# The present and future of microbial inoculants for silages

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**Abstract**

Silage making is not a novel technique. However, the agricultural industry has made great strides in improving our understanding of — and efficiency in — producing high-quality silage for livestock. Silage microbiology research has been using the newest molecular techniques to study microbial diversity and metabolic changes. This chapter reviews important research that has laid the foundation for field-based utilization of silage inoculants. We also outline areas of current, and future, research that will improve global livestock production through the use of silage.

**Keywords:** additives, forage, inoculants, silage

## 1. Introduction

Fermentation of forage is harder to control when compared to other fermentation processes such as industrial fermentation of food, for example. Whole plants cannot be manipulated to remove contaminating microorganisms, and this can lead to important variations in the quality of the forage. Harvesting machinery can also contribute to the inclusion of soil or manure particles as contaminants. Other factors have an impact on silage quality include harvesting management, packing rate, weather events during harvest, selection of the ensiling structure, and selection of a microbial or chemical additive to preserve the crops. Figure 1 provides an overview of the interactions between the main parameters involved in the production of high-quality silage.

This chapter will evaluate the recent published literature and will expand on the current knowledge in the study of the microbiota, search for silage inoculants, issues with aerobic instability, and understanding non-users of forage inoculants. We will also review important research areas for microbial inoculants: fiber digestibility, analyzing "big data" functional studies, co-ensiling with by-products or food-processing wastes, and how lactic acid bacteria (LAB) used as forage additives influence animal performance.

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## 2. Microbiota diversity during ensiling

Characterization of the different microbial species observed throughout the different phases of the ensiling process was traditionally performed using culture-dependent methods, following isolation of strains and determination of their taxonomic classification. The use of selective media has several shortcomings, including limited knowledge on how composition of the different defined culture media influences the growth of organisms within the targeted species range. Dormant or inactive cells (viable but nonculturable) may not have been accurately measured [1].

New techniques based on DNA profiling have helped the industry's understanding of the microbial diversity of silage within specific families or genera. [2]. These techniques were diverse and included denaturating gel electrophoresis [3] or metabolic fingerprinting by Fourier Transform infrared spectroscopy [4].

Next generation sequencing (NGS) technologies provide more complete details on microbiota diversity. The first application of NGS in silage was performed on ensiled grass to help understand how inoculation would influence the microbial communities [5]. Three years passed before a second paper would be published using NGS studying spatial and temporal microbial variations in commercial bunkers [6]. Several more papers or communications were performed afterwards (see Table 1).

One of the complexities facing ensiling of forage is that several factors will influence the size and diversity of the microbial community on the forage at harvest. Microbial diversity will change according to the plant species, weather during growth and prior to harvesting, fertilization management, physiological state of the forage, etc. As an example of the potential variation, important differences in the composition of the epiphytic bacterial population were observed from different organs of whole plant corn in the weeks prior harvesting (Figure 2). Leaves, silk, and tassels harbored different proportions of the main epiphytic bacterial families even though the variation in microbiota composition was small between sampling periods. *Cytophagaceae* and *Methylobacteriaceae* were mainly observed on the leaves, while *Enterobacteriacaea* and *Pseudomonadaceae* were observed on silk, cob, and tassel [7].

Published results of microbiome analysis were performed from varied forages, including pure strands of legumes or grasses and mixed forages. The forages were from temperate as well as tropical regions. Several studies performed time-based samplings to describe changes in the microbial communities in relation to the fermentation periods

[7-9] (Table 1). Generally, the relationship between the time of fermentation and the microbial composition was similar to the general succession pattern previously reported by culture-dependent microbiological techniques. For example, with corn silage inoculated with either *Lactobacillus plantarum* or *Lactobacillus buchneri* and/or *Lactobacillus hilgardii*, it was possible to observe that the succession to *Firmicutes* was rapid, in a matter of hours after sealing the experimental mini-silos. A second observation was that *Leuconostocaceae* (mainly *Weissella* sp.) was the dominant operational taxonomic unit (OTU) during early fermentation. In both studies, important changes in bacteria richness during the fermentation, with values below 50 OTUs after incubation of 30 days [9] or decreasing throughout fermentation to a similar level of OTUs [8]. In both studies, fungal richness dropped throughout fermentation.

These changes in microbial population were also observed in samples collected on farms. Under commercial conditions, comparing silage made from the same forage between sites is difficult since differences in dry matter (DM), packing density, and other physical parameters will influence efficiency of the fermentation and the microflora. Associating those parameters to NGS studies could improve the understanding of this process. It will then be possible to understand how other physical variables may contribute, e.g. the impact of high or low temperature on microbial succession, the impact of length of storage, length of time at a high temperature, and the impact of DM variations within the same forage.

To date, most of the data collected from experimental silos was performed with incubation periods shorter than 100 days and at a temperature around 20 to 25°C. These conditions offer an initial set of parameters but must be expanded to simulate real-life conditions in silos, which could include variances of more than 20°C above ambient during fermentation and long fermentation periods [11].

Most of the published studies included a comparison between control and a microbial silage additive or between different strains of LAB. The general trend on microbial diversity is that inoculation with LAB reduces the microbial diversity, but the impact differs in relation with the forage and the species of LAB. As observed by Wang et al. [12], microbial diversity was influenced by the inoculation of *Moringa oleifera* independently from each of the four species used as well as from the temperature of incubation.

Comparisons between studies tend toward similar changes in microbial composition. To facilitate comparisons, it will be necessary to standardize DNA

isolation and preparation of the amplicons prior to sequencing. By summarizing the main methodology information from different trials (Table 1), it was observed that some studies did not include fungal diversity, and the amplified DNA region differed. Most bacterial studies were performed following amplification of the V3-V4 region, but there was a trend toward using the V4 or V4-V5 region, which offers potential for longer DNA strand and improving comparison scores against the database. Using a good quality database is a critical step that is often overlooked during analysis [13]. The drawback of the current methodology for amplicons based metasequencing is that the amplified region is short and does not provide enough coverage of the complete 16S rRNA gene. Two published studies were able to gather near complete fragments by sequencing the 16S rRNA gene on a PacBio sequencer instead of the Illumina model [13-14]. This expanded the analysis of diversity to the species, or even subspecies, level.

Currently, no study has tried to mix the potential offered by polymerase chain reaction (PCR)-based profiling technology — like PCR-DGGE — with NGS sequencing capacities. Instead of amplifying with universal primers, primers targeting regions of lower variations could provide more precise results allowing higher similarity scores at the species level.

Microbial communities continuously evolve during the storage period, even during the anaerobic stable phase. By improving our knowledge on the succession between communities, genus, species, and even strains, it will be possible to refine how strains are selected as microbial silage additives. This could easily allow selection of strains for particular forages species or climatic conditions.

3. **Searching for new forage inoculants in temperate and tropical forages**

The fermentation capability — or the acidification potential — depends directly on the DM content, the level of water-soluble carbohydrates (WSC) and, inversely, on the buffering capacity of a given forage [16]. Due to their compositions, the ensiling potential is completely different among the different families of forages: tropical (C4), temperate (C3) grasses, and legumes.

Studies conducted by Wilkinson [17] with C3 grasses have concluded the minimal concentration of the WSC should be at least 2.5 to 3.0% of the fresh forage. Below 2% of WSC of fresh crop weight, forages are prone to undesirable fermentations. The average level of WSC found by Zopollatto et al. [18] in a review of microbial

additives in Brazil for tropical grasses was only 1.6%, far from the minimum for a good fermentation.

Tropical grasses provide large quantities of DM, which can reach up to 30 tons of DM per hectare. This great yield, however, comes, at the better stage of maturity in terms of nutrients, with other types of challenges: wilting is an issue and the excess moisture can lead to important losses of nutrients through effluent production [19]; additionally its nutritive value sharply declines as maturity advances.

The microflora existing on the vegetative parts of plants consists mainly of microorganisms considered undesirable from the point of view of the fermentation process. These include anaerobic bacilli of the genus Clostridium; aerobic bacteria of the genus *Bacillus*; coliform bacilli, including *Escherichia coli*, *Enterobacter* spp., *Citrobacter* spp., and *Klebsiella* spp.; as well as bacteria of the genus *Listeria*, *Salmonella*, *Enterococcus* (*E. faecium*, *E. faecalis*, *E. mundtii*, *E. casseliflavus*, *E. avium*, and *E. hirae*); and the occurrence of actinomycetes. Species of *Clostridium* are responsible for large losses because they produce $CO_2$ and butyric acid instead of lactic acid. Yeast and molds also form a large group [20].

