Methods to Study the Relationship between Forage Composition and Silage Fermentation and Aerobic Stability

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Cover: experimental silos from 40 g to 2.5 kg
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Abstract

This thesis is based on the following hypotheses: (I) under controlled ensiling conditions, forage chemical and microbial composition explain variation in silage end-product concentrations (II) sterilized forage inoculated with parent microflora gives similar fermentation result as untreated control forage and (III) this methodology enables studying interactions between forage source and field flora on silage fermentation and aerobic stability.

Hypothesis (I) was evaluated by constructing regression models of end-products on common forage chemical variables (e.g. dry matter, crude protein) and lactic acid bacterial numbers using data from 66 maize and 52 perennial crop silages. To address hypothesis (II), grass, grass-clover, white clover and maize samples were sterilized by heating, inoculated with parent microfloras and ensiled. Validation was made against untreated control silages. To test hypothesis (III), grass, red clover and maize samples were sterilized by heating and each sterile sample was inoculated with each of the three microfloras, derived from intact samples, before ensiling.

I On average, the models captured 43% and 52% of total variation in end-product concentrations in perennial crop and maize silages, respectively.

II Reconstituted silages contained lower amounts of lactic acid, ethanol and ammonia (i.e. 18%, 20% and 37%, respectively) but similar amounts of volatile fatty acids and 2,3-butanediol and had similar microbial profiles compared to control silages.

III Forage source had marked effects on both end-product formation and silage aerobic stability whereas, field flora mainly affected the aerobic stability.

It is concluded that the new *in vitro* methodology, in spite of needs for further improvement, allows hypothesis testing on relative effects of field flora and forage species on silage quality. The results also suggest that more detailed information on forage chemical composition, such as data on water activity, pectin, organic acids, etc., could provide additional information for the prediction of ensiling outcome.

Keywords: silage, *in vitro*, methodology, sterilization, heat, reconstitution, inoculation, fermentation, aerobic stability, field flora, prediction, regression, modelling

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Dedication

To my beloved wife for her endless love, support and encouragements and to my beautiful daughter for giving a new meaning to my life

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Mogodiniyai Kasmaei, K., Rustas, B-O., Spörndly, R. and Udén, P. (2013). Prediction models of silage fermentation products on crop composition under strict anaerobic conditions: A meta-analysis. *Journal of Dairy Science* 96(10), 6644-6649.

II Mogodiniyai Kasmaei, K., Spörndly, R. and Udén, P. (2013). Research Note: A sterilization technique with application to silage research and inoculant evaluation. *Grass and Forage Science* 69(4), 724-728.

III Mogodiniyai Kasmaei, K., Passoth, V., Spörndly, R. and Udén, P. (2014). A new sterilization and inoculation method in silage research. *Grass and Forage Science* 70(4), 668-673.

IV Mogodiniyai Kasmaei, K., Dicksved, J., Spörndly, R. and Udén, P. Separating the effects of forage source and field flora on silage fermentation quality and aerobic stability (submitted).

Papers I-III are reproduced with the permission of the publishers.

The contribution of Kamyar Mogodiniyai Kasmaei to the papers included in this thesis was as follows:

I Data collection and analysis, manuscript preparation jointly with co-authors, manuscript submission and correspondence with the scientific journal

II Experimental design jointly with co-authors, performing the experiment, manuscript preparation jointly with co-authors, manuscript submission and correspondence with the scientific journal

III Experimental design jointly with co-authors, performing the experiment, manuscript preparation jointly with co-authors, manuscript submission and correspondence with the scientific journal

IV Experimental design jointly with co-authors, performing the experiment, manuscript preparation jointly with co-authors, manuscript submission and correspondence with the scientific journal

Abbreviations

ADIN	Acid detergent insoluble N
BSN	Buffer soluble N
BC	Buffering capacity
CP	Crude protein
DM	Dry matter
Y_{ATP}	Energy yield
EtOH	Ethanol
EEA	Exogenous electron acceptor
LAB	Lactic acid bacteria
NAD	Nicotinamide adenine dinucleotide
ND	Neutral detergent
NDF	Neutral detergent fibre
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCR	polymerase chain reaction
redox	Reduction-oxidation
E_0'	Reduction potential
NaClO	Sodium hypochlorite
VFA	Volatile fatty acid
WSC	Water soluble carbohydrate

1 Introduction

1.1 Forage preservation

Forages constitute the major part of dairy cow rations. Successful preservation of forages, in which losses of nutrients are minimal and hygienic quality remains high, is therefore essential. Large populations of microorganisms, including obligate aerobic bacteria, facultative anaerobic bacteria and fungi collectively known as epiphytic microbes, inhabit standing crops. During harvest and wilting, there is also a contamination with soil bacteria such as clostridia. The harvested biomass will therefore deteriorate unless proliferation of these microorganisms is prevented. Three preservation methods dominate in animal production: (i) ensiling, i.e. anaerobic storage of the biomass at a dry matter (DM) level between 25 and 50%, (ii) haylage making, i.e. anaerobic storage of the biomass at a DM level between 50 and 60% and (iii) hay making, i.e. aerobic storage of the biomass at a DM level ≥80%.

In ensiling, the means of preservation is a reduced pH of the biomass, from ~6 to ~4, primarily due to the activity of epiphytic lactic acid bacteria (LAB). The domination of LAB can additionally be assured by inoculation with commercial LAB or addition of sugar containing by-products such as molasses. Application of organic acids (e.g. formic and propionic acids) or organic salts (e.g. sodium nitrite and potassium sorbate) may also be used to prevent clostridial and fungal growth. In hay making, microbial proliferation is prevented by a reduced water activity (a_w) level (~<0.880), which is contrary to ensiling where, high a_w levels of the biomass are needed to ensure sufficient LAB growth. Haylage making is a marriage between methods (i) and (ii) and preservation is achieved through combined effects of the reduced pH and a_w of the biomass. However, pH in haylage is not reduced to a same extent as in silage due to a limited LAB activity caused by the lower a_w of the haylage biomass. The choice of preservation method is essentially a function of climatic conditions and cost.

1.2 Silage

The pickled forage produced from the ensiling process contains mainly lactic acid but also varying amounts of other waste products of microbial catabolism including volatile fatty acids (VFAs), alcohols and ammonia. The fermentation quality of silage can affect animal performance considerably. For instance, high levels of ammonia and VFAs can decrease intake (Huhtanen *et al*., 2007) and lactic acid or total acids can reduce milk fat and protein concentrations (Huhtanen *et al*., 2003). Feeding silages containing high amounts of ethanol can also cause milk off-flavour (Randby *et al*., 1999). Today, variation in fermentation quality is unfortunately still large.

