Proceedings of the VI International Symposium on Forage Quality and Conservation

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Thursday – November 07th, 2019

- 07:40-08:10 – Registration
- 08:10-08:30 – Opening Ceremony
- 08:30-09:10 – Interpretation of chemical, microbial, and organoleptic components of silages (Limin Kung Jr. – University of Delaware, USA)
- 09:10-09:50 – Present and future of microbial inoculants for silages (Lucas Mari – Lallemand, Brazil)
- 09:50-10:20 – Coffee Break
- 10:20-10:35 – Volunteered paper
- 10:35-10:50 – Volunteered paper
- 10:50-11:30 – Chemical additives for silages: When to use and what options we have (Horst Auerbach –KONSIL Europe GmbH, Germany)
- 11:30-11:40 – Sponsortime
- 11:40-12:30 – Poster exhibition
- 12:30-14:00 – Lunch
- 14:00-14:40 – Strategies to explore the potential of corn hybrids (Thiago Bernardes – Federal University of Lavras, Brazil)
- 14:40-14:50 – Sponsortime
- 14:50-15:20 – Coffee Break
- 15:20-16:00 – Recent advances and future technologies for silage harvesting (Brian Luck – University of Wisconsin, USA)
- 16:00-16:40 – Profiling of metabolome and bacterial community dynamics in silages (Xusheng Guo – Lanzhou University, China)

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Contents

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] *Lallemand Animal Nutrition*

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.

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.

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 CO2 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.

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 H2O, CO2 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 pKa between these weak acids and lactic acid, which is a stronger acid, with a pKa of 3.86. At higher pKa, 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 pKa 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 CO2 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.

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.

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.

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 105 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 (105 vs. 106 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.

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].

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.

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)

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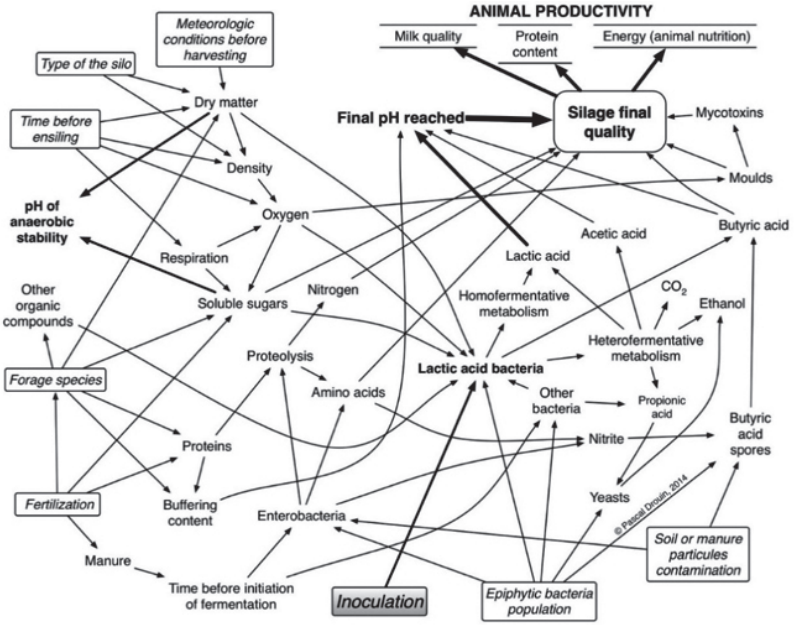

Figure 1. Ensiling involves several biochemical and microbiological descriptors that are influencing silage quality and could be control by different management criteria (boxed elements) which are directly influencing the main fermentation parameters of forage as well as animal productivity.

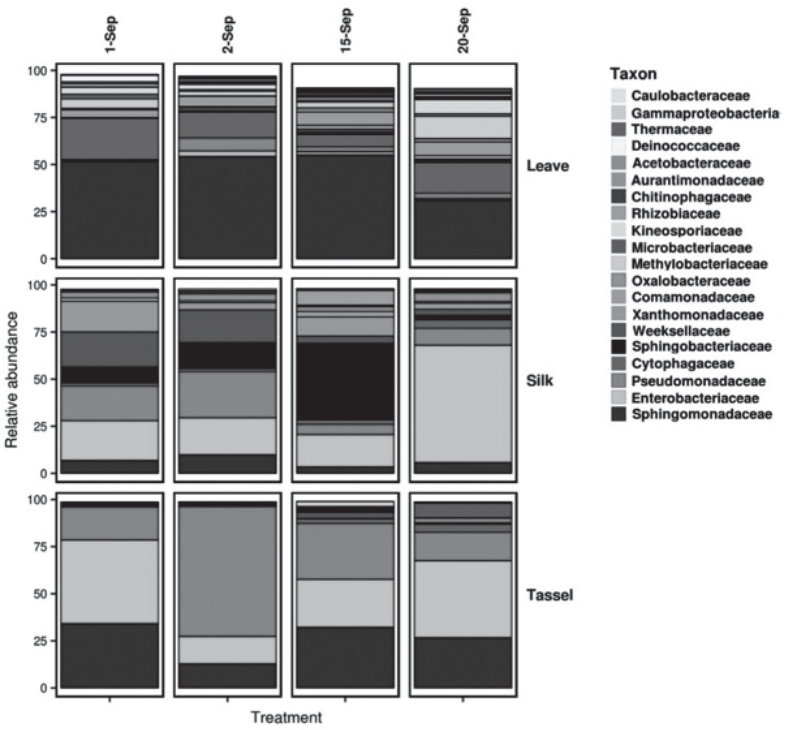

Figure 2. Bacterial microbiome from different corn organs (leave, silk, tassel) at four time points prior harvesting (unpublished results).

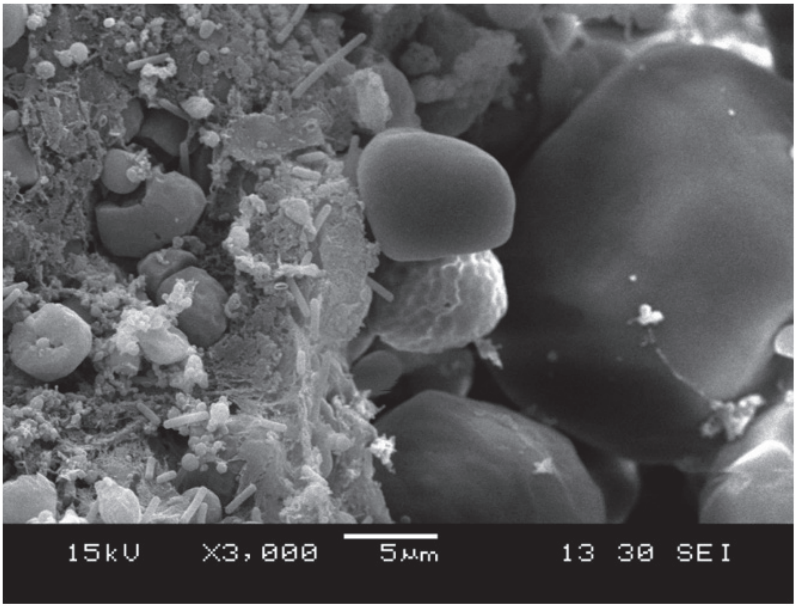

Figure 3. Starch granules of corn after several months of ensiling. Rod shape bacteria, putatively LAB, were thriving on fibers particles surrounding the starch granule but not on the granules. Micrograph provided by Lallemand Specialities Inc.

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 10^5 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 10^5 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 (vacuum bags)	86 %	V3-V4	No	[88]
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	57%	V4	ITS1	[11]

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

Chemical additives for silages: When to use it and what are the options?

HORST AUERBACH[1]
ELISABET NADEAU[2]

In memory of Professor Dr. Friedrich Weissbach

Introduction

Maintaining the highest possible quality of silage from field to trough poses one of the biggest challenges for dairy and beef producers worldwide as it requires thorough management to reduce dry matter (DM) losses to a minimum from field to feed-out. This is necessary to keep high animal performance and to ensure farm profitability. In addition to the well-known general principles of silage making, the use of additives has become a strategic management tool in silage production. Biological additives, mainly using different genera and species of lactic acid bacteria, have become the dominant additive type but chemical additives still play a major role in certain regions, especially in Europe. Published research provides evidence that there also has been an increasing interest in other areas of the world. This paper aims at providing guidance on which of the available active ingredients that can be used to solve a specific problem in silage production, and under which conditions those should be used.

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Chemical additives to overcome the principal challenges for silage quality

Silage quality can be compromised by the activity of a range of undesired microorganisms, which grow and thrive under different conditions. In silages made from crops of high protein content and buffering capacity, especially if ensiled at low DM levels, the risk for secondary fermentation during the storage phase caused mainly by clostridia is most prominent. Their activities result in high DM losses, proteolysis as reflected by high concentrations of ammonia-N, and the formation of biogenic amines (Scherer et al., 2015; Auerbach et al., 2016; König et al., 2016; Borreani et al., 2018; König et al., 2018). However, sometimes high anaerobic DM losses can be associated primarily with the development of yeasts forming ethanol in excess of 10 g/kg DM, which can be observed mainly in high DM grass, whole-crop cereals and corn (Driehuis and van Wikselaar, 1996; Nadeau et al., 2013; Weiss et al., 2016; Auerbach et al., 2018,), although the contribution of other ethanol producers, e.g. heterofermentative lactic acid bacteria (LAB), should not be neglected (Rooke and Hatfield, 2003). Data depicted in Figure 1 show the relationship between butyric acid or ethanol concentrations and anaerobic DM losses (Auerbach et al., 2016; Auerbach et al., 2018). Additional DM losses by heating processes may be incurred during feed-out, which is usually initiated by yeasts. At later stages, bacilli, other aerobic bacteria and molds contribute to deterioration, thereby increasing temperature further and leading to reduced nutritive value and performance (Wilkinson and Davies, 2013; Auerbach and Nadeau, 2018a; Borreani et al., 2018), and mycotoxin formation (Auerbach and Theobald, 2018; Ferrero et al., 2019).

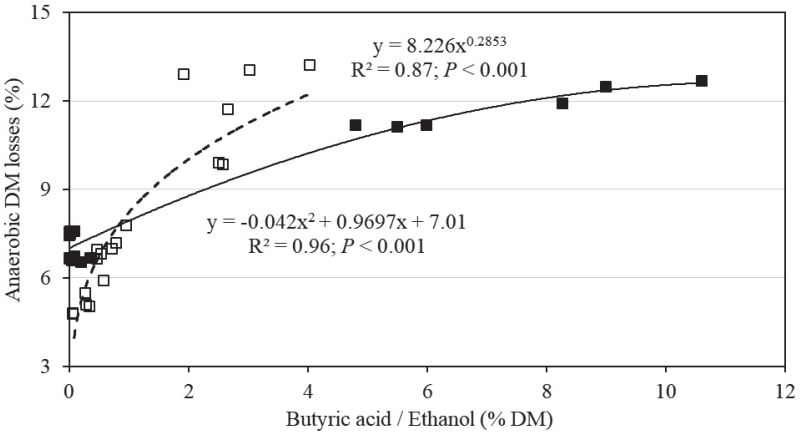

Figure 1. Relationships between the concentrations of butyric acid in silage from alfalfa and orchardgrass (▪, solid regression line, n=24, Auerbach et al., 2016) or ethanol in silage from whole-crop rye (▪, dotted regression line, n=18, Auerbach et al., 2018) and anaerobic DM losses.

There is a variety of chemicals available that are used as silage additives. These are usually classified according to their inhibitory effects on target microorganisms to improve fermentation or increase aerobic stability (ASTA) (Table 1). This classification has some drawbacks and should be looked at from a practical perspective as already Paracelus (1538) stated that "All things are poisons, and nothing is without poison; only the dosage is it that makes a thing not a poison". This means that we will see effects of typical fungal inhibitors on undesired bacteria and of typical bacteria inhibitors on fungi given that the application rate is high enough (Table 2). However, cost per kg of active ingredient in combination with high dosage will ultimately increase treatment costs, rendering the use as economically unfeasible.

Aim/type	Ingredient/species	Effects
Improving the fermentation process		
acids	formic	direct acidification, suppression of undesired spoilage bacteria
salts	calcium formate, sodium formate, ammonium formate, sodium nitrite, hexamethylene tetramine	suppression of undesired spoilage bacteria
Improving aerobic stability		
acids	sorbic, benzoic, propionic, acetic	inhibition of yeasts and molds
salts	sodium benzoate, potassium sorbate, ammonium propionate, calcium propionate, sodium propionate, sodium acetate	inhibition of yeasts and molds after release of respective acid during fermentation

Table 2. Proportion of silages free of butyric acid or with low DM losses as affected by silage additive use (10 trials with 3 replicates per treatment, adapted from Weissbach, 2010b).

Treatment	Proportion (%)	
	Silages free of butyric acid (< 0.3% of DM)[1]	Low fermentation losses (DM loss < 8%)[2]
Control	33.3a	20.0a
Formic acid (85%, 4 l/t)	60.0a	82.8b
Sodium nitrite/Hexamine (3 l/t)[3]	100.0b	82.8b
Sodium benzoate (2 kg/t)	100.0b	100.0c
Sodium benzoate (2 kg/t) + Sodium nitrite (0.6 kg/t)	100.0b	100.0c
Significance[4]	$P < 0.001$	$P < 0.001$

[1]dry matter corrected for the loss of volatiles during drying; [2]n=29 for DM loss, [3]mixture of sodium nitrite (300 g/L) and hexamine (200 g/L); [4]Likelihood ratio Chi-Square test, frequency values with different superscripts differ (Fisher´s Exact Test).

Chemical additives to improve fermentation

Fermentability of the crop to be ensiled is one of the most important factors affecting the choice of additive type. Results of an evaluation adapted from Honig and Thaysen (2002) including 673 comparisons between treated and untreated silage are presented in figure 2. The underlying official German silage additive evaluation scheme (Pauly and Wyss, 2019) distinguishes between three classes within the aim-of-action (AoA) 1 – Improving the fermentation process – based on the crop´s fermentability coefficient (FC). This parameter is calculated using the following equation: FC = DM (%) x 8 WSC/BC, where WSC is water-soluble carbohydrate concentration and BC is buffering capacity expressed as g lactic acid/kg DM required to acidify the silage to pH 4.0: 1) difficult to ensile, crops with insufficient WSC content and/or low DM, FC < 35; 2) moderately difficult to easy to ensile in the lower DM range, FC ≥ 35, DM < 35%; 3) moderately difficult to easy to ensile in the upper DM range, FC ≥ 35, DM ≥35%. It can clearly be seen that chemical additives, including acids or salts solely or mixtures of acids and salts, outperformed homofermentative LAB inoculants. The scoring system back in 2002 considered the parameters pH, acetic acid, butyric acid (sum of n- and iso-butyric acid, n- and iso-valeric acids and n-caproic acid all expressed in % of DM) and ammonia-N (% of total N), with 100 points being the maximum score.

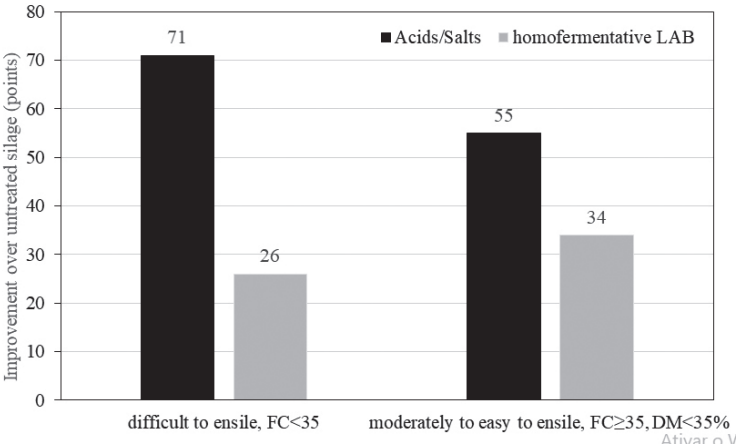

Figure 2. Effects of silage additives on fermentation quality as affected by fermentability based on the silage evaluation scheme of 2002 by The German Agricultural Society (DLG) (max. score 100 points) (Honig and Thaysen, 2002).

However, studies such as this one, or meta-analyses (Kleinschmit and Kung, 2006; Morais et al., 2017; Oliveira et al., 2017) although being very helpful regarding the assessment of general effects and their consistency, have serious limitations as they always use chemical and biological additives of different compositions applied to different crops stored under different conditions, which certainly has a significant effect on the results. Thus, this type of investigation is useful to characterize the potential of additive types, but not of given products. To evaluate commercially available products in terms of consistency of their effect on silage quality, a rather large number of trials (we advise to carry out at least 10 but the more the better) have to be conducted, covering a range of crops for which the additive is intended to be used, and DM concentrations.

From the historical point of view, formic acid has been the most important chemical additive to improve fermentation

and numerous studies have been published on the positive effects on fermentation, and animal performance (Haigh and Parker, 1985; Steen, 1990; Nadeau et al., 2000a; Nadeau et al., 2000b; Broderick et al., 2007), which is still the main additive used in northern Europe. However, due to its corrosive nature on metal and skin/eyes, partial buffering with ammonia or sodium has become very popular, and frequently it is blended with other chemicals, e. g. propionic and benzoic acids. However, improving handling properties comes at the expense of efficacy so that higher application rates are required when compared with pure formic acid (Randby, 2000).

Despite these developments, certain risks remain such as anectodal reports of machinery operators complaining about eye and lung irritation due to pungent formic acid smell when applied on the chopper, or while compacting. Usually, applications of acid-based products are done at the chute`s rear end in the top-deflector of self-propelled harvesters in order to avoid corrosion of the expensive machinery. However, this application location resulted in the largest variation of additive application rate and increased the frequency of samples that had received less than the intended dosage of 5 L/t, although the average dosage did not differ between application locations (Nysand and Suokannas, 2012). Moreover, effluent production posing an environmental risk is increased by pure and buffered formic acid-based products (O`Kiely, 1993; Jones and Jones, 1995,). Heavy compaction may stimulate effluent production and seepage during the early stages of fermentation. This is supported by empirical observations from the field where effluent is produced already during the filling period, which can last several days on large farms, and at DM contents at which seepage is not to be expected.

The aforementioned disadvantages of using formic-acid based products have been overcome by the use of combinations

of sodium nitrite, one of the most potent clostridia inhibitors, and hexamethylene tetramine (hexamine), which was first introduced in the Eastern German market in the mid 1980ies after very thorough research under the brand name CEKAFUSIL, containing 300 g/L of sodium nitrite and 200 g/L of hexamine with a maximum application rate of 3 L/t of fresh matter (FM), depending on the crop DM at ensiling (Reuter and Weissbach, 1991). As demonstrated in table 3, this combination applied at 3 L/ t FM was as efficient as pure formic acid (85%, 4 L/t FM), but has no corrosive properties and does not stimulate effluent production.

Table 3. Effects of chemical additives on DM losses during fermentation, butyric acid production and clostridia counts (Weissbach, 2010a).

Parameter	Control	Nitrite/ Hexamine[1] (3 l/t)	Formic acid (85%) (4 l/t)
DM loss[2] (%)	11.0	7.1*	6.2*
Frequency of silages with low DM losses[3] (%)	28	70*	73*
Butyric acid content (% FM)[2]	1.17	0.35*	0.45*
Proportion of butyric acid-free-silages[4](%)	27	74*	67*
Proportion of silages with low spore counts[5] (%)	44	71*	43

[1]containing sodium nitrite (300 g/L) and hexamine (200 g/L); [2]n=363, [3]DM loss ≤ 8%, n=291; [4]butyric acid < 0.2% of fresh matter (FM);[5]≤ 1,000 clostridia (MPN)/g, n=75; *denotes significant differences of treatment vs. control at $P< 0.05$.

Both formic acid/propionic acid-based additives and nitrite-containing additives in various blends with hexamine, benzoate, sorbate and propionate have been shown to be efficient to eliminate butyric acid production and decrease ammonia-N concentration in grass-clover silage contaminated with soil

containing clostridia (Nadeau and Auerbach, 2014). Frequently, nitrite/hexamine-containing additives have been shown to be superior to formic acid-based products in terms of reducing clostridia contamination and butyric acid formation (Lättemäe and Lingvall, 1996; Lingvall and Lättemäe, 1999; Knicky and Lingvall, 2004; Knicky and Spörndly, 2009; König et al., 2016; König et al., 2018). The overall effect of the combination of nitrite and hexamine is caused by eradicating clostridia and their spores by nitrite and its decomposition products, nitric oxides, during the early phases and, at later stages, by the release of formaldehyde from hexamine, which is caused by a drop in pH. The findings by Weissbach (2010a) that hexamine addition to nitrite improved silage quality over that of untreated and silage that received nitrite only was recently opposed by König et al. (2018), who could not find an additional effect. However, this statement warrants caution as it was based on one experiment, where only one crop was ensiled at two different DM contents and the untreated silages contained very low butyric acid concentrations (<0.5 g/kg DM). Unpublished results by the University of Maringa, Paraná, Brazil on tropical grass, which is usually low in sugar and low in DM, showed that also in this crop chemical additives have the potential to improve the efficiency of the fermentation process as reflected by lower DM losses during fermentation by up to 60% when compared with untreated silage, and that the combination of sodium nitrite and hexamine was superior to the sole use of sodium nitrite (Table 4).

Table 4. Effects of additives on fermentation and DM losses of tropical grass (*Panicum maximum cv.* Mombaça) (University of Maringá, Paraná, Brazil, unpublished).

Item	Control	Soybean hulls[1]	Sodium nitrite[2]	Sodium nitrite+ Hexamine[3]	Formic acid[4]	SEM	P value
pH	4.60$_{bc}$	4.89^{a}	4.66^{b}	4.79^{ab}	4.44c	0.053	< 0.01
DM loss, %	15.8^{a}	12.9^{b}	9.2c	6.5^{d}	7.1^{d}	0.07	< 0.01
Lactic acid (% of DM)	0.20$_{c}$	0.92^{b}$_{c}$	1.72^{b}	2.99^{a}	3.71^{a}	0.323	< 0.01
NH3-N (% of DM)	0.26^{a}	0.24^{a}	0.21^{a}	0.22^{a}	0.13^{b}	0.014	< 0.01
NH3-N (% of DM)[5]	0.26^{a}	0.24^{a}	0.17^{b}	0.09$_{c}$	0.13^{b}c	0.014	< 0.01

[1]100 kg/t; [2]1 kg/t; [3]1 kg/t sodium nitrite + 0.65 kg/t hexamine; 485%, [4] L/t; 5corrected for addition of nitrogen by additives.

Hexamine, which provides a long-lasting effect against clostridia, which may not have been eradicated by sodium nitrite. According to Auerbach et al. (2016), it can likely be replaced by other components, which remain in the silage. In their studies on alfalfa and orchardgrass ensiled at low DM (20-23%) with high ash contents (13-18% of DM), the sodium nitrite concentration was kept identical in all three different additives tested, but hexamine was replaced in two treatments by either sodium formate/sodium benzoate or ammonium formate/potassium sorbate with no difference in DM loss, fermentation pattern and biogenic amine formation. As shown by Nadeau et al. (2016) and Nadeau et al. (2019), both nitrite-containing and formic/propionic-based additives decrease proteolysis during ensiling of grass and grass-legume forages, as indicated by decreased concentrations of nonprotein N and ammonia-N content but increased proportion of cell-wall bound protein compared to untreated silage. Because additives containing sodium nitrite and/or hexamine and ammoniated acid mixtures directly add ammonia, for instance hexamine decomposes under acidic conditions into formaldehyde and ammonia, and nitrite is converted into nitric oxides and ammonia, the correction of the ammonia-N concentrations must be carried out in order

to avoid false results because ammonia production originating from these aforementioned reactions is not caused by proteolysis (Table 5). It is obvious that the magnitude of the effect of NH3-correction is bigger in low-nitrogen crops (Auerbach et al., 2012).

Table 5. Ammonia-N concentrations in silages as affected by correction for the ammonia applied with the additive (Auerbach et al., 2012).

Crop	Crude protein of fresh crop (% DM)	NH3-N (% total N)		
		Untreated	Nitrite/Hexamine[1]	
			uncorrected	corrected[2]
Green rye, ear emergence	15.2	18.5	13.0	6.8
Grass, not fertilized	12.1	9.0	10.9	5.7
Grass, not fertilized	9.6	9.4	12.8	5.7
Grass/clover	15.3	9.2	11.6	8.6
Grass, not fertilized	9.5	10.7	6.9	1.0
Grass/clover	15.0	6.7	9.4	6.6
Alfalfa	15.5	13.4	12.2	9.5
Grass, late cut	8.9	7.7	10.5	4.9
Whole-crop barley	4.8	10.5	14.0	4.1
Grass, late cut	6.0	8.1	10.8	3.8
Whole-crop barley	4.9	10.1	11.5	3.6
Whole-crop barley	4.6	8.0	10.6	3.2
Grass, late cut	7.1	9.4	9.4	4.7
Grass, late cut	7.9	8.3	8.8	4.1
Alfalfa	22.9	8.3	6.8	5.2
Grass, fertilized	20.5	14.1	10.8	8.4
Grass, fertilized	15.1	17.1	14.2	9.8
Mean	12.1	10.5	10.1	5.3

[1]containing sodium nitrite (300 g/L) and hexamine (200 g//L) applied at 3 L/t; 2corrected for the addition of NH3-forming chemicals.

When additives are used to improve the fermentation process, also their effects on ASTA cannot be ignored as well-fermented silages tend to be particularly prone to aerobic instability. Particularly under challenging farm conditions of low feed-out rate, it is important to maintain the nutritive value until the silage is ingested by the animal (Auerbach and Nadeau, 2018a; Auerbach and Nadeau, 2018b). In series of 20 trials by Bader (1997), the use of nitrite/hexamine was shown to be superior to the application of a homofermentative LAB inoculant in terms of DM loss and clostridia contamination (Table 6). However, although the mean ASTA was not affected by treatment, inoculation increased the frequency of silages with low ASTA (≤ 3 days) from 24% in untreated silage to 42% ($P < 0.05$), whereas only 16% of the silages that received nitrite/hexamine had low ASTA ($P < 0.05$), and no difference to untreated silage was found. These data were confirmed by Honig and Thaysen (2002), who also did not observe an effect of acid/salt use on ASTA use but reported a significant decline by 1 day by treatment with homofermentative LAB.

Table 6. Effects of sodium nitrite/hexamine and homofermentative LAB on DM losses during fermentation, clostridia contamination and aerobic stability from 20 trials (Bader, 1997).

Parameter	Control	Nitrite/ Hexamine[1] (3 l/t)	LAB^{ho}[2]
DM loss[3] (%)	6.1	4.6*	5.8*
Frequency of silages with DM losses < 5% (%)[3]	17	65*	23*
Frequency of silages with low spore counts (%)[4]	70	90*	60*

[1]containing sodium nitrite (300 g/L) and hexamine (200 g/L); [2]combination of two *Lactobacillus plantarum* strains applied at a total inoculation rate of 100,000 cfu/g herbage; [3]n=60, 4≤ 10,000 clostridia (MPN)/g; *denotes significant differences between nitrite/hexamine or homofermentative LAB vs. control ($P < 0.05$).

Our own results from a total of six trials on grasses (three trials, 23.5 to 31.2% DM) and early-cut rye harvested before ear emergence (three trials, 25.0 to 38.8% DM) are presented in figure 3. They prove that inoculation with homofermentative LAB across trials reduced ASTA by 76 hours when compared with untreated silage ($P < 0.001$), and the use of nitrite/hexamine had no effect. Inoculation with homofermentative LAB, whose aim is to dominate the fermentation, results in a shift in metabolic end-products towards more lactic acid at the expense of butyric and acetic acids (Nadeau et al., 2018), which have an antifungal effect, but lactic acid can be utilized by a variety of yeasts as carbon and energy source (Jonsson and Pahlow, 1984; Santos et al., 2016). On the contrary, chemical additives including nitrite and hexamine do not selectively stimulate homofermentative LAB but allow the whole epiphytic LAB flora, which is never exclusively composed of homofermentative species, to develop and produce acetic acid/ethanol, in addition to lactic acid, given that the application rate is not too high to generally inhibit LAB.

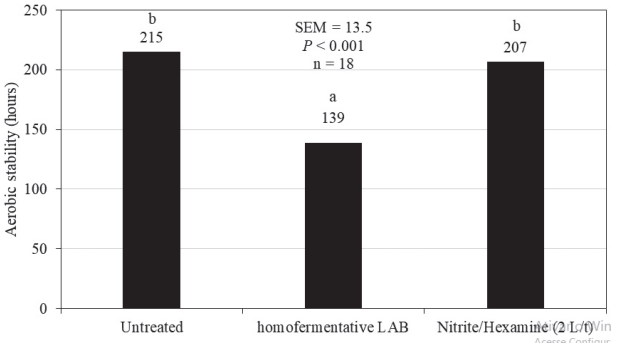

Figure 3. Effects of a liquid blend of sodium nitrite (300 g/L) and hexamine (200 g/L) applied at 2 L/t and a homofermentative LAB inoculant, applied at 1 g/t forage supplying 100,000 cfu/g forage of *Lactobacillus plantarum* DSM 16627 and 50,000 cfu/g forage of Lactobacillus paracasei NCIMB 30151, on the aerobic stability of silages from grasses (three trials) and early-cut rye (three trials) stored for > 90 days; a-bbars bearing unlike superscripts differ at $P < 0.05$ (Tukey`s test) (Auerbach et al., unpublished).

The magnitude of the effect of chemical additives not only depends on the composition but also on the application rate. Any approach taken by the farmer "to purposely applying less than the recommended rates of application to save money is a dubious practice because it only increases the probability that the additive will not be successful" (Kung, 2009). It has been well known that application rate is crucial regarding the magnitude of the effect. Randby (2002) showed on grass silage that increasing application rates of a liquid unbuffered acid blend containing 640 g/kg formic acid, 93 g/kg propionic acid and 19 g/kg benzoic acid applied at 2, 3, 4 and 5 L/t forage increased residual water-soluble carbohydrate concentrations and ASTA. Similar dose effects on butyric acid concentrations were obtained by Custodio et al. (2016) on sugarcane silage, which was treated with a combination of lime (CaCO3 at 15 kg/t) and graded doses of sodium nitrite (0.5, 1.0, 1.5 kg/t forage). This is in line with observations on a wide range of crops used in 21 trials by Weissbach (2010a) using a combination of sodium nitrite (300 g/L) and hexamine (200 g/L) applied at either 2 or 3 L/t (Table 7).

Table 7. Effects of application rate of a chemical additive on butyric acid formation in silages made from a variety of crops (n=63, Weissbach, 2010a).

		Sodium nitrite / Hexamine[1]			
Parameter	Control	2 L/t		3 L/t	
Butyric acid (% FM)					
Mean	1.07	0.36		0.15	
SD[2]	0.90	0.60		0.37	
Difference to control		0.71	*	-0.92	*
Difference to lower dosage				-0.21	
Frequency of butyric acid-free (< 0.2% of FM) silage					
Mean	30	67		81	
Difference to control		37	*	51	*
Difference to lower dosage				14	

[1]containing sodium nitrite (300 g/L) and hexamine (200 g/L); *denotes significant difference at $P < 0.05$; [2]standard deviation.

Chemical additives to improve aerobic stability

Aerobic deterioration of silage has become a major problem in silage production on many farms in the world, with corn silage being considered the most vulnerable silage type but also silages from grasses with high sugar concentrations or from whole-crop cereals can undergo severe spoilage process during feed-out. Since its introduction to the markets in the late 20th century, the heterofermentative *Lactobacillus buchneri* either applied solely or in combination with homofermentative LAB have become very popular globally due to the improvements in ASTA (Kung et al., 2003; Kleinschmit and Kung, 2006; Muck et al., 2018). In addition to acetic acid production from sugar, which is facilitated by all obligate heterofermentative LAB species, *Lactobacillus buchneri* also anaerobically converts lactic acid to antifungal acetic acid, 1,2-propanediol and ethanol (Oude-Elferink et al., 2001). This metabolic pathway increases DM losses during the anaerobic fermentation phase and requires a minimum of 6-8 weeks of storage before silo opening in order to have sufficiently high acetic acid concentrations to inhibit fungi consistently. In a meta-analysis by Kleinschmit and Kung (2006), an overall decrease in DM recovery by *Lactobacillus buchneri* inoculation was observed in silages from corn or grass/small grain silages, which depended on the inoculation rate. Usually though, the increased DM losses during storage offset those incurred by fungi under aerobic conditions. Honig and Thaysen (2002) found a significant increase in DM losses by *Lactobacillus buchneri* treatment by 10% (n=173) compared with untreated, whereas the application of chemical additives of unspecified composition did not have an effect. No difference in the magnitude of the effect on ASTA were found, ranging between +2.4 days for heterofermentative LAB and +2.2 days for chemicals when compared with untreated silages.

Under certain circumstances, such as DM concentrations below 30% (Auerbach and Weiss, 2012; Gomes et al., 2018) excessive acetic acid concentrations can be produced. Occasionally, also at typical DM contents ranging between 30 and 40%, very high concentrations of this organic acid can be detected, leading to much higher DM losses than usually seen compared with untreated (Driehuis et al., 1999; Kleinshmitt et al., 2013; Auerbach and Nadeau, 2018b). On the contrary, lower DM losses were found when untreated silages underwent ethanolic fermentations, which can be prevented by *Lactobacillus buchneri*, as reported for sugarcane silage by Rabelo et al. (2018) and for whole-crop rye by Auerbach et al. (2018). When tested in the same trial, however, chemical additives outperformed *Lactobacillus buchneri*-type products regarding anaerobic DM losses (Auerbach and Weiss, 2012; Auerbach and Nadeau, 2018a; Auerbach and Nadeau, 2018b).

The question as to which chemical is best to improve silage ASTA is not quite easy to answer. It has been well documented that the minimum inhibitory concentration (MIC) of organic acids and their salts depend on the microorganism tested, and the pH of the medium (Woolford, 1975a; Woolford, 1975b; Auerbach, 1996; Stanojevic et al., 2009). Reducing pH from 5 to 4 decreased MIC (mmol/L) against yeasts and molds for each chemical investigated, but the following order remained unchanged: potassium sorbate < sodium benzoate < propionic acid < acetic acid < formic acid (Woolford, 1975a, Woolford, 1975b). Although *in vitro* screening tests are very useful to determine the relative differences between chemicals, the MIC values derived from those tests should be treated with caution, and due to the complexity of the silage ecosystem they can very likely not be directly extrapolated to a silage environment. Moreover, the tested microorganisms may not even be found in silage. Driehuis and van Wikselaar (1996) conducted one trial on

grass and maize silage using equimolar concentrations of formic (3.3 kg/t), acetic acid (4.3 kg/t) or propionic acid (5.3 kg/t) and concluded that formic acid improved ASTA in maize silage but not in grass silage, in which propionic acid had the best effect. Acetic acid failed to increase ASTA in both trials. The lack of response to propionic acid in maize silage, despite the lowest yeast count, was explained by the fact that acetic acid bacteria and not yeasts caused silage to deteriorate, whereas in grass silage acetic acid bacteria were below the detection limit in all treatments. However, only testing the organic acids at the same application (kg/t) would have made a direct comparison possible. Our own data presented in Figure 4 on corn ensiled at 41% DM and stored for 59 days with air ingress for 24 hours on day 28 and 52 show that already the lowest concentration of potassium sorbate (500 g/t) improved ASTA, which substantiates recent findings by Huenting et al. (2018) using potassium sorbate at 400 g/t. On the contrary, 1000 g/t of sodium benzoate were required to show an effect on ASTA, supporting data by Kleinschmit et al. (2005) who detected a similar effect for potassium sorbate at 500 g/t and sodium benzoate at 1000 g/t.

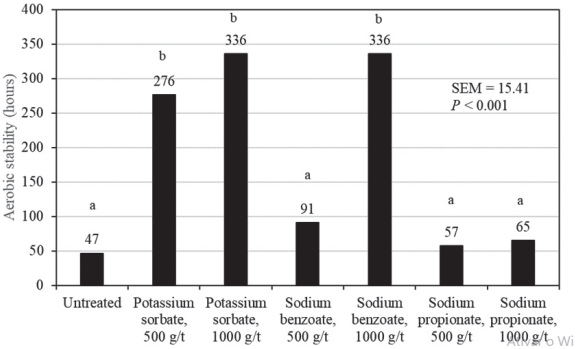

Figure 4. Effects of antimycotic chemical additives applied at 500 or 1000 g/t on the ASTA of corn silage ensiled at 41% DM and stored for 59 days with air ingress for 24 hours in day 28 and 52 during subsequent air exposure for 336 hours after silo opening. a-bbars with unlike superscripts differ at $P < 0.001$ (Tukey´s test, n = 3) (Auerbach et al., unpublished).

Moreover, our data confirm those by Teller et al. (2012) who found improved ASTA in corn silage by adding potassium sorbate applied at 1000 g/t, but the magnitude of the effect was lower in their trial (+121 hours higher ASTA compared with untreated). Da Silva et al. (2014) reported higher ASTA of corn silage treated with sodium benzoate applied at 2000 g/t when compared with untreated silage, or those that were inoculated with *Lactobacillus buchneri*. Furthermore, Bernardes at al. (2014) tested 1000 g/t and 2000 g/t of either potassium sorbate or sodium benzoate in corn silage ensiled at 37% DM and found a dose-response regarding the onset of aerobic instability (silage temperature +2 °C above ambient), but no difference between the chemicals within application rate. However, yeast development differed. At the end of aerobiosis, silage treated with potassium sorbate had the lowest yeast counts indicating that growth was retarded. Sodium propionate did not improve ASTA in our study regardless of the application rate. In a trial by Kung et al. (2000) on corn, a dose-response to the application of a buffered propionic acid-based product (1000 to 3000 g/t) was observed but only the highest dosage had a significant effect over that of untreated silage.

Single-component chemical additives to minimize aerobic deterioration are rarely used in practical farming. It has been much more common to combine different active ingredients, which makes it very difficult, if not impossible, to directly compare the results. Blending different ingredients aims at optimizing costs due to large differences in price between raw materials, at extending the range of microorganisms to be inhibited due to potential differences between substances, and sometimes at creating synergistic effects, but these have only been reported for a mixture of sodium nitrite and sodium benzoate on Candida albicans and not for other important silage yeasts or mold species (Stanojevic et al., 2009).