Concerning the presence of LAB, Pahlow et al. [21] found in grasses that *L. plantarum*, *L. casei*, *E. faecium*, and *Pediococcus acidilactici* were the most frequently observed species. However, with the development and the use of DNA sequencing profile techniques, it is possible to identify hundreds of species as mentioned earlier. Most of the studies done by scientific groups were based on the efforts to find any microorganisms, especially bacteria, able to drive a good fermentation and inhibit undesirable and detrimental microorganisms.

Zielińska et al. [22] demonstrated that microbial inoculants altered many parameters of silages, but the strength of the effects on fermentation depends on specific characteristic of an individual strain. Several research teams have been searching for new strains able to perform better than the ones currently on the market. For example, Agarussi et al. [23] searched for new promising strains for alfalfa silage inoculants and isolated *Lactobacillus pentosus* 14.7SE, *L. plantarum* 3.7E, *Pediococcus pentosaceus* 14.15SE and a mixture of *L. plantarum* 3.7E, and *P. pentosaceus* 14.15SE. The authors concluded all of the tested strains had a positive effect on at least one chemical feature of the silage during the fermentation process, although the most promising strain found in that trial was the *P. pentosaceus* 14.15SE.

Moreover, Saarisalo et al. [24] searched for LAB capable of lowering the pH of grass silages with low proteolytic activity. The researchers found a potential strain of *L. plantarum*, which was effective in reducing the deamination in silages.

Besides aiming to enhance silage fermentation, aerobic stability has been an important topic in the last 20 years. During silage feedout, accelerated growth of spoilage organisms (yeasts) results in high temperatures and nutrients and DM losses, leading to increased silage deterioration [25]. According to McDonald et al. [26], even though yeasts can grow from 5 to 50°C, the optimum growth of most species occurs at 30°C. Other spoiling microorganisms, like molds and *Clostridium* bacteria, grow between 25 and 37°C, respectively. Considering the specific temperature and humidity ranges of different microbes for growth, it is possible to see that tropical climates are more prone to spoilage than temperate ones.

## 4.   Improving aerobic stability using forage inoculants

Silage feedout is the final phase of the ensiling process. At that moment, oxygen can slowly diffuse inside the silage mass. Diffusion speed will be influenced by different factors, including the level of humidity, porosity, and temperature of the silage [27].

The process of aerobic deterioration of silage involves a shift to aerobic metabolism in some microorganisms and the reactivation of strict aerobes that were dormant. Oxidation of the fermentation products and residual carbohydrates will reduce nutritional value due to oxidation of carbohydrates, amino acids, and lipids to $H_2O$, $CO_2$ and heat. Simultaneously, the higher metabolic activity will increase the silage temperature, accelerating microbial growth. Several microorganisms are involved, but yeast and acetic acid bacteria are adapted to tolerate the initially low pH conditions, thus able to exploit this niche before pH increases following the catabolism of the organic acids. Crops with higher levels of easily accessible carbohydrates are more prone to aerobic deterioration, i.e. corn, sorghum, and sugarcane, since these sugars can be readily fermented by spoilage microorganisms in the presence of oxygen.

Following the isolation of a *L. buchneri* strain [28], researchers described its unique metabolic pathway, which consisted of converting moderate amounts of lactate under low pH to equal parts of acetate and 1,2-propanediol [29]. The latter chemical is an intermediate in the potential synthesis of propionic acid. *L. buchneri* does not have the gene to complete the reaction, so another species of LAB has to be involved to

convert 1,2-propanediol to an equimolar amount of 1-propanol and propionic acid [30]. This conversion was initially observed in silage by *Lactobacillus diolivorans* [31], but other members of the buchneri group also possess the genetic system [32], like *Lactobacillus reuteri* [30].

Compared to lactic acid, the key feature of acetic and propionic acids in improving aerobic stability of silage is based on the difference in $pK_a$ between these weak acids and lactic acid, which is a stronger acid, with a $pK_a$ of 3.86. At higher $pK_a$, 4.76 for acetic acid and 4.86 for propionic acid, these weak organic acids will have a low dissociation level under most ensiling conditions, thus allowing for passive diffusion inside the yeast or other microorganisms cytoplasm. Once inside the cytoplasm, propionic acid will dissociate to the corresponding salt since internal pH is above $pK_a$ value. The same process is also possible for acetic acid. Constant pumping of the protons released inside the cytoplasm causes physiological stresses impacting several metabolic pathways in yeast cells [33].

Length of fermentation and establishment of heterofermentative LAB population are now considered critical toward the establishment of a good aerobic stability level. The facultative, or obligate heterofermentative, strains of LAB have lower growth rates than homofermentative strains, including rods like *L. plantarum* or coccids of the genera *Leuconostoc*, *Enterococcus*, or *Lactococcus*. The growth conditions after several days of ensiling are also more restrictive for physiological activities considering the low pH usually encountered. The strains succeeding the earlier colonizer need to be more tolerant to both acidity and osmotic stresses, simultaneously. Observation of the succession of different species of LAB during the anaerobic stability phase often lead to high abundance of LAB belonging to the *L. buchneri* taxonomical group [15], leading to specific adaptation to this ecological niche by these strains. Although few physiological studies on *L. buchneri* strains had been published, Heinl and Grabherr recently published a complete analysis of the genetic potential of the strain CD034 compared to other genomes from public databases [34]. One of the comparisons performed aimed to describe how the genetic system of this species could cope with high concentration of organic acids, including lactic acid. The anaerobic conversion system of lactic acid to 1,2-propanediol (to acetic acid and $CO_2$ under aerobic condition) represents one of those properties. It is possible to extend these observations to the results gathered from transcripts analysis on the strain *L. buchneri* CD034 [35] following aeration of culture grown under anaerobic conditions. The team described the functions of 283 genes

induced by the presence of oxygen. They also observed physiological adaptation related to changing oxygen concentration. Genes required by lactic acid fermentation systems were hardly affected.

Co-inoculation with different heterofermentative strains has recently been tested in the field or in commercialized conditions. This was the case for *L. buchneri* and *L. diolivorans*, tested on the fermentation of sourdough [36]. The authors showed an increase in the accumulation of propionic acid following inoculation with both strains together. Co-inoculation of *L. buchneri* and *L. hilgardii* was tested in different ensiling trials [37-38] inducing better fermentation and higher aerobic stability level. *L. hilgardii*, an obligate heterofermentative strain, was previously observed as a contaminant of wine but also represents one of the dominant LAB strains in water kefir [39]. Strains of this species are often observed in sugar cane silage [40-41] and provide increased aerobic stability levels for this challenging crop. Improvement in fermentation and aerobic stability of sugarcane silage allowed increasing DM intake and milk yield [42].

Two recent meta-analysis [43-44] provided a complete overview of the impact of inoculation of LAB and described the importance of fermentation and aerobic stability in relation to the specificities of the forages and the activity of homofermentative, facultative heterofermentative, and obligate heterofermentative strains. In particular, the meta-analysis of Blajman et al. [43] analyzed the role of inoculation on reducing the amount of yeast in silage.

Improving aerobic stability to reduce overall losses during the storage and feed-out is one of the main reasons to apply microbial inoculants on the forage at the time of ensiling. The value of silage inoculants is important, but optimal management of silos at all steps of the ensiling process is critical.

5. **Improving adoption of forage inoculant use by increasing awareness of the economic value of forage inoculants**

According to the 2017 National Agricultural Statistics Survey [45] census report, approximately 120,000,000 tons of whole-plant corn alone was harvested for silage in the United States. Even with this huge quantity of silage, there is little reliable survey data about the use of forage inoculants.

Based on an independent market survey of U.S. beef and dairy producers, two thirds of respondents indicated that forage additives used on their operations are

microbial based. The main reason for their use is to minimize mold and spoilage in silage. Other reasons cited include preventing heat damage and increasing herd productivity [46]. Most inoculant users plan on continuing using and investing in this technology each year [47].

Product performance, ease of use, and cost are the main influencers on the purchasing decision of inoculants. In addition, nutritionists and consultants are important sources for providing information on forage inoculants and the most involved outside sources in the purchase decision [47].

Most producers do not have a detailed understanding of the different types of inoculant products but recognize the value and return on investment (ROI) that these technologies can bring to their operation. Value-added services and education offered by inoculant companies are also reasons to purchase, especially for larger producers.

Producers may often choose not to purchase forage inoculants due to the cost of the products. Other top reasons that influence purchase decisions are (1) not believing inoculants work, (2) lack of knowledge, or (3) lack of specific equipment for inoculating the forage. With all these factors in mind, there is a strong need for proper education on the application and showing the cost-to-benefit calculation of these forage additives [48].

Even though some producers are non-users, they believe inoculants have the potential to improve consistency of silage quality, enhance ration quality, and increase feedout stability. In the same question, just 40% answered that improving ROI is one of the most important benefits of purchasing inoculants. Even though some producers do not associate inoculants with contributing to overall herd ROI and profitability, the word "fresh" is shown to have a positive association with good smell and palatability [48].

