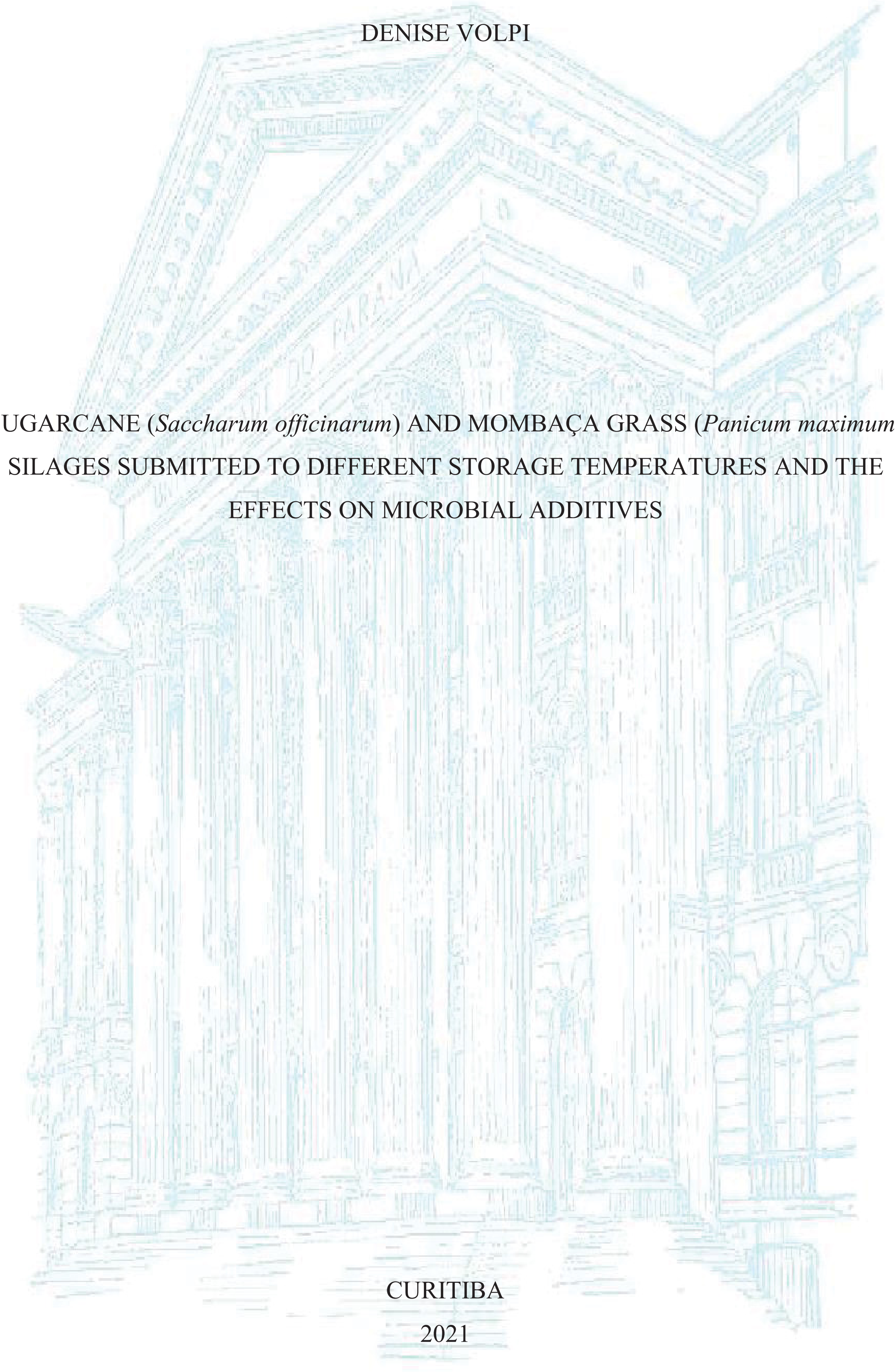
FEDERAL UNIVERSITY OF PARANA

DENISE VOLPI



SUGARCANE (*Saccharum officinarum*) AND MOMBAÇA GRASS (*Panicum maximum*) SILAGES SUBMITTED TO DIFFERENT STORAGE TEMPERATURES AND THE EFFECTS ON MICROBIAL ADDITIVES

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Tese apresentada como requisito parcial para a obtenção do título de Doutor(a) em Zootecnia, do Programa de Pós-Graduação em Zootecnia, Setor de Ciências Agrárias, da Universidade Federal do Paraná.

Orientadora: Dra. Maity Zopollatto

CURITIBA
2021

Volpi, Denise
Sugarcane (Saccharum officinarum) and mombaça grass (Panicum maximum) silages submitted to different storage temperatures and the effects on microbial additives. / Denise Volpi. - Curitiba, 2021.

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Zootecnia.
Orientação: Maity Zopollatto.

1. Cana-de-açúcar. 2. Capim-guiné. 3. Silagem - Aditivos. I. Zopollatto, Maity. II. Título. III. Universidade Federal do Paraná.

Sistema de Bibliotecas/UFPR
Guilherme Luiz Cintra Neves - CRB9/1572

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIÊNCIAS AGRÁRIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ZOOTECNIA -
40001016082P0

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOTECNIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **DENISE VOLPI** intitulada: **Sugarcane (Saccharum officinarum) and mombaça grass (Panicum maximum) silages submitted to different storage temperatures and the effects on microbial additives** , sob orientação da Profa. Dra. MAITY ZOPOLLATTO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 12 de Março de 2021.

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ACKNOWLEDGMENT

A Deus, por absolutamente TUDO em minha vida! Por me mostrar que as coisas no tempo Dele são melhores e maiores do que no meu querer.

Aos meus pais, por nunca sequer terem pensado em medir esforços para que esse momento chegasse. Embora um “calma, tenha paciência” não fosse exatamente o que eu quisesse ouvir em alguns momentos, tentar ter calma e paciência me trouxeram até aqui e eu sou muito grata por isso. Esse documento/título é mais de vocês do que meu.

À profa Maity, novamente agradeço por ter aceitado me orientar desde o mestrado. Lembro-me que quando entrei em 2015 eu tinha 2 resumos, mas de alguma forma a sra viu além do currículo e também ensinou além. Tenho sorte por ter me mostrado que inteligência emocional é tão importante quanto a inteligência intelectual em todos os momentos da vida, inclusive esse.

Ao Centro de Pesquisa em Forragicultura, incluindo todos os “CPMembers”. Nunca imaginei defender uma tese sobre silagem, nunca, e veja só como as coisas são. O tanto que eu aprendi no CPFOR não tem proporção foi como um intensivão de 4 anos sobre forragicultura onde em muitos casos o conhecimento não era passado por osmose hahah Obrigada!

Ao meu irmão e amigos (novos e de longa data), o incentivo e a colaboração de vocês (não só braçal, aliás, isso é o de menos) também foram fundamentais para a conclusão dessa fase.

À Universidade Federal do Paraná, especialmente ao Programa de Pós-Graduação em Zootecnia pela oportunidade de estudo e suporte para a realização desse sonho.

À Lallemand, pelo auxílio financeiro para que esse estudo se concretizasse e por colaborar/incentivar a pesquisa nacional.

Ao meu pai Nelson e à minha mãe Elizabeti
Pelo amor verdadeiro e apoio incondicional,
Dedico.

“Eu não sou o que me aconteceu,
Eu sou o que escolho me tornar”
Carl Juna

RESUMO

É sabido que a temperatura de armazenamento pode influenciar o processo de fermentação. No entanto, como isso ocorre e quais variáveis são afetadas nas silagens de gramíneas tropicais é pouco conhecido. Objetivou-se avaliar o efeito de duas temperaturas de armazenamento sobre o desempenho de aditivos bacteriano-enzimáticos, perfil fermentativo e perdas durante a conservação de silagens de cana-de-açúcar (*S. officinarum*) e capim Mombaça (*P. maximum*). Três tratamentos foram avaliados no ensaio com cana-de-açúcar: controle (sem aditivo); Hetero (*P. acidipropionici* (1 x 10^5 ufc/g de forragem fresca (FF)) e enzima celulase); e Homo (*L. plantarum, P. acidilactici, P. pentosaceus* (1 x 10^5 ufc/g de FF), enzimas e benzoato de sódio). Por sua vez, cinco tratamentos foram avaliados no ensaio com capim Mombaça: controle (sem aditivo); hoBACT (*L. plantarum, L. salivarius, P. acidilactici* e *E. faecium* a 2,1 x 10^5 ufc/g de FF); BACT (*L. plantarum, P. acidilactici, P. pentosaceus* e *P. acidipropionici* a 5 x 10^3 ufc/g de FF); BACT2 (*L. plantarum, P. acidilactici, P. pentosaceus* e *P. acidipropionici* a 1 x 10^6 ufc/g de FF); e BACT4 (*L. plantarum, P. acidilactici, P. pentosaceus* e *P. acidipropionici* a 2 x 10^5 ufc/g de FF). Todos os aditivos continham enzimas celulase, amilase, xilanase and glucanase (exceto hoBACT que possuía hemicelulase ao invés de glucanase). A concentração das enzimas foi crescente nos aditivos BACT, BACT2 e BACT4, nessa ordem. Os silos foram mantidos em salas com temperatura controlada a 26°C e a 18°C. O delineamento experimental foi inteiramente casualizado em esquema fatorial (tratamentos (3 ou 5) x 2 temperaturas). As silagens de cana-de-açúcar com o aditivo Hetero foram semelhantes às silagens controle para a maioria das variáveis estudadas. A 26ºC essas silagens praticamente dobraram ($p<.0001$) as produções de efluente (+20,5 kg/t FF) e gases (+16,2 mL/kg MS) devido à fermentação alcoólica e uma possível melhora na atividade enzimática dos aditivos, enquanto que sob 18ºC nenhuma levedura se desenvolveu ($p=.0002$). Em relação às silagens de capim Mombaça, todas aquelas armazenadas a 26ºC apresentaram maior ($p<.0001$) pH (+0,6) e concentração de ácidos oriundos de fermentação heterolática, além dessa temperatura aparentemente prejudicar o crescimento de BAL homofermentativas por reduzir ($p<.0001$) em 2,5 e 1,6% a contagem nas silagens hoBACT e BACT4, respectivamente. Apesar disso, a produção de gases foi 103% superior ($p=.009$) nas silagens mantidas a 18ºC. Silagens de cana-de-açúcar no início da fase de maturação possuem perfil fermentativo heterolático e sem o crescimento de leveduras quando armazenadas a 18ºC. Por sua vez, silagens de capim Mombaça mantidas nessa temperatura possuem melhor fermentação e alto crescimento de BAL homoláticas dos aditivos. Em ambas silagens, resultados opostos são observados a 26ºC, especialmente em relação à alta concentração de ácidos propiônico e butírico nas silagens de capim com *P. acidipropionici* inoculado, o qual pode ser prejudicial à boa fermentação.

Palavras-chave: Aditivo. Bactéria ácido lática. Clima tropical. Fermentação. Inoculação. Microflora epifítica.

ABSTRACT

It is known that temperature can influence the fermentation process. However, how this occurs and which variables are affected in tropical grass silages is few investigated. The objective of this study was to evaluate the effect of two storage temperatures on the performance of bacterial-enzymatic additives, fermentation profile and losses during the conservation of sugarcane (*S. officinarum*) and Mombaça grass (*P. maximum*) silages. Three treatments were evaluated in the sugarcane trial: Control (without additive); Hetero (*P. acidipropionici* (1 x 10^5 cfu/g of fresh forage (FF)) and cellulase enzyme); and Homo (*L. plantarum, P. acidilactici, P. pentosaceus* (1 x 10^5 cfu/g of FF), enzymes and sodium benzoate). In turn, five treatments were evaluated in the trial with Mombaça grass: Control (without additive); hoBACT (*L. plantarum, L. salivarius, P. acidilactici* and *E. faecium* at 2.1 x 10^5 cfu/g of FF); BACT (*L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* at 5 x 10^3 cfu/g of FF); BACT2 (*L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* at 1 x 10^6 cfu/g of FF); and BACT4 (*L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* at 2 x 10^5 cfu/g of FF). All additives contained cellulase, amylase, xylanase, and glucanase enzymes (except hoBACT that had hemicellulase instead of glucanase). The enzymes concentration was increasing in the BACT, BACT2 and BACT4 additives, in that order. The silos were kept in rooms with controlled temperatures at 26°C and 18°C. The experimental design was completely randomized in a factorial arrangement (treatments (3 or 5) x 2 temperatures). The sugarcane silages with the Hetero additive were similar to Control silages for most of the studied variables. At 26ºC these silages practically doubled ($p<.0001$) the effluent (+20.5 kg/t FF) and gas (+16.2 mL/kg DM) production due to alcoholic fermentation and possible improvement in the enzymatic activity of the additives, while at 18ºC there was no yeast growth ($p=.0002$). In relation to Mombaça grass silages, all those stored at 26ºC showed high ($p<.0001$) pH (+0.6) and concentration of acids from heterolactic fermentation, in addition to this temperature apparently impairing the LAB homofermentative growth due to reducing ($p<.0001$) in 2.5 and 1.6% the count in the hoBACT and BACT4 silages, respectively. Despite this, the gas production was 103% higher ($p=.009$) in silages maintained at 18ºC. Sugarcane silages at the beginning of maturation phase have heterolactic fermentation profile, with no yeast growth when stored at 18ºC. In turn, Mombaça grass silages maintained in this temperature have better fermentation and high LAB homolactic growth from additives. In both silages, opposite results are observed at 26ºC, especially in relation to high propionic and butyric acids concentrations in the grass silages with *P. acidipropionici* inoculated, which can be detrimental to good fermentation.

Keywords: Additive. Epiphytic microflora. Fermentation. Inoculation. Lactic acid bacteria. Tropical climate.

FIGURES

TABLES

ABBREVIATIONS

ADF	Acid detergent fiber
CFU	Colony-forming unit
CHO	Carbohydrate
CO_2	Carbon dioxide
CP	Crude protein
DM	Dry matter
DML_{AE}	Total dry matter losses during air exposure
DML	Total dry matter losses during fermentation
FF	Fresh forage
GP	Gas production
LA	Lactic acid
LAB	Lactic acid bacteria
NaCl	Sodium chloride
NDF	Neutral detergent fiber
$N\text{-}NH_3$	Ammoniacal nitrogen
O_2	Oxygen gas
RNA	Ribonucleic acid
SEM	Standard error of the mean
sp.	Specie
spp.	Several species
TMR	Total mixed ration
VOC	Volatile organic compounds
WSC	Water-soluble carbohydrates

SUMMARY

1 GENERAL INTRODUCTION

The influence of storage temperature on the silage's quality is incipient in the national literature with discreet advances about how and which way this factor impacts the silages production. Despite this, the international literature affirms through several methodologies that this factor is important and influences food quality.

Adesogan and Kim (2005), in a trial with different temperature ranges; Cao et al. (2011), evaluating the influence of different regions and seasons; Liu et al. (2011), using controlled storage temperature; Wang and Nishino (2013), relating temperature and storage period... All these studies and others discussed below, in general, agree that temperature interferes on the reactions occurred inside the silo, being in a greater or lesser extent, in a few or many variables.

However, most of these studies were not done in Brazil or in tropical conditions and, therefore, use typical crops or total mixed ration silage with specific ingredients from the study place, which do not represent or make it difficult to infer for tropical climates. Seeing the vast Brazilian territory, composed of different climates and the increasing silage use in the ruminants nutrition, this subject cannot remain superficially discussed because it can positively impact three areas: scientific (avoiding outcome bias, making the experimentation more homogeneous, and expanding inferences), commercial (sale of products developed especially for a specific condition and, consequently, consistent results), and on the farm (greater economic return and nutritional quality of silages).

The inoculants use is a controversial topic (Weissbach, 2011) due to divergent results when evaluating, for example, doses of the same strain in different crops. As a consequence, in order to increase the results precision, it is tried to strictly control the factors that may influence data collection and generate inconsistent information. However, many times the environmental temperature is few considered as an interference source and maybe therefore the conclusions of some studies are precision, but few accurate.