At feed-out, extensive spoilage of ensiled materials may often occur. The spoilage is mainly associated with the presence of lactate-assimilating yeasts and levels of acetic and butyric acid (Jonsson & Pahlow, 1984; Wilkinson & Davies, 2013). However, others have found no relationship between these factors and spoilage (Muck & O'Kiely, 1992; O'Kiely & Muck, 1992). Interestingly, new evidence has emerged suggesting an association between aerobic spoilage and certain species of LAB (Li & Nishino, 2011).

1.2.1 Ecosystems

Leaves and shoots of plants are covered with a waxy layer known as the cuticle, which prevents desiccation and nutrient leaching (Schreiber *et al*., 2008; Hussain, 2008). The three-dimensional structure and hydrophobic nature of the cuticle give roughness and dryness to the cuticle surface and make aerial parts of the plant a hostile environment for microbial colonization (Schreiber *et al*., 2008).

As the cuticle roughness decreases with age by erosion, bacteria, yeasts and moulds (in this order) initiate colonization and this in turn increases wettability of the cuticle which facilitates microbial colonization (Knoll & Schreiber, 2000; Schreiber *et al*., 2008). This might explain increases in epiphytic microbial counts at later harvests (Müller, 2009). Other factors affecting the population of epiphytic microbes include plant species, humidity, temperature and ultraviolet radiation (Leben, 1988; Lin *et al*., 1992; Schreiber *et al*., 2008).

In a silo, the anoxic condition favours growth of facultative (LAB, enterobacteria) and strict (clostridia) anaerobes. The system is closed and compared to the rumen, it allows accumulation of end-products from microbial catabolism. This in turn reduces pH which enables the pH tolerant LAB to become dominant. Substrate availability in the silo under temperate conditions is usually not a limiting factor for microbial growth, as residual sugars are normally present after several weeks of fermentation. During the course of ensiling, there can even be a release of sugars from dead microbial cells and a

utilization of hemicellulose (Pitt *et al.*, 1985; Muck, 1988), starch and pectin (Peter Udén, unpublished data). Unfortunately, little is known about the magnitude of the contribution of these sources to metabolism.

The acidic condition of silage creates a stressful environment for most silage microorganisms. The acid stress is mainly attributed to a passive entrance of undissociated forms of propionic, acetic and lactic acids into microbial cells followed by an internal dissociation due to a higher pH (De Angelis & Gobbetti, 2004; Piper, 2011). This can result in a decrease of intracellular pH to detrimental levels. Factors affecting the amount of undissociated forms of organic acids in the silage medium include acid concentration, acid pK_a value and silage buffering capacity (Pitt *et al.*, 1985). The main defensive mechanism against acid stress is active transport of intracellular protons (Sanders *et al*., 1999; De Angelis & Gobbetti, 2004). This requires energy and silage microorganisms require effective strategies to cope with inflated energy demands if they are to survive or continue growth under low pH conditions.

1.2.2 Catabolic processes

Silage microorganisms obtain energy by respiration, fermentation or both. In both respiration and fermentation, energy is released by means of reduction-oxidation (redox) reactions in which compounds with a lower reduction potential (E_0') donate electrons to compounds with a higher E_0'. As the difference between E_0' of donor and acceptor increases, energy efficiency also increases. Translocation of electrons between donor and acceptor is mediated by electron carrier molecules such as nicotinamide adenine dinucleotide (NAD). Availability of these molecules in an oxidized form is the main limiting factor in energy conservation pathways (Madigan *et al.*, 2012).

In fermentation, the redox balance is primarily reached internally by transferring electrons onto intermediates (e.g. pyruvate and acetyl-CoA) formed during the initial steps of substrate breakdown. However, in respiration an exogenous electron acceptor (EEA) is used, allowing complete utilization of the chemical energy in the substrate (Madigan *et al*., 2012). During fermentation, the released energy from the redox reactions is captured via substrate-level phosphorylation whereas in respiration, oxidative phosphorylation is also employed (Stryer, 1981; Madigan *et al*., 2012). Oxidative phosphorylation, carried out across the cytoplasmic membrane in prokaryotes (Madigan *et al.*, 2012) or inner mitochondrial membrane in eukaryotes (Stryer, 1981), gives a significant edge to respiration, as 89% of the total ATP gained from aerobic respiration of a glucose molecule is derived from this process.

Respiration is often, mistakenly, associated only with the presence of O_2. However, under anoxic conditions and presence of other EEAs (e.g. nitrate), certain bacteria (e.g. *Escherichia coli*) can still respire, a phenomenon known as anaerobic respiration (Madigan *et al.*, 2012). Reduction of nitrate by enterobacteria results in the formation of nitrite (NO_2^-), which can further be decomposed to nitric oxide (NO) under acidic conditions, inhibiting the growth of clostridia in silage (Spoelstra, 1983; Spoelstra, 1985). In a well-covered silo, only a small amount of O_2 is available. If O_2 together with other electron acceptors are available, utilization of O_2 is favoured due to a higher energy yield (Y_{ATP}) (Madigan *et al*., 2012).

Some silage microorganisms can also switch from fermentation to respiration and *vice versa*, as commonly observed in yeasts. Crabtree negative and positive species of yeasts (e.g. *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae*, respectively) switch to respiratory catabolism in the presence of O_2 (Passoth *et al.*, 2006; Madigan *et al*., 2012). The Crabtree positive species, however, initiate respiration only under a low concentration of fermentable carbon sources (Van Urk *et al*., 1990). Silage yeasts are generally classified into lactate/non-lactate assimilators, mainly to explain flourishment of yeasts in response to the ingress of air into a silo (Jonsson & Pahlow, 1984). However, most yeasts classified as non-lactate assimilators are indeed able to utilize lactate, provided culturing conditions similar to silage environments, e.g. pH 4 and presence of complex N sources (Middelhoven & Franzen, 1986). Aerobic growth of yeasts in a silo can be explained by an increase in Y_{ATP} from respiratory catabolism which greatly facilitates survival and growth of yeasts.

Similar strategies of increasing Y_{ATP} can also be found in microorganisms classified as strictly fermentative. Under the presence of O_2, nitrate or even available sugars acting as EEAs, additional ATP can be obtained from the acetyl phosphate-to-acetate pathway, referred to as 'externally balanced fermentation' in the thesis. This pathway theoretically results in one extra mole of ATP per mole of glucose (Rooke & Hatfield, 2003). Heterofermentative LAB therefore tend to produce mostly acetic acid instead of ethanol under these conditions (Lucey & Condon, 1986; McDonald *et al*., 1991; Weissbach, 1996). When fructose acts as an EEA, it is converted to mannitol (McDonald *et al.*, 1991) which can further be catabolized to lactic acid in the presence of a number of electron acceptors including acetic or citric acid (McFeeters & Chen, 1986). When serving as an EEA, acetic acid is reduced to ethanol (McFeeters & Chen, 1986). *Clostridium* spp. can also improve Y_{ATP} by forming acetic acid instead of butyric acid when nitrate is present (Spoelstra, 1985).

