METHODS TO CHARACTERIZE MICROORGANISMS OF INTEREST PRESENT IN SILAGES

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INTRODUCTION

Silage is a major component of the diet fed to ruminants and the ensiling process relies on the anaerobic conversion of fermentable water-soluble carbohydrates to organic acids in forage crops with adequate moisture (McDonald et al., 1991). Lactic acid bacteria (LAB) usually dominate the ensilage and produce mainly lactic acid, though fermentation is also dependent on a variety of other epiphytic microbes (often detrimental); for instance, forage dry-matter content, water-soluble carbohydrates concentration, hybrid selection or stage of maturity (Bolsen et al., 1992). Additionally, enterobacteria, clostridia, yeasts and molds can be involved depending on the conditions at the time of ensiling. Hence, the use of microbial inoculants to suppress these microbes and ensure good silage quality is a recommended practice.

Traditional inoculants, which normally contain species of classical homofermentative LAB, often claim to promote a faster fermentation, shifting the end-products towards lactic acid and away from ethanol and acetic acid, thus preserving nutrients by inhibiting plant enzymes and detrimental microorganisms. In contrast, other bacteria are specifically marketed as enhancers of aerobic stability such as *Lactobacillus buchneri* and species of Propionibacteria, primarily by the production of acetic and propionic acids, respectively (Kung et al., 2003).

For a long time, the populations of these microorganisms that dictate the ensilage were enumerated by classical plating, where the microorganisms are grown on a medium that supports its growth. However, one important shortcoming of plating is that the cultivable fraction of most microbial ecosystems is still a minority.

Microscopic counts were done as means of comparison to the total viable counts done by plating, and the higher total microscopic counts were believed to be caused by the number of dead cells (Apatalahti et al., 2003). Nonetheless, molecular techniques

indicated that the majority of the bacteria could not be cultured. Unknown nutrient and/or growth factor requirements, dormant or latent organisms, the selectivity of the media that are used, the stress due to the cultivation procedures, the need of anaerobic conditions, and inability to reproduce the interactions with other microorganisms and host cells are some explanations for the unsuccessful cultivation (Zoetendal et al., 2004).

Therefore, in order to answer the most essential questions for environmental microbiology – "Who are there? What are they doing?" – It is essential to precisely investigate the microbial groups present in that environment and their roles.

The development of culture-independent techniques based on the analysis of nucleic acids has been increasing in popularity. The 16S rDNA encoding gene is a highly conservative region that can be amplified with universal primers and compared with a reference-sequence database to evaluate phylogenetic relationships. Since the bases of this conserved region are not neighboring each other, there is flexibility to design a universal or specific prime pair (Baker et al., 2003). Molecular protocols to identify, quantify and access the microbial population in silages such as denaturing gradient gel electrophoresis, terminal restriction fragment analysis, 16S rDNA cloning and sequencing are replacing phenotypic methods (Figure 1).

This short review aimed to provide a summary of the most common techniques to identify and compare the composition and structure of microorganisms in silages.

THE USE OF TRADITIONAL METHODS TO MONITOR MICROBIAL POPULATIONS IN SILAGES

Classical or traditional methods used for identifying microbial species have been based on microbial culturing, microscopy or phenotypic traits such as substrate utilization, fermentation products and enzyme activity. Nevertheless, these techniques are expensive and time-consuming, and often cannot clearly differentiate among the LAB species (Mackay, 2004).

Dutkiewicz et al. (1989) studied the concentration and species composition of the microflora found in the surface and upper layers of bulk and aerosolized corn silages. The authors used selective media, morphology, Gram reaction, substrate preference tests,

and microscopic, biochemical, and serological methods selected from *Bergey's Manual of Systematic Bacteriology* (Sneath et al., 1986) as tools for differentiation of the microorganisms. *Aspergillus fumigatus* prevailed among the fungi, while *Bacillus* and aerobic gram-negative organisms predominated among bacteria.

Lin et al. (1992b) reported that lactic acid bacteria comprised only a small fraction (< 0.5%) of the epiphytic microbial population in whole-plant corn and alfalfa, measured by traditional plating. The group *Enterobacteriaceae*, enumerated on violet red bile agar, was predominant in both standing crops, and yeasts and molds were also major epiphytic microbial groups but only in whole-plant corn.