The meta-analysis on the effects of chemical additives carried out by Morais et al. (2017) on high-moisture corn (HMC) or high-moisture winter cereal grains from wheat, barley and rye (HMWCG) revealed improvements in ASTA by a range of additives of different compositions and application rates by 131 hours in HMC ($P < 0.01$) and by 116 hours in HMWCG ($P = 0.10$) but this study also does not allow to draw conclusions on the superiority of any tested product. Combinations of sodium benzoate and potassium sorbate alone (Auerbach and Weiss, 2012; Auerbach and Nadeau, 2013; Weiss et al., 2016), or with sodium nitrite (Knicky and Spörndly, 2009, Knicky and Spörndly, 2011, Auerbach and Nadeau, 2013; Knicky and Spörndly, 2015; da Silva et al., 2015; Kung et al., 2018,); or ammonium propionate (Auerbach et al. 2015; Nadeau et al., 2015; Schneider et al., 2018) have consistently been shown to improve ASTA, and a dose-response was usually observed, highlighting the importance of the added application regarding the magnitude of the effect. In a series of corn silage trials on the effects of storage conditions and the addition of a mixture of potassium sorbate (134 g/L), sodium benzoate (257 g/L) and ammonium propionate (57 g/L) applied at 1 and 2 L/t by Auerbach et al. (unpublished) (Figure 5), it was found that under challenging conditions (four trials, air ingress for 24 hours on day 28 and one week before silo opening, max. storage length 63 days) only the dosage of 2 L/t was successful to improve ASTA. On the contrary, if corn silage was stored strictly anaerobically for > 90 days (two trials), already the lower application rate increased ASTA, highlighting the interaction between storage conditions and required dosage (Weber et al., 2006). Despite positive effects of formic acid-based additives on ASTA of corn silage, their use should be scrutinized because of frequently detected stimulation of ethanol formation, leading to higher anaerobic DM losses (Auerbach et al., 2012; Weiss and Auerbach, 2012; Weiss et al., 2016).

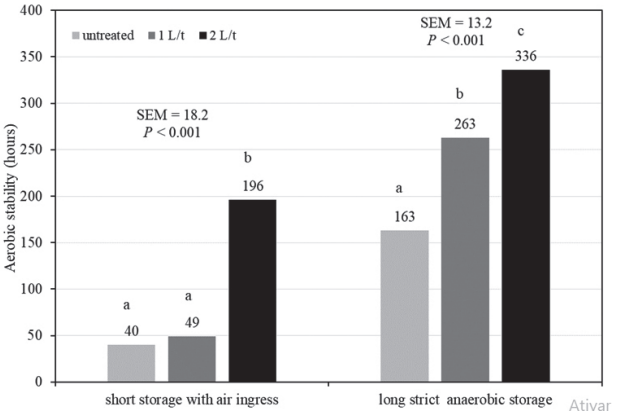

Figure 5. Effects of the application rate of an antimycotic chemical silage additive composed of 257 g/L sodium benzoate, 134 g/L potassium sorbate and 57 g/L ammonium propionate on the aerobic stability of corn silages stored under different conditions (short storage of max. 63 days with air ingress for 24 hours on day 28 and one week prior to silo opening (n = 12) or strict anaerobic storage for > 90 days (n = 6)). a-cbars with unlike superscripts differ at $P < 0.05$ (Tukey´s test) (Auerbach et al., unpublished).

In order to overcome the effects on ASTA by qualitative and quantitative differences in composition between commercial chemical additives, and to explain why certain products perform better than others, Auerbach and Nadeau (2013) have introduced the concept of "sodium benzoate equivalents" (SBE). This concept is based on results by Auerbach (1996) who studied the effects of sorbate, benzoate and propionate at pH 4 on the growth of the most important silage mold in temperature climate, *Penicillium roqueforti*. It assumes a relative effect size of 0.5:1:2 for potassium sorbate, sodium benzoate and sodium propionate. The relationship between SBE and ASTA was first described by Auerbach and Nadeau (2013) using data from a corn silage trial. The power of the curvilinear relationship (R2 = 0.85, $P < 0.01$) supported the author´s assumptions. More recently, we applied this approach to another corn silage

trial (Auerbach et al., 2017), in which two sodium benzoate/ potassium sorbate containing additives supplemented with either sodium nitrite or ammonium propionate were applied at different dosages and confirmed previous findings (Figure 6). With increasing application rate of SBE, the count of yeast was reduced and, concurrently, the ASTA was improved. However, further studies are warranted to substantiate the general validity of this interesting approach.

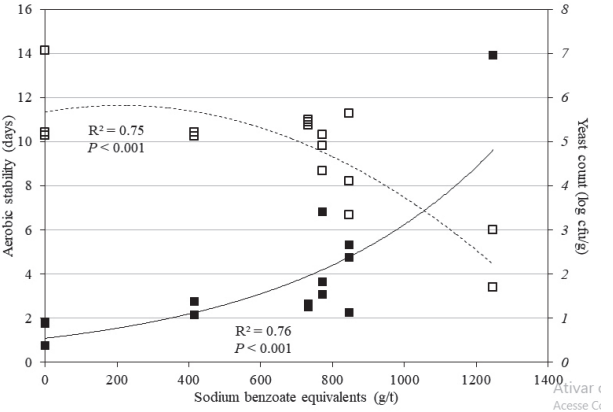

Figure 6. Relationship between the concentration of sodium benzoate equivalents and the counts of yeasts (☒, dotted regression line, n = 18) and the aerobic stability (▪, solid regression line, n = 18) in corn silage ensiled at 30% DM and stored for 63 days with air ingress for 24 hours on day 28 and 57 followed by air exposure for 14 days after silo opening (adapted from Auerbach et al., 2017).

Under conditions of short storage length, the use of antimycotic chemical additives seems warranted due to the high risk of aerobic spoilage during feed-out. Data by Kung et al. (2018) from corn silage and da Silva et al. (2015) from HMC using a chemical blend of potassium sorbate, sodium benzoate and sodium nitrite support our own observations on corn silage ensiled at 27% DM and stored for up to 142 days before aeration

(Figure 7). The ASTA was improved over untreated silage by the use of a liquid mixture of potassium sorbate, sodium benzoate and ammonium propionate already after 7 days and this effect persisted until day 34. With increasing storage length ASTA increased ($P < 0.001$), but an additive-by-fermentation length interaction was observed ($P < 0.01$). Delayed sealing, however, had a pronounced effect on ASTA, highlighting the importance of fast sealing of silos.

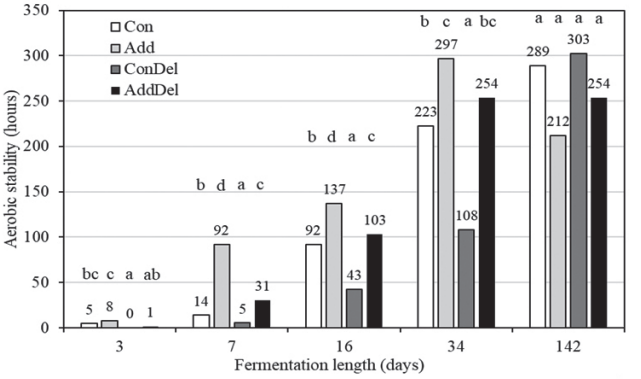

Figure 7. Effect of delayed sealing and the use of a chemical additive composed of 257 g/L sodium benzoate, 134 g/L potassium sorbate and 57 g/L ammonium propionate applied at 2 l/t on aerobic stability of corn silage ensiled at 27% DM and stored for 3, 7, 16, 34 and 142 days. Con = untreated, sealed immediately, Add = chemical additive, sealed immediately, ConDel = untreated, sealed with a delay of 24 hours, AddDel = chemical additive, sealed with a delay of 24 hours; Effects of treatment: day 3, SEM = 0.75, $P < 0.001$; day 7, SEM = 0.94, $P < 0.001$; day 16: SEM = 1.73, $P < 0.001$; day 34: SEM = 15.73, $P < 0.001$; day 142, SEM = 34.94, $P = 0.326$. a-dbars with unlike superscript within fermentation length differ at $P < 0.05$ (Tukey´s test, n = 3) (Auerbach et al., unpublished).

Recent studies on the use of obligate heterofermentative LAB other than *Lactobacillus buchneri*, e.g. *Lactobacillus diolivorans* and *Lactobacillus hilgardii*, to evaluate whether silos could be opened earlier than 6-8 weeks, have shown their potential to improve ASTA (Huenting et al., 2018; Thaysen

and Kramer, 2018). However, their effects were highly variable (Ferrero et al., 2018a; Ferrero et al., 2018b) and seemed to be largely affected by crop and DM concentration, and the effect averaged over five corn silage studies was low (<1 day) when compared with untreated silages stored for up to 30 days before aeration.

Chemical additives and animal performance

In addition to decreased DM losses and improved aerobic stability, chemical additives have the potential to improve animal performance. Formic acid restricts fermentation and protein degradation during ensiling, which have shown to increase intake by 1.0 kg/day and daily live-weight gain (LWG) by 270 g of steers when fed direct-cut grass silage treated with 3.3 L/t of formic acid (Winters et al., 2001). Likewise, compared to untreated alfalfa silage, Broderick et al. (2007) reported decreased proteolysis and, thereby, lower contents of soluble nonprotein N, ammonia N and free amino acid N in alfalfa silage treated with ammonium tetraformate (7 L/t). When fed to dairy cows, the daily DM intake increased by 1.0 kg and the 3.5% fat-corrected milk increased by 2.1 kg. Content and yield of milk true protein and nitrogen efficiency in milk N per unit of N intake were also increased (Broderick et al., 2007). However, this production response was not observed in a second trial. In an experiment by Agnew and Carson (2000), beef steers were fed unwilted grass silage untreated or treated with a blend of ammonium hexamethanoate, ammonium hexapropionate and octanoic acid (6 L/t) ad libitum. The additive increased silage intake, which resulted in an increased carcass gain. Also, carcass conformation and fat grade were higher in steers fed the treated silage (Agnew and Carson, 2000). In a dairy cow experiment, where ammonium propionate at 5 L/t was applied to corn silage,

no effect on intake or milk yield was observed (Levital et al., 2009). Furthermore, Diaz et al. (2013) found no improvements in diet digestibility, nitrogen balance, liveweight gain (LWG) or carcass quality of finishing steers fed ammoniated high-moisture ear corn. However, Nadeau and Arnesson (2016) reported increased live weight at birth (6.0 vs. 5.2 kg) and a tendency to increased LWG until weaning (442 vs. 409 g/day) in lambs suckling ewes fed grass-clover silage treated with an additive, containing sodium nitrite, hexamine, sodium benzoate, potassium sorbate and sodium propionate applied at 2 L/t. The same additive (2 L/t) was used on grass-legume silage fed to dairy cows (Nadeau et al., 2014). The additive decreased milk urea content (230 vs. 240 mg/L, $P < 0.001$) and tended to increase the excretions of purine derivatives in urine (115 vs. 95 g/day), suggesting an increase in the microbial protein flow to the duodenum. Furthermore, cows fed the treated silage had lower somatic cell counts in milk (52,000 vs. 92,000 per mL, $P < 0.05$). The improved performance of the cows was attributed to decreased proteolysis and increased sugar content of treated as compared to untreated silage (Nadeau et al., unpublished; Nadeau et al., 2014). In a later experiment (Nadeau et al., 2015b), dairy cows were fed grass-clover silage treated with the same additive or untreated silage in diets differing in rumen undegradable protein (RUP, 4.9 vs. 2.9% of DM at 15% CP of DM). The additive-treated silage produced 3.1 and 3.4 kg higher yields of milk and ECM, respectively, compared to untreated without affecting intake in the low RUP diets. The improved performance of the cows on additive-treated silage when fed a diet with low RUP could partly be related to the additive decreasing proteolysis and increasing sugar content of the silage as compared to untreated silage (Nadeau et al., unpublished; Nadeau et al., 2015b).

Conclusions

Chemical silage additives play an important role in ensuring high silage quality from field to trough. The decision on what additive to use needs to be based on the target microorganism to be inhibited. Formic-acid based products and mixtures containing sodium nitrite and hexamine are recommended to improve the fermentation process by inhibiting clostridia, whereas potassium sorbate, sodium benzoate and salts of propionic acid improve the ASTA of silages by suppressing yeasts and molds. It is crucially important to use a sufficient quantity of active ingredients in order to make the best use of the potential of the additives. The additive that is most suitable to achieve the intended goals will not only depend on its effect and consistency, but also on factors such as storage conditions, handling properties and safety to animal, user and environment, and the cost per tonne of treated forage. If applied properly, chemical additives will minimize qualitative and quantitative losses, thereby improving animal performance and farm profitability.

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Recent advances and future technology for silage harvesting

BRIAN D. LUCK[1]

Introduction

Silage harvest is a very time and machinery intensive process that requires specific harvest timing to produce high quality animal feed. In the United States, over 4 million hectares were dedicated to silage/haylage production in 2018 (USDA-NASS, 2019). The agricultural sector has been the beneficiary of gains in efficiency and production quality through the development and implementation of new technologies in recent years. Advancements in machine control, sensors, and crop monitoring have improved silage production efficiency and feed quality. This discussion will cover the current state-of-the-art as well as look to new technologies that have the potential to revolutionize silage production in the future.

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Forage Harvest Logistics and Compaction

Unlike other crops, silage harvest is unique in that there is no material storage on-board the harvest machine. This requires a fleet of support vehicles to collect the harvested material and transport it to the storage facility. This harvest method presents challenges for machinery movement and coordination during harvest along with soil health and compaction. Harrigan (2003) assessed cycle times of transport vehicles during corn silage harvest. This work showed that the harvester worked 85% of the available time for harvest. Buckmaster and Hilton (2005) developed a computer model to simulate silage harvest and assess different machinery configurations to improve silage harvest efficiency. This work developed equations to define harvester utilization and transport vehicle capacity requirement. Harmon et al. (2018) assessed two differing silage harvest operations for harvest efficiency. Total utilization of the forage harvester was found to be 65%. Transport vehicle efficiency found that semi-tractor/trailer transport vehicles were the most efficient with medium sized straight trucks and tractor-towed carts to be slightly less efficient. Dudenhoeffer et al. (2018) simulated the forage harvest cycle and developed an online tool for assessing machine performance and the effect of the addition of new machines to the fleet. This body of work shows that cycle times and machinery movement during forage harvest is not always optimized. Previous research has also focused on the optimization of forage harvester cycle times. While the forage harvester is the most expensive piece of machinery in the process, an argument should be made that the material flow at the storage site is more influential to optimizing feed quality.

Utilization of many machines to achieve alfalfa silage harvest has an impact on soil compaction and re-growth of the

crop. A current study at the University of Wisconsin-Madison is investigating the effect of wheel traffic on alfalfa re-growth. Alfalfa plots were grown during the 2019 growing season with three blocked tillage treatments. One block received fall and spring tillage, one block received spring tillage only, and the third block was planted with no tillage applied. A fourth replication of the machinery traffic passes was completed in an established field of alfalfa that was in its second growing season. Seven machinery traffic treatments were applied after each harvest (table 1).

Table 1: Machinery traffic treatments applied to alfalfa plots in 2019 to assess the effect on yield and quality parameters.

Treatment	Name	Description
1	Single Pass Silage	One application of compaction immediately after harvest covering the entire plot.
2	Three Pass Silage	Three applications of compaction. One immediately after harvest, one 24 hours after harvest and one 26 hours after harvest. Full plot application.
3	Five Pass Silage	Five applications of compaction. One immediately after harvest, two passes 24 hours after harvest, and two passes 26 hours after harvest. Full plot application.
4	Simulated Silage	Two wheel tracks applied within the plot. One pass immediately after harvest, one pass 24 hours after harvest, and two passes 26 hour after harvest.
5	Three Pass Hay	Three applications of compaction. One immediately after harvest, one 48 hours after harvest and one 56 hours after harvest. Full plot application.
6	Five Pass Hay	Five applications of compaction. One immediately after harvest, two passes 48 hours after harvest, and two passes 52 hours after harvest. Full plot application.
7	Zero Compaction (control)	No machine traffic applied.

In addition to yield measurements, samples were collected to determine moisture content and forage quality, soil compaction was measured prior to application of compaction and post application of compaction, and Unmanned Aerial Vehicle based remote sensing data was collected prior and post compaction application. Data from this study is currently undergoing analysis. Preliminary results utilizing the Mixed Procedure in SAS 9.3 show that traffic application had an impact on alfalfa yield (P-value = 0.0023, alpha = 0.05). Table 2 shows the yield results from the compaction study. There were no significant statistical differences found between no-compaction, simulated silage, and single pass silage. Three and five compaction treatments yielded between 4.0 Mg ha-1 and 1.2 Mg ha-1 less than the lower compaction treatments. Moisture content was approximately 75% wet basis at harvest. These preliminary results show that minimizing wheel traffic or implementing controlled traffic could have a substantial impact on alfalfa yield.

Table 2. Yield results from alfalfa compaction treatments during the 2019 growing season.

Treatment	Mean Yield (Mg/ha)	Standard Error (Mg/ha)
7. No-Compaction	16.8[a]	0.51
1. Single Pass Silage	14.6[ab]	0.48
4. Simulated Silage	14.3[ab]	0.54
5. Three Pass Hay	13.1[b]	0.51
3. Five Pass Silage	13.1[b]	0.51
2. Three Pass Silage	13.1[b]	0.51
6. Five Pass Hay	12.8[b]	0.51

* Letters denote statistically significant differences (alpha = 0.05)

Remote Sensing and Unmanned Aerial Vehicles

Those in production agriculture currently have a lot of interest in monitoring crop health during the growing season using remote sensing and Unmanned Aerial Vehicles (UAVs). Utilizing these tools, producers and crop consultants can identify problem areas within their fields and make adjustments to crop inputs. The goal of this in-season data collection is to optimize crop production and/or quality. Figure 1 shows two different types of UAVs. Fixed-wing and hybrid UAVs fly horizontally similarly to an airplane (Figure 1, left). These devices fly approximately 97 km h-1 and cover more area at generally lower resolution. Rotor type UAVs fly more like a helicopter and cover less area at a slower speed (Figure 1, right). These machines are able to hover in a single location and collect high resolution imagery of smaller areas within the field.

Figure 1. Deployment of rotor-type and hybrid fixed-wing Unmanned Aerial Vehicles for remote sensing data collection in Wisconsin, U.S.A. Undergraduate and graduate students obtained Remote Pilot Licesnes from the U.S. Federal Aviation Administration in order to conduct this research.

Utilizing the visible light spectrum and spectra within the Near-Infrared range, we can produce vegetative indices that provide indication of crop health and vigor during the growing season. Figure 2 shows a Normalized Difference Vegetative Index (NDVI) image of the alfalfa compaction study described above.

This image was collected with a rotor-type UAV approximately 10 days after compaction was applied. Red areas show locations of high crop stress, yellow areas are medium crop stress, and green areas are low crop stress. This data can provide producers valuable information regarding plant health and give them the opportunity to adjust inputs to correct problem areas.

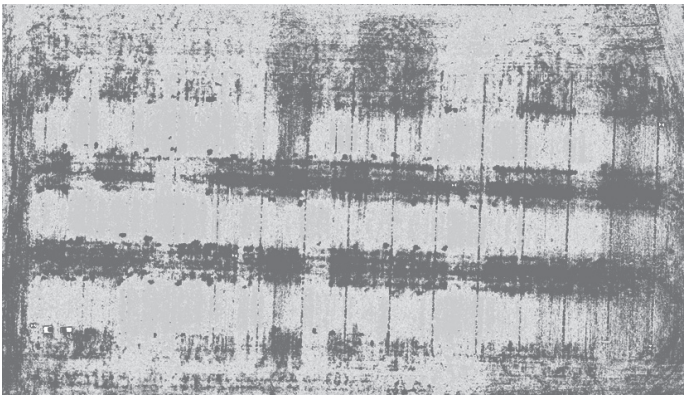

Figure 2. Orthomosaic image of Normalized Difference Vegetative Index (NDVI) for the alfalfa compaction wheel traffic study. High traffic plots can be seen in red and yellow compared to green areas with low traffic.

Kernel Processing

Corn silage is an important crop for dairy producers in Wisconsin, U.S.A. Utilizing forage harvesters to chop and process this crop provides a feed supply for animals over the entire year. Kernel processing on the machine is a very important process to producing high-quality corn silage. Reducing the kernel size allows for easier digestion, higher starch availability, and increased milk production (Cooke & Bernard, 2005; Johnson et al., 2003; Shinners et al., 2000; Weiss & Wyatt, 2000).

Image processing can be effectively used for particle size analysis of agricultural materials (Igathinathane &

Leslie, 2007; Igathinathane et al., 2008; Igathinathane et al., 2009; Savoie et al., 2014). Drewry et al. (2019) utilized image processing to determine Kernel Processing Score for chopped and processed corn silage. This method was found to be well correlated with traditional sieving methods and showed that mechanical sieving reduced the particle size of the corn kernels during measurement. From this effort a smart phone app was developed so that producers and custom harvesters can check the performance of their kernel processors during harvest rather than relying on sending a sample to the lab. Figure 3 shows the home screen of the app called SilageSnap that can be used for kernel processor performance determination. This app utilizes a size reference within the image (a U.S. coin or 1 Euro or ½ Euro coin) to determine the size of every particle in the image. Maximum inscribed circle diameter is applied to each particle to so that results correlate well to sieving results. To date the app has nearly 1,000 downloads, is free to use, and is available in the iTunes and Google Play stores.

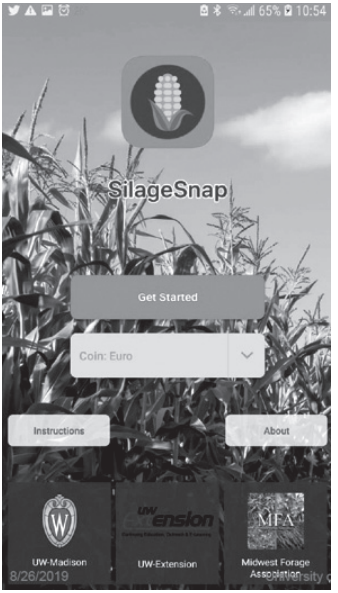

Figure 3. SilageSnap smart phone app for determining corn silage kernel processing score during harvest.

Conclusion

There have been many technological advancements in hay and forage harvest in recent years. Utilizing advancements in machine controls, machine communication, and novel data collection methods producers are better equipped to manage crop and feed production. Future advancements will only improve these management strategies and optimize forage production.

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Profiling metabolome and bacterial community dynamics in whole-plant corn silage inoculated without or with homo - or heterofermenters

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Abstract

Corn silage has become the predominant forage used in ruminant diets, especially those used for dairy cattle. Although the importance of the silage fermentation quality and methods to improve it are well established, little is known about the complex microbiome and metabolome and subsequent biofunctions silage may have. Inoculating homo- or hetero-fermentative cultures to fresh whole crop corn at ensiling resulted in substantial differences in microbial and metabolome dynamics and composition in ensiled corn. Inoculants altered the correlations of microflora in different manners and various keystone species were identified in silages treated differently. Many metabolites with biofunctional activities like bacteriostatic, antioxidant, central nervous system inhibitory and anti-inflammatory were found in corn silage with potential benefits for animal health and welfare. A constitutive difference in microbiota dynamics was found for several pathways which were upregulated by specific taxa in middle stage of fermentation.

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The concomitant alterations of the microbiome and metabolome suggested the presence of widespread associations between metabolites with biofunctions and the species of lactic acid bacteria dominating in silage. The data generated in the current study improves our understanding of the complicated biological process underlying silage fermentation, and provides a framework to re-evaluate silages with biofunctions, which may contribute to target-based regulation methods to produce functional silage for animal production.

Keywords: Untargeted metabolomics, Microbiome, 16S rRNA amplicon sequencing, Metabolome-microbiome network, Functional silage, Lactic acid bacteria inoculants

1. Introduction

The advantage of ruminants in the global food system evolves from their ability to convert fibrous forages into high quality milk and meat for human consumption. The forages can be grazed by the animals but in most environments and production systems, conserved forages are needed. The anaerobic lactic acid fermentation of moist forages such as grasses, legumes, small grain cereals or corn provides an efficient way to preserve the forages (Grant and Ferraretto, 2018; Wilkinson, 2018). Corn silage is an important source of ensiled forage, which makes up over 40% of forage fed to dairy cows (Kolver et al., 2001). The dairy sector is growing fast and according to FAO (FAO, 2013), world milk production is projected to increase by 177 million tonnes by 2025, at an average growth rate of 1.8% per annum in the next 10 years (from 2016). About half of global ruminant meat and two thirds of global milk demand has been estimated to be produced in developing countries by 2050, especially in

China and India (Rosegrant, 2009; Gerber, 2013). There are about 133 million dairy cattle worldwide (FAO, 2013) which means that at least 665 million tonnes of silage is consumed per annum. Hence, nutritionally and hygienically high quality silage is a crucial prerequisite for developing ruminant husbandry able to provide efficiently high quality ruminant products to the growing global population.

The biochemistry of ensiling is complex and there are interactions among plant enzymes and the activities of microbial species (Pahlow et al., 2003). Numerous metabolites are produced during ensiling and there are important interactions between the metabolites and the microbes (Pahlow, 1991; Broberg et al., 2007). In addition, many metabolites are produced by lactic acid bacteria (LAB) during fermentation, such as vitamins, oligosaccharides, amino acids, aromatic compounds and fatty acids (Sun et al., 2012). Certain metabolites have conventionally been investigated to assess the fermentation quality of ensiled forage, but only few studies have focused on metabolites potentially affecting animal health and welfare. Therefore, improving understanding of silage microbiome and metabolome will provide a scientific foundation for producing high-quality silage and potentially even silages with active metabolites that may positively impact animal health and welfare.

Among the silage additives currently available, LAB strains have great potential in modulating the microbial community dynamics and end products during ensiling process. The LAB inoculants are divided into homofermentative and heterofermentative cultures based on their fermentation pattern. It is still unclear how the homofermentative or heterofermentative LAB affect the bacterial community and metabolite dynamics in whole crop corn silage. Advanced

molecular biological techniques have recently been taken into use to help understand the complex microbial communities and their succession (Pang et al., 2011; Keshri et al., 2018). However, to the best of our knowledge, no study has so far reported the population dynamics of whole crop corn silage during ensiling at species level with PacBio single molecule in conjunction with real-time sequencing technology (SMRT). The objective of the current study was to determine changes in the metabolome and microbiome during ensiling of whole crop corn, with or without homo- and heterofermentative lactic acid bacterial inoculants in order to obtain an improved insight on the effects of such changes on silage quality and biofunction.

2. Materials and Methods

2.1 Ensiling and characteristics of corn silages

The whole crop maize (*Zea mays* L.; from a commercial farm of Anding county, Dingxi city, Gansu province, China.) harvested at the stage of half milk-line was chopped into 2-cm pieces and ensiled in vacuum-sealing polyethylene plastic bags (30 cm × 23 cm). Three inoculant treatments were applied, including a control without inoculation, *Lactobacillus plantarum* MTD/1 (Vita Plus, Madison, MI, US) and *Lactobacillus buchneri* 40788 (Vita Plus, Madison, MI, US). The inoculants were mixed into distilled water and applied at a rate of 1 × 106 cfu g-1 fresh matter (FM), and an equal volume of distilled water was sprayed in the fresh maize for control. The bags (with about 300 g of fresh maize crop) were then stored at ambient temperature (22-25°C) and sampled after 3, 7, 14, 45 and 90 d of fermentation using three replicates per treatment and time.

2.2 Microbial composition SMRT analysis

The DNA extraction, PCR amplification of the full-length 16S rRNA gene for SMRT sequencing, analysis of 16S rRNA amplicon sequencing data were performed as described in our previous study (Xu et al., 2019). After the comparison with the Silva (Release132 http://www.arb-silva.de) database (classified at a bootstrap threshold of 0.8) using the Mothur (https://mothur.org/wiki/Classify.seqs) software, the reads belonging to unclissifiedd *Lactobacillus* were subjected to the best BLAST hit method to gain species level information (Ovaskainen et al., 2010; Quast et al., 2013) (using BLASR software). Sample ordination based on beta diversity was examined by means of principal coordinate analyses (PCoA) with phylogeny-based (UniFrac) unweighted and weighted distances (using QIIME). Linear discriminant analysis effect size (LEfSe) method was used to determine the genes most likely to explain differences between treatments by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance (Segata et al., 2011). Microbial networks were used to statistically identify keystone taxa and we recommend that the combined score of high mean degree and low betweenness centrality should be used as a threshold for defining keystone taxa in microbial communities (Berry and Widder, 2014). PICRUSt was used to predict the metagenome in terms of Kegg Orthology (KO) terms for each 16S rRNA sample (Langille et al., 2013). Microbiome functional shifts and phylotype-level contributions to functional shifts were obtained using the FishTaco framework (Manor and Borenstein, 2017).

2.3 Metabolomics using GC-TOF-MS

The method of extraction was described in our previous study (Xu et al., 2019). The system used a DB-5MS capillary column coated with 5% diphenyl and cross-linked with 95% dimethylpolysiloxane (30 m × 250 μm inner diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). Samples (1 μl) were injected in split mode (split ratio 20:1), with helium used as the carrier gas at a flow rate of 1.0 ml min−1. The oven temperature ramp was as follows: initial temperature was 50°C for 1 min, then raised to 310°C at a rate of 10°C min−1, and finally kept at 310°C for 8 min. The injection, transfer line, and ion source temperatures were 280, 280, and 250°C, respectively. The energy was -70 eV in electron impact mode. The mass spectrometry data was acquired in full-scan mode with an m z-1 range of 50-500 at a rate of 12.5 spectra per second, after a solvent delay of 6.17 min.

Raw peak exaction, data baseline filtration and calibration of the baseline, as well as peak alignment, deconvolution analysis, peak identification and integration of the peak area and biochemicals that differed significantly between experimental groups identified according to the methods of our previous study (Xu et al., 2019).

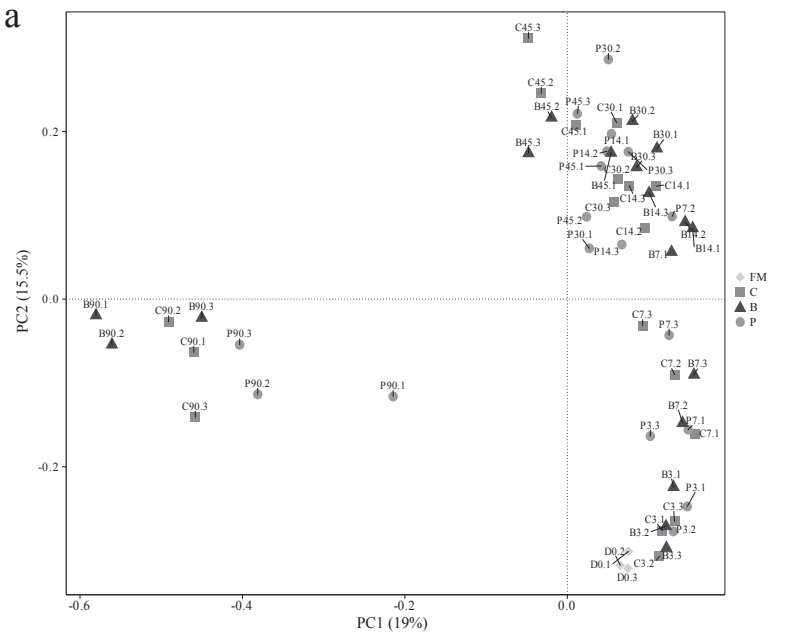
3. Results and Discussion

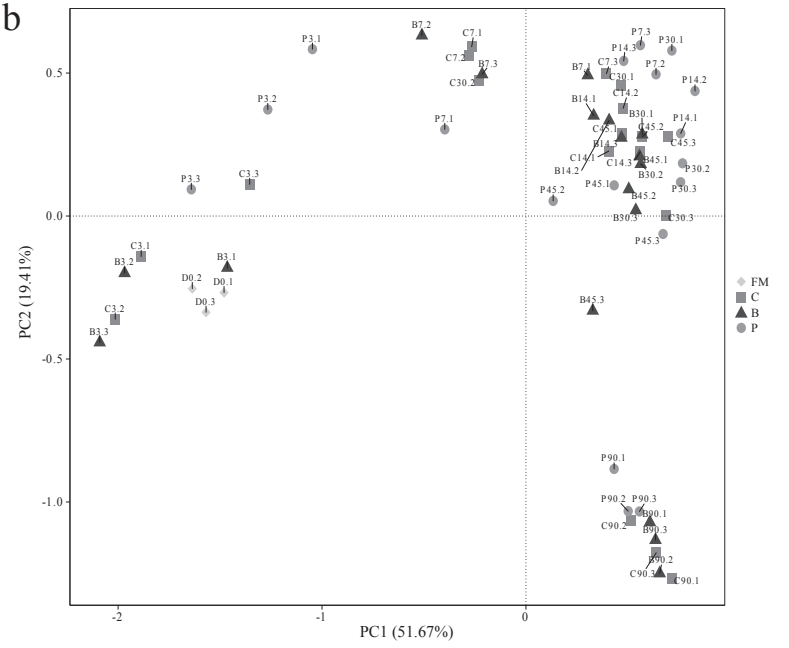
3.1 The inoculants altered the composition of the corn silage microbiome

Bacteria composition and dynamics was inferred from the analysis of 16S rRNA amplicon sequencing data. Based on SMRT sequencing in silage bacteria, an average of 11948 Circular Consensus Sequencing sequences (CCS) were obtained from each sample. The principal co-ordinates analysis (PCoA) based on unweighted UniFrac distances and weighted

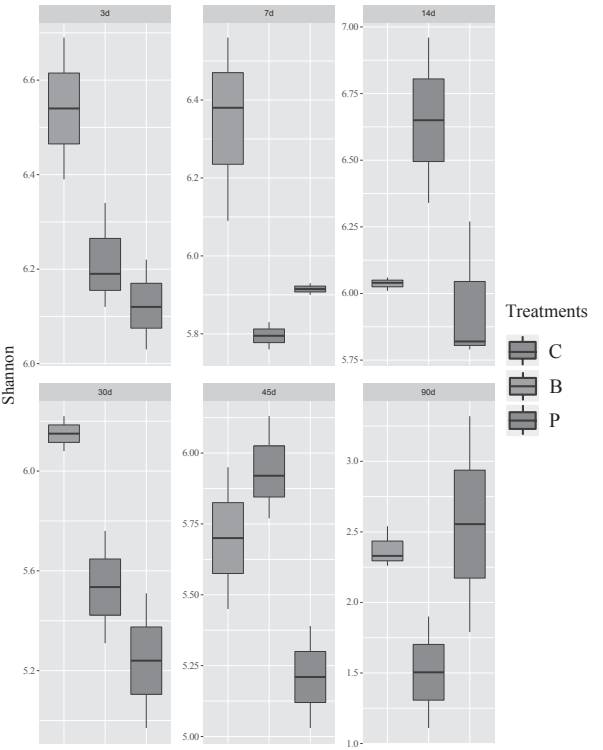
UniFrac distances was applied to identify factors that shape the differences between corn silage microbiomes (beta diversity). The results indicated a significant bacteria succession according to fermentation time while they were indistinguishable among silage inoculated without or with *L. plantarum* and *L. buchneri*. (Figure 1a, 1b). However, three distinct clusters were indentified on silages fermented for 3 to 7 d, 14 to 45 d and 90 d, respectively. Especially, the microbial diversity of silages fermented for 90 d was clearly separated from that of other fermentation times. The α-diversity analysis revealed decrease of the bacterial biodiversity from prolonged fermentation process in whole crop corn silage (Figure 1c). The *L. buchneri* increased the Shannon index at fermentation times of 3, 7 and 30 d compared with other treatments (P value < 0.013) but there was no difference on 90 d of fermentation (P value = 0.089). The *L. plantarum* decreased the Shannon index at fermentation times of 14 (P value = 0.023) and 45 d (P value = 0.013) compared with control.

