



## Silage review: Using molecular approaches to define the microbial ecology of silage<sup>1,2</sup>

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### ABSTRACT

Ensiling of forages was recognized as a microbial-driven process as early as the late 1800s, when it was associated with the production of “sweet” or “sour” silage. Classical microbiological plating techniques defined the epiphytic microbial populations associated with fresh forage, the pivotal role of lactic acid-producing bacteria in the ensiling process, and the contribution of clostridia, bacilli, yeast, and molds to the spoilage of silage. Many of these classical studies focused on the enumeration and characterization of a limited number of microbial species that could be readily isolated on selective media. Evidence suggested that many of the members of these microbial populations were viable but unculturable, resulting in classical studies underestimating the true microbial diversity associated with ensiling. Polymerase chain reaction-based techniques, including length heterogeneity PCR, terminal RFLP, denaturing gradient gel electrophoresis, and automated ribosomal intergenic spacer analysis, were the first molecular methods used to study silage microbial communities. Further advancements in whole comparative genomic, metagenomic, and metatranscriptomic sequencing have or are in the process of superseding these methods, enabling microbial communities during ensiling to be defined with a degree of detail that is impossible using classical microbiology. These methods have identified new microbial species in silage, as well as characterized shifts in microbial communities with forage type and composition, ensiling method, and in response to aerobic exposure. Strain- and species-specific primers have been used to track the persistence and contribution of silage inoculants to the ensiling process and the role of specific species of yeast and fungi in silage spoil-

age. Sampling and the methods used to isolate genetic materials for further molecular analysis can have a profound effect on results. Primer selection for PCR amplification and the presence of inhibitors can also lead to biases in the interpretation of sequence data. Bioinformatic analyses are reliant on the integrity and presence of sequence data within established databases and can be subject to low taxonomic resolution. Despite these limitations, advancements in molecular biology are poised to revolutionize our current understanding of the microbial ecology of silage.

**Key words:** silage, microbial ecology, biotechnology, microbiome

### INTRODUCTION

Ensiling of forages has become a global practice for forage preservation and is particularly prevalent in wet climates, where the conservation of dried forage is difficult (Pahlow et al., 2003). The microbiome associated with freshly harvested forage plays a critical role in the ensiling process and is composed of a complex mixture of bacteria, yeasts and molds with colony-forming units ranging from  $10^5$  to  $10^9$  cfu·g<sup>-1</sup> (Langston and Bouma, 1960a,b,c). Once introduced into the silo, the diversity of the microbiome decreases as obligate aerobes and acid-sensitive microbes are killed or fail to grow as oxygen is excluded and acid production reduces pH. Under these conditions, epiphytic lactic acid-producing bacteria (**LAB**), enterobacteria, yeasts, and molds can enter a viable but unculturable state precluding their characterization through traditional plating methods. Epiphytic and spoilage microbial populations associated with the ensiling process are exceedingly complex, requiring significant effort to characterize members in detail on the basis of morphological, physiological, and biochemical traits. To assess the composition of bacterial communities associated with plants, a wide variety of techniques, both culture-based (e.g., most probable number, selective media, biochemical profiling, and so on) as well as culture-independent analyses of microbial

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communities using techniques such as denaturing gradient gel electrophoresis (**DGGE**), single strand conformation polymorphisms, terminal RFLP (**T-RFLP**), automated ribosomal intergenic spacer analysis, or length heterogeneity PCR have been employed for the past decade. In addition, polyphasic/multipronged approaches, using multiple techniques in parallel, have also been used to thoroughly characterize the structure and diversity of silage microbial communities (Brusetti et al., 2008).

Advances in deep sequencing using a variety of platforms (e.g., Illumina, Roche 454, Ion Torrent, PacBio) now make it possible to undertake metagenomic sequencing of microbial DNA extracted from freshly harvested forage (Eikmeyer et al., 2013), during ensiling (Bao et al., 2016), upon aerobic exposure (Dunière et al., 2017), and even from the rumen after consumption of silage by ruminants (Huws et al., 2015). These techniques have generated new insight into the complexity of the microbial ecology of ensiling, characterizing the role of epiphytic populations in silage quality and how silage additives can generate microbiomes more conducive to the production of high-quality silage. Quantitative PCR (**qPCR**) can be used to specifically quantify and document the persistence of specific phylogenies, species, or even strains of bacteria and fungi during ensiling and feed out. Consequently, the growth and survival of inoculated bacteria can now be differentiated from their epiphytic counterparts, generating a greater understanding of how these specific strains favorably alter the ensiling process. Using these techniques to identify mycotoxin-producing fungal populations or potential pathogens such as *Listeria* spp. in silage could provide new approaches to excluding these undesirable microorganisms from silage.

Presently, most studies have been confined to study the phylogeny of microbes using 16S rRNA or 18S rRNA/internal transcribed spacer (**ITS**) regions for the classification of bacterial and fungal populations, respectively. Although these studies provide a wealth of information on the microbial ecology of silage, they provide limited information on the function of the microbial species involved in the ensiling process. Future use of metatranscriptomics to characterize the ensiling process could shed insight into the expression of genes coding for enzymes involved in silage acid production and metabolism, proteolysis, or the production of mycotoxins during silage spoilage.

Although molecular techniques are poised to revolutionize our understanding of the role of microorganisms in ensiling, the scientific integrity of the information generated depends on the representativeness and purity of the nucleic acids that are extracted from the forage.

The nature of the primers selected for amplification of genetic regions of interest, the presence of PCR inhibitors and the selected sequencing platform can all influence the nature of the results generated. Once sequence information is obtained, the breadth and depth of the bioinformatic pipelines and limits of the gene databases used in the interpretation of data can affect predicted outcomes. The objective of this review is to outline some of the molecular methodologies currently being employed in the area of silage ecology and how they will advance the field of silage science both now and in the future.

## **SAMPLING, STORAGE, AND EXTRACTION METHODOLOGIES**

### ***Sampling Methods***

Many of the same sampling principles of acquiring silages for nutrient analysis are applicable to collecting silage samples for molecular analysis (Undersander et al., 2005). For fresh forage, 3 to 5 samples can be collected after the truck or wagon has been unloaded. Samples can be mixed and a single sample collected for storage as described below. This procedure can be repeated 4 to 5 times at regular intervals as silage is delivered for ensiling throughout the day. For bunker or bag silos, 150 to 300 cm of silage should be removed from the silo face to create a pile to sample from. If spoiled silage is not the subject of interest in the study, care should be taken to ensure that it is not collected during the sampling procedure. Five to 8 samples can be selected from the pile and mixed within a bucket before sampling and storage as described below. A total of 3 to 6 samples should be collected in this manner. If a microbial profile of the silage within the entire silo is desired, this procedure should be repeated at regular intervals during feed out.

### ***Extraction Methods and Storage***

One of the important factors in planning PCR-based studies or undertaking amplicon- or shotgun-based metagenomic or metatranscriptomic sequencing is to ensure that the extraction protocol employed obtains the quality and yield of nucleic acids needed to generate high-quality sequences. Ideally, samples should be frozen immediately after collection and stored in an ultra-low-temperature freezer to impede microbial activity, sustain cellular integrity, and ensure that the microbial diversity profile remains stable during storage. Short-term storage (14 d) of biological samples at temperatures ranging from  $-80$  to  $20^{\circ}\text{C}$  has been

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