Soon, recommendation of additives for each region will be a reality as is the recommendation by crop. For that, microbial inoculation must overcome the forage, storage, and climate challenges. This will only be possible uniting the knowledge of the basic science with the understanding of the complex interactions.

The objective of this study was to evaluate the influence of two ambient temperatures during the storage period of sugarcane (*Saccharum officarum*) and Mombaça grass (*Panicum* *maximum* cv. Mombaça) silages, as well as their influence on the performance of bacterial-enzymatic additives.

2 LITERATURE REVIEW

2.1 MOMBAÇA GRASS AND EPYPHYTIC MICROFLORA

Panicum maximum was introduced in Brazil in 1980 from Africa. This is a perennial grass, of C4 metabolism, and with great potential for production in semi-arid regions (Ramos et al., 2016). Mombaça grass as well as other cultivars of this specie has higher growth in the hot and rainy season, which makes it an option mainly for direct-cut or silage with the objective of better-taking advantage of the high yield period (20-30 t DM/ha) (Daniel et al., 2019).

However, in tropical grasses the effluent production needs attention because the forage will hardly reach the ideal dry matter (DM) content for ensilage (>30%) so that these losses are reduced. It is known that wilting technique increases DM content of the grass and reduces fermentative losses (Loures et al., 2005; Carvalho et al., 2007; Nussio and Ribeiro, 2008). Furthermore, it is possible to increase the quality of the grass silages not only by wilting, but also assisting for an adequate fermentation process through the use of inoculants or additives.

The main water-soluble carbohydrates present in forage crops are fructose, glucose and sucrose (Woolford, 1984), in which sucrose and fructose are rapidly hydrolyzed in their monomers after cutting the forage. These carbon sources are readily used by epiphytic microflora. The common bacteria specie found in grass are *L. plantarum, L. casei, P. acidilactici,* and *E. faecium*. Some heterofermentative lactic acid bacteria (LAB) can also be found (Santos et al., 2013) such as *Lc. pseudomesenteroides* and *L. brevis*.

These LAB are important during fermentation but usually are present in the grass in 1000 times less than its main competitors, fungi and enterobacteria (Dellaglio, 1985). In 1989, Pahlow isolated approximately 10^4 to 10^5 cfu/g of LAB in the grass epiphytic microflora. In 2003, Pahlow et al. found another value in perennial grass, 10^1 to 10^6 cfu/g. Regardless of the quantity is essential to promote an adequate environment so that these bacteria, even in smaller numbers, can establish and dominate fermentation.

In general, after fermentation beginning, *L. plantarum* and species of the genus *Pediococcus* prevail in untreated silages. However, the reported results are divergent (Silva et al., 2011; Santos et al., 2013). According to Beck (1972), in well-preserved silages, acidification was initiated by homofermentative strains, mainly of the *L. plantarum* and *L. curvatus*, and after four days 85% were heterofermentative, for example, *L. buchneri* and *L. brevis*, which dominated until the end. For Dellaglio and Torriani (1986), fermentation was also initiated by homofermentative LAB, but it dominated until 60 days of ensiling, when there were a gradual decline and concomitant increase of the heterofermentative sp.

Most studies (Ávila et al., 2010, 2009; Zhang et al., 2015) that isolated and inoculated autochthonous species obtained results as good as selected commercial strains, which leads us to believe that other factors beyond the simple microbial metabolism can influence for a good fermentation. The specificity between plant and its epiphytic microflora must be considered when deciding to use additives and which is the most appropriate (Silva et al., 2011), as well as the whole environment (microorganism-plant-climate) together.

2.2 SUGARCANE AND EPIPHYTIC MICROFLORA

Sugarcane is cultivated predominantly in tropical and subtropical countries of Asia, Latin America, and Africa. World production is projected to grow 1.1% p.a., which Brazil, India, and Thailand will contribute to 74% of global volume. For Brazil, this forage is especially important because it gives the country flexibility to switch between the use for sugar or ethanol production according to relative profitability. In addition to the food industry, sugarcane can also be used in the energy sector, in the pharmaceutical industry, in the transformation of materials, among other applications (OECD/FAO, 2020).

As silage, sugarcane is a crop that needs intervention for adequate fermentation. Some plant characteristics that favor its use are high production per area, varieties adapted to different environments, and low cost per ton of DM (Santos et al., 2008). However, due to the large amount of sugars, the yeasts present in the medium convert them into ethanol, which can cause losses up to 49% of DM (McDonald et al., 1991). Therefore, the use of additives in sugarcane ensilage is considered an obligatory practice (Schmidt et al., 2014).

Among the LAB species of sugarcane epiphytic microflora, Leuconostocs are the main responsible for losses in the sugar and alcohol industry due to formation of dextrans that, among other problems, result in precipitates in cachaça. For this reason, it was one of the first bacterial groups studied due to cause commercial losses (Van Tieghem, 1878). During harvesting, sugarcane is contaminated with *L. mesenteroides* subsp *mesenteroides* present in the soil and that grow within the cut stalks, acidifying the sugarcane juice (Tilburry, 1975). The high sugar content (about 15%) and the initial pH of 5.0-5.5 make sugarcane juice a suitable substrate for LAB. In hot and humid climates, the growth of *L. mesenteroides* subsp *mesenteroides* in harvested sugarcane may result in losses of 1-5% of total sugar/day between harvesting and processing (Tilbury, 1975). Moreover, large amount of dextran may be synthesized from sucrose by this sp, causing problems during refinement (Pivnick, 1980).

Other spores of sugar-tolerant lactobacilli (capable of multiplying by 15% sucrose) and acidophilic, mainly consist of *L. confusus* (Sharpe et al., 1972) and occasionally *L. plantarum* and *L. casei.* Most strains multiply in the sugarcane juice causing acidification and deterioration, in addition to also producing large amounts of dextran from sucrose (Tilbury, 1975). Contamination by these bacteria comes from the sugarcane itself and from contaminated equipment.

In relation to fungi, in temperate regions the sugarcane can have *Fusarium poae*, and in subtropical and tropical regions can be found *Fusarium verticilloides* (Chang et al., 2014). A survey carried out in the early 1970s on Haitian distilleries showed that *Schizosaccharomyces* yeast strains were the main alcoholic fermentation agents (Fahrasmane et al., 1988). Of 60 strains collected, there were 44 *Schizosaccharomyces pombe*, 4 *Shizosaccharomyces malidevorans* and 1 *Shizosaccharomyces japonicus*. In some conditions, strains from this genus could have higher ethanol productivity than *Saccharomyces cerevisiae* (Haraldson and Björling, 1981).

In Brazil, sugarcane has an epiphytic microflora rich in yeasts that can reach 1 x 10^6 cfu/g FF (Ávila et al., 2010). The main microorganisms isolated in this condition are shown in Table 1. Particularly, the genus *Sporobolomyces* has been relatively rare in studies of yeasts in tropical habitats as well as the identification of *S. cerevisiae* in leaves, stems and rhizosphere (Azeredo et al., 1998). About to bacteria, all species found by Rodrigues et al. (2016) in Goiás belonged to Enterobacteriaceae family, which differ from findings by Pereira et al. (2019) in São Paulo, where the genus *Bacillus*, *Pseudomonas, Arthrobacter, Streptomyces*, *Microbacterium* and *Falsibacillus* were identified in all samples. In addition to temperature, drought stress and its duration can also change the proportion of microorganism genera (Pereira et al., 2019).

Table 1. Main microorganisms isolated from sugarcane in Brazil and their habitats (%).

	Leaf	Stem	Rhizosphere	Root
Fungi[1]				
Cryptococcus spp.	51.2	25.6	23.2	-
Rhodothorula spp.	40	47.5	12.5	-
Trichosporon spp.	81.8	9.1	9.1	-
Bacteria[2]				
Klebsiella spp.	66.7	-	-	33.3
Enterobacter spp.	-	-	66.7	33.3
Pantoea spp.	-	-	100	-

Adapted from [1]Azeredo et al. (1998); [2]Rodrigues et al. (2016).

2.3 SOME SPECIES OF BACTERIA USED IN ADDITIVES

According to Morlon-Guyot et al. (1998), the microorganisms must meet the following criteria to be used as inoculants in silages:

1. Have vigorously growth, able to compete and dominate other organisms;
2. Be homofermentative (produces L-lactate as the main product);
3. Be acid tolerant and capable of reducing pH to at led 4.0 as quickly as possible;
4. Be able to ferment glucose, fructose, sucrose, fructosans and pentoses;
5. Not produce dextran from sucrose or mannitol from fructose. If mannitol appears, it should be able to ferment it rapidly;
6. Not act on organic acids;
7. Have a growth temperature range extending to 50ºC;
8. Be able to grow at high dry mass contents.

However, today some of these factors are no longer essential (such as the obligation to be homofermentative LAB) because not all starter cultures are suitable for all kinds of forages or will act similarly in all environmental conditions.

Weinberg and Muck (1996) already had a more pioneering vision, indicating other factors that should be considered in the manufacture of inoculants, e.g., LAB specific to some crops, the inclusion of heterofermentative bacteria to inhibit molds and yeasts, selection and modification of LAB strains, and cloning and gene expression of LAB capable of using polysaccharides.

In general, the use of heterofermentative LAB strains or their combination with homofermentative LAB is preferable for sugar-rich silages (Driehuis et al., 2001; Holzer et al., 2003). In turn, the use of homofermentative LAB in crop silages with a low content of water-soluble carbohydrates (WSC) is preferable in order to quickly and efficiently use the substrate to rapidly decrease the pH (Weinberg and Muck, 1996; Holzer et al., 2003).

Table 2 shows the bacterial species applied in the main crops used for silage production in Brazil. Due to having a countless number of genera, species and strains used as inoculants for silages, only the microorganisms used in the additives of this research, their history, application in the industry and mode of action on silages will be described in detail below.

Table 2. Frequency (%) of inoculants use in Brazil according to crop, bacterial species, and their preferential route in silages.

Item[1]	Alfalfa	Corn	Grass	Sugarcane	Route[2]
One specie					
Bacillus subtilis	-	3.4	-	-	HE
Lactobacillus brevis	-	-	-	15.0	HE
Lactobacillus buchneri	-	13.6	6.7	34.4	HE
Lactobacillus hilgardii	-	-	-	5.6	HE
Lactobacillus kefiri	-	-	-	0.6	HE
Lactobacillus paracasei	-	-	-	1.1	HE
*Lactobacillus plantarum**	-	6.8	15.1	32.7	HO
Leuconostoc mesenteroides	-	0.8	-	-	HE
Streptococcus bovis	-	-	11.8	-	HO
Streptococcus faecium	-	-	2.5	-	HO
Two species					
L. buchneri + *L. kefiri*	-	-	-	0.5	HE + HE
L. buchneri + *P. acidipropionici*	-	-	-	-	HE + HE
L. casei + *S. faecalis*	-	-	-	-	HO + HO
L. plantarum + *B. subtilis*	-	0.8	-	-	HO + HE
L plantarum + *L. buchneri*	-	3.4	-	0.5	HO + HE
L. plantarum + *P. acidilactici*	-	5.1	14.3	0.5	HO + HO
L. plantarum + *P. pentosaceus**	3.3	13.6	2.5	3.8	HO + HO
L. plantarum + *P. acidipropionici*	-	1.7	1.7	2.1	HO + HE
L. plantarum + *S. faecium*	40.0	11.8	10.1	1.6	HO + HO
Combo[1]	26.7	39.0	35.3	1.6	

Adapted from Rabelo et al. (2016).

[1]Combination of three or more bacteria.

[2]HE: heterofermentative; HO: homofermentative.

*Facultative heterofermenters.

2.3.1 Genus *Enterococcus*

2.3.1.1 *Enterococcus faecium*

Enterococcus were classified as Streptococci group D until 1970, when they became a distinct genus. Although they are phenotypically similar to other related genera, the presence of Gram-positive cocci in pairs or chains and the growth in a medium with NaCl are some discriminatory characteristics for most species of the genus Enterococcus. In 1985, the distinction was confirmed by 16S rRNA sequence analysis (Ludwig et al., 1985).

The Streptococci and the Lactococci to which the Enterococci have been linked in the past are more distantly related, as are the Lactobacilli. Within the genus there are four groups with the respective specie described below, which are related as shown in Figure 1:

1. *E. faecium* group with *E. faecium* (Schleifer and Kilpper-Balz, 1984), *E. durans* (Collins et al., 1984), *E. hirae*, and *E. mundtii* (Collins et al., 1986) sp;
2. *E. avium* group with *E. avium*, *E. malodoratus* (Collins et al., 1984), *E. raffinosus*, and *E. pseudoavium* (Collins et al., 1989) sp;
3. *E. gallinarum* group (Collins et al., 1984) with *E. gallinarum* and *E. casseliflavus* sp;
4. *E. cecorum* group with *E. cercorum* (Williams et al., 1989) and *E. columbae* (Devriese et al., 1990) sp.

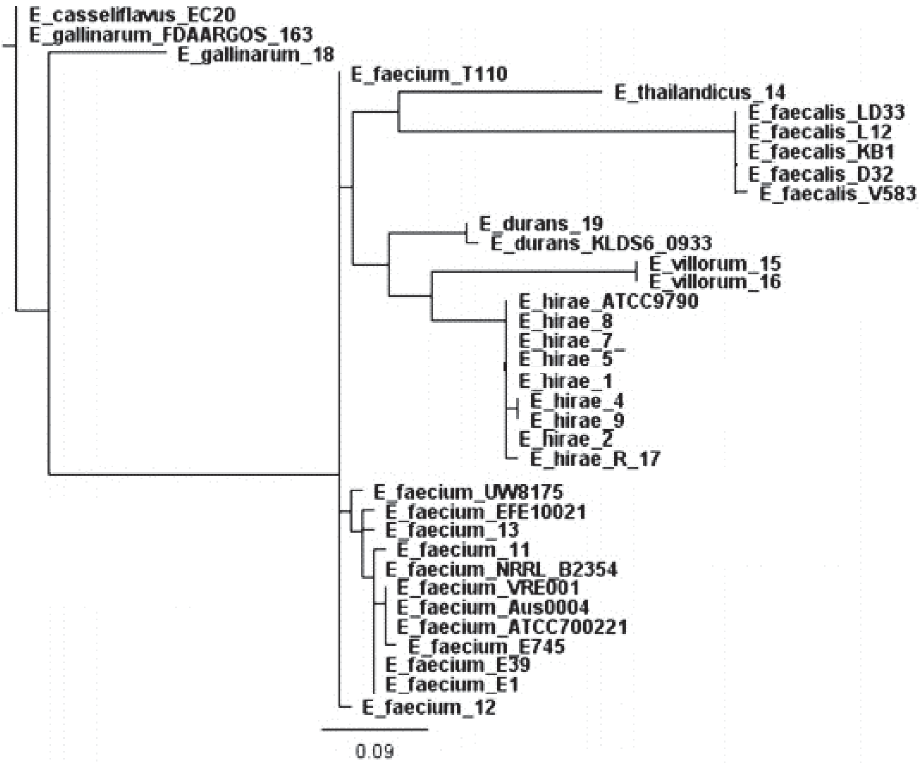

Figure 1. Dendrogram of the Enterococci species based on analysis of single-nucleotide polymorphisms (SNPs).
Adapted from Beukers et al. (2017).

The species of this genus can be found in the most varied habitats, from fermented sausages, dairy products and plants to blood, bile and faeces (Zhong et al., 2017). However, it is in medical bacteriology where it stands out due to its growing role in hospitals-acquired infections, acting as opportunistic pathogens in different extra-intestinal compartments of the body, and due to its natural and acquired resistance to antibiotics (Devriese et al., 2006).

