-plant corn silage in Eastern Canada. The purpose was to determine the effects of temperature, ranging from 5 to 25 °C, on the fermentation and storage of whole-plant corn silage. The hypothesis was that low temperatures affect silage fermentation not only by lowering enzymatic activities and microbial metabolism, but also by influencing the diversity of microbial populations, and in particular the lactic acid bacteria (LAB) involved at ensiling. Two ensiling trials were designed to study this hypothesis. In the first trial, the diversity of LAB population during corn ensiling process was monitored at five different temperatures (5, 10, 15, 20 and 25 °C)

(Chapter II), and biochemical and microbiological parameters of these silages were measured after fermentation period of 60 days (Chapter III). In the second trial, biochemical patterns and microbiological populations were determined while storage temperature was gradually increased from 5 to 25 °C. These silages were previously fermented at either 10 or 20 °C and stored at 5 °C for two months (Chapter IV).

In chapter II, it was observed that temperature has a selective effect on epiphytic LAB species that are involved at ensiling. At warmer temperatures (20 and 25 °C), *Lactobacillus plantatum, Pediococcus pentosaceus, Lactobacillus brevis* and *Lactobacillus buchneri* were predominant species identified during the fermentation process. For the treatments incubated at low temperatures (5 and 10 °C), *Lactobacillus sakei* and *Lactobacillus curvatus* prevailed. In addition, a shift from homo- to hetero-fermentative LAB species during the ensiling process was observed at warmer temperatures (20 and 25 °C). Corn silage stored at 15 °C acted as a transition between higher and lower fermentation temperatures, in terms of both LAB diversity and their corresponding successions.

In Chapter III, we studied the biochemical and microbiological parameters of the silage treatments from the first experiment (Chapter II). Results showed that lower temperatures contributed to hamper silage fermentation resulting in delayed acidification, low rates of pH decline, higher pH, low production of organic acids, and high concentration of residual WSC. At all temperatures, fermentation patterns demonstrated heterolactic fermentations after 60 days. These fermentation patterns were explained by epiphytic LAB species as shown with LAB diversity analysis in Chapter II.

In Chapter IV, whole-plant corn was previously fermented at 20 or 10 °C for 60 days and then stored at 5 °C for two months. After these treatments, highly acidic conditions were achieved at both fermentation temperatures (20 and 10 °C). When temperature was increased from 5 to 25 °C, rather little changes occurred in silages initially fermented at 20 °C for most biochemical parameters as well as bacterial and moulds populations. However, yeasts and enterobacteria were detected at a rather high level in these silages as temperature reached 15 °C and above. On the other hand, for silages initially fermented at 10 °C, fermentation

resumed and *L. buchneri* started to dominate the bacterial flora as incubation temperature increased from 5 to 25 °C.

5.2 Practical implications

Many homofermentative bacteria such as *L. plantarum* and *P. pentosaceus* have been used as inoculants for decades to enhance silage fermentation (Kung, *et al.*, 2003; Muck, 2010). However, results obtained with these inoculants were not always satisfactory. Muck and Kung (1997) summarized over 230 trial comparisons (inoculated versus untreated silage) published between 1990 and 1995, and found that inoculation with homofermentative LAB reduced pH in approximately 60% of the cases. Low temperature at ensiling could be a factor causing the failure of these inoculants (Weinberg and Muck, 1996). For instance, Tanaka *et al.* (2000) found that inoculation with *L. plantarum* had no effect on the fermentation of alfalfa stored at 4 °C. Our results confirmed these observations as homofermentative *L. plantarum* and *P. pentosaceus* were uncommon at temperatures lower than 15 °C.

Also, our results clearly demonstrated that *L. buchneri* was not able to compete at temperatures lower than 15 °C. However, heterofermentative *L. buchneri* has been widely applied to improve the aerobic stability of silages (Combs and Hoffman, 2001; Driehuis, *et al.*, 1999; Kung and Taylor). Thus, it would be important to test if silage inoculants presently sold on the market can work at low temperatures, and moreover, if these inoculants are able to compete with epiphytic LAB populations found on crops ensiled at low temperatures. This is particularly important for crops such as corn in Eastern Canada.

5.3 Future research recommendations

Overall, the investigation presented in this study has not only provided a better understanding of the effects of low temperatures on the fermentation and storage of whole-plant corn silage, but also demonstrated the effects of low temperature on the microbial flora in particular epiphytic LAB populations during the corn ensiling process. Our suggestions for further research include the following:

5.3.1 Possibility of developing silage inoculants with *L. sakei* or *L. curvatus* strains for cool climates

Our results showed that *L. sakei* and *L. curvatus*, were able to grow at low temperatures. This finding suggests the possibility of developing these species as LAB inoculants for cool climates. In fact, *L. sakei* proved to contain several transporters for cryo-protective substances and to have more cold stress proteins than other lactobacilli (Eijsink and Axelsson, 2005). One strain of *L. curvatus* was isolated from sorghum silage stored at 4 °C, and ensiling trials suggested that it could be used as a silage inoculant at low temperatures (Tanaka, *et al.*, 2000). In the present study, biochemical and microbiological results showed that, at 10 and 5 °C where *L. sakei* and *L. curvarus* prevailed, forage acidification was delayed and the decline of pH was slower resulting in less fermented silage. And moreover, these LAB species are heterofermenters at such low temperature conditions. This is in contradiction with the common criteria to select homofermentative LAB strains for silage inoculants to assure a rapid acidification and to reduce dry matter losses (Saarisalo, *et al.*, 2007; Weinberg and Muck, 1996). Effects of these two LAB species on aerobic stability should also be tested, as our results showed that low temperature during fermentation and storage prolonged the survival of spoilage microbes such as yeasts. However, if these species used as inoculant can lower pH more rapidly, this should help to inhibit spoilage microbial populations as they are pH dependant.

5.3.2 Study of the mechanisms of LAB succession during the ensiling process

Lactic acid bacteria are essential microorganisms responsible for silage fermentation, and LAB populations involved at ensiling dictate silage fermentation and, accordingly, the quality of silage (Cai, *et al.*, 1998; Lin, *et al.*, 1992b). Our study clearly demonstrated that a shift in LAB population took place during the ensiling process. For instance, at 20 and 25 °C, the predominant homofermentative species *L. plantarum* and *P. pentosaceus* were replaced by heterofermentative species *L. buchneri* as ensiling proceeded. This phylogenetic change of LAB populations during ensiling has also been observed in other studies (Brusetti, *et al.*, 2006; Lin, *et al.*, 1992b; McEniry, *et al.*, 2008; Stevenson, *et al.*, 2006). This might be a survival strategy dictated by pH but our results suggested that temperature might also be a

factor. Unfortunately, no study so far has been done to look into the biochemical mechanisms of this shift of LAB populations during the ensiling process. Therefore, in order to better understand the ensiling process, more research is needed to gain comprehensive knowledge of these underlying mechanisms.

5.3.3 Aerobic stability study of the effects of low temperature on silage fermentation

Aerobic deterioration is a major issue in silage production, in particular for carbohydrate-rich silages such as whole-plant corn silage (Filya and Sucu, 2010; Li and Nishino, 2011b; Schmidt and Kung, 2010). It usually manifests itself by heating of the silage (Pahlow, *et al.*, 2003). An aerobic stability test may be carried out to evaluate the susceptibility of silages to aerobic spoilage while exposed to air. It is usually done by exposing silage to air and determining the time before silage temperature rises 2 °C above ambient temperature (Kim and Adesogan, 2006; McEniry, *et al.*, 2007). Other variables may be measured as microorganism metabolism linked to aerobic stability, such as change in pH, evolution of CO_2, development of yeast and mould counts, and visual appraisal of moulds (Weinberg, *et al.*, 2001).

Unfortunately, due to practical limitations, an aerobic stability test was not conducted in the present study. However, in both trials, restricted fermentation was observed at low temperature, which resulted in high residual WSC, low production of organic acids. Moreover, yeasts survived for a longer period in these silages. All these measures indicated higher susceptibility of silage to aerobic spoilage. However, this may differ from the real case when silages are exposed to oxygen. Thus, future work on the effects of low temperature on whole-plant corn silage should include the determination of silage aerobic stability, in order to gain knowledge of factors influencing aerobic stability under these particular conditions.

5.3.4 Applying metagenomic approaches in future silage microbiology study

Until about a decade ago, our knowledge of silage microbiology was restricted to the small fraction of species that would grow in the laboratory. Identification of species was done using microscopy and culture techniques. This work was tedious and laborious (Muck, 2013). DNA

sequencing technology has revolutionized how we study microbes. Recently, a variety of culture-independent molecular techniques based on the amplification and sequencing of portions of DNA have been widely used to study the microbial community in silages. A portion of bacterial 16S rRNA gene (e.g., McEniry, *et al.* (2008), Naoki, *et al.* (2008)) and fungal 18S rRNA (e.g., Li and Nishino (2011b)) or ITS genes (e.g., Mansfield and Kuldau (2007)) have been most commonly used and other genes such as *rec*A genes (e.g., Stevenson, *et al.* (2006)) have also been used. These techniques have largely expanded our view of silage microorganisms far beyond those that will grow on various selective media. PCR-DGGE is one of the most widely used techniques and proves to be an effective approach to study microbial community in silages (Naoki and Yuji, 2008; Wang, *et al.*, 2006; Yang, *et al.*, 2006). In this study, PCR-DGGE was chosen on merit previously described. Recently, microbial diversity study has gained a step further because of the development of metagenomics. Researchers can now sequence a metagenome, i.e., the complete genome of every microbe directly from environmental samples. Metagenomics offers deeper sets of information from the microbial world. As analytical prices are going down, metagenome analyses have only recently been applied in the agriculture domain (Fierer, *et al.*, 2012; Zakrzewski, *et al.*, 2012). Eikmeyer, *et al.* (2013) analyzed a grass ensiling process and corresponding microbial communities with a metagenomic approach. But, unfortunately, in the present study, it was not financially possible to use that approach because of the high number of samples in our experimental design. In the future, this approach should be a useful tool to study microbial ecology in silages.

REFERENCES

ADESOGAN A. T. and NEWMAN Y. C. (2010) Silage harvesting, storing, and feeding. Gainesville, Florida: Agronomy Department, University of Florida IFAS Extension.

ALLEN M. S., COORS J. G. and ROTH G. W. (2003) Corn Silage. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage science and technology*, pp. 547-608. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

ANDRIEU J. P. and GOUET J. (1991) Variations and modifications of epiphytic microflora on standing crops and after their harvest for silage. Proceedings of the Conference on Forage Conservation towards 2000, Braunschweig, Germany, 23-25 January 1991.

AOAC (1990) Official methods of analysis of the association of official analytica chemists, 15th edn. Washington, DC: Association of Offical Anlaytical.

ASHBELL G. and WEINBERG Z. G. (1992) Top silage losses in horizontal silos. *Canadian Agricultural Engineering*, **34**, 171-175.

ASHBELL G., WEINBERG Z. G., HEN Y. and FILYA I. (2002) The effects of temperature on the aerobic stability of wheat and corn silages. *Journal of Industrial Microbiology and Biotechnology*, **28**, 261-263.

ATLAS R. M. (1995) *Principles of microbiology*: Mosby.