During typical field and harvest management conditions, silage losses are easily reported between 15 to 20%. If inoculant use can reduce DM losses by a 5 percentile points, there would be savings of $2,000 (US$) per thousand tons of silage, assuming the silage is valued at $40.00 (US$) per ton FM. Moreover, silage with high degree of deterioration not only has less overall tonnage to be fed, but the feed is also of lower nutritional quality.

## 6.    Optimizing fiber and carbohydrate digestibility

The main metabolic activity of LAB during the ensiling process consists of reducing soluble carbohydrates to organic acids to acidify and preserve the forage for

long-time storage. It has been observed that animal performance has been increased following use of microbial inoculants, even if no or small changes in silage fermentation parameters were observed [2]. Future research is needed to explain why these improvements are observed. Yet, past research has made several important advancements.

As discussed previously, inoculation with LAB contributes to important modifications of the silage microbiota, for both the bacterial and the fungal communities. Some of these modifications could partly explain the contribution of the inoculant to one or more nutritional characteristics of silage. This also could support the theory of an indirect positive impact of these nutritional characteristics to the rumen microbial population and functions.

The rumen environment may also be affected by LAB forage inoculants. Some strains of LAB used as inoculants were shown to survive in the rumen fluid [49] and shift gas production toward other products or microbial cells [50]. Weinberg et al. [51] observed that LAB inoculants applied at ensiling, or into the rumen, had the potential to increase DM and fiber digestibility.

Studies using different inoculants showed increases in animal performance and milk production [52]. Mohammed et al. was also able to quantify elevated levels of *L. plantarum* in the rumen of cows eating the treated silage [53].

To help explain this improved animal performance, results from studies of LAB used as a human probiotic may offer some clues. In a review of the metabolism of oligosaccharides and starch by lactobacilli, Gänzle and Follador [54] described limitations of the conversion of oligosaccharides since most related enzymes in LAB are active intracellularly and their substrates must be transported inside the cells to hydrolyze (Figure 3). By studying the genome of several LAB species, they report that most lactobacilli could generally metabolize α-glucans. They would require contribution of a trans-membrane transporter in order to hydrolyze small oligosaccharides. Like some other lactobacilli, *L. plantarum*, has gene encoding for an extracellular amylase with endoamylases activity. Presence of this amylase in the genome is strain specific as reported by Hattingh [55] for a strains of *L. plantarum* isolated from barley.

Selecting strains with a functional trait, for example fiber- or starch-degrading functions, represents the initial step in the development of a new inoculant. The strain has to cope with the different stresses of silage and also compete against epiphytic LAB and other microorganisms. The function also has to be expressed under the targeted

microbial niche. The extracellular enzymes then have to be optimized for the acidic conditions and cope with the specific nature of polysaccharide substrates.

Access by fibrolytic enzymes to cellulose due to steric hindrance of the lignin-hemicellulose matrix was the target in isolating a LAB strain producing ferulate esterase [56]. This enzyme releases ferulic acid from arabinoxylans, improving access to other fibrolytic enzymes of the lignin-cellulose layer within cell walls. Initial *in situ* ruminal incubations were able to show interesting potential of these strains, but further testing was not conclusive [57], showing the positive effect of the lactobacilli on fermentation, but the enzymatic function did not seem to contribute to improvement of the nutritional properties as would have been expected.

More research is needed in this area. The complexity and dynamic of the microbial communities following inoculation provide an important challenge in understanding the impact and role of the key players involved in this beneficial effect of microbial silage additive.

## 7. Improving animal performance with LAB forage inoculants

The expected effects of using a LAB forage additive are improved fermentation and enhanced feedout stability, which, in turn, leads to better recovery of nutrients and DM. However, expectations from producers are often beyond better silage characteristics, such as improvements in feed efficiency and, subsequently, animal performance.

Scientific evidence shows the use of microbial inoculants for ensiling increases animal performance and production, in addition to enhancing the fermentation. However, these improvements are difficult to quantify.

Some of the existing theories are the effects of these beneficial bacteria may have positive influence in the rumen environment including: altering the fermentation profile and interacting with the animal's existing digestive microbiota [49], and inhibiting undesirable microorganisms, which subsequently help reduce the potential for toxin production [58].

Oliveira et al. [44] analyzed 31 studies — including animal performance results. This meta-analysis showed that microbial inoculation at a rate of at least $10^5$ colony-forming units (CFU) of LAB per gram of forage significantly increased milk production by 0.37 kg/d, increased DM intake, and had no effect on feed efficiency and total tract DM digestibility. Furthermore, the contents of milk fat and milk protein tended to be

higher for cows fed inoculated silage. The effects on increased milk production due to LAB inoculation happened regardless of the type of forage and diet, inoculant bacterial species and application rate ($10^5$ vs. $10^6$ CFU/g of forage), and level of milk production.

Among the animal performance trials, there are cases when the inoculant had no effect on the silage fermentation compared to untreated silage, although animal productivity was increased [59]. Therefore, this indicates that some LAB strains are positively affecting the rumen microbial community and the digestive tract environment, resulting in improved effects on animal performance.

Recent research has described these effects by evaluating the impact of inoculated silages in the populations of the rumen microbial community, but no significant changes were observed [54]. However, nitrogen efficiency seemed to be improved due to lower levels of milk urea nitrogen in cows fed inoculated silage and greater ruminal DM digestibility on the inoculated silage ration [60]. Since LAB were shown to attach to the fiber inside the rumen [61], isolation methodology needs to be adapted to target the correct ecological niche.

Changes in nitrogen compounds during ensiling are expected. For example, over half of the true protein in alfalfa is degraded to soluble non-protein compounds initially by the plant's own proteases, and then later by microbial activity within the cow, resulting in inefficient nitrogen use to the cow [62].

Specifically, in the corn kernel or other cereal grain, a protein matrix (prolamins) around the starch granules partially prevents ruminal starch digestion. It has been reported that a slow and continuous breakdown of the prolamins during the storage phase, makes the starch more digestible with longer storage time [63]. The authors explained that this effect is due to natural proteolytic mechanisms. This event, however, requires months of storage for optimum level of starch digestibility in the rumen, which it is not always feasible in commercial operations. One alternative solution would be to shorten the time necessary for storage to help enhance starch digestibility by inoculation with bacteria that possess high proteolytic activity but to date, limited research has been reported and results are inconsistent.

Improvement of fiber digestibility has to be considered in relation to the activity of silage inoculants. Some strains of LAB have been reported to produce the enzyme ferulic acid esterease, which breaks the esterease bond between lignin and the hemicellulose fraction, leading to more digestible fiber portions for the rumen microorganisms [64]. However, data from animal performance or production studies did

not show consistencies in the improvements [64-65]. While *in vitro* and *in situ* effects may be conceivable, the expression of this phenomenon within *in vivo* environments needs additional research to be better understood.

There is still a need to better understand how the microbial additives for ensiling positively affect animal performance, so this should be used as criteria for a new generation of this type of additive.

## 8. Understanding the impact of ensiling on a global scale

Silage represents an important part of animal diets. Challenges in production, reducing losses, and the impact on agricultural practices are often overlooked compared to other nutritive benefits provided. Microbial activity during fermentation produce several compounds besides the desirable organic acids; some of those compounds were identified as negatively influencing air quality around farms. They are classified as alcohols, esters, and aldehydes [66-67]. Production and volatilization of these compounds contributes to a reduction in quality of the stored feed, inducing ground-level ozone, and influences emission of greenhouse gases by the agricultural sector [68].

Forage characteristics and yield potential are influenced by several factors, including geographic and meteorological conditions. New analytical technologies and statistical methodologies now allow more comprehensive understanding of ensiling techniques and analyze productivity and nutritional quality on a broader scale.

Comparison between farms is always challenging, even between neighboring farms, since they could differ on animal husbandry, genetics of the herd, field management, harvesting periods, type and size of silos, management of the silos, etc. On a broader geographic area, these differences will be minimized by the inclusion of higher numbers of farms, up to a point that patterns of variations could be analyzed. This type of analysis was performed by Gallo et al. in two recent studies [68-69]. The team used a multivariate analysis technique, Principal Component Analysis (PCA), to evaluate ensiling of corn silage on 68 dairy farms [69] and generated a fermentation quality index to rank the silage [70]. Using 36 variables measured on every individual samples, they were able to group the silage according to quality parameters in relation to silo management techniques to discriminate between well-preserved and poorly preserved forages.

At the farm level, quality parameters from silage and feed analysis reports could be analyzed to identify trends in animal health and performance. Different types of data

could be collected and analyzed to understand the main variations in milk quality and yield on a yearly or multi-year basis. Linking milk quality parameters to farm management practices was performed following the analysis of milk constituent using Fourier transformed mid-infrared spectroscopy results gathered from 33 farms [71]. The difference between observed high and low de novo fatty acid composition of milk allowed characterizing differences in feeding management (one or two feeding periods - fresher silage) and higher animal management scores (free-stall stocking - lower housing density).