In the industry, specifically *E. faecium* does not have an expressive application like the other LAB. However, this bacteria can be used for the fermentation of starch-containing feedstocks (Nolasco-Hipolito et al., 2012); in the mature Italian type I sourdough, where it is used during the first steps of preparation as a starter culture to promote stronger and rapid acidification, more efficient, e.g., than *L. sanfrancisensis* strains (Settani, 2017); and also in cheeses, which when made with *E. faecium* strains had better consistency and more pronounced aroma that generally fulfilled criteria demanded in Tolminc cheese specification (Majhenic et al., 2015).

In animal production, *E. faecium* is widely used as a probiotic for poultry and piglets. In forage conservation, this species did not improve quality of alfalfa and guinea grass silages when inoculated at 1 x 10^5 cfu/g FF (Cai, 1999), nor associated with *L. plantarum* at 1.5 x 10^5 cfu/g FF in corn silages (Bolsen et al., 1992). On the other hand, Filya et al. (2007), using alfalfa silages at a rate of 1 x 10^6 cfu/g FF obtained lower NDF, acid detergent lignin and hemicellulose concentrations, but high pH (average 4.9).

The variability of the results can be justified based on the meta-analysis made by Oliveira et al. (2017). These authors observed that only 5.7% of the studies used *P. pentosaceus*, *E. faecium* or *L. rhamnosus* as an individual inoculant and that this can make it difficult to attribute a result to a certain specie when in association with others. *E. faecium* also rapidly grows at high pH (Table 3) and therefore dominates fermentation in the early stages. With the gradual reduction in pH by other LAB, the growth of this specie is inhibited (Kung et al., 2011) and this may be another reason for the subtle results: the short period of time which it acts.

In relation to ruminant performance, animals fed with *E. faecium* (isolated or not) can increase milk production (Gomez Basauri et al., 2001), DM intake (Nocek and Kautz, 2006), the percentage of milk fat on first lactation cows (Oetzel et al., 2007), and the average daily gain of feedlot steers (McAllister et al., 1998).

Table 3. Characteristics of interest of *Enterococcus faecium* in the silage production.

Stimulus	Response[1]
Optimal temperature	35ºC
Optimal pH	6-7
Optimal carbon source	Dextrose
O_2 tolerance	Low
Metabolism in silages	Homofermentative
Metabolic balance	Glucose (or fructose) + 2ADP + 2Pi → 2 lactate + 2ATP + 2 H_2O
Ribose fermentation	+
Starch fermentation	+
Sucrose fermentation	+
Cellobiose fermentation	+

[1]Can be changed according to strain.

2.3.2 Genus *Lactobacillus*

The genus *Lactobacillus* constitutes together with the genus *Pediococcus* the family Lactobacillaecea, that comprises 80 recognized sp and 15 subsp; which the relationship between them is shown in Figure 2. The increased interest in its ecology and the availability of accurate taxonomic methods created a marked increase in the number of new sp and subsp within the genus *Lactobacillus* (Hammes and Hertel, 2006).

This genus may be homofermentative, producing more than 85% lactic acid (LA) from glucose, or heterofermentative, producing LA, CO_2, ethanol, and/or acetic acid in equimolar amount (Hammes and Hertel, 2006). Some important facultative heterofermentative, such as, *L. casei, L. plantarum* and *L. paracasei*, under glucose limitation or in presence of the pentoses can produce these by-products in addition to LA (Idler et al., 2015).

In general, Lactobacilli can be involved in spoilage, but also in food production (sauerkraut, silage, and dairy, meat and fish products) and beverages (beer, wine, juices; Hammes et al., 1991); participate in the beet sugar production (Tilbury, 1975); and are widely used as probiotic products (Dworkin et al., 2006).

Bacteria of this genus are also fundamental for the fermentation and production of good silage, therefore of considerable economic importance. The main species isolated in this medium has been *L. mesenteroides* subsp *mesenteroides*, *L. diolivorans, L. paracasei, L. plantarum, L. buchneri*, unclassified “streptobacteria”, *L. fermentum, L. acidophilus* and *L.*

salivarius. They dominate the silage microflora after the multiplication of streptococci and leuconostoc (Langston et al., 1962).

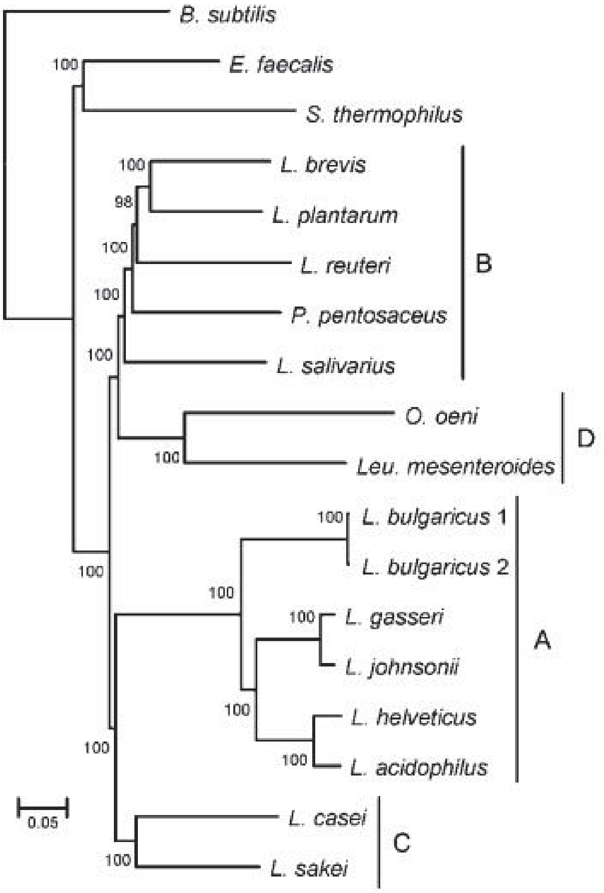

Figure 2. Dendrogram of the Lactobacilli species based on sequences of 141 concatenated core proteins.
From Claesson et al. (2008).

2.3.2.1 *Lactobacillus plantarum*

L. plantarum was usually classified as homofermentative LAB (Ray and Bhunia, 2008) for producing higher amounts of LA (both isomers D and L). However, in 1992 this specie was reclassified as facultative heterofermentative by Nout and Rombouts due to being homofermentative from glucose, but also ferment others carbohydrates heterofermentatively. This is one of the most economic interest LAB due to its great adaptability to different use conditions and therefore it is also one of the most studied specie of the genus *Lactobacillus* over time (Figure 3).

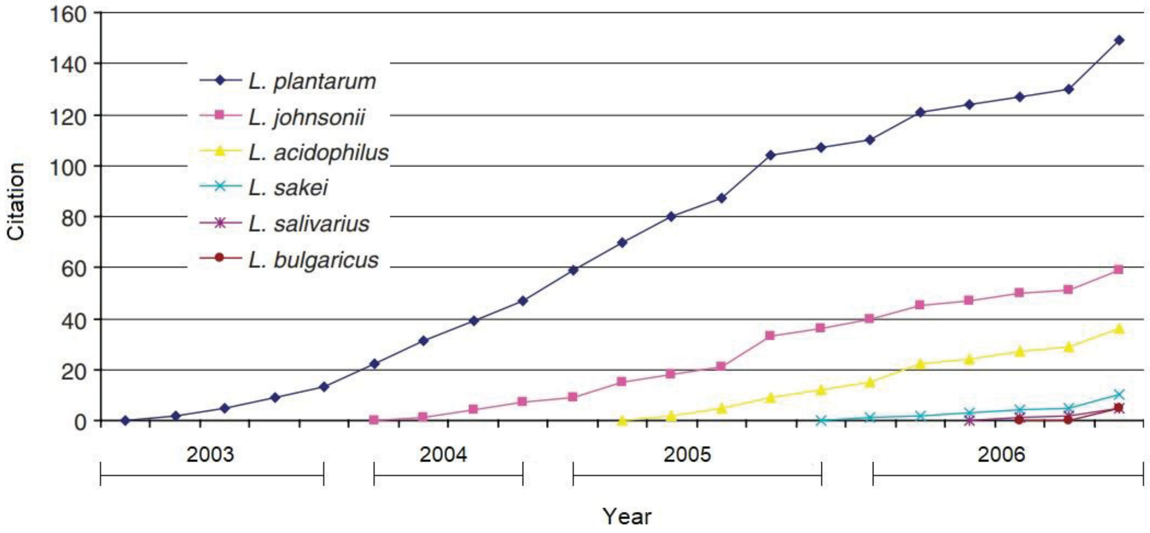

Figure 3. Accumulated number of citations of some *Lactobacillus* species.
Adapted from Claesson et al. (2007).

Although the *L. plantarum* genome is one of the best known among the LAB, the strains are dynamic and change during transition from log to stationary growth (Nigam and Singh, 2014), limiting the consolidation of genetic knowledge about it.

Due to its flexibility and versatility, *L. plantarum* is found in meats, processed and fermented foods, and in anaerobic plant matter (Lorenzo et al., 2018). Still, some strains have antifungal activity, which increases the commercial interest for these bacteria since it inhibits the growth of deteriorating microorganisms as *A. flavus, A. fumigatus, A. petrakii, A. ochraceus, A. nidulans, Epicoccum nigrum,* and *C. gossypiicola* (Yang and Chang, 2008).

In the production of kimchi, *L. plantarum* predominates in the later stage of vegetable fermentation due to its high acid tolerance and metabolic versatility, being a probable choice when homolactic fermentation is desired (Litopoulou-Tzanetaki and Tzanetakis, 2014). This specie is also present in the spontaneous fermentation of cocoa bean (Camu et al., 2007); in the baking, prolonging the shelf-life of sourdough bread (Lavermicocca et al., 2000); in the production of sauerkraut; in the late stage of cucumber fermentation (Pederson, 1961); as a starter culture for functional probiotic vegetable juices (Lahtinen et al., 2012); and other diverse applications.

Regarding its application in silages, the results in the current literature begin to diverge, leading to believe that although widely used and with good characteristics for this fermentation type (Table 4), *L. plantarum* has better performance in conjunction with other LAB than isolated.

Table 4. Characteristics of interest of *Lactobacillus plantarum* in the silage production.

Stimulus	Response[1]
Optimal temperature	42ºC
Optimal pH	4.7
Optimal carbon source	Glucose
O_2 tolerance	Medium
Metabolism in silages	Homofermentative
Metabolic balance	Glucose (or fructose) + 2ADP + 2Pi → 2 lactate + 2ATP + 2 H_2O
Ribose fermentation	+
Starch fermentation	+
Sucrose fermentation	+
Cellobiose fermentation	+

[1]Can be changed according to strain.

According to a meta-analysis made by Bernardi et al. (2019), 27% of studies used *L. plantarum* alone, while 55% used this bacterium in combination with others. This result is similar to Blajman et al. (2018) who found 29 and 71% of the studies using *L. plantarum* isolated and in combo, respectively.

In general, inoculation with *L. plantarum* reduced the acetate concentration and the fungi count (meta-analysis made by Oliveira et al. (2017) with different silages); increased the DM content in alfalfa silages; and reduced ethanol, propionate and acetate concentrations (Blajman et al., 2020). However, in sugarcane silages, inoculation of *L. plantarum* was not recommended. In these silages, ethanol concentrations and DM losses increased and the digestibility decreased as well as no effect was observed on aerobic stability at an application rate of 2.5×10^4 to 2.5×10^{10} cfu/g FF (Rabelo et al., 2016). Blajman et al. (2018) also found no effects on the WSC, ammoniacal nitrogen ($N\text{-}NH_3$) and fungi counts in corn silages that could be attributed to *L. plantarum* at a rate of $1 – 5 \times 10^{10}$ cfu/g.

2.3.2.2 *Lactobacillus salivarius*

L. salivarius is native to human oral cavity (Rogosa et al. 1953) and can be found in various parts and organs of the human body as well as in other animals. This diversity of hosts/habitats results from a long-term evolutionary process and consequent specialization at species level (Duar et al., 2017).

In human health, the administration of *L. salivarius* can improve periodontal health and treat dermatitis. In turn, in animal production this specie has been used as a probiotic to improve the immune status, reduce pathogenic bacteria and even improve the poultry and swine performance (Chaves et al., 2017).

L. salivarius is also widely used in the alcoholic beverage industry, especially those called "brown juice". These strains are considered very promising for acidification due to its potential to reduce pH to 4 in only 4 hours after the end of the lag phase, with LA productivities of more than 7 g/L/h in a dilution rate of 1/h (Thomsen and Kiel, 2008).

In fact, corn silages inoculated with *L. salivarius* and stored at 35ºC showed the lowest pH since beginning (with 7 days of ensiling) in relation to those kept at 45ºC (Guan et al., 2020). This is an example of how the environment can influence fermentation, because in this case the temperature of 35ºC favored the development of the bacteria whose optimum temperature is 37ºC (Table 5). Low pH was also observed by Guo et al. (2020) in alfalfa silages which resulted in better quality compared to commercial inoculant. In addition, the LA concentration and the lactic acid:acetic acid ratio were also higher, characterizing the homofermentative profile. Apparently, this specie can also favor ruminal development. Frizzo et al. (2010) found that young calves supplemented with *L. casei, L. salivarius* and *Pediococcus* had higher feed intake, weight gain and final weight, which contributes to earlier weaning and with less risk.

Table 5. Characteristics of interest of *Lactobacillus salivarius* in the silage production.

Stimulus	Response[1]
Optimal temperature	37ºC
Optimal pH	6.5
Optimal carbon source	Maltose
O_2 tolerance	Low
Metabolism in silages	Obligate homofermentative
Metabolic balance	Glucose (or fructose) + 2ADP + 2Pi → 2 lactate + 2ATP + 2 H_2O
Pentose fermentation	–
Starch fermentation	–
Sucrose fermentation	+
Cellobiose fermentation	–

[1] Can be changed according to strain.

2.3.3 Genus *Pediococcus*

This genus is phylogenetically heterogeneous, formed by distinct groups with small similarities between them. The species of *Pediococcus* (Figure 4) differ in their sugar fermentation ranges, arginine hydrolysis, growth at different pH (4.5-7.0), configuration of the produced LA (Axelsson, 2004) and ribotyping (Satokari et al., 2000).

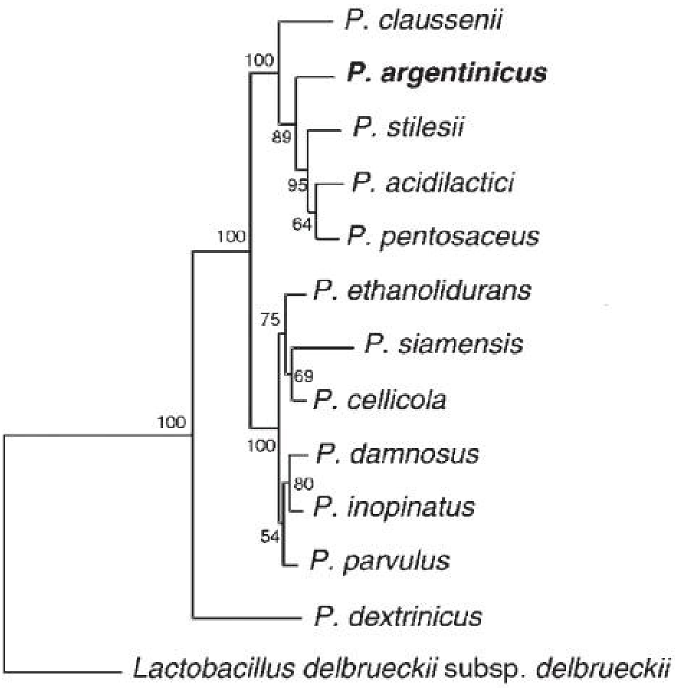

Figure 4. Dendrogram of the genus *Pediococcus* based on 16S rRNA gene sequence analysis. Bars, 1% sequence divergence.
Adapted from De Bruyne et al. (2008).