Different possible strategies for obtaining energy are summarized in Figure 1. Aerobic respiration and internally balanced fermentation result in the highest and lowest Y_{ATP}, respectively. Microorganism capability and availibility of EEAs are factors determining the possible pathways. There are also some evidence that certain species of LAB can further improve their internally balanced fermentation. For example, *Streptococcus lactis* improves Y_{ATP} by switching from homolactic fermentation to forming acetic acid, ethanol and formic acid when glucose becomes scarce (Thomas *et al.*, 1979). One extra molecule of ATP is obtained from the acetyl phosphate-to-acetate pathway and the ethanol production results in formation of two NAD^+ molecules and a redox balance (Thomas *et al.*, 1979; McDonald *et al.*, 1991).

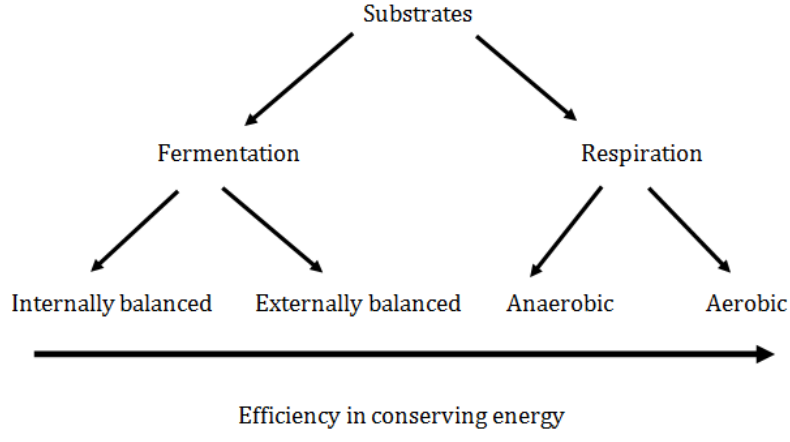

Figure 1. An overview of possible catabolic pathways employed by silage microorganisms.

The strategy of increasing Y_{ATP} can result in oxidative stress in many species of LAB. Due to the absence of catalase and superoxide dismutase in these species, O_2 is not completely reduced to H_2O, resulting in accumulation of superoxide and hydrogen peroxide (Pahlow, 1991; Sanders *et al.*, 1999; Madigan *et al.*, 2012). This condition is usually manifested in lower numbers of cultureable LAB on standing, compared to machine harvested, forages (Pahlow, 1991). In the so called 'chopper inoculation', viable counts of LAB are increased 100 folds or more after the release of catalase, superoxide dismutase and Mn^{2+} from ruptured plant cells (Daeschel *et al.*, 1987; Pahlow, 1991). It is worthwhile investigating how LAB incorporate these external compounds and use them in defence against oxidative stress.

1.2.3 Metabolic losses

Even under good ensiling practice (i.e. fast silo filling, proper compaction and coverage), good fermentation and high outtake rates, losses of DM can reach 12% (Köhler *et al*., 2013). Major parts of these losses are due to aerobic metabolism, even though some contribution from anaerobic metabolism may also occur. Under complete airtightness in laboratory silos and over a period of 99 days of storage, DM losses of ~4% in silages (n=189) with an average DM content of 33% and a pH of 4 were observed (own unpublished data). The presence of acid tolerant species of LAB, yeast and/or a slow rate of pH reduction probably decides the magnitude of anaerobic metabolic losses.

It is often argued that losses of energy during fermentation are negligible and there can even be cases with increased energy concentration in silage from ethanol production (McDonald *et al*., 1973; Kung *et al*., 2003). It should be noted, however, that this refers to the gross energy content of ensiled materials. Under extensive silage fermentation, utilization of energy by the animal can become inefficient or even impaired. For instance, VFAs have minimal nutritional value for rumen microbes (Huhtanen *et al*., 2003; Wilkinson & Davies, 2013) and high levels of organic acids and ammonia can reduce rumen microbial protein synthesis (Huhtanen *et al*., 2003). High levels of ethanol can also cause metabolic stress to the animal (Randby *et al*., 1999).

1.2.4 Modelling

Prediction of ensiling results from forage chemical and microbial composition has been of a great interest in silage research. It allows correct treatments of forages prior to ensiling (e.g. wilting or additive application or both) based on the predicted ensiling outcomes. Prediction models can also be used for research or educational purposes.

One of the first attempts to predict silage end-product composition was made by Wilkinson *et al*. (1983). The regression of end-products on DM and ratio of water soluble carbohydrate (WSC):buffering capacity (BC) was constructed. This work was inspired by an earlier work (Weissbach *et al*., 1974) where minimum levels of DM were estimated by the WSC:BC ratio to achieve successful fermentation of biomass.

A dynamic model was proposed by Neal & Thornley (1983) to simulate interactions between LAB and clostridia under anaerobic ensiling conditions to predict silage pH, butyric and lactic acid concentrations. Pitt *et al*. (1985) modelled microbial and biochemical processes in lactate silages during the course of ensiling. The model included growth and death of LAB, pH dynamics and proteolysis. Other examples of dynamic models include effects

of homofermentative inoculants (Pitt & Leibensperger, 1987) and aerobic deterioration of silage at feed-out (Pitt & Muck, 1993).

1.2.5 Research challenges

One likely reason for the variability of ensiling results could be the variation in field floral composition. Field flora is defined as the flora entering a silo in the form of epiphytic plus soil microorganisms. It is difficult to test hypotheses regarding field floral variation mainly due to technical difficulties in obtaining sterile forage materials for experimental inoculations. Sterilization methods investigated include gamma-irradiation (Heron *et al*., 1986) and growing forage aseptically (Playne *et al*., 1967). There has also been an attempt to simulate chemical and physical properties of forages by use of artificial substrates (Woolford & Wilkins, 1975). Gamma-irradiation is expensive and often impractical and growing forages aseptically results in alterations of normal chemical composition. Use of artificial substrates is definitely a more convenient method, but it has not yet been validated. The possibility to separate the effects of substrate and microbial composition on silage quality is definitely of great interest and should, for instance, also enable a more effective evaluation of inoculants.