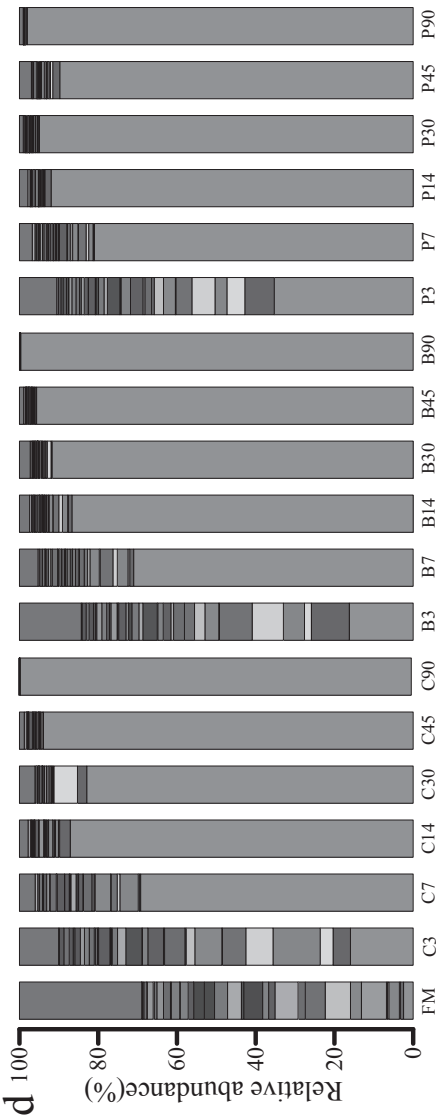
The composition dynamics of microbiota are shown in Figure 1d and Figure 1e. At genus level (Figure 1d), epiphytic microflora of fresh maize was mainly comprised of undesirable bacteria for ensiling like *Agrobacterium* (6.42%), *Microbacterium* (6.35%), *Sphingobacterium* (5.88%), *Chryseobacterium* (5.05%) and others (31%), which will be restrained promptly with start of fermentation. After 3 d of fermentation, most bacterial reads were derived from *Lactobacillus, Acinetobacter, Klebsiella, Methylobacterium* and *Citrobacter* in all treatments. From d 7 to d 90 of fermentation, *Lactobacillus* dominated the ensiling process. The dominating species were *L. farciminis, L. parabrevis, L. brevis, L. parafarraginius, L. heilongjiangensis, L. acetotolerans* and *L. silage* (Figure 1e). From these LAB species, *L. acetotolerans* and *L. silagei* dominated the process on 90 d of fermentation. Interestingly, the inoculants *L. plantarum* and *L. buchneri* did not appear as dominating species during the fermentation process.


a
PC2 (15.5%)
PC1 (19%)
FM
C
B
P


b
PC2 (19.41%)
PC1 (51.67%)
FM
C
B
P

C




Relative abundance(%)
0
20
40
60
80
100
FM
C3
C7
C14
C30
C45
C90
B3
B7
B14
B30
B45
B90
P3
P7
P14
P30
P45
P90
Lactobacillus
Acinetobacter
Brevundimonas
Klebsiella
Methylobacterium
Citrobacter
Microbacterium
Gammaproteobacteria_unclassified
Agrobacterium
Chryseobacterium
Enterobacteriaceae_unclassified
Sphingobacterium
Rahnella
Delftia
Candidatus Phytoplasma
Aureimonas
Enterobacterales_unclassified
Sphingomonas
Stenotrophomonas
Leuconostoc
Anoxybacillus
Enterobacter
Bacteroidetes_unclassified
Bacillus
Frigoribacterium
Ochrobactrum
Pseudomonas
Lactococcus
Serratia
Achromobacter
Pantoea
Enterobacteriaceae_Raoultella
Proteobacteria_unclassified
Rhizobiales_unclassified
Enterobacteriaceae_Raoultella
Kluyvera
Lactobacillaceae_unclassified
Others

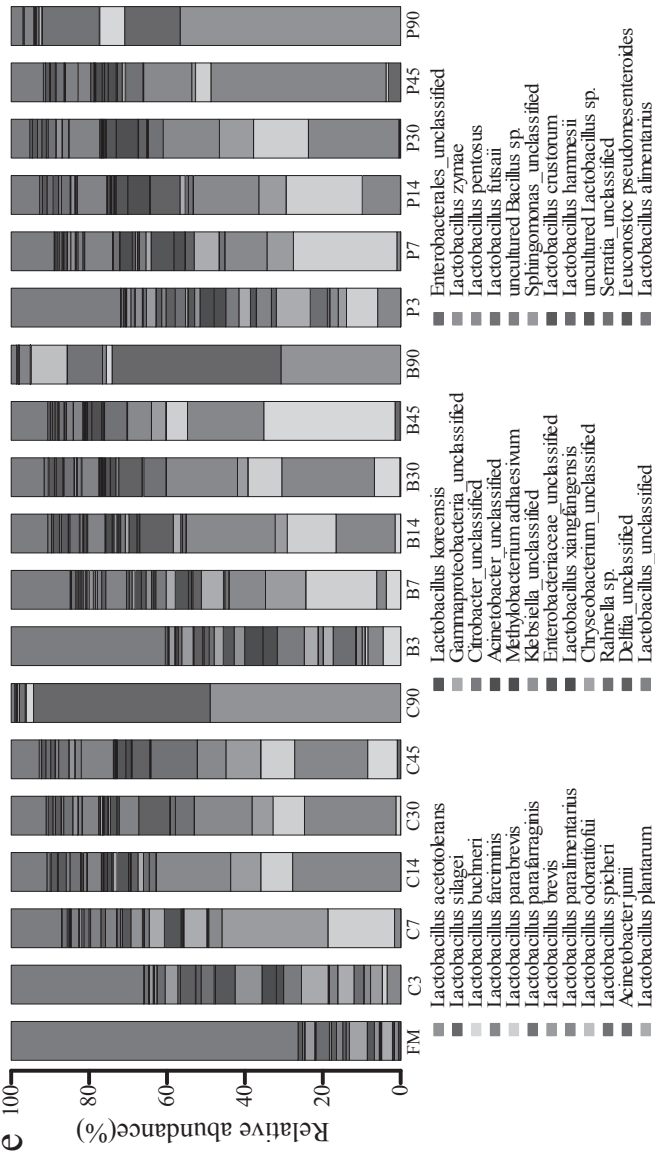
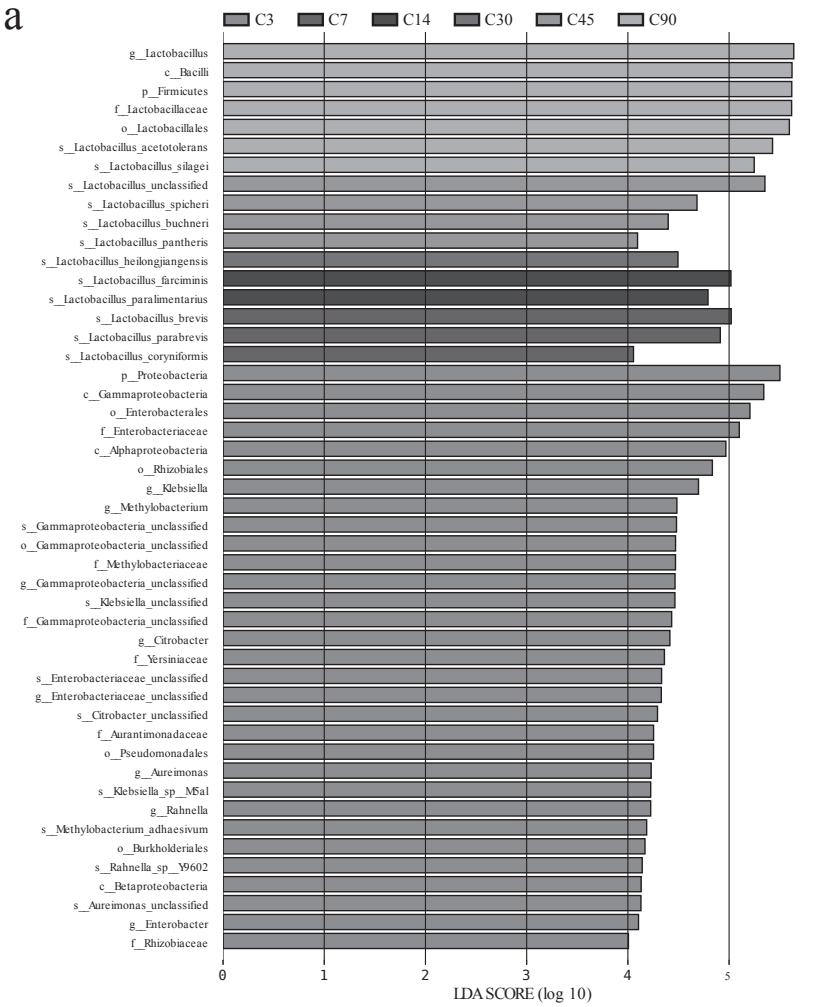

e
Relative abundance(%)
100
80
60
40
20
0
FM
C3
C7
C14
C30
C45
C90
B3
B7
B14
B30
B45
B90
P3
P7
P14
P30
P45
P90
Lactobacillus acetotolerans
Lactobacillus silagei
Lactobacillus buchneri
Lactobacillus farciminis
Lactobacillus parabrevis
Lactobacillus parafarraginis
Lactobacillus brevis
Lactobacillus paralimentarius
Lactobacillus odoratitofui
Lactobacillus spicheri
Acinetobacter junii
Lactobacillus plantarum
Lactobacillus heilongjiangensis
Brevundimonas diminuta
Lactobacillus pontis
Lactobacillus koreensis
Gammaproteobacteria_unclassified
Citrobacter_unclassified
Acinetobacter_unclassified
Methylobacterium adhaesivum
Klebsiella_unclassified
Enterobacteriaceae_unclassified
Lactobacillus xiangfangensis
Chryseobacterium_unclassified
Rahnella sp.
Delftia_unclassified
Lactobacillus_unclassified
Klebsiella sp.
Lactobacillus coryniformis
Aureimonas_unclassified
Enterobacterales_unclassified
Lactobacillus zymae
Lactobacillus pentosus
Lactobacillus futsaii
uncultured Bacillus sp.
Sphingomonas_unclassified
Lactobacillus crustorum
Lactobacillus hammesii
uncultured Lactobacillus sp.
Serratia_unclassified
Leuconostoc pseudomesenteroides
Lactobacillus alimentarius
Others

Figure 1 Microbial community dissimilarities and diversities of corn silage. C, Corn silage without inoculants; B, Corn silage inoculated with *L. buchneri*; P, Corn silage inoculated with *L. plantarum*; FM, Fresh material. (a) The community dissimilarities in different treatments and fermentation time, calculated by unweighted UniFrac distances, with coordinates calculated by principal co-ordinates analysis (PCoA). (b) The community dissimilarities in different treatments and fermentation time, calculated by weighted UniFrac distances, with coordinates calculated by principal co-ordinates analysis (PCoA). (c) The variations of community alpha-diversities (Shannon index). (d) Relative abundances of corn silage bacterial genus across different treatments and fermentation time. (e) Relative abundances of corn silage bacterial species across different treatments and fermentation time.

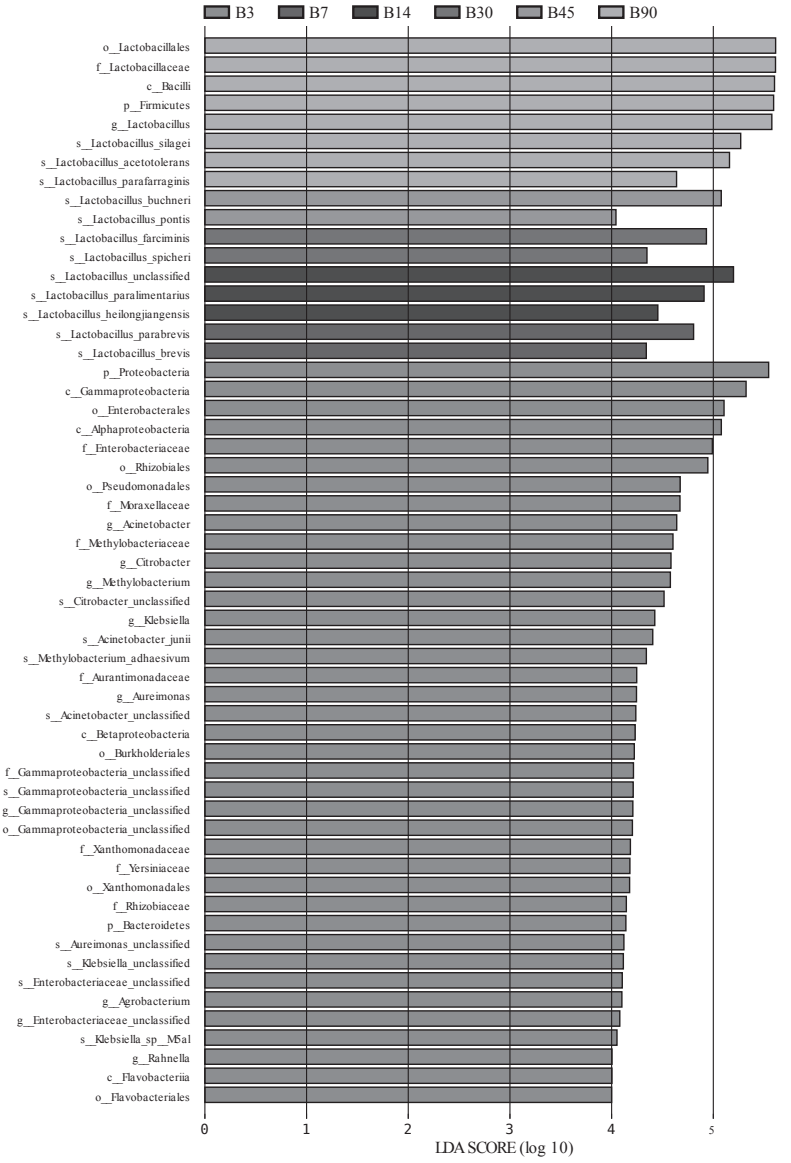
To explain the effect of differentiating fermentation process of silages treated with or without inoculants by bacterial taxa, LDA Effect Size (LEfSe) analysis was conducted (Figure 2a-2c). In control silage, many undesirable bacteria like *Proteobacteria*, *Enterobacteriales*, *Rhizobiaceae* and *Methylobacteriaceae* were abundant at the early stage of fermentation (3 d). Compared with control silage, *L. plantarum*, *Clostridium beijerinckii* and *Agrobacterium* were significantly enriched in silage inoculated with *L. plantarum* while Bacteroidetes and Acinetobacter were enriched in *L. buchneri* treated silage on 3 d of fermentation. When silages fermented for 7 d, *L. coryniformis* and *L. plantarum* were enriched in silage, while *L. plantarum* and *L. xiangfangensis* were enriched in samples treated with *L. buchneri* and *L. plantarum*, respectively. On 14 d of fermentation, *L. farciminis* was enriched in control silage, while *L. heilongjiangensis* was enriched in samples treated with inoculants and *L. pontis* also enriched in *L. plantarum* treated silage. On 30 d of fermentation, *L. heilongjiangensis* was enriched in control silage, *L. farciminis* and *L. futsaii* were enriched in *L. buchneri* treated silage and *L. brevis* was enriched in *L. plantarum* treated silage. On 45 d of fermentation, *L. buchneri* and *L. pantheris* were enriched in control silage, *L. buchneri* and *L. pontis* were enriched in *L. buchneri* inoculated silage and *L. farciminis* and *L. coryniformis*

were enriched in *L. plantarum* inoculated silage. On 90 d of fermentation, the dominating species were *L. acetotolerans* and *L. silagei*, and on top of those, *L. odoratitoful* and *L. parafarraginis* were enriched in *L. buchneri* inoculated silage and *L. parafarraginis* and *L. buchneri* were enriched in *L. plantarum* inoculated silage. Inoculants *L. buchneri* and *L. plantarum* were not the dominant strains during ensiling, but they modulated the various microbial communities and metabolome dynamics in different ways compared to the silage without inoculation.

Microbial network was used to assess correlation between various species and to statistically identify the bacteria species that were keystone taxa for modulating the fermentation process disproportionately large relative to its abundance (Power et al., 1996). This study is the first to identify keystone taxa with network topological properties in silage. The results indicated that inoculants obviously changed the correlations within microbiota (Figure 2d-2f). The putative drivers of keystone taxa in microbial communities of corn silages with or without different inoculants were defined with the combined score of high degree centrality and low betweenness centrality (data not show). The results showed that *L. buchneri*, *L. parafarraginis*, *L. hammesii* and *Agrobacterium* larrymoorei in silage without inoculants; *L. panis* and unclassified Enterobacteriaceae in silage inoculated with *L. buchneri;* and *L. crustorum* and *Agrobacterium* larrymoorei in silage inoculated with *L. plantarum* can be considered as keystone taxa.


a
C3
C7
C14
C30
C45
C90
g__Lactobacillus
c__Bacilli
p__Firmicutes
f__Lactobacillaceae
o__Lactobacillales
s__Lactobacillus_acetotolerans
s__Lactobacillus_silagei
s__Lactobacillus_unclassified
s__Lactobacillus_spicheri
s__Lactobacillus_buchneri
s__Lactobacillus_pantheris
s__Lactobacillus_heilongjiangensis
s__Lactobacillus_farciminis
s__Lactobacillus_paralimentarius
s__Lactobacillus_brevis
s__Lactobacillus_parabrevis
s__Lactobacillus_coryniformis
p__Proteobacteria
c__Gammaproteobacteria
o__Enterobacterales
f__Enterobacteriaceae
c__Alphaproteobacteria
o__Rhizobiales
g__Klebsiella
g__Methylobacterium
s__Gammaproteobacteria_unclassified
o__Gammaproteobacteria_unclassified
f__Methylobacteriaceae
g__Gammaproteobacteria_unclassified
s__Klebsiella_unclassified
f__Gammaproteobacteria_unclassified
g__Citrobacter
f__Yersiniaceae
s__Enterobacteriaceae_unclassified
g__Enterobacteriaceae_unclassified
s__Citrobacter_unclassified
f__Aurantimonadaceae
o__Pseudomonadales
g__Aureimonas
s__Klebsiella_sp__M5a1
g__Rahnella
s__Methylobacterium_adhaesivum
o__Burkholderiales
s__Rahnella_sp__Y9602
c__Betaproteobacteria
s__Aureimonas_unclassified
g__Enterobacter
f__Rhizobiaceae
0
1
2
3
4
5
LDA SCORE (log 10)

b


B3
B7
B14
B30
B45
B90
o__Lactobacillales
f__Lactobacillaceae
c__Bacilli
p__Firmicutes
g__Lactobacillus
s__Lactobacillus_silagei
s__Lactobacillus_acetotolerans
s__Lactobacillus_parafarraginis
s__Lactobacillus_buchneri
s__Lactobacillus_pontis
s__Lactobacillus_farciminis
s__Lactobacillus_spicheri
s__Lactobacillus_unclassified
s__Lactobacillus_paralimentarius
s__Lactobacillus_heilongjiangensis
s__Lactobacillus_parabrevis
s__Lactobacillus_brevis
p__Proteobacteria
c__Gammaproteobacteria
o__Enterobacterales
c__Alphaproteobacteria
f__Enterobacteriaceae
o__Rhizobiales
o__Pseudomonadales
f__Moraxellaceae
g__Acinetobacter
f__Methylobacteriaceae
g__Citrobacter
g__Methylobacterium
s__Citrobacter_unclassified
g__Klebsiella
s__Acinetobacter_junii
s__Methylobacterium_adhaesivum
f__Aurantimonadaceae
g__Aureimonas
s__Acinetobacter_unclassified
c__Betaproteobacteria
o__Burkholderiales
f__Gammaproteobacteria_unclassified
s__Gammaproteobacteria_unclassified
g__Gammaproteobacteria_unclassified
o__Gammaproteobacteria_unclassified
f__Xanthomonadaceae
f__Yersiniaceae
o__Xanthomonadales
f__Rhizobiaceae
p__Bacteroidetes
s__Aureimonas_unclassified
s__Klebsiella_unclassified
s__Enterobacteriaceae_unclassified
g__Agrobacterium
g__Enterobacteriaceae_unclassified
s__Klebsiella_sp__M5a1
g__Rahnella
c__Flavobacteriia
o__Flavobacteriales
0
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LDA SCORE (log 10)


c
P3
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o__Lactobacillales
f__Lactobacillaceae
c__Bacilli
p__Firmicutes
g__Lactobacillus
s__Lactobacillus_acetotolerans
s__Lactobacillus_parafarraginis
s__Lactobacillus_silagei
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s__Lactobacillus_heilongjiangensis
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s__Lactobacillus_unclassified
s__Lactobacillus_futsaii
s__Lactobacillus_parabrevis
s__Lactobacillus_koreensis
s__Lactobacillus_xiangfangensis
p__Proteobacteria
c__Gammaproteobacteria
c__Alphaproteobacteria
o__Enterobacterales
o__Rhizobiales
f__Enterobacteriaceae
s__Lactobacillus_plantarum
f__Methylobacteriaceae
g__Methylobacterium
s__Acinetobacter_junii
f__Yersiniaceae
c__Betaproteobacteria
o__Burkholderiales
g__Citrobacter
s__Methylobacterium_adhaesivum
s__Citrobacter_unclassified
f__Aurantimonadaceae
g__Rahnella
g__Gammaproteobacteria_unclassified
g__Aureimonas
o__Gammaproteobacteria_unclassified
g__Klebsiella
f__Gammaproteobacteria_unclassified
s__Gammaproteobacteria_unclassified
f__Rhizobiaceae
s__Rahnella_sp__Y9602
s__Clostridium_beijerinckii
g__Agrobacterium
s__Aureimonas_unclassified
0
1
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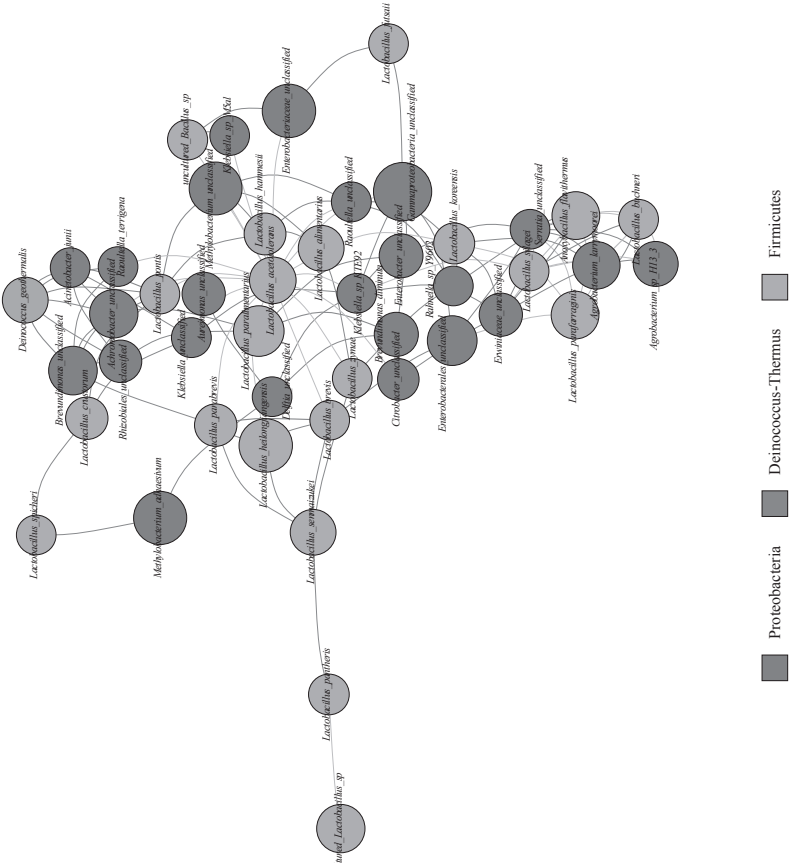
LDA SCORE (log 10)

d



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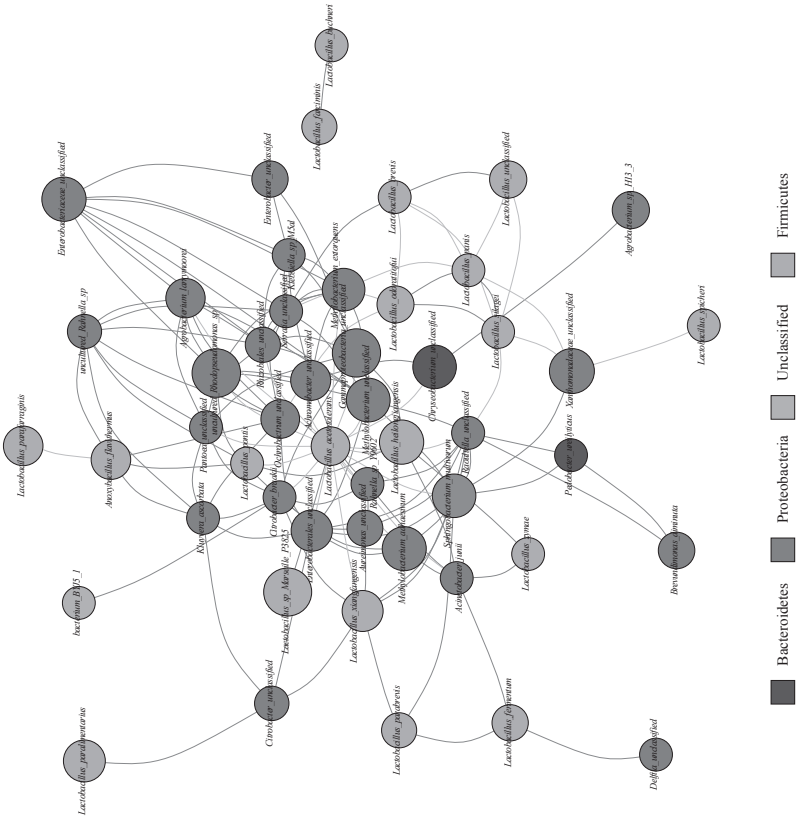
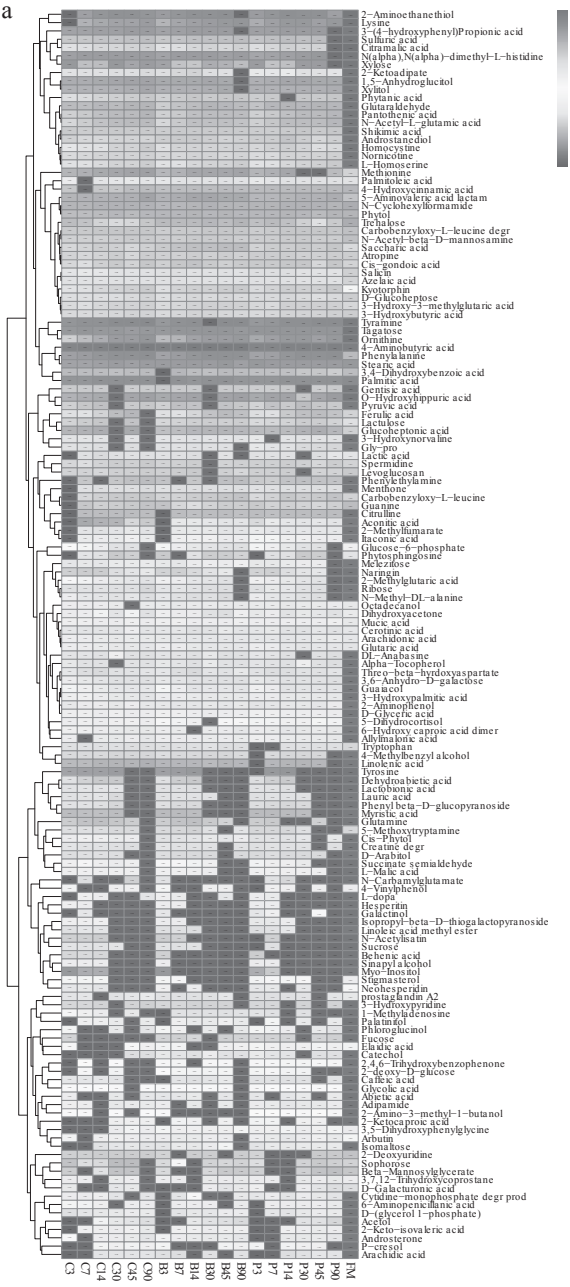


f

Figure 2 Differences of bacterial taxa in corn silage with different treatments. C, Corn silage without inoculants; B, Corn silage inoculated with *L. buchneri*; P, Corn silage inoculated with *L. plantarum*. Latent Dirichlet Allocation Effect Size (LEfSe) analysis of corn silage bacterial biomarkers associated with inoculants for different fermentation time (a-c). Histogram of the Latent Dirichlet Allocation (LDA) scores computed for bacteria differentially abundant among six fermentation time. LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between features in the six fermentation time of analyzed microbial communities. The histogram thus identifies which bacteria taxa among all those detected as statistically and biologically differential explain the greatest differences between communities. (a) Corn silage without inoculants. (b) Corn silage inoculated with *L. buchneri*. (c) Corn silage inoculated with *L. plantarum*. Interaction networks of the whole crop corn silage microbiota (d-f). 16S rRNA gene-based correlation network of the whole crop corn silage microbiota, displaying statistically significant interactions with absolute value of correlation coefficients > 0.6. Node size is scaled based on the overall abundance of each taxa in the microbiota. Edge width is proportional to the strength of association between each metabolite-phylotype pair (as measured by the correlation), red edge indicates the positive correlation and green edge indicates the negatively correlation. (d) Corn silage without inoculants. (e) Corn silage inoculated with *L. buchneri*. (f) Corn silage inoculated with *L. plantarum*.

The inoculants altered the metabolome of the corn silage

To evaluate the changes of the corn silage metabolome we used the untargeted metabolomic approach. In total, 643 metabolites were identified (data not show). The heatmap of sum of significantly different compounds in various treatments and various fermentation times is shown in Figure 3a. The plot showed that many metabolites were produced after fermentation such as amino acids, carbohydrates, organic acids, tocopherol, pantothenic acid and tyramine. Some metabolites including amino acids, sugar acids and polyhydric alcohols disappeared faster (on d 30) from inoculated silages compared to the control silage (on d 45).


a
0
−2
−4
−6
2−Aminoethanethiol
Lysine
3−(4−hydroxyphenyl)Propionic acid
Sulfuric acid
Citramalic acid
N(alpha),N(alpha)−dimethyl−L−histidine
Xylose
2−Ketoadipate
1,5−Anhydroglucitol
Xylitol
Phytanic acid
Glutaraldehyde
Pantothenic acid
N−Acetyl−L−glutamic acid
Shikimic acid
Androstanediol
Homocystine
Normicotine
L−Homoserine
Methionine
Palmitoleic acid
4−Hydroxycinnamic acid
5−Aminovaleric acid lactam
N−Cyclohexylformamide
Phytol
Trehalose
Carbobenzyloxy−L−leucine degr
N−Acetyl−beta−D−mannosamine
Saccharic acid
Atropine
Cis−gondoic acid
Salicin
Azelaic acid
Kyotorphin
D−Glicoheptose
3−Hydroxy−3−methylglutaric acid
3−Hydroxybutyric acid
Tyramine
Tagatose
Ornithine
4−Aminobutyric acid
Phenylalanine
Stearic acid
3,4−Dihydroxybenzoic acid
Palmitic acid
Gentisic acid
O−Hydroxyhippuric acid
Pyruvic acid
Ferulic acid
Lactulose
Glucoheptonic acid
3−Hydroxynorvaline
Gly−pro
Lactic acid
Spermidine
Levoglucosan
Phenylethylamine
Menthone
Carbobenzyloxy−L−leucine
Guanine
Citrulline
Aconitic acid
2−Methylfumarate
Itaconic acid
Glucose−6−phosphate
Phytosphingosine
Melezitose
Naringin
2−Methylglutaric acid
Ribose
N−Methyl−DL−alanine
Octadecanol
Dihydroxyacetone
Mucic acid
Cerotinic acid
Arachidonic acid
Glutaric acid
DL−Anabasine
Alpha−Tocopherol
Threo−beta−hydroxyaspartate
3,6−Anhydro−D−galactose
Guaiacol
3−Hydroxypalmitic acid
2−Aminophenol
D−Glyceric acid
5−Dihydrocortisol
6−Hydroxy caproic acid dimer
Allylmalonic acid
Tryptophan
4−Methylbenzyl alcohol
Linolenic acid
Tyrosine
Dehydroabietic acid
Lactobionic acid
Lauric acid
Phenyl beta−D−glucopyranoside
Myristic acid
Glutamine
5−Methoxytryptamine
Cis−Phytol
Creatinine degr
D−Arabitol
Succinate semialdehyde
L−Malic acid
N−Carbamylglutamate
4−Vinylphenol
L−dopa
Hesperitin
Galactinol
Isopropyl−beta−D−thiogalactopyranoside
Linoleic acid methyl ester
N−Acetylisatin
Sucrose
Behenic acid
Sinapyl alcohol
Myo−Inositol
Stigmasterol
Neohesperidin
prostaglandin A2
3−Hydroxypyridine
1−Methyladenosine
Palatinitol
Phloroglucinol
Fucose
Elaidic acid
Catechol
2,4,6−Trihydroxybenzophenone
2−deoxy−D−glucose
Caffeic acid
Glycolic acid
Abietic acid
Adipamide
2−Amino−3−methyl−1−butanol
5−Ketocaproic acid
3,5−Dihydroxyphenylglycine
Arbutin
Isomaltose
2−Deoxyuridine
Sophorose
Beta−Mannosylglycerate
3,7,12−Trihydroxycoprostane
D−Galacturonic acid
Cytidine−monophosphate degr prod
6−Aminopenicillanic acid
D−(glycerol 1−phosphate)
Acetol
2−Keto−isovaleric acid
Androsterone
P−cresol
Arachidic acid
C3
C7
C14
C30
C45
C90
B3
B7
B14
B30
B45
B90
P3
P7
P14
P30
P45
P90
FM

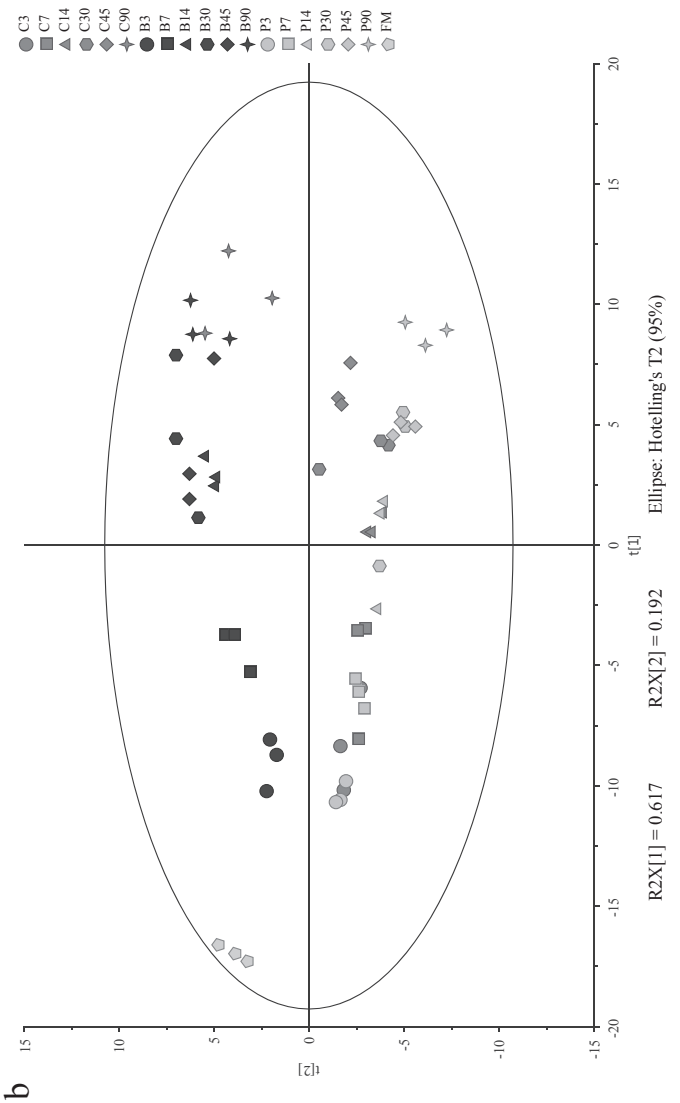

b
C3
C7
C14
C30
C45
C90
B3
B7
B14
B30
B45
B90
P3
P7
P14
P30
P45
P90
FM
t[2]
t[1]
R2X[1] = 0.617
R2X[2] = 0.192
Ellipse: Hotelling's T2 (95%)

Figure 3 Untargeted metabolomic profile of the whole crop corn silage metabolome. C, Corn silage without inoculants; B, Corn silage inoculated with *L. buchneri*; P, Corn silage inoculated with *L. plantarum*; FM, Fresh material. (a) A heatmap of the relative concentrations of sum of differentially expressed metabolites. (b) Principal component analysis (PCA) of metabolic profiles in whole crop corn silage inoculated withoutinoculation (control) or inoculated with *L. plantarum* or *L. buchneri* (n = 3) for different fermentation time. Input data were the total mass of the signal integration area of each sample, and the signal integration area was normalized with a method of internal standard normalization for each sample.

The PCA of metabolome showed that the samples inoculated with *L. buchneri* were clearly separated by PC2, while the differences with control were not significant in samples fermented for 90 d (Figure 3b). The control silage and samples inoculated with *L. plantarum* were separated until 45 d of fermentation. The differences of fermentation process were contributed by PC1, which represented 61.7% of metabolites in ensiled forages at different fermentation times. The end products during fermentation are either directly produced by microbial activity or indirectly as a result of degradation and transformation of substances present in the material. Plant enzymes in aerobic silo stage (Pahlow et al., 2003) and variable dynamic abundances of LAB strains resulted in dynamics of metabolome, which contributed to variations of many metabolites, particularly amino acids, in whole crop corn silage in the current experiment. At the same time, many metabolites with biofunctional activities like bacteriostatic (naringin and 3,4-dihydroxybenzoic acid), antioxidant (ferulic acid and catechol), central nervous system inhibitory (4-aminobutyric acid) and anti-inflammatory (salicin) were found with potential benefits fo animal health and welfare in corn silage.

3.3 Microbial alterations contributed to functional shifts after fermentation

In order to determine if the observed variations in bacterial community succession contribute to community-wide functional shifts, we used KEGG (Kyoto Encyclopedia of Genes and Genomes) database (specify full pathway in level 2) with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) approach (Figure 4a-4c). In comparison with the control, the inoculation by *L. plantarum* and *L. buchneri* modulated the microbial communities, which resulted in marked differences in functional shift. The results predicted that the pathways closely related with silage fermentation were metabolism of carbohydrates, amino acids, energy, cofactors and vitamins, glycan biosynthesis and metabolism, biosynthesis of other secondary metabolites, and xenobiotics biodegradation and metabolism. The relative abundances of certain function genes were also dynamic.

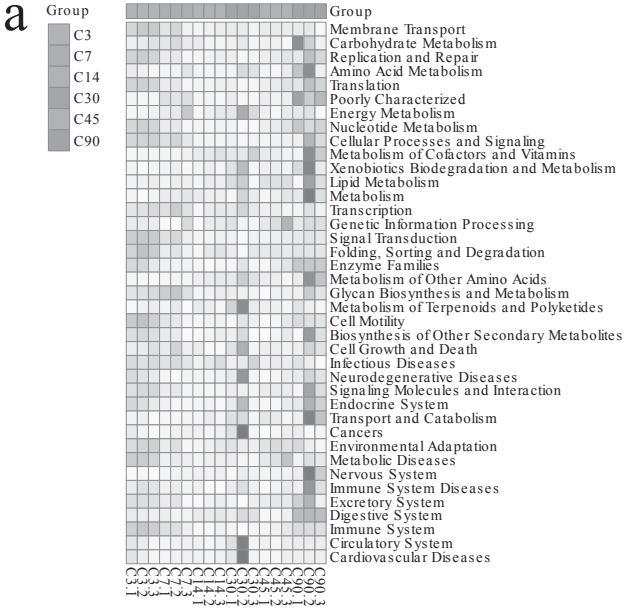
Predicted functional shifts were further examined for their association with the relative extinction or blooming of specific phylotypes. All differences between each fermentation time could not be identified statistically, so we divided the fermentation process into early period (before 7 d of fermentation, aerobic in-silo phase in the early stage), middle period (7 to 45 d of fermentation, anaerobic fermentation) and late period (after 45 d of fermentation, anaerobic storage). We observed driving or attenuating functional shifts of flavones and flavonol biosynthesis, polycyclic aromatic hydrocarbon degradation, glycosaminoglycan degradation and D-alanine metabolism (Figure 4d-4f).