Among all species of this genera, *P. acidilactici* (Skerman et al., 1980) and *P. pentosaceus* (Mees, 1934) are the most intensely studied due to their ecological importance and biotechnological potential (Dicks and Endo, 2014). Despite this, it is not always possible to differ *P. acidilactici* and *P. pentosaceus* strains based on morphology, physiology and culture medium. Both sp normally occur in relatively small numbers in fresh plants, but participate effectively in the spontaneous fermentation of several vegetables where they establish a stable microbial community with Lactobacilli, Leuconostocs and other LAB (Sade and Bjorkroth, 2019).

In general, they are used as starter microorganisms in the silage (*P. acidilactici, P. pentosaceus*); in the preparation of soya sauce (*P. halophilus*) (Konig and Frohlich, 2017); in the sauerkraut production; during the ripening of cheeses (Hutkins, 2019); and in the dry sausages production due to controlling undesired pathogens growth (Hugas and Monfort, 1997). In the animal nutrition, *P. acidilactici* strains have been used in farming of broilers and shrimp as probiotics to promote growth performance and strengthening defense mechanisms (Lee et al., 2007; Castex et al., 2010) as well as in the nursery phase of piglets.

2.3.3.1 *Pediococcus acidilactici*

The first starter culture marketed and used in the meat industry was a strain of *P. cerevisiae*, after being classified as *P. acidilactici*. This culture was marketed in 1957, by Merck (USA), for use in the manufacturing of summer sausages and spreads. Currently, different species from LAB are commercialized as starter cultures for fermented raw sausages (ripened or not), being *L. sakei* and *P. acidilactici* the most frequently sp used in Europe and USA, respectively (Centeno and Carballo, 2014). Table 6 shows some other characteristics of this species.

Table 6. Characteristics of *Pediococcus acidilactici*.

First isolation	Lindner (1887)
Synonyms	*P. lindneri, P. cerevisiae*
Habitats	Fermenting plant material, fruits, salami
Pathogenicity	No
Growth conditions	Complex requirements: various vitamins, organic acids, biotin, riboflavin stimulates the growth
Remarks	Some strains produce the bacteriocin pediocin

Adapted from Idler et al. (2015).

The new strains of *P. acidilactici* are characterized by high temperature tolerance, resistance to inhibitors derived from lignocellulose and high LA production in combination with saccharification and fermentation of high levels of corn straw solids (Zhao et al., 2013).

Wang et al. (2019) evaluated the effects of three isolated *P. acidilactici* strains and one commercial additive of *L. plantarum* (both at 1 x 10^5 cfu/g FF) on silages of different forages (Italian ryegrass, Tall fescue, and Oat). In general, the results showed that *P. acidilactici* strains were similar to *L. plantarum* in most fermentation characteristics in all silages, in addition to reducing the pH below 5 in less time (6 vs 10 hours). In turn, Uezen et al. (2020) observed that corn and sorghum silages inoculated with *P. acidilactici* (1 x 10^5 cfu/g FF) exhibited the lowest pH (3.45 and 3.65, respectively) and highest WSC content (10.0 and 70.2

g/kg DM, respectively) when compared to control silages and that this sp can be used to improve aerobic stability, since fungi activity was impaired in silages inoculated with *P. acidilactici* combined with *L. fermentum*.

Another fact refers to the heat tolerance of *P. acidilactici* strains in relation to other homofermentative LAB, making them promising for use in silage at high temperatures due to this characteristic (Cai, 1999; Zhang et al., 2000). This means that commercial strains of *P. acidilactici* are effective at fermentation temperatures above 25ºC (Lücke, 2014) precisely because they have excellent growth around 40ºC (Rutkins, 2018). For this reason, the use of inoculants of *P. acidilactici* strains would be preferred in countries with high environmental temperatures, when it is necessary to quickly drop the pH (Zhang et al., 2000) or when the ensiled forage can naturally reach high temperatures due to fermentation. In addition to the tolerance to high temperatures, *P. acidilactici* also does not tolerate ethanol and does not ferment sucrose. Other characteristics of this sp in silages are shown in Table 7.

Table 7. Characteristics of interest of *Pediococcus acidilactici* in the silage production.

Stimulus	Response[1]
Optimal temperature	40ºC
Optimal pH	6.0-6.5
Optimal carbon source	Glucose
O_2 tolerance	High
Metabolism in silages	Homofermentative
Metabolic balance	Glucose (or fructose) + 2ADP + 2Pi $\rightarrow$ 2 lactate + 2ATP + 2 H_2O
Ribose fermentation	+
Starch fermentation	–
Sucrose fermentation	–
Cellobiose fermentation	+

[1] Can be changed according to strain.

2.3.3.2 *Pediococcus pentosaceus*

Most strains of *P. pentosaceus* are characterized for the ability to hydrolyze arginine (although rare); for the inability to ferment sucrose (Holzapfel et al., 2006); and for fermenting arabinose, ribose, maltose, fructose, galactose, and glucose to produce DL-lactate (Ray, 1995). Another characteristic but few reported is the production of exopolysaccharides in some strains of *P. pentosaceus* isolated from Argentine wine (Nadra and Saad, 1995) and from Thai fermented pork sausage (Smitinont et al., 1999).

P. pentosaceus differs from *P. acidilactici* by the lowest optimal growth temperature, 28-32ºC and 40ºC, respectively, although both grow at 50ºC (Raccach, 2014). Another difference is in the ability of *P. pentosaceus* to ferment maltose (Table 8). In relation to *P. dextrinicus*, *P. pentosaceus* does not hydrolyze starch. The absence of sucrose and melizitosis fermentation and its ability to hydrolyze arginine differs this sp from all *Pediococcus*, except *P. acidilactici* (Simpson and Taguchi, 1995).

Table 8. Some characteristics of *Pediococcus acidilactici* and *Pediococcus pentosaceus* strains.

Traits	Percentage Positive	
	P. acidilactici	*P. pentosaceus*
Growth		
At 10ºC	17.0	99.5
At 45ºC	100.0	96.5
In pH 5.0	100.0	92.6
In pH 8.0	26.5	74.3
Fermentation of		
Glucose	100.0	100.0
Fructose	100.0	100.0
Maltose	6.9	94.5
Lactose	7.3	66.7
Glycerol	0	0

Adapted from Ray (1995).

This specie is one of the most recommended as starter culture because it reduced the pH to 4.9 by 10 h at 30ºC, while *P. acidilactici* and *L. plantarum* required 14 h. This occurs because *P. pentosaceus* grows at a rapid rate at 30ºC, while *P. acidilactici* needs 37-40ºC for faster growth and consequent efficient activity (Ray, 1995). This factor makes the selected strains of this specie potential inoculants for various fermentation processes, including cucumber and green bean fermentations and soy milk, besides it, can also be used in a traditional fermented Chinese dish tou-pan-chiang (Simpson and Taguchi, 1992).

P. pentosaceus normally dominate the natural fermentation of *Pennisetum glaucum* and alfalfa. However, when it comes to commercial strains, apparently the development is more sensitive, since the results of the literature are divergent. In the fermentation of triticale silage under different humidity conditions, Soundharrajan et al. (2019) state that the addition of 1 x 10^5 cfu/g of *P. pentosaceus* TC48 had a positive impact on all parameters analyzed, especially for the greater LA production and the consequent pH reduction, and that this strain still showed potent antibacterial activity with high probiotics properties, which resulted in better silage quality. However, Kleinschmit and Kung (2006), using corn silages with *L. buchneri* (4 x 10^5 cfu/g FF) and *P. pentosaceus* (1 x 10^5 cfu/g FF), and Filya et al. (2007), with alfalfa silages at a rate of 1 x 10^6 cfu/g FF, did not obtain consistent results of the action of this LAB.

Other factors of possible interference are the origin of the strain and the storage conditions. The *P. pentosaceus* 6.16 strain (1 x 10^6 cfu/g FF) obtained from a tropical legume proved to be a potential inoculant for alfalfa silages due to its ability to grow fast, dominate fermentation, produce greater LA concentrations (> 30 g/kg DM), and quickly reduce pH (Silva et al., 2016). As shown in Table 9, *P. pentosaceus* has a relatively high optimum temperature. However, the similar fermentative characteristics of the study made by Silva et al (2016) were also observed with the Q6 strain inoculated in the same proportion in *E. nutans* silages stored at 10 to 15°C (Xu et al., 2019).

Table 9. Characteristics of interest of *Pediococcus pentosaceus* in the silage production.

Stimulus	Response[1]
Optimal temperature	28-32ºC
Optimal pH	6.0-6.5
Optimal carbon source	Glucose
O_2 tolerance	High
Metabolism in silages	Homofermentative
Metabolic balance	Glucose (or fructose) + 2ADP + 2Pi $\rightarrow$ 2 lactate + 2ATP + 2 H_2O
Ribose fermentation	+
Starch fermentation	–
Sucrose fermentation	–
Cellobiose fermentation	+

[1] Can be changed according to strain.

In relation to animal performance, one of the most cited studies (Saleem et al., 2016) reported that supplementation of lambs with diets containing *P. pentosaceus* (1.3 x 10^6 cfu/g) and *P. acidilactici* (1 x 10^6 cfu/g) reduced cholesterol blood in the post-weaning (-1 mg/dL) and increased digestibility and nutritional value of most nutrients until 13.5%, except for crude fiber and ether extract. In addition, (Kumar et al., 2015), studying the *in vitro* inoculation of *P. pentosaceus* in the buffered rumen liquor of buffaloes, concluded that the strain evaluated can increase digestibility and total volatile fatty acids beyond reducing methane production, and considers *P. pentosaceus* as an option for use such as direct-fed microbials for ruminants feeding.

2.3.4 Genus *Propionibacterium*

2.3.4.1 *Propionibacterium acidipropionici*

Propionibacterineae is a suborder contained in the Actinobacteria class (Stackebrandt et al., 1997). In this is the family Propionibacteriaceae (Delwiche, 1957) with the genera: *Propionibacterium* (Orla-Jensen, 1909), *Luteococcus* (Tamura et al., 1994), *Microlunatus* (Nakamura et al., 1995), *Propioniferax* (Yokota et al., 1994), *Friedmanniella* (Schumann et al., 1997), *Tessaracoccus* (Maszenan et al., 1999), *Micropruina* (Shintani et al., 2000) and *Propionimicrobium* (Stackebrandt et al., 1999). Figure 5 shows the genetic relationship between some species of the family Propionibacteriaceae.

Propionibacterium are divided them into two principal groups: (1) the classical or dairy Propionibacteria, and (2) the acnes or cutaneous Propionibacteria. Five species of milk Propionibacteria are currently recognized: *P. freudenreichii* subsp *freudenrichii*, *P. freudenreichii* subsp *shermanii*, *P. thoenii*, *P. acidipropionici* and *P. jensenii*. The classical Propionibacteria are important starter organisms in dairy fermentations, can contribute to natural silage and olives fermentation, and can produce a variety of industrially important products.

The Propionibacteria are probably best known for their role as dairy starter cultures, in which they produce the “eyes” characteristics and flavor of Swiss-type cheeses (Langsrud and Reinbold, 1973). The fermentation of lactose to LA by the starter Streptococci and Lactobacilli provides the substrate for fermentation by the Propionibacteria. The propionic and acetic acids produced by Propionibacteria are inhibitory to molds, yeasts, and some bacteria. The presence of these organic acids is known to improve the shelf life of fermented products (Hettinga and Reinbold, 1972). Other application is the production of vitamin B12

(Piwowarek et al., 2018), producing enough of this vitamin to be used as a commercial source. Propionic acid, the primary metabolite of Propionibacteria, has many uses as additive in cellulosic plastics, herbicides, pharmaceutical industry, perfumes, and others (Boyaval and Corre, 1995).

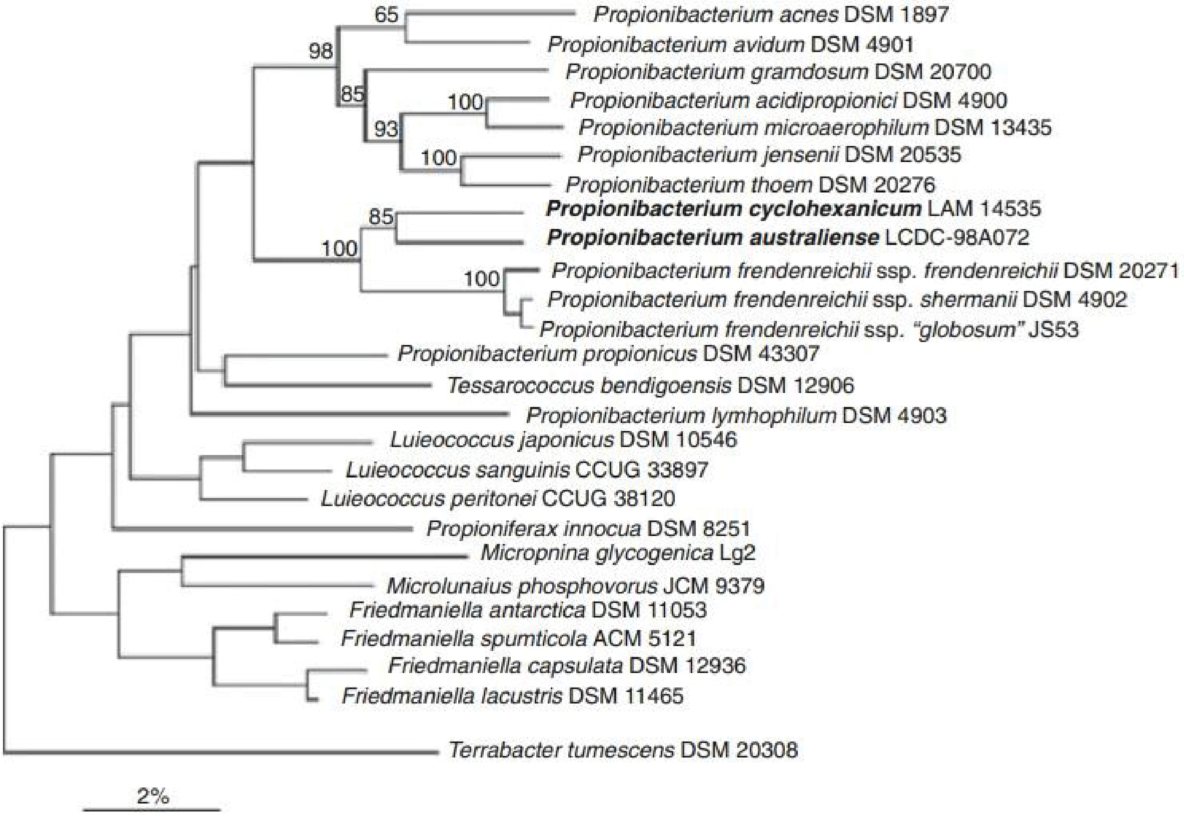

Figure 5. Dendrogram of the family Propionibacteriaceae based on 16S rDNA sequences analysis. Numbers refers to bootstrap values (500 resamplings).
From Stackebrandt et al. (2006).

Specifically, in relation to *P. acidipropionici* there is a consensus about their use in silages: greater aerobic stability. This because, like human foods, the non-dissociated propionic acid present in silages also has fungicidal and fungistatic properties, which reduce the growth of fungi and, consequently, improve the hygienic and nutritional quality of the silages when exposed to air (Filya et al., 2004). However, because it is not a starter culture, longer fermentation periods (minimum 3 months) are necessary to observe these effects (Silva et al., 2015). Table 10 shows some action characteristics of *P. acidipropionici* on silages that can support more assertive decision-making in relation to its use.

Table 10. Characteristics of interest of *Propionibacterium acidipropionici* in the silage production.

Stimulus	Response[1]
Optimal temperature	35ºC
Optimal pH	6-7
Optimal carbon source	Glucose
O_2 tolerance	High
Metabolism in silages	Obligate heterofermentative
Metabolic balance	2 lactate + 2ADP + 2Pi $\rightarrow$ Propionate + acetate + CO_2 + H_2 + 2ATP + $2H_2O$
Ribose fermentation	+
Starch fermentation	+
Sucrose fermentation	+
Cellobiose fermentation	+

[1] Can be changed according to strain.