AXELSSON L. T. (2011) Lactic acid bacteria: classification and physiology. In: SALMINEN S., VON WRIGHT A., LAHTINEN S. and OUWEHAND A. (eds.) *Lactic Acid Bacteria: Microbiology and Functional Aspects*, pp. 1-72. New York: CRC Press.

BAUER S., THOLEN A., OVERMANN J. and BRUNE A. (2000) Characterization of abundance and diversity of lactic acid bacteria in the hindgut of wood- and soil-feeding termites by molecular and culture- dependent techniques. *Archives of Microbiology*, **173**, 126-137.

BECK T. (1972) The quantitative and qualitative composition of the lactic acid population of silage. *Landwirtschaftliche Forschung*, **27**, 55-63.

BECK T. and GROSS F. (1964) Causes of the differences in the keeping properties of silage. *Wirtschaftseigene Futter*, **10**, 298-312.

BEHRENDT U., ULRICH A. and SCHUMANN P. (2008) *Chryseobacterium gregarium* sp. nov., isolated from decaying plant material. *International Journal of Systematic and Evolutionary Microbiology*, **58**, 1069-1074.

BERTHIAUME R., IVAN M. and LAFRENIERE C. (2007) Effects of sodium bentonite supplements on growth performance of feedlot steers fed direct-cut or wilted grass silage based diets. *Canadian Journal of Animal Science*, **87**, 631-638.

BONITO G., ISIKHUEMHEN O. S. and VILGALYS R. (2010) Identification of fungi associated with municipal compost using DNA-based techniques. *Bioresource Technology*, **101**, 1021-1027.

BORREANI G. and TABACCO E. (2010) The relationship of silage temperature with the microbiological status of the face of corn silage bunkers. *Journal of Dairy Science*, **93**, 2620-2629.

BOUKHORS A. (2006) *Développement d'un silo expérimental à l'échelle du laboratoire à partir du matériel végétal sec* Maîtrise en biologie, Université du Québec à Montréal.

BOYSEN M. E., JACOBSSON K. G. and SCHNÜRER J. (2000) Molecular identification of species from the *Penicillium roqueforti* group associated with spoiled animal feed. *Applied and Environmental Microbiology*, **66**, 1523-1526.

BRUSETTI L., BORIN S., MORA D., RIZZI A., RADDADI N., SORLINI C. and DAFFONCHIO D. (2006) Usefulness of length heterogeneity-PCR for monitoring lactic acid bacteria succession during maize ensiling. *FEMS Microbiology Ecology*, **56**, 154-164.

BRUSETTI L., BORIN S., RIZZI A., MORA D., SORLINI C. and DAFFONCHIO D. (2008) Exploration of methods used to describe bacterial communities in silage of maize (*Zea mays*) cultivars. *Environmental Biosafety Research*, 7, 25-33.

CAI Y., BENNO Y., OGAWA M., OHMOMO S., KUMAI S. and NAKASE T. (1998) Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. *Applied and Environmental Microbiology*, **64**, 2982-2987.

CAPLICE E. and FITZGERALD G. F. (1999) Food fermentations: role of microorganisms in food production and preservation. *International Journal of Food Microbiology*, **50**, 131-149.

CHAN R. K., WORTMAN C. R., SMILEY B. K. and HENDRICK C. A. (2003) Construction and use of a computerized DNA fingerprint database for lactic acid bacteria from silage. *Journal of Microbiological Methods*, **55**, 565-574.

CHARMLEY E. (2004) Making good corn silage. In: BITTMAN S. and KOWALENKO C. G. (eds.) *Advanced silage corn management: a production guide for coastal British Columbia and the Pacific Northwest.* Agassiz, British Columbia, Canada: Pacific Field Corn Association.

COLOMBATTO D., MOULD F. L., BHAT M. K., PHIPPS R. H. and OWEN E. (2004) In vitro evaluation of fibrolytic enzymes as additives for maize (*Zea mays* L.) silage. I. Effects of ensiling temperature, enzyme source and addition level. *Animal Feed Science and Technology*, **111**, 111-128.

COMBS D. K. and HOFFMAN P. C. (2001) *Lactobacillus buchneri* for silage aerobic stability. *Focus on Forage*, **3**, 1-3.

COURTIN M. G. and SPOELSTRA S. F. (1990) A simulation model of the microbiological and chemical changes accompanying the initial stage of aerobic deterioration of silage. *Grass and Forage Science*, **45**, 153-165.

DE CÁCERES M., LEGENDRE P. and MORETTI M. (2010) Improving indicator species analysis by combining groups of sites. *Oikos*, **119**, 1674-1684.

DE VRIES W., KAPTEIJN W. M., VAN DER BEEK E. G. and STOUTHAMER A. H. (1970) Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. *Journal of General Microbiology*, **63**, 333-345.

DELLAGLIO F. and TORRIANI S. (1986) DNA-DNA homology, physiological characteristics and distribution of lactic acid bacteria isolated from maize silage. *Journal of Applied Bacteriology*, **60**, 83-92.

DEMARQUILLY C. (1986) L'ensilage et l'évolution récente des conservateurs. *Bulletin technique - Centre de Recherches Zootechniques et Vétérinaires de Theix* **63**, 5-12.

DONALD S., FENLON D. R. and SEDDON B. (1993) A novel system for monitoring the influence of oxygen tension on the microflora of grass silage. *Letters in Applied Microbiology*, **17**, 253-255.

DRIEHUIS F., OUDE ELFERINK S. J. W. H. and SPOELSTRA S. F. (1999) Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. *Journal of Applied Microbiology*, **87**, 583-594.

DROUIN P. and LAFRENIÈRE C. (2012) Clostridial spores in animal feeds and milk. In: CHAIYABUTR N. (ed.) *Milk Production - An up-to-date overview of animal nutrition, management and health*, pp. 375-394: Intech.

DUARTE S., CÁSSIO F. and PASCOAL C. (2012) Denaturing Gradient Gel Electrophoresis (DGGE) in microbial ecology — Insights from freshwaters. In: MAGDELDIN S. (ed.) *Gel electrophoresis — principles and basics*, pp. 173–195. Rijeka, Croatia: InTech.

DUBOIS M., GILLES K. A., HAMILTON J. K., REBERS P. A. and SMITH F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, **28**, 350-356.

DYKES G. A., CLOETE T. E. and VON HOLY A. (1995) Taxonomy of lactic acid bacteria associated with vacuum-packaged processed meat spoilage by multivariate analysis of cellular fatty acids. *International Journal of Food Microbiology*, **28**, 89-100.

EDWARS U., ROGALL T., BLOCKER H., EMDE M. and BOTTGER E. C. (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research*, **17**, 7843-7853.

EIJSINK V. G. H. and AXELSSON L. (2005) Bacterial lessons in sausage making. *Nature Biotechnology*, **23**, 1494-1495.

EIJSINK V. G. H., BRURBERG M. B., MIDDELHOVEN P. H. and NES I. F. (1996) Induction of bacteriocin production in *Lactobacillus sakei* by a secreted peptide. *Journal of Bacteriology*, **178**, 2232-2237.

EIKMEYER F. G., KÖFINGER P., POSCHENEL A., JÜNEMANN S., ZAKRZEWSKI M., HEINL S., MAYRHUBER E., GRABHERR R., PÜHLER A., SCHWAB H. and SCHLÜTER A. (2013) Metagenome analyses reveal the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community involved in grass ensiling. *Journal of Biotechnology*, **167**, 334-343.

ENDO A. and OKADA S. (2005) Monitoring the lactic acid bacterial diversity during Shochu fermentation by PCR-denaturing gradient gel electrophoresis. *Journal of Bioscience and Bioengineering*, **99**, 216-221.

ENNAHAR S., CAI Y. and FUJITA Y. (2003) Phylogenetic diversity of lactic acid bacteria associated with paddy rice silage as determined by 16S ribosomal DNA analysis. *Applied and Environmental Microbiology*, **69**, 444-451.

ENVIRONMENT-CANADA Canadian climate normals 1971-2000 http://climate.weatheroffice.gc.ca/climate_normals/index_e.html [11 April 2010].

ERCOLINI D. (2004) PCR-DGGE fingerprinting: novel strategies for detection of microbes in food. *Journal of Microbiological Methods*, **56**, 297-314.

ERCOLINI D., RUSSO F., TORRIERI E., MASI P. and VILLANI F. (2006) Changes in the spoilage-related microbiota of beef during refrigerated storage under different packaging conditions. *Applied and Environmental Microbiology*, **72**, 4663-4671.

FENLON D. R. (1986) Growth of naturally occurring *Listeria* spp. in silage: a comparative study of laboratory and farm ensiled grass. *Grass and Forage Science*, **41**, 375-378.

FIERER N., LEFF J. W., ADAMS B. J., NIELSEN U. N., BATES S. T., LAUBER C. L., OWENS S., GILBERT J. A., WALL D. H. and CAPORASO J. G. (2012) Cross-biome metagenomic analyses of soil microbial communities and their functional attributes. *National Academy of Sciences*, **109**, 21390-21395.

FILYA I. and SUCU E. (2010) The effects of lactic acid bacteria on the fermentation, aerobic stability and nutritive value of maize silage. *Grass and Forage Science*, **65**, 446-455.

FILYA I., SUCU E. and KARABULUT A. (2006) The effects of *Propionibacterium acidipropionici* and *Lactobacillus plantarum*, applied at ensiling, on the fermentation and aerobic stability of low dry matter corn and sorghum silages. *Journal of Industrial Microbiology and Biotechnology*, **33**, 353-358.

FLIPOT P., MOWAT D. N., PARKINS J. J. and BUCHANAN-SMITH J. G. (1976) Ensiling characteristics of silage treated with sodium hydroxyde. *Canadian Journal of Plant Science*, **56**, 935-940.

FRASER M. D., WINTERS A., FYCHAN R., DAVIES D. R. and JONES R. (2001) The effect of harvest date and inoculation on the yield, fermentation characteristics and feeding value of kale silage. *Grass and Forage Science*, **56**, 151-161.

FRAZIER W. C. and WESTHOFF D. C. (1978) *Food microbiology*: McGraw-Hill, Inc.

FUSSELL R. J. and MCCALLEY D. V. (1987) Determination of volatile fatty acids (C2-C5) and lactic acid in silage by gas chromatography. *The Analyst*, **112**, 1213-1216.

FYCHAN R., FRASER M. D. and JONES R. (2002) Effect of ensiling method on the quality of red clover and lucerne silage. Proceedings of the XIIIth International Silage Conference, pp. 104-105.

GAO L., YANG H., WANG X., HUANG Z., ISHII M., IGARASHI Y. and CUI Z. (2008) Rice straw fermentation using lactic acid bacteria. *Bioresource Technology*, 99, 2742-2748.

GARCIA A. D., OLSON W. G., OTTERBY D. E., LINN J. G. and HANSEN W. P. (1989) Effect of temperature, moisture and aeration on fermentation of alfalfa silage. *Journal of Dairy Science*, **72**, 93-103.

GOERING H. K., VAN SOEST P. J. and HEMKEN R. W. (1973) Relative susceptibility of forages to heat damage as affected by moisture, temperature, and pH. *Journal of Dairy Science*, **56**, 137-143.

GREEN S. J., LEIGH M. B. and NEUFELD J. D. (2009) Denaturing gradient gel electrophoresis (DGGE) for microbial communitiy analysis In: TIMMIS K. N. (ed.) *Handbook of Hydrocarbon and Lipid Microbiology*, pp. 4137-4158. Heidelberg, Germany: Springer.