Silage microbiology has always been a cornerstone of silage research. Most of our knowledge has been derived from culture dependent techniques where forage microfloras are grown on various media. The assumption made during sampling is that the floral extract is representative of the microbial population. But, to what extent this assumption is valid is yet unknown. A further drawback of culture dependent techniques is that they often provide a limited spectrum of the microbial population as culturing conditions of most species are still unknown (Nishino, 2015). The rapid development of culture independent techniques has provided a great opportunity to broaden the current knowledge but these methods also have problems. They are expensive and need further adaptation for silage research purposes before reliable conclusions can be drawn. It seems that a combination of culture dependent and independent techniques is currently the best available option.

2 Objectives

The overall objective of this thesis is to obtain a deeper understanding of the relationship between forage composition and silage quality. The specific objectives include:

- Constructing prediction models of fermentation end-products from forage composition (**Paper I**)

- Developing a methodology that enables sterilization of forage and inoculation of the sterile forage with microfloras from other sources (**Paper II-IV**)

- Testing the feasibility of the new methodology for separating the effects of forage source and field flora on silage quality (**Paper IV**)

3 Materials and methods

The specific objectives were addressed in four experiments carried out at the Department of Animal Nutrition and Management of the Swedish University of Agricultural Sciences (SLU) in Uppsala. Most forage samples were obtained from fields within or nearby Uppsala. Chemical and microbial analyses were done at the laboratory unit of the same department. Detailed descriptions of the materials and methods used can be found in **Papers I-IV**.

3.1 Prediction models of end-product formation

In **Paper I**, a meta-analysis was conducted on data collected from laboratory silage trials (1.5 to 25 L silos) with no additive application and no air stress. The trials had been carried out between 1994 and 2011 at the Department of Animal Nutrition and Management, SLU. A total number of 118 observations from 66 whole-crop maize and 52 perennial forage samples were included in a database. The database comprised information on forage DM, WSC, BC (only for perennial forages), crude protein (CP), neutral detergent fibre (NDF) (only for maize), ash (only for maize) and starch (only for maize) and silage fermentation end-products including lactic acid, VFAs, alcohols and ammonia. The DM ranges of perennial forage and maize samples were 131-623 and 200-406 g/kg, respectively.

3.2 Methodology development

In **Paper II**, several sterilization techniques were tested on grass samples to identify a potential method for further evaluation. The sterilizing agents tested included ethanol (EtOH), combined effects of EtOH and sodium hypochlorite (NaClO), neutral detergent (ND) buffer, cold shock, dry heat and moist heat. For the chemical agents, a ~200-g sample was formed into a bundle and dipped

into the agent. This was followed by dipping in water and sample aeration (15-20 min). For the cold shock treatment, sample was frozen at -80°C either directly or after freezing first at -20°C and then -60°C. Sample subjected to heating was first wilted to ~400 g/kg DM content, freeze-dried (>900 g/kg DM) and ground in a hammer mill. The dry heat treatment included heating at 121°C for 20 min in a force-drought oven and the moist heat treatment was autoclaving at 121°C for 20 min under 320 kPa air pressure. After heating, sample was reconstituted to the wilted DM content by addition of sterile distilled water. Sterilization efficacy of all tested methods was assessed by culture dependent microbial assays for LAB, enterobacteria, yeasts and moulds. Both moist and dry heat were found effective sterilizing agents but after chemical analysis of the heated samples, the dry heat treatment was chosen as the preferred method based on changes in WSC, buffer soluble N (BSN) and acid detergent insoluble N (ADIN) concentrations (Figure 4, below).

In **Paper III**, the dry heat treatment was further developed as enterobacteria had shown some resistance (see Table 1 in **Paper II**) and the technique had also not been tested against spore forming species. After a series of preliminary trials on grass samples, dry heating at 60°C for 3 h + 103°C for 15 h reduced LAB, enterobacteria, yeasts, moulds and spores of clostridia below detection limits (see Table 1 in **Paper III**).

A further aim in **Paper III** was to assess ensileability of sterile materials in comparison with untreated controls. An amount of 1.5 kg of grass, grass-clover mixture, white clover and maize was inoculated with spores of *Clostridium tyrobutyricum* at 10^3 cfu/g fresh matter and divided into two parts. From each replicate, ~75 g was ensiled in 100 mL glass tubes and 50 g was used for inoculum preparation. The rest was dried and ground before sterilizing 35 g of the material as described above. Inoculum (15 mL) was prepared by pummelling samples in a 0.25-strength Ringer solution (300 mL) fortified with Tween 80 (0.5 mL/L), centrifugation (15500 g for 40 min) of the microbial suspensions, filtration (0.45 and 0.22 µm porosity) of the supernatants and homogenization of the microbial pellets and filters. The sterile samples were inoculated and reconstituted with water so as to simulate the control forages with respect to DM concentration and theoretical ratio of DM to microbes. An amount of ~37 g of the reconstituted forages was then ensiled in 50 mL Falcon tubes (Figure 2). Both the control and reconstituted silages were stored at room temperature for 60-63 d.

In **Paper IV**, the methodology evaluated in **Paper III** was further fine-tuned. Modifications done to enhance the practicality included: (i) at inoculum preparation, the length of centrifugation was increased to 90 min and filtration

step was abandoned and (ii) samples were reconstituted to a higher ratio of DM to microbes compared to parent forages.

Figure 2. An example of a control (left) and reconstituted (right) silage in **Paper III**.

3.3 Influence of forage source and field flora

Single source samples of grass, red clover and maize were used in **Paper IV**. An amount of 150 g sample was used to prepare a 15-mL inoculum as described above. Each inoculum was divided in three and 5-mL inoculum was added to 27 g of sterile DM. According to calculation (see **Paper IV** for more details), the 5-mL inoculum should have been added to ~10 g of DM in order to maintain the theoretical ratio of DM to microbes as in parent forages. Each sterile sample received each of the three types of inoculum. Samples were reconstituted to a DM level of 400 g/kg and ensiled in 100 mL glass tubes for 71 d. After unsealing, 30 g of silage was aerated at 20°C for 8 d (Figure 3). The temperature was recorded at 2 h intervals and pH before and after aeration was measured. The aeration vessel comprised of a glass filter crucible insulated with foam polyethylene pipe insulation and aluminium foil. Silage end-product composition and bacterial community of forage and silage were also analysed.

Figure 3. Aeration vessel and online temperature measurement in **Paper IV**.