Among the species of the *Propionibacterium* genus, *P. acidipropionici* are naturally present in the rumen in large quantities and use the LA to produce propionate. Thus, provide these bacteria via food or promote conditions for their development means increasing the propionate production (Lehloenya et al., 2008), which is the main precursor of gluconeogenesis in ruminants (Huntington 1990).

2.4 SILAGE VARIABLES INFLUENCED BY STORAGE TEMPERATURE

Several factors can contribute to the good or bad development of the bacteria. During the inoculant's production, we can say that especially the components of the physical environment (Table 11) will have a great effect on microbial performance, since the biological environment, which involves the interaction between different microorganisms, is controlled under laboratory conditions, e.g., reducing or avoiding competition for resources.

All the stressors exemplified in the previous table plus the competition with other microorganisms occur in the silage (= ecosystem) and will determine the food quality (= sustainability of the ecosystem). Therefore, for silage to have good quality, microorganisms (inoculated or from epiphytic microflora) must be in an optimum medium for growth, which includes no thermal stress.

Table 11. Major microbial stress inducers and possibilities for their reduction.

Stress	Inducer	Reduction options
Starvation	Lack of nutrients	Medium formulation
pH stress	pH changes Inadequate maintenance	Medium formulation
Oxidative stress (anaerobic microbes)	Reactive oxygen species (such as free radicals)	Anaerobic conditions Medium formulation
Osmotic stress	Ionic strength	Rapid processing Preservation agents Medium formulation
Thermal stress	Temperature beyond the ideal range	Low-temperature drying Short heating periods

Adapted from Taskila (2017).

The good quality mentioned is related to preservation of nutrients during the fermentation period. However, after opening the silo, other acids such as acetic and propionic, due to their antifungal properties, will also contribute to control spoilage microorganisms and, thus, make silages less susceptible to aerobic deterioration (Driehuis et al., 1999; Weinberg et al., 2001). The following review will be based on the survey results compiled in Table 12.

Table 12. Chemical and microbiological characteristics of silages subjected to different storage temperatures.

Reference	Temperature (ºC)	pH	N-NH$_3$ (% TN)	g/kg DM			log cfu/g	
				Lactic acid	Acetic acid	Ethanol	LAB	Yeasts
Maize silage								
Weinberg et al., 2001	28	4.1	1.1	28	16	04	7.9	< 2.0
	37	4.4	1.5	14	17	04	6.7	< 2.0
Kim and Adesogan, 2006	20	3.7	17.6	75	26	09	-	7.3
	40	4.3	19.7	45	19	15	-	6.1
Weiss et al., 2016	20	-	-	52	18	04	-	5.8
	35	-	-	25	53	03	-	< 2.0
Alfalfa silage								
Muck and Dickerson, 1988	15	5.0	9.4	65	23	12	-	-
	25	4.7	10.3	83	26	11	-	-
	35	4.7	8.9	70	24	10	-	-
	15	5.2	5.7	41	14	06	-	-
	25	5.0	7.6	38	10	04	-	-
	35	5.2	8.9	24	07	03	-	-
Garcia et al., 1989	38	5.0	2.0	26	08	-	-	-
	65	5.0	2.0	12	05	-	-	-
	38	5.7	2.0	09	06	-	-	-
	65	5.2	1.0	04	05	-	-	-
Oshima et al., 1997	20	5.6	20.8	01	07	-	2.5	-
	30	5.5	34.4	03	10	-	1.0	-
	40	4.8	7.8	10	02	-	5.4	-
Stylosante silage								
Liu et al., 2011	10	5.4	33.1	11	12	-	8.3	0
	20	5.4	39.5	19	29	-	7.5	0
	30	4.8	45.6	23	48	-	7.3	0

	40	4.9	24.9	20	23	-	5.8	0
Liu et al., 2012	20	5.4	39.5	19	29	-	-	-
	30	4.8	45.6	23	48	-	-	-
	40	4.9	24.9	20	23	-	-	-
Sorghum silage								
Tjandraatmadja et al., 1991	20	4.6	4.2	52	15	-	6.5	-
	30	5.1	4.0	42	11	-	5.3	-
	40	5.7	4.0	26	08	-	4.6	-
Wheat silage								
Weinberg et al., 1998	25	4.3	-	43	07	-	7.6	3.9
	41	4.6	-	19	05	-	5.3	3.5
Weinberg et al., 2001	24	3.9	1.0	40	20	04	6.3	-
	41	4.1	1.0	26	11	05	4.8	-
Napier grass silage								
Tamada et al., 1999	30	4.5	11.3	17	03	-	-	-
	40	4.4	9.0	10	02	-	-	-
Total mixed ration silage								
Wang and Nishino, 2013	5	4.3	-	43	10	14	8.8	< 2.0
	15	4.3	-	57	15	23	7.7	< 2.0
	25	4.3	-	57	17	11	5.0	< 2.0
	35	4.2	-	50	16	04	3.8	< 2.0

In maize silages, the studies by Weinberg et al. (2001), Kim and Adesogan (2006), and Weiss et al. (2016) indicate that storage temperatures close to or above 35ºC impair fermentation due to reducing the LA production and, consequently, the silage quality. In general, studies agree that high temperatures, according to respective contrasts, cause high pH (+0.5), N-NH_3 (+ 1.3% TN), and acetic acid concentration (+9.7 g/kg MS). On the other hand, there was less LA content (-23.6 g/kg DM) and less LAB (-1.2 log cfu/g) and yeast count (-1.7 log cfu/g).

The complexity of the interaction between silage variables and storage temperature can be exemplified by the ethanol content, which for maize silages did not have a linear relation, whereas in alfalfa silages as the temperature increased, the ethanol decreased. That is, it is not possible to state that at determined temperature hetero or homolactic fermentation will prevail, regardless of the ensiled crop.

Another interesting fact refers to pH. In maize silages, pH increased with increasing temperature, whereas in alfalfa silages the opposite occurred in most studies, pH and temperature had an inverse relation.

Muck and Dickerson (1988), in a study with alfalfa silages conclude that the temperature was more important than the DM content of the ensiled plant and showed that variations of only 5ºC, during cutting or silage storage, are already sufficient to positively or negatively influence fermentative variables. For silages from the same culture, Garcia et al. (1989) also emphasizes the importance of storage temperature and compares it to the influence of air and DM content to obtain adequate fermentation.

Tjandraatmadja et al. (1991) were even more categorical to conclude that in the tropics, the temperature effects would be largely responsible for silage success or failure, and stated that temperatures above 30°C can limit the LAB count and species that will dominate the fermentation. These conclusions came from his study with sorghum silage, which showed lower LAB count (-1.9 log NMP/g DM) and LA concentration (-26.1 g/kg DM), and higher pH (+1.1) and FDA (+ 24 g/kg DM) in silages maintained at 20ºC in relation to those at 40ºC.

Again, it is not possible to generalize that temperatures above 30ºC will impair the silages fermentation. Tamada et al. (1999) demonstrate that napier grass silage can be preserved with good quality (high WSC (+0.9% DM), low pH (4.4) and low N-NH_3 (-2.3% TN)) at 40ºC, including providing better conditions for the cellulase action. Major losses have also not occurred in stylosant silages (*S. guianensis* Sw.) stored at this temperature (Liu et al., 2011, 2012).

It is clear that changes in the fermentative profile due to temperature affect aerobic stability, favoring or not some microorganisms. In the studies by Kim and Adesogan (2006), Weiss et al. (2016), and Wang and Nishino (2013), the silages stored at higher temperatures had greater stability. In the latter, the authors found that total mixed ration silages (TMR) stored above 25ºC did not deteriorate after air exposure, while those that were stored below 15ºC showed deterioration (visual aspect). In conclusion, Wang and Nishino (2013) suggest that *L. panis,* naturally present in silages, may be associated with changes in fermentation products and greater aerobic stability since the optimum temperature for bacterium growth is above 30ºC, and that in this condition the 1.3-propanediol concentration (which has antifungal action) is higher (Kang et al., 2013ab). In fact, Kim and Adesogan (2006), already stated that high temperatures can favor heterolactic fermentation and, consequently, increase aerobic stability.

After analysis of the studies, it is understood that fermentation is extremely susceptible to ensiled crop and its region of origin (will influence the epiphytic microflora), and the temperature at which this process will occur; all of these factors together may favor or inhibit some microorganisms. In addition, different results can also be obtained including other sources of variation, e.g., inoculated LAB rate and/or species association. There are several genera and strains that can be used in the silage production and, consequently, different temperatures and optimum medium (Liu et al., 2012). Following this principle, it is possible that high or low temperatures will also dictate the speed of reactions and the chemical and enzymatic additives performance. Therefore, assessing the effects of temperature on additive silages is as important as the assessment on untreated silages since more appropriate the medium should be to meet the requirements of the selected strains and allow them to reach their potential.

In general, the data point to favoring heterolactic fermentation at higher temperatures (>35ºC) and for a tendency to homolactic fermentation at temperatures below this threshold. Although there is speculation about the temperature influence on the different ensilage phases, the most consistent hypothesis is the modulation of microbial activity according to predominant temperature during fermentation.

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3 LOWER STORAGE TEMPERATURES MAY CONTROL YEASTS IN SUGARCANE SILAGES

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Abstract

Sugarcane is a crop that requires intervention to be ensiled due to a large amount of sugar and epiphytic yeast. In addition to the challenge of the culture, additives are also influenced by external conditions such as ambient temperature. The objective of this study was to evaluate two storage temperatures of sugarcane silage on additives performance, gas production and fermentative profile. Fifteen silos were maintained in a room with controlled temperature at 26°C (warm) and fifteen kept under 18°C (cool). The treatments consisted of a control (without additive) and two additives: Hetero (*P. acidipropionici* (1 x 10^5 cfu/g of FF) and cellulase enzyme) and Homo (*L. plantarum, P. acidilactici, P. pentosaceus* (1 x 10^5 cfu/g of FF), enzymes and sodium benzoate). The experimental design was a factorial arrangement with 3 treatments and 2 factors (storage temperature), with 5 replications each. The temperature effects were analyzed by the Student's t test ($p<.05$). For the treatment effects, Tukey's test ($p<.05$) was applied. The sugarcane silages with the Hetero additive were similar to untreated silages for most studied parameters. The treatments differed in terms of DM, CP, pH, lactic and acetic acid. In its turn, the warm and cool storage temperature influenced ash (45; 47 g/kg DM), NDF (712; 698 g/kg DM), pH (3.4; 3.6), lactic acid (10.8; 5.1 g/kg DM) and acetic acid (16.1; 24.3 g/kg DM). Only NDF ($p=.029$) and pH ($p=.10$) were influenced by the two factors (storage temperature and treatments). It was observed that at 18ºC no yeast grew while the opposite occurred in the treatment with homolactic bacteria and sodium benzoate (Homo additive). Despite the high ($p<.0001$) yeast count (3.3 log cfu/g FF) in the Homo treatment, this showed the lowest values of DML ($p<.0001$), gas losses ($p=.001$) and gas production ($p=.004$). Silages under 26ºC practically doubled ($p<.0001$) effluent losses (+20.5 kg/t FF) and gas production (+16.2 mL/kg DM) due to alcoholic fermentation and a possible improvement in enzymatic activity of additives. Additives composed of heterolactic bacteria control yeasts growth but increase fermentative losses. Additives with homolactic bacteria reduce losses during fermentation and do not generate significant DM losses during air exposure. The cool storage temperature favors heterofermentation and inhibits yeasts.

Keywords: bacteria, enzyme, fermentation, nutritional value

3.1 INTRODUCTION

The use of additives makes possible the ensiling of various materials as well as improves the quality of feed provided to the animals. Sugarcane is a crop that requires intervention to be ensiled, because despite having positive characteristics such as high production potential per area, varieties adapted to different environments, and for being an excellent source of effective fiber (Harrison, 2016), has as limitation a large amount of sugar and yeast, which results in ethanol production and can cause losses of up to 532 g/kg of dry matter (Jacovaci et al., 2017).

The ambient temperature where fermentation occurs can also be challenging for the development of inoculated bacteria and enzymatic action. Several studies with preserved foods show the relationship between storage temperature, fermentation profile and aerobic stability (Wang and Nishino, 2013; Weiss et al., 2016; Sun et al., 2020). That is, the environmental conditions are responsible for a large part of inoculant action, since it favors the development of certain microorganisms or enzymes, according to temperature range which they are submitted, and consequently may impact on fermentation (Wang et al., 2019).

However, the results of these studies do not allow to estimate with precision how it would occur in tropical grasses, because most of them used unusual or specific materials of a certain region, e.g., wheat, cauliflower leaf, and oat (Weinberg et al., 2001; Ren et al., 2020; Li et al., 2021). In addition, recommending ideal temperature ranges for bacterial growth are no longer a parameter. Factors such as pH, ethanol concentration and previous contact of strains for acclimatization during production by the industry can modify the tolerance to stressors and consequently change the lethal effect of temperature (Casadei et al., 2001; Silva et al., 2005). Therefore, the study of inoculants action in response to environmental temperature is necessary when considering the different climates and materials to be ensiled and the variety of commercial genus and strains used in the world.

The objective of this study was to evaluate two ambient temperatures during the storage period of sugarcane silage on the additive’s performance, gas production and fermentative profile.

3.2 MATERIAL AND METHODS

The trial was carried on Curitiba, south region of Brazil, in June 2017, which monthly precipitation and air temperature was 142 mm and 13.9ºC, respectively. The culture used for silage production was *Saccharum* spp. (sugarcane) harvested at the beginning of maturation phase of the middle third (14.5 ºBrix), about 10 cm from the soil, and cultivated without agrochemicals through manual crops traits. Two forage samples of about 0.3 kg were collected at the harvesting time and before ensiling for pH, dry matter (DM), chemical composition and total soluble solids content (ºBrix) analysis. These values are shown in Table 13.

Table 13. Chemical composition and microbiology of sugarcane plants before ensiling.

Variables[1]	Means	SEM[2]
pH	5.0	0.40
ºBrix	14.5	0.72
Dry matter (g/kg)	236.5	3.45
g/kg DM		
Ash	40.4	0.65
Crude protein	29.3	1.10
NDF	542.5	0.50
ADF	329.0	4.00
log cfu/g FF		
LAB	2.9	0.09
Yeast	5.2	0.03
Molds	5.2	0.17

[1]DM: dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber; FF: fresh forage; LAB: lactic acid bacteria.

[2]Standard error of the mean.

3.2.1 Experimental Units and Treatments

The silos that represented the experimental units were PVC silos with a capacity of 8.5 L (50 cm height x 15 cm diameter) equipped with apparatus to direct measurement of gas production, as described by Bueno (2020), and for the gravimetric determination of total dry matter (DML), gas and effluent losses (Jobim et al., 2007).

Two additives were evaluated: Hetero (*Propionibacterium acidipropionici* (5.0 x 10^{10} cfu/g) and cellulase (10 CMCU/g of inoculant)) and Homo (*Lactobacillus plantarum* (≥ 4.0 x 10^8 cfu/g), *Pediococcus acidilactici* (≥ 2.0 x 10^8 cfu/g), *Pediococcus pentosaceus* (≥ 6.7 x 10^7 cfu/g), cellulase (≥ 0.39 CMCU/g), amylase (≥ 12.00 BAU/g of inoculant), and benzoate, sorbate and dextrose (≥ 0.15 g/kg of FF)). All these were diluted in 100 mL of deionized water and applied using hand sprayers to achieve a concentration of 1 x 10^5 cfu/g of fresh forage (FF) for all treatments. In the Control treatment only 100 mL of deionized water (without additive) was applied. After homogeneous mixing of the forage with additive, an amount to fill the silos (about 5.4 kg) plus 10% over was weighed. Thus, all the experimental units showed an average bulk density of 634 ± 8 kg/m^3. All care was taken to avoid contamination across treatments.