GRIFFITHS R. I., MANEFIELD M., WHITELEY A. S. and BAILEY M. J. (2004) DNA and RNA extraction from soil In: KOWALCHUK G. A., DE BRUIJN F. J., HEAD L. M., AKKERMANS A. D. and VAN ELSAS J. D. (eds.) *Molecular Microbial Ecology Manual Second Edition-Volume 1*, pp. 149-158. Dordrecht, The Netherlands: Kluwer Academic Publishers.

HAGI T., TANAKA D., IWAMURA Y. and HOSHINO T. (2004) Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture*, **234**, 335-346.

HAIGH P. M. and PARKER J. W. G. (1985) Effect of silage additives and wilting on silage fermentation, digestibility and intake, and on liveweight change of young cattle. *Grass and Forage Science*, **40**, 429-436.

HASSANAT F., MUSTAFA A. F. and SEGUIN P. (2006) Chemical composition and ensiling characteristics of normal and brown midrib pearl millet harvested at two stages of development in southwestern Québec. *Canadian Journal of Animal Science*, **86**, 71-80.

HASSANAT F., MUSTAFA A. F. and SEGUIN P. (2007) Effects of inoculation on ensiling characteristics, chemical composition and aerobic stability of regular and brown midrib millet silages. *Animal Feed Science and Technology*, **139**, 125-140.

HEUER H., KRSEK M., BAKER P., SMALLA K. and WELLINGTON E. M. H. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Applied and Environmental Microbiology*, **63**, 3233-3241.

JOHNSON H. E., MERRY R. J., DAVIES D. R., KELL D. B., THEODOROU M. K. and GRIFFITH G. W. (2005) Vacuum packing: a model system for laboratory-scale silage fermentations. *Journal of Applied Microbiology*, **98**, 106-113.

JONES B. A., HATFIELD R. D. and MUCK R. E. (1995) Characterization of proteolysis in alfalfa and red clover. *Crop Science*, **35**, 537-541.

JONES B. A., SATTER L. D. and MUCK R. E. (1992) Influence of bacterial inoculant and substrate addition to lucerne ensiled at different dry matter contents. *Grass Forage Science*, **47**, 19-27.

JONSSON A. (1990) Enumeration and confirmation of *Clostridium tyrobutyricum* in silages using neutral red, D-cycloserine, and lactate dehydrogenase activity. *Journal of Dairy Science*, **73**, 719-725.

JULIEN M. C., DION P., LAFRENIÈRE C., ANTOUN H. and DROUIN P. (2008) Sources of clostridia in raw milk on farms. *Applied and Environmental Microbiology*, **74**, 6348-6357.

KANDLER O. (1983) Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek*, **49**, 209-224.

KEBLI H., DROUIN P., BRAIS S. and KERNAGHAN G. (2011) Species composition of saproxylic fungal communities on decaying logs in the boreal forest. *Microbial Ecology*, **61**, 898-910.

KIM S. C. and ADESOGAN A. T. (2006) Influence of ensiling temperature, simulated rainfall, and delayed sealing on fermentation characteristics and aerobic stability of corn silage. *Journal of Dairy Science*, **89**, 3122-3132.

KIM T. W., LEE J. H., KIM S. E., PARK M. H., CHANG H. C. and KIM H. Y. (2009) Analysis of microbial communities in doenjang, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis. *International Journal of Food Microbiology*, **131**, 265-271.

KIZLLSIMSEK M., SCHMIDT R. J. and KUNG JR L. (2007) Effects of a mixture of lactic acid bacteria applied as a freeze-dried or fresh culture on the fermentation of alfalfa silage. *Journal of Dairy Science*, **90**, 5698-5705.

KLOCKE M. and MUNDT K. (2004) Development of a 16S rDNA-targeted PCR assay for monitoring of *Lactobacillus plantarum* and *Lact. rhamnosus* during co-cultivation for production of inoculants for silages. *Letters in Applied Microbiology*, **39**, 267-273.

KLOCKE M., MUNDT K., IDLER C., MCENIRY J., O'KIELY P. and BARTH S. (2006) Monitoring *Lactobacillus plantarum* in grass silages with the aid of 16S rDNA-based quantitative real-time PCR assays. *Systematic and Applied Microbiology*, **29**, 49-58.

KOBAYASHI S. Heat Damage in Silage and Hay http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/for4906 [28 June 2013].

KOC F., COSKUNTUNA L., OZDUVEN M. L., COSKUNTUNA A. and SAMLI H. E. (2009) The effects of temperature on the silage microbiology and aerobic stability of corn and vetch-grain silages. *Acta Agriculturae Scandinavica A: Animal Sciences*, **59**, 239-246.

KOWALCZYK M. and BARDOWSKI J. (2007) Regulation of sugar catabolism in *Lactococcus lactis*. *Critical Review in Microbiology* **33**, 1-13.

KROONEMAN J., FABER F., ALDERKAMP A. C., OUDE ELFERINK S. J. H. W., DRIEHUIS F., CLEENWERCK I., SWINGS J., GOTTSCHAL J. C. and VANCANNEYT M. (2002) *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 639-646.

KUNG L. (2010a) Anaerobic stability of silage. Proceedings of the California Alfalfa & Forage Symposium and Corn/Cereal Silage Conference, Visalia, CA, December 1-2

KUNG L. (2010b) Understanding the biology of silage preservation to maximize quality and protect the environment. Proceedings of the California Alfalfa & Forage Symposium, Visalia, CA, 1-2 December 2010.

KUNG L. (2011) Silage temperatures: how hot is too hot? Newark, DE: Dairy Research, Teaching and Extension. University of Delaware.

KUNG L., ROBINSON J. R., RANJIT N. K., CHEN J. H., GOLT C. M. and PESEK J. D. (2000) Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. *Journal of Dairy Science*, **83**, 1479-1486.

KUNG L., SCHMIDT R. J., EBLING T. E. and HU W. (2007) The effect of *Lactobacillus buchneri* 40788 on the fermentation and aerobic stability of ground and whole high-moisture corn. *Journal of Dairy Science*, **90**, 2309-2314.

KUNG L. and SHAVER R. (2001) Interpretation and use of silage fermentation analysis reports. *Focus on forage*, **3**, 1-5.

KUNG L., SHEPERD A. C., SMAGALA A. M., ENDRES K. M., BESSETT C. A., RANJIT N. K. and GLANCEY J. L. (1998) The effect of preservatives based on propionic acid on the fermentation and aerobic stability of corn silage and a total mixed ration. *Journal of Dairy Science*, **81**, 1322-1330.

KUNG L., STOKES M. R. and LIN C. J. (2003) Silage Additives. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage science and technology*, pp. 305-360. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

KUNG L. and TAYLOR C. Questions and answers on *Lactobacillus buchneri* 40788, a new microbial inoculant that improves the aerobic stability of silages http://ag.udel.edu/anfs/faculty/kung/articles/questions_and_answers_on_lactoba.htm [28 October 2013].

LAHTINEN S., OUWEHAND A. C., SALMINEN S. and WRIGHT A. V. (2011) *Lactic acid bacteria: microbiological and functional aspects, fourth edition*. New York: CRC Press.

LANGSTON C. W. and BOUMA C. (1960) Types and Sequence Change of Bacteria in Orchardgrass and Alfalfa Silages. *Journal of Dairy Science*, **43**, 1575-1584.

LEISNER J. J., VANCANNEYT M., GORIS J., CHRISTENSEN H. and RUSUL G. (2000) Description of *Paralactobacillus selangorensis* gen. nov., sp. nov., a new lactic acid bacterium isolated from chili bo, a Malaysian food ingredient. *International Journal of Systematic and Evolutionary Microbiology*, **50**, 19-24.

LI Y. and NISHINO N. (2011a) Bacterial and fungal communities of wilted Italian ryegrass silage inoculated with and without Lactobacillus rhamnosus or Lactobacillus buchneri. *Letters in Applied Microbiology*, **52**, 314-321.

LI Y. and NISHINO N. (2011b) Effects of inoculation of *Lactobacillus rhamnosus* and *Lactobacillus buchneri* on fermentation, aerobic stability and microbial communities in whole crop corn silage. *Grassland Science*, **57**, 184-191.

LIN C., BOLSEN K. K., BRENT B. E. and FUNG D. Y. C. (1992b) Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. *Journal of Applied Bacteriology*, **73**, 375-387.

LIN C., BOLSEN K. K., BRENT B. E., HART R. A., DICKERSON J. T., FEYERHERM A. M. and AIMUTIS W. R. (1992a) Epiphytic microflora on alfalfa and whole-plant corn. *Journal of Dairy Science*, **75**, 2484-2493.

LIU S. Q. (2003) Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. *International Journal of Food Microbiology*, **83**, 115-131.

MANDA T., SHINODA M., IWASAKI K., HASEGAWA N., SAITO H., OKAMOTO M., SUDA T. and AOTANI H. (1985) Preparation of roll bale silage. 1 Effects of baling methods and vertical distribution of fermentation quality. *Grassland Science (Suppl.)*, **31**, 160-161.

MANSFIELD M. A. and KULDAU G. A. (2007) Microbiological and molecular determination of mycobiota in fresh and ensiled maize silage. *Mycologia*, 99, 269-278.

MARI L. J., SCHMIDT R. J., NUSSIO L. G., HALLADAS C. M. and KUNG JR L. (2009) Short communication: an evaluation of the effectiveness of *Lactobacillus buchneri* 40788 to alter fermentation and improve the aerobic stability of corn silage in farm silos. *Journal of Dairy Science*, **92**, 1174-1176.

MARSH R. (1979) The effects of wilting on fermentation in the silo and on the nutritive value of silage. *Grass and Forage Science*, **34**, 1-10.

MARTINS S. I. F. S., JONGEN W. M. F. and VAN BOEKEL M. A. J. S. (2000) A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science & Technology*, **11**, 364-373.

MAY L. A., SMILEY B. and SCHMIDT M. G. (2001) Comparative denaturing gradient gel electrophoresis analysis of fungal communities associated with whole plant corn silage. *Canadian Journal of Microbiology*, **47**, 829-841.

MCDONALD P., HENDERSON A. R. and WHITTENBURY R. (1966) The effect of temperature on ensilage. *Journal of the Science of Food and Agriculture*, **17**, 476-480.

McDONALD P., HENDERSON N. and HERON S. (1991) Microorganisms *The Biochemistry of Silage*, 2nd edn, pp. 81-151. Marlow, Bucks, UK: Chalcombe Publications.

McENIRY J., O'KIELY P., CLIPSON N. J. W., FORRISTAL P. D. and DOYLE E. M. (2007) The relative impacts of wilting, chopping, compaction and air infiltration on the conservation characteristics of ensiled grass. *Grass and Forage Science*, **62**, 470-484.

McENIRY J., O'KIELY P., CLIPSON N. J. W., FORRISTAL P. D. and DOYLE E. M. (2008) Bacterial community dynamics during the ensilage of wilted grass. *Journal of Applied Microbiology*, **105**, 359-371.

McKERSIE B. D. (1981) Proteinases and peptidases of alfalfa herbage. *Canadian Journal of Plant Science*, **61**, 53-59.

MEROTH C. B., WALTER J., HERTEL C., BRANDT M. J. and HAMMES W. P. (2003) Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **69**, 475-482.