Bolsen et al. (1992) evaluated the impact of additives on the microbial succession of alfalfa and corn silages using selective media. Overall counts of LAB were higher for one of the two treatments with microbial inoculants when compared to control; other microorganisms – *Enterobacteriaceae*, yeasts and molds, and clostridial spores – were not affected by treatment for either crop.

Lin et al. (1992a) estimated only the lactic acid bacterial population at species level during the pre-ensiling and ensiling periods of alfalfa and maize. *Lactobacillus plantarum, Pediococcus pentosaceus, Enterococcus faecium and E. faecalis*, enumerated on Rogosa SL medium, were predominant on both standing crops and, shortly after ensiling, the species most involved in the microbial succession were *L. plantarum* and *P. pentosaceus*. After 7 d of storage, *L. brevis, L. gasseri* and *L. homohiochii* became prevalent depending on the crop. In addition to selective media, Gram reaction, morphology, catalase production, soluble protein patterns, deamination of arginine, and tests for lactic acid configuration, optimum growth temperature, gas production from glucose and fermentation of 32 carbohydrates were conducted.

Grazia and Suzzi (1984) harvested LAB colonies from MRS agar plates and used the same phenotypic tests as the previous authors. They reported that lactobacilli were the predominant lactic acid bacteria, with *L. buchneri* being the most frequently recovered bacterium in 13 samples of maize and 10 samples of alfalfa silages. Among the homofermentative LAB, strains of *L. plantarum* and *L. casei* were the most recovered.

Torriani et al. (1992) studied the natural populations of LAB in growing maize and lucerne. The phenotypic characterization indicated *L. plantarum* and *Leuconostoc*

paramesenteroides as dominant species for both forages and *L. buchneri* as a major species in lucerne. The identification of strains was done by analysis of total cell proteins with sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE).

USING MOLECULAR TECHNIQUES TO MONITOR MICROORGANISMS IN SILAGES

The use of phenotypic methods for species-specific identification and quantification of bacteria are labor intensive and time-consuming and, furthermore, there is currently no selective media available to enumerate microorganisms in mixed cultures; therefore, genetic approaches are required. Specifically, ribosomal RNA genes are extensively used to classify bacteria and have already been used to identify and quantify lactic acid bacteria present in silages (Klocke et al., 2006; Schmidt et al., 2008).

Moreover, several methods were developed for the assessment of genetic diversity and, particularly, random amplification of polymorphic DNA (RAPD), terminal restriction length polymorphism (T-RFLP), temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) and length heterogeneity polymerase chain reaction (LH-PCR) have been used to determine the microbial diversity of silages. Each technique has its advantages and limitations and these factors need to be considered when choosing which method is best suited for a particular study (Table 1).

The extraction of the microbial DNA dictates the success of the analysis

The variability in DNA extraction efficiencies and the presence of inhibitory compounds are the major obstacles for successful PCR analysis of environmental samples (Mumy and Findlay, 2004). Inconsistency in the yield and purity of the DNA can significantly affect the result of the microbial community analysis (vonWintzingerode et al., 1997).

Specifically, lactic acid bacteria are Gram-positive rod or cocci, hence the cells are more resistant to lyses than and DNA extraction from Gram-negative bacteria. Furthermore, the isolation and purification of the microbial DNA from silage samples can magnify this challenge.

Classical protocol for extraction of DNA from Gram-positive bacteria relies on lysis of the cells by physical, enzymatic and/or chemical treatments. Lysozyme and mutanolysin function by attacking peptidoglycans, a component of the cell walls of Gram-positive bacteria that is organized as a multilayer network (Kämpfer, 1995). Krsek and Wellington (1999) observed beneficial effects of lysozyme on purity of isolated DNA by acting directly on humic acids. Furthermore, they reported high yields of DNA by treatment with SDS and precipitation with ethanol. DNA isolated using lysozyme and SDS lysis is of higher molecular weight and can further be used for a wide variety of applications, including microbial community and diversity analyses. The drawbacks of this protocol are the labor and the time required.