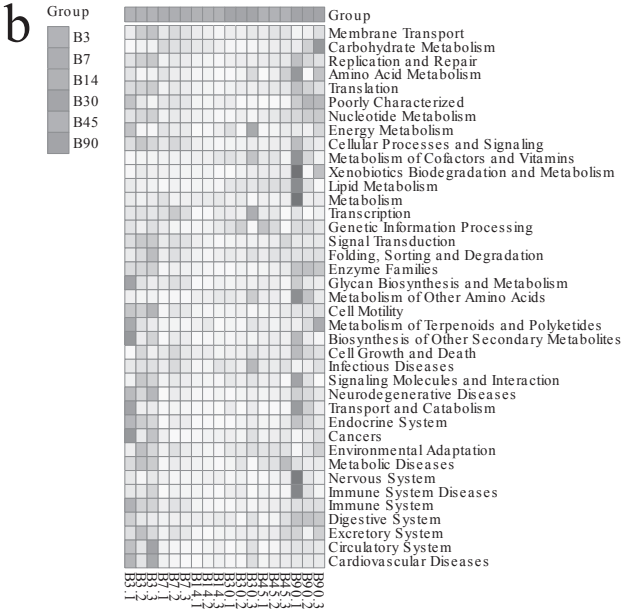
Although bacterial taxa contributed to reducing functional shifts, the statistical data showed that marked upregulation of these pathways in middle period of fermentation

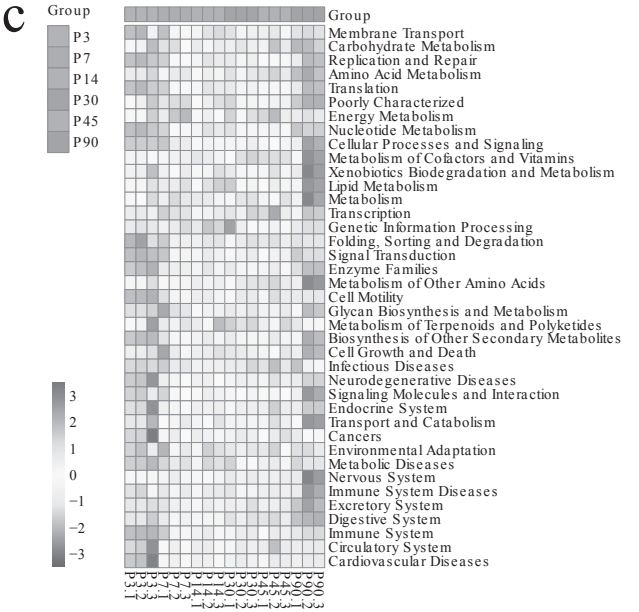
are largely dependent on *Lactobacillus* reactions (data not show). In the majority of silage fermentation reactions, the substrates are fructose and glucose. Once the crop is cut, fructose and glucose concentrations can only increase by degradation of polysaccharides (Pahlow et al., 2003). The glycosaminoglycan degradation pathway was significantly upregulated by *Lactobacillus* at middle stage of fermentation in non-inoculated corn silage, but not in the inoculated silages. However, fructose and glucose were not detected with GC-TOF-MS. The glucose-6-phosphate is the key intermediate to understand the glucose metabolism, which increased with start of fermentation in all treatments. The present study with metabolome approach verified the previous research theories (Pahlow et al., 2003), but this pathway suggested that polysaccharides were hydrolyzed until middle stage of maize fermentation.

The D-alanine metabolism pathway was upregulated by *Lactobacillus* in corn silage inoculated with *L. buchneri*. D-alanine is an amino acid that occurs only in the peptidoglycan of bacterial cell walls (Schleifer and Kandler, 1972), which can be a as a marker of bacteria. Deamination can be induced by alanine to product acetate (Li et al., 2018) from amino acid metabolism. Thus, inoculants of *L. buchneri* can improve aerobic stability (Reich and Kung, 2010) via upregulation of this pathway to increase the production of acetate. However, acetic acid was produced via other pathways in the present study as indicated by the similar and increasing trend in concentration of alanine and acetic acid with progressing fermentation process. The inconsistency may partly be due to technical difficulties in extraction and detection of some metabolites using the same oven temperature of GC-TOF-MS. On the other hand, the gene expression is impacted by many factors in silage fermentation like changing pH, substrates for microbiota fermentation, other metabolite regulations and interactions between microbes

in the system (Guo et al., 2018; Song and Chan, 2019). As for flavones and flavonol biosynthesis, differences were shown in all treatments. Compared with control silage, samples inoculated with *L. buchneri* increased the upregulation degree and the opposite result was observed in samples inoculated with *L. plantarum*. Compared with control silage, the inoculation of *L. plantarum* also decreased the upregulation degree of polycyclic aromatic hydrocarbon degradation pathway.




b
Group
B3
B7
B14
B30
B45
B90
Group
Membrane Transport
Carbohydrate Metabolism
Replication and Repair
Amino Acid Metabolism
Translation
Poorly Characterized
Nucleotide Metabolism
Energy Metabolism
Cellular Processes and Signaling
Metabolism of Cofactors and Vitamins
Xenobiotics Biodegradation and Metabolism
Lipid Metabolism
Metabolism
Transcription
Genetic Information Processing
Signal Transduction
Folding, Sorting and Degradation
Enzyme Families
Glycan Biosynthesis and Metabolism
Metabolism of Other Amino Acids
Cell Motility
Metabolism of Terpenoids and Polyketides
Biosynthesis of Other Secondary Metabolites
Cell Growth and Death
Infectious Diseases
Signaling Molecules and Interaction
Neurodegenerative Diseases
Transport and Catabolism
Endocrine System
Cancers
Environmental Adaptation
Metabolic Diseases
Nervous System
Immune System Diseases
Immune System
Digestive System
Excretory System
Circulatory System
Cardiovascular Diseases


c
Group
P3
P7
P14
P30
P45
P90
Group
Membrane Transport
Carbohydrate Metabolism
Replication and Repair
Amino Acid Metabolism
Translation
Poorly Characterized
Energy Metabolism
Nucleotide Metabolism
Cellular Processes and Signaling
Metabolism of Cofactors and Vitamins
Xenobiotics Biodegradation and Metabolism
Lipid Metabolism
Metabolism
Transcription
Genetic Information Processing
Folding, Sorting and Degradation
Signal Transduction
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Metabolism of Other Amino Acids
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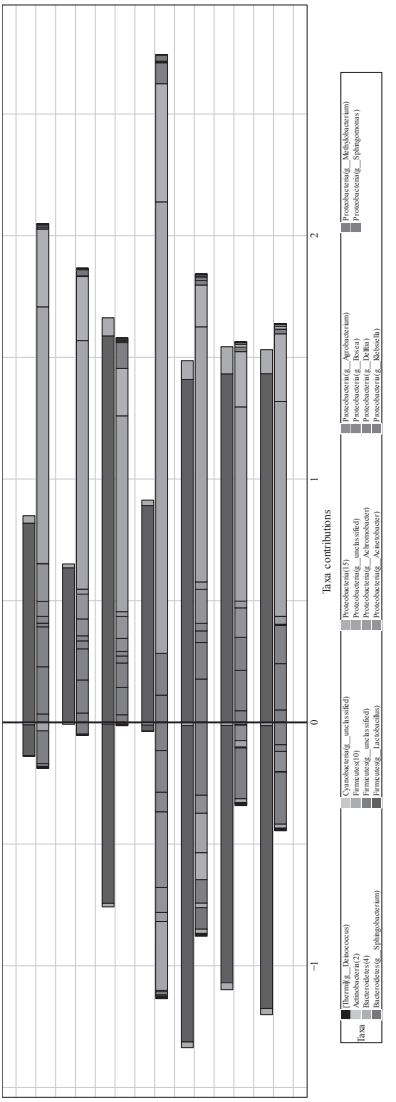
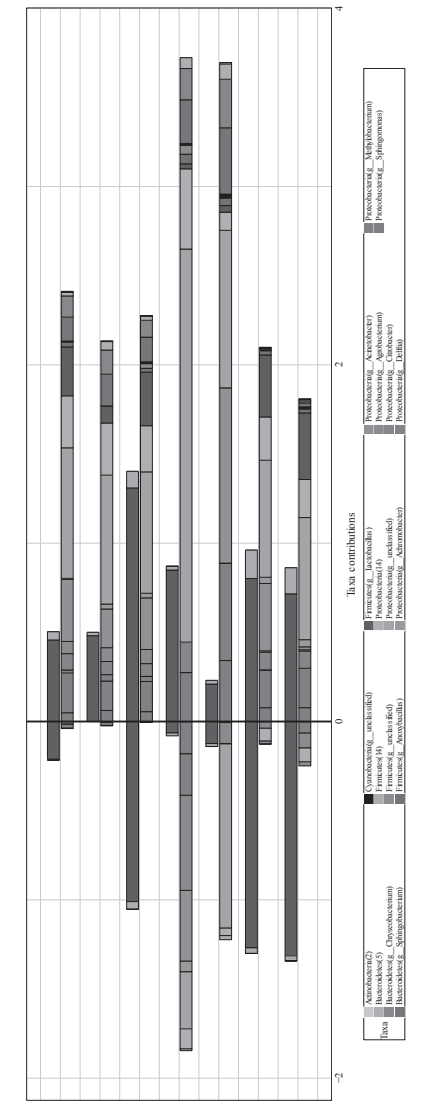
Glycan Biosynthesis and Metabolism
Metabolism of Terpenoids and Polyketides
Biosynthesis of Other Secondary Metabolites
Cell Growth and Death
Infectious Diseases
Neurodegenerative Diseases
Signaling Molecules and Interaction
Endocrine System
Transport and Catabolism
Cancers
Environmental Adaptation
Metabolic Diseases
Nervous System
Immune System Diseases
Excretory System
Digestive System
Immune System
Circulatory System
Cardiovascular Diseases
3
2
1
0
−1
−2
−3

d


Staphylococcus aureus infection
RNA polymerase
Flavone and flavonol biosynthesis
Polycyclic aromatic hydrocarbon degradation
Glycosaminoglycan degradation
Secondary bile acid biosynthesis
Primary bile acid biosynthesis
−1
0
1
2
Taxa contributions
Taxa
Thermi(g__Deinococcus)
Actinobacteria(2)
Bacteroidetes(4)
Bacteroidetes(g__Sphingobacterium)
Cyanobacteria(g__unclassified)
Firmicutes(10)
Firmicutes(g__unclassified)
Firmicutes(g__Lactobacillus)
Proteobacteria(15)
Proteobacteria(g__unclassified)
Proteobacteria(g__Achromobacter)
Proteobacteria(g__Acinetobacter)
Proteobacteria(g__Agrobacterium)
Proteobacteria(g__Bosea)
Proteobacteria(g__Dyella)
Proteobacteria(g__Methylobacterium)
Proteobacteria(g__Sphingomonas)

e


Staphylococcus aureus infection
RNA polymerase
Flavone and flavonol biosynthesis
Polycyclic aromatic hydrocarbon degradation
D-Alanine metabolism
Secondary bile acid biosynthesis
Primary bile acid biosynthesis
−2
0
2
4
Taxa contributions
Taxa
Actinobacteria(2)
Bacteroidetes(5)
Bacteroidetes(g__Chryseobacterium)
Bacteroidetes(g__Sphingobacterium)
Cyanobacteria(g__unclassified)
Firmicutes(4)
Firmicutes(g__unclassified)
Firmicutes(g__Anoxybacillus)
Firmicutes(g__Lactobacillus)
Proteobacteria(4)
Proteobacteria(g__unclassified)
Proteobacteria(g__Achromobacter)
Proteobacteria(g__Achromobacter)
Proteobacteria(g__Acinetobacter)
Proteobacteria(g__Citrobacter)
Proteobacteria(g__Delftia)
Proteobacteria(g__Methylobacterium)
Proteobacteria(g__Sphingomonas)

f

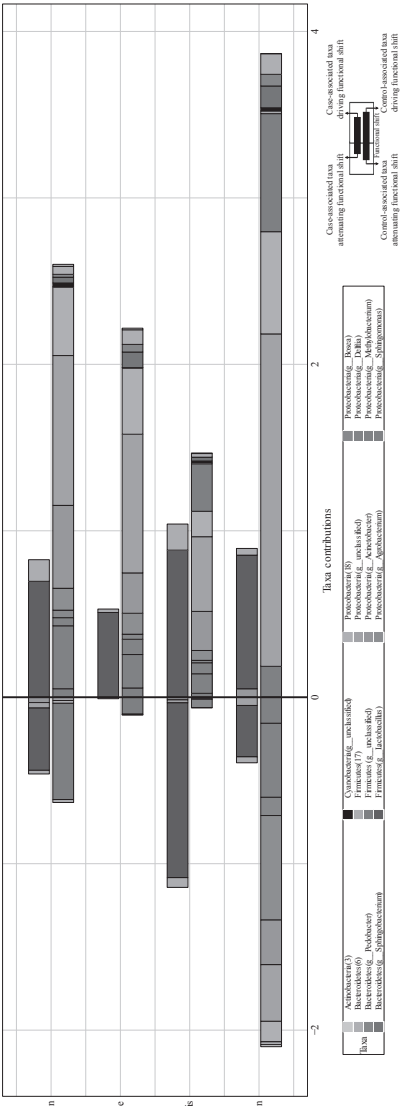

Staphylococcus aureus infection
RNA polymerase
Flavone and flavonol biosynthesis
Polycyclic aromatic hydrocarbon degradation
−2
0
2
4
Taxa contributions
Taxa
Actinobacteria (3)
Bacteroidetes (6)
Bacteroidetes(g__Pedobacter)
Bacteroidetes(g__Sphingobacterium)
Cyanobacteria(g__unclassified)
Firmicutes (3)
Firmicutes (f__unclassified)
Firmicutes(g__Lactobacillus)
Proteobacteria (8)
Proteobacteria (f__unclassified)
Proteobacteria(g__Acinetobacter)
Proteobacteria(g__Agrobacterium)
Proteobacteria(g__Bosea)
Proteobacteria(g__Bulkholderia)
Proteobacteria(g__Methylobacterium)
Proteobacteria(g__Sphingomonas)
Case-associated taxa attenuating functional shift
Case-associated taxa driving functional shift
Control-associated taxa attenuating functional shift
Control-associated taxa driving functional shift
Functional shift
Case
Control

Figure 4 Microbial alterations contribute to functional shifts after fermentation with or without inoculants. C, Corn silage without inoculants; B, Corn silage inoculated with *L. buchneri*; P, Corn silage inoculated with *L. plantarum*. Summary of significant functional shifts predicted by the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) approach (a-c). For each KEGG pathway, the second level of the predicted functional shift is shown with respect to fermentation process and treatments. (a) Corn silage without inoculants. (b) Corn silage inoculated with *L. buchneri.* (c) Corn silage inoculated with *L. plantarum*. Comparing taxon-level contribution profiles of functional shifts in fermentation process by FishTaco approach. (d-f). For each KEGG pathway, the third level of the predicted functional shift is shown with respect to fermentation process and treatments. Comparison of middle stage (7-45 d of fermentation) and early stage (3 d of fermentation) of fermentation of corn silage without inoculants (d), inoculated with *L. buchneri* (e) and inoculated with *L. plantarum* (f).

3.4 Correlations between silage bacteria and well-predicted metabololites with biofunctions

The widespread associations between bacteria and well-predicted metabolites with biofunctions across the silages with or without inoculants are presented (data not show). The correlations between bacterial species and well-predicted metabolites with biofunctions (lysine, methionine, phenylalanine, naringin, 3,4-dihydroxybenzoic acid, 4-aminobutyric acid, salicin, L-malic acid, ferulic acid, linolenic acid and catechol) in silages with different treatments were clearly exhibited (Figure 5a-5c; the absolute value of correlation coefficients was > 0.6 between LAB and metabolites; the absolute value of correlation coefficients was > 0.9 between other bacteria and metabolites). Network analysis showed various associations between well-predicted metabolites with biofunction activity and lactic acid bacteria species in corn silages with or without inoculants.

In control silage, essential amino acids lysine, methionine and phenylalanine were positively correlated with *L. buchneri*, *L. silagei* and *L. parafarraginis*. Inoculants, especially *L. plantarum*,

decreased the positive correlation between essential amino acids and LAB. The correlations between metabolites with bacteriostatic activity (naringin and 3,4-dihydroxybenzoic acid) and LAB were markedly negative. A previous study reported that *L. plantarum* with antifungal property produced a phenolic-related antibiotic or 3-hydroxy fatty acids (Sjögren et al., 2003; Valan Arasu et al., 2013). In the current study, *L. plantarum* also positively correlated with the two metabolites irrespective of treatments used. Thus, *L. plantarum* could be considered a species for screening inoculants with potential of producing high quality silage.

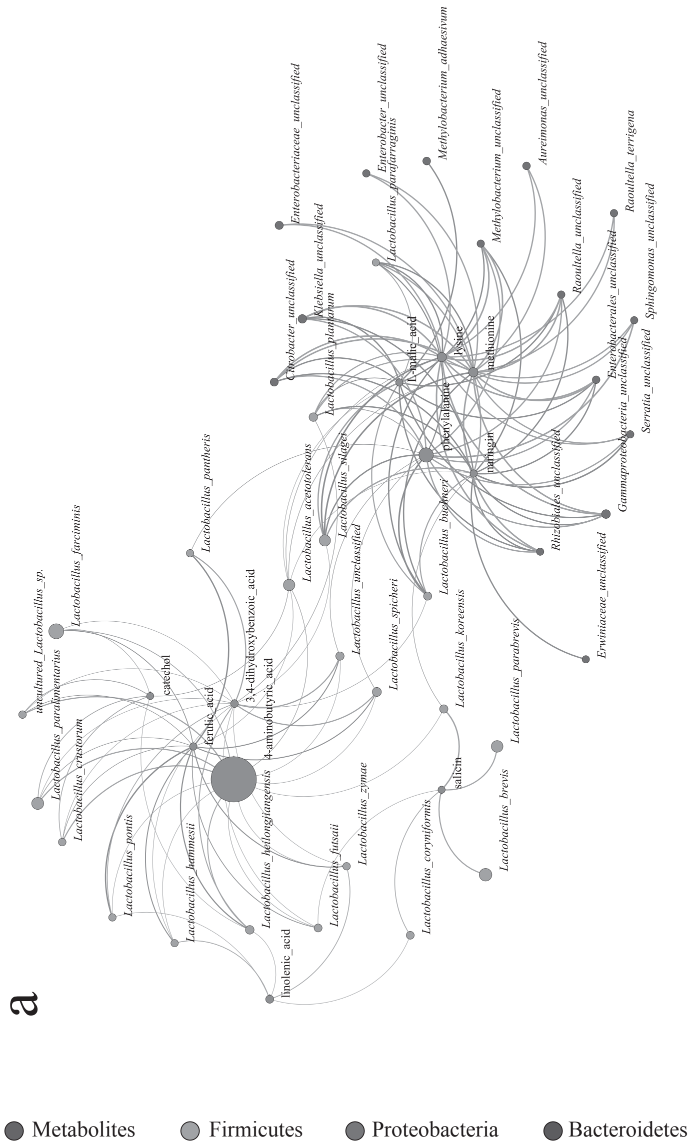
Metabolites like ferulic acid and catechol with antioxidant activity were found in the present study. The correlation between ferulic acid and LAB was markedly positive, and seven LAB species positively correlated with ferulic acid ($r > 0.8$), but inoculants decreased the positive correlations. It might be because the increased ferulic acid inhibited the growth and viability (Rodríguez et al., 2009) of some species in inoculant-treated corn silage. Ferulic acid was positively correlated with *L. silagei*, *L. panis* and *L. kefiri* in samples inoculated with *L. buchneri* and *L. plantarum*, respectively. Catechol is a member of flavonoids, which can be metabolized from protocatechuic acid by *Lactobacillus* spp. (Filannino et al., 2015). However, LAB species indicated only low positive correlations with catechol in this study. It might be because of lack of LAB species and protocatechuic acid to produce catechol in the corn silage fermentation ecosystem.

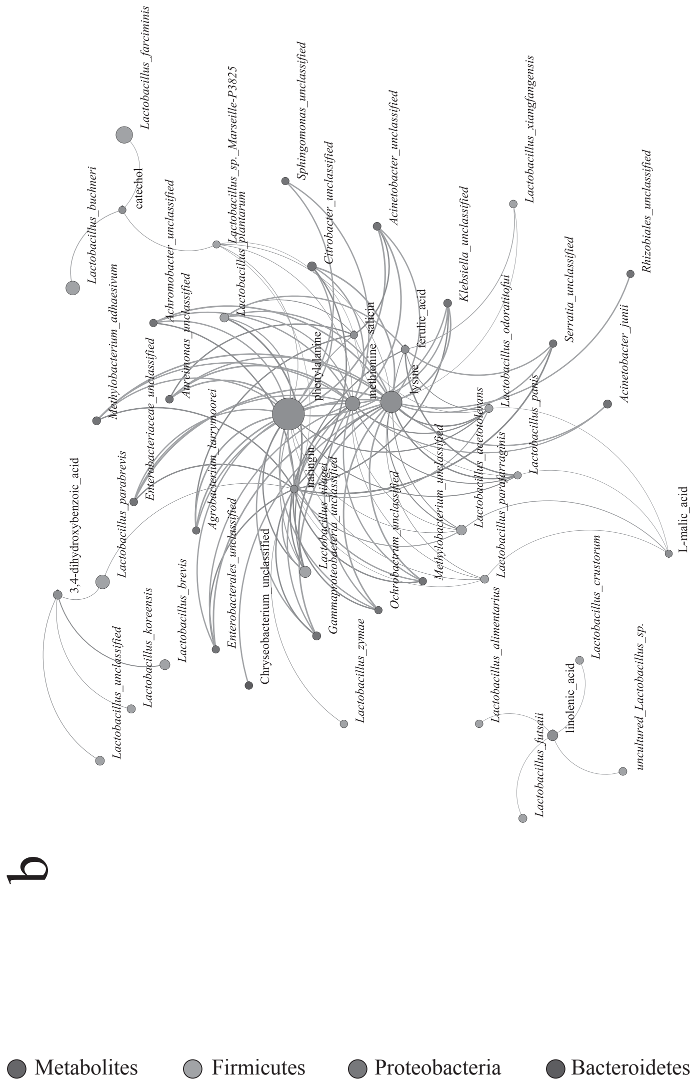
Metabolite 4-aminobutyric acid or gamma-aminobutyric acid with central nervous system inhibitory activity can decrease blood pressure and insulin secretion and was identified in the present study. Low positive correlations between LAB and 4-aminobutyric acid were observed in corn silage, and only *L. acetotolerans* ($r = 0.655$) positively correlated with it in control silage while *L. buchneri* and *L. parafarraginis* were positively correlated with it in samples inoculated with *L. plantarum*. A

number of bacteria and fungi have been reported to produce 4-aminobutyric acid (Kono and Himeno, 2000; Lu et al., 2008). The most common microorganisms for 4-aminobutyric acid production are LAB. In addition, different fermentation factors affect the 4-aminobutyric acid production by microorganisms, the most important ones being pH, temperature and substrate availability of culture (Dhakal et al., 2012). Therefore, we speculate that many microorganisms and their dynamics as well as fermentation factors co-affected the production of 4-aminobutyric acid rather than abundance of certain bacterial species.

Linolenic acid is an essential fatty acid with well-established health benefits. Corn silage inoculated with *L. plantarum* increased the positive correlation between LAB and linolenic acid. Twelve LAB species were positively correlated with linolenic acid in L. plantarum inoculated silage but no LAB species was positively correlated with it in samples inoculated with *L. buchneri* and in control silage. Linked to the dynamics of linolenic acid and microbial community, we found that the changes of relative abundances of *L. heilongjiangensis* and *L. pontis* were accordant with concentration of linolenic acid. Thus, the linolenic acid could be produced by *L. heilongjiangensis* and *L. pontis*.

Salicin with anti-inflammatory activity decreased with progressing time of fermentation, although inoculation with *L. buchneri* relieved the decrease. It might be because some bacterial species utilized salicin (Wang et al., 2009; Cai et al., 2012) and the community structure and dynamics resulted in lower positive correlations between salicin and LAB strains. In addition, salicylic acid is a metabolite from salicin with antipyretic activity (Mackowiak, 2000). The relative concentration of salicylic acid decreased with prolonged ensiling time as well. If the silage contains these two substances, it can be used to treat inflammatory conditions in periparturient or heat stressed dairy cows (Trevisi and Bertoni, 2008).


a
Metabolites
Firmicutes
Proteobacteria
Bacteroidetes
Enterobacteriaceae_unclassified
Enterobacter_unclassified
Lactobacillus_parafarraginis
Methylobacterium_adhaesivum
Methylobacterium_unclassified
Aureimonas_unclassified
Raoultella_terrigena
Raoultella_unclassified
Sphingomonas_unclassified
Enterobacterales_unclassified
Klebsiella_unclassified
Citrobacter_unclassified
Lactobacillus_plantarum
L-malic_acid
lysine
methionine
phenylalanine
naringin
Serratia_unclassified
Gammaproteobacteria_unclassified
Rhizobiales_unclassified
Erwiniaceae_unclassified
Lactobacillus_buchneri
Lactobacillus_silagei
Lactobacillus_acetotolerans
Lactobacillus_pantheris
Lactobacillus_unclassified
Lactobacillus_spicheri
Lactobacillus_koreensis
Lactobacillus_parabrevis
Lactobacillus_brevis
salicin
Lactobacillus_coryniformis
Lactobacillus_zymae
Lactobacillus_futsaii
4-aminobutyric_acid
3,4-dihydroxybenzoic_acid
ferulic_acid
catechol
Lactobacillus_farciminis
uncultured_Lactobacillus_sp.
Lactobacillus_paralimentarius
Lactobacillus_crustorum
Lactobacillus_pontis
Lactobacillus_hammesii
Lactobacillus_heilongjiangensis
linolenic_acid


b
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catechol
Lactobacillus_buchneri
Lactobacillus_sp._Marseille-P3825
Lactobacillus_plantarum
Sphingomonas_unclassified
Citrobacter_unclassified
Acinetobacter_unclassified
Lactobacillus_xiangfangensis
Rhizobiales_unclassified
Klebsiella_unclassified
Serratia_unclassified
Acinetobacter_junii
Lactobacillus_odoratitofui
Lactobacillus_panis
ferulic_acid
lysine
methionine
salicin
phenylalanine
Achromobacter_unclassified
Aureimonas_unclassified
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Lactobacillus_crustorum
linolenic_acid
Lactobacillus_futsaii
uncultured_Lactobacillus_sp.
Metabolites
Firmicutes
Proteobacteria
Bacteroidetes

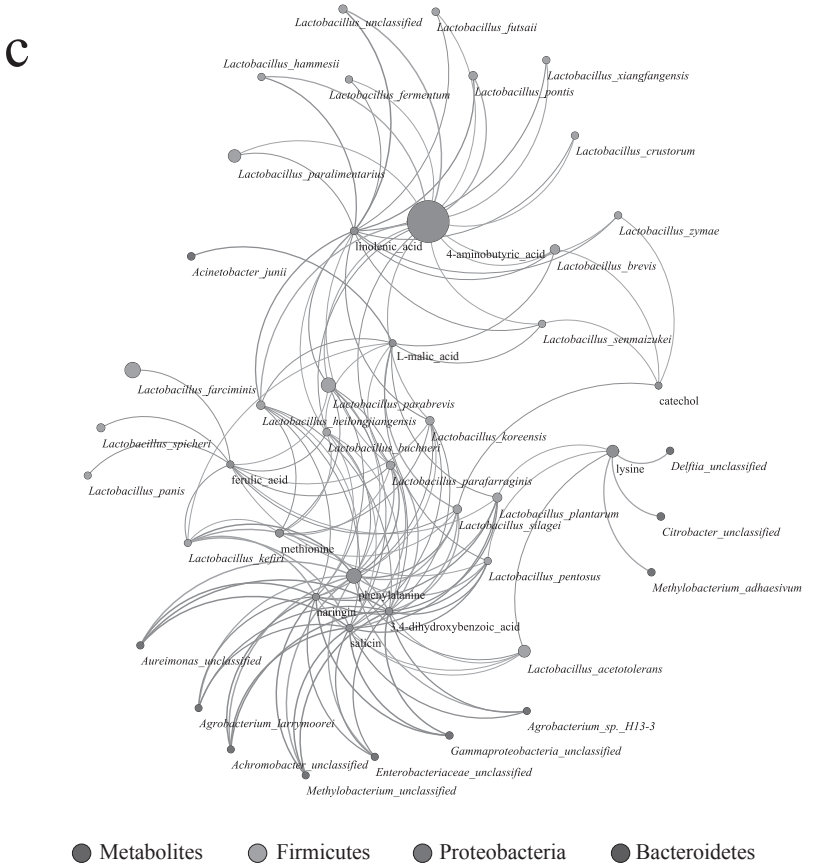

Figure 5 Correlation analysis of the bacteria and metabolites with biofunctional activity Correlation visualization of significant associations between well-predicted metabolites with biofunctions (similarity > 500, a total of 643 compounds) and bacterial phylotypes (the absolute value of correlation coefficients between metabolites and LAB > 0.6, the absolute value of correlation coefficients between metabolites and bacterial species except for LAB > 0.8). Node size is proportional to the relative abundance of the corresponding metabolite (from GC-TOF-MS) or phylotype (from 16S amplicon data). Edge width is proportional to the strength of association between each metabolite-phylotype pair (as measured by the correlation), red edge indicates the positive correlation and green edge indicates the negatively correlation. (a) Corn silage without inoculants. (b) Corn silage inoculated with *L. buchneri*. (c) Corn silage inoculated with *L. plantarum*.

With many beneficial activities, malic acid is also a key intermediate in the citric acid cycle of biological tissues, and has been used as a feed additive for ruminants to improve performance and efficiency (Ke et al., 2018). However, malic acid is metabolized to lactic acid, and can be consumed during forage fermentation (Ke et al., 2017). L-malic acid has not been detected in fresh maize but was present in trace amounts during ensiling in this study. It might be because the extraction and test conditions of GC-TOF-MS were not optimal for precisely detecting some substances, especially trace metabolites. In this regard, targeted metabolomic analysis should be profiled in further studies.

4. Conclusions

This work gives insights into the complicated biological processes underlying silage fermentation. It also provides a framework to re-evaluate ensiled forages with regard to metabolome and microbiome, which may contribute to target-based regulation methods to produce functional silages for various animal groups based on their particular needs to guarantee high quality and safety of animal products.

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Fiber quality and forage allocation throughout lactation

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Introduction

Optimal forage allocation and supplementation strategies for dairy cattle must consider variation in forage quality characteristics as well as cow's responses to their diet throughout lactation. Forage quality characteristics can affect diet cost, energy intake and partitioning, feed conversion efficiency (FCE), and animal health. These characteristics include neutral detergent fiber concentration and digestion characteristics, the concentration, digestibility, and site of digestion of starch, particle size and fragility, and crude protein concentration and composition. Forages are unique among diet ingredients fed to ruminants because they provide fiber that is effective at retaining feed particles in the rumen, increasing their digestibility and increasing digesta mass and volume. Increased digesta mass in the rumen can reduce risk of ruminal acidosis and abomasal displacement but can also limit feed intake, depending upon the physiological state of animals. Control of feed intake by ruminal distension increases with milk yield and diet forage NDF concentration. This paper will discuss the relative importance of forage quality characteristics to cows and how they change throughout the lactation cycle.

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Feed intake

Eating is controlled by the integration of signals in brain feeding centers. Whereas cow diets must contain a minimal concentration of relatively low-energy roughages for proper rumen function, signals from ruminal distension can control feed intake when the drive to eat is high and metabolic control of feed intake is diminished (e.g. cows at peak lactation). Signals derived from metabolism of fuels dominate the control of feed intake when signals from distension diminish (e.g. cows in late lactation). Therefore, effects of diet on feed intake vary with the physiological state of the animal.

Filling effect of forages

The extent to which ruminal distention limits feed intake is positively related with milk yield. This was shown in two studies from our laboratory in which groups of cows with a wide range of milk yield were offered diets differing in rumen fill. The first study compared brown midrib corn silage to control corn silage (Oba and Allen, 1999a). The two silages had similar DM and NDF concentrations but *in vitro* NDF digestibility (30 h) was nearly 10 units higher for the low-lignin brown midrib corn silage. When both forages were offered to a group of cows with a wide range of milk yield, response in DMI and FCM to the brown midrib corn silage compared with the control corn silage increased linearly with milk yield. Whereas the lower producing cows with ~30 kg/d milk yield had similar DMI and FCM for the two silages, FCM increased ~8 kg/d for the highest producing cows with ~55 kg/d milk yield. The second study compared diets differing in forage to concentrate ratio (Voelker and Allen, 2000). Diets contained either 44% forage (24% NDF and 34% starch) or 67% forage (31% NDF and 23% starch). Response in DMI to the lower forage diet increased linearly (up

to ~4.5 kg/d for the highest producing cows) and FCM yield increased ~2.2 kg per kg increase in DMI for cows producing over ~40 kg FCM/d. However, cows producing less than ~40 kg/d this had similar FCM for the two treatments. Therefore, high-producing dairy cows should be fed less-filling diets to maximize feed intake.

The filling effect of a diet is determined primarily by the initial bulk density of feeds, as well as their filling effect over time in the rumen. The overall filling effect is determined by forage NDF content, forage particle size, fragility of forage NDF affected by forage type (legumes, perennial grasses, annual grasses), and NDF digestibility within a forage family (Allen, 2000). Forage NDF is less dense initially, digests more slowly, and is retained in the rumen longer than other diet components. Increasing diet forage NDF concentration can dramatically reduce feed intake of high producing cows. Several studies in the literature reported a decrease in DMI of up to 4 kg/d when diet NDF content was increased from 25 to 35% by substituting forages for concentrates (Allen, 2000). Although most studies reported a significant decrease in DMI as forage NDF increased, the DMI response was variable, depending upon the degree to which intake was limited by ruminal fill and the filling effect of the forage NDF. Higher producing cows are limited by fill to the greatest extent and the filling effect of forage fiber varies depending upon particle size and fermentation characteristics.

Experiments that have evaluated effects of forage particle size have generally shown small effects on DMI (Allen, 2000). However, one experiment showed little effect of particle size of alfalfa silage when fed in high grain diets but a large reduction in DMI for the diet containing longer alfalfa silage when fed in a high forage diet (Beauchemin et al., 1994). Feed intake might have only been limited by ruminal fill in the high forage diet, which could explain the interaction observed.

Increasing diet NDF concentration by substituting non-forage

fiber sources (NFFS) for concentrate feeds has shown little effect on DMI in studies reported in the literature (Allen, 2000). Non-forage fiber sources include byproduct feeds with significant concentrations of NDF such as soyhulls, beet pulp, cottonseeds, corn gluten feed, almond hulls and distiller's grains. Fiber in NFFS is much less filling than forage NDF because it is less filling both initially (smaller particle size) and over time in the rumen because it digests and passes from the rumen more quickly.

Forage NDF has a much longer ruminal retention time than other major dietary components. Retention time in the rumen is longer because of longer initial particle size, and greater buoyancy in the rumen over time, which differs greatly across forages. As forages mature, the NDF fraction generally becomes more lignified. Lignin is a component of plant cell walls that helps stiffen the plant and prevent lodging. It is also essentially indigestible by ruminal microbes and limits fermentation of cellulose and hemicellulose. Within a forage type, the degree to which NDF is lignified is related with the filling effects of the NDF. Fiber that is less lignified generally digests and clears from the rumen faster, allowing more space for the next meal. However, ruminal retention time of NDF from perennial grasses is generally longer than for legume NDF despite being less lignified (Oba and Allen, 1999b; Voelker Linton and Allen, 2008; Kammes and Allen, 2012a). Forage NDF from alfalfa was more fragile with a greater rate of breakdown of large particles than NDF from orchardgrass, allowing faster rate of passage of particles from the rumen (Kammes and Allen 2012b). Because of this, forage NDF from perennial grasses is more filling and should not be included in high concentrations in diets of cows for which feed intake is limited by ruminal fill, unless it is of exceptionally high quality. Corn is an annual grass, and corn silage NDF digests and passes from the rumen quickly compared to perennial grasses and can be an excellent source of forage NDF for high producing cows.

Ruminal starch fermentation

Diets with greater ruminal starch fermentability can depress feed intake and forages can greatly affect diet starch concentration and fermentability. Increasing ruminal starch fermentation by substituting a more fermentable starch source for a less fermentable starch source decreased feed intake of cows by more than 3 kg/d in several studies reported in the literature (Allen, 2000). Grain containing forages such as corn, sorghum, and small grain silages vary greatly in both starch concentration and fermentability. Starch concentration and fermentability vary by forage type, genetics, and maturity at harvest and starch fermentability also varies by moisture concentration and time ensiled. Other forages varying in NDF concentration can affect diet fermentability by affecting diet starch concentration; low NDF forages require higher forage diets to meet optimum diet forage fiber concentration, leaving less dietary space for grain possibly limiting dietary starch.

Depression in feed intake by a more fermentable diet is more likely for fresh cows and cows in late lactation when oxidation of fuels in the liver dominates control of feed intake. A more fermentable starch source (high moisture corn) decreased DMI and milk yield compared with dry ground corn when fed to fresh cows and the reduction in DMI and milk yield was greater when fed in diets containing 28% starch compared with 22% starch (Albornoz and Allen, 2018). Ruminal propionate production increases as diet fermentability increases and propionate might limit feed intake by stimulating more complete oxidation of mobilized fatty acids in the liver of fresh cows or by extending hepatic oxidation over time when its supply to the liver exceeds its rate of utilization which likely increases as milk yield declines through lactation (Allen et al., 2009). Forages with high concentrations of starch that is highly fermentable should be limited in diets of fresh cows and late lactation cows to enhance feed intake.

Importance of maintaining ruminal fill

Whereas ruminal distention becomes a primary limitation to feed intake as milk yield increases, it likely has less effect on feed intake when it is controlled primarily by oxidation of mobilized fatty acids in the liver during the transition period (Allen et al., 2009). Glucose demand of fresh cows is high when glucose utilization for milk production outpaces gluconeogenesis by the liver. Whereas cows require diets with adequate glucose precursors (i.e. starch from grains), it is important to also maintain rumen fill. Formulating diets to maintain rumen fill with ingredients that are retained in the rumen longer, and have moderate rates of fermentation and high ruminal digestibility will likely benefit transition cows several ways. Increased ruminal digesta mass can provide more energy over time when feed intake decreases at calving or from metabolic disorders, mastitis or infectious disease. This will help maintain plasma glucose and insulin concentrations preventing even more rapid mobilization of body reserves compared with when diets are formulated with ingredients that disappear from the rumen quickly. Ruminal digesta is very important to buffer fermentation acids and buffering capacity is directly related with the amount of digesta in the rumen. Therefore, diets formulated with ingredients that increase the amount of digesta in the rumen will have greater buffering capacity and will maintain buffer capacity longer if feed intake decreases. Inadequate buffering can result in low ruminal pH, decreasing fiber digestibility and acetate production, and increasing propionate production, possibly stimulating oxidation in the liver and decreasing feed intake. Low ruminal pH also increases risk of health problems such as ruminal ulcers, liver abscess, and laminitis, and causes stress, likely increasing mobilization of body reserves even further. Diets formulated with ingredients that maintain digesta in the rumen longer when feed intake decreases will likely decrease risk of abomasal displacement.