MERRY R. J., LOWES K. F. and WINTERS A. L. (1997) Current and future approaches to biocontrol in silage, In: JAMBOR V., KLAPIL L., CHROMEC P. and PROCHAZKA P. (eds). Proceedings of the 8th Internaltional Symposium of Forage Conservation, Brno, Czech Republic, 29 September - 1 October, pp. 17-27.

MHEEN T. and KWON T. (1984) Effect of temperature and salt concentration on *Kimchi* fermentation. *Korean Journal for Food Science Technology*, **16**, 443-450.

MIDDELHOVEN W. J. and FRANZEN M. M. (1986) The yeast flora of ensiled whole-crop maize. *Journal of the Science of Food and Agriculture*, **37**, 855-861.

MIDDELHOVEN W. J. and VAN BAALEN A. H. M. (1988) Development of the yeast flora of whole-crop maize during ensiling and during subsequent aerobiosis. *Journal of the Science of Food and Agriculture*, **42**, 199-207.

MOON N. J. (1983) Inhibition of the growth of acid tolerant yeasts by acetate, lactate and propionate and their synergistic mixtures. *Journal of Applied Microbiology*, **55**, 453-460.

MUCK R. (2013) Recent advances in silage microbiology. *Agriculture and Food Science*, **22**, 3-15.

MUCK R. E. (1990) Dry matter level effects on alfalfa silage quality. II. Fermentation products and starch hydrolysis. *Transactions of the American Society of Agricultural Engineers*, **33**, 373-381.

MUCK R. E. (2010) Silage microbiology and its control through additives. *Revista Brasileira de Zootecnia*, **39**, 183-191.

MUCK R. E. and DICKERSON J. T. (1988) Storage temperature effects on proteolysis in alfalfa silage. *Transactions of the American Society of Agricultural Engineers*, **31**, 1005-1009.

MUCK R. E. and L. KUNG J. (1997) Effects of silage additives on ensiling. Proceedings of the Silage: Field to Feedbunk North American Conference, Hershey, Pennsylvania, Feburary 11-13, pp. 187-199.

MUCK R. E., MOSER L. E. and PITT R. E. (2003) Postharvest factors affecting ensiling. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage Science and Technology*, pp. 283-284. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

MUYZER G. (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, **2**, 317-322.

MUYZER G., DE WAAL E. C. and UITTERLINDEN A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695-700.

MUYZER G. and SMALLA K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, **73**, 127-141.

NAOKI N. and YUJI T. (2008) Variations in bacterial communities in laboratory-scale and big bale silos assessed by fermentation products, colony counts and denaturing gradient gel electrophoresis profiles. *Letters in Applied Microbiology*, **46**, 283-288.

NCYC (2013) 2450-*Candida humilis* http://www.ncyc.co.uk/print-ncyc-2450.html. UK: National Collection of Yeast Cultures.

NEVES A. R., POOL W. A., KOK J., KUIPERS O. P. and SANTOS H. (2005) Overview on sugar metabolism and its control in *Lactococcus lactis* - The input from in vivo NMR. *FEMS Microbiology Reviews*, **29**, 531-554.

NISHINO N., WADA H., YOSHIDA M. and SHIOTA H. (2004) Microbial counts, fermentation products, and aerobic stability of whole crop corn and a total mixed ration ensiled with and without inoculation of *Lactobacillus casei* or *Lactobacillus buchneri*. *Journal of Dairy Science*, **87**, 2563-2570.

NKOSI B. D., MEESKE R., PALIC D., LANGA T., LEEUW K. J. and GROENEWALD I. B. (2009) Effects of ensiling whole crop maize with bacterial inoculants on the fermentation, aerobic stability, and growth performance of lambs. *Animal Feed Science and Technology*, **154**, 193-203.

NOUT M. J. R., BOUWMEESTER H. M., HAAKSMA J. and VAN DIJK H. (1993) Fungal growth in silages of sugarbeet press pulp and maize. *Journal of Agicultural science*, **121**, 323-326.

O'BRIEN M., EGAN D., O'KIELY P., FORRISTAL P. D., DOOHAN F. M. and FULLER H. T. (2008) Morphological and molecular characterisation of *Penicillium roqueforti* and *P. paneum* isolated from baled grass silage. *Mycological Research*, **112**, 921-932.

OCPA (2010) Corn & the environment: corn and canada. http://www.ontariocorn.org, *accessed 11/04/*, **2010**.

OGIER J. C., LAFARGE V., GIRARD V., RAULT A., MALADEN V., GRUSS A., LEVEAU J. Y. and DELACROIX-BUCHET A. (2004) Molecular fingerprinting of dairy microbial ecosystems by use of temporal temperature and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **70**, 5628-5643.

OHMOMO S., NITISINPRASART S. and HIRANPRADIT S. (2002) Silage-making and recent trend of dairy farming in Thailand. *Japan Agricultural Research Quarterly*, **36**, 227-234.

OLSTORPE M., LYBERG K., LINDBERG J. E., SCHNU¨RER J. and PASSOTH V. (2008) Population diversity of yeasts and lactic acid bacteria in pig feed fermented with whey, wet wheat distillers' grains, or water at different temperatures. *Applied and Environmental Microbiology*, **74**, 1696-1703.

OUDE ELFERINK S. J. W. H., KROONEMAN E. J., GOTTSCHAL J. C., SPOELSTRA S. F., FABER F. and DRIEHUIS F. (2001) Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Applied and Environmental Microbiology*, **67**, 125-132.

PAHLOW G., MUCK R. E. and F. D. (2003) Microbiology of ensiling. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage science and technology*, pp. 31-94. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

PANG H., TAN Z., QIN G., WANG Y., LI Z., JIN Q. and CAI Y. (2012) Phenotypic and phylogenetic analysis of lactic acid bacteria isolated from forage crops and grasses in the Tibetan Plateau. *Journal of Microbiology*, **50**, 63-71.

PANG H., ZHANG M., QIN G., TAN Z., LI Z., WANG Y. and CAI Y. (2011) Identification of lactic acid bacteria isolated from corn stovers. *Animal Science Journal*, **82**, 642-653.

PARVIN S. and NISHINO N. (2009) Bacterial community associated with ensilage process of wilted guinea grass. *Journal of Applied Microbiology*, **107**, 2029-2036.

PARVIN S. and NISHINO N. (2010) Succession of lactic acid bacteria in wilted rhodesgrass silage assessed by plate culture and denaturing gradient gel electrophoresis. *Grassland Science*, **56**, 51-55.

PARVIN S., WANG C., LI Y. and NISHINO N. (2010) Effects of inoculation with lactic acid bacteria on the bacterial communities of Italian ryegrass, whole crop maize, guinea grass and rhodes grass silages. *Animal Feed Science and Technology*, **160**, 160-166.

PAULY T. M., HANSSON I. B. and THAM W. A. (1999) The effect of mechanical forage treatments on the growth of *Clostridium tyrobutyricum* and *Listeria monocytogenes* in grass silage. *Animal Feed Science and Technology*, **78**, 127-139.

PEDERSON C. S. and ALBURY M. N. (1953) Factors affecting the bacterial flora in fermenting vegetables. *Journal of Food Science*, **18**, 290-300.

PEDERSON C. S. and ALBURY M. N. (1954) The influence of salt and temperature on the microflora of sauerkraut fermentation. *Food technology*, **8**, 1-5.

PEDROSO A. F., NUSSIO L. G. and PAZIANI S. F. (2005) Fermentation and epiphytic microflora dynamics in sugar cane silage. *Scientia Agricola*, **62**, 427-432.

PEREIRA O. G., SANTOS M. C. and KUNG L. (2009) The effects of microbial inoculants and enzymes on the fermentation of fresh alfalfa or alfalfal that was freshly frozen and thawed before ensiling. Proceedings of the XV International Silage Conference, Madison, Wisconsin, USA, 27-29 July, pp. 293-394.

PITT R. E., MUCK R. E. and PICKERING N. B. (1991) A model of aerobic fungal growth in silage. *Grass and Forage Science*, **46**, 301-312.

RANJIT N. K. and KUNG JR L. (2000) The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. *Journal of Dairy Science*, **83**, 526-535.

REICH L. J. and KUNG L. (2010) Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Animal Feed Science and Technology*, **159**, 105-109.

RHEE S. K. and PACK M. Y. (1980) Effect of environmental pH on fermentation balance of *Lactobacillus bulgaricus*. *Journal of Bacteriology*, **144**, 217-221.

ROOKE J. A. and HATFIELD R. D. (2003) Biochemistry of ensiling. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage science and technology*, pp. 95-139. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

ROSSI F. and DELLAGLIO F. (2007) Quality of silages from Italian farms as attested by number and identity of microbial indicators. *Journal of Applied Microbiology*, **103**, 1707-1715.

RUXTON I. B., CLARK B. J. and MCDONALD P. (1975) A review of the effects of oxygen on ensilage. *Grass and Forage Science*, **30**, 23-30.

SAARISALO E., SKYTTÄ E., HAIKARA A., JALAVA T. and JAAKKOLA S. (2007) Screening and selection of lactic acid bacteria strains suitable for ensiling grass. *Journal of Applied Microbiology*, **102**, 327-336.

SCHMIDT R. J., EMARA M. G. and KUNG JR L. (2008) The use of a quantitative real-time polymerase chain reaction assay for identification and enumeration of *Lactobacillus buchneri* in silage. *Journal of Applied Microbiology*, **105**, 920-929.

SCHMIDT R. J., HU W., MILLS J. A. and KUNG JR L. (2009) The development of lactic acid bacteria and *Lactobacillus buchneri* and their effects on the fermentation of alfalfa silage. *Journal of Dairy Science*, **92**, 5005-5010.

SCHMIDT R. J. and KUNG L. (2010) The effects of *Lactobacillus buchneri* with or without a homolactic bacterium on the fermentation and aerobic stability of corn silages made at different locations. *Journal of Dairy Science*, **93**, 1616-1624.

SCHROEDER J. W. Dairy focus: cold temperatures challenge corn silage production http://www.ag.ndsu.edu/news/columns/dairy-focus [28 February 2013].

SEALE D. R., HENDERSON A. R., PETTERSSON K. O. and LOWE J. F. (1986) The effect of addition of sugar and inoculation with two commercial inoculants on the fermentation of lucerne silage in laboratory silos. *Grass and Forage Science*, **41**, 61-70.

SEBASTIAN S., PHILLIP L. E., FELLNER V. and IDZIAK E. S. (1996) Comparative assessment of bacterial inoculation and propionic acid treatment on aerobic stability and microbial populations of ensiled high-moisture ear corn. *Journal of Animal Science*, **74**, 447-456.

SPOELSTRA S. F., COURTIN M. G. and VAN BEERS J. A. C. (1988) Acetic acid bacteria can initiate aerobic deterioration of whole crop maize silage. *Journal of Agicultural science*, **111**, 127-132.

STEVENSON D. M., MUCK R. E., SHINNERS K. J. and WEIMER P. J. (2006) Use of real time PCR to determine population profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. *Applied Microbiology and Biotechnology*, **71**, 329-338.

STIEGLMEIER M., WIRTH R., KMINEK G. and MOISSL-EICHINGER C. (2009) Cultivation of anaerobic and facultatively anaerobic bacteria from spacecraft-associated clean rooms. *Applied and Environmental Microbiology*, **75**, 3484-3491.