Up to now, few data analysis included data specific to silage fermentation beside the main fermentation acids. This is truer for other parameters related to silage production and management, including yield from the field, management of the silos, losses during fermentation, or type of silage additive used. This is to be addressed considering important changes to the microbiota following inoculation discussed previously and to differentiate in other fermentation chemicals or their relationship with the nature of the additives applied, as observed by Daniel et al. [72].

## 9. Increasing understanding of the fermentation process

Compared to other research domains in agricultural and environmental sciences, using new sequencing technologies to understand the dynamics of the microbial communities in silage is recent. McAllister et al. [13] published a review providing a technological and methodological overview. Currently, the number of trials performed using this technique is small enough that repetitions between geographical regions or over time are non-existent.

Amplicons-based metasequencing represents the entry level of the -omics techniques. For silage research, the industry could also consider metagenomic, proteomic, transcriptomic, or epigenomic as a potential area of study. A review of the possibilities offered by metabolomics in agriculture was recently published [73].

Since ensiling is based on the fermentation of forage crops, knowledge of the metabolic activity of the forage prior to ensiling would be useful. A review by Rasmussen et al. [74] provides insight of how plants are coping with physiological changes due to breeding strategies, associations with endophytes or rhizobia, responses to nutrients, and, more interestingly, on the metabolic responses to the osmotic stress. Harvesting and wilting will directly influence plant cell activities and nutrient cycling. The authors reported amino acids, fatty acids, and phytosterols generally decrease

following water stress, while sugars and organic acids increased. Since the fermentation process requires fermentable sugars for optimal acidification of the forage, wilted plants may respond positively toward ensiling. We need to consider the speed of those changes in concentration of metabolites during wilting compared to propose a model of the response to an osmotic stress. Ould-Ahmed et al. [75] provided some knowledge on this response to wilting while studying changes in fructan, sucrose, and some associated hydrolytic enzymes, concluding there is a positive effect toward ensiling requirements from the different metabolites.

Metabolomic profiling of silage was performed in a study aiming to understand the role of inoculation with *L. plantarum* or *L. buchneri* in alfalfa silage against a non-inoculated control [14]. The authors were able to distinguish all three inoculation treatments by a PCA of the 102 metabolites surveyed. The major metabolites observed were related to amino acids, organic acids, polyhydric alcohols, and some derivatives. One of the main observations was an increase in free amino acids and 4-aminobutyric acid following inoculation with *L. buchneri* and a decrease in cadaverine and succinic acid following inoculation with *L. plantarum*.

Testing the same two LAB strains on whole plant corn silage instead of alfalfa, Xu et al. [15] observed a total of 979 chemical substances, from which 316 were identified and quantified. The PCA allowed separating the three inoculation treatments along the first axis, representing nearly 80% of the variations between samples. The second axis was able to further distinguish how inoculation with *L. buchneri* influenced the fermentation. Inoculation with either *L. plantarum* or *L. buchneri* contributes to increase the concentration of amino acids and phenolic acids, 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, glycolic acids, and other organic acids. Inoculation with *L. buchneri* also induces higher concentration of 2-hydroxybutanoic acid, saccharic acid, mannose, and alpha-D-glucosamine-1-phosphate, among others. Other substances were increased by ensiling without specific impact of the inoculants, like catechol and ferulic acid that could have antioxidant functions.

Metabolomic studies can also be used in defining a metabolomic signature specific of different forage and silage on feed efficiency of ruminants. With the aim of identifying feed efficiency traits in beef cattle, Novais et al. [76] investigated how serum metabolomic profiles could be used to predict feed intake and catabolism. They identified different molecules having feed efficiency role. Two molecules from the

retinol pathway, vitamin A synthesis, were significantly associated with feed efficiency (higher concentration of retinal and lower concentration of retinoate).

Beside the studies of Guo et al. [14] and Xu et al. [15], one other study combined different -omic techniques in understanding ensiling process. The first glimpse of that study was presented at the International Silage Conference in 2018 [8] with data on microbiota dynamic between 1 and 64 days of fermentation of corn silage. Multi-omics analysis of the amplicon-based metagenomic, metagenomic, and metabolomic data set is currently underway.

The potential of transcriptomic was also shortly covered by the *in vitro* trial of Eikmeyer et al. [35], which aimed to understand induction of genes in *L. buchneri* CD034 under different incubation settings. It is expected that additional studies performed directly under ensiling conditions may be published in the next few years.

Metabolomic data have shown how inoculation of LAB strains induces changes to the ensiled forage that goes beyond the simple production of lactic and acetic acid from the fermentation of sugars under anaerobic conditions. Increases in a whole array of molecules were observed, but the change also extends to the fibers and is either a direct or an indirect effect of the inoculant. Inoculation of alfalfa by *L. plantarum* or *Pediococcus pentosaceus* strains increased the release of different hemicellulose polysaccharides, including homogalacturonan, rhamnogalacturonan, and arabinogalactan from the cell walls [77].

These new technologies will allow greater understanding of the impact of bacterial inoculants on improvements of the silage and their contribution in induction of specific genes and proteins by other members of the microbial community at different stages of the ensiling process.

## 10. Co-ensiling forage with food processing waste and TMR conservation

Food processing residues represent high-energy organic material already used in some way that could include food-processing residues either from food industries or distiller's grains from the ethanol production. These residues could easily be used by farms closely located to the production site, but their relatively high humidity content renders them prone to a rapid deterioration. New ensiling techniques allow mixing them with low moisture forage or grain in order to perform a fermentation that in enclosed into a kind of total mixed ration (TMR) acidic conservation.

Aiming to use a bakery co-product waste, Rezende et al. [78] tested possibilities of re-hydration, treating it with acid whey or water and levels of urea. The authors found that the resulting silages had reduced populations of molds and yeast by acidification process. However, the initial population of these microorganisms were high, mainly accounting of *Penicillium* and *Aspergillus* spp. Inoculating with a bacteria that could produce antifungal chemicals, including acetic and propionic acids might be considered for this kind of co-product.

TMR silage is an important source of ruminant feed. This practice has been more common in some places where companies or producers mix wet co-products with dry feeds to prepare TMR that are then preserved as silage. Based on conventional criteria, aerobic deterioration could occur easily in TMR silage, because lactic acid prevails during fermentation and any sugars remaining unfermented can serve as substrates for the growth of yeasts. However, some trials [80-81] have been shown that when added concentrate, the brewer's grains or soybean curd residue, main co-products used in TMR preserved, do not show heating in the TMR. For the trial with brewers' grain-based TMR the main bacteria found in the stable silages where *L. buchneri*, but for the soybean curd-based TMR, the main LAB found were *P. acidilactici* and *L. brevis* [82], showing potential association of those bacteria to preserve TMR silages. A similar trial was performed by Ferraretto et al. [83] to test how the process influenced luminal *in vitro* starch digestibility. They used dry ground corn to adjust the humidity level of wet brewers' grain and observed an increased in digestibility of the starch from the combined feed.

Nishino & Hattori [79] evaluated two bacterium-based additives in wet brewer's grains stored as a TMR in laboratory silos with alfalfa hay, cracked corn, sugar beet pulp, soya bean meal, and molasses. The additives tested were the homofermentative LAB, *L. casei*, and the heterofermentative LAB *L. buchneri*. This last one was responsible for controlling yeast growth and the homolactic one helped in the fermentative profile of the ensiled TMR.

**Final comments**

General microbiology techniques have helped to better understand the basic dynamic of microbial communities, the diversity of species, the biochemical pathways involved at each phase of the fermentation process and the metabolic functions of the main spoiling agents involved in degrading the nutritional quality of the silage. NGS

helped observe microbial communities and metabolic profiling do not cease to evolve. This fact directly influences the nutritional characteristics of the silage.

In this text, the authors reviewed the main research activities that helped the agricultural industry understand silage as it is known today and also pointed to experimental techniques that will continue to improve the understanding of metabolic pathways and functional aspects of the ensiling process. It is clear these techniques will allow the scientific community to discover new inoculants that will combine our knowledge of silage fermentation, nutritional quality, improve rumen function, and contribute to better animal health. We look forward to the third generation of forage inoculants and seeing their positive impact.