Five replicates of each treatment were maintained in a room with controlled temperature at 26 ± 2°C (warm) and the other five kept in a room with controlled temperature at 18 ± 2°C (cool) for 131 days. The ambient temperatures were maintained through air conditioning and recorded by dataloggers thermometer configured to measure the temperature every 30 minutes. Figure 6 shows how each ambient temperature fluctuated within a range, during the fermentation period, and how the temperature inside the corresponding silo was affected by this variation.

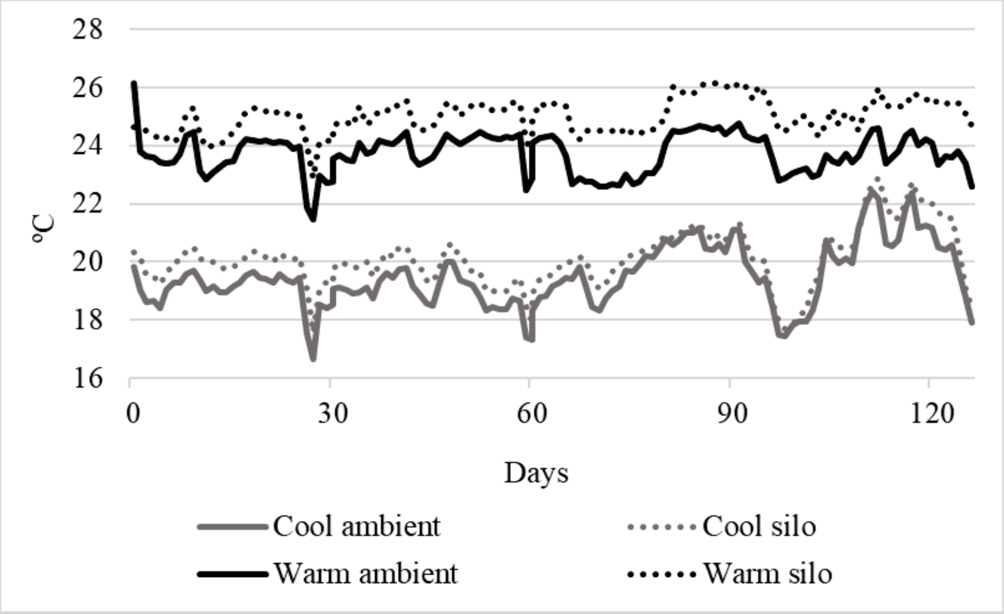

Figure 6. Ambient temperatures (cool (18 ± 2°C) and warm (26 ± 2°C)) and the respective internal temperatures of silos with untreated silages during fermentation period.

3.2.2 Sampling and Analysis

The gas production volume was measured from start to finish (until ceases), which corresponded to 111 days of the storage period. The measurement intervals were determined according to the speed of gas production, every hour for the first weeks after closing the silos and gradually reducing.

After the ensiling period, the silos were weighed, opened and all silage content was placed in individual plastic bags identified and homogenized for sampling. Chemical composition analysis were performed by the following methods: Crude Protein (CP), by DUMAS; Ash and DM as described by Silva and Queiroz (2002); Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) by ANKOM sequential method described by Holden (1999) and; partly DM at 65ºC for 72 h according to AOAC (1990).

Three other samples were collected for pH (Kung Jr et al., 2000); volatile organic compounds (VOC) such as lactic, acetic, propionic, and butyric acids (Daniel et al., 2013); and microbiology analysis for lactic acid bacteria (LAB), yeasts and molds determination as adapted from Kung and Ranjit (2001). In this last, from the filtrate, sequential dilutions were carried out in MRS broth for LAB growth and saline solution for yeasts and molds. LAB was plated in Petrifilm® plates (AC) and placed in the incubator for microbiological cultures at 30 ºC. The colony forming units (cfu) count was done 48 ± 4 h after. The yeast and molds were plated in Petrifilm® plates (YM) and placed in the BOD incubator at 23ºC. The population count was done 72 h (for yeasts) and 120 h (for molds) after.

After collecting all the samples, the aerobic stability test was started with a duration of 240 h in a controlled temperature room (25 ± 5ºC). A 3.5 kg portion of silage from each replicate was placed, without compaction, in individual plastic buckets. At the end of the evaluation period the buckets were weighed to determine DML during aerobic stability. Then, the material was individually homogenized and samples were collected at five different points to compose a final sample used to determine the DM content, in triplicate.

3.2.3 Mathematical Treatment and Statistical Analysis

A completely randomized design in a 3 (additives) x 2 (temperatures) factorial arrangement was used, totalizing 30 experimental units.

The raw data were submitted to Shapiro-Wilk test to normality, and the homogeneity of variance was evaluated and corrected by Box-Cox method when necessary. In the case of microbiology, the cfu count was log-transformed before running the statistical model.

The variables that presented significant differences with a probability of error less than 5% in the ANOVA were submitted to Tukey's comparison test at 5% of significance. For the unfolding of the temperature effects, the Student's t test ($p<0.05$) was applied.

The JMP® pro 13 software was used to carry out the statistical analysis.

3.3 RESULTS

The chemical composition of the Control silages is in line with what was expected for the sugarcane maturity stage (except for the high NDF content). There was no statistical difference between treatments for the variables: ash, NDF, ADF, N-NH_3 and propionic and butyric acids (Table 14) and for lactic and acetic acid concentrations between Control and Hetero treatments. However, although there was no treatment effect, storage temperature influenced ash and NDF, which presented an inverse relationship with higher NDF at 26ºC. The storage temperature also influenced the organic acids concentration. Silages stored in cool room showed a heterofermentative pattern (lactic:acetic = 0.21) when compared to warm room (lactic:acetic = 0.67). The Homo additive showed lactic acid concentration about 30% higher than others ($p<0.05$), consequently, also one of the lowest pH and acetic acid values.

Table 14. Chemical composition of sugarcane silages with or without additives after 131 days of storage in two temperatures.

Variables[1]	Treatments[2]				Temperatures (ºC)			Interaction Effects[3]		
	Control	Hetero	Homo	SEM[4]	26	18	SEM[4]	Treat	Temp	Treat x Temp
DM (g/kg)	207.4^{ab}	201.3^{b}	215.6^{a}	2.42	210.6	205.6	1.98	0.001	0.085	0.081
Ash (g/kg DM)	45.7	45.4	46.5	0.63	45.3^{b}	47.1^{a}	0.51	0.054	0.024	0.937
CP (g/kg DM)	36.6^{b}	36.2^{b}	40.0^{a}	0.74	38.5	36.8	0.60	0.002	0.062	0.116
NDF (g/kg DM)	701.3	710.2	702.5	4.24	711.7^{a}	697.6^{b}	3.47	0.293	0.008	0.029
ADF (g/kg DM)	407.0	455.2	443.1	23.25	452.5	417.7	18.99	0.329	0.208	0.325
$N\text{-}NH_3$ (% TN)	0.9	0.8	0.9	0.05	0.9	0.9	0.04	0.327	0.775	0.298
pH	3.5^{a}	3.4^{ab}	3.3^{b}	0.03	3.4^{b}	3.6^{a}	0.03	0.035	<.0001	0.010
Lactic acid (g/kg DM)	7.5^{b}	6.8^{b}	9.5^{a}	0.45	10.8^{a}	5.1^{b}	0.37	0.001	<.0001	0.569
Acetic acid (g/kg DM)	19.9^{ab}	23.9^{a}	16.9^{b}	1.75	16.1^{b}	24.3^{a}	1.43	0.032	0.001	0.102
Propionic acid (g/kg DM)	1.5	1.4	2.4	0.67	2.3	1.3	0.55	0.521	0.216	0.726
Butyric acid (g/kg DM)	0.1	0.0	0.0	0.05	0.1	0.0	0.04	0.383	0.327	0.383

Means followed by different letters in rows differ statistically between treatments and temperatures by Tukey and Student test ($p<0.05$), respectively.

[1] DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; TN: total nitrogen.

[2] Hetero: *P. acidipropionici* (1.0 x 10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0 x 10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[3] Temp: ambient temperatures (26 ± 2ºC and 18 ± 2ºC); Treat: treatment.

[4] Standard error of the mean.

Interaction between storage temperature and additives occurred for NDF and pH variables (Table 15). In the warm room, the treatments did not differ for the NDF content, while Homo additive presented worse results for this variable (+14.4 g/kg DM) at 18ºC. Another interesting fact refers to the Hetero silages under 18ºC that increased about 27 g/kg DM of NDF ($p<0.05$). That is, in a silo with 10 t FF and 24% DM, there would be an increase of approximately 65 kg in fiber content (2.7% of the total ensiled) just changing the condition of use of the same additive.

Table 15. The unfolding of treatment and temperature interaction in the neutral detergent fiber and pH of sugarcane silages with or without additives.

Temperatures (ºC)	Treatments[1]			
	Control	Hetero	Homo	SEM[3]
		Neutral detergent fiber (g/kg DM)[2]		
26	711.8	723.6^{A}	699.8	7.73
18	690.8^{b}	696.8^{Bab}	705.2^{a}	3.51
		pH		
26	3.4^{Bab}	3.5^{a}	3.3^{Bb}	0.04
18	3.7^{Aa}	3.4^{b}	3.5^{Aab}	0.05

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Tukey and Student test ($p<0.05$), respectively.

[1] Hetero: *P. acidipropionici* (1.0 x 10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0 x 10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[2] DM: dry matter.

[3] Standard error of the mean.

Despite the high lactic acid concentration in the Homo treatment, the pH did not differ from the Control silage, although both have reduced 8% at 26ºC. The opposite was observed in the Hetero, which did not differ between temperatures for this variable.

Although the Homo additive presented the best general results on the bromatological composition of the silages and good performance in warm room, it was not efficient to reduce yeast count (Table 16).

Table 16. Lactic acid bacteria, yeasts, and molds count of sugarcane silages with or without additives in two temperatures.

Variables[1]	Treatments[2]				Temperatures (ºC)			Interaction Effects[3]		
	Control	Hetero	Homo	SEM[4]	26	18	SEM[4]	Temp	Treat	Temp x Treat
LAB (log cfu/g FF)	7.1	> 8.0	7.7	0.29	7.5	7.7	0.23	0.119	0.388	0.196
Yeasts (log cfu/g FF)	2.3^b	2.4^b	3.3^a	0.16	5.2^a	0.0^b	0.20	0.0002	<.0001	0.0002
Molds (log cfu/g FF)	4.7	4.5	5.1	0.59	4.4	5.1	0.47	0.696	0.306	0.391

Means followed by different letters in rows differ statistically between treatments and temperatures by Tukey and Student test ($p<0.05$), respectively.

[1] LAB: lactic acid bacteria; FF: fresh forage.

[2] Hetero: *P. acidipropionici* (1.0 x 10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0 x 10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[3] Temp: ambient temperatures (26 ± 2ºC and 18 ± 2ºC); Treat: treatment.

[4] Standard error of the mean.

The inefficiency of the Homo additive to reduce yeast growth (about 30% more log cfu/g FF compared to Control silages) was observed at 26ºC (Table 17). On other hand, temperatures below 20°C appear to inhibit yeast development in sugarcane silages independently of treatment.

Table 17. The unfolding of treatment and temperature interaction in the yeast count (log cfu/g of fresh forage) of sugarcane silages with or without additives.

Temperatures (ºC)	Treatments[1]			
	Control	Hetero	Homo	SEM[2]
26	4.6^{Ab}	4.8^{Ab}	6.5^{Aa}	0.34
18	0.0^{Ba}	0.0^{Ba}	0.0^{Ba}	0.00

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Tukey and Student test ($p<0.05$), respectively.

[1] Hetero: *P. acidipropionici* (1.0×10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0×10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[2] Standard error of the mean.

Contrary to what was expected due to the presence of sodium benzoate, the largest yeast population was found in the Homo silages. The high acetic acid concentration in the Control and Hetero treatments were more efficient in reducing yeast count, although the fermentative losses were not lower in these treatments (Table 18). In addition, DML during aerobic stability were not affected by treatments. Silages treated with Homo additive produced fewer gas than Hetero (-9.31 mL/kg DM), but both did not differ from the Control.

Table 18. Gas production, fermentative losses and total dry matter losses during air exposure of sugarcane silages with or without additives in two temperatures.

Variables[1]	Treatments[2]				Temperatures (ºC)			Interaction Effects[3]		
	Control	Hetero	Homo	SEM[4]	26	18	SEM[4]	Treat	Temp	Treat x Temp
DML (%)	17.7^{a}	20.2^{a}	13.7^{b}	0.81	17.2	17.2	0.66	<.0001	0.947	0.016
Gas (% DM)	14.6^{a}	17.2^{a}	11.1^{b}	0.94	13.4	15.2	0.77	0.001	0.107	0.052
Effluent (kg/t FF)	35.0	34.9	28.2	2.72	43.0^{a}	22.5^{b}	2.22	0.153	<.0001	0.655
GP (mL/kg DM)	34,8^{ab}	40,9^{a}	31,5^{b}	1782.8	43,8^{a}	27,6^{b}	1455.6	0.004	<.0001	0.468
DML$_{AE}$ (%)	7.3	7.4	8.7	1.34	7.9	7.7	0.38	0.092	0.694	0.549

Means followed by different letters in rows differ statistically between treatments and temperatures by Tukey and Student test ($p<0.05$), respectively.

[1] DML: dry matter losses; DM: dry matter; FF: fresh forage; GP: gas production (directly measurement); DML$_{AE}$: dry matter losses during air exposure.

[2] Hetero: *P. acidipropionici* (1.0 x 10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0 x 10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[3] Temp: ambient temperatures (26 ± 2ºC and 18 ± 2ºC); Treat: treatment.

[4] Standard error of the mean.

The effluent and gas production were affected by the storage temperature, which were about 2 times higher in silages kept under 26ºC in comparison to those silages kept in the cool room (Table 18). However, it is possible to observe in Figure 7 that the curves of gas production were similar in both temperatures, differing only for the quantity produced. Although the figure shows the values of the initial weeks, it was observed that the silages stored at 18ºC remained producing gas for a longer time.

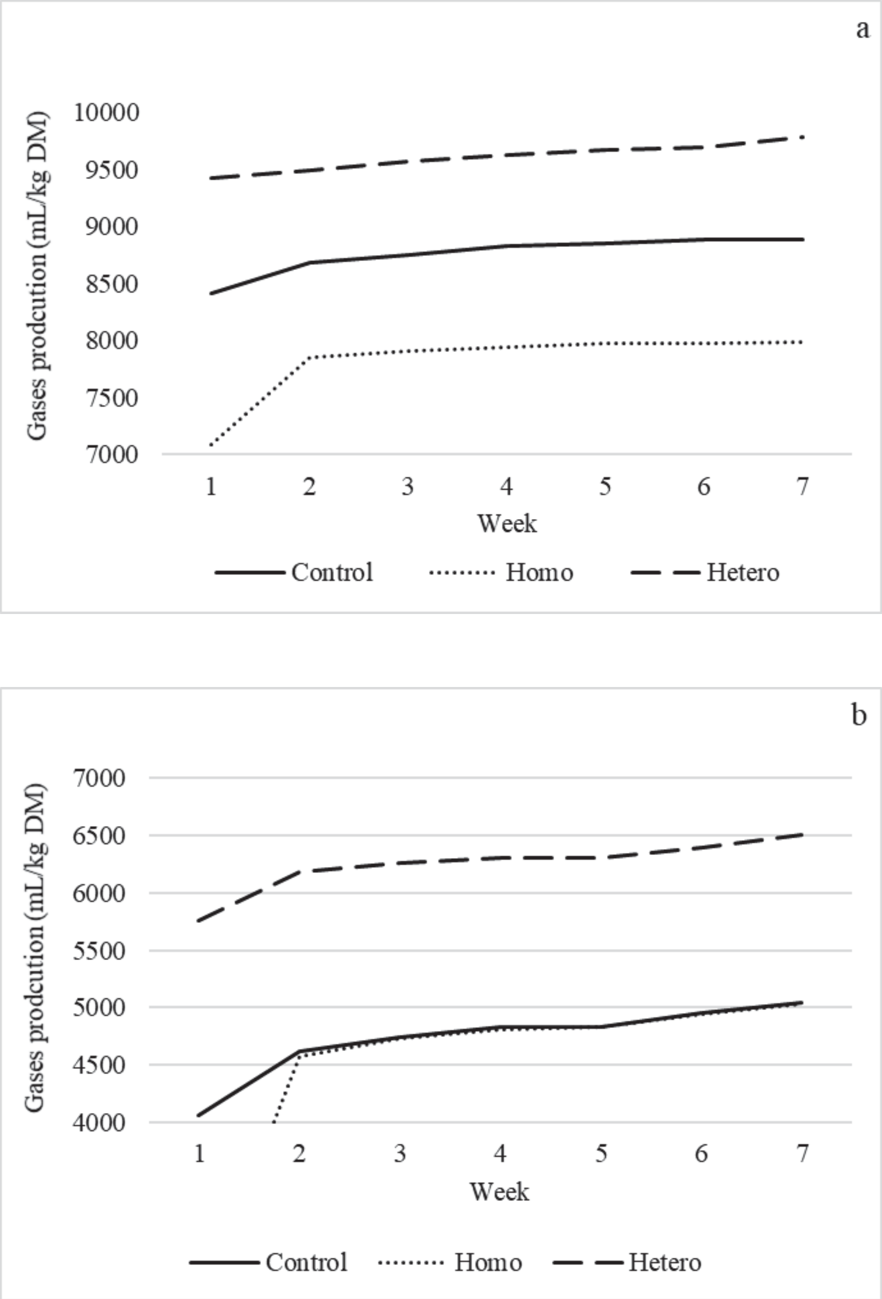

Figure 7. Gas production kinetics of sugarcane silages with or without additives stored at 26ºC (a) and 18ºC (b)

Interaction (temperature x treatment) was observed in DML where again the Homo treatment was favored at the highest temperature by reduce 8.5% of losses. At 18ºC this treatment also presented a favorable result but did not differ from the Control (Table 19). Despite the cool room control yeasts growth during fermentation, this only favored the Control silages.