3.4 Chemical analyses

Samples were dried at 60°C in a forced-drought oven overnight and milled in a hammer mill to pass a 1-mm screen. Sample DM was estimated by drying at 103°C overnight. A correction was made for silage volatiles according to our in-house correction: 1.577 + 0.992 × DM (%). In **Paper I**, starch and WSC concentration was measured by an enzymatic method and in **Papers II** and **III**, WSC was estimated by Fourier transform mid-infrared transmission (FTIR) spectroscopy after hot water extraction (Udén, 2010). The Kjeldahl method with Cu as a catalyst was used to measure N concentration. Contents of BSN were measured according to a 60-min extraction and centrifugation-passive filtration procedure of Udén & Eriksson (2012). The method described by Van Soest (1973) was used to determine ADIN and BC was estimated as described by McDonald & Henderson (1962). In **Paper I**, NDF was measured according to Chai & Udén (1998). Short chain fatty acids and alcohols in silage extracts were measured by High Performance Liquid Chromatography (Ericson & André, 2010). The method of Broderick & Kang (1980) or the Flow Injection technique (ASN 50-01/92) was used for ammonia-N quantification.

3.5 Microbial analyses

In **Papers I-III**, culture dependent assays (Seale *et al*., 1986; Jonsson, 1990) were used to enumerate LAB on Rogosa agar, enterobacteria on crystal-violet neutral-red bile glucose agar, fungi on malt extract agar and clostridia on reinforced clostridial agar.

In **Paper IV**, bacterial community was analysed by the amplification of the V4 region of the *16S rRNA* gene. In short, samples were freeze-dried, ground and incubated in 30 g/L CTAB buffer (cetyltrimetylammoniumbromid, 2 mM EDTA, 150 mM Tris-HCl, 2.6 M NaCl, pH 8) fortified with polyvinylpyrrolidone at 20 g/L. The extracted DNA in suspensions was purified with chloroform, precipitated with cold 2-propanol, washed with cold EtOH, dried at room temperature overnight and re-suspended in MilliQ water. Amplicon library was prepared according to Hugerth *et al*. (2014). The 515´F (Hugerth *et al*., 2014) and 805´R (Herlemann *et al*., 2011) primers equipped with linker sequences were used for polymerase chain reactions (PCRs) and the thermocycling program was: an initial denaturation at 95°C for 5 min; 35 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec) and elongation (72°C for 1 min); a final elongation at 72°C for 5 min. In a second PCR, the linker region of the PCR products was targeted using primers equipped with sample specific barcodes and Illumina adaptors (see Hugerth *et al*., 2014 for primer description). The thermocycling protocol was: an initial denaturation at 95°C for 5 min; 9 cycles of denaturation (95°C for 30 sec), annealing (62°C for 30 sec) and elongation (72°C for 1 min); a final elongation at 72°C for 5 min. Amplicons were sequenced on an Illumina MiSeq sequencer (Illumina Inc., San Diego, CA, USA) at the Science for Life Laboratory, Stockholm, Sweden.

3.6 Statistical and bioinformatic analyses

In **Paper I,** stepwise multiple regression models of end-products on forage variables were constructed using the REG procedure of SAS (v. 9.2; SAS Institute Inc., Cary, NC, USA). The model selection criterion was chosen at $P < 0.15$. The CORR procedure of SAS was also used to study correlation between variables. In **Paper II,** the General Linear Model (GLM) and the Tukey method of Minitab 16 (Minitab Inc., State College, PA, USA) was used to assess the effects of treatments on microbial and chemical variables. In **Paper III,** the MIXED procedure of SAS was used to test the overall effects of the sterilization procedure on forage microbial and chemical variables, with forage type as a random factor. Likewise, fermentation quality and microbial profiles of control and reconstituted silages were compared. In **Paper IV**, the

effect of inoculum, forage and their interaction on response variables was assessed by the GLM and Tukey method of Minitab 16. Significant level was declared at $P < 0.05$ for main effects and at $P < 0.10$ for interactions. When observations in microbial and chemical assays were below detection limits, values equal to half the detection limits were used. Values are reported as the least square means ± SEM.

Bioinformatic analysis was done in the Quantitative Insights into Microbial Ecology (QIIME, 1.7.0/1.8.0) pipeline (Caporaso *et al*., 2010). High quality paired-end reads were assembled and operational taxonomic units (OTUs) were assigned by the open-reference method against the Greengenes core set (gg_13_8). Chimeric sequences were detected and filtered out. The Ribosomal Database Project classifier was used to assign taxonomy to OTUs and the number of reads was equalized among libraries. In **Paper IV**, distribution of bacterial species across forage and silage samples was assessed by the Principal Component Analysis (PCA) of PAST (Hammer *et al*., 2001).

4 Main results

4.1 Prediction models of end-product formation

Formation of end-products during ensiling was negatively related to the DM content of perennial forages in **Paper I** (Table 1). More than 74% of the captured variation in concentration of ammonia and VFAs was explained by the DM content. Water soluble carbohydrate level was the best predictor of ethanol formation and the second best predictor of ammonia and butyric acid production. The best prediction model was for acetic acid where DM, LAB and CP explaining 63% of the variation (Table 1). The residuals of lactic acid regression severely deviated from normal distribution and log transformation did not alleviate the problem and hence, the model is not reported.

Contrary to the perennial crop silages, DM was not a powerful predictor of end-product formation in maize silages and was only included in the models of ethanol and 2,3-butanediol with marginal contributions to total explained variation. The CP concentration, on the other hand, appeared as a good predictor and was included in the models of lactic acid, acetic acid, propionic acid and the two alcohols. Interestingly, formation of ammonia was only related to the starch concentration. The model for lactic acid had the greatest explanatory power (R^2=0.84) and included CP, ash, NDF, LAB and WSC as predictors (Table 1).

Table 1. ***Paper I****: regression of fermentation end-products on forage variables. Variables are expressed in g/kg dry matter unless otherwise stated*.