**Acronyms and Abbreviations**

aerobic stability (AS)

colony-forming units (CFU)

dry matter (DM)

lactic acid bacteria (LAB)

next generation sequencing (NGS)

operational taxonomic unit (OTU)

polymerase chain reaction (PCR)

principal component analysis (PCA)

total mixed ration (TMR)

water-soluble carbohydrate (WSC)

**References**

1.  Pinto D, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms. *Critical Reviews in Microbiology.* 2015;41:61-76.

2.  Muck RE. Recent advances in silage microbiology. *Agricultural and Food Science.* 2013;22:3-15.

3.  Julien MC, Dion P, Lafrenière C, Antoun H, Drouin P. Sources of Clostridia in raw milk on farms. *Appl Environ Microbiol.* 2008;74:6348-6357.

4.  Johnson HE, Broadhurst D, Kell DB, Theodorou MK, Merry RJ, Griffith GW. High-throughput metabolic fingerprinting of legume silage fermentations via Fourier Transform infrared spectroscopy and chemometrics. *Appl Environ Microbiol.* 2004;70:1583-1592.

5. Eikmeyer F, Köfinger P, Poschenel A et al. Metagenome analyses reveal the influence of the inoculant *Lactobacillus buchneri* CD034 on the microbial community involved in grass ensiling. *Journal of Biotechnology.* 2013;167:334-343.

6. Kraut-Cohen J, Tripathi V, Gatica J et al. Temporal and spatial assessment of microbial communities in commercial silages from bunker silos. *Applied Microbiology and Biotechnology.* 2016

7. Drouin P, Tremblay J, Chaucheyras-Durand F. Dynamic succession of microbiota during ensiling of whole plant corn following inoculation with *Lactobacillus buchneri* and *Lactobacillus hilgardii* alone or in combination. (submitted for publication)

8. Gerlach K, Südekum K-H, editors. How do time of fermentation and lactic acid bacteria inoculation influence microbial succession during ensiling? 2018; Bonn, Germany: University of Bonn; 2018.

9. Keshri J, Chen Y, Pinto R, Kroupitski Y, Weinberg ZG, Sela S. Microbiome dynamics during ensiling of corn with and without *Lactobacillus plantarum* inoculant. *Applied Microbiology and Biotechnology.* 2018;102:4025-4037.

10. Lianhua L, Yongming S, Zhenhong Y et al. Effect of microalgae supplementation on the silage quality and anaerobic digestion performance of Manyflower silvergrass. *Bioresource Technology.* 2015;189:334-340.

11. Romero JJ, Zhao Y, Balseca-Paredes MA, Tiezzi F, Gutierrez-Rodrigues E, Castillo MS. Laboratory silo type and inoculation effects on nutritional composition, fermentation, and bacterial and fungal communities of oat silage. *J Dairy Sci.* 2017;100:1812-1822.

12. Wang Y, He L, Xing Y et al. Bacterial diversity and fermentation quality of *Moringa oleifera* leaves silage prepared with lactic acid bacteria inoculants and stored at different temperatures. *Bioresource Technology.* 2019;284:349-358.

13. McAllister TA, Dunière L, Drouin P et al. Silage Review: Using molecular approaches to define the microbial ecology of silage. *J Dairy Sci.* 2018;101:4060-4074.

14. Guo XS, Ke WC, Ding WR et al. Profiling of metabolome and bacterial community dynamics in ensiled *Medicago sativa* inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. *Nature Scientific Reports.* 2018;8:357.

15. Xu D, Ding W, Ke W, Li F, Zhang P, Guo X. Modulation of metabolome and bacterial community in whole crop corn silage by inoculating homofermentative *Lactobacillus plantarum* and *Lactobacillus buchneri*. *Frontiers in Microbiology.* 2019;9:3299.

16. FAO. Silage fermentation processes and their manipulation. 2000; Rome.

17. Wilkinson JM. Silage. Shedfield, UK: Chalcombe Publications; 1990

18. Zopollatto M, Daniel JLP, Nussio LG. Aditivos microbiológicos em silagens no Brasil: revisão dos aspectos da ensilagem e do desempenho de animais. *Revista Brasileira de Zootecnia.* 2009;38:170-189.

19. Ferrari EJ, Lavezzo W. Qualidade da silagem de capim-elefante (*Pennisetum purpureum*, Schum) emurchecido ou acrescido e de farelo de mandioca. *Revista Brasileira de Zootecnia.* 2001;30:1424-1431.

20. O'Brien M, O'Kiely P, Forristal PD, Fuller HT. Quantification and identification of fungal propagules in well-managed baled grass silage and in normal on-farm produced bales. *Animal Feed Science and Technology.* 2007;132:283-297.

21. Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH, Spoelstra SF. Microbiology of ensiling. In: Buxton DR, Muck RE, Harrison JH, editors. Silage science and technology. Madison, Wisconsin, USA: *American Society of Agronomy;* 2003. p. 31-93.

22. Zielińska K, Fabiszewska A, Stefańska I. Different aspects of *Lactobacillus* inoculants on the improvement of quality and safety of alfalfa silage. *Chilean Journal of Agricultural Research.* 2015;75:298-306.

23. Agarussi MCN, Pereira OG, da Silva VP, Leandro ES, Ribeiro KG, Santos SA. Fermentative profile and lactic acid bacterial dynamics in non-wilted and wilted alfalfa silage in tropical conditions. *Molecular Biology Reports.* 2019;46:451-460.

24. Saarisalo E, Skyttä E, Haikara A, Jalava T, Jaakkola S. Screening and selection of lactic acid bacteria strains suitable for ensiling grass. *J Appl Microbiol.* 2007;102:327-336.

25. Bernardes TF, Daniel JLP, Adesogan AT et al. Silage Review: Unique challenges of silages made in hot and cold regions. *J Dairy Sci.* 2018;101:4001-4019.

26. McDonald P, Henderson N, Heron S. The biochemistry of silage. Marlow Bottom: Chalcombe Publications; 1991:340.

27. Wilkinson JM, Davies DR. The aerobic stability of silage: key findings and recent development. *Grass For Sci.* 2013;68:1-19.

28. Driehuis F, Elferink SJWHO, Spoelstra SF. Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. *J Appl Microbiol.* 1999;87:583-594.

29. Oude Elferink SJWH, Krooneman J, Gottschal JC, Spoelstra SF, Faber F, Driehuis F. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri. Appl Environ Microbiol.* 2001;67:125-132.

30. Sriramulu DD, Liang M, Hernandez-Romero D et al. *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. *J Bacteriol.* 2008;190:4559-4567.

31. Krooneman J, Faber F, Alderkamp AC et al. *Lactobacillus diolivorans* sp. nov., a 1,2-propanediol-degrading bacterium isolated from aerobically stable maize silage. *Int J Syst Evol Microbiol.* 2002;52:639-646.

32. Zielińska K, Fabiszewska A, Świątek M, Szymanowska-Powalowska D. Evaluation of the ability to metabolize 1,2-propanediol by heterofermentative bacteria of the genus *Lactobacillus. Elec J Biotechnol.* 2017;26:60-63.

33. Lourenco AB, Ascenso JR, Sá-Correia I. Metabolic insights into the yeast response to propionic acid based on high resolution 1H NMR spectroscopy. *Metabolomics*. 2011;7:457-468.

34. Heinl S, Grabherr R. Systems biology of robustness and flexibility: *Lactobacillus buchneri* - A show case. *Journal of Biotechnology*. 2017;257:61-69.

35. Eikmeyer FG, Heinl S, Marx H, Pühler A, Grabherr R, Schlüter A. Identification of oxygen-responsive transcripts in the silage inoculant *Lactobacillus buchneri* CD034 by RNA sequencing. PLOS ONE. 2015e0134149.

36. Zhang C, Brandt MJ, Schwab C, Gänzle MG. Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiology*. 2010;27:390-395.

37. Ferrero F, Piano S, Tabacco E, Borreani G. Effects of conservation period and *Lactobacillus hilgardii* inoculum on the fermentation profile and aerobic stability of whole corn and sorghum silages. *J Sci Food Agric*. 2019;99:2530-2540.

38. Reis CB, de Oliveira dos Santos A, Carvalho BF, Schwan RF, da Silva Ávila CL. Wild *Lactobacillus hilgardii* (CCMA 0170) strain modifies the fermentation profile and aerobic stability of corn silage. *J Appl Anim Res*. 2018;46:632-638.

39. Waldherr FW, Doll VM, Meißner D, Vogel RF. Identification and characterization of a glucan-producing enzyme from *Lactobacillus hilgardii* TMW 1.828 involved in granule formation of water kefir. *Food Microbiology*. 2010;27:672-678.

40. Carvalho BF, Ávila CLS, Miguel MGCP, Pinto JC, Santos MC, F. SR. Aerobic stability of sugar-cane silage inoculated with tropical strains of lactic acid bacteria. *Grass For Sci*. 2014;70:308-323.

41. Ávila CLS, Carvalho BF, Pinto JC, Duarte WF, Schwan RF. The use of *Lactobacillus* species as starter cultures for enhancing the quality of sugar cane silage. *J Dairy Sci*. 2014;97:940-951.