Physically effective fiber

Optimum particle length of individual forages depends upon several factors including forage type, silo type (if ensiled), other forage(s) in the diet, the characteristics of the cow consuming the forage, stocking density/competition for feed, and diet fermentability. An adequate concentration of long particles is required to form a rumen mat to retain small particles that would otherwise escape, increasing diet digestibility, rumen fill, and buffering capacity. Some forages that are particularly fragile such as brown midrib corn silage benefit by chopping longer. Forages that are resilient to packing might have to be chopped shorter, particularly where packing is difficult (e.g. upright silos). Forages lacking physically effective fiber must be limited in diets and combined with forages with adequate particle length. When overcrowding causes competition at the feed bunk and slug feeding, diets with more physically effective fiber can limit rate of eating, decreasing risk of low ruminal pH, especially for highly fermentable diets.

Energy partitioning

Energy partitioning between milk production and body condition varies as physiological state changes throughout lactation. As lactation proceeds past peak, insulin concentration and sensitivity of tissues increases and energy is increasingly partitioned to body condition, sometimes at the expense of milk yield. Whereas high-starch diets can increase milk yield of high producing cows, they can result in excessive gain in body condition as milk yield declines and insulin sensitivity of tissues increase. We showed that a 69% forage diet (0% corn grain) containing brown midrib corn silage increased energy partitioned to milk, decreasing body weight gain while maintaining yield of milk compared to a 40% forage diet (29 % corn grain) containing control corn silage (Oba and Allen, 2003). *In vitro* NDF digestibility of the brown midrib corn silage

was ~20% higher (55.9 vs 46.5%) than the control corn silage. In contrast, DMI and milk yield was reduced when the control corn silage was fed in the higher forage diets. We also showed that beet pulp decreased BCS without decreasing yields of milk or milk fat when substituted for high-moisture corn up to 12% of diet DM (Voelker and Allen, 2003). Similarly, an experiment conducted with cows in the last 2 months of lactation showed that substitution of beet pulp for barley grain linearly decreased body condition score, maintained milk yield and linearly increased milk fat yield (Mahjoubi et al., 2009). Decreased body condition score and increased milk fat yield might have been because of a linear decrease in plasma insulin concentration which linearly increased plasma NEFA concentration. However, lower ruminal pH was reported as starch concentration of the diet increased, which might have caused the milk fat depression through CLA production in the rumen (not measured). Harvatine et al. (2009) reported that CLA-induced milk fat depression decreased gene expression for enzymes and regulators of fat synthesis in adipose tissue. Decreasing fermentability of diets by increasing fiber from forages or NFFS can maintain milk yield while decreasing gain in body condition.

Feed conversion efficiency

The efficiency for which feed is converted to milk is affected primarily by feed intake, digestibility, and energy partitioning. Greater milk yield, which is affected by energy intake and partitioning, increases FCE by diluting maintenance costs. Diet digestibility is affected by initial forage quality, forage supplementation, and animal characteristics. Therefore forage allocation and supplementation can have large effects on FCE.

Protein

Protein concentration and ruminal degradability vary greatly

among forages and should be considered when allocating forages to different groups of animals. Overfeeding protein decreases efficiency of N utilization, increases excretion of N waste, increases feed costs and costs energy to excrete excess N as urea. Whereas forages with high protein concentrations are typically allocated to cows with higher protein requirements, forages with low NDF and high protein concentrations such as immature alfalfa can result in excessive diet protein concentration unless forage(s) with lower protein concentrations are also used in the diet.

Specific recommendations

Testing: All forages should be tested for concentrations of DM, NDF, CP, as well as lignin or *in vitro* NDF digestibility. Some laboratories report lignin as a % of DM, which isn't useful because lignin only limits digestion of fiber and not other fractions of DM. Therefore, lignin (% of DM) should be divided by NDF (% of DM) to determine the extent to which the NDF is lignified. There are several measures of lignin used although the predominant measure is acid detergent sulfuric acid lignin (ADL). Acid detergent lignin as a percent of NDF ranges from ~3 to 9 % for corn silage and from ~11 to 20% for alfalfa (hay and silage). Within a forage type, forage NDF with the lowest ADL/NDF is likely the least filling. Additionally, mixed grass-legume forages should be tested for ADF to help determine the fraction of grass and legume in the forage; ADF/NDF is ~0.8 for legumes and ~0.6 for grasses. Mixed forages with more grass are more filling and should be limited for high producing cows with intake limited by rumen fill. Ensiled forages should be tested for pH, ammonia, and fermentation profile as well.

In vitro NDF digestibility (NDFD) varies greatly by NDF source (from forage and NFFS). NDFD is the percentage of initial NDF that is fermented *in vitro* by rumen microbes over a specific period of time such as 30 h (NDFD30) or 48 h (NDFD48). The

time selected is supposed to represent the retention time of the feed in the rumen of the target cow. However, it is affected by forage source, diet and dry matter intake (DMI) as discussed below. The opposite of NDFD is uNDF, which is the percentage of initial NDF that remains after *in vitro* fermentation for a specific time period such as 30 h (uNDF30) or 240 h (uNDF240). The very long fermentation time of 240 h is used to approximate the percentage of NDF that is completely indigestible. This fraction is called indigestible NDF (iNDF) and is the same as uNDF240. The NDFD30 and uNDF30 percentages add to 100% of NDF and because they are perfectly related, they explain the same amount of variation in rumen fill and DMI. Similarly, the percentages of potentially digestible NDF (pdNDF) and iNDF sum to 100%. The pdNDF fraction is used to calculate NDF digestibility using models based on rates of digestion and passage.

Dietary forage NDF percentage is recommended as a primary basis for diet formulation because it is an actual measurement that has been demonstrated to be related with feed intake and rumen pH in published, peer-reviewed research. However, it must be adjusted for various factors including forage particle size (particularly when forages are chopped very finely) when formulating diets. Other measures are affected by the rates of digestion in, and passage from the rumen; both of which vary with source of feed, diet composition, animal characteristics, and their interactions. The peNDF fraction is typically determined based upon the NDF comncentration and particle size of the feed. However, it is also affected by the NDFD and fragility of forages, both of which vary greatly both among, and within, forage families. Forage fragility affects the rate of particle size reduction during eating and rumination. Increased forage fragility will decrease the physical effectiveness of the NDF by decreasing ruminal retention time.

Measures of NDFD or uNDF are determined *in vitro* with dried, ground feeds. However, grinding increases rate of digestion by increasing the surface area available to microbes

compared with feeds as fed. In addition, the retention times selected (e.g. 30 h or 48 h) are only estimates and actual rumen retention times in the cow differ greatly among feeds. Therefore, NDFD30 and uNDF30 do not accurately represent the digested or undigested fractions in the rumen. For instance, forage legumes such as alfalfa are more fragile, and pass from the rumen more quickly than cool-season grasses so using the same retention time for both over-predicts digestibility of the legume and under-predicts digestibility of the grass. Despite this, the digestibility of NDF measured *in vitro* or *in situ* (such as NDFD30) within a forage family (grass or legume) is a useful measurement because it is positively related with DMI of lactating cows. However, it is very important to note that it is *negatively* related with DMI across these forages. This is because grasses have higher NDFD than legumes but lower DMI, as discussed below. In addition, NDFD30 is not necessarily related with NDF digestibility *in vivo*, which depends upon the effect of diet on DMI and rumen retention time. Therefore, it should not be used to adjust the energy content of feeds. The positive relationship between NDFD and DMI (and milk yield) within, but not across forage family (legumes, grasses) has been shown in individual studies as well as several statistical analyses of treatment means from the literature. uNDF240 has been proposed as proxy for DMI potential with the idea that forages with low uNDF240 should allow greater DMI. However, cool-season grasses have lower uNDF240 and lower DMI compared with legume forages. This is because legumes digest and pass from the rumen more quickly, increasing ruminal clearance rate and decreasing rumen fill compared with grasses. Therefore, like NDFD, uNDF240 cannot be used to rank forages for intake potential across forage family. Whereas uNDF240 might be related with DMI within forage family, there is not enough data reported to conduct a statistical analysis to verify this claim or to compare it with NDFD30. It is important to note that all measures of NDFD or uNDF should be measured on individual feeds and not the entire diet. This is

because NDF from non-forage sources have little effect on DMI, regardless of their digestibility or indigestibility.

Stage of lactation

Far-off dry cows: Goal is to maintain body condition score and limit visceral fat accumulation.

Allocation: Forages with high NDF and low crude protein concentrations such as mature grass hay or silage and straw to limit energy intake close to requirements. Forages with lower NDF digestibility and long ruminal retention times can be utilized. Limit corn silage with high grain concentration.

Supplementation: Add grain to meet energy requirements and limit body condition gain.

Close-up dry cows: Goal is to maintain rumen fill through the transition period. The pool of ruminal digesta will provide energy, buffering capacity and distention to reduce risk of ketosis, acidosis, and displaced abomasums, respectively.

Allocation: Wheat straw digests and likely passes from the rumen slowly and it has been used to dilute energy density of corn silage in TMRs for dry cows. Grass silage or hay is likely more beneficial because the fiber is more digestible and it provides energy for a longer time when feed intake decreases at calving. However, grass with high potassium concentrations might require anionic salts in prepartum diets to reduce milk fever following calving. Avoid finely chopped silages (to ensure adequate rumen retention time) and forages with high protein concentration (to avoid excessive protein in diets).

Supplementation: Include a limited amount of moderately fermentable grains to stimulate insulin secretion and limit fat mobilization while maintaining rumen fill. Non-forage fiber sources do not provide glucose precursors or rumen fill and should be avoided.

Fresh cows: Goal is to maintain rumen fill to reduce risk of displaced abomasum and acidosis and to provide glucose

precursors in a form that will maximize energy intake.

Allocation: Forages with moderate to high NDF concentration with high NDF digestibility but long ruminal retention times such as grass hay or silage. Use of forage with higher NDF concentration will allow adequate dietary space for grain while maintaining rumen fill. Avoid finely chopped silages. Some long fiber particles are necessary to form a mat and increase digesta retention in the rumen, but excessive length of cut can increase sorting, particularly for dry diets. Corn silage can be used but highly fermentable corn silage (e.g. aged corn silage ensiled more than one year, corn silage less than 30% DM, over-processed corn silage) should be limited.

Supplementation: Avoid feeding highly fermentable starch sources to fresh cows because rapid production and absorption of propionate will stimulate oxidation in the liver and suppress feed intake (Allen et al., 2009; Albornoz and Allen, 2018). Starch sources with moderate ruminal fermentability and high digestibility in the small intestine, such as dry ground corn, will provide glucose precursors and less propionate to stimulate oxidation and suppress feed intake. Dry ground corn is the preferred starch source because rumen fermentability is moderate but whole-tract digestibility is high. Non-forage fiber sources can be used to dilute starch when high NDF forages are used but should otherwise be limited because they provide few glucose precursors and little rumen fill.

High producing cows Goal is to feed a low-fill, highly fermentable diet as gut fill begins to dominate control of feed intake. This might be only 7 to 10 days after calving for some cows in the herd or more than 3 weeks for others and is likely indicated by lower plasma NEFA and ketone concentrations, visual observation of cow gut distension, and steadily increasing feed intake.

Allocation: Forages with fiber that is broken down by digestion and chewing quickly (increased fragility) such as brown-midrib corn silage and hay and silage from legumes will

clear from the rumen and allow greater feed and energy intake than forages that have long retention times in the rumen (e.g. mature grasses). Adequate long particles are needed to retain potentially fermentable particles, increasing diet digestibility. Forages with low NDF concentrations (except corn silage) might limit diet space for starch (needed to provide glucose precursors) and should be used sparingly.

Supplementation: High producing cows respond favorably to highly fermentable diets and low-density steam-flaked corn, high moisture corn and rolled barley work well in these diets. However, starch sources that are very rapidly fermented such as ground wheat should be limited. Because feed intake is limited to a large extent by ruminal fill, feed ingredients that can depress ruminal motility such as fat and sugar sources should be limited. Non-forage fiber sources can be used to dilute starch, if needed, but should otherwise be limited because they provide few glucose precursors.

Maintenance group Goal is to maintain body condition score (preventing further gain) while also maintaining or increasing milk yield. Feed a more filling, less fermentable diet as milk yield declines. As lactation progresses past mid-lactation, the highly fermentable diet that is optimal for high-producing cows can depress feed intake as milk yield and glucose demand decreases. Therefore, cows should be switched to a less fermentable and more filling diet as milk yield declines. This will increase feed intake and provide a more consistent supply of fuels, partitioning more energy to milk rather than body condition. Furthermore, the less fermentable, more filling diet will increase ruminal pH and decrease risk of milk fat depression and late lactation abomasal displacement.

Allocation: Forages with a wide range of NDF concentration can be used in these diets but the NDF should be potentially digestible. More grass can be included in these diets; although grass fiber may have longer retention time in the rumen and be more filling, it is also more digestible. Highly

fermentable corn silage should be limited to avoid partitioning energy to body condition. High-protein forages should be limited to avoid feeding excess protein.

Supplementation: Limit highly fermentable starch sources (e.g. high moisture corn, ground barley, wheat) by substituting less fermentable feeds such as dry ground corn or NFFS. These "flex-fuel" cows have lower requirements for glucose precursors and can better utilize non-starch feeds to provide energy in a form to spare glucose. Unsaturated fats likely decrease feed intake and increase risk of milk-fat depression and subsequent partitioning of energy to body condition and should be avoided.

Conclusions

Consideration of physiological changes occurring through lactation and the physical and digestion characteristics of diets beyond their nutrient composition is required to optimize forage allocation and supplementation for lactating cows. Whereas more research is needed to better understand animal response to diets, the concepts presented in this paper will help to formulate diets to improve animal health and farm profitability.

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Ensiling total mixed ration for ruminants

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Introduction

Complete or total mixed rations (TMR) are produced by mixing forages, byproducts, concentrates, minerals, vitamins, and additives. From this mix, animals consume the nutrients necessary to meet the requirements of maintenance and production (Schingoethe, 2017).

Ensiling TMR is not a recent practice (Owen e Howard, 1965); meanwhile, the ruminant production industry has shown a renewed interest in TMR silage in a number of countries (e.g., Japan, China, South Korea, Vietnam, Thailand, Indonesia, Nepal, Israel, Iran, South Africa, and Argentina), including Brazil (see Schmidt et al., 2017 in the proceedings of the V Intl. Symp. Forage Qual. Conserv.).

Several benefits have been associated with TMR silages, such as a reduced requirement for labor and machinery (if TMR silage is purchased), uniform composition during storage under farm

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conditions, potential for incorporating unpalatable byproducts (if their flavors and odors are altered by fermentation), and high aerobic stability after feedout (Nishino et al., 2003; Weinberg et al., 2011; Schmidt et al., 2017). Additionally, TMR silages, similar to other silages, have the potential for commercialization, especially if stored in smaller structures (e.g., bales, bags, and pouches).

However, in our view, the main motivation for resuming the use of TMR silages has been the great availability of wet residues coproduced by the agroindustry. Byproducts from the industrialization of cereal grains (including breweries and distilleries), soybean (including the biodiesel industry), sugarcane, cotton, peanut, cassava, vegetables, legumes and fruits (including citrus growers and wineries) are among the valuable residues available in Brazil. Therefore, marketing TMR silages (containing byproducts) has been a promising business opportunity for agroindustry. In small farms, TMR silage has also been proposed as a strategy to improve the conservation of wet forages (Gusmão et al., 2018).

Feeding a conventional TMR (prepared daily) allows the nutritional composition of the diet to be adjusted by using different feedstuffs and supplements [e.g., sources of rumen undegraded protein (RUP) and vitamins] in addition to conserved feeds (hays and whole-crop or grain silages). On the other hand, ensiling a TMR directs all nutrients to fermentation, which ultimately modifies the nutritive value of the diet by altering nutrient content and availability.

This review attempts to nutrient changes during the ensiling of complete rations and their feeding value for ruminants. In many cases in which studies investigating TMR silage were not available (to the best of our knowledge), we discussed data from other silage types (whole-crop or grain silages). Partial mixed ration (PMR) silage has also gained

prominence in ruminant production systems (e.g., dairy cows receiving concentrates in feed stations or automatic milking systems) but is beyond the scope of this paper.

Nutrient changes during silage fermentation

Carbohydrates

Soluble carbohydrates and fermentation end-products

Water-soluble carbohydrates (primarily sucrose, glucose and fructose) are the main substrates for microbial growth during silage fermentation (Rooke and Hatfield, 2003). After ensiling, a reduction in soluble carbohydrates is expected at the expense of the formation of fermentation end-products.

In TMR ensiled in round bales, Weinberg et al. (2011) reported a continuous decrease in soluble carbohydrates during 144 d of storage. Wang et al. (2016) reported a marked decrease of soluble carbohydrates in a TMR silage after 7 or 56 d of storage compared with the fresh TMR (3.0 vs. 6.3% of DM, respectively). Ning et al. (2017) observed losses of soluble carbohydrates by 57% and 54% TMR silages containing alfalfa hay or *Leymus chinensis* hay stored for 56 d (11.1 to 4.93% and 11.7 to 5.58%, respectively). Compared with fresh TMR, Kondo et al. (2016) found lower concentrations of soluble carbohydrates in TMR silage (5.3 vs. 0.7% of DM, respectively), regardless of the length (30 d or 90 d) and temperature (15ºC or 30ºC) of storage. Hence, the final content of soluble carbohydrates in TMR silages will depend on their initial contents and the course of fermentation, but most soluble carbohydrates are consumed during the first weeks of fermentation.

The fermentation end-products are essential for the preservation of TMR silages during storage and feedout.

Moreover, these compounds may contribute to animal nutrition by supplying nutrients, altering feed intake and changing rumen and host metabolism. As in conventional silages, LAB dominate the fermentation, and lactic acid is the main end-product in TMR silages; whereas several other compounds may be formed during fermentation (e.g., volatile fatty acids, alcohols, esters, aldehydes and ketones). Of course, the final profile of fermentation products will depend on ration ingredients, DM content, storage length and temperature and application of additives (Wang and Nishino, 2013; Chen et al., 2014; Hao et al., 2015). Not rarely, TMR silages present high concentrations of lactic acid (e.g., >8% DM) (Weinberg et al., 2011; Chen et al., 2014; Chen et al., 2017; Chen et al., 2019; Restelatto et al., 2019). Although this finding is seldomly discussed, the inclusion of urea, mineral mixtures, limestone and buffers (e.g., sodium bicarbonate) certainly increases the buffering capacity and stimulates the formation of lactic acid in TMR silages, as previously reported for corn and sugarcane silages (Klosterman et al., 1961; Byers et al. 1964; Custódio et al., 2016). The intake of lactic acid has been associated with a greater proportion of propionic acid and higher pH (*in vivo*) (Jaakkola and Huhtanen, 1989; Daniel et al., 2013) and lower methane production (*in vitro*) (Wagner et al., 2018) in the rumen fluid, which might be beneficial to animal performance.

Starch

Once most bacteria responsible for silage fermentation have not been associated with amylolytic activity, a decrease in starch content is generally not expected due to ensiling (Rooke and Hatfield, 2003; Der Bedrosian, 2012; Ferrareto et al., 2015). However, Ning et al. (2017) observed that amylase activity from silage microorganisms decreased by 75% and 57% of the

original activity in TMR silages containing alfalfa hay or *Leymus chinensis* hay, respectively, and that starch losses occurred until the end (56 d) of storage (by 20.5.% for alfalfa hay TMR and by 17.1% for *Leymus chinensis* hay TMR). Miyaji et al. 2017 reported a starch loss by 22.8% (on average) in TMR silages containing steam-flaked corn or brown rice and stored for 210 d. Nevertheless, in TMR silage, the starch concentration tends to be similar to the fresh TMR due to the consumption of other nutrients during fermentation.

In addition to starch loss, ensiling often increases starch digestibility, especially in cereals with a higher content of prolamins in their endosperm (e.g., flint corn and sorghum) (Benton et al., 2005; Hoffman et al., 2011). Prolamins are hydrophobic proteins that surround the starch granules, impairing starch digestion. Protease activity during ensiling reduces prolamin concentration and increases starch availability (Hoffman et al., 2011; Der Bedrosian et al., 2012; Junges et al., 2017). In TMR silages, Miyaji et al. (2017) reported an increase in ruminal starch degradation with a greater benefit in a TMR silage containing steam-flaked corn (6.83%) than brown rice (1.57%). A pronounced enhancement in starch digestibility is expected for dry ground or dry rolled corn (or sorghum) when ensiled as part of a TMR, especially for flint hybrids. Such an increase in starch digestibility has been associated with higher feed efficiency in animals fed TMR silages (Hibbs and Conrad, 1976; Lazzari et al., unpublished data from the State University of Maringá).

Cell wall polysaccharides

The plant cell wall is a complex matrix of polysaccharides, mainly consisting of cellulose, hemicellulose and pectin. Although soluble carbohydrates from the cell content are the

main fuel for microbes during ensiling, in a minor proportion, the constituents of the cell wall may also be cleaved and used as substrate for fermentation (Rooke and Hatfield, 2003). Such partial breakage of cell wall polymers is likely favored in silages with higher moisture content. For instance, in alfalfa silage, Jones et al. (1992) reported a decrease in constituents of cell wall carbohydrates (e.g., uronics, rhamnose, arabinose and galactose) in low DM silage (29% DM), whereas in high DM silage (40% DM), no changes were observed.

Weinberg et al. (2011) reported NDF loss (recovery of 88%) and a lower content of NDF (38% vs. 34%) in a TMR ensiled for 140 d compared with the fresh TMR (both with 50% of DM). The same authors did not find loss of NDF or alterations in NDF concentration (38% of NDF on average) in a similar TMR ensiled with higher DM content (64.8% of DM).

Ning et al. (2017) examined the occurrence of carbohydrate losses and hemicellulase activity in TMR silages. They did not observe hemicellulase activity after 14 d of storage, but hemicellulose loss continuously increased in both silages up to 56 d of storage, probably due to acid hydrolysis (by 19.9% in alfalfa hay TMR and by 23.5% in *Leymus chinensis* hay TMR). Compared to the fresh TMR, Wang et al. (2016) observed a reduction in NDF content of ensiled TMR formulated with corn silage during 56 d of storage (46% vs. 43% of NDF), whereas ADF concentration (27% on average) did not change during storage. Kondo et al. (2016), on the other hand, did not observe changes in NDF concentration after ensiling a TMR, regardless of the length (30 d or 90 d) and temperature (15°C or 30°C) of storage.

In alfalfa and orchardgrass silages, Yahaya et al. (2001) observed that ensiling slightly reduced the concentrations of hemicellulose and pectin, whereas changes in cellulose concentrations were inconsistent. Compared to fresh forage,

hemicellulose and pectin decreased by 2.9% and 1.5% in alfalfa silage and by 4.1% and 1.2% in orchardgrass silage, respectively. In Napier grass silage, Desta et al. (2016) reported a slight decrease of fiber entities during 90 d of storage (NDF by 2.2%, ADF by 1.2%, hemicellulose by 3% and cellulose by 1.9%).

Although lignin is not a carbohydrate, it is closely related to polysaccharides in the cell wall. Classically, lignin has been assumed to be undegradable under anaerobic conditions. In fact, most silages have higher lignin concentrations than their fresh crops due to the disappearance of soluble nutrients. However, lignin is not completely recovered after ensiling. Recently, Machado et al. (unpublished data from the State University of Maringá) examined the effects of an enzymatic complex on the recovery of nutrients in whole-plant corn and sugarcane silages. For lignin (assayed as acetyl bromide lignin), these researchers reported recoveries of 84% and 81% for untreated corn and sugarcane silages, respectively. In both silages, lignin recovery linearly decreased with enzyme dose (from 84 to 54% and from 81 to 69%, respectively), while the contents of flavonoids and polyphenols steeply increased, resulting in silages with greater antioxidant capacity. Most likely, those molecules formed during silage fermentation would promote health in animals and, perhaps, consumers of animal products.

Proteins

During silage fermentation, N losses may occur; however, they are normally less expressive than other soluble fractions with minor effects on the total content of crude protein (CP) (Rooke and Hatfield, 2003). Silages with slightly higher CP contents than their crops are not rare due to the consumption of other nutrients, primarily soluble carbohydrates. On the other hand, protein breakdown undergoes extensively during

fermentation. The partial transformation of true protein into nonprotein compounds due to ensiling is an inevitable process performed by plant and microbial enzymes (McDonald et al., 1991).

The occurrence of proteolysis in high-CP forages (e.g., legumes, temperate grasses) is undesirable and leads to poorer N use efficiency (NUE) (Huhtanen et al., 2008; Hymes-Fecht et al., 2013). However, in corn and sorghum silages (whole plant or grain silages), proteolysis has been positively associated with starch digestibility due to the degradation of hydrophobic proteins (prolamins) that surround the starch granules (Hoffman et al., 2011). Such an increase in starch digestibility often results in greater synthesis of ruminal microbial protein and, in turn, higher NUE (Wilkerson et al., 1997; Valadares et al., 1999; San Emeterio et al., 2000). Nevertheless, in conventional (non-ensiled) TMR, protein sources (e.g., heat-treated soybean meal) are incorporated into the ration to balance any loss of true protein during the storage of ensiled ingredients and, in turn, meet the requirements of metabolizable protein.

In TMR silages, however, all ingredients are ensiled. Therefore, all protein sources are exposed to proteolysis. During ensiling, protein degradation occurs in two phases. Primarily, protein hydrolysis occurs due to plant and microbial proteases, resulting in peptides and free amino acids (AA) (Klembe, 1956; Heron et al., 1986; McDonald, 1991; Rooke and Hatfield, 2003). Afterwards, the decarboxylation of AA leads to the formation of biogenic amines and carbon dioxide, whereas deamination of AA results in NH3 and organic acids (Oshima and McDonald, 1978; Scherer et al., 2015).

Heron et al. (1986) and Klembe (1956) observed that ryegrass and timothy silages sterilized before ensiling still present high amounts of free AA, indicating a large contribution of plant enzymes to proteolysis. In wilted alfalfa

silage, Ding et al. (2013) reported that ensiling increased the proportion of nonprotein N (NPN) from 26% to 73% of total N and that plant enzymes contributed approximately 2/3 of the proteolysis, whereas microorganisms contributed 1/3. Interestingly, most NPN remained as peptides (~70% of NPN), which is more efficiently used by both ruminal microorganisms and animal than NH3-N (Broderick et al., 2013). In rehydrated corn grain silage, however, Junges et al. (2017) reported that bacterial enzymes were the major responsible for proteolysis (60%) followed by plant proteases (30%). Fungi enzymes and fermentation products contributed approximately 5% of the proteolysis during fermentation. In TMR silages, which are mixtures of ingredients, we did not find any report that accounted for the contribution of microorganisms and plant proteases to proteolysis. Meanwhile, enterobacteria, clostridia and bacilli are the main candidates involved in protein breakdown during silage storage (McDonald et al., 1991; Pahlow et al., 2003).

The extent of protein transformation during ensiling is determined by factors capable of altering enzyme and microbial activities, such as pH, moisture, temperature, and storage length. In general, a rapid pH decrease below 4 notably reduces protein breakdown (Virtanen, 1933; McKersie, 1985; Heron et al., 1986; Heron and Philips 1989). However, protein degradation may continue during extended storage (Hoffman et al., 2011).In TMR silages, ingredient sources may also affect proteolysis.

Hao et al. (2015) evaluated moisture contents (40%, 45% and 50% moisture) and storage times (from 0 to 56 d) and reported no differences in total N content in TMR silages. Nevertheless, higher moisture content increased the contents of NPN, free AA and NH3-N during fermentation. No effect was observed on the content of peptides. Although proteolysis continuously increased during storage, major transformations in N fractions were observed within the first week of storage. According to the

authors, on d 56 of storage, 43% of N was NPN. Kondo et al. (2016) observed that storage length and temperature influenced the concentrations of soluble protein (SP) and NH3-N in TMR silage. The authors reported no differences of SP in ensiled TMR stored for 30 d compared with fresh TMR, whereas extended storage (90 d) increased SP concentration. In addition, silage stored at 30°C had a higher content of SP than silage stored at 15°C. The NH3-N increased with temperature and length of storage.

Nishino et al. (2007) reported lower synthesis of biogenic amines (histamine, cadaverine, tyramine and putrescine) in TMR silage compared with whole plant corn and wilted festulolium silages, possibly because of the ingredients used to formulate TMR (dry grains and heat-treated byproducts) were less susceptible to proteolysis, reducing substrates (free AA) for biogenic amine synthesis.

Recently, we compared the N fractionation in TMR silages containing different sources of CP (urea, soybean grain and soybean meal) for finishing beef cattle (Lazzari, unpublished). As expected, ensiled TMR had higher proportions of the A1 fraction (NH3-N) than fresh TMR, but the values remained within acceptable levels (<10%), except for the TMR silage formulated with urea. The proportion of the A2 fraction (non-ammonia SP, including soluble true protein) also increased in ensiled TMR due to the proteolytic process. On the other hand, the insoluble N fractions [insoluble true protein (B1), fiber-bound protein (B2) and indigestible protein (C)] decreased during ensiling (Figure 1).

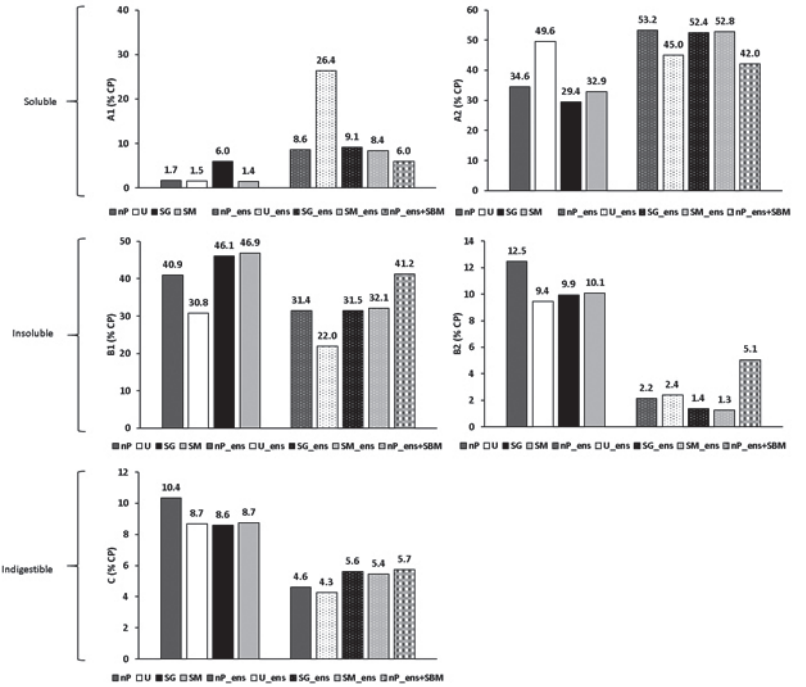

Figure 1. Nitrogen fractionation (CNCPS; Higgs et al., 2015) in fresh and ensiled TMR with difference sources of CP. nP: fresh TMR without a protein supplement; U: fresh TMR with urea; SG: fresh TMR with soybean grain; SM: fresh TMR with soybean meal; nP_ens: ensiled TMR without a protein supplement; U_ens: ensiled TMR with urea; SG: ensiled TMR with soybean grain; SM: ensiled TMR with soybean meal; nP_ens+SBM: nP_ens supplemented with soybean meal before feeding.

Source: Lazzari (unpublished).

Combining N fractionation outcomes enabled the content of rumen-degraded protein (RDP) of each ration to be estimated (Figure 2). Except for the TMR silage containing urea, all ensiled rations contained similar concentrations of RDP, including the ensiled ration supplemented with soybean meal before each feeding. The estimated values of RDP were relatively close to the estimated requirements of RDP (TDN × 0.13) with a slightly higher difference for the TMR formulated with soybean grain

due to the greater TDN value of this fat-rich feedstuff (although fat is not fuel for ruminal fermentation). Higher values of TDN were assumed for ensiled TMR (than fresh TMR) due to the improved starch digestibility in ensiled corn grain. Supplying (nonfermented) soybean meal at feeding or ensiling it with other ingredients altered only 0.2%-units the content of RDP or RUP.

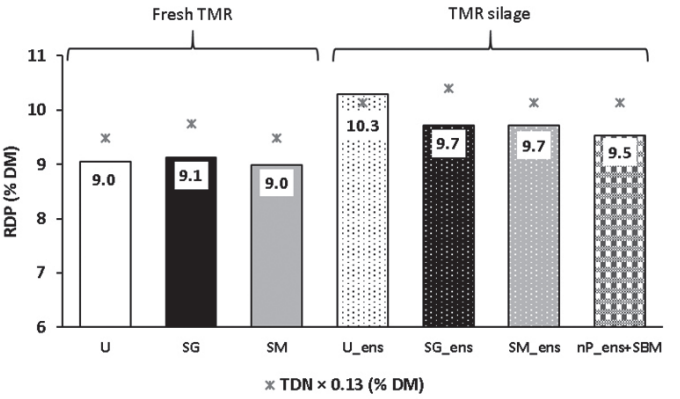

Figure 2. Estimates of rumen-degraded protein (RDP) from N fractionation, for finishing beef cattle. Ruminal degradability was calculated using the first-order approach [kd / (kd + kp)] (CNCPS; Van Amburgh et al., 2015). Fractional passage rates (liquid, concentrate and forage) were estimated using actual values of DM intake, dietary forage level and shrunk BW (Tylutki et al., 2008; NRC, 2016). Requirements of RDP are indicated as TDN × 0.13 (NRC, 2016).

Source: Lazzari (unpublished).

In brief, proteolysis extension seems to be lower in TMR silages (except for urea-containing TMR) than we usually see in high-CP forages (e.g., alfalfa and temperate grasses), mainly due to the higher DM content. Additionally, the use of byproducts that have undergone washing and/or heating during food processing (at agroindustry) might also decrease protein fractions that are more susceptible to proteolysis, both during ensiling and rumen

fermentation. Of course, the content of RUP in TMR silage is lower than in fresh TMR, but it does not mean that RDP/RUP cannot be balanced in the diet. From our perspective, more research is merited to define and predict patterns of proteolysis for a given ingredient or a combination of ingredients and, ultimately, allow the supply of adequate amounts of metabolizable protein to meet animal requirements.

Lipids

A vast group of molecules with different biological functions sharing a common characteristic, 'insolubility in water', are called lipids. Fatty acids (FA), for instance, are synthesized as a form to store energy, whereas galactolipids and phospholipids are the main components of biological membranes in forages (Nelson and Cox, 2015). Overall, linolenic acid (C18:3 n-3) is the main FA in forages, whereas cereals and oilseeds are rich in linoleic acid (C18:2n-6). Palmitic acid (C16:0), stearic (C18:0) and oleic (C18:1) acids are also representative FA in forages and concentrates (Alves et al., 2011; Liu et al., 2018; Liu et al., 2019). In complete rations, grains, oilseeds and byproducts contribute most to lipid content.

Overall, FA are not usual fuels for fermentation (Mackie et al., 1991). In well-sealed silos, the lack of oxygen generates an excess of reducing equivalents (e.g., NADH), which constrains the use of reduced molecules to produce energy, such as FA (Nelson and Cox, 2015). In this way, well-conserved silages will present similar content of total fat as in fresh material (Alves et al., 2011; Liu et al., 2019). However, changes in proportions of individual FA have been observed in silages (Liu et al., 2018; Liu et al., 2019).

A higher proportion of free (non-esterified) FA has been reported for silages in comparison with the corresponding

fresh crop. Elgersma et al. (2003) reported that only 2% of the FA were in their free form in fresh ryegrass, whereas after ensiling, this level increased to 50% in ryegrass silage. Lipases and lipoxygenases are associated with lipolytic activity during ensiling. Lipases (carboxylic ester hydrolase) are present in fresh forages and grains (Barros et al., 2011, Liu et al., 2018; Liu et al., 2019), and they can cleave ester bonds of triglycerides, thereby releasing glycerol and free FA (Gadge et al., 2011). Under favorable conditions, the free FA may undergo lipoxygenase action. Lipoxygenases can oxidize free FA, mainly linoleic and linolenic acids, initially producing hydroperoxides that are later cleaved in aldehydes and ketones (Feussner and Wasternack, 2002; Senger et al., 2005). Lipase and lipoxygenase activities are elevated soon after harvesting or in poorly fermented silages. However, lipase and lipoxygenase activities are altered by pH or temperature variation. Optimum conditions for lipase activity are near 24ºC and pH 8 (Gadge et al., 2011). For lipoxygenase, optimal activity occurs in neutral to basic pH conditions (6.5 to 8.0) (Malekian et al., 1999). This finding means that rapid acidification might preclude the cleavage of FA and the formation of some aldehydes in silages.

Although plant enzymes seem to be primarily responsible for lipolysis, some strains of lactic acid bacteria (LAB) found in silage possess biohydrogenation activity, reducing the content of unsaturated FA (Liu et al., 2019). Ding et al. (2013) observed a decrease in unsaturated FA (C18:2n-6 and C18:3n-6) in control (43%) or sterile silages (28%), whereas autoclave-treated silages (to inhibit plant enzymes and microbial activity) had a similar profile of FA compared to fresh forage; however, control silage presented a higher level of FA C16:0 compared to fresh forage (alfalfa). Liu et al. 2019 also reported more FA C16:0 in oat silage compared to fresh herbage. Han and Zhou (2013) observed alterations in the main FA present in corn silage (C16:0, C18:0,

C18:1, C18:2 and C18:3) during the first 2 d after ensiling. According to the authors, saturated FA increased in favor of a decrease of unsaturated FA. These results were attributed to lipoxygenase activity, which benefited from the high pH observed during the first hours after ensiling.

Liu et al. (2018) evaluated changes in the FA composition of alfalfa silage in relation to temperature (15ºC, 30ºC and 45ºC) and length of storage (0 to 65 d). The decrease observed in the total FA content, as well as in C18:1 and C18:3n3, was larger on the first day of fermentation than on the remaining 65 d of storage. In addition, losses of FA were higher at 45ºC than at lower temperatures. The authors attributed those results to the thermolability of FA, as well as an increase in LAB development at higher temperatures. However, on d 65 of storage silages kept at 15°C had the lowest amount of total FA. The authors attributed this result to an enhanced activity of aerobic bacteria, yeasts and plant lipoxygenase.