	Cumulative R^2	Regression equation
Perennial crop silages		
Ammonia-N	0.43	$295.68 - 0.45 \times DM\ (0.34) - 0.42 \times WSC\ (0.9)$
Acetic acid	0.63	$8.52 - 0.10 \times DM\ (0.47) + 4.89 \times LAB (0.11) + 0.13 \times CP (0.05)$
Butyric acid	0.48	$77.92 - 0.15 \times DM\ (0.41) - 0.12 \times WSC\ (0.07)$
Propionic acid	0.30	$1.28 - 0.003 \times DM$
Ethanol	0.46	$1.09 + 0.003 \times WSC\ (0.29) - 0.001 \times DM (0.17)$
2,3-Butanediol	0.29	$2.24 - 0.003 \times DM\ (0.14) - 0.27 \times LAB\ (0.15)$
Maize silages		
Ammonia-N	0.43	$60.43 + 0.12 \times starch$
Lactic acid	0.84	$-87.41 + 1.15 \times CP\ (0.67) + 2.18 \times ash\ (0.11) - 0.20 \times NDF (0.03) + 4.99 \times LAB\ (0.02) + 0.06 \times WSC (0.01)$
Acetic acid	0.45	$-0.42 + 0.02 \times CP$
Butyric acid	0.37	$-3.31 + 0.004 \times starch\ (0.25) + 0.04 \times ash\ (0.12)$
Propionic acid	0.44	$-3.29 + 0.004 \times starch\ (0.31) + 0.02 \times CP\ (0.13)$
Ethanol	0.61	$-2.49 + 0.03 \times CP\ (0.52) + 0.003 \times DM (0.09)$
2,3-Butanediol	0.47	$4.35 - 0.01 \times WSC\ (0.34) - 0.02 \times CP (0.08) - 0.004 \times DM (0.05)$

Values in the parentheses are proportions of the variables to cumulative R^2.
Ammonia-N (g/kg total N).
DM=dry matter (g/kg); WSC=water soluble carbohydrates; LAB=lactic acid bacteria (log cfu/g fresh matter); CP=crude protein; NDF=neutral detergent fibre.

4.2 Methodology development

The EtOH treatment reduced, in most cases, counts of LAB below the detection limit but had no effect on enterobacteria or only minor effects on fungal populations (see Table 1 in **Paper II**). Moulds were particularly susceptible to the EtOH-NaClO treatment (see Table 1 in **Paper II**). Cold shock or mechanical removal of microbes by ND solution had only limited antimicrobial effects (see Table 1 in **Paper II**).

Chemical analyses of the sample subjected to heat treatments in **Paper II** are presented in Figure 4. Moist heat resulted in a 5-fold increase in ADIN concentration and a 56% reduction in BSN concentration whereas, dry heat only reduced the BSN concentration, i.e. for 22%.

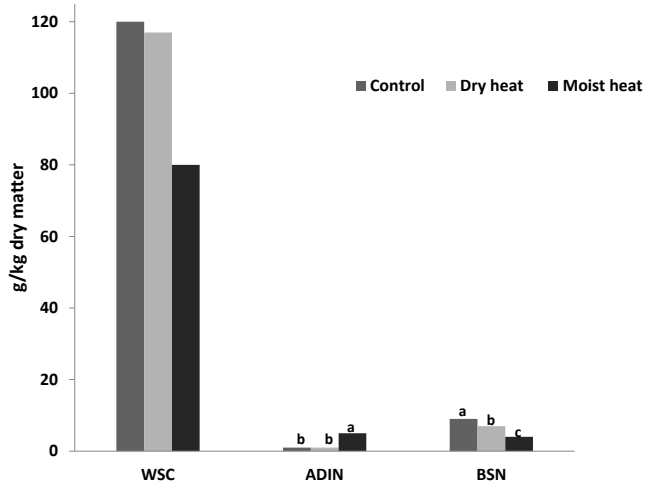

Figure 4. **Paper II:** effect of dry (121°C, 20 min) and moist (121°C, 320 kPa, 20 min) heat on water soluble carbohydrates (WSC), acid detergent insoluble N (ADIN) and buffer soluble N (BSN) of a grass sample. Comparisons with different labels differed at $P < 0.05$.

The final sterilization procedure evaluated in **Paper III** (i.e. dry heating at 60°C for 3 h + 103°C for 15 h) reduced WSC and BSN concentrations by 49% and 22%, respectively and increased ADIN content by 53% (Figure 5). In spite of this, fermentation end-products of the reconstituted silages and control silages were comparable. Some differences were however found in the concentrations of lactic acid, ethanol and ammonia-N (Figure 6). The two silage treatments had similar counts for LAB, enterobacteria, yeasts, moulds and clostridia (see Table 4 in **Paper III**).

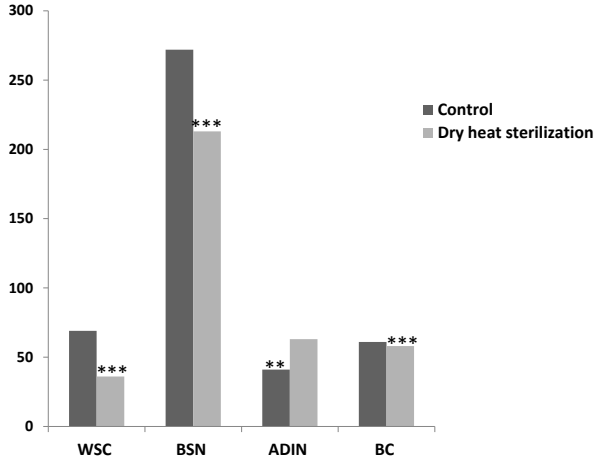

Figure 5. **Paper III:** effect of dry heat sterilization (60°C for 3 h + 103°C for 15 h) on water soluble carbohydrates (WSC; g/kg dry matter), buffer soluble N (BSN; g/kg total N), acid detergent insoluble N (ADIN; g/kg total N) and buffering capacity (BC; g lactic acid/kg dry matter) of grass, grass-clover mixture, white clover and maize. $^{**}P < 0.01$; $^{***}P < 0.001$

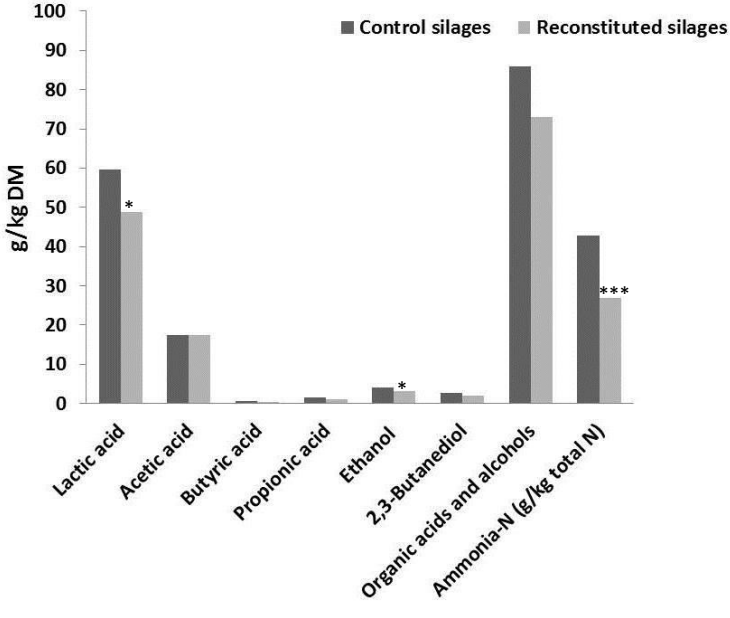

Figure 6. **Paper III:** fermentation end-product composition of reconstituted silages. Dried samples were sterilized (60°C for 3 h + 103°C for 15 h), reconstituted to original dry matter (DM) content, inoculated with parent microfloras and ensiled. $^{*}P < 0.05$; $^{***}P < 0.001$