42. Santos WP, Ávila CLS, Pereira MN, Schwan RF, Lopes NM, Pinto JC. Effect of the inoculation of sugarcane silage with *Lactobacillus hilgardii* and *Lactobacillus buchneri* on feeding behavior and milk yield of dairy cows. *J Anim Sci*. 2017;95:4613-4622.

43. Blajman JE, Páez RB, Vinderola CG, Lingua MS, Signorini ML. A meta-analysis on the effectiveness of homofermentative and heterofermentative lactic acid bacteria for corn silage. *J Appl Microbiol*. 2018;125:1655-1669.

44. Oliveira AS, Weinberg ZG, Ogunade IM et al. Meta-analysis of effects of inoculation with homofermentative and facultative heterofermentative lactic acid bacteria on silage fermentation, aerobic stability, and the performance of dairy cows. *J Dairy Sci*. 2017;100:4587-4603.

45. Service USDANAS. Geographic area series - Part 51. Washington: United States Department of Agriculture; 2019:820.

46. Kung LJ, Shaver RD, Grant RJ, Schmidt RJ. Silage Review: Interpretation of chemical, microbial, and organoleptic components of silages. *J Dairy Sci*. 2018;101:4020-4033.

47. Vance Publishing. 2015. Lallemand Silage inoculant study. page 92.

48. G&S Business Communications. 2019. Forage inoculants portfolio research: United States. page 83.

49. Weinberg ZG, Muck RE, Weimer PJ. The survival of silage inoculant lactic acid bacteria in rumen fluid. *J Appl Microbiol.* 2003;94:1066-1071.

50. Muck RE, Filya I, Contreras-Govea FE. Inoculant effects on alfalfa silage: *In vitro* gas and volatile fatty acid production. *J Dairy Sci.* 2007;90:5115-5125.

51. Weinberg ZG, Shatz O, Y. C et al. Effect of lactic acid bacteria inoculants on *in vitro* digestibility of wheat and corn silages. *J Dairy Sci.* 2007;90:4754-4762.

52. Contreras-Govea FE, Muck RE, Mertens DR, Weimer PJ. Microbial inoculant effects on silage and *in vitro* ruminal fermentation, and microbial biomass estimation for alfalfa, BMR corn, and corn silages. *Animal Feed Science and Technology.* 2011;163:2-10.

53. Mohammed R, Stevenson DM, Beauchemin KA, Muck RE, Weimer PJ. Changes in ruminal bacterial community composition following feeding of alfalfa ensiled with a lactic acid bacterial inoculant. *J Dairy Sci.* 2012;95:328-339.

54. Gänzle MG, Follador R. Metabolism of oligosaccharides and starch in lactobacilli: a review. *Frontiers in Microbiology.* 2012;3:340.

55. Hattingh M, Alexander A, Meijering I, Van Reenen CA, Dicks LMT. Amylolytic strains of *Lactobacillus plantarum* isolated from barley. *African Journal of Biotechnology.* 2015;14:310-318.

56. Nsereko VL, Smiley BK, Rutherford WM et al. Influence of inoculating forage with lactic acid bacterial strains that produce ferulate esterase on ensilage and ruminal degradation of fiber. *Animal Feed Science and Technology.* 2008;145:122-135.

57. Jin L, Dunière L, Lynch JP, McAllister TA, Baah J, Wang Y. Impact of ferulic acid esterase producing lactobacilli and fibrolytic enzymes on conservation characteristics, aerobic stability and fiber degradability of barley silage. *Animal Feed Science and Technology.* 2015;207:62-74.

58. Ellis JL, Hindrichsen IK, Klop G et al. Effects of lactic acid bacteria silage inoculation on methane emission and productivity of Holstein Friesian dairy cattle. *J Dairy Sci.* 2016;99:7159-7174.

59. Daniel JLP, Morais G, Junges D, Nussio LG, editors. Silage additives: Where are we going? 2015; Piracicaba, Brazil: University of Sao Paulo; 2015.

60. Kuoppala K, Rinne M, Vanhatalo A, editors. Lactating cow response to lucerne silage inoculated with *Lactobacillus plantarum*. 2012; Hameenlinna, Finland: MTT Agrifood Research Finland, University of Helsinki, Helsinki, Finland; 2012.

61. Yang HE, Zotti CA, McKinnon JJ, McAllister TA. Lactobacilli are prominent members of the microbiota involved in the ruminal digestion of barley and corn. *Frontiers in Microbiology.* 2018;9:718.

62. Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC, Kung LJ. Silage Review: Recent advances and future uses of silage additives. *J Dairy Sci.* 2018;101:3980-4000.

63. Hoffman PC, Esser NM, Shaver RD et al. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. *J Dairy Sci.* 2011;94:2465-2474.

64. Lynch JP, Jin L, Lara EC, Baah J, Beauchemin KA. The effect of exogenous fibrolytic enzymes and a ferulic acid esterase-producing inoculant on the fibre degradability, chemical composition and conservation characteristics of alfalfa silage. *Animal Feed Science and Technology.* 2014;193:21-31.

65. Lynch JP, Baah J, Beauchemin KA. Conservation, fiber digestiblity, and nutritive value of corn harvested at 2 cutting heights and ensiled with fibrolytic enzymes, either alone or with a ferulic acid esterase-producing inoculant. *J Dairy Sci.* 2015;98:1214-1224.

66. Hafner SD, Howard C, Muck RE et al. Emission of volatile organic compounds from silage: Compounds, sources, and implications. *Atmospheric Environment.* 2013;77:827-839.

67. Weiss K. Volatile organic compounds in silages - Effects of management factors on their formation: a review. *Slovak Journal of Animal Science.* 2017;50:55-67.

68. Åby BA, Randby ÅT, Bonesmo H, Aass L. Impact of grass silage quality on greenhouse gas emissions from dairy and beef production. *Grass For Sci.* 2019;74:in press.

69. Gallo A, Bertuzzi T, Giuberti G et al. New assessment based on the use of principal factor analysis to investigate corn silage quality from nutritional traits, fermentation end products and mycotoxins. *J Sci Food Agric.* 2015

70. Gallo A, Giuberti G, Bruschi S, Fortunati P, Masoero F. Use of principal factor analysis to generate a corn silage fermentative quality index to rank well- or poorly preserved forages. *J Sci Food Agric.* 2015;96:1686-1696.

71. Woolpert ME, Dann HM, Cotanch KW et al. Management practices, physically effective fiber, and ether extract are related to bulk tank milk de novo fatty acid concentration on Holstein dairy farms. *J Dairy Sci.* 2017;100:5097-5106.

72. Daniel JLP, Weiß K, Custódio L et al. Occurence of volatile organic compounds in sugarcane silages. *Animal Feed Science and Technology.* 2013;185:101-105.

73. do Prado RM, Porto C, Nunes E, de Aguiar CL, Pilau EJ. Metabolomics and agriculture: what can be done. *mSystems.* 2018;3:e00156-17.

74. Rasmussen S, Parsons AJ, Jones CS. Metabolomics of forage plants: a review. *Annals of Botany.* 2012;110:1281-1290.

75. Ould-Ahmed M, Decau M-L, Bertrand A, Prud'homme M-P, Lafrenière C, Drouin P. Fructan, sucrose and related enzyme activities are preserved in timothy (*Phleum pratense* L.) during wilting. *Grass For Sci.* 2015;72:64-79.

76. Novais FJ, Pieres PRL, Alexandre PA et al. Identification of a metabolomic signature associated with feed efficiency in beef cattle. *BMC Genomics.* 2019;20:8.

77. Drouin P, Ordaz S, Verrastro L, Sivakumar P. Impacts of silage bacterial additives on forage neutral detergent-soluble fiber. 2018; American Dairy Science Association Annual Meeting, Knoxville, TN.

78. Rezende AVD, Rabelo CHS, Sampaio LDM et al. Ensiling a dry bakery by-product: effect of hydration using acid whey or water associated or not at urea. *Revista Brasileira de Saúde e Produção Animal.* 2016;17:626-641.

79. Nishino N, Hattori N. Resistance to aerobic deterioration of total mixed ration silage inoculated with and without homofermentative or heterofermentative lactic acid bacteria. *J Sci Food Agric.* 2007;87:2420-2426.

80. Nishino N, Harada H, Sakaguchi E. Evaluation of fermentation and aerobic stability of wet brewers' grains ensiled alone or in combination of various feeds as a total mixed ration. *J Sci Food Agric.* 2003;83:557-563.

81. Wang F, Nishino N. Ensiling of soybean curd residue and wet brewers grains with or without other feeds as a total mixed ration. *J Dairy Sci.* 2008;91:2380-2387.