STILES M. E. and HOLZAPFEL W. H. (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*, **36**, 1-29.

STRINGINI M., COMITINI F., TACCARI M. and CIANI M. (2008) Yeast diversity in crop-growing environments in Cameroon. *International Journal of Food Microbiology*, **127**, 184-189.

SYLVESTRE M.-A. (2011) *Facteurs qui influencent la cinétique de fermentation chez la fléole des prés (Phleum pratense L.)* Maîtrise en biologie, Université du Québec en Abitibi-Témiscamingue.

TANAKA O., MORI K. and OMOMO S. (2000) Effect of inoculation with *Lactobacillus curvatus* on ensiling. *Grassland Science*, **46**, 148-152.

TANAKA O. and OHMOMO S. (1995) A simple method of laboratory silage fermentation by using a plastic pouch for packing. *Grassland Science*, **41**, 55-59.

TANNOCK G. W., TILSALA-TIMISJARVI A., RODTONG S., NG J., MUNRO K. and ALATOSSAVA T. (1999) Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S-23S rRNA gene intergenic spacer region sequence comparisons. *Applied and Environmental Microbiology*, **65**, 4264-4267.

TAYLOR C. C., RANJIT N. J., MILLS J. A., NEYLON J. M. and KUNG JR L. (2002) The effect of treating whole-plant barley with *Lactobacillus buchneri* 40788 on silage fermentation, aerobic stability, and nutritive value for dairy cows. *Journal of Dairy Science*, **85**, 1793-1800.

TAYLOR K. A. C. C. (1996) A simple colorimetric assay for muramic acid and lactic acid. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology*, **56**, 49-58.

TE GIFFEL M. C., WAGENDORP A., HERREWEGH A. and DRIEHUIS F. (2002) Bacterial spores in silage and raw milk. *Antonie van Leeuwenhoek*, **81**, 625-630.

THANH V. N., MAI L. T. and TUAN D. A. (2008) Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. *International Journal of Food Microbiology*, **128**, 268-273.

THOMAS T. D., ELLWOOD D. C. and LONGYEAR V. M. C. (1979) Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *Journal of Bacteriology*, **138**, 109-117.

USDA (2010) Major world crop areas and climatic profiles. *http://www.usda.gov/oce/weather/pubs/Other/MWCACP/world_crop_country.htm, accessed data: 11/04/2010.*

USDANASS (2013) Corn, silage - acres harvested: U.S. Department of Agriculture NASS.

VAN RANST G., FIEVEZ V., DE RIEK J. and VAN BOCKSTAELE E. (2009) Influence of ensiling forages at different dry matters and silage additives on lipid metabolism and fatty acid composition. *Animal Feed Science and Technology*, **150**, 62-74.

VAN SAUN R. and HEINRICHS A. J. (2008) Troubleshooting silage problems: how to identify potential problems. Proceedings of the Mid-Atlantic Conference Pittsburgh, Pennsylvania, USA,, May 26-28, 2008.

VAN SAUN R. and JUD HEINRICHS A. (2008) Troubleshooting silage problems: how to identify potential problems. *Penn State College of Agricultural Sciences and Cooperative Entension*

WALTER J., HERTEL C., TANNOCK G. W., LIS C. M., MUNRO K. and HAMMES W. P. (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* Species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, **67**, 2578-2585.

WANG F. and NISHINO N. (2009) Association of *Lactobacillus buchneri* with aerobic stability of total mixed ration containing wet brewers grains preserved as a silage. *Animal Feed Science and Technology*, **149**, 265-274.

WANG P., BAI C., LIU L. and CAO B. (2011) Effects of lactic acid bacteria inoculant on the fermentation quality of reed grass (*Phragmites australis* Cav.Trin.ex Sterd.) at low temperature. *Acta Agrestia Sinica*, **19**, 127-131.

WANG X., HARUTA S., WANG P., ISHII M., IGARASHI Y. and CUI Z. (2006) Diversity of a stable enrichment culture which is useful for silage inoculant and its succession in alfalfa silage. *FEMS Microbiology Ecology*, **57**, 106-115.

WARD R. T. and DE ONDARZA M. B. (2008) Fermentation analysis of silage: use and interpretation: Cumberland Valley Analytical Services (CVAS).

WEINBERG Z. G., ASHBELL G., HEN Y. and AZRIELI A. (1993) The effect of applying lactic acid bacteria at ensiling on the aerobic stability of silages. *Journal of Applied Bacteriology*, **75**, 512-518.

WEINBERG Z. G. and MUCK R. E. (1996) New trends and opportunities in the development and use of inoculants for silage. *FEMS Microbiology Reviews*, **19**, 53-68.

WEINBERG Z. G., SZAKACS G., ASHBELL G. and HEN Y. (1998) The effect of temperature and *Lactobacillus amylovorus* and *Lact. plantarum*, applied at ensiling, on wheat silage. *Journal of Applied Microbiology*, **84**, 404-408.

WEINBERG Z. G., SZAKACS G., ASHBELL G. and HEN Y. (2001) The effect of temperature on the ensiling process of corn and wheat. *Journal of Applied Microbiology*, **90**, 561-566.

WHITE J. Solutions for silage problems http://todaysfarmermagazine.com/mag/livestock/724-solutions-for-silage-problems [28 June 2013].

WIERINGA G. W. (1969) Influence of moisture and nutrient content of forage plants on fermentation processes. Proceedings of the 3rd General Meeting of the European Grassland Federation, Braunschweig, Germany, June 9–14, pp. 133-137.

WILKINSON J. M., BOLSEN K. K. and J. L. C. (2003) History of silage. In: BUXTON D. R., RICHARD E. M. and HARRISON J. H. (eds.) *Silage science and technology*, pp. 1-31. Madison, Wisconsin, USA: ASA, CSSA, SSSA.

WILKINSON J. M. and DAVIES D. R. (2013) The aerobic stability of silage: key findings and recent developments. *Grass and Forage Science*, **68**, 1-19.

WILKINSON J. M. and TOIVONEN M. I. (2003) *World silage*. Painshall, Lincoln,UK: Chalcombe Publications

WILLIAMS A. G., HOXEY R. P. and LOWE J. F. (1997) Changes in temperature and silo gas composition during ensiling, storage and feeding-out grass silage. *Grass and Forage Science*, **52**, 176-189.

WOOLFORD M. K. (1990) The detrimental effects of air on silage. *Journal of Applied Bacteriology*, **68**, 101-116.

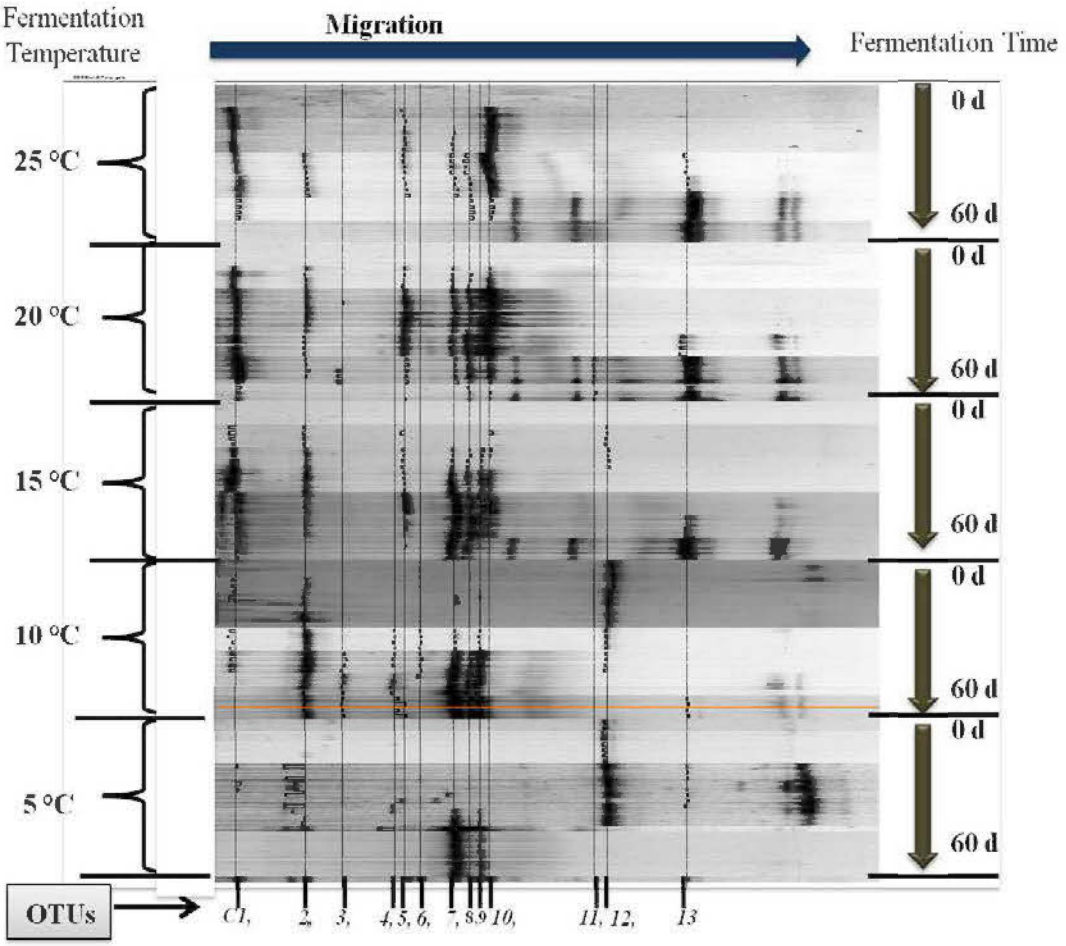
XING L., CHEN L. J. and HAN L. J. (2009) The effect of an inoculant and enzymes on fermentation and nutritive value of sorghum straw silages. *Bioresource Technology*, **100**, 488-491.

YANG H. Y., WANG X. F., LIU J. B., GAO L. J., ISHII M., IGARASHI Y. and CUI Z. J. (2006) Effects of water-soluble carbohydrate content on silage fermentation of wheat straw. *Journal of Bioscience and Bioengineering*, **101**, 232-237.