4.3 Influence of forage source and field flora

All fermentation variables, except ethanol, were affected by forage source, while only pH and acetic acid were affected by inoculum source (see Table 1 in **Paper IV**). Silages made from the maize sample contained the lowest levels of lactic and acetic acids but pH in these silages was also the lowest. On the other hand, silages made from the clover sample contained the highest levels of lactic and acetic acids but these silages also had the highest pH. Inoculum from the grass or maize source resulted in a greater formation of acetic acid than the inoculum from the clover source. The maize inoculum also resulted in the lowest silage pH. An effect of forage × inoculum was observed on acetic acid formation, with the clover sample and inoculum derived from the maize or grass sample containing the highest level.

As far as aerobic stability concerns (see Table 1 in **Paper IV**), silages made from the clover and maize samples had, respectively, the best and worst performances, whereas the maize inoculum resulted in the best aerobic stability profile. The grass × grass, maize × grass and maize × clover silage combinations (i.e. forage source × inoculum source) were aerobically unstable.

Each sample library contained 5740 sequences from 846 OTUs. Less than 1% of the OTUs was assigned as ‘no match’. The relative abundance of bacteria (at family level) on the forage samples is shown in Figure 7. The figure suggests that the three inoculum sources differed in relative abundance of some bacterial families. For instance, abundance of *Enterobacteriaceae* was higher on the maize sample than on the grass or clover sample. On the other hand, *Sphingomonadaceae* was more abundant on the grass or clover sample than on the maize sample. The maize sample had a higher relative abundance of LAB than the grass or clover sample (see pattern bars in Figure 7) but generally, a small proportion of the reads belonged to LAB. A relatively large within replicate variation was found for the clover sample.

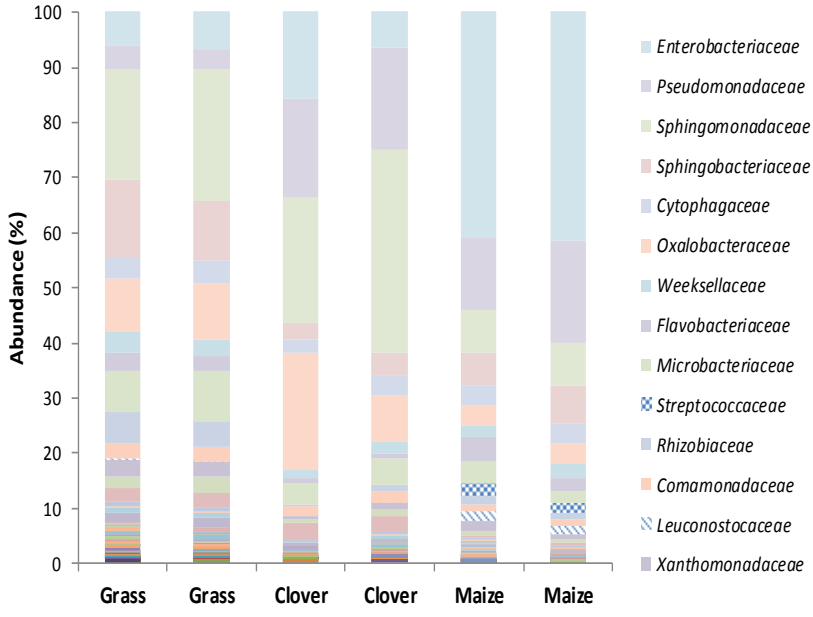

Figure 7. **Paper IV**: relative abundance of bacteria, at family level, on replicate forage samples. Only the top 14 families are shown here.

The first two components of PCA captured 71% of the variation in distribution of bacterial species across silages (see Figure 3 in **Paper IV**). Species from *Lactobacillaceae* family dominated the silage samples. Silages inoculated with the maize inoculum tended to form a cluster. Silages inoculated with the grass inoculum had a higher relative abundance of a species from the *Pediococcus* genus, with two out of three combinations (i.e. grass × grass, maize × grass) becoming unstable.

5 General discussion

5.1 Prediction models of end-product formation

Under controlled ensiling conditions, it is generally agreed that WSC, DM and BC mainly determine silage end-product composition (Weissbach *et al*., 1974; Wilkinson *et al*., 1983), partly evidenced from regressions of end-products in perennial crop silages (Table 1). Dry matter content was included in all the models and WSC had contributions in half of the constructed models. The absence of BC in regressions was probably due to a large number of missing value for this variable in the database.

In maize samples, CP content was positively correlated with LAB numbers ($r = 0.54$, $P < 0.001$). This could explain why CP had a positive weight as well as a great explanatory power in regressions of lactic acid, acetic acid and ethanol (Table 1). There was a positive association between starch and formation of ammonia in maize silages. At higher starch contents, in which a_w and WSC are limiting factors for fermentation (starch vs. DM: $r = 0.81$, $P < 0.0001$; starch vs. WSC: $r = -0.77$, $P < 0.0001$), silage pH did not prevent enterobacteria from growing. In silage, both clostridia and enterobacteria play roles in ammonia formation but clostridia are only active at low silage DM levels.

The overall poor prediction models obtained in **Paper I** suggest that conventional chemical variables do not seem to be sufficient to predict fermentation end-product composition. It is possible that information on forage a_w, pectin, organic acids, etc. are needed for quantitative modelling. It is also likely that a simple model, without consideration of microbial interactions and regulatory mechanisms of silage ecosystem, may also not be able to predict end-product formation.

5.2 Methodology development

In silage studies, the most common *in vitro* technique includes ensiling small quantities of forages in mini-silos. However, this technique alone does not provide the means to study interaction between forage species and field flora on silage quality. In **Papers II-IV**, efforts were therefor made to develop a methodology for such purposes.

The results from sterilization treatments in **Paper II,** which included treatments with chemical agents (e.g. EtOH, NaClO), heat and cold, proved again that microbes are able to withstand very harsh conditions. Enterobacteria and LAB appeared as the most and the least resilient groups, respectively. Lactic acid bacteria are known to have a limited biosynthetic ability and to be heavily dependent on the supply of minerals, amino acids and vitamins (McDonald *et al*., 1991; Pahlow *et al*., 2003; Madigan *et al*., 2012). On the other hand, members of enterobacteria are diverse and not as fastidious as other bacterial groups including LAB (McDonald *et al*., 1991; Pahlow *et al*., 2003). Whether these factors had anything to do with the response of LAB and enterobacteria to sterilization treatments in **Paper II** is yet to be elucidated.