82. Li Y, Wang F, Nishino N. Lactic acid bacteria in total mixed ration silage containing soybean curd residue: their isolation, identification and ability to inhibit aerobic deterioration. A*sian Australasia Journal of Animal Science.* 2016;29:516-522.

83. Ferraretto LF, Silva Filho WI, Fernandes T, Kim DH, Sultana H. Effect of ensiling time on fermentation profile and ruminal *in vitro* starch digestiblity in rehydrated corn with or without varied concentrations of wet brewers grains. *J Dairy Sci.* 2018;101:4643-4649.

84. Gharechahi J, Kharazian ZA, Sarikhan S, Jouzani GS, Aghdasi M, Salekdeh GH. The dynamics of the bacterial communities developed in maize silage. *Microbial Biotechnology.* 2017;10:1663-1676.

85. Guan H, Yan Y, Li X et al. Microbial communities and natural fermentation of corn silages prepared with farm bunker-silo in Southwest China. *Bioresource Technology.* 2018;265:282-290.

86. Wang M, Wang L, Yu Z. Fermentation dynamics and bacterial diversity of mixed lucerne and sweet corn stalk silage ensiled at six ratios. *Grass For Sci.* 2019;in press

87. Romero JJ, Joo Y, Park J, Tiezzi F, Gutierrez-Rodrigues E, Castillo MS. Bacterial and fungal communities, fermentation, and aerobic stability of conventionnal hybrids and brown midrib hybrids ensiled at low moisture with or without a homo- and heterofermentative inoculant. *J Dairy Sci.* 2018;101:3057-3076.

88. Wang C, He L, Xing Y et al. Fermentation quality and microbial community of alfalfa and stylo silage mixed with *Moringa oleifera* leaves. *Bioresource Technology.* 2019;284:240-247.

89. Peng K, Jin L, Niu YD et al. Condensed tannins affect bacterial and fungal microbiomes and mycotoxin production during ensiling and upon aerobic exposure. *Appl Environ Microbiol*. 2018;84:e02274-17.

90. Dunière L, Xu S, Long J et al. Bacterial and fungal core microbiomes associated with small grain silages during ensiling and aerobic spoilage. *BMC Microbiology*. 2017;17:50.

91. Ni K, Zhao J, Zhu B et al. Assessing the fermentation quality and microbial community of the mixed silage of forage soybean with crop corn or sorghum. *Bioresource Technology*. 2018;265:563-567.

92. Ren F, He R, Zhou X et al. Dynamic changes in fermentation profiles and bacterial community composition during sugarcane top silage fermentation: A preliminary study. *Bioresource Technology*. 2019;285:121315.

93. Kreshri, J, Chen Y, Pinto R, Kroupitski Y, Weinberg ZG, Saldinger SS. Bacterial dynamics of wheat silage. *Frontiers Microbiol*. 2019;10:1532.
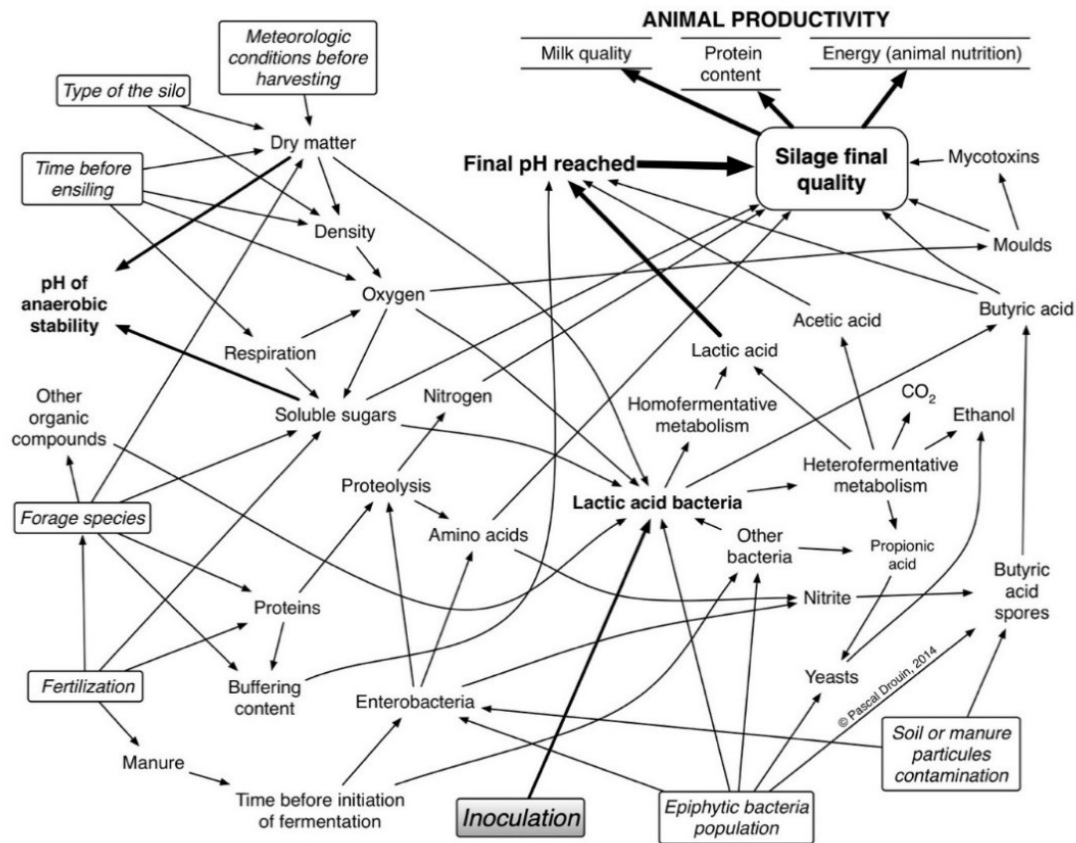
**Figure 1.** Ensiling involves several biochemical and microbiological descriptors that are influencing silage quality and could be control by different management criteria (boxed elements) which are directly influencing the main fermentation parameters of forage as well as animal productivity.
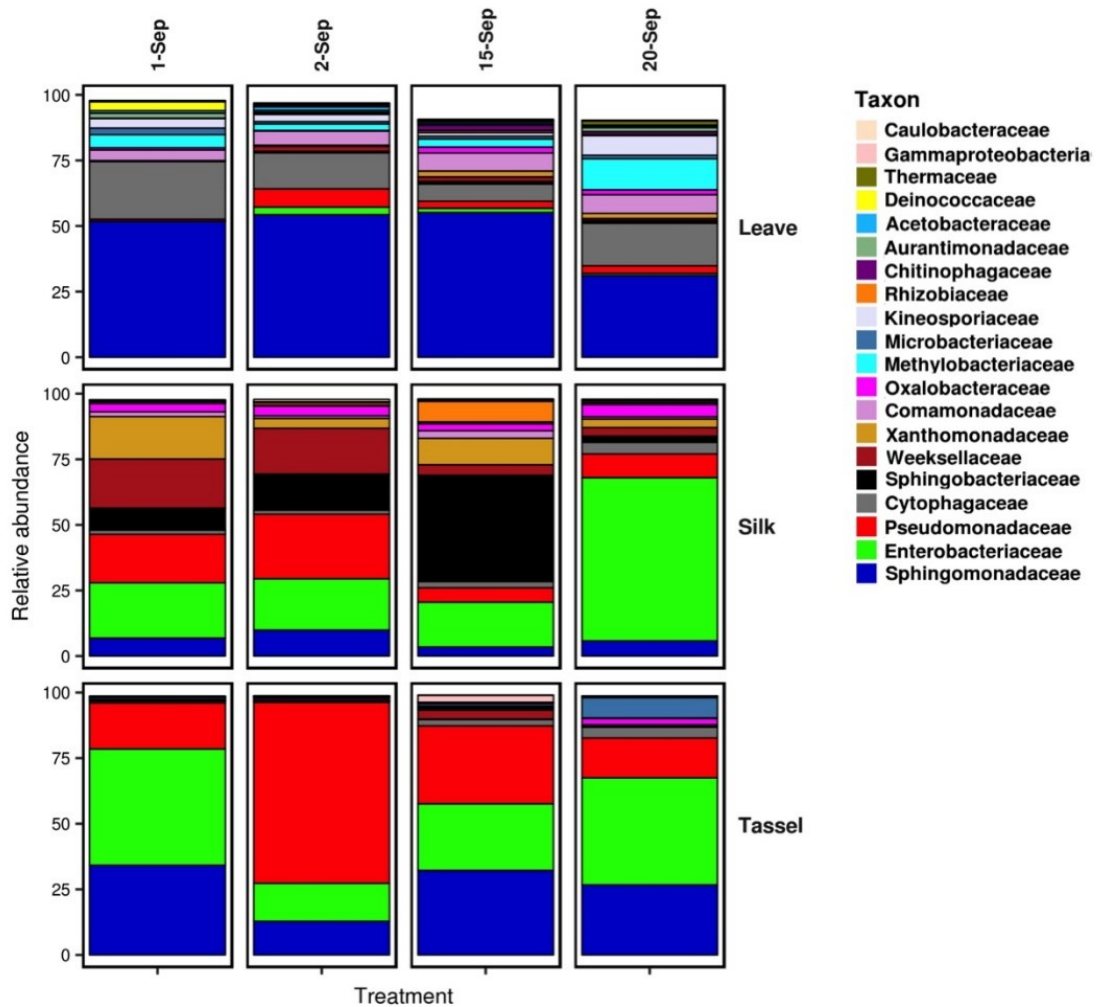