Commercial DNA extraction kits currently available enable rapid recovery of PCR-ready nucleic acids from a large number of samples, but most researchers prefer to use more traditional methods to obtain higher yields of purer DNA, especially from complex samples (Wilson, 1997). Schmidt et al (2008) compared commercial kits to lysozyme and SDS, aided by overnight incubation with proteinase K and mutanolysin, and purified with phenol and chloroform. Compared to commercial kits, the DNA yield from lysozyme/SDS methodology was the highest and with good purity.

PCR and Real-time quantitative PCR

Polymerase chain reaction is an *in vitro* technique for the enzymatic synthesis of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank that region of interest in the target DNA (Saiki, 1995). Primarily used to produce copies of a specific sequence, PCR is also a powerful and precise way of altering a particular template sequence.

Utilization of PCR technology has proved to be sensitive and specific for rapid detection of lactic acid bacteria (Dubernet et al., 2002) and the 16S rDNA targeted end-point PCR technique has been shown to be a valuable technique to detect the presence of specific bacteria in silages (Romanov et al., 2004). Further down, the quantification of PCR products with the aid of the non-specific double-stranded DNA binding dye SYBR Green I can be accomplished with only minor changes in the protocol from the classic PCR. In addition, the SYBR Green I method allows high resolution of DNA fragments

100-1000 bp in size and has a large linear detection range, which is an essential requirement for quantification (Skeidsvoll and Ueland, 1995).

Real-time quantitative polymerase chain reaction (real-time qPCR) has been applied to monitor the dynamics of the specific LAB populations rapidly in a single process (Deng et al., 2008) but more research needs to be conducted to evaluate specific bacterial species, minimize the variations during preparation and purification of genomic DNA, and determine the influence of different types of silage crops under investigation.

Klocke et al. (2006) monitored the dynamics of selected bacteria (*L. plantarum*) in grass silages for the first time with a real-time qPCR. However, after 20 and 40 d of ensilage large variations were observed between the 16S rDNA copy number calculated by real-time qPCR and the LAB colony-forming units.

In contrast, Stevenson et al. (2006) quantified seven LAB species in alfalfa, treated or not with commercial silage inoculant formulations, and corn stover silages using primers designed based on *recA* gene sequences. At 4 days of storage, *L. brevis* was the most abundant LAB in alfalfa silages while modest populations of *L. plantarum* and *P. pentosaceus* were observed. The authors noted a lack of persistence of the silage inoculant strains, which varied according to the inoculant and the environmental conditions. High epiphytic populations of *L. plantarum* and *L. brevis* were detected in corn stover after 9 months of storage. *Lactococcus lactis* and *L. buchneri* comprised a small but consistent LAB population in all silages.

Schmidt et al. (in press) measured the population of *L. buchneri* in corn silage treated or not with it at the moment of ensiling, with the study replicated in different locations. After 120 days of storage, the specific fermentation profile from silages treated with *L. buchneri* matched the treatments and sites that had higher populations of this organism.

Schmidt et al. (2007) observed a small natural population of *L. buchneri* in alfalfa silage, not able to dominate the fermentation process. When applied at the moment of ensiling, the population of *L. buchneri* increased markedly after 2 and 5 days of ensilage. After 45, 90 and 180 days of storage, *L. buchneri* appeared to be the predominant bacterium in inoculated silages based on the fermentation profile of those silages.

DNA-DNA hybridization and DNA sequencing

DNA-DNA hybridization measures the degree of genetic similarity between complete genomes by measuring the amount of heat required to melt the hydrogen bonds between the base pairs that form the links between the two strands of the double helix of duplex DNA (Theron and Cloete, 2000). Under experimental conditions, "hybrid" double-stranded DNA molecules may be formed from the single strands of the DNAs of two species. The hybrid molecules are then dissociated in order to measure the melting temperature of the hybrid duplex. The experimental conditions are set so that only homologous sequences, i.e. sequences derived from a common ancestor, can form double-stranded structures.