Heat has long been known as the most effective sterilization agent for non-heat sensitive objects. For heat sensitive objects like plant materials, application of heat can result in chemical alterations, as for instance through Maillard reactions. The ADIN fraction is commonly used as an indicator of products from Maillard reactions. The presence of water is essential for the formation of Maillard products and samples were therefore gently dried prior to heating in **Papers II-IV**. The initial grinding of the dried samples was to facilitate handling of dried materials and to remove the need for grinding small quantities prior to chemical analysis. In **Papers III-IV**, the additional drying at 60°C for 3 h prior to heating at 103°C was to remove any moisture absorbed during storage.

A 22-unit (g/kg N) increase in ADIN in heat sterilized samples (Figure 5) only accounts for 37% of the 59-unit (g/kg N) reduction in BSN content. If it is assumed that only one mole of N reacts with one mole of hexose sugar in Maillard reactions, the increase in ADIN after sterilization accounts for 26% of the 33-unit (g/kg DM) reduction in WSC (Figure 5). Caramelization of sugars and denaturation of protein were therefore probably the main causes of WSC and BSN loss. A possible way to reconstitute a heat sterilized forage could be the addition of sugars and soluble protein. Nevertheless, the similar fermentation quality (Figure 6) and microbial profiles of the two silage treatments suggest that the methodology evaluated in **Paper III** can be useful to study relative impacts of field flora or forage source on ensiling results.

During sample reconstitution in **Paper III**, maintenance of the theoretical ratio of DM to microbes as in control forages was believed to be essential for a similar fermentation result. However, this may not be necessary and inoculation at lower levels could still give similar end-results after an initial lag phase. Therefore, in **Paper IV**, forages were reconstituted to a somewhat lower inoculation level for practical reasons. The removal of the filtration step during inoculum preparation in **Paper IV** was justified for reasons that preliminary observations showing that ≥90% of a microbial population in a microbial suspension is recovered by a 90-min centrifugation at 15500 *g* (data not shown).

To the knowledge of the author, this is the first attempt to separate the effects of forage source and field flora on silage quality. Further work is still needed to improve the methodology. The extent of heat damage can perhaps be mitigated through evaluating different heating regimes for different forage types. Although the sterilization method introduced in this thesis provides a simple and inexpensive tool, new techniques such as non-thermal plasma, commonly used to sterilize heat sensitive objects, can also be of interest.

5.3 Influence of forage source and field flora

Despite the fact that the chemical composition of forage samples was not measured in **Paper IV**, it is reasonable to consider them having contrasting chemical composition, supported by the effect of forage source on end-product formation and silage aerobic stability (see Table 1 in **Paper IV**). The poor explanatory power of chemical variables on end-product formation in **Paper I** (Table 1), as opposed to significant effects of forage source on end-products in **Paper IV**, therefore, supports the idea that the presently used chemical variables are insufficient in explaining end-product formation.

The higher abundance of LAB on the maize sample in **Paper IV** (Figure 7) is in agreement with previous observations (Andrieu & Gouet, 1991; Lin *et al.*, 1992; **Paper I**) and suggests that growth of LAB is probably better supported on maize, compared to other silage crops. A similar difference may also exist for other epiphytic microbial groups (e.g. enterobacteria and fungi) among silage crops, which is worthwhile of further investigations. For the fully matured clover sample, the relatively large within replicate variation in relative abundance of bacterial population (Figure 7) was most likely due to non-uniformity of replicates. The positive effect of the maize inoculum on silage pH and acetic acid concentration might have been related to a higher abundance of LAB and/or a different composition of LAB species.

Fungal community was not analysed in **Paper IV.** This could possibly have provided more solid evidence as to why some treatments became aerobically unstable or why maize inoculum improved stability. Currently, 454-sequening of the internal transcribed spacer (ITS) regions of ribosomal genes provides the highest data resolution for fungal community analysis (Ihrmark *et al*., 2012). However, this technique is expensive compared to other Next Generation Sequencing techniques (e.g. Illumina and Ion Torrent).

6 Conclusions

The general conclusion of this thesis is that relationship between forage composition and silage quality is complex. More elaborate analyses of forage chemical composition and field flora are needed for a better understanding of what drives the ensiling process. The specific conclusions are:

- In **Paper I**, data on presently used chemical variables (e.g. DM, WSC and CP) and LAB explained more than 60% of the variation in lactic acid and ethanol formation in maize silages and acetic acid formation in perennial crop silages. Prediction models of end-product formation were generally weak.

- The final sterilization method (heating at 60°C for 3 h + 103°C for 15 h) evaluated in **Paper III** resulted in a 49% reduction in WSC, a 22% reduction in BSN and a 53% increase in ADIN. However, the reconstituted forages, i.e. sterilized and inoculated with parent microflora, produced silages with comparable fermentation composition and microbial profile to untreated forages.

- In **Paper IV**, formation of end-products were more influenced by forage source than by field flora, whereas, silage aerobic stability was affected by both factors.

- The results from **Paper III** and **IV** indicate that the new *in vitro* methodology with sterilization of forages and inoculation with selected floras can be useful to study the relative effects of field flora or forage species on silage quality.

7 Future perspectives

Silage nutritive and hygienic quality play important roles in dairy production. Further improvements in silage technology are however needed to assure production of high quality silages. The main obstacle in silage research is, like in many other disciplines, lack of suitable analytical tools. This includes analytical tools in silage microbiology and biochemistry.

Silage microbiology should benefit from new methodological developments and emerging molecular methods. Amplicon sequencing, in which molecular markers are amplified by PCR, is currently the most widely used method in studies of microbial community analysis. An interesting alternative can be PCR-independent methods, based on the sequencing of whole genomic or transcriptomic materials of a sample, particularly in the light that information biases introduced by PCR are eliminated.

Ecology of silage microorganism is an area with the least research attempts. Deeper insights into the ecology of silage microorganisms can help us to better understand how silage ecosystems function, resulting in more effective additives or perhaps even new generations of silage additives.

Although the methodology introduced and the results obtained in this thesis are preliminary, the current version of the methodology still allows studying the effects of variation in field flora on silage quality. It can also allow evaluation of chemical or microbial additives against different field floras. An updated methodology particularly with respect to the sterilization procedure should also enable hypothesis testing as to the effects of variation in forage chemical composition, e.g. by season, maturity, climate, location, etc., on silage quality.

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