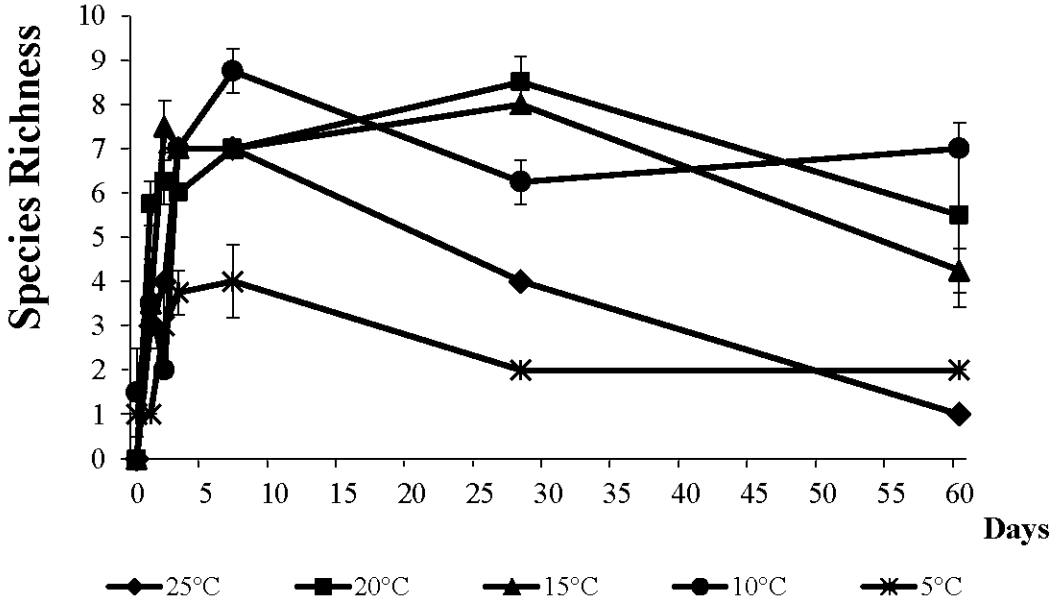
ZAKRZEWSKI M., GOESMANN A., JAENICKE S., JÜNEMANN S., EIKMEYER F., SZCZEPANOWSKI R., AL-SOUD W. A., SØRENSEN S., PÜHLER A. and SCHLÜTER A. (2012) Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. *Journal of Biotechnology*, **158**, 248-258.

ANNEXE A

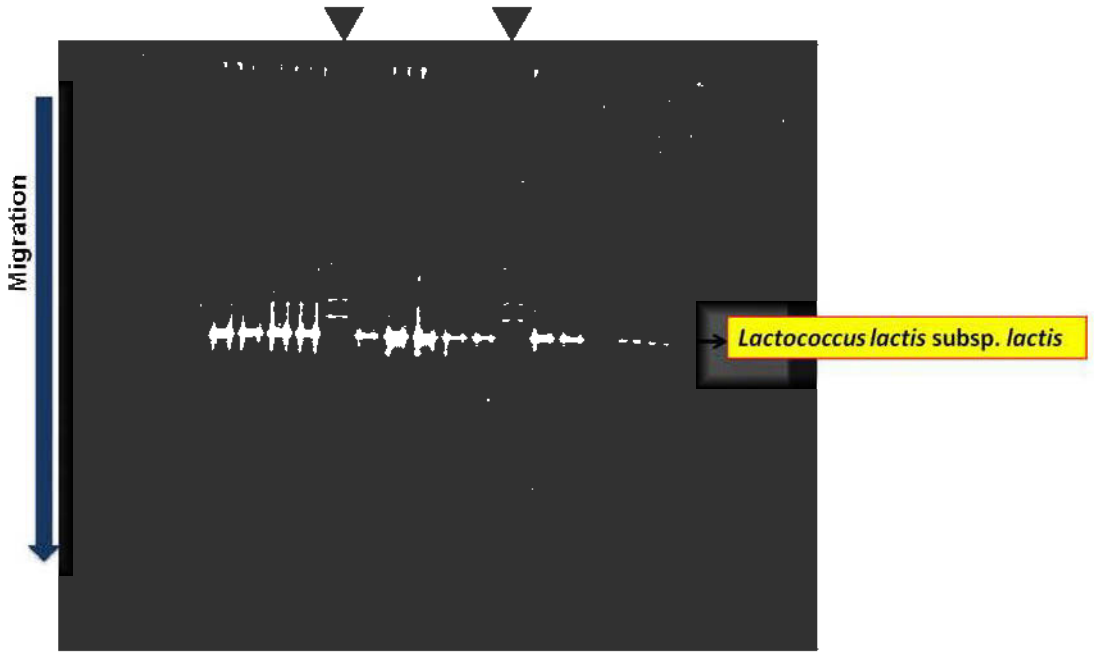
SUPPLEMENTARY INFORMATION FOR CHAPTER II



A.1 Succession of LAB population (OTUs) during the ensiling process of corn silage stored at different temperatures. †OTUs C1 ~ 13 represents the same LAB-associated OTUs; C1: *L. plantarum*, C2: *Leuconostoc citreum*, C3: *Leuconostoc mesenteroides*, C4: Unidentified bacterium A, C5: *L. brevis*, C6: *Leuconostoc lactis*, C7: *L. sakei*, C8, *L. coryniformis subsp. torquens*, C9, *L. curvatus*, C10, *P. pentosaceus*, C11, Unidentified bacterium B, C12, Uncultured bacterium C, C13, *L.buchneri*. Four repetitions of every experimental treatment are included, and a total of 140 silage samples were analysed in this DGGE profile.



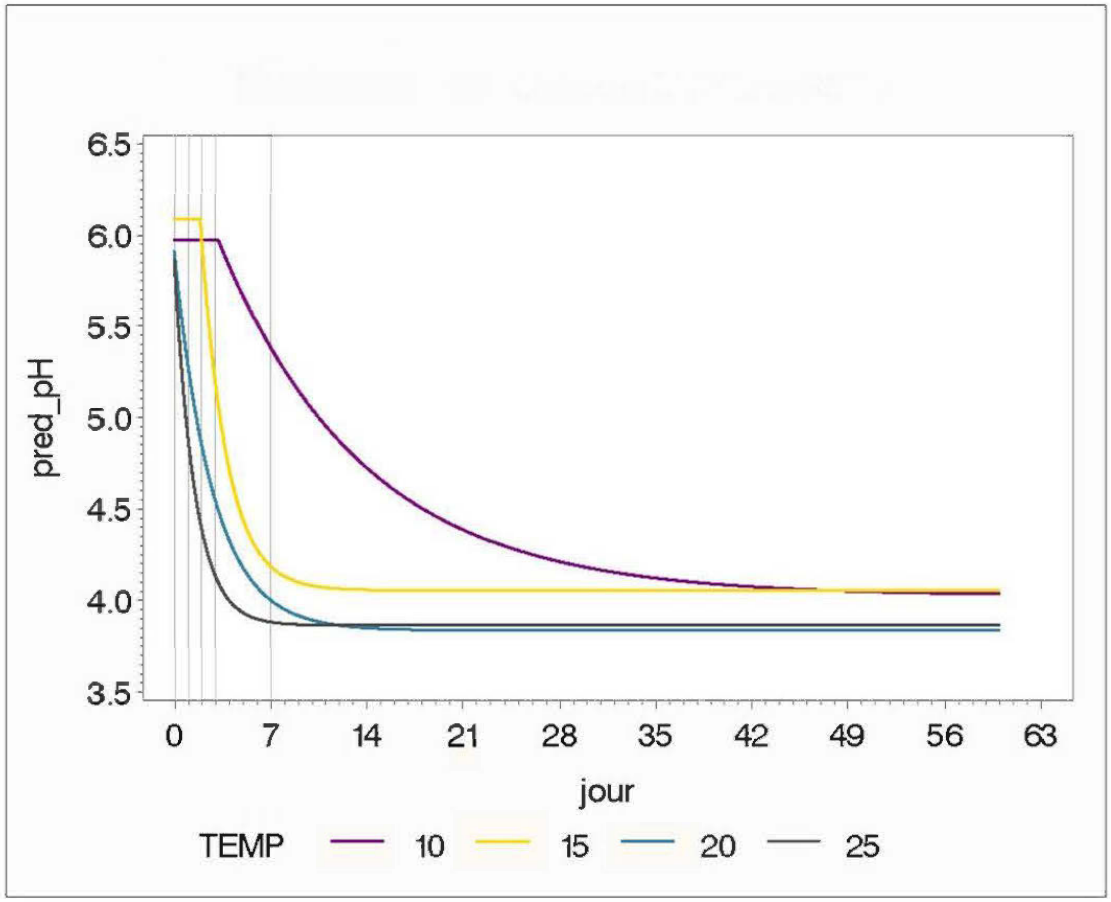
A.2 Species richness of LAB population during ensiling of corn silage stored at different temperatures. Species Richness was described as mean value ± sd. Four repetitions of every experimental treatment are included.



A.3 DGGE analysis of *Lactococcus*-group LAB in silages was performed usimg primer Lac3 / Lac2GC. A 8% acrylamide gel with a linear denaturing range of 30-70% was used, and electrophoresis was performed at 75 V and 60 °C for 20 h. Each lane represents one specific silage sample with different treatment, except the two ladders which are marked with '▼'. This profile demonstrates that there was no apparent development in *Lactococcus*-group LAB during the ensiling process. Additionally, the distinct band was successfully excised, cloned and sequenced, and further identified as *Lactococcus lactis* subsp. *lactis*.

ANNEXE B

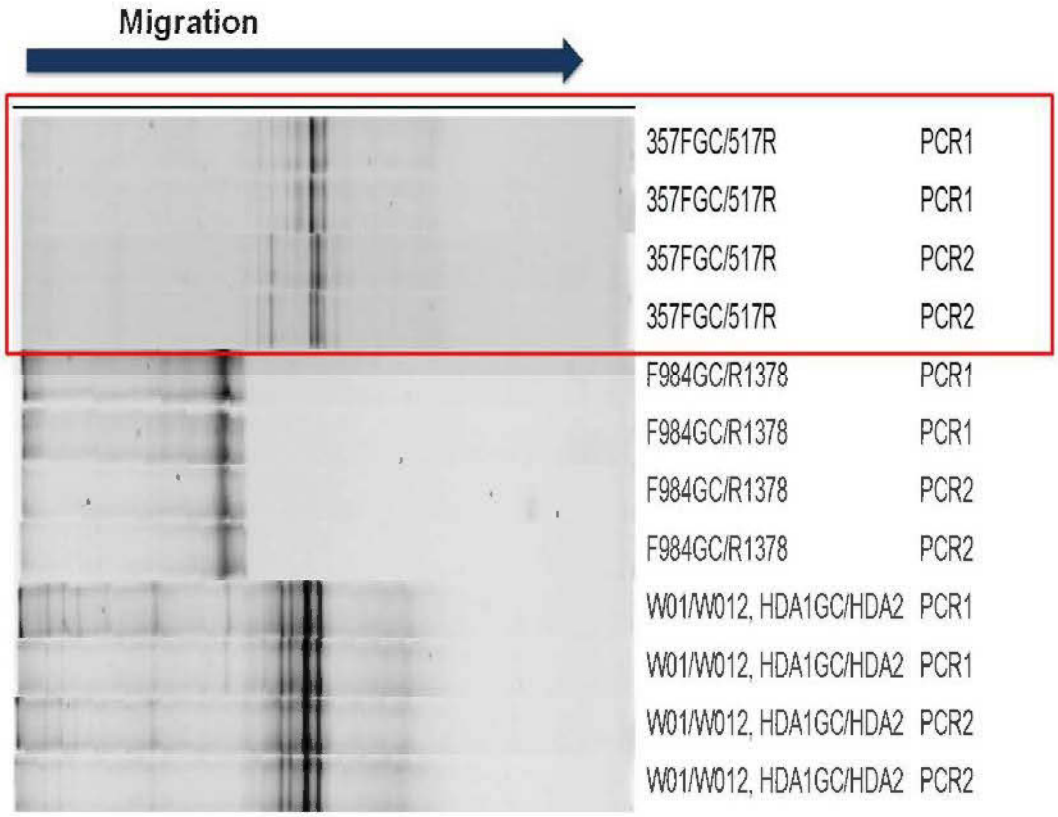
SUPPLEMENTARY INFORMATION FOR CHAPTER III



B.1 Curves of the predicted pH values based on a non-linear regression model developed by Jones *et al.* (1992) for whole-plant corn ensiled at different temperatures. This model was able to fit to the pH data at 10, 15, 20 and 25°C, but not at 5°C. The lag time (lag, time prior to the rapid decline, day) and the rate of pH decline (k, per day) were accordingly calculated.