Table 19. Unfolding of treatment and temperature interaction in the total dry matter losses (%) of sugarcane silages with or without additives.

Temperatures (ºC)	Treatments[1]			
	Control	Hetero	Homo	SEM[2]
26	19.5^{Aa}	20.3^{Aa}	11.9^{Bb}	1.31
18	15.9^{Bb}	20.1^{Aa}	15.5^{Ab}	0.95

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Tukey and Student test ($p<0.05$), respectively.

[1] Hetero: *P. acidipropionici* (1.0 x 10^5 cfu/g FF) and enzyme cellulase; Homo: *L. plantarum, P. acidilactici, P. pentosaceus* (1.0 x 10^5 cfu/g FF), sodium benzoate and enzymes amylase and cellulase; Control: without additive.

[2] Standard error of the mean.

3.4 DISCUSSION

The Homo treatment had greater control of DML, gas losses and gas production (Table 18). This effect can be explained by the homolactic fermentation profile observed in these silages (Table 14), which apparently controlled yeasts growth during anaerobiosis because, despite the higher count, it did not lead to greater losses (Table 16). This hypothesis is supported by the fact that the losses during air exposure did not differ between treatments, otherwise, the Homo treatment would be significantly worse for this variable when the yeast had contact with air again, which did not happen.

The Homo treatment also had sodium benzoate, which antimicrobial properties are well described in the literature (Valdés et al., 2017). Its effectiveness of use as an antifungal in silages (Queiroz et al., 2013) can be affected at high pH conditions, but this fact did not occur in our test, since the pH of the Homo silages was low in both temperatures (average 3.4). High yeast count and low DM fermentative losses from sugarcane silages stored during 180 days were also found by Carvalho et al. (2014) in the treatments combining calcium oxide + *L. plantarum*. The authors justify that the silage conditions may have affected the bacteria performance because the results differed from previous studies with the same strain. These conditions also may have influenced our results.

The heterofermentative behavior of the bacteria in the Hetero additive resulted in higher gas production in this treatment during the initial seven weeks in both conditions (Figure 7), although the final total volume was similar to Control treatment. Another fact refers to smaller and lasting production in the cool room. Possibly the insignificant presence of yeasts in this condition favored a lower gas generation as well as there was substrate available for fermentation for longer. The greatest losses by effluent and gas production in the warm room corroborate with Ashbell et al. (2002), that also observed greater losses in the corn and wheat silages stored between 20 – 30ºC, as well as Weinberg et al. (2001) in corn silages with or without inoculant under 41ºC. The authors point to the highest yeast growth and high pH occasioned the greater losses. In our case, the results can be based on the expressive yeast growth at 26ºC and consequently increase losses through the fermentation pathway of this microorganism because high pH was not observed in this condition.

Under 26ºC, DML of the Control silages were higher because storage temperature favored epiphytic yeast, which although with lower count (Table 17), are adapted to higher temperatures (tropical climate) and therefore are more active in these conditions. According to Robert et al. (2015), despite the low correlation between sucrose assimilation and temperature, the strain geographical origin is related to tolerance to temperature changes and it is possible to divide into two large groups: those who prefer temperatures between 4 - 21°C and the other from 25 - 45°C.

This explanation can be affirmed by the fact that yeast did not differ from the Hetero treatment at 26ºC (Table 17) and, consequently, also reflected in the interaction effects for DML (Table 18). The yeast count in the Control and Hetero silages from this condition is consistent with values normally found in the literature (Carvalho et al., 2014; Jacovaci et al., 2017; Rabelo et al., 2019).

The higher DM content in the Homo treatment is related to greater preservation of nutrients in these silages due to less heterolactic activity, although this variable was similar to Control. Only Homo treatment showed higher CP (Table 14), which may indicate the use of carbon source for bacterial growth and the proportionally increasing the value of this nutrient. Cardoso et al. (2019) also did not observe significant differences between Control silages and those inoculated with *L. plantarum* and *P. acidipropionici* with 180 days of ensiling for most of the studied variables, changing only ethanol content. Propionibacteria have some difficulties to multiply in ensiling conditions due to delay or lethality at pH below 4.0, inability to develop when the pH decreases rapidly, and late growth, usually after 12 days (Merry and Davies, 1999). All these situations happened in our trial, which can then justify the absence of differences for Hetero treatment in some variables.

The intermediate results of the Control silages for DM and acetic acid concentration (Table 14) again demonstrate that heterolactic fermentation was controlled. Although the Homo treatment and the warm temperature provided lactic acid content higher than expected for the ºBrix used (Magalhães et al., 2012); in general, this content is below the lowest reported value (17 g/kg DM) for untreated sugarcane silages (Carvalho et al., 2014). In turn, the storage temperature of 18ºC apparently stimulated the growth of bacteria capable to use other substrates in addition to glucose for acetic acid production, such as *L. mesenteroides*, and members of the *L. brevis-buchneri* complex, for example, because in the cool room the content of this acid was bigger. According to Li et al. (2019), the acetic acid concentration of the king grass (*Pennisetum purpureum* × *P. americana*), paspalum (*Paspalum plicatulum*) and stylo (*Stylosanthes guianensis*) silages did not differ between the temperatures evaluated (28 and 40ºC) during 60 days of ensiling; in addition, the values of this acid for king grass silages were higher (37.8 g/kg DM) than those in our study. The understanding of the reason for these divergences is still limited, but in general involve speculation about bacterial acclimatization, geographic origin, and interaction with pH. So, emphasizing the influence of temperatures on the fermentative process is a good practical information to guide the use of additives or crops for ensiling according to region.

The DM was not influenced by storage temperature, which lead us to believe that despite the distinct fermentative pattern of each temperature, undesirable fermentations were controlled and therefore they also did not affect DML. In turn, the NDF and ash contents were changed. It was expected that the higher storage temperature would favor enzymatic activity, resulting in lower NDF concentrations in silages kept under 26ºC, which did not happen. The optimum temperature for cellulase and amylase enzymes action is 50ºC and 60ºC, respectively, while the pH of silages is far below the optimum values for full enzymatic activity, which are 5 for cellulase and 7 for amylase (Acer et al., 2016; Pachauri et al., 2018). As the pH in the warm room was also lower, this may be a possible explanation.

When we analyze the Table 4, it is noted that the pH is not related to NDF because the treatment that differed between temperatures for this variable did not change the pH and vice versa. Our results differ from those found by Colombatto et al. (2004) with maize silages, who observed lower pH and higher initial organic matter degradation in silages with enzymes stored at elevated temperatures. The authors cite that this effect is not easy to explain, since the optimal pH range of the same enzyme can vary 10 times, depending on the commercial product, and suggest that, in addition to activity, hydrolytic capacity may also vary according to pH x temperature interaction. Homo silages showed the highest NDF values because, although they contain cellulolytic enzymes, most of their composition is amylolytic enzymes and, therefore, have no influence on sugarcane silages. However, the Hetero treatment (only cellulolytic enzymes) was similar to Control and Homo, which leads us to believe that the amount of enzymes in this additive was not sufficient to promote significant changes.

The low pH is directly related to higher and lower lactic and acetic acids concentration, respectively. It is likely that temperature influenced the fermentation pathway or determined hetero or homofermentative LAB development because there was no significant difference for LAB count between treatments (Table 16), but the silages differed in relation to fermentative profile. Silages with higher acetic acid concentration (Control and Hetero treatments) had a lower yeast count, which was expected due to the antifungal effect of this acid (Kung et al., 2018). The acetic acid concentration of about 7 g/kg DM less in Homo treatment may have contributed to the increasing of yeast in approximately 1 cfu/g FF at the opening.

3.5 CONCLUSIONS

At both temperatures (18 and 26ºC), additives composed of heterolactic LAB control yeasts growth but increase fermentative losses, which from a practical point of view requires long storage periods to observe this effect. In turn, additives with homolactic LAB reduce loss during the fermentation and do not generate significant DM losses during air exposure also in both temperatures, but especially at 26ºC. Sugarcane silage at the beginning of maturation phase does not show yeast grow at storage temperature of 18ºC.

Acknowledgments

To Lallemand Animal Nutrition.

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4 HIGH STORAGE TEMPERATURE PRODUCES MOMBAÇA GRASS SILAGES WITH LOWER LACTIC ACID: ACETIC ACID RATIO

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Abstract

The objective of this study was to evaluate the effect of two storage temperatures on the profile and fermentative losses of *P. maximum* silages with bacterial-enzymatic additives. The Mombaça grass was wilted before ensiling, which occurred when the plants presented 248.2 g/kg of dry matter (DM). The treatments were: Control (without additive); hoBACT: *L. plantarum, L. salivarius, P. acidilactic* and *E. faecium* (2.1 x 10^5 cfu/g of FF); BACT: *L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* (5 x 10^5 cfu/g of FF); BACT2: *L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* (1 x 10^6 cfu/g of FF); BACT4: *L. plantarum, P. acidilactici, P. pentosaceus* and *P. acidipropionici* (2 x 10^5 cfu/g of FF). All additives had cellulase, hemicellulase, amylase, xylanase and glucanase enzymes. Four replicates per treatment were maintained in a room with controlled temperature at 26°C (warm) and the other four were kept in a room at 18°C (cool) for 107 days. A completely randomized design in 5 x 2 factorial arrangement was used. The variables were submitted to Student's T-Test ($p<.05$). The silages of the treatments with *P. acidipropionici* showed +5.3, +0.7, and +3 g/kg DM of acetic, propionic, and butyric acids, respectively, in relation to Control and hoBACT silages, which had 2 times more lactic acid ($p<.002$) and lower pH ($p<.0001$). However, all silages stored at 26ºC had higher pH compared to those kept at 18ºC ($p<.0001$). Despite the silages at warm ambient presented higher acids content from heterolactic fermentation, the lactic acid: acetic acid ratio in silages at 18ºC was about 1:3 while at 26ºC this ratio was about 1:2. LAB counts of the Control treatment (7.99 log cfu/g FF) were among the highest at both temperatures ($p<.0001$). At 26ºC, only the BACT additive (8.08 log cfu/g FF) was similar, about +2.5% in relation to the others. In addition, storage temperature at 26ºC appeared to impair homofermentative LAB ($p<.0001$) because it reduced the hoBACT and BACT4 silages count by 2.5% and 1.6%, respectively. Gas production was the only loss variable influenced by temperature ($p=.009$); for each 1 mL produced at 26ºC, the silages stored at 18ºC produced 2 mL/kg DM, i.e., +103% in the total volume. Mombaça grass silages stored at 18ºC have better fermentation and high LAB homolactic growth from additives. At 26ºC the opposite occurs, heterolactic fermentation predominates in this temperature, especially in the silages with *P. acidipropionici* inoculated, which can be detrimental to good fermentation.

Keywords: effluent; epiphytic microflora; gas; inoculants; *Panicum maximum*; tropical grass.

4.1 INTRODUCTION

The environment where the silage is made can interfere with microflora, e.g., acid lactic bacteria, enterobacteria, and clostridia. Studies show that lower temperatures (15ºC) could limit the microbial diversity, metabolism, and enzyme activity of some microorganisms like Clostridium and Enterobacter (Wang et al., 2019b). In turn, high temperatures (40ºC) can favor other genera such as Bifidobacterium, which gradually increases with increasing temperature (Li et al., 2019). Yeasts and molds are also affected by the storage temperature, but with divergent results in aerobic stability assessments (Liu et al., 2011; Wang and Nishino, 2013; Weiss et al., 2016) since the air deterioration depends on the fermentative profile of the silage and, in turn, is affected by factors such as ensiled crop and environment.

Due to the microflora modification, it is expected that the storage temperature also changes the silage's chemical composition. A study by Li et al. (2019) showed that several tropical grass silages kept at 40°C had higher lactic acid content and lower pH after 30 days of ensilage, however small variations of 5ºC already were sufficient to totally change the chemical composition of cauliflower leaf silages (Ren et al., 2020). These studies indicate how this area is little explored and the need for more research to generate assertive interpretations for the different crops and materials.

Grass silages have always been associated with higher fermentative losses, even with inoculants or ensiled with crops suitable for this purpose such as corn (Paludo et al., 2020). Emissions of volatile organic compounds and effluent production are the main environmental issues associated with silage (Daniel et al., 2019) and are expressive characteristics of forage silages with high moisture content. Thus, since the storage temperature influences the microorganism community in the silage and, consequently, the type of fermentation that will occur, it is possible to assume that the preservation of dry matter is also affected.

The environmental temperature as a modulator of the inoculants' response is a recent topic in the literature and of great importance when considering different regions, climates, the environment and financial losses that the incorrect ensilage can cause.

The objective of this study was to evaluate the effect of two storage temperatures on the profile and fermentative losses of *Panicum maximum* silages with or without bacterial-enzymatic additives.

4.2 MATERIAL AND METHODS

The trial was carried on Curitiba, south region of Brazil, in December 2016, which average monthly precipitation and air temperature were 98.6 mm and 19.7ºC, respectively. The culture used for silage production was *Panicum maximum* cv. Mombaça harvested on average with 70 days of growth, about 10 cm from the soil, and without crops traits.

4.2.1 Experimental Units and Treatments

The silos that represented the experimental units were PVC silos with 8.5 L capacity (50 cm height x 15 cm diameter), equipped with apparatus to direct measurement of gas production, as described by Bueno et al. (2020), and for the gravimetric determination of total dry matter (DML), gas and effluent losses (Jobim et al., 2007).

All additives (Table 20) were diluted in 100 mL of deionized water and applied using hand sprayers aiming concentration of cfu/g of FF according to recommendation from the manufacturer. In the Control treatment, only 100 mL of deionized water (without additive) was applied. After homogeneous mixing of the forage with additive, an amount to fill the silos (about 5.5 kg) plus 10% over was weighed. Thus, all the experimental units showed an average bulk density of 527 ± 2 kg/m^3. All care to avoid contamination across treatments was done.

Table 20. Additive's composition.