In brief, in well-fermented silages, the content of total FA is not expected to change expressively, once FA are not usual fuels for fermentation. However, an increase in free FA and changes in the concentration of specific FA, such as a decrease in unsaturated FA (e.g., linoleic and linolenic) and an increase in the proportion of saturated FA, are expected.

Minerals

Minerals are highly important to the maintenance of vital processes, as well as animal productivity, despite the lower requirements compared to other nutrients. In general, well-preserved silages have slightly higher ash contents than their fresh crops (Meschy et al., 2005; Baumont et al., 2011). However, the contents of specific minerals may change during ensiling.
Schlegel et al. (2018) observed higher concentrations of Mn

(65%), Na (33%), Se (27%), Zn (13%) and Mg (12%) in grass and legume silages compared with their respective herbages. No changes were observed in the Ca, P, K, Cl, S and Cu contents. Overall, macro- and micromineral concentrations increased by an average of 6% and 31%, respectively, explained by the loss of fermentable nutrients.

Microbial development and low pH conditions have been related to higher availability of minerals in silage. Lee et al. (2019) reported that some LAB strains possess the ability to increase the bioavailability of inorganic selenium during silage fermentation by converting sodium selenite into organic selenium. Hansen and Spears (2009) observed that ensiling increased Fe availability in whole-plant corn silage, probably because of the acidic conditions in silage. Ibrahim et al. (1990) evaluated the ruminal solubility of minerals in feedstuffs used in ruminant nutrition. They reported higher ruminal availability of minerals (Ca, Mg, P, Na, K, Cu, Zn) in corn silage, despite the lower content of ash in corn silage compared to other tested feeds (4.9% DM vs. 10.7% DM on average). Overall, the ruminal availability of minerals in corn silage was above 80% (from 70% to 100%), which was also attributed to the effect of low silage pH (3.7 in the mentioned study). Rooke et al. (1983) also reported high availability of minerals (K, Ca, Na, P, Mg and Cu) in grass silage (average above 93%).

Although there is lack of information on the fate of minerals in ensiled TMR, one would expect minor changes in their concentrations but an increase in their availability, as previously reported for other silages.

Vitamins

Vitamins are complex molecules essential for different metabolic pathways, the immune system and gene expression

(NRC, 2001). Vitamins are classified as fat-soluble (A, D, E and K) and water-soluble (B and C) (McDowell, 2000). Adult ruminants are less dependent on exogenous sources of vitamins than nonruminants. Vitamins from B and K complexes are synthesized within the rumen by microorganisms; vitamin D is produced from steroids present in skin after sunlight exposure; glucose and galactose are converted in vitamin C when required (NRC, 2001).

In this way, fat-soluble vitamins, mainly A and E, are more required from exogenous sources. The carotenes (provitamin A) and tocopherols (vitamin E) present in fresh forages are the main sources of those vitamins, although grains may also contribute (Kalac, 2012; Nozière et al., 2006; Lindquisvist et al., 2011; Liu et al., 2016).

Forage conservation (haying and ensiling) has been generically linked to losses of carotenoids and tocopherols, but losses related to ensiling are much smaller than for wilted forages and hay (Carter, 1960). Carotenes and tocopherols are prone to oxidation after harvesting. Exposition to ultraviolet radiation (even in the dark), oxygen, high temperature and lipoxygenase activity are the primary cause of carotene destruction (Kalac and McDonald, 1981; Cardinault et al., 2004; Nozière et al., 2006; Kalac, 2013). Tocopherols are natural antioxidants protecting other molecules from oxidation; however, due to this protective activity, tocopherol may also be degraded (McDowell, 2000).

Kalac and Kyzlink (1979) reported that carotenoids are rapidly degraded when exposed to oxygen under low pH conditions (pH 3.7 to 4.2). In the same study, carotenoid degradation was greater within the temperature range of 30 to 40ºC. On the other hand, a rapid lactic acid synthesis in the absence of oxygen had a positive correlation with β-carotene and α-tocopherol concentration, which means that a rapid pH drop under anoxic conditions is beneficial to the conservation of

(pro)vitamins as found in well-preserved silages (Muller et al., 2006; Lindqvist, Nadeau and Jensen, 2011). In well-fermented crops, Nozière et al. (2006) and Liu et al. (2019) reported losses of carotenes during ensiling by 20% (for silages in general) and 25.5% (for oat silage). Carotene loss during ensiling was attributed to lipoxygenase activity (Liu et al., 2019). Tocopherol, on the other hand, was less degraded (11.6%) than carotene (Liu et al., 2019). This phenomenon has been attributed to a higher stability of tocopherol in anoxic conditions (Liu et al., 2019), as well as a possible activity of tocopherol-producing bacteria (Tani and Tsumura, 1989).

Silo structure, length of storage, additives and temperature during storage are also important factors related to vitamin conservation. Nadeau et al. (2004) observed a reduction of vitamins in grass-legume silage stored in round bales. α-Tocopherol and β-carotene decreased by 49% (from 35 to 18 mg/kg DM) and 37% (from 19 to 12 mg/kg DM), respectively, during 3 months of storage. However, vitamin contents in silages stored in bunker and tower silos were similar to vitamin contents in the fresh herbage. These results were attributed to a greater risk of oxygen infiltration and oxidation of vitamins in round-bale silos.

Jares (2018) evaluated the concentrations of tocopherols (α, γ and δ) in rehydrated corn grain silages (32% moisture) during storage (0, 21, 38, 63 100 and 185 d) and observed a decrease over time (e.g., α-tocopherol decreased from 9.7 μg/g at ensiling to 6.91 μg/g at 185 d of storage). Most of the reduction occurred during the first 21 d of fermentation. A reduction of tocopherols was also observed in dry grains, but ensiled grains had lower concentrations of tocopherols than dry grains.

Liu et al. (2016) compared the effects of additives (fibrolytic enzyme, tert-butylhydroquinone and tea polyphenols) and temperature (15, 30 and 45ºC) on vitamin concentrations

in Napier grass silage. They found that silage stored at 30°C had more tocopherol than fresh forage or silage kept at 15°C. Ensiling reduced the content of carotenes independent of temperature, but carotene was higher in silage kept at 30°C than at 15 or 45°C. According to the authors, silage stored at 30°C presented more tocopherol because of the probable activity of microorganisms capable of producing this molecule. Additives had no beneficial effect, except for tert-butylhydroquinone, which is an antioxidant. Lindqvist et al. (2011) reported that bacterial inoculation (*Lactobacillus plantarum* and *Pediococcus acidilactici*) improved the tocopherol concentration in red clover silage (50.1 vs 34.2 mg/kg DM). In contrast, Liu et al. (2019) observed a reduction in carotene due to *Lactobacillus plantarum* inoculation in oat silage. The use of propionic acid (4 kg/t as fed), however, was effective in controlling carotene loss because carotene concentration in treated silage did not differ from fresh forage.

In brief, losses of carotene and tocopherols are expected during ensiling; however, tocopherol seems more stable than carotenes in silages. The TMR silages often contain exogenous sources of vitamins which, in terms of stability, may differ from natural sources of vitamins (or provitamins) present in feeds. To the best of our knowledge, the fate of vitamins in TMR silage is unknown.

Feed additives

Knowledge on the course of feed additives during ensiling is rare (e.g., ionophores, essential oils, tannins, probiotics, prebiotics, sodium bicarbonate, β-adrenergic agonists, etc.). Some studies have examined the effects of feed additives on silage conservation, but the fate of those additives is unknown. Hoon and Meeske (2011) investigated the effect of lasalocid

(an ionophore antibiotic) on the conservation of corn silage. It is worth noting that LAB are gram-positive and ionophore-sensitive. Compared with untreated silage, lasalocid sodium (0.15 g/kg as fed) decreased lactic acid concentration and increased fermentative losses, resulting in silage with poorer *in vitro* organic matter digestibility. The treated silage also had a more intense aerobic deterioration, as indicated by the higher production of CO2 during air exposure. No information was provided on the concentration/activity of lasalocid at silage feedout.

Kung et al. (2008) examined the effect of a commercial blend of essential oils (40 or 80 mg/kg as fed) on the conservation of corn silage. The blend of essential oils did not affect the populations of yeasts, molds, lactic acid bacteria, or enterobacteria, the fermentation end-products or the aerobic stability of the corn silage. Foskolos et al. (2016) investigated the effects of essential oils on protein degradation during ensiling. Five essential oil compounds (thymol, eugenol, cinnamaldehyde, capsaicin and carvacrol) in four doses (from 0 to 2 g/kg as fed) were sprayed on ryegrass forage before ensiling. Thymol, eugenol, and cinnamaldehyde at 2 g and carvacrol at 0.5 and 2 g/kg inhibited deamination. Cinnamaldehyde at 2 g/kg resulted in silages with approximately 10% more true protein than other silages. However, the highest dose of the essential oils (2 g/kg) *negatively* affected the ensiling process by decreasing LAB counts and lactic acid concentration and increasing silage pH.

Recently, Pereira (2018) studied the effects of essential oils (thymol and carvacrol) as additives in corn and sugarcane silages. In sugarcane silages, the addition of thymol, carvacrol or a combination of thymol and carvacrol led to higher concentrations of soluble carbohydrates. The combination of thymol and carvacrol decreased the lactic acid concentration.

Carvacrol alone decreased the DM loss during fermentation, whereas thymol alone decreased the counts of LAB and ethanol concentration, whereas it increased the aerobic stability of sugarcane silage. In corn silage, LAB counts were reduced by thymol alone or combined with carvacrol. Carvacrol alone or in combination with thymol decreased the lactic acid concentration. All treated silages had less ethanol, lower DM loss during fermentation, and slightly higher aerobic stability upon air exposure. As in the aforementioned studies, no information was provided on the concentration/activity of essential oils at silage feedout.

Even as discussed for nutrients in previous topics, feed additives may be altered during fermentation. In the meantime, the capacity of the additives to act in the rumen and/or host metabolism after undergoing fermentation warrants further investigation.

Nutritive value of TMR silages for ruminant

Ensiled TMR for dairy cows

During the 1960s, TMR increased in dairy operations in the US, as milk production per cow increased, herds became larger, freestall and large-group handling of cows became more common, and milking parlors became more prevalent (McCoy et al. 1966; Schingoethe, 2017). At nearly the same time, the first studies on TMR silages appeared in the literature.

Owen and Howard (1965) evaluated the effects of ensiled TMR with different moisture levels (68%, 53% and 47%), obtained by varying the period of alfalfa wilting, on the performance of dairy cows. The rations were composed of 50% alfalfa and 50% cracked corn (DM basis) and contained approximately 17% CP. Cows fed low- and medium-moisture

rations (47% and 53%) had higher DM intake than cows fed high-moisture rations. However, milk fat content was higher for the high-moisture ratio, which counterbalanced the differences in milk yield, resulting in similar yield of fat-corrected milk (FCM) among treatments. The authors concluded that the use of TMR silage is a good alternative to simplify feeding management.

Marshall and Voigt (1975) compared the nutritive value of an ensiled TMR formulated with whole plant corn and a fresh TMR formulated with corn silage and moistened concentrate mix. The rations contained 61% corn silage and 39% concentrate mix (citrus pulp, cottonseed meal, urea and minerals). The authors found no difference among treatments for cow performance (20.2 kg of FCM, 4.8% milk fat, DM intake of 3.2% of BW).

Since the pioneering studies, ensiling TMR formulated with cereal grain was associated with higher feed efficiency. Hibbs and Conrad (1976) compared fresh with ensiled TMR for dairy cows. In both diets, a rate of 0.45 kg of concentrate was mixed with 3.18 kg of corn silage (as fed basis). The concentrate mix contained 29.1% CP and was composed of 57.32% corn, 30.00% soybean meal, 5.00% dehydrated alfalfa meal, 3.52% urea, 3.20% bone meal and 0.96% salt. Both diets contained 14% CP. Total-tract DM digestibility increased (76.7% vs. 79.1%) and DM intake decreased (18.1 vs. 16.3 kg/d) for cows fed TMR silage, whereas there was no difference in milk yield (18.8 vs. 19.0 kg/d). There was no difference in milk fat content and FCM among treatments (18.3 vs. 18.7 kg/d). Therefore, feed efficiency was higher for cows fed TMR silage (1.01 vs. 1.15 FCM/DM intake for fresh and ensiled TMR, respectively).

In a second trial, Hibbs and Conrad (1976) used the same proportion of corn silage and concentrate as in the previous trial to compare fresh and ensiled TMR, but they altered the concentrate composition (40.80% of corn, 50.00% of soybean

meal, 5.00% of dehydrated alfalfa, 3.20% of bone meal and 1.00% of salt). Diet CP was similar to the first trial (14.00%). As in the first trial, DM intake was lower (17.1 vs. 15.6 kg/d), and feed efficiency was greater (FCM/DM intake 1.17 vs. 1.32) for cows fed ensiled TMR without differences in FCM (19.9 vs. 20.4 kg/d, for fresh and ensiled TMR, respectively).

Pardue et al. (1975) evaluated the effect of ensiling on the nutritive value of a TMR for dairy cows. Two groups of ten Holstein cows (approximately 84 d postpartum) were assigned to a switch-back design with three 28-d periods to compare the ensiled TMR with the same fresh ingredients (silage and concentrate) but fed separately at each milking (Ctrl). Although the performance was similar, the ruminal concentration of volatile fatty acids was greater in cows fed the ensiled TMR (85.4 vs. 95.2 mM, for Ctrl and TMR silage, respectively), suggesting a higher ruminal degradability of the TMR silage.

After a temporal gap, the ruminant production industry has shown renewed interest in TMR silage, probably due to the availability of moisture byproducts. In 2009, Wongnen et al. evaluated the nutritive value of fresh or ensiled TMR, containing whole or cracked cottonseed, for dairy cows. Four multiparous Holstein Friesian crossbred cows (48 ± 12 DIM and 450 ± 13 kg BW) were assigned to a 4 × 4 Latin square design (21-d periods) with 4 dietary treatments in a 2 × 2 factorial arrangement (fresh or ensiled TMR × whole or cracked cottonseed). Diets contained (% DM): chopped rice straw (20.0%), cassava chip (40.0%), soybean meal (7.0%), cottonseed (10.0%), dried brewers grain (5.0%), tomato pomace (5.0%), molasses (8.0%), urea (1.5%), salt (0.5%), oyster shell (0.3%), di-calcium phosphate (0.2%), mineral-vitamin mix (0.3%), sulfur (0.2%), tallow (1.0%) and sodium bicarbonate (1.0%). Diet composition was approximately 63% DM, 16% CP and 69% TDN. Ruminal fermentation parameters (pH, NH3 and VFA) and performance

were similar among diets (15.5 kg/d DM intake, 18.9 kg/d FCM, 4.30% milk fat, 3.34% milk protein). However, TMR silage led to a lower proportion of oleic acid (C18:1) in milk fat (27.0 vs. 24.4% of total FA, for fresh vs. ensiled TMR). This response was likely due to the partial biohydrogenation of unsaturated FA during silage fermentation.

Recently, Myyaji and Nonaka (2018) compared the digestion and performance of dairy cows fed fresh or ensiled TMR. The TMR contained rye silage, beet pulp, soybean meal, mineral-vitamin mix, and dry-rolled or steam-flaked hulled rice. Ensiling TMR improved starch (91.6 vs. 97.2%) and DM digestibility (72.3 vs. 77.6%), tended to increase DM intake (22.4 vs. 23.0 kg/d) and resulted in greater milk yield (36.4 vs. 37.6 kg/d, for fresh and ensiled TMR, respectively). The ruminal concentration of VFA (97.1 vs. 101.8 mM) was slightly higher in cows fed ensiled TMR. Despite the higher ruminal fermentability, TMR silages led to slightly greater concentrations of ruminal NH3 (7.27 vs. 8.81 mg/dL) and higher urinary N excretion (124 vs. 152 g/d). In a companying paper, the authors reported higher ruminal degradability of CP for those TMR silages (Miyaji et al., 2017). Meanwhile, compared with the fresh TMR, daily secretion of milk protein was higher in cows fed TMR silage (1.24 vs. 1.31 kg/d, respectively) (Myyaji and Nonaka, 2018). A potential of TMR silages for high-producing cows was demonstrated in this study.

Ensiled TMR for growing ruminants

The TMR silages have also been used for feeding growing sheep and cattle. Cao et al. (2010) reported the effects of ensiling a TMR on N balance, ruminal fermentation and methane production in sheep. Compared with fresh TMR, ensiled TMR had higher contents of digestible energy (13.8 vs. 14.6 MJ/kg

DM) and digestible CP (94 vs. 105 g/kg DM), which contributed to lower fecal N excretion (8.21 vs. 7.08 g/d). On the other hand, ensiling the TMR increased the urinary N excretion (9.76 vs. 11.81 g/d). Meanwhile, N retention was similar between treatments (5.2 g/d). The ruminal concentration of total VFA (2 and 4 h after feeding) was higher in sheep fed TMR silage (88.4 and 87.4 mM vs. 127 and 116 mM, for fresh and ensiled TMR, respectively). Compared to fresh TMR, the ensiled TMR reduced methane emissions by 10 L/d by 9.84 L per kg of DM intake, by 17.3 L per kg of digestible DM intake, by 0.54 L per kg of metabolic BW and by 20.9 J per kJ gross energy intake. The authors argued that lower methane production was due to the conversion of lactic acid (formed during silage fermentation) to propionic acid, an electron-consuming reaction in the rumen.

Meenongyai et al. (2017) examined the effect of ensiling on the nutritive value of a grass-based TMR for beef cattle. Thirty crossbred Zebu-Holstein steers were blocked and received one of three experimental diets for 188 d. The treatments were 1) fresh TMR with fresh Napier grass, 2) fresh TMR with Napier silage, and 3) TMR silage (containing the Napier grass). For treatments 1 and 2, forage and concentrate were mixed before feeding the animals. Experimental diets contained (DM basis): Napier grass (41.15%), cassava pulp (6.00%), cassava chip (5.00%), corn (9.97%), rice bran (14.00), palm kernel meal (6.91%), soybean meal (5.00%), sugar (9.16%), salt (0.50%), urea (2.00%) and minerals (0.30%). On average, diets had 47% DM and 15% CP. The pH values of rations 1, 2 and 3 were 4.7, 4.0 and 3.5, respectively. Total-tract digestibility was lower for the fresh TMR containing grass silage (60.54%) compared with fresh TMR containing fresh Napier grass (71.13%) or TMR silage (65.51%). The DM intake, ADG and feed efficiency were similar across treatments.

Recently, our research group carried a trial to compare

the nutritive value of TMR silages containing different protein sources for finishing beef cattle (Lazzari et al., unpublished). Thirty-two Nellore heifers were blocked by initial BW and received one of four dietary treatments. The TMR silages contained (DM basis): sugarcane bagasse (13%), rolled corn (59.4 to 68.4%), corn gluten feed (15%), limestone (0.6%), mineral mix (2%) and one of the following protein sources: urea (1%), soybean meal (ensiled with all ingredients) (7.1%), soybean meal (omitted at ensiling but supplied at TMR feeding = nonfermented) (7.1%) or rolled soybean grain (10%). All diets contained 13% CP and 10.5% roughage NDF (DM basis). The TMR silage formulated with soybean grain contained approximately 4.7% ether extract (EE), whereas the remaining diets contained approximately 3.0% EE. There was no difference in animal performance [DM intake, average daily gain (ADG), carcass gain, dressing and feed efficiency] among diets formulated with urea or soybean meal (ensiled or nonfermented). However, compared with other treatments, TMR silage containing soybean grain improved the ADG (1.22 vs. 1.49 kg/d) and carcass gain (0.890 vs. 1.010 kg/d), probably due to the trend of higher DM intake (8.02 vs. 9.17 kg/d), higher energy supply by EE and, perhaps, lower ruminal methane emission due to the unsaturated FA from the soybean grain. Hence, balancing protein in TMR silage with urea or soybean meal (ensiled or not) does not affect the performance of finishing cattle. On the other hand, the inclusion of soybean grain (and perhaps other oilseeds) is a feasible strategy to improve the nutritive value of TMR silages for beef cattle.

Final remarks

Marketing TMR silages represents a promising business opportunity for agroindustry, whereas feeding TMR silages is an alternative to simplify nutritional management.

Among nutritional changes during TMR storage, a decrease in true-protein content and an increase in starch digestibility (which often leads to higher feed efficiency) have been consistently reported. Further research is warranted to fine-tune the supply of metabolizable protein, vitamins and additives via TMR silages.

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New Physically Effective Fiber Recommendations for High Producing Dairy Cows

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Introduction

Keeping the rumen environment "healthy" helps high producing dairy cows to meet their genetic potential for milk production. Cows with a healthy rumen ruminate extensively throughout the daytime and nighttime, the pH of their rumen contents is maintained above 5.8, and their rumen contractions are strong. These conditions help maximize fiber digestion by the rumen microorganisms and passage of undigested feed residues from the rumen, thereby promoting high dry matter intake (**DMI**) to meet the energy requirements of the cow.

Heathy rumen function is often attributed to the chemical and physical characteristics of forage fiber consumed by cows, but new information indicates that other factors such as diet fermentability and feeding management of the cow are also important. Chemical fiber, measured as neutral detergent fiber (**NDF**), supplies energy substrates for the rumen microbes, while the physical attributes of fiber stimulate eating, ruminating, and rumen motility that contribute to feed digestion and

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passage from the rumen. Maintaining healthy rumen function in high producing dairy cows is challenging because rations are comprised of grains and forages that are extensively processed (with small particle size) and highly fermentable in the rumen. Lack of physical fiber combined with highly fermentable carbohydrates increases the risk of sub-acute ruminal acidosis (**SARA**), which is characterized by repeated drops in pH <5.8. The objective of this paper is to discuss the factors that promote healthy rumen function in dairy cows, and provide new information for diet formulation and feeding management.

Healthy Rumen Function

The Importance of Chewing

Eating and ruminating play a vital role in maintaining high levels of feed intake and efficient digestive function in high-producing dairy cows (Figure 1). Ruminants chew their feed initially during eating, with swallowed feed later regurgitated and remasticated during rumination. As feed is chewed, particles are reduced in size and saliva is secreted. Saliva is an important buffer for the rumen, and thus chewing plays a key role in maintaining optimum rumen pH for microbial digestion of feed. Furthermore, physical breakdown of feed during chewing facilitates microbial colonization of ingested feed and passage of small particles from the rumen to the lower gastrointestinal tract. Thus, promoting chewing helps minimize the risk of SARA, enhances fiber digestion, and promotes high DMI.

Eating. On average, dairy cows spend about 4.7 h/d eating, ranging from 2.3 to 8.5 h/d (Table 1; Beauchemin, 2018). Time spent eating is highly variable because it is affected by feeding management, DMI, diet composition, and inherent variability among animals, but the main factors affecting eating

time are particle size of the ration and feeding management. Long particles, defined as particles retained on the 19-mm sieve of the Penn State Particle Separator (**PSPS**), slow the eating rate and increase eating time (Table 2). Starch and crude protein content decrease eating time because they are mainly contributed by concentrates, which are consumed more rapidly than forages. Greatest feeding activity typically occurs after feed is delivered or pushed-up in front of the cows. Thus, frequent delivery of feed tends to promote feeding activity and a more even distribution of feeding time throughout the day, which helps stabilize rumen pH.

Table 1. Summary of treatment means for chewing activity (min/day) of dairy cows from peer reviewed publications (from Beauchemin, 2018)

	N	Mean	Min	Max
White et al., 2017a				
Eating	**182**	**284**	**141**	**507**
Ruminating	**179**	**436**	**236**	**610**
Total chewing	**175**	**717**	**396**	**973**
Zebeli et al., 2006				
Eating	**NR**	**NR**	**NR**	**NR**
Ruminating	**99**	**434**	**151**	**632**
Total chewing	**99**	**691**	**425**	**969**

NR, not reported.

Rumination. Rumination time is also highly variable with an average of 7.2 h/d, but ranging from 3.9 to 10.2 h/d (Table 1; Beauchemin, 2018). Rumination time is mainly influenced by DMI, forage-NDF intake (i.e., the intake of NDF from forage sources) and particle size of the diet (particles retained on 8- and 19 mm sieves), and to a lesser extent fragility (hardness) of the feed that imparts resistance to chewing and the digestibility of the fiber. There are complex interactions among these factors; thus, the correlations between rumination time and individual dietary factors are only low to moderate (Table 2).

Table 2. Correlations between diet variables and eating and ruminating (from White et al., 2017a)

	Eating time, min/d	Ruminating time, min/d
DMI	**-0.06**	**-0.06**
Starch, % DM	**-0.22***	**-0.22***
NDF, % DM	**0.12**	**0.12**
Crude protein, % DM	**-0.22***	**-0.22***
Forage-NDF, % DM	**0.10**	**0.10**
Forage, % DM	**0.12**	**0.12**
TMR particles retained on sieve		
19-mm, % DM	**0.45***	**0.45***
8-mm, % DM	**0.03**	**0.03**
Silage, % DM	**-0.13***	**-0.13***

**P ≤ 0.05; *P ≤ 0.10. DM, dry matter; DMI, dry matter intake; NDF, neutral detergent fiber; TMR = total mixed ration.*

Saliva Secretion, Rumen Buffering, and Rumen pH

For healthy rumen function, rumen pH needs to be maintained in the range of 5.8 to 7.0, with a mean rumen pH above 6.0. SARA occurs when rumen pH drops below 5.6 for more than 3 h/d (Plaizier et al., 2008) or below 5.8 for more than 5 to 6 h/d (Zebeli et al., 2012). Saliva plays an important role in buffering the pH of the rumen (Figure 1), accounting for 35 to 50% of the bicarbonate flow into the rumen of dairy cattle. The most important source of rumen buffering is absorption of short chain fatty acids (**SCFA**) from the rumen. Absorption of SCFA from the rumen stabilizes ruminal pH by removing protons during passive diffusion of undissociated SCFA and by secretion of bicarbonate during absorption of dissociated SCFA (Aschenbach et al., 2011).

Increased bicarbonate flow into the rumen can be achieved through increased chewing time to promote saliva secretion. Additionally, the need for rumen buffering can be

lowered by decreasing or slowing the production of SCFA by reducing diet fermentability (primarily by lowering starch content). Although promoting chewing time helps increase the flow of bicarbonate into the rumen, keeping the rumen healthy needs to consider both increasing total buffering of the rumen as well as managing SCFA production in the rumen.

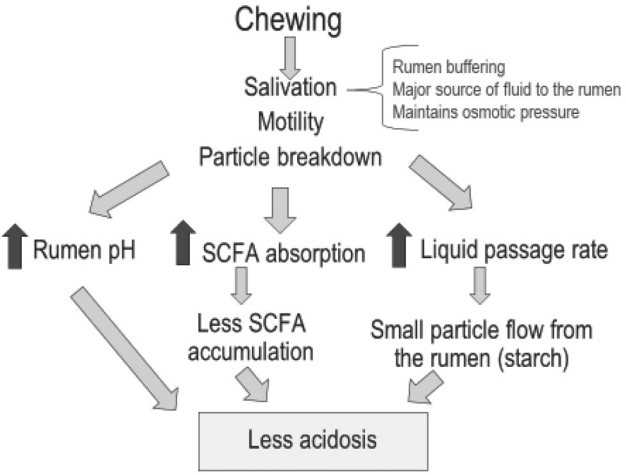

Figure 1. *Overview of the role of eating and ruminating in preventing ruminal acidosis.*

Raft Formation, Motility, and Particulate Passage from the Rumen

The ruminal contents are stratified into a liquid phase, a floating mat (raft), and a pool of small particles dispersed within the fluid phase ventrally to the floating mat. Ruminal mat formation enhances the microenvironment needed by the fiber-digesting microorganisms. It also acts as a filter bed, which helps retain forage particles in the rumen increasing the time allowed for fiber digestion. Mat formation strongly depends on intake of forage-NDF and long particles.

Muscular contractions of the rumen promote mixing of the digesta and SCFA absorption, while pushing the indigestible material out of the rumen. The number and strength of the contractions are increased significantly during eating and ruminating. Contractions tend to be smaller when cows are fed finely processed feeds and during SARA.

Saliva, Liquid Passage and Site of Starch Digestion

Increasing forage-NDF content and particle size of the diet increases fractional passage rate of liquid from the rumen due to increased salivation and motility associated with chewing. Faster liquid passage increases the flow of grain particles from the rumen, decreasing ruminal digestion of starch and the production of SCFA. Thus, increasing forage-NDF content of the diet, and to a lesser extent increasing particle size of diets, shifts the site of starch digestion such that less starch is digested in the rumen and a greater proportion of starch is digested in the intestine. The shift in site of starch digestion is a major contributing factor that reduces the risk of SARA with increased intake of physically effective fiber. In addition to shifting the site of digestion of starch, increasing forage-NDF content of the diet typically decreases the starch content of the diet by dilution. As a result, SCFA production in the rumen is also decreased, which helps minimize the postprandial drop in pH.

Capturing the Characteristics of Fiber in Ration Formulation

Long fiber particles are important in dairy cow rations to promote healthy rumen function, yet it is very difficult to capture the characteristics of physical fiber in ration formulation. Mertens (1997) proposed the term physically effective fiber

(**peNDF**) to reflect the effects of NDF content and particle length and indicate the overall potential of the feed to stimulate chewing. The particle size of the feed can be assessed using the PSPS. A physical effectiveness factor (**pef**, ranging from 0 to 1) is then calculated as the proportion of feed or TMR (on an as-fed or dry matter [**DM**] basis) that is retained on sieves ≥ 1.18 mm (pef1.18) or sieves ≥ 8 mm (pef8). The pef is then multiplied by NDF content to determine peNDF content (peNDF≥1.18mm and peNDF≥8mm). Thus, peNDF relates to chewing, but does not account for rumen fermentability of the feeds.

Recently, White et al. (2017a) evaluated the literature on chewing, rumen pH and fiber, and concluded there was no advantage of peNDF compared with using particle size and NDF individually in ration formulation. Thus, they proposed using particle size and NDF content separately along with other dietary components to predict chewing time and rumen pH. The concept is referred to as physically adjusted fiber (**paNDF**). Equations were developed to predict eating time, ruminating time, and mean rumen pH using individual factors as inputs (including DMI, dietary forage content, NDF content, forage-NDF content, particles retained on various sieves of the PSPS, starch content, and so forth). Outputs from these models were used to develop recommendations for NDF content, forage-NDF content and particle size for diets that differ in fermentability (White et al., 2017b).

Recommendations

Forage-NDF, Particle Size and Starch Levels

NRC (2001) recommends a minimum of 25% total dietary NDF, with 75% of this fiber from forages, which equates to 19% forage-NDF. It was stated that the amount of forage-NDF could

be decreased to as low at 15% if total dietary NDF is increased and the non-fiber carbohydrate level (mainly starch) is lowered from 44% to 36%. These recommendations for minimum fiber levels are based on diets that contain alfalfa or corn silage as the predominant forage, with the silages being coarsely chopped. Thus, particle size of the forage is not accounted for.

Over the past couple of decades, there has been a lot of research to better understand the dietary factors affecting rumen pH and rumen function. Based on a meta-analysis of the literature, Zebeli et al. (2012) suggested that 31.2% peNDF>1.18 or 18.5% peNDF>8 in the diet (DM basis) is needed to prevent SARA. They stated that feeding < 14.9% peNDF>8 results in an imminent risk of SARA, while feeding ≥ 14.9% peNDF>8 in the diet DM may lower DMI and consequently the production of high-producing dairy cows.

Based on the meta-analysis of White et al. (2017a), response surfaces for variables affecting mean rumen pH were developed (While et al., 2017b). The response surfaces examine the interactions between particle size (measured as particles retained on the 8- and 19-mm sieves of the PSPS) of the total mixed ration (**TMR**), starch content, and forage-NDF content of the diet. Note that the particle size measurements are expressed on a DM basis, but in practice most on-farm measurements with the PSPS are on an as-fed basis. From a practical standpoint, the difference between the two methods is relatively small.

My interpretation of the results from White et al. (2017b) is shown in Table 3. The minimum forage-NDF required in the diet varies from 12 to 27% DM, depending upon starch content and particle size of the TMR. As starch content of the diet increases, the need for forage-NDF increases to offset the increased rate of production of SCFA. As the proportion of 8-mm particles in the TMR increases, the minimum amount of forage-NDF decreases. Similarly, as the proportion of 19-mm

particles in the TMR increases, the minimum amount of forage-NDF decreases. For high starch diets (> 25% DM) that are low in forage proportion and therefore have ≤30% particles on the 8-mm screen, attaining the minimum level of forage-NDF required (26 to 27% of DM) may not be feasible when using good quality forages. This example illustrates the potential risk of SARA when feeding high starch diets that are finely chopped, as it can be difficult to supply the required minimum level of forage-NDF. In that case it is critical to implement feeding and management strategies that help prevent SARA (see **Other Considerations that Help Prevent SARA** below).

Table 3. Minimum forage-NDF required in a total mixed ration (dry matter [DM] basis) to maintain average rumen pH of 6.1 (adapted from White et al., 2017b).

Starch, % DM	Particles retained on 8 mm sieve (% of TMR DM)	Particles retained on 19 mm sieve (% of TMR DM)			Range in forage-NDF required
		6	12	16	12-21
15	>50	14	13	12	
	50	16	16	14	
	40	19	18	17	
	≤30	21	20	19	17-24
20	>50	19	18	17	
	50	21	20	19	
	40	24	22	21	
	≤30	24	24	23	19-26
25	>50	21	20	19	
	50	23	23	21	
	40	25	24	23	
	≤30	26	26	24	20-27
30	>50	22	21	20	
	50	24	24	22	
	40	26	25	25	
	≤30	27	27	26	

Several important concepts are apparent from Table 3:

a) At a particular dietary starch content, the minimum level of forage-NDF required increases as proportion of 8- and 19-mm particles decrease.
b) Increasing the starch content of the diet increases the minimum concentration of forage-NDF required.
c) Increasing the proportion of material captured on the 8-mm sieve has a greater impact on reducing the minimum level of forage-NDF required, than does increasing the proportion of material on the 19-mm sieve. Furthermore, rations with greater proportion of particles retained on the 19-mm sieve are prone to sorting by cows at the feed bunk, thus increasing the long particles in the diet is not a very effective way of promoting healthy rumen function.
d) As the proportion of particles retained on the 8-mm sieve increases, less long particles on the 19-mm screen are needed to maintain rumen pH.

Evaluation of Fiber Recommendations

The data from Yang and Beauchemin (2007) can be used to compare the different recommendations for fiber (Table 4). The diets in that study varied in forage:concentrate ratio (**F:C**; 35:60, 60:40) and particle size was altered by using short and long-chopped alfalfa silage. The diets provided a large range in contents of starch, NDF, forage-NDF, and peNDF. Continuous measurement of rumen pH indicated that the cows fed the 35:65 F:C diets, regardless of silage chop length, experienced SARA. Although the mean pH of the coarsely chopped 35:65 F:C diet was 6.17, the pH dropped undesirably below 5.8 for 10.1 h/day. In contrast, the cows fed the 60:40 F:C diets did not experience SARA. The 35:65 F:C diets were below the recommendations for fiber for all models (NRC, 2001; Zebeli et al., 2012; White et al., 2017b). In contrast, the 60:40 F:C diets exceeded the recommendations of NRC (2001) and White et al. (2017b), but the diet contained less peNDF8 than required according to Zebeli et al. (2012; 18.5% required versus 13.9% actual). Based on this single study, the recommendations for peNDF8 by Zebeli et al. (2012) may be too high.

Table 4. Evaluation of fiber recommendations using results from diets varying in forage:concentrate ratio (F:C) and chop length of alfalfa silage (from Yang and Beauchemin, 2007).

F:C: / Chop length:	35:65 short	35:65 long	60:40 short	60:40 long	Minimum required	
TMR, % DM						
19-mm sieve	3.6	7.1	7.1	13.1		
8-mm sieve	27.8	34.5	33.3	41.1		
1.18-mm sieve	63.0	52.8	52.9	39.1		
pan	5.6	5.7	6.6	6.7		
DM content, %	72.5	71.1	66	62		
CP, % DM	19.9	20.5	21.6	21.8		
NDF, % DM	30.3	30.6	34.5	36.4		
Forage NDF, % DM	16.0	16.5	26.6	27.5		
ADF, % DM	20.4	21.6	26.9	27.4		
Starch, % DM	30.6	30.6	17.8	17.8		
NEL, Mcal/kg	1.65	1.65	1.65	1.65		
Eating time, min/d	203	211	225	218		
Ruminating time, min/d	441	444	446	522		
Mean rumen pH	5.86*	6.17	6.46	6.55		
Time pH < 5.8, h	11.5	10.1	1.1	1.3		
Assessment						
Actual forage-NDF, % TMR DM	16.0	16.5	26.6	27.5	19.0	NRC, 2001
Actual peNDF1.18, % TMR DM	28.6	28.9	32.2	34.0	31.2	Zebeli et al., 2012
Actual peNDF8, % TMR DM	9.5	12.7	13.9	19.7	18.5	Zebeli et al., 2012
Forage-NDF required (White et al., 2017b)	27	26	20	19		

**Bold red indicates below requirement*

Other Considerations that Help Prevent SARA

Straw and Supplemental Fat

As mentioned above, the recommendations for minimum forage-NDF levels in high starch diets are difficult to achieve when using high quality forages that are low in NDF

concentration. In that case, supplementing diets with a limited amount of chopped straw can be very beneficial (Kahyani et al., 2019). However, it is important to compensate for the lower net energy content of straw by supplementing with rumen-protected fat. Additionally, supplemental fat can be used to lower the starch content (and fermentability) of the diet. The NRC (2001) recommends a maximum total concentration of 6 to 7% fat in the diet DM (basal diet + supplemental fat).

Diet Adaptation/Transition Cows

Any major change in diet composition that affects carbohydrate fermentability requires an adaptation of both the rumen epithelium and the rumen microbiota. Such is the case in early lactation where the diet fermentability is increased due to the use of high quality forages and grain, while the particle size of the TMR decreases, compared with the close-up diet. The cow and its rumen must quickly adapt after parturition. The microbial community must transition from being primarily cellulose degraders to a more complex community of starch and cellulose degraders. This transition takes about 3 weeks to occur and during that time the rumen microbial community is highly unstable, and the risk of SARA is high (Humer et al., 2018). The rumen epithelium must also adapt to increase the functional capacity for absorption. This transition takes about 4 to 6 weeks (Humer et al., 2018). During dietary transition, there is a risk that the rate of SCFA production will exceed the rate of SCFA absorption and neutralization, thereby leading to SARA.