**Figure 2.** Bacterial microbiome from different corn organs (leave, silk, tassel) at four time points prior harvesting (unpublished results).
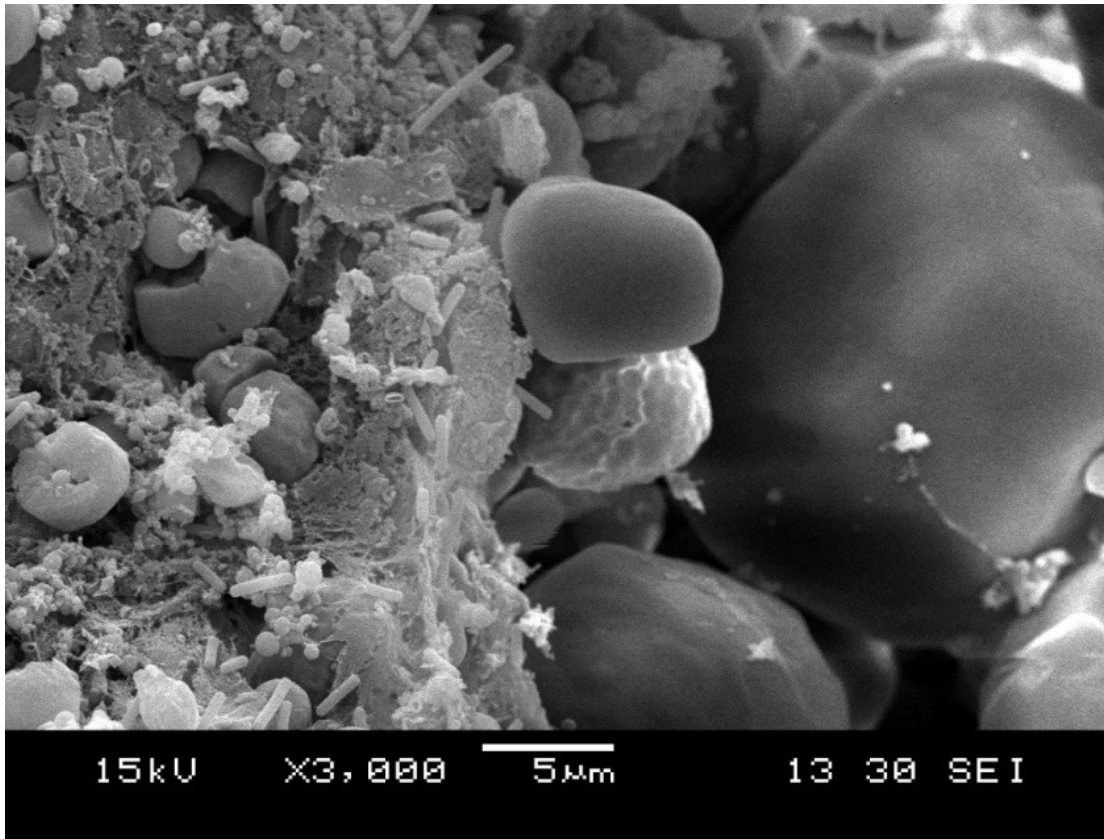
**Figure 3.** Starch granules of corn after several months of ensiling. Rod shape bacteria, putatively LAB, were thriving on fibers particles surrounding the starch granule but not on the granules. Micrograph provided by Lallemand Specialities Inc.

**Table 1.** Characteristics of the silage and experimental design from publications using amplicon based metasequencing to study the microbiome.

| Forage | DM (g kg) | Time of fermentation | Temperature | Inoculation and rate | Abundance of *Lactobacillus* (max) | 16S rDNA amplicons | ITS amplicons | Reference |
|---|---|---|---|---|---|---|---|---|
| **Time related dynamic** | | | | | | | | |
| Alfalfa-grass | 395 | 7 periods, up to 64 days | 20 °C | *L. buchneri* and *L. hilgardii* (4 x $10^5$ CFU g FM) | 61 % | V3-V4 | ITS1-4 | [7] |
| Alfalfa | 421 | 4 periods, up to 90 days | 22 - 25 °C | *L. plantarum* or *L. buchneri* (1 x $10^6$ CFU g FM) | 93 % | Full 16S - PacBio | No | [14] |
| Corn | 381 | 9 periods, up to 90 days | n.a. | *L. plantarum* MTD1 ($10^6$ CFU g FM) | 97 % | V3-V4 | ITS1-2 | [9] |
| Corn | 352 | 8 periods, up to 64 days | 20 °C | *L. buchneri* and *L. hilgardii* (4 x $10^5$ CFU g FM) | 95 % | V3-V4 | ITS1-4 | [7] |
| Manyflower | 410 | 6 periods, up to 30 days | Ambient | No | 75 % | V4-V5 | No | [10] |
| Oat | 456 | 6 periods, up to 90 days | n.a. | *L. plantarum* (1 x $10^6$ CFU g FM) | 97% | V4-V5 | No | [93] |
| **Commercial silos** | | | | | | | | |
| Corn (bunker) | n.a. | Vary | n.a. | n.a. | 96 % | V1-V3 | No | [84] |
| Corn (bunker) | 212 to 373 | 60 days | n.a. | n.a. | 8 to 90 % | V4 | No | [85] |
| Corn-Sorghum (bunker) | 320 to 510 | Vary | n.a. | n.a. | > 90 % | V4 | No | [6] |
| Corn (bag silo) | 383 | 150 | n.a. | *L. buchneri* and *L. hilgardii* (3 x $10^5$ CFU g FM) | | V3-V4 | ITS1-4 | unpublished |
| Corn (bunker) | 360 | 150 | n.a. | *L. hilgardii* (1.5 x $10^5$ CFU g FM) | | V3-V4 | ITS1-4 | unpublished |
| **Experimental silos** | | | | | | | | |
| Alfalfa and sweet corn | 187 to 222 | 65 days | 25 °C | No | 91 to 96 % | V3-V4 | No | [86] |
| Corn (whole) | 380 | 100 days | 23 °C | *L. buchneri* 40788 (4 x $10^5$ CFU g FM) and *P. pentosaceus* (1 x $10^5$ CFU g FM) | 34 (con) to 99 % | V4 | ITS1 | [87] |
| Corn | 234 | 90 days | 22 – 25 °C | *L. plantarum* or *L. buchneri* (1 x $10^6$ CFU g FM) | > 98 % | Full 16S – PacBio | No | [15] |
| Grass (not further defined) | 368 | 14 and 58 days | n.a. | *L. buchneri* CD034 ($10^6$ CFU g FM) | 35 to 67 % (inoculated) | V3-V4 | No | [5] |
| High moisture corn | | 10, 30 and 90 days | 20 – 22 °C | *L. buchneri* and/or *L. hilgardii* (4 x $10^5$ CFU g FM) | 95 % | V3-V4 | ITS1-4 | unpublished |
| *Moringa oleifera* | n.a. | 60 days | 15 and 30 °C | 4 species of LAB (individual) ($10^5$ CFU g FM) | 61 to 97 % | V3-V4 | No | [12] |
| *Moringa oleifera* | 233 | 60 days | 25 to 32 °C | No (vaccum bags) | 86 % | V3-V4 | No | [88] |

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|---|---|---|---|---|---|---|---|---|
| Purple prairie clover | 300 | 76 days | 22 °C | No | 30 % | V3-V4 | V4-V5 | [89] |
| Small grain (mix) | 385 | 90 days | 22 °C | No | 82 % | V3-V4 | V4-V5 | [90] |
| Oat | 450 | 217 days | 23 °C | *L. buchneri* 40788 (4 x 10$^5$ CFU g FM) and *P. pentosaceus* (1 x 10$^5$ CFU g FM) | 57% | V4 | ITS1 | [11] |
| Soybean + corn | 340 | 60 days | 15 to 30 °C | No | 60 to 80 % | V3-V4 | No | [91] |
| Sugar cane | n.a. | 90 days | 20 to 35 °C | No | 50 % | V4 | No | [92] |