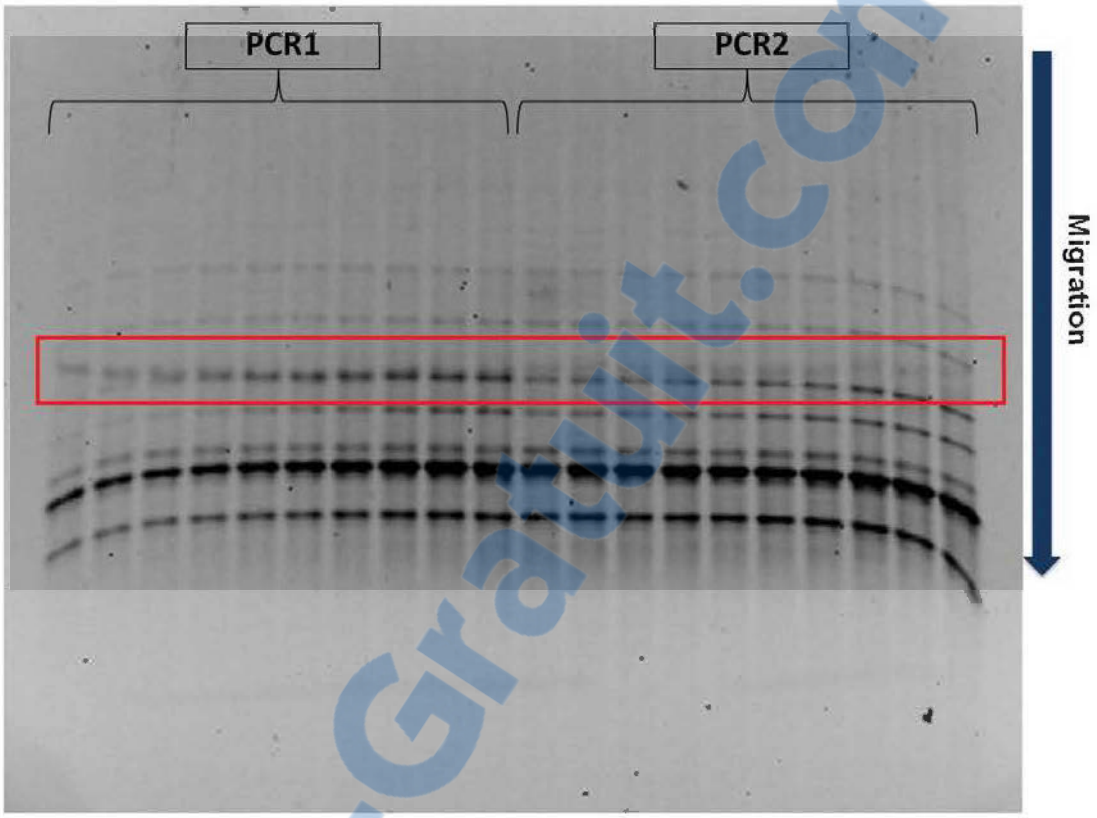
ANNEXE C

SUPPLEMENTARY INFORMATION FOR CHAPTER IV

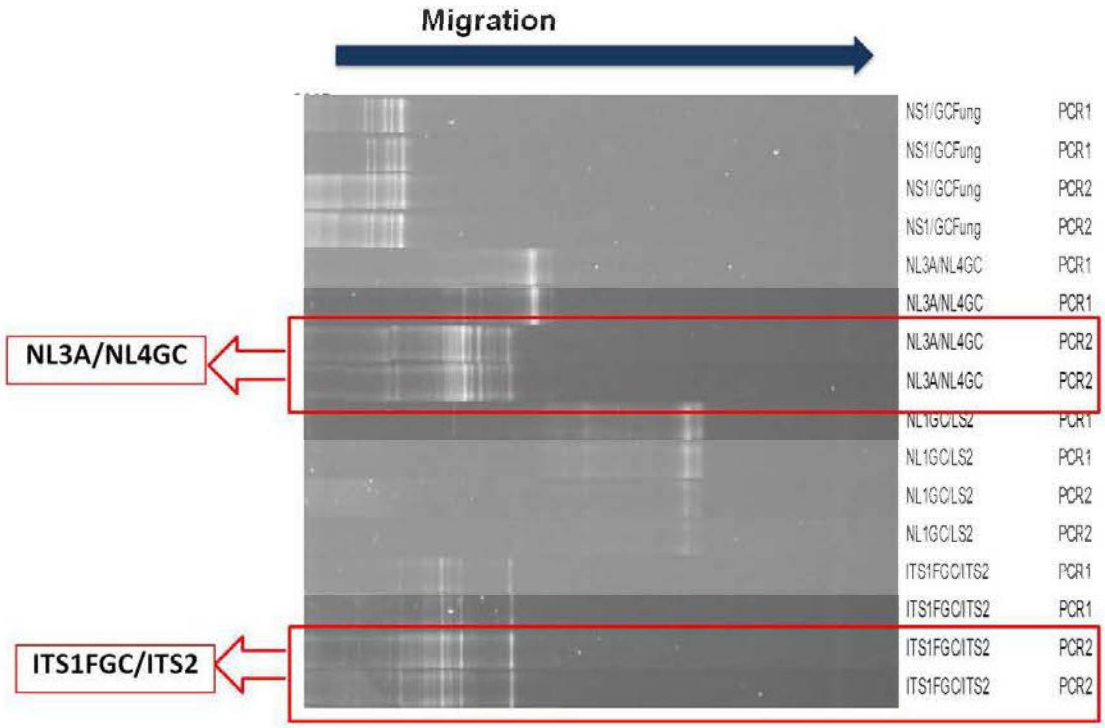


C.1 To analysis the bacterial diversity in silage, we tested four primer sets including 357FGC / 517R (Gao, *et al.*, 2008), F984GC / R1378 (Heuer, *et al.*, 1997), 16F945 / 16R1401GC (Thanh, *et al.*, 2008) and W01 / W012 – HDA1GC / HDA2 (a nested PCR) (Ogier, *et al.*, 2004), with same PCR mixture [15 µL containing 1 µL of DNA template (50 ng), 1 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.3 µM of each primer and 0.025 U μL^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit, New England BioLabs, Canada)], but two PCR programs for each primer set, i.e., PCR1 (according to the reference) and PCR2 (recommended by the *Taq* PCR kit, with the annealing temperature from the reference). DGGE results showed that the amplification failed with 16F945/16R1401GC. Primer set 357FGC / 517R produced more distinct bands and therefore was selected for further optimization. A 8% acrylamide gel with a linear denaturing range of 20-80% was used, and

electrophoresis was performed at 75 V and 60 °C for 16 h. One silage sample was used for the test. For 357FGC / 517R, PCR1 consists of an initial DNA denaturation at 95 °C for 10 min, 30 cycles of 93 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 5 min; PCR2 consists of an initial step at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 48 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min. For F984GC/R1378, PCR1 consists of an initial step at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and a final step at 72 °C for 10 min; PCR2 consists of an initial step at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 54 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min. For W01/W012-HDA1GC/HDA2, PCR1 consists of an initial 96 °C for 5 min, 30 cycles of 96 °C for 10 sec, 50 °C for 30 sec, 72 °C for 1 min, and a final step at 72 °C for 2 min for W01/W02, plus a program for HDA1GC/HAD2 of an initial 94 °C for 4 min, 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec, 68 °C for 1 min, and a final step at 68 °C for 7 min. For W01/W012-HDA1GC/HDA2, PCR2 consists of an initial 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 50 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min for W01/W02, plus a program for HDA1GC/HAD2 of an initial 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 58 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min.

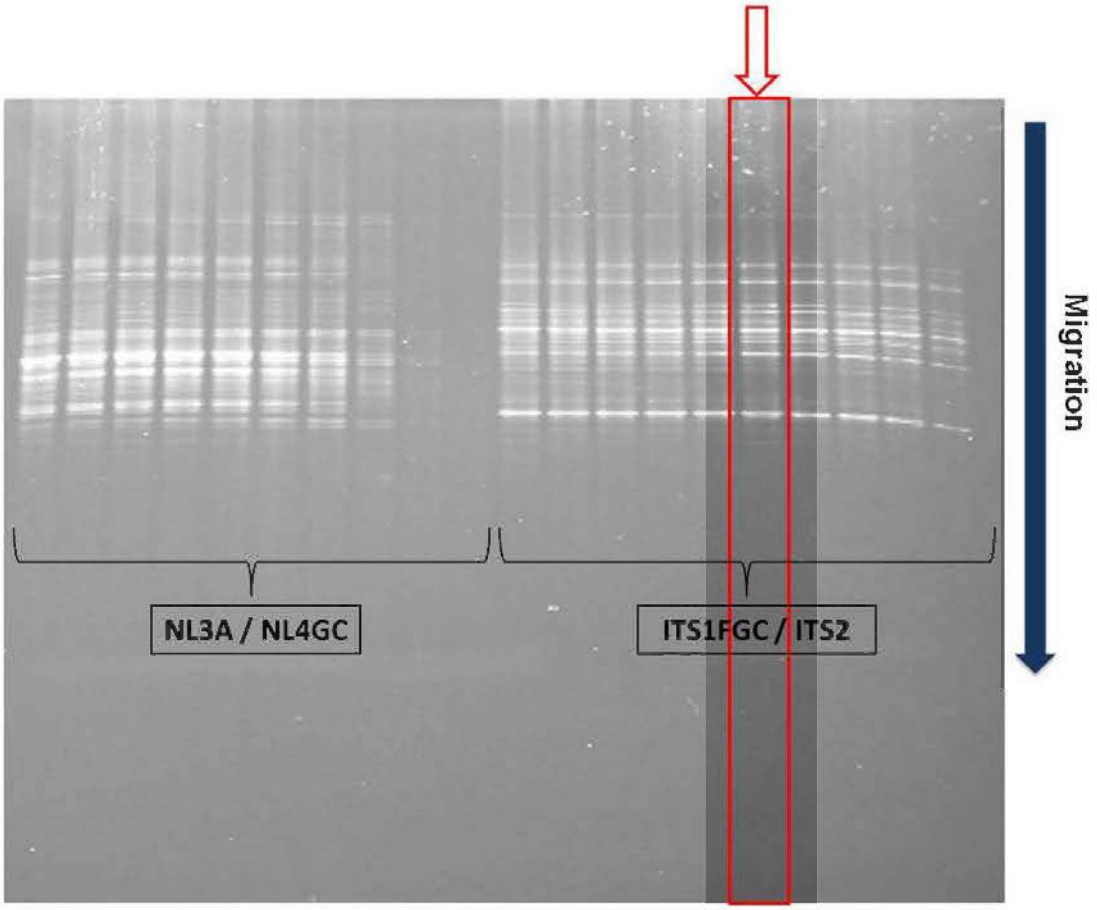


C.2 A gradient PCR with an annealing temperature from 43 to 54°C was performed with 357FGC / 517R with two amplification programs, i.e., PCR1 and PCR2 as previously described in C.1, respectively. Program PCR1 with an annealing temperature of 48 °C was selected for universal bacteria analysis. A 8% acrylamide gel with a linear denaturing range of 35-60% was used, and electrophoresis was performed at 75 V and 60 °C for 16 h. One silage sample was used for the test.