Primiparous Cows

Primiparous cows are at higher risk for SARA than multiparous cows because they: 1) are less well adapted to highly fermentable diets, 2) have lower chewing time, 3) eat more slowly and thus require more time for meals, and 3) are less dominant than mature cows and thus tend to eat larger meals because they have trouble getting access to the feed bunk

(Beauchemin, 2018). When possible, it is advantageous to group primiparous cows separately from multiparous cows, or at the very least ensure pens are not overcrowded and that bunk space does not limit intake and meal frequency (minimum of 60 cm per primiparous cow; Rioja-Lang et al., 2012).

Feeding Management

Greatest feeding activity typically occurs after feed is delivered or pushed-up throughout the day. Thus, frequent delivery of TMR tends to promote feeding activity and a more even distribution of feeding time throughout the day, which helps minimize the post-prandial drop in rumen pH. Once again it must be stated that it is important to provide sufficient space at the feed bunk, because increased competition increases eating rate and decreases chewing time (Crossley et al., 2017).

Even when a TMR is fed, cows will preferentially select the grain and leave the longer forage particles. Sorting of the ration can increase the risk of SARA. Increasing the frequency of feed delivery or feed push-up reduces feed sorting (Miller-Cushon and DeVries, 2017). Overfeeding such that there are excessive orts left in the feed bunk also increases sorting.

Feed Additives

Buffers: Incorporating sodium bicarbonate into the diet at 0.5 to 1% of DM increases the total bicarbonate flow to the rumen by 3 to 4%. The effects of adding sodium bicarbonate on rumen pH are highly variable, but on average pH is expected to increase by about 0.13 units (Hu and Murphy, 2005). Thus, dietary buffers will not eliminate SARA, but they do help stabilize the overall acid load in the rumen.

Yeast: There is overwhelming evidence that including active dry yeast and yeast cultures (from *Saccharomyces cerevisiae*) **in the diet of dairy cows** can improve milk production and feed conversion efficiency, partially due to beneficial effects on rumen pH (Chaucheyras-Durand et al., 2008). However,

effects of yeast on rumen pH can depend on a number of conditions, such as whether the yeast is viable or non-viable, the strain of yeast, dose rate, and diet. Many *in vitro* and animal studies have examined the effects of yeast and provide evidence for changes in rumen fermentation, stabilization of rumen pH, stimulation of the growth and metabolism of lactate-utilizing bacteria, scavenging of oxygen present in the ingested feed particles (active dry yeast only), promotion of growth of protozoa, and improvement in fiber digestion (Chaucheyras-Durand et al., 2008; McAllister et al., 2011).

Mohammed et al. (2017) demonstrated that some cows benefit more from supplemental yeast than others. They fed cattle a diet with 50:50 forage:concentrate ratio that did not include ionophore. Based on continuous measurements of rumen pH, the cattle were characterized as being more or less susceptible to SARA. The more susceptible cattle benefited when fed active dry yeast especially when the yeast was viable (Table 5). However, when the cattle were subsequently subjected to grain overload to destabilize the rumen, yeast failed to attenuate severe acidosis (data not shown). The study demonstrates that yeast can help stabilize rumen pH of cattle that are prone to SARA, but yeast will not prevent acute acidosis.

Table 5. Effectiveness of viable and non-viable yeast (ADY) for cattle grouped by susceptibility to SARA during a baseline period (Mohammed et al., 2017)

	Most susceptible cattle			Least susceptible cattle		
	Control	Viable ADY	Non-viable ADY	Control	Viable ADY	Non-viable ADY
Lactate, mM	0.53	0.09	0.19	0.03	0.02	0.01
Minimum pH	5.26	5.55	5.53	6.03	6.08	5.99
Mean pH	5.93	6.30	6.18	6.55	6.68	6.56
pH<5.8, h/day	12.9	2.0	5.8	2.5	0.03	0
pH<5.5, h/day	9.0a	0.3b	1.1b	0.5b	0b	0b

a-b Values within a row with different letters differ (P < 0.05).

Conclusions

Cows with a healthy rumen ruminate extensively, have a mean rumen pH > 6 with very short infrequent dips below pH 5.8, have strong rumen mixing contractions, and their rumen microbiota are well adapted to a mixed diet of cellulose and starch. Maintaining healthy rumen function can be particularly challenging for cows in early lactation and primiparous cows because they are at a higher risk for developing SARA. New recommendations for the minimum amount of forage fiber needed to maintain healthy rumen function depend on particle size and fermentability of the ration, with starch content used as a proxy for fermentability. Diet formulation, feeding management, and feed additives can also help reduce the risk of acidosis.

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Baled silage management

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Keywords: bale silage, machine innovation, oxygen barrier film, agricultural compactor.

Introduction

Bale silage has proved to be a good alternative to haymaking for silage on small-to-medium farms in lowlands and highlands to produce higher quality forage than hay (Hancock and Collins, 2006; Borreani et al., 2007a). Over the last 25 years, baled silage has become an economical alternative to other harvesting systems in Europe (Tabacco et al., 2011), and this has led to a remarkable increase in the amount of herbage stored as silage (from 30% to 80% of the total harvested dry matter, depending on the country considered) (Wilkinson and Toivonen, 2003) and has been gaining popularity in the US over the last decade (Arriola et al., 2015; Coblentz and Akins, 2018).

Big bale silage is by now a well-established conservation system for storing excellent quality forage, and provides an opportunity to maintain the high feeding value of young herbage (Hancock and Collins, 2006; Borreani et al., 2007a; Coblentz and Akins, 2018). Forage for baled silage is often wilted extensively and therefore presents more limited

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fermentation than conventional silage stored in horizontal silos (Borreani and Tabacco, 2018). Moisture is known to influence silage fermentation and the production of fermentation acids (Coblentz and Akins, 2018). A greater dry matter (DM) content than 35% (moisture < 65%) is commonly adopted by farmers to avoid effluents, to minimize bale deformation and to reduce the number of bales per hectare and the plastic consumption per tonne of stored DM (Han et al., 2006; McEniry et al., 2007; Tabacco et al., 2013). This results in a restricted fermentation and consequently in a higher pH than precision-chopped silages with the same DM content, as reported in Figure 1 (Borreani and Tabacco, 2018). Figure 2 reports the relationships between lactic acid and silage pH for corn silage and for baled alfalfa and grass silages. It appears that the buffering capacity of grassland forages reduces the acidification effect due to lactic acid, compared to well conserved corn silages. The high DM content of baled silages and the reduced release of solubles from plant tissues in a few cases has resulted in a final pH of less than 4.2 being achieved, which is considered sufficient to reduce the risks of clostridial fermentation in all forages. Moreover, the reduced moisture content at harvest, due to the wilting process, reduces the water activity (aw) of the forage and this results in a synergistic inhibitory effect with pH on clostridial development, as can clearly be seen in Table 1 (Pahlow et al., 2003).

Another factor that could influence clostridial growth in baled silages is a delay in wrapping after baling, which leads to a reduction in the carbohydrates that are available for fermentation, due to plant tissue and microbial respiration, and results in a higher final pH (Ciotti et al., 1989; Niyigena et al., 2019).

Table 1. Indicative critical pH values for anaerobically stable silages, as influenced by water activity in the absence of free nitrates in the forage at ensiling (from Pahlow et al., 2003).

	DM content (%)						
	20	25	30	35	40	45	50
Moisture (%)	80	75	70	65	60	55	50
Water activity (a^w)	0.999	0.995	0.990	0.966	0.960	0.956	0.950
Maximum pH for stable silage	4.20	4.35	4.45	4.60	4.75	4.85	5.00

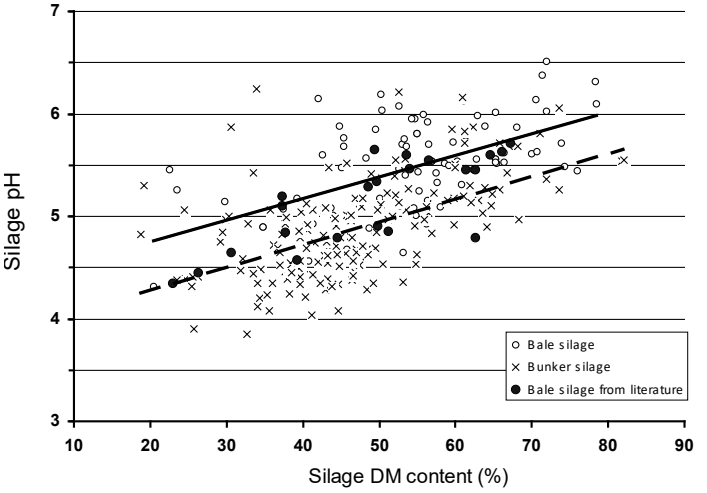


Figure 1. Relationship between silage pH and DM content of grass and legume silages conserved in bunker silos (dotted regression line; pH = 0.0223 DM content + 3.839; R2 = 0.309**) or in wrapped bales (continuous regression line; pH = 0.0212 DM content + 4.327; R2 = 0.292*) on dairy farms in northern Italy (n = 277) (adapted from Borreani and Tabacco, 2018). The black circles refer to data from Coblentz and Akins (2018).

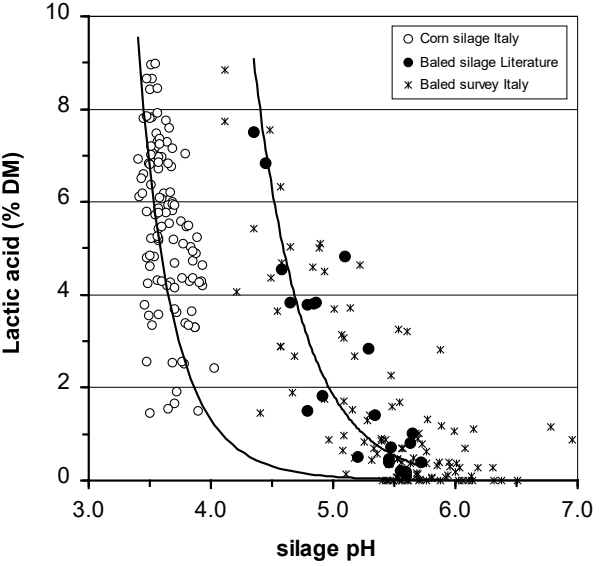


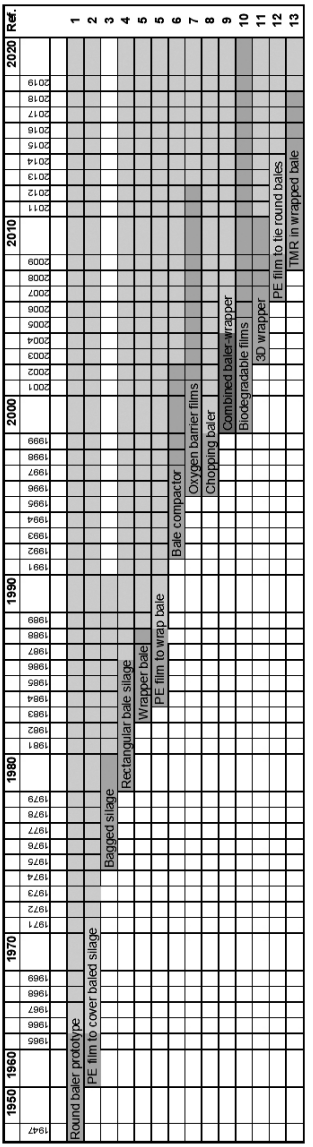
Figure 2. Relationship between lactic acid and silage pH in corn silage (the open circles refer to data from Borreani and Tabacco, 2010); in baled silage from literature (black circles, alfalfa and grass baled silages from Coblentz and Akins, 2018), and in baled silages from an Italian survey on farm (asterisks, alfalfa and grass bales from Nucera et al., 2016).

The main innovation milestones concerning bale silage technology are reported in Figure 3. The concept of conserving grass as silage in big round bales originated in the early '70s and it involved ensiling individual or two bales together in polyethylene plastic bags or rectangular bales covered with plastic films (Marshall and Howe, 1989). Defective sealing, especially at the neck of the bag, allowed air to penetrate the bale and, as a result, bagged silages often showed mold development and extensive aerobic deterioration (Wilkinson et al., 2003). For these reasons, bagged bales were quickly replaced by a new wrapper developed by Silawrap in 1982 and they had completely disappeared by the beginning of 1990s. In 1985, Tom Golden,

marketing director of Silawrap (Kverneland group, Norway), said, about a newly developed bale wrapping machine: "*It'll revolutionize round bale silage making*" as it was a fast, low-cost way of wrapping bales in spoilage-proof plastic (Anonymous, 1985). In the 90s, improvements were made to baler machines with the introduction of chopping devices and improved forage compaction in the bale chamber, which led to high density and well-shaped bales being obtained (Tremblay et al., 1997). New machines that integrate both a baler and a wrapper are now available on the market, and they are able to increase productivity and reduce operation costs, compared to the use of two separate machines (Münster, 2001).

In 2017, the Kverneland Group introduced the non-stop bale production process onto the market with the introduction of a film-on-film applicator option and two bale chambers arranged in series – a full sized main chamber and a pre-chamber, located directly above the intake rotor, which is about two thirds the size of the main chamber. This design results in an extremely compact machine with a non-stop capability. Earlier this year (2019), this system was combined with a wrapping machine. The newly proposed system, named "*FastBale*", is a non-stop round baler wrapper that integrates a pre-chamber with a main chamber and a wrapper (https://ien.kverneland.com/Kverneland-brand-Corporate-site/Bale-Equipment/Round-Balers/Kverneland-FastBale).

Another innovation aspect regards the improvement of the quality of film applications during wrapping (Borreani et al., 2007b; Bisaglia et al., 2011) and the development of stretch films with a high barrier to oxygen (Borreani and Tabacco, 2008).



References: 1 = https://en.wikipedia.org/wiki/Baler; 2 = Wilkinson et al., 2003; 3 = Marshall and Howe, 1989; 4 = Shinners et al., 1996; 5 = Anonymous, 1996; 6 = Anonymous, 1990, 1992; 7 = Borreani and Tabacco, 2017; 8 = Tremblay et al., 1997; 9 = Münster, 2001; 10 = Keller et al., 2000; 11 = Borreani et al., 2007b; 12 = Bisaglia et al., 2011; 13 = Wang et al., 2010.

Figure 3. Main innovations in baled silage from the '50s onwards. In gray = prototypes and setting of the technology; in green = commercial development and diffusion.

2. Factors that affect bale silage quality

In addition to the crop type and morfological stage at cutting, the moisture content, pH drop, density, porosity and air-tightness are the main factors that influence the quality of silage, and they have been found to be influenced by the wilting process before baling (moisture and sugar contents), the type of baler (density and porosity), the wrapping delay (respiration of fermentable carbohydrates), the characteristics of the stretch film and number of layers applied (to achieve the best anaerobic environment), the length of storage and by mechanical damage to the plastic cover (lost air-tightness) (Borreani and Tabacco, 2018; Coblentz and Akins, 2018).

The higher DM content and the increased porosity of forages conserved in wrapped bales increase the risk of fungal growth (O'Brien et al., 2008; Tabacco et al., 2013), and consequently increase the risk of mycotoxicosis (O'Brien et al., 2007; McElhinney et al., 2016) and Listeria contamination (Fenlon et al., 1989; Nucera et al., 2016). Even though the baled silage system is based on a well-established procedure, the fact that the incidence of mold spoilage can be relatively high (O'Brien et al., 2008; Borreani and Tabacco, 2010) suggests that the current bale ensiling practices may be considered only partially satisfactory (McEniry et al., 2011). Air-tightness has to be maintained for extended conservation periods to keep the molded surface as low as possible, because more than 40% of the stored DM in baled silage is within a space of 120 mm from the film cover, and the reduced total thickness of the combined layers of stretch-film on the bale side (from 80 μm for 4 layers to 120 μm for 6 layers) makes wrapped bales more susceptible to oxygen ingress than horizontal silos (Tabacco et al., 2013).

Furthermore, the stretch polyethylene wrapping system has shown some limits, with regards to sealing efficiency

(Jacobsson et al., 2002), concerning the high permeability to oxygen of stretch films (Borreani and Tabacco, 2008; 2010) and the non-uniform distribution of plastic films between the ends and the curved surface of the bale (Borreani et al., 2007b). These problems lead to undesirable air exchanges over the conservation period, and it has been suggested that an increasing number of plastic film layers is required. A significant reduction in mold growth and an improvement in silage conservation quality are obtained when six or more layers of plastic film are applied instead of four, especially for high DM content baled forages (Keller et al., 1998; Borreani and Tabacco, 2008). Increasing the layers of plastic film contributes to increasing the hygienic quality of the overall silage, as the bale surface covered by mold is reduced, especially when the baled silage is conserved for long conservation periods (Figure 4). Several layers of stretch film ensure a better airtight cover, but also lead to prohibitive increases in costs, in plastic usage and in environmental concerns, due to the necessity of disposing of the additional plastic (Lingvall, 1995; Borreani and Tabacco, 2017).

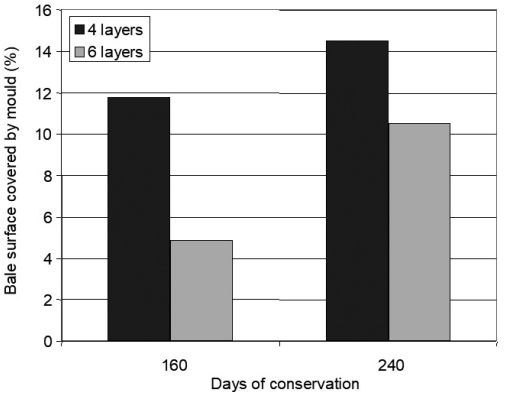


Figure 4. Bale surface covered by mold in relation to the days of conservation and number of layers of PE films for grass and legume silages from a farm survey in Northern Italy (G. Borreani and E. Tabacco, University of Turin, unpublished data).

The technical solutions that have been introduced on the market to improve bale silage quality in the last few decades have involved the following aspects: speeding up the wilting process to reduce the field period and mechanical losses during harvesting and to improve bale densities, especially for high DM content silages; increasing the uniformity of the plastic distribution over the bale surface; and reducing plastic permeability to oxygen (Borreani and Tabacco, 2018). The rapid development of the wrapping bale technology has led to a great improvement in the ensiling process, and this has been achieved by increasing bale densities using round balers equipped with chopping-devices (Tremblay at al., 1997; Borreani and Tabacco, 2006), reducing the working times, thanks to the use of combined baler-wrapper machines (Münster, 2001), improving the uniformity of the plastic distribution on the bale surface using a new-concept 3D wrapping system (Borreani et al., 2007b) or round balers equipped with film-tying attachments to replace the standard net-tying system with a film tying system, in order to improve the airtightness of the coverage on the curved bale surfaces (Bisaglia et al., 2007), and the use of oxygen barrier films (Borreani and Tabacco, 2009).

2.1. *Bale density*

Improving the bale density, in order to reduce forage porosity and plastic use, has been one of the main goals of baled silages since the '90s (Shinners, 2003). Herbage is rolled during baling, but this does not give the bale a high density and it makes oxygen exclusion more difficult. Silage compaction has been improved as a result of the introduction of variable chamber balers and the addition of chopping devices to balers (Tremblay et al., 1997). Round balers with a cutting system behind the pickup are available on the market and could provide

the following advantages: density increases of up to 15% (Figure 5), with subsequent improvements in baler productivity and silage quality (Borreani and Tabacco, 2006), and bales that are more readily processed by TMR mixer-feeders (Shinners, 2003). The technique of cutting herbage into shorter lengths on entry to the bale chamber could facilitate the release of plant sugars and provide an aid to obtaining a better bale density (Shinners, 2003). It has been found, from a literature review covering research works from 1984 to 2019, that the bale DM density is related to the moisture content of the forage at baling, with densities increasing as the moisture content decreases till 50% (silage), plateauing for moisture contents ranging between 50% and 20% (haylage) and then decreasing again for a moisture content lower than 20% (hays) (Figure 6). Within each moisture range, the use of variable chamber balers and of a cutting system before the baler chamber could increase the DM density of the bales.

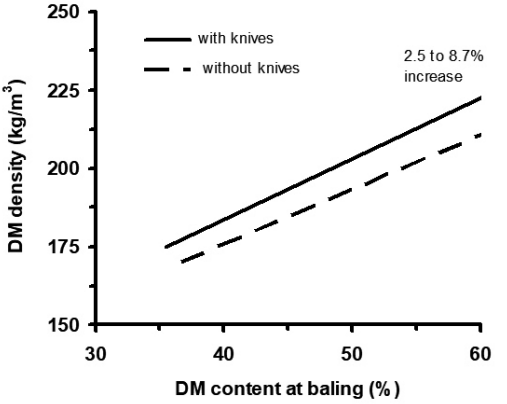


Figure 5. Bale DM density in relation to the DM content at baling of alfalfa and Italian ryegrass with and without a baler cutting device (adapted from Borreani and Tabacco, 2006).

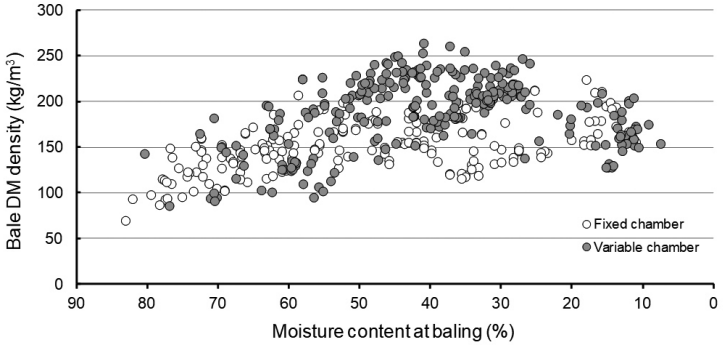


Figure 6. Scatter plot of the moisture at baling and bale DM density with variable and fixed chamber balers. The data were obtained from a review of the literature on bale silage, haylage and hay from 1984 to 2019.

2.2. *Improving the uniformity of the plastic distribution*

Traditionally, four layers of polythene are applied in two subsequent and complete rotations of a bale, with an overlap of 50% between layers. A significant reduction in mold growth and an improvement in silage conservation quality is obtained by increasing the number of layers, but this causes a waste of plastic film in conventional wrapping systems, due to the higher proportion of plastic distributed on the flat ends (Figure 7 - Conventional wrapper).

In order to increase the uniformity of the plastic distribution, two different solutions have recently appeared on the market: a selective 3D wrapper and round-balers equipped with a tying system to secure large round bales with polyethylene tying-film in the baler chamber. The improvement in efficiency that may be obtained with the new selective wrapper concept (3D), based on a biaxial rotation of film applicators, is reported in Figure 6. This concept is of great interest because it reduces the amount of plastic used per bale, while improving the uniformity of the plastic distribution on the surface and increasing the

number of layers in the areas that are most at risk to damage (Borreani et al., 2007b). In a conventional wrapper bale, which is nominally wrapped with four layers of plastic film, the flat ends have as many as 16-20 layers in the center, a number which gradually decreases to four layers at the outer edge and on the curved surface.

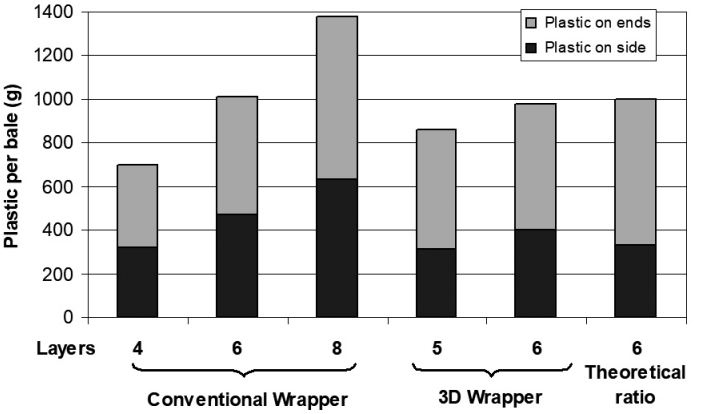


Figure 7. Plastic distribution on the bale surface (bale diameter 1.2 m). Theoretical ratio means the uniform distribution of 120 m of plastic film (6 layers) over the whole bale surface (adapted from Borreani et al., 2007b).

Some new-generation balers have recently been equipped with tying systems that allow a bale to be secured in the press chamber using twine, net or polyethylene film. Replacing the standard net used to secure bales with a polyethylene film represents an innovative alternative to net-tying when preparing round bales for silage, and it has been shown to improve the airtightness of the coverage on the curved surface of the bale and to reduce the bale surface covered by mold (Bisaglia et al., 2011). Tabacco et al. (2013) studied the possibility of reducing mold growth on the surface of low-moisture baled silage of grass-legume mixtures for a long conservation period (8 months), without increasing plastic costs, by replacing polyethylene net with polyethylene tying-film to secure large round bales in the

baler chamber. The high DM content of the silages restricted fermentation and resulted in low concentrations of acids, with pH values in the inner part of the bale ranging from 5.41 to 5.70. The use of tying film, compared to net, led to a reduction in the number of holes and an improvement in the anaerobic status of the baled silage, even with just four layers of stretch-film, and resulted in a decrease in mold counts and visible mold growth over the bale surface (Table 2). The Authors of this study concluded that, with similar costs for plastic and the same amount of plastic used to secure the bale with net and wrapping it in four layers of stretch-film, it is possible, using a tying film of 16 µm in the baler chamber, to obtain more than six effective layers of plastic on the curved side and on the edges of the bale, and therefore to reduce the risk of cover puncturing and the incidence of mold growth over the bale surface to a similar level to that of baled silage wrapped in six layers of polyethylene and secured with net.

Items	Tying Method			
	Net-tying		Film-tying	
	4 Layers	6 Layers	4 Layers	6 Layers
Surface covered by mold (%)	25.3	2.6	3.3	0.8
DM losses (g/kg DM)	56	30	29	28
Net/film per bale for tying (g)	216	209	260	271
Stretch-film wrap per bale (g)	901	1275	901	1256
Total plastic per bale (g)	1117	1484	1161	1527
Thickness of stretch-film wrap (µm)	79	121	81	119
Thickness of tying film (µm)	-	-	43	46
Micro-holes in the plastic cover (n)	14	5	7	3
Cost of the tying film/net (€/bale)	0.81	0.78	0.71	0.75
Cost of the stretch-film wrap (€/bale)	2.92	4.12	2.91	4.06
Plastic cost per bale (€)	3.72	4.91	3.63	4.81
Plastic cost per tonne of harvested DM (€)	10.97	14.00	9.58	13.30

Table 2. Bale weight, bale density, plastic consumption, plastic thickness on the curved side of the bale, plastic damage, surface covered by mold, DM losses, and costs of plastic in relation to the tying method and the number of plastic layers applied (from Tabacco et al., 2013).

2.3. *Oxygen barrier films to wrap bale silages*

Improving the oxygen impermeability of stretch film has been identified as one of the most effective ways of obtaining significant improvements in the conservation quality of baled silage (Borreani and Tabacco, 2008; 2017). New plastic manufacturing technologies, coupled with new low oxygen permeability polymers that can be coextruded with PE, offer the possibility of producing multilayer stretch films for the wrapping of bale silages at costs that are competitive with those of the conventionally used PE on farms (Borreani and Tabacco, 2017). Most plastic films for stretch-wrap silage production are made of coextruded, linear, low-density polyethylene, and are 25 um thick before being stretched 50% or more during application. The high O2 permeability of PE films seems to be one of the main drawbacks of wrapped silage, especially for long conservation periods (Borreani and Tabacco, 2008; 2017). The new generation of high oxygen barrier (HOB) films improve oxygen impermeability 374-fold compared to standard PE films, and maintain similar mechanical properties to those of the best performing PE stretch films (Borreani and Tabacco, 2017). When tested at a farm scale, the HOB stretch films were effective in reducing the DM losses during conservation to values of around 2% for alfalfa baled silage with a DM content ranging from 55 to 65% (Figure 8). Other authors have reported DM losses of 6% (Hancock and Collins, 2006), or of 7% of the total harvested DM (Shinners et al., 2009b; Borreani and Tabacco, 2008) for alfalfa silage baled at similar DM contents and wrapped with standard PE stretch films.

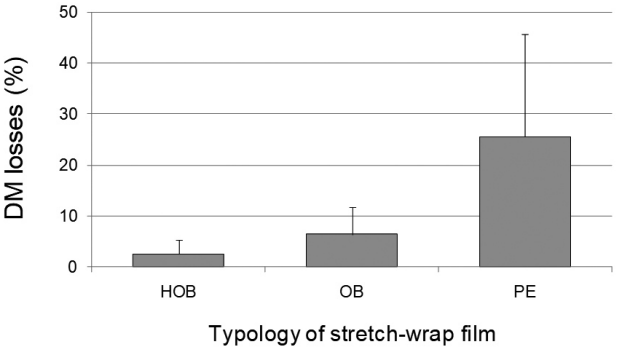


Figure 8. DM losses in relation to the oxygen impermeability of stretch films on alfalfa baled silages after 420 d of conservation (average of three trials in Northern Italy). HOB, high barrier film (4 layers); OB, medium barrier film (4 layers); PE, standard polyethylene film (6 layers) (adapted from Borreani and Tabacco, 2010).

Oxygen barrier films also have a remarkable influence on the evolution of the surface covered by mold: the higher the barrier properties of the plastic film utilized to wrap the bales are, the greater the reduction in mold growth on the bale surface over the conservation period (Figure 9).

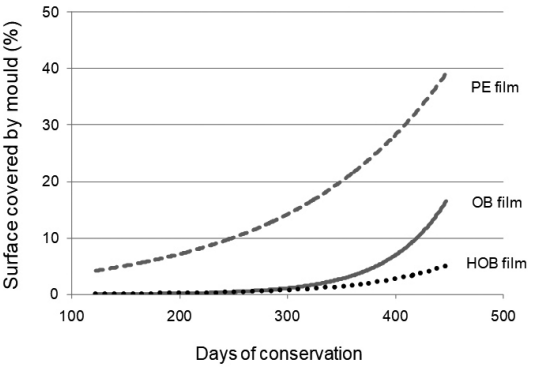


Figure 9. Surface covered by mold in relation to the days of conservation in baled alfalfa silage wrapped with stretch film with different oxygen impermeability. HOB, high barrier film; OB, medium barrier film; PE, standard polyethylene film (adapted from Borreani and Tabacco, 2010).

2.4. Storage management and plastic bale damage

Bales are usually wrapped in the field immediately after baling and before being transported to the storage site and are invariably stored outdoors (McNamara et al., 2001). If the integrity of the stretch film is damaged during storage, the subsequent ingress of oxygen will permit the growth of filamentous fungi and other microorganisms, thus resulting in extensive quantitative and qualitative losses (McNamara et al., 2001; Kawamoto et al., 2012). The plastic stretch-film surrounding baled silage is prone to damage during storage, prior to being fed to livestock, by many vertebrates. Damage by birds (McNamara et al., 2002) and by rats (Kawamoto et al., 2012) has been reported to be the most frequent on farms, while that caused by cats, dogs and other farm livestock is comparatively limited (McNamara et al., 2002). Direct physical barriers to bird access, as opposed to scaring devices, such as the use of nets securely positioned 1 m above and beside the bales, appear to be the most reliable way of preventing damage (McNamara et al., 2002). Whole cereal baled silages result to be particularly attractive to rats, which could easily damage bales stored under a masking situation (Kawamoto et al., 2012). It has been suggested that creating open spaces between the bales and not covering bales with plastic sheets reduce the number of hiding places that are available for rats, thereby decreasing their potential damage.

The storing position of in-line bales on the ground plays an important role in preventing mold development on the bale surface (Figure 10). The storing position has been shown to influence the amount of surface covered by mold to a great extent, for both Italian ryegrass and alfalfa forages, by reducing the area of visible mold on the curved surface of bales that were stored on their flat ends (Table 3) (Bisaglia et al., 2011).

These data are in agreement with McCormick et al. (2002) who reported that the appearance of mold was virtually absent on end-stored bales, whereas mold damage was more prevalent on bales stored on their curved surface.

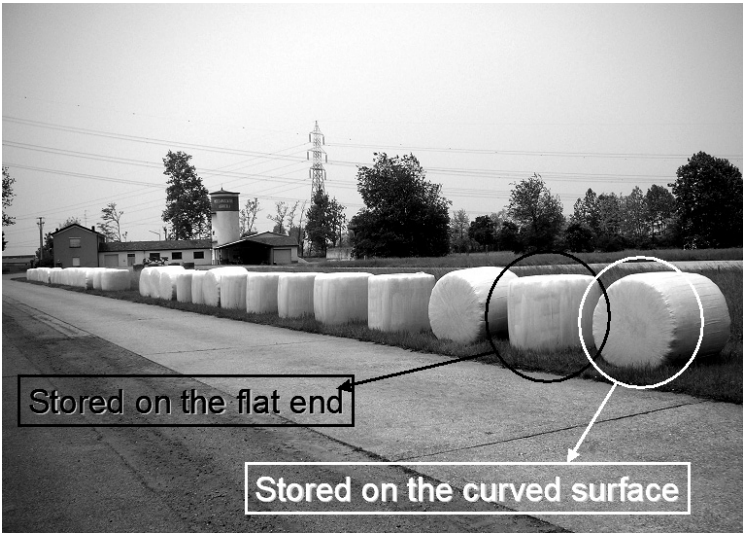


Figure 10. Bale storing position.

Table 3. The storing position and of number of layers were found to affect the bale surface covered by mold in alfalfa and Italian ryegrass baled silages after 180 d of conservation outdoors (from net-tying treatment, Bisaglia et al., 2011). END = stored on the end; SITE = stored on the curved surface.

		Bale surface covered by mold (%)		DM losses (%)	
Crop	PE film layers	END	SITE	END	SITE
Italian ryegrass (45% DM content)	4	11.1	23.1	6.5	5.8
	6	5.8	11.8	5.8	6.4
Alfalfa (56% DM content)	4	22.3	24.5	2.6	2.8
	6	9.4	13.7	2.8	2.7

3. Ensiling fine chopped material in wrapped bales

The need to ensile fine chopped forages or milled grains has become important to reduce the production costs of milk and meat, even for small to medium farms. The agricultural compactors that have recently appeared on the market are able to transform bulk material, such as biomass and fine particle materials, into easy-to-handle, high-density round bales. These baler-wrapper combined machines are able to bale more than 30 different materials, such as corn silage, high moisture grain silage, grass and legume chopped forages, sugar pulp, total mixed rations (TMR), cotton, grape marc, etc, by tying the bale with plastic film and then wrapping the bale with stretch plastic film. To the best of the authors' knowledge, the compactors available on the market for fine-chopped material are made by Orkel, Goweil and Hisarlar. Many of these new machines have adopted some or all of the technical solutions described above, such as film tying in the compression chamber and two film dispensers to speed up the wrapping operation. The first machine that was able to bale fine-chopped material was developed in Norway (Anonymous, 1990), and it was designed to be top-filled by a forage chopper.

Weinberg et al. (2011) showed the possibility of ensiling TMR in square bales and presented the results of an established commercial process that is based on the production of dense bales of silage under high pressure, followed by packing and wrapping with 8 to 9 layers of polyethylene stretch film. Weinberg et al. (2011), Miron et al. (2012) and Shaani et al. (2015), utilizing the same machine, indicated that the DM density of a bale was above 400 kg/m3, which is more than twice the average DM density of silage in a bunker silo (Savoie and Jofriet, 2003), that the fermentation process takes place during storage even for already ensiled material, and that the forage quality can be maintained outdoors for a long period of time, even under hot

summer conditions. Furthermore, the preserved TMR showed an increase in aerobic stability, compared to that of the fresh TMR.

This technology has been successfully used for the preservation of high-moisture by-products stored with dry feeds (Miron et al., 2012; Shaani et al., 2015), or as completely finished TMR for lactating dairy cows (Wang et al., 2010; Weinberg et al., 2011). Ensiling TMR is becoming a wide-spread practice, and the advantages attributed to it include: the supply of homogeneous feed over time to the animals, labor savings during preparation and the opportunity of including otherwise perishable moist by-products (Weinberg et al., 2011; Shaani et al., 2015). Forage crops conserved as silage in round bales undergo a slight reduction in particle size during harvest (Muck, 2006), are baled at a higher DM concentration, are stored at a lower bulk density, and are less fermented than silages stored in bunker silos (Weinberg et al., 2011). In the last few years, stationary compactor machines have been developed to suitably conserve, apart from finished TMR, fine chopped forage or ground grain that were previously only conserved in stack silos, thus allowing them to be stored until needed and to be transported like any other commodity. Many chopped forages, such as whole corn silage, whole ear corn silage and whole crop soybean silage, could be profitably preserved in wrapped bales as feeds for lactating cows on small-medium sized farms, as well fine chopped corn stover, rice straw and other lignocellulosic wastes, as ensiled biomass that could be used to produce bioenergy and biofuels (Borreani G. and Tabacco E., University of Turin, pers. com., 2017; Anonymous, 2017).

Our group (Forage Team, University of Turin, Italy) carried out a trial on a farm in Northern Italy in 2018 to compare bunker silage and a bale compactor on the first cut of Italian ryegrass. The forage was wilted for 2 days till a DM content of around 45% was reached, and it was harvested and chopped

using a conventional forage harvester to a 30-mm theoretical length of cut and then ensiled in both a bunker silo and with an agricultural compactor (Dens-X, Orkel, Fannrem, Norvay). The silages were opened after two conservation periods (64 and 142 d) and the fermentative and microbial parameters were analyzed (Table 4). The fermentative silage quality was comparable for the two ensiling methods, with the bales having a lower pH and higher lactic acid content than the bunker silages. Moreover, the microbial reduction of yeast and the mold count were very similar for both conservation methods and after both conservation times. An agricultural compactor that produces wrapped bales could hence be an alternative solution to ensiling fine-chopped forages or other fine particle materials mixed together, such as TMR, thus overcoming the problem of the lower DM densities and lower level of fermentation that characterize conventional baled silages. These machines are, at the moment, managed by contractors and are able to produce from 40 to 60 bales per hour at a cost per bale of around €18-20 (Italian contractor costs for baling, wrapping, hauling and storing in 2019) for a 900 to 1200 kg 120 mm diameter bale.

Table 4. Chopped Italian ryegrass silage ensiled in both a bunker silo and in bales produced by a compactor (Orkel, Norvay) opened after 64 and 142 days of conservation (Tabacco et al., unpublished data).