Dellaglio and Torriani (1986) were some of the pioneers to use genetic tests to determine LAB populations in silage. The total 100 LAB strains that were identified in maize silages could be divided in four groups: *L. plantarum, L. casei* and *L. coryniformis*; *L. buchneri, L. brevis, L. fermentum* and *Le. paramesenteroides*; *P. pentosaceus* and *P. acidilactici*; and *E. faecium*, *Streptococcus lactis* and *S. bovis*. The authors found good correlation between the DNA-DNA hybridization and the phenotypic tests done.

Cai et al. (1998) harvested LAB colonies from forage crops and studied their impact on silage fermentation. DNA base composition and DNA-DNA hybridization were determined to identify the strains besides morphological, physiological and biochemical tests. Two epiphytic strains assigned as *Weissella paramesenteroides* and *Le. mesenteroides* and a commercial strain of *L. casei* were applied to alfalfa and Italian ryegrass at the time of the harvest. Only the commercial inoculant improved the silage quality and reduced fermentation loss, since it is a homofermentative LAB. A study with similar methodology and design was conducted by Cai (1999), evaluating *Enterococcus* spp. from forage crops and their effects on alfalfa and guine grass silages but no improvements in silage quality from inoculation were noticed.

Cai et al. (1999) repeated the previous model with *Pediococcus* spp. isolated from forage crops and applied as inoculant to alfalfa and Italian ryegrass. In addition to DNA-DNA hybridization, the authors used 16rRNA sequencing to identify 3 epiphytic strains used for the ensilage trial. Two strains of *P. acidilactici* were more efficient than the strain of *P. pentosaceus* and therefore, more suitable as silage inoculants. Initially,

methods to analyze microbial population relied on DNA sequencing from environmental samples. The rRNA genes can be amplified directly from the total sample DNA, cloned and sequenced. Universal primers can be used to amplify genes from all domains, or specific primers from a particular genus or species.

The automated fluorescent DNA sequencing machine relies on the use of a different colored dye for each of the four DNA bases. The Sanger method is based on the use of dideoxynucleotide triphosphates as DNA chain terminators and results in all fragments ending in one of the four labeled terminators correspondent to the dideoxy bases. The fragments are sorted by electrophoresis and the use of four different dyes permits the sequencing reaction to be performed in a single tube. The obtained sequence is then compared to sequences stored in online databases, which is done using a search algorithm such as FASTA (Temmerman et al., 2004).

Ennahar et al. (2003) sequenced 161 Gram-positive bacteria isolated from paddy rice silage to confirm the results obtained by phenotypic analyses. The molecular data was in agreement with the phenotypic results, except for one species. The homofermentative bacteria totalized 2/3 of total LAB, and the predominant species were *L. plantarum* (24%), *Lc. lactis* (22%), *Le. pseudomesenteroides* (20%), *P. acidilactici* (11%) and *L. brevis* (11%).

Although the majority of published microbial silage research has been done with LAB, Mansfield and Kuldau (2007) studied the mycobiota of fresh and ensiled maize with culturing techniques and a DNA sequence-based approach. Comparing the two methods, the molecular techniques detected a greater number of species than selective plating. Yeast was the majority of the detected fungi and *Fusarium* and *Penicillium* were the dominant mycotoxigenic fungi in silage. The abundance of *Penicillium* in spoiled silages is well-known. O'Brien et al. (2008) characterized the morphological, cultural and molecular characters of 237 isolates of *P. roqueforti* and 78 isolates of *P. paneum* from over 900 colonies cultured from baled grass silage. The molecular description was based on the partial sequences of β-tubulin and acetyl-co-enzyme A synthetase genes.

Random amplification of polymorphic DNA

RAPD protocols rely on the random amplification of anonymous DNA segments with short, arbitrary 8 to 10 bases-pair identical primers under low annealing temperature, in order to amplify multiple products that would represent different locus (Liu and Cordes, 2004).

Advantages of RAPD include: no previous DNA knowledge is required, the commercially available primers will bind somewhere in the sequence, although it is not certain exactly where. It is an inexpensive though powerful method for many bacterial species. Limitations of this technique include the inability to distinguish between homozygote (2 copies) and heterozygote (1 copy), the prerequisite of large and intact DNA template sequences, and low reproducibility due to the low annealing temperature used in the PCR amplification.