C.3 To analysis the fungal diversity in silage, we tested five primer sets including NS1 / FungGC (May, *et al.*, 2001), NL1GC / LS2 (Stringini, *et al.*, 2008), NL3A / NL4GC (Thanh, *et al.*, 2008), ITS1GC / ITS4 (Bonito, *et al.*, 2010), and ITS1FGC / ITS2 (Kebli, *et al.*, 2011), with same PCR mixture [15 µL containing 1 µL of DNA template (50 ng), 1 X standard *Taq* reaction buffer, 200 µM of each deoxynucleotide, 0.3 µM of each primer and 0.025 U μL^{-1} of *Taq* DNA polymerase (*Taq* PCR Kit, New England BioLabs, Canada)], but two amplification programs for each primer set, i.e., PCR1 (recommended by the *Taq* PCR kit with the annealing temperature from the reference) and PCR2 (according to the reference). DGGE results showed that the amplification failed with ITS1GC/ITS4. Primer set NL3A / NL4GC and ITS1FGC / ITS2 with amplification program of PCR2 produced more distinct bands and therefore were selected for further optimization. A 8% acrylamide gel with a linear denaturing range of 20-80% was used, and electrophoresis was performed at 75 V and 60 °C for 16 h. One silage sample was used for the test. For NS1/GCFung, PCR1 consists of an

initial DNA denaturation at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 50 °C for 1 min, 68 °C for 1 min, and a final elongation step at 68 °C for 5 min; PCR2 consists of an initial step at 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 3 min, and a final step at 68 °C for 10 min. For NL3A/NL4GC, PCR1 consists of an initial step at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 60 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min; PCR2 consists of an initial step at 95 °C for 2 min, 30 cycles of 94 °C for 30 sec, 60 °C for 40 sec, 72 °C for 1 min, and a final step at 72 °C for 7 min. For NL1GC/LS2, PCR1 consists of an initial step at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 52 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min; PCR2 consists of an initial step at 95 °C for 5 min, 30 cycles of 95 °C for 1 sec, 52 °C for 45 sec, 72 °C for 1 min, and a final step at 72 °C for 7 min. For ITS1FGC/ITS2, PCR1 consists of an initial step at 95 °C for 4 min, 30 cycles of 95 °C for 30 sec, 55 °C for 1 min, 68 °C for 1 min, and a final step at 68 °C for 5 min; PCR2 consists of an initial step at 95 °C for 3 min, 30 cycles of 94 °C for 45 sec, 55 °C for 45 sec, 72 °C for 1 min 15 sec, and a final step at 72 °C for 8 min.



C.4 A gradient PCR with an annealing temperature from 54 to 66°C was performed with NL3A / NL4GC, as well as a gradient PCR with an annealing temperature from 50 to 60°C with ITS1FGC / ITS2, with amplification program of PCR2 as previously described in C.3. Primer set ITS1FGC / ITS2 with program PCR2 with an annealing temperature of 54.0 °C were selected for universal fungal analysis. A 8% acrylamide gel with a linear denaturing range of 15-70% was used, and electrophoresis was performed at 75 V and 60 °C for 16 h. One silage sample was used for the test.

C.5 Effects of temperature on the chemical composition of corn silages (g kg^{-1} DM ± se)

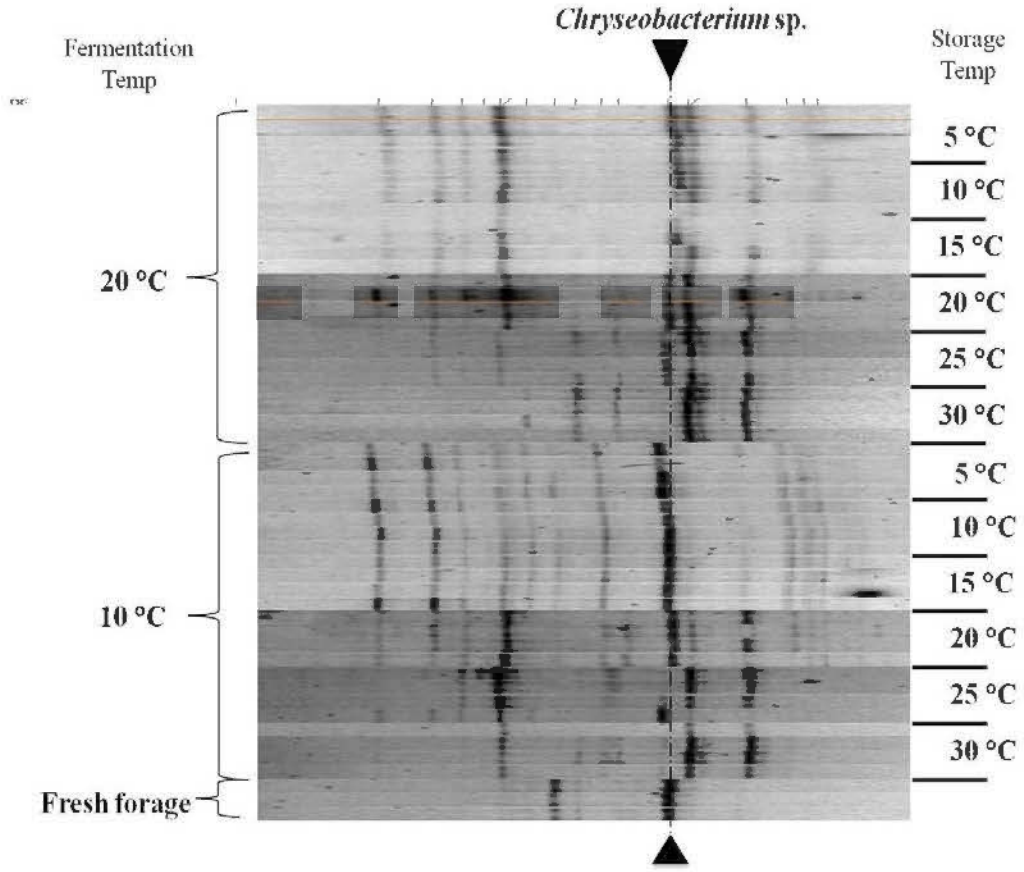
Fermentation temp-Storage temp	Total N	DM (g kg^{-1} FM)	Lactic acid	Acetic acid	Ethanol	NH_3-N/ Total N (%)	Propionic acid	nButyric acid	Isobutyric acid	nValeric acid	Isovaleric acid
20°C- 5°C	13.78	299.4^{ab}	59.24^{a}	80.01^{ab}	10.66^{bcd}	5.41±0.18	1.17±0.60	0.53±0.10	0.28±0.18	0.65±0.33	0.84±0.08
20°C-10°C	13.87	307.9^{b}	55.92^{a}	70.64^{a}	7.50^{b}	5.35±0.11	1.81±0.87	1.32±0.25	0.47±0.40	0.74±0.67	1.02±0.40
20°C-15°C	13.86	306.9^{b}	59.01^{a}	93.22^{bc}	8.10^{bc}	5.42±0.09	0.90±0.28	1.64±0.68	0.10±0.04	0.12±0.02	0.61±0.10
20°C-20°C	13.53	303.9^{b}	50.98^{a}	107.63^{c}	13.45^{cd}	5.25±0.38	0.76±0.28	1.02±2.87	0.13±0.06	0.08±0.03	0.64±0.06
20°C-25°C	13.59	306.6^{b}	61.33^{a}	107.86^{c}	15.25^{d}	6.16±0.10	1.21±0.16	1.85±0.46	0.28±0.08	0.56±0.23	0.98±0.18
10°C- 5°C	13.80	308.3^{b}	34.99^{b}	78.41^{ab}	2.83^{a}	3.13±0.11	0.95±0.05	0.45±0.05	0.23±0.04	0.24±0.04	0.63±0.02
10°C-10°C	13.63	314.0^{b}	40.49^{b}	80.96^{ab}	2.79^{a}	3.40±0.14	1.10±0.05	0.22±0.08	0.19±0.07	0.17±0.08	0.65±0.04
10°C-15°C	13.70	314.8^{ab}	36.08^{b}	80.17^{ab}	3.16^{a}	3.44±0.09	1.07±0.07	0.21±0.06	0.17±0.03	0.17±0.03	0.52±0.04
10°C-20°C	13.68	304.8^{b}	44.39^{b}	81.16^{ab}	2.52^{a}	3.55±0.26	2.42±1.02	0.54±0.21	0.76±0.41	0.93±0.44	0.79±0.13
10°C-25°C	13.97	305.2^{b}	45.41^{b}	139.17^{d}	4.08^{a}	4.95±0.40	1.27±0.14	2.21±1.65	0.30±0.12	0.78±0.17	1.50±0.43
SEM	0.60	11.14	1.822	3.465	0.772	0.178	0.155	0.349	0.063	0.094	0.072
Fermentation temp	NS	*	***	NS	***						
Storage temp	NS	NS	NS	***	***						
Interaction	NS	NS	NS	***	*						

Note: values with different letters within the same column are statistically different ($P < 0.05$); se: standard error; SEM: standard error of the mean; NS, not significant; *, ** and ***: significant at $P < 0.05$, 0.01 and 0.001. The data of Ethanol and WSC were log transformed for the verification of the two assumptions before ANOVA analysis.

C.6 Effects of temperature on the microbiological composition of corn silages (log 10 CFU g FM^{-1} ± se)

Fermentation temp-Storage temp	LAB	Clostridia spores	Enterobacteria	Yeasts	Moulds
20°C- 5°C	7.85^{a}	2.80^{a}	ND	ND	ND
20°C-10°C	7.82^{a}	3.21^{ab}	ND	ND	ND
20°C-15°C	7.67^{ac}	3.52^{ab}	4.02±0.07	3.93±0.08	ND
20°C-20°C	7.81^{a}	3.43^{ab}	4.71±0.47	4.85±1.21	ND
20°C-25°C	8.19^{d}	3.68^{b}	4.78±0.10	4.44±1.11	ND
10°C- 5°C	5.80^{b}	3.12^{ab}	ND	ND	ND
10°C-10°C	5.85^{b}	3.37^{ab}	ND	ND	ND
10°C-15°C	5.85^{b}	3.28^{ab}	ND	ND	ND
10°C-20°C	7.43^{c}	3.54^{ab}	ND	ND	ND
10°C-25°C	8.54^{e}	3.58^{ab}	ND	ND	ND
SEM	0.159	0.061	0.214	0.304	0
Fermentation temp	***	NS			
Storage temp	***	**			
Interaction	***	NS			

Note: values with different letters within the same column are statistically different ($P < 0.05$); ND, not detected (< 2.00 $\log_{10}$ CFU g FM^{-1}); SEM: standard error of the mean; NS, not significant; ** and ***: significant at $P < 0.01$ and 0.001.



C.7 Occurrence of DGGE OTU corresponding to *Chryseobacterium* sp. in corn silage and fresh corn forage prior to ensiling. Four repetitions of every experimental treatment are included, and a total of 52 silage samples were analysed in this DGGE profile.