Items[1]	Conservation period							
	64 d				142 d			
	Compactor				Compactor			
	Bunker	bale	SE	P-value	Bunker	bale	SE	P-value
DM content (%)	48.0	49.1	0.91	NS	42.8	44.7	0.67	*
pH	4.41	4.27	0.031	***	4.40	4.33	0.021	**
Lactic acid (g/kg DM)	45.9	54.5	2.14	*	66.6	71.4	1.45	*
Acetic acid (g/kg DM)	16.7	16.9	0.37	NS	23.1	25.8	0.81	NS
1,2 propanediol (g/kg DM)	3.05	4.14	0.49	NS	5.17	6.86	0.52	NS
Ethanol (g/kg DM)	2.79	3.69	0.25	NS	7.89	13.24	1.60	*
Butyric acid (g/kg DM)	<0.01	<0.01	-	-	<0.01	<0.01	-	-
Yeasts (log cfu/g/ silage)	<1.00	0.93	-	-	<1.00	1.67	-	-
Mold (log cfu/g/ silage)	1.68	1.58	0.15	NS	0.98	0.90	0.26	NS
LAB (log cfu/g/ silage)	8.57	7.17	0.41	**	3.00	5.84	1.42	***

[1]cfu = colony forming units; LAB = Lactic acid bacteria.

Over 2019, the Forage Team (University of Turin, Italy) have conducted several extension trials on farms with agricultural compactors that produced bale silages of whole crop cereals (corn, sorghum, winter cereals), wilted chopped forages (alfalfa, Italian ryegrass, and grass-legume mixtures), high moisture ear corn and TMR. Each forage was sampled during ensiling, the bales were weighed immediately after baling, the bale dimensions were measured, and the FM (fresh matter) and DM density were calculated. The relationship between the DM density of the bales obtained using the compactors and those reported in Figure 5 from conventional balers is reported in Figure 11. It can be observed that, for any moisture content, the bales made with the compactor were denser than those obtained with conventional balers, with higher values than 450 kg DM/m3 for high moisture ear corn (highest value 591 kg DM/m^3) and ranging from 324 to 499 kg DM/m^3 for TMR. These DM densities are comparable with the highest values obtained in bunker silos (Savoie and Jofriet, 2003; Borreani et al., 2018). However, further research is needed to evaluate the fermentative profiles, aerobic stabilities, DM recoveries, nutritional qualities and economic feasibility that may be obtained from ensiling with compactors, compared to conventional balers.

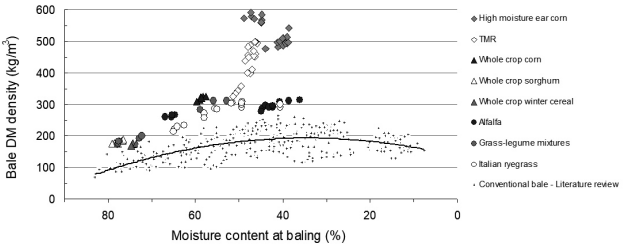


Figure 11. Bale densities of baled silages produced on commercial farms in Northern Italy with bale compactors plotted against the moisture content at ensiling and compared with the DM density of conventional round bales obtained from the literature review (black line regression equation - reported in Figure 5).

Conclusions

The technical and research innovations that have been developed over the last few decades in the field of wrapped bales provide an opportunity to successfully plan farm silage making, while maximizing silage quality and minimizing losses. The reported new technical solutions will improve the feasibility of producing high DM content baleage and of maintaining the nutritional and microbial quality of the forage, while reducing the cost per tonne of stored DM. The improvement in the uniformity of baled silage, in terms of nutritional and hygienic quality, is a priority to make this technique successful in terms of the economic sustainability of dairy production systems.

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Challenges and perspectives of tropical grasses silages

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Introduction

In tropical regions, ruminant production systems are based in the use of adapted grasses. The use of tropical grasses in the pastures provides the possibility of productivity intensification in those regions. Unlike temperate grasses, tropical grasses present high dry matter (DM) yield due to its C4 photosynthetic pathway, which assimilates CO2 with higher efficiency and produces more biomass than C3 species. In addition, the high tillering capacity of tropical grasses assures its perennity and can provide at least of harvests over the year, depending on the cultivar and environmental conditions (rainfall, fertilization, soil conditions). In general, tropical grasses vary with phenology and growth, which are related with the productive potential.

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[4] *Federal University Paraiba, Areia-PB, Brazil*

Tropical grasses are commonly used for grazing in the tropical regions. However, due to the lower DM allowance during the dry season, and the increasingly demand for high quality forage for more efficient livestock production, tropical grasses can be used as conserved forage (silage, hay and haylage). The most productive species are also used as fresh chopped forage in some farms, however in the dry season it may provide lower nutritional value than when properly conserved.

Tropical grasses have high ensiling potential due to its lower liability on climatic conditions when compared with hay and haylage production. When compared to other silage crops (e.g. corn) some tropical grasses such as *Panicum maximum* and *Pennisetum purpureum* cultivars are more adapted to faster growth rate at high temperatures and rainfall. In this way, they can provide greater forage mass to be used in feedlot diets or in supplementation of grazing animals. In Brazil, tropical grass silages are used also as a tool for pasture management, when the grass overcome the target height for grazing, which decreases grazing efficiency. In this situation, the grass can be ensiled to adjust the canopy height and consequently provide proper residues and increase forage accumulation.

Although the described potentialities of ensiling tropical grasses, the combination of nutritive value and time of ensiling may be difficult to manage. Harvesting tropical grasses for combined high productivity and nutritive value may result in low DM content (150 to 200 g/kg), low concentration of water-soluble carbohydrates (WSC; lower than 100 g/kg of DM) and high buffering capacity (Bernardes et al., 2018). The concentration of WSC can be affected by specie, cultivar, phenological stage, environment and fertilization (McDonald et al., 1991). The phenological stage is the most important factor that influences the forage quality, which decreases as the grass grows (Harrison et al., 2003). Thus, the high availability of

tropical grasses cultivars and the diversity of management and harvesting strategies leads to a wide perspective in development of strategies to improve efficiency of silage production.
In this text, we will discuss the challenges based on the papers published and discuss the perspectives and new directions to silage production of tropical grasses.

Overview of papers on tropical grass silage in the world

The first review about grass silages was published in 1939 by Bender and Bosshardt. These authors mentioned challenges observed during the silage production of temperate grasses, especially in the fermentation processes. A few decades later, similar challenges were observed with tropical grasses in Brazilian conditions.

In Brazil, the production of grass silage started with the cultivation of elephant grass (*Pennisetum purpureum*), which was introduced in 1920, with significant diffusion after 60's. Later, the expansion of the *Urochloa* and *Panicum* grasses and the development of specific machines for grass harvest, increased the silage production of tropical grasses to store the forage produced, as explained before. A survey performed in dairy farms showed tropical grasses in the third position of crops used for silage production Bernardes & do Rêgo (2014). The impact of this type of silage can be expressed by the numbers of published papers that will be discussed.

To evaluate the changes and the challenges related to tropical grasses silage, we found 115 papers from 1999 to 2019 published in indexed journals (Figure 1). The following search terms were used, both alone and in combination: grass silage, tropical grass silage, silage additive, additives in grass silage, warm-season grass silage. In the Science Direct, Oxford

Academic, Cambridge Oxford, ASM journals, Wiley on Library, Scopus, and Google Scholar platforms. From the total of 115 observed papers, 13 papers were excluded: 12 because of lack of information about the silage and one literature review, totaling 102 papers.

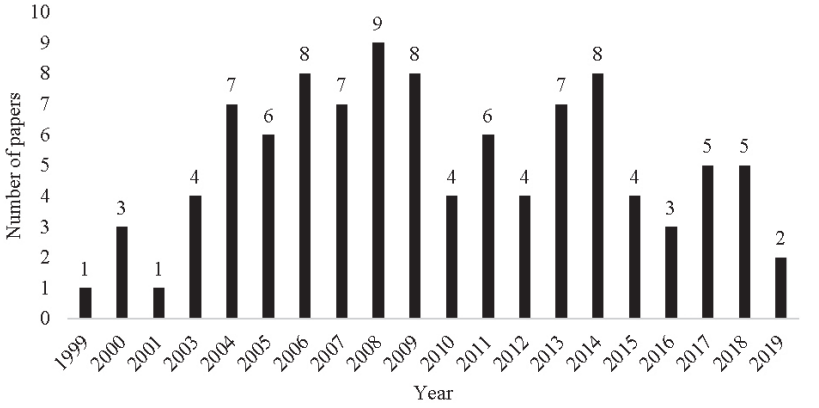


Figure 1: Number of papers with tropical grass silage carried out by year (total number of papers accessed = 102)

The papers with tropical grasses silages comprise different countries from different continents, as South America, North America, Africa, Europe and Asia (Figure 2). Most of the papers were published by Brazilian researchers (73% of the total). It is important to show the common name of some tropical grasses stated by the authors from different countries, which shows some similarities and local variation. The denominations are Napier grass (*Pennisetum purpureum* - JAPAN; Khota et al., 2016), Bana grass (*Pennisetum purpureum* - ZIMBABUE; Manyawu et al., 2003), King grass (*Pennisetum purpureum* – CHINA, INDONESIA; Li et al., 2014; Ridwan et al., 2015), Guinea grass (*Panicum maximum* – JAPAN, TAILAND, BRAZIL; Li and Nishino et al., 2013; Khota et al., 2016; Zanine

et al., 2018), Rhodes grass (*Chloris gayana*- JAPAN; Parvin and Nishino, 2009), Ruzi grass (*Urochloa ruziziensis*- TAILAND; Bureenok et al., 2011), Signal grass (*Urochloa decumbens* – BRAZIL; Santos et al., 2011), Palisade grass (*Urochloa brizantha* – BRAZIL; da Silva et al., 2017), Pasto Saboya (*Megathyrsuss maximus* - ECUADOR; Espinoza Guerra et al., 2016), Kikuyu grass (*Pennnisetum clandestinum* – BRAZIL, Guzzati et al., 2017).

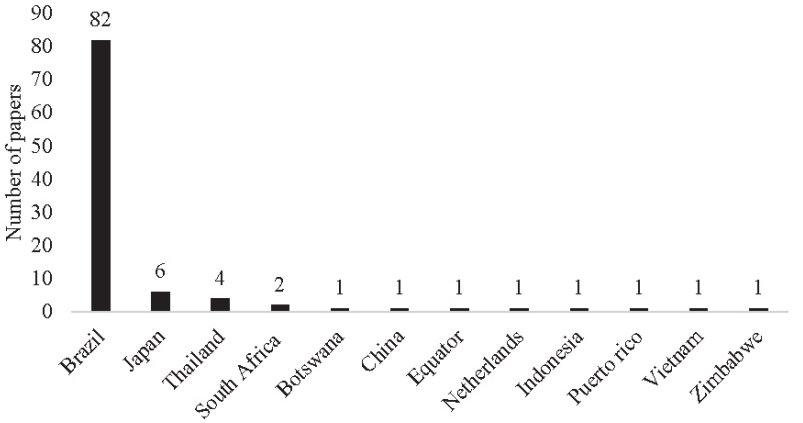


Figure 2: Number of papers with tropical grass silage carried out by country (total number of papers accessed = 102)

When the genera of the tropical grasses were grouped (Figure 3), we observed that 51% of the papers used the *Pennisetum genus*, followed by *Megathyrsus* (21%) and *Urochloa* (16%). The class "others" includes grasses not included in the genera *Pennisetum*, *Megathyrsus*, *Urochloa*, and *Cynodon*, representing 5% of the total papers.

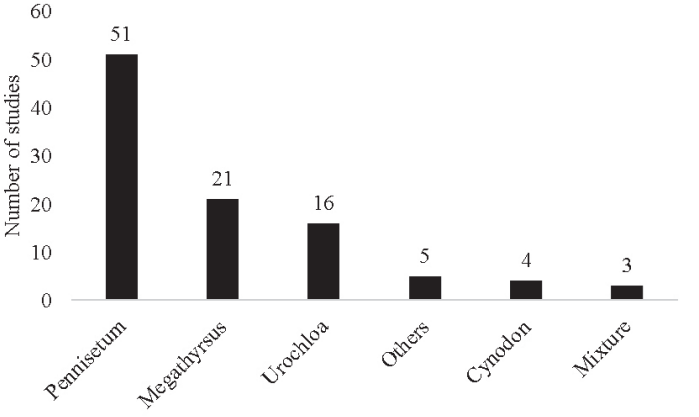


Figure 3: Number of papers with tropical grass silage carried out per genus of grass (total number of papers accessed = 102).

The nomination "mixture" includes papers with two different tropical grasses species for ensiling, or two species from the same genus. The *Pennisetum* is the most studied grass because of its high productive potential and its adaptation to different climates. Most of the papers with *Megathyrsus*, *Urochloa* and *Cynodon* grasses are recent because these grasses have been used for silage production among the farmers. When the forage canopy overgrows the target height for grazing, these grasses can be used for silage production.

It is known that most of the papers with tropical grass silage have evaluated additives in order to improve fermentation and decrease effluent and DM losses. When the treatments of each study were grouped (Figure 4), we observed that 66% of the papers used additive as main factor. The combination additive+wilting (AD+wilting) was second most evaluated among the summarized papers (12.37%). Other important factors (cutting age, wilting, cultivar, fertilization) were observed in lower percentual.

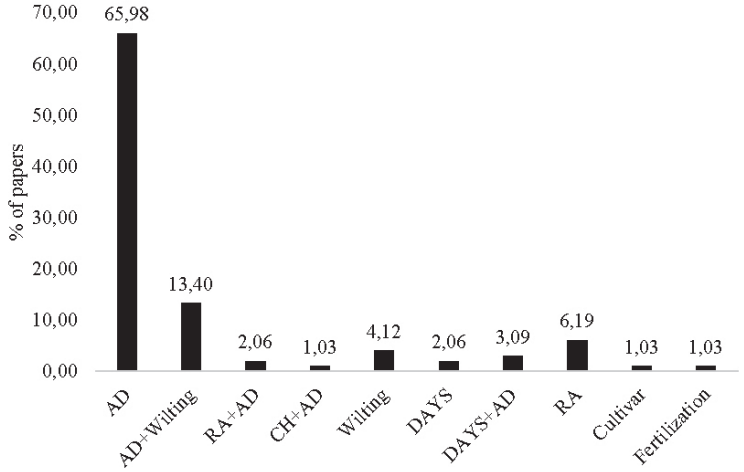


Figure 4: Treatments used in the papers evaluated of tropical grass silage (total number of papers accessed =102).

The use of additives was the main factor evaluated according the papers. Based on that, we grouped them by classes, considering the number of observations: chemical, moisture absorbent, microbial, enzymatic, and others (Table 1). Each class of additive was also grouped by the tropical grass genus or specie, and in case of mixtures including tropical grasses. We observed highest number observations for the use of moisture absorbent additives in *Cynodon* and *Pennisetum purpureum* grasses. The level of inclusion of moisture absorbent additives ranged from 0.1 to 40% (fresh basis). *Pennisetum purpureum* is the most studied specie considering all classes of additives, followed by *Megathyrsus* and *Urochloa*. However, considering the genus *Megathyrsus*, the most used additive was microbial inoculant.

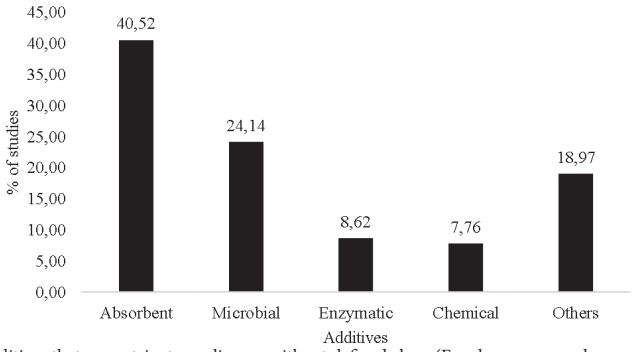
Given that the main challenges of production of tropical grasses silages are the low moisture at harvest and high buffering capacity, most of the papers evaluated moisture absorbent

additives and microbial inoculants (Figure 5). It demonstrates the greater efforts of the scientific community to improve silage fermentation and decrease DM losses in tropical grasses silages.

Table 1: Nunber of observations and papers with tropical grass silage by type of additive (total number of papers accessed = 102)

Number of observations					
Grass	Absorbent	Microbial	Enzymatic	Chemical	Others[1]
Urochloa	19	29	11	7	22
Cynodon	10	1	0	0	2
Mixture[2]	1	0	12	0	2
Others[3]	4	6	6	0	9
Megathyrsus	15	68	42	17	16
Pennisetum	200	53	34	28	78
Total	249	157	105	52	129
Number of papers					
Grass	Absorbent	Microbial	Enzymatic	Chemical	Others[1]
Urochloa	5	8	3	3	4
Cynodon	4	1	0	0	1
Mixture	1	0	1	0	1
Others3	0	1	1	0	2
Megathyrsus	3	10	2	4	4
Pennisetum	34	8	3	2	10
Total	47	28	10	9	22

[1]Others additives- Additives that are nutrient suppliers or without defined class. (Ex: glucose, ground corn, corn kernel, molasses, forage juice, salt, ground sorghum and whey). [2]Mixture grass- (70% *Megathyrsus maximum* + 30% *Sorghum halapense* (Gonzalez e Rodriguez, 2003), *Urochloa ruziziensis* + *Urochloa brizantha* + *Urochloa decubens* (Lukkananukool, et al. 2013), *Pennisetum purpureum* + *Pennisetum americano* (Khota et al, 2018). [3]Others grass- (*Digitaria eriantha*, *Chloris gayana*, *Cenchrus ciliaris*, *Lolium multiflorum*).



Others: Additives that are nutrient suppliers or without defined class. (Ex: glucose, ground corn, corn kernel, molasses, forage juice, salt, ground sorghum and whey).

Figure 5: Percentage of use of the additives in the papers evaluated (total number of papers accessed = 102).

The microbial additives used included homolactic lactic acid bacteria (LAB), with the main species: Inoculant microbial used in papers: *Streptoccocus faescium*, *Enterococcus faecium*, *Lactobacillus plantarum*, *Lactobacillus salivaris*, *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Pediococcus pentosaceus* and *Pediococcus acidilactici*. In addition, some of them would be combined or not with enzymes, as: celulase, hemicelulase e amilase, acremonium cellulase, maicelase, sucrase, xylanase, trichoderma celulase. The use of homolactic LAB indicates the goal of increase lactic acid production and accelerate the drop in pH, improviing fermentation process and decreasing DM losses. Theoretically, the use of fibrolytic enzymes usually releases sugars from fiber solubilization which can be used as substrates by the inoculants. These effects will be discussed in this text.

Once we know the range of additives, it is important to state some factors that can influence all the evaluations of them, as wilting (h), cutting age (days), cutting height (cm), particle size (cm), days of storage and packing density (kg of

fresh matter/ m^3; Table 2). Wilting was evaluated as a treatment in 4% of the papers but was also applied in other papers for all the treatments, which would affect the results of a moisture absorbent additive. The regrowth age showed a large variation among the papers, considering the fact that less than 10% of the have evaluated it as a treatment. The variation in regrowth age could be related to the size of the grass specie, since it varied from 30 to 120 days. Stoloniferous and decumbent grasses showed the regrowth age varied from 30 to 45 and from 30 to 90 days, respectively. The regrowth age for tussock or bunch grasses, as *Megathyrsus* and *Pennisetum* varied from 30 to 120 days. We also observed considerable variations on particle size, days of storage and packing density.

Table 2. Range of factors of ensiling of tropical grasses

Item	n*	Mean	SD	Min	Max
Wilting (h)	163	9.38	10.25	1	48.0
Regrowth age (days)	551	61.66	16.10	30	120.0
Particle size (cm)	425	1.96	0.98	1	6.0
Moisture absorbent additive level (% of FM[1])	92	10.50	11.50	0	60
Days of storage	706	54.43	44.32	1	250.0
Packing density (kg of FM/ m^3)	230	562.96	58.15	450	796.0

[1]Fresh matter * Number of observations for each variable in the 102 papers evaluated

The variation observed in the Table 2 reveals the difficult to perform a comparison among papers because the efficacy of additives varies depending on regrowth age for example. In addition, it is known that the variation on regrowth age affects the chemical composition of the grass, as the concentration of WSC and the nutritive value (Santos et al., 2014). For example, if a microbial inoculant is used in guinea grass harvested with 100 days of regrowth its effect will be lower or absent because the WSC concentration in the fresh forage can be enough to provide an adequate fermentation. On the other hand, the same inoculant would have a different effect if the same grass is harvested with 60 days of regrowth. The variation observed

in the concentration of WSC confirm this hypothesis. These facts affect the chemical composition at ensiling, which shows significant variation (Table 3).

Table 3. Range of fermentative characteristics, microbiology and chemical composition of tropical grass at ensiling (total number of papers accessed = 102)

At ensiling					
Item[1]	n	Mean	SD	Min	Max
Yeasts (log cfu/g)	5	6.59	0.91	5.1	7.38
Molds (log cfu/g)	1	4.0	-	4.0	4.0
Enterobacteria (log cfu/g)	18	5.50	0.78	4.26	6.94
Clostridia (log cfu/g)	2	3.10	2.83	1.1	5.11
LAB (log cfu/g)	18	4.71	0.95	1.52	5.72
DM (g/kg)	225	280	110	124	787.9
Ash (g/kg)	71	84.92	26.79	34.9	143
CP (g/kg)	179	89.6	58.13	25.9	743
NDF (g/kg)	169	666.27	112.76	252.3	861.5
ADF (g/kg)	158	396.53	87.03	119	603.3
NIDN (g/kg of TN)	28	346.31	127.41	152	631.23
NIDA (g/kg of TN)	50	346.31	145.74	9.2	593.86
Celulose (g/kg)	55	364.61	58.16	73.5	465.8
Hemicellulose (g/kg)	96	285.37	73.94	122.1	741.4
Lignin (g/kg)	71	60.32	39.59	3.04	186
NFC (g/kg)	27	122.36	96.84	12.8	340.5
WSC (g/kg)	70	50.99	34.54	1.8	156
BC eq.mg HCl/100 g MS	35	59.77	70.70	5	243
IVDMD (%)	38	64.96	9.54	40.45	89.34

* Number of observations for each variable in the 102 papers evaluated; DM- dry matter; CP- crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NDIN- neutral detergent insoluble N; ADIN- acid detergent insoluble N; NFC- Non fibrous carbohydrates; IVDMD- *in vitro* DM digestibility;

Average DM was 280 g/kg, with a range from 124 to 787.9 g/kg. In general, we observed significant variations in the chemical composition, which can be related to the factors described in Table 2, but also with other treatments evaluated (as moisture absorbent additives). Considering the 551 observations, we can state that the average regrowth age is around 60 days (Table 2), which shows the balance between DM yield and nutritive value,

and DM concentration above 200 g/kg. In general, it is related in the averages observed for chemical composition (Table 3).
Based on the collection of published papers, the challenges of ensiling tropical grasses are related to the harvest time and to the fermentation process, because of the characteristics at ensiling. Many efforts have been made to overcome these limitations, but we still observed a low number of papers and a lack of standardization.

In order to visualize the effects of additives and wilting, we grouped the papers considering the averages of the treatments and the percentual of positive responses (Table 4).

Table 4. Positive responses obtained and number of papers that evaluated fermentative characteristics, microbiology and chemical composition of published papers that used absorbent additives

		Absorbent additive	Mean	
Item	nº of papers	% positive responses	With additive	Without additive
Yeasts (log cfu/g)	1	0	2.30	1.81
Molds (log cfu/g)	1	0	2.48	2.03
Enterobacteria (log cfu/g)	0	0	-	-
Clostridia (log cfu/g)	0	0	-	-
LAB (log cfu/g)	1	100	9.26	6.95
LA (g/kg)	12	91.6	47.87	33.24
AA (g/kg)	11	27.2	7.25	7.16
PA (g/kg)	9	22.2	1.03	0.64
BA (g/kg)	10	50	0.90	1.47
pH	24	66.6	4.09	4.37
N-NH3 g/kg of TN	20	75	76.2	103.8
Ethanol (% of DM)	2	0	1.25	0.18
GL (% of DM)	7	71.4	2.13	2.64
EL (kg/ton of MV)	6	83.3	10.06	27.57
DML (g/kg of DM)	0	0	-	-
DMR (% of DM)	5	100	91.94	81.67
DM (g/kg)	36	97.2	290.44	197.98
Ash (g/kg)	6	50.0	81.54	84.23
CP (g/kg)	33	6.0	86.18	61.89
NDF (g/kg)	32	9.3	662.87	727.58
ADF (g/kg)	32	9.3	433.45	455.96
NIDN (g/kg of TN)	8	25.0	342.28	277.51
NIDA (g/kg of TN)	15	33.3	160.91	151.02
Celulose (g/kg)	13	76.9	343.80	378.23
Hemicellulose (g/kg)	19	84.2	233.06	261.26
Lignin (g/kg)	13	15.3	102.51	65.42
NFC (g/kg)	5	100	111.91	83.56
WSC (g/kg)	6	100	77.07	32.03
IVDMD 48h (%)	5	40.0	54.38	56.07

LAB- Lactic acid bacteria; LA- Lactic acid; AA- Acetic acid; PA- Propionic acid; BA- Butiric acid; GL- Gas losses; EL- Efluent losses; DML- Dry matter losses; DMR- Dry matter recovery; DM- dry matter; CP- crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NDIN- neutral detergent insoluble N; ADIN- acid detergent insoluble N; NFC- Non fibrous carbohydrates; WSC- Water soluble carbohydrates; IVDMD- *in vitro* DM digestibility;.

We can observe that important parameters as microbial populations (yeasts, molds, and lactic acid bacteria) and nutritive value (*in vitro* dry matter digestibility (IVDMD)) are evaluated in a low number of papers that evaluated moisture absorbent additives as the main factor.

Silages treated with moisture absorbent additives showed higher concentration of lactic acid (90% of the papers) and lower pH concentration of butyric acid and ammonia nitrogen compared with untreated silages. In addition, the concentration of fiber components (neutral detergent fiber, acid detergent fiber and lignin) reduced with the addition of moisture absorbent additives, because they are usually concentrates or by-products with low fiber content. As the main effect of moisture absorbent additives, effluent losses were reduced in 75% of the four summarized papers. As a consequence of improvements in fermentation, DM recovery was increased in 100% of the papers when these additives were used.

The second most used class of additives used in the tropical grass silages were the microbial inoculants. Among the 102 papers, we grouped only 10 papers that exclusively evaluated microbial inoculants (Table 5). In their other papers the microbial inoculants were combined with moisture absorbent additives, enzymes, harvest time or wilting. As observed before, a few numbers of papers have evaluated the microbial populations. However, the effect of microbial inoculants on the fermentation profile by increasing lactic acid and decreasing pH was consistent in more than 80% of the papers.

The treatment with microbial inoculants reduced the concentrations of ammonia nitrogen and butyric acid in 80 and 85% of the papers, respectively. Considering both DM loss and DM recovery we observed positive results in 100% of five papers, which is an important achievement as a consequence of improvements in the fermentation. Despite the low number of papers with microbial inoculants, the compilation of papers showed positive results. However, these results must be verified under farm conditions with large amounts of silage and greater environmental influence, which is a key challenge.

By performing an overview of the published papers, we observed that the majority of research with tropical grasses silage is performed by Brazil, followed by other tropical countries. In addition, the number of published papers about this topic has decreased over the last five years. The use of additives proved to be a suitable practice to improve silage fermentation and decrease DM losses. However, it has not been tested in farm conditions, neither considering the costs.

Table 5. Positive responses obtained and number of papers that evaluated fermentative characteristics, microbiology and chemical composition of works that used Inoculant microbial additive

Treated with Inoculant microbial additive				
			Mean	
Item	n° of papers	% positive responses	Inoculated	Untreated
Yeasts (log cfu/g)	0	0	-	-
Molds (log cfu/g)	0	0	-	-
Enterobacteria (log cfu/g)	2	100	2.93	3.99
Clostridia (log cfu/g)	0	0	0.44	1.23
LAB (log cfu/g)	2	50.0	7.94	6.72
LA (g/kg)	6	83.3	60.4	47.95
AA (g/kg)	6	83.3	7.39	11.58
PA (g/kg)	4	75.0	1.86	2.29
BA (g/kg)	5	80.0	0.97	1.19
pH	10	100	4.46	4.61
N-NH3 g/kg of TN	7	85.7	42.77	47.18
Ethanol (% of DM)	1	0	47.18	1.03
GL (% of DM)	4	75.0	2.77	4.44
EL (kg/ton of MV)	3	66.6	41.5	41.1
DML (g of DM)	1	100	60.9	127
DMR (% of DM)	4	100	92.64	90.62
DM (g/kg)	10	90.0	238.68	240.01
Ash (g/kg)	3	0	107.62	109.46
CP (g/kg)	10	70.0	86.63	76.38
NDF (g/kg)	9	55.5	725.76	720.51
ADF (g/kg)	9	66.6	447.74	447.61
NIDN (g/kg of TN)	1	0	176.45	185
NIDA (g/kg of TN)	2	0	150.62	137.77
Celulose (g/kg)	2	50.0	137.77	330.16
Hemicellulose (g/kg)	6	83.3	306.35	309.46
Lignin (g/kg)	3	0	90.25	82.78
NFC (g/kg)	0	0	-	-
WSC (g/kg)	3	100	23.28	17.83
IVDMD 48h (%)	6	33.3	62.8	61.03

LAB- Lactic acid bacteria; LA- Lactic acid; AA- Acetic acid; PA- Propionic acid; BA- Butiric acid; GL- Gas losses; EL- Efluent losses; DML- Dry matter losses; DMR- Dry matter recovery; DM- dry matter; CP- crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; NDIN- neutral detergent insoluble N; ADIN- acid detergent insoluble N; NFC- Non fibrous carbohydrates; WSC- Water soluble carbohydrates; IVDMD- *in vitro* DM digestibility;

By grouping published papers, we could discuss the main challenges of tropical grasses silages and bring an overview of this topic. Despite the large variation observed and the low number of studies, the use of moisture absorbent additives and microbial inoculants can improve the fermentation of tropical grasses silages in most of the cases. However, considering the influence of many factors, these technologies may not be efficient at a farm level. Based on that, we observe quite good experiences and unsuccessful ones. Based on that, the adequate harvest time remains as a challenge for researchers, consultants and farmers. In addition, the use of tropical grasses silages in the diets for ruminants has not been studied as it should be, which is also an important topic to discuss.

Impacts of tropical grasses silages utilization on animal performance

The number of studies evaluating animal performance with animals fed tropical grasses silages is considerably low. Despite the advances in the ensiling technologies, the effects of additives and management practices on animal performance has not been studied extensively. The studies performed evaluating animal performance have testes the effects of microbial inoculants (Restle et al., 2003; Paziani et al.,2006; Cezario et al., 2015), moisture absorbent additives, as citric pulp, coffee hulls. cocoa meal, cassava meal and dehydrated passion-fruit peel (Carvalho Junior et al., 2009; da Cruz et al. 2011), and other additives as urea and cassava bagasse (Carvalho et al., 2006); Silva et al., 2006).

The three studies evaluating microbial inoculants have not observed effects on intake and productive performance of beef cattle. The species and the forage:concentrate ratio used in these studies were *Urochloa plantaginea*, *Megathyrsus maximus*, *Urochloa brizantha* cv Marandu and 65:35, 55:45 e 50:50 for Restle et al. (2003), Paziani et al. (2006) and Cezario et al. (2015), respectively. These results show that the use of additives has high impact on the fermentation process than the intake

and performance. It means that they will not always improve weight gain even though they have improved fermentation process. However, if the DM losses are reduced by improving fermentation, the microbial additive has reached its goal, which will reduce the feeding cost. When moisture absorbent additives were evaluated, Carvalho Junior et al. (2009) and Cruz et al. (2011) observed that some additives increased nutrient intake and productive performance, as cassava meal (15%, fresh basis) and dehydrated passion-fruit peel (up to 30%, fresh basis). The addition of cassava bagasse levels during ensiling of *Pennisetum purpureum* did not affect animal performance in diets containing 40% of concentrate. The impact of using moisture absorbent additives depends on the composition of the concentrate, plant, by-product and others that is used, which can be checked in small scale trials. Some moisture absorbent additives, as coffee hulls and some fruit by products may not improve animal performance because of the high concentration of lignin. All effects of additives, as discussed before, will depend on many effects, specially the forage:concentrate ratio and harvest time.

The harvest time was studied by Daniel et al. (2016) and Cezario et al. (2015). Cezario et al. (2015) observed that harvesting the *U. brizantha* with 35 or 70 days of regrowth did not affect intake of total digestibly nutrients, nutrient digestibility and productive performance. Daniel et al. (2016) stated that feeding lactating cows with bermudagrass ensiled after 4 weeks of regrowth improved energy intake and milk production compared with the cows fed bermudagrass ensiled after 7 weeks of regrowth. Missio et al. (2019) showed that the dietary inclusion of 100 and 400 g/kg of Mulato II grass (Urochloa sp.) silage resulted in similar productive performance of young Nellore bulls and dairy crossbreeds. The decision of harvesting tropical grasses for silage production should be based on the proportion of the diet that grass silage will be used and on the fermentation characteristics of the grass specie, and other factors that will be discussed.

Perspectives about production and utilization of tropical grasses silages

After an overview about tropical grasses silages we will discuss some perspectives as provocations for researchers and consultants. Despite the lower nutritive value compared with corn, ensiled tropical grasses can be used to reduce feeding costs, to help pasture management, and as a forage source in feedlots or dairy farms. As discussed before, tropical grasses have a great potential to be used in diets for ruminants, but to be fairly compared with other crops, we suggest quantifying the digestibly energy produced by hectare. However, we still need to define what should base the decision of harvesting the grass. For example, when the dietary inclusion of tropical grasses silages is low (eg. feedlots), the harvest can be directed to obtain more DM per hectare because the silage is going to act as effective fiber in diets with high proportion of concentrate. On the other side, lactating cows need the maximum of nutritive value from the grass silage because they are an important energy source in the diet.

This is a wide discussion and we would say that there is not just the only specific time of harvest. In this context, some criteria of harvesting tropical grasses for silage production can be cited: the growth phase, days of regrowth, light interception and sward height. It will depend on many factors, but the purpose that the grass silage will be used. In addition, when considering harvest for silage production, the balance between DM yield and nutritive value would vary depending on the final purpose of the system that the silage will be used.

The researches about pasture management in rotational grazing systems has considered the maximum yield of leaves in order to provide an efficient harvest by the animal and to improve productive performance (Burns and Sollenberg, 2002; Mezzalira et al. 2014). The main recommendation to reach this harvest point is when the sward intercepts 95% of the incident light, which is correlated with sward height. This is considered the threshold point where the plants have more proportion

of leaves than stem and dead material, which is related with the nutritive value (da Silva et al., 2015). Based on that, recommendations of grazing target height for maximum yield without compromising nutritive value were generated and they also match with animal behavior and productive performance. This recommendation could be used for harvesting tropical grasses for silage production.

However, the amount of DM harvested by hectare is important for silage production because it affects the costs per stored DM (Busano et al., 2019). Tropical grasses are quite efficient on producing biomass in short periods, especially when submitted to adequate management practices (da Silva et al., 2015). If we consider the harvest by machines for silage production, the concepts of grazing efficiency would not be directly applied.

By using machine harvesters, in order to improve efficiency, the grasses can be harvested at higher heights than those recommended for grazing, in order to increase harvested DM and decrease production costs. The research conducted by Thomas et al. (2018) with *M. maximus* cv. Mombaca drives new directions for harvesting tropical grasses for silage production. They recommended harvesting the grass with 130 cm height and 20 cm from the soil to achieve the maximum fermentability coefficient and to maximize DM yield. In this case, they remove 85% of the sward, while the recommendations for grazing are between 40 to 60% of removal and considering a lower height (90 cm). Thus, we can state that we need to generate new target heights for the purpose of silage production. These new goals should consider the characteristics for ensiling process (buffering capacity, DM content and concentration of WSC) and they should be directed to the maximum harvest of total digestible nutrients or digestible DM per hectare.

Based on that, the adequate harvest time for each cultivar of grass should be determined and the farmers and consultants should base this decision on the percentual of dietary inclusion of tropical grass silage and on the animals that will be fed with this silage.

Final remarks

Efficiency of the production and utilization of tropical grasses is essential for livestock production in tropical regions, and silage production is a key strategy for efficient production systems. Ensiling tropical grasses as a suitable tool of pasture management and for animal supplementation is extremely important for the sustainability of livestock systems and to reduce production costs.

Most of the research were conducted in Brazil and they focused more on the fermentation profile. The use of moisture absorbent and or microbial inoculants additives provides significative beneficial effects for tropical grass silage fermentative process. Chemical additives have not been significantly evaluated in tropical grasses. The height target for ensiling tropical grasses can be higher than the ones used for grazing. However, these recommendations for all cultivars of grasses do not exist yet.

As tropical grasses are a heterogeneous group both regarding to species/cultivars variabilities and regarding to phenological stage and management, studies combining and not combining these factors with additives are needed. Studies at farm level, evaluating new cultivars and additives, measuring digestibility and animals' performance are relevant gaps to be fulfilled.

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SOCIAL PROGRAM

Thursday – November 07th, 2019

- 19:00-22:00 – Conference Dinner – Sal e Grill Churrascaria

Friday – November 08th, 2019

- 08:30-09:10 – Fiber quality and forage allocation throughout lactation (Michael Allen – Michigan State University, USA)
- 09:10-09:50 – Ensiling total mixed ration for ruminants (João Daniel – State University of Maringa, Brazil)
- 09:50-10:20 – Coffee Break
- 10:20-10:35 – Volunteered paper
- 10:35-10:50 – Volunteered paper
- 10:50-11:30 – Physically effective fiber recommendations for high producing dairy cows (Karen Beauchemin – Agriculture and Agri-Food, Canada)
- 11:30-11:40 – Sponsortime
- 11:40-12:30 – Poster exhibition
- 12:30-14:00 – Lunch
- 14:00-14:40 – Baled silages management (Giorgio Borreani – University of Torino, Italy)
- 14:40-14:50 – Coffee Break
- 14:50-15:25 – Volunteered paper
- 15:25-15:40 – Volunteered paper
- 15:40-16:20 – Challenges and perspectives of tropical grasses silages (Thiago Silva – Federal Rural University of Amazônia, Brazil)
- 16:20-16:40 – Summary of The VI ISFQC 2019

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