Rossi and Dellaglio (2007) studied the quality of farm-made silages in Italy by examining populations of LAB, clostridia, lactate-fermenting yeasts and propionibacteria using RAPD-PCR, sequencing of the V2-V3 16S rRNA gene region, 5.8 ITS rDNA RFLP and species-specific PCR. Among the LAB isolates, *L. plantarum* was the predominant species in alfalfa, maize and Italian ryegrass silages, but strains of *L. buchneri* were isolated in many samples. Anaerobic spore-producing organisms included 6 species of the genus *Clostridium* and *Paenibacillus macerans* and yeasts were identified as *Candida apicola*, *C. mesenterica* and *Pichia fermentans*.

Walker et al. (2009) used RAPD-PCR to discriminate between different strains of *L. buchneri*. Although some strain differences could not be picked by using 16S rDNA sequencing, RAPD-PCR was able to distinguish to the strain level.

Restriction fragment length polymorphism

This technique is based on an enzymatic restriction digestion of total DNA and separation by gel electrophoresis; the DNA fragments are transferred to a membrane via Southern blotting, hybridized to a labeled DNA probe to a final radioactive pattern. The RFLP analysis was developed before DNA sequencing techniques but it is still being used due to its low cost, although it is slow and cumbersome, and requires a large amount

of template DNA. More recent protocols utilize PCR instead of the Southern blot method (Liu and Cordes, 2004)

Chan et al. (2003) utilized specialized computer software to compare RFLP pattern from gels instead of the Southern blot of the total DNA digest. Electrophoretic patterns of DNA fragments from *Eco*RI digests of total DNA were utilized to analyze over 10,000 isolates of LAB from fresh and ensiled corn, alfalfa and ryegrass, and constructed a database of approximately 700 unique patterns. The database contained large homogenous clusters of *L. plantarum, E. faecium, L. buchneri, L. brevis* and *Pediococci*.

Denaturing gradient gel electrophoresis

Analogous to Temperature gradient gel electrophoresis (TGGE), DGGE is a form of electrophoresis on acrylamide gel where DNA fragments of identical length but different in sequence composition are separated by their denaturing profile (from double- to single stranded) when subjected to a chemical gradient. For each kind of application the optimum denaturing gradient has to be adjusted, although theoretically differences of a single base pair could be detected (Temmerman et al., 2004).

Major advantages of this well-established technique are low cost and simple interpretation of the results; furthermore, individual bands can be extracted from the gel and identified by sequencing. Shortcomings of this method include the presence of small fragments that contain insufficient information, lack of reproducibility and low sensitivity because of gel staining (Justé et al., 2008).

May et al. (2001) used DGGE on fungal rDNA amplified from total silage to identify fungi in maize silage; however, closely related rDNA sequences were complex to resolve. Yeasts were the predominant fungi and *P. anomala* was the dominant species present in silages after 2-3 months of fermentation.

Wang et al. (2006) evaluated a LAB community (Al2) from well-preserved alfalfa silage through continuous restricted subcultivation, containing *L. plantarum, L. kimchii, L. pentosus* and *L. faciminis*. The Al2 community was then used to inoculate alfalfa and, at different lengths of storage, the researchers studied the LAB diversity with DGGE. The DGGE bands showed that *P. pentosaceus* was the predominant species in untreated

silage; however, the components of the Al2 community, particularly *L. plantarum*, dominated the fermentation and were the prevailing bacterial species in treated silage.

Terminal restriction fragment length polymorphism

Similar to DGGE, T-RFLP is a technique based on PCR amplification that aims to produce a profile of an unidentified microbial community. In this method, the sets of primers used for the PCR assay have their 5' end labeled with a fluorescent molecule. The amplicons are subjected to a reaction using a restriction enzyme, and the resulting fragments are sorted using a DNA sequencer. Different from RFLP, only the terminal (and end-labeled) fragments are read because they are analyzed in a sequencer. The sizes of the different fragments are determined depending on the emitted fluorescence (Liu et al. 1997).

The use of a sequencer provides reproducible results for repeated samples and the digital output allows easy data storage for later comparison with other samples or experiments. Nonetheless, distinct sequences that share a terminal restriction site will result in only one peak on the electropherogram, and the possibility of false restriction fragments are some disadvantages. Another drawback is the impossibility to recover the sequences; thus, parallel analysis of clone libraries have to be done (Egert and Friedrich, 2003).

McEniry et al. (2008) described the bacterial profile of wilted perennial ryegrass ensiled as baled and precision-chop silage using culture-based and culture-independent T-RFLP with robust statistical analysis. The ensiling system had little effect on the overall community composition, except that *Enterobacteria* sp. was more abundant in the baled system compared to precision chop silage. *Leuconostoc mesenteroides, Le. carnosum, L. sakei and L. graminus* were present after 2 and 6 days of ensiling. Nevertheless, the authors suggested additional research involving the application of more-species-specific molecular techniques.

Length heterogeneity PCR

While DGGE/TGGE and T-RFLP target base changes in DNA sequence, LH-PCR uses naturally occurring sequence length-based differences to distinguish between

communities. The LH-PCR is a sensitive and reproducible technique that does not require post-PCR treatment; therefore the products can be directly loaded onto the genetic analyzer. Nevertheless, the software can impose a limitation when the profiles often present adjacent amplicon distributions complex to resolve; also, if one amplicon can represent more than one taxon that are distinct but produce the same length amplicon; moreover, clones libraries need to be constructed given that individual fractions or peaks cannot be collected (Mills et al., 2007).

Brusetti et al. (2006) monitored LAB succession in maize silages under ideal and spoilage-simulating conditions. During 30 days of storage, the LAB community peaked at 13 d in silages under optimal ensiling conditions. *Pediococcus* and *Weissella* predominated in the early days of fermentation, *Lc. lactis* and *L. brevis* were the dominant organisms after 6 d. The presence of *L. plantarum* was noticed at all stages of fermentation but it was only a fraction of the total LAB population.

CONCLUSIONS

The field of silage and environmental microbiology has observed a huge jump in qualitative and quantitative knowledge over the past 20 years. Due to limitation on probe/primers and the artifacts associated with the reaction, there is a need of improving accuracy, specificity and quantitative detection. Beneficially, the development of probes and primers is being reinforced by the increasing discovery of new sequences. Furthermore, to investigate the gene functions and the ecological purpose of the organisms that enclose them is a significant task.

Moreover, the development of new techniques for cultivation of microorganisms cannot be forgotten and has to be investigated. The successful cultivation of these microorganisms is still the most efficient way to study and characterize the genotypes and phenotypes of novel species.

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Table 1. Techniques that can be used to study silage microorganisms [adapted from Gilbride et al. (2006) and Zoetendal et al. (2004)].

Technique	Benefits	Limitations
Cultivation/	Easy to carry out	Not representative, laborious, slow
Media-based methods	Identification of individual microbes of interest	Majority of bacteria cannot be easily cultivated
		Selective media are not specific
Phenotypic test systems	Easy to carry out	Laborious, slow
	Identification of individual microbes of interest	Limited accuracy
PCR	Fast detection of specific microbes at species or genus level	Subject to DNA extraction biases
16S rDNA sequencing	Phylogenic identification	Subject to DNA extraction and PCR biases
		Laborious
RAPD	Knowledge of the DNA sequence of the target organism not required	Subject to DNA extraction and PCR biases
		Requires large, intact DNA template sequence
	Inexpensive and powerful	
LH-PCR	Allows a rapid analysis of a wide range of microbes and the monitoring of population shifts	Subject to DNA extraction and PCR biases
		Individual peaks cannot be separated
	Post-PCR treatment not required	Accuracy of the peak detection for longer domains
	Reproducible and fast	
DGGE	Allows a rapid analysis of a wide range of microbes and the monitoring of population shifts	Subject to DNA extraction and PCR biases
		Identification requires clone library
	Powerful and robust	
T-RFLP	Allows a rapid analysis of a wide range of microbes and the monitoring of population shifts	Subject to DNA extraction and PCR biases
		Identification requires clone library
	Potential for high throughput	

Figure 1. Flow diagram of methods used singularly or in combination to analyze microbial ecosystems [adapted from Justé et al. (2008) and Zoetendal et al. (2004)].