Components[1]	Treatments			
	hoBact	BACT	BACT2	BACT4
cfu/g of inoculant				
Lactobacillus plantarum	2.1 x 10^{10}	5.0 x 10^8	2.0 x 10^9	3.3 x 10^{10}
Lactobacillus salivarius		-	-	-
Enterococcus faecium		-	-	-
Pediococcus acidilactici		2.0 x 10^8	8.0 x 10^8	3.3 x 10^{10}
Pediococcus pentosaceus	-	2.0 x 10^8	8.0 x 10^8	3.3 x 10^{10}
Propionibacterium acidipropionici	-	1.0 x 10^8	4 x 10^8	1.0 x 10^9
Cellulase (CMCU/g)	242 U/g	0.6	1.2	150
Hemicellulase		-	-	-
Amylase (BAU/g)		36	72	9.000
Xylanase (IU/g)		15	30	3750
Glucanase (IU/g)	-	10	20	2500
cfu/g of fresh forage	2.1 x 10^5	5 x 10^5	1 x 10^6	2 x 10^5

[1] cfu: colony forming units; CMCU: carboxymethycellulose activity unit; BAU: bacterial amylase unit; IU: international units.

The Mombaça grass was previously wilted for 24 hours before ensiling in ambient temperature, which occurred when the plants presented about 248.2 g/kg of dry matter (DM). One forage sample of each treatment was collected before ensiling for chemical analysis, pH, DM, and LAB count. For the fungi count, a single sample was collected at the beginning (before Control ensilage). These values are shown in Table 21. The ensiling sequence of the treatments starting with the Control and ending with the hoBact.

Four replicates of each treatment were maintained in a room with controlled temperature at 26 ± 2°C (warm) and the other four were kept in a room with controlled temperature at 18 ± 2°C (cool) for 107 days. The room temperatures were maintained through air conditioning and recorded by a datalogger thermometer configured to measure the temperature every 30 minutes.

Table 21. Chemical and microbiological composition of Mombaça grass before ensiling of each treatment.

Variables[1]	Treatments				
	Control	hoBACT	BACT	BACT2	BACT4
Dry matter (g/kg FF)	231.51	268.69	239.41	275.37	225.95
Ash (g/kg DM)	112.70	101.50	109.80	101.00	114.20
CP (g/kg DM)	113.60	110.30	122.70	104.10	112.60
NDF (g/kg DM)	695.70	741.50	712.40	718.50	705.60
ADF (g/kg DM)	398.80	425.50	403.80	405.50	397.10
pH	6.80	6.60	6.60	6.71	6.40
LAB (log cfu/g FF)	4.96	7.30	10.10	10.29	10.06
Yeasts (log cfu/g FF)	6.14	-	-	-	-
Molds (log cfu/g FF)	5.58	-	-	-	-

[1] FF: fresh forage; DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; LAB: lactic acid bacteria.

4.2.2 Sampling and Analysis

During nine days of the storage period, the gas production volume was measured until ceases. The measurement intervals were determined according to the speed of gas production, every hour for the first days and then gradually reducing.

After the ensiling period (107 days), the silos were weighed, opened and all silage content was placed in individual plastic bags, identified, homogenized and two portions of about 300 g were collected by one person from each replicate for chemical composition analysis according to the following methods: Crude Protein (CP), by DUMAS; Ash and DM as described by Silva and Queiroz (2002); Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) by ANKOM sequential method described by Holden (1999) and; partly DM at 65ºC for 72 h according to AOAC (1990).

Two other samples of 50 g were collected for pH (Kung Jr et al., 2000); volatile organic compounds (VOC) such as lactic, acetic, propionic, and butyric acids (Daniel et al., 2013); and microbiology analysis for lactic acid bacteria (LAB), yeasts and molds determination as adapted from Kung and Ranjit (2001). In this last, from the filtrate, sequential dilutions were carried out in MRS broth for LAB growth and saline solution for yeasts and molds. LAB was plated in Petrifilm® plates (AC) and placed in the microbiological incubator at 30ºC. The colony forming units (cfu) count was done 48 ± 4 h after. The fungi were plated in Petrifilm®

plates (YM) and placed in the BOD incubator at 23ºC. The population count was done 72 h (for yeasts) and 120 h (for molds) after.

After collecting all the samples, the aerobic stability was started with a duration of 240 h in a controlled temperature room (25 ± 5ºC). A 3 kg portion of silage from each replicate was placed, without compaction, in individual plastic buckets. At the end of the evaluation period, the buckets were weighed to determine total DML during air exposure (DML_{AE}). Then, the material was individually homogenized, and five different points were collected to compose the final sample used to determine the DM content in duplicate.

4.2.3 Mathematical Treatment and Statistical Analysis

A completely randomized design in a 5 (treatments) x 2 (temperatures) factorial arrangement was used, totalizing 40 experimental units (4 per treatment).

The raw data were submitted to Shapiro-Wilk test to normality, and the homogeneity of variance was evaluated and corrected by Box-Cox method when necessary. In the case of microbiology, the cfu count was log-transformed before running the statistical model.

The variables that presented significant differences with a probability of error less than 5% in the ANOVA were submitted to Student's T-Test at 5% of significance.

The JMP$^{®}$ pro 13 software was used to carry out the statistical analysis.

4.3 RESULTS

The chemical composition of Mombaça silages is presented in Table 22. The DM, ash, pH, and all variables of VOC were significantly different between treatments. The hoBACT and BACT2 silages showed 47 g/kg of DM higher when compared to the others. These same silages had on average approximately 10 g/kg DM of ash lower, since an inverse relation between DM and ash was observed in all silages. The inverse relation also occurred between lactic acid and those from heterolactic fermentation (acetic, propionic, and butyric acids). The silages of the treatments composed by *P. acidipropionici* showed an average of +37.4% of acetic acid, +71.1% of propionic acid, and +564.7% of butyric acid compared to Control and hoBACT silages, although the BACT2 treatment have been similar to these two.

The silages composed exclusively by homofermentative bacteria (hoBACT) had about 2 times more lactic acid than BACT4 treatment and, as expected, high concentrations of this acid reduced pH. Although BACT silages did not differ from the Control, the difference of 25% of lactic acid may have contributed to the lower pH in the untreated silages. Another fact refers to the relationship between pH and butyric acid, where the treatments with the highest pH were the same with the highest concentrations of butyric acid and vice versa. Thus, if there was a positive linear relationship between these variables, we could conclude that the increase of 0.32 in the pH above 4.66 would be enough to octuple the butyric acid content (from 0.71 g/kg DM (Control treatment) to 5.71 g/kg DM (BACT treatment)). This pattern was maintained between temperatures, where both pH and butyric acid were 1 and 14.5 times higher, respectively, in silages stored at 26ºC.

Table 22. Chemical composition of Mombaça grass silages, with or without additives, after 107 days of storage at different temperatures.

Variables[1]	Treatments[2]						Temperatures (ºC)			Interaction Effects[4]		
	Control	hoBact	BACT	BACT2	BACT4	SEM[3]	26	18	SEM[3]	Temp	Treat	Temp x Treat
DM (g/kg)	221.37^{b}	265.57^{a}	226.85^{b}	274.57^{a}	221.75^{b}	3.67	241.35	242.69	2.32	0.685	<0.0001	0.738
g/kg DM												
Ash	114.35^{a}	104.55^{b}	117.88^{a}	107.66^{b}	114.26^{a}	2.27	113.0	110.5	1.44	0.232	0.001	0.866
CP	112.90	108.53	109.39	110.26	111.01	1.13	109.49	111.35	0.72	0.077	0.095	0.013
NDF	621.81	644.25	647.74	632.27	644.26	11.97	637.40	638.74	7.57	0.902	0.525	0.035
ADF	378.05	377.24	386.30	371.05	373.76	8.58	376.50	378.06	5.43	0.840	0.770	0.049
pH	4.64^{b}	4.66^{b}	4.98^{a}	4.62^{b}	5.03^{a}	0.04	5.08^{a}	4.49^{b}	0.02	<0.0001	<0.0001	<0.0001
Lactic acid	48.74^{ab}	53.06^{a}	38.99^{b}	49.49^{ab}	27.73^{c}	4.70	43.37	43.83	2.84	0.907	0.002	<0.0001
Acetic acid	15.19^{bc}	13.20^{c}	20.60^{a}	17.48^{ab}	20.42^{a}	1.43	18.76^{a}	16.00^{b}	0.91	0.040	0.003	0.623
Propionic acid	1.06^{c}	0.92^{c}	2.13^{a}	1.18^{c}	1.79^{b}	0.11	1.74^{a}	1.09^{b}	0.07	<0.0001	<0.0001	<0.0001
Butyric acid	0.71^{b}	0.35^{b}	5.71^{a}	0.62^{b}	4.24^{a}	0.83	4.35^{a}	0.30^{b}	0.52	<0.0001	<0.0001	<0.0001

Means followed by different letters in rows differ statistically between treatments and temperatures by Student's T-Test ($p<0.05$).

[1] DM: dry matter; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber.

[2] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1×10^5 cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5×10^3 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4×10^4 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2×10^5 cfu/g FF), amylase, xylanase, cellulase and glucanase.

[3] Standard error of the mean.

[4] Temp: temperatures (26 ± 2ºC, 18 ± 2ºC); Treat: treatments (control, hoBact, BACT, BACT2, BACT4).

The treatments were influenced by storage temperature for most of the chemical composition variables as shown in Table 23.

Table 23. Chemical composition variables of Mombaça grass silages with significant temperatures x treatments interaction.

Temperatures	Treatments[1]					
(ºC)	Control	hoBact	BACT	BACT2	BACT4	SEM[2]
Crude protein (g/kg DM)						
26	114.28^{a}	107.58^{b}	107.18^{b}	111.28^{ab}	107.15^{bB}	1.60
18	111.53^{ab}	109.48^{b}	111.60^{ab}	109.25^{b}	114.88^{aA}	1.60
Neutral detergent fiber (g/kg DM)						
26	610.03^{b}	624.90^{b}	681.95^{aA}	629.37^{b}	640.75^{ab}	16.92
18	633.60^{ab}	663.60^{a}	613.53^{bB}	635.18^{ab}	647.78^{ab}	16.92
Acid detergent fiber (g/kg DM)						
26	376.73^{ab}	367.43^{b}	409.90^{aA}	365.20^{b}	363.25^{b}	12.14
18	379.38	387.05	362.70^{B}	376.90	384.28	12.14
pH						
26	4.88^{cA}	4.82^{cA}	5.30^{bA}	4.85^{cA}	5.54^{aA}	0.07
18	4.40^{bB}	4.50^{bB}	4.65^{aB}	4.38^{bB}	4.51^{abB}	0.07
Lactic acid (g/kg DM)						
26	50.37^{ab}	44.17^{b}	23.52^{dB}	68.55^{aA}	30.24^{bd}	7.10
18	47.11^{ab}	61.95^{a}	54.45^{aA}	30.43^{bcB}	25.22^{c}	6.15
Propionic acid (g/kg DM)						
26	1.12^{c}	0.91^{c}	2.99^{aA}	1.32^{c}	2.36^{bA}	0.22
18	0.99	0.93	1.27^{B}	1.05	1.21^{B}	0.22
Butyric acid (g/kg DM)						
26	0.71^{b}	0.69^{b}	11.14^{aA}	0.98^{b}	8.21^{aA}	1.65
18	0.71	0.00	0.27^{B}	0.27	0.27^{B}	1.65

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Student's T-Test ($p<0.05$).

[1] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1 x 10^{5} cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5 x 10^{3} cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4 x 10^{4} cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2 x 10^{5} cfu/g FF), amylase, xylanase, cellulase and glucanase.

[2] Standard error of the mean.

For the CP content, only the BACT4 silages differed between temperatures with about 8 g/kg DM more in cool ambient. The storage temperature drastically changed the way the treatments interacted. Under 26ºC only the Control and BACT2 silages had the highest values, while at 18 ºC the BACT2 silages were not even among the best results as well as the hoBACT treatment, which was average of 5.5 g/kg DM of CP lower compared to BACT4 silages of better absolute value.

The warm ambient resulted in about +68.5 g/kg DM of NDF of the BACT silages in relation to those kept at 18ºC during the fermentation period. This increase of approximately 10% at 26ºC made the BACT silages one of the highest NDF values as well as those of the BACT4 treatment, which due to having about -40 g/kg DM of NDF were intermediate. In the cool ambient, the hoBACT and BACT silages had the highest and lowest NDF values, respectively, with a difference of 50 g/kg DM. Similarly, for the ADF variable, only the BACT treatment differed between temperatures, again with an increase of about 10% in silages kept at 26ºC. This made it the treatment with the higher value as well as the Control which, in turn, did not differ from the others.

All silages stored at 26ºC had higher pH in relation to those were kept at 18ºC. Even the silages with lower pH on the warm ambient (average 4.9) were higher than the maximum pH of the cool ambient (average 4.6). This variable was not related to the lactic acid content because only BACT and BACT2 treatments differed between temperatures. Due to the large formation of lactic acid in the BACT2 silages in warm ambient, this treatment differed from the others (except Control) with +36 g/kg DM of lactic acid on average, that is, only this difference exceeds the total value of BACT and BACT4 silages. However, in the cool ambient the opposite occurred, silages with low concentrations of lactic acid stored at 26ºC presented the highest values when fermented at 18ºC as is the case of the hoBACT and BACT treatments that, although similar to Control, produced on average 2 times more lactic acid when compared to treatments BACT2 and BACT4.

In relation to acids from heterolactic fermentation, only the warm ambient differed the treatments as well as resulted in a higher concentration of propionic and butyric acids of the BACT (+1.7 and +10.9 g/kg DM, respectively) and BACT4 (+1.2 and +7.9 g/kg DM, respectively) additives when compared to silages kept at 18ºC. Under 26ºC, the BACT silages had an average of 2.7 times more propionic acid than others, except the BACT4 silages, which had 0.6 g/kg DM less in relation to the highest concentration and therefore resulted in intermediate values. In turn, butyric acid also showed high values in the silages of these same additives (BACT and BACT4), but with an even greater difference, equivalent to about 16 times the value of hoBACT silages.

Although the treatments in warm ambient had highest pH, fungi population was not influenced by this variable, because the yeast and mold count of silages stored at 26ºC corresponded to 88 and 66%, respectively, of the values of silages at 18ºC (Table 24).

Table 24. Lactic acid bacteria, yeasts, and molds count of Mombaça grass silages, with or without additives, after 107 days of storage at different temperatures.

Variables[1]	Treatments[2]						Temperatures (ºC)			Interaction Effects[4]		
(log cfu/g FF)	Control	hoBact	BACT	BACT2	BACT4	SEM[3]	26	18	SEM[3]	Temp	Treat	Temp x Treat
LAB	7.99^{ab}	7.81^{c}	8.05^{a}	7.73^{c}	7.93^{b}	0.03	7.92	7.88	0.02	0.137	<0.0001	<0.0001
Yeasts	2.86	2.85	3.18	2.95	3.04	0.09	2.79^{b}	3.17^{a}	0.06	<0.0001	0.091	0.612
Molds	2.02	1.63	2.36	2.44	1.75	0.35	1.63^{b}	2.45^{a}	0.21	0.011	0.343	0.242

Means followed by different letters in rows differ statistically between treatments and temperatures by Student's T-Test ($p<0.05$).

[1] cfu: colony forming units; FF: fresh forage; LAB: lactic acid bacteria.

[2] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1 x 10^5 cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5 x 10^3 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4 x 10^4 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2 x 10^5 cfu/g FF), amylase, xylanase, cellulase and glucanase.

[3] Standard error of the mean.

[4] Temp: temperatures (26 ± 2ºC, 18 ± 2ºC); Treat: treatments (control, hoBact, BACT, BACT2, BACT4).

Only LAB were influenced by treatments and interacted with temperature. Counts of Control treatment were among the highest at both temperatures and at 26ºC only the BACT treatment was similar, about +2.5% in relation to the others. The BACT treatment was the only that was not influenced by storage temperature for this variable (Table 25).

Table 25. Lactic acid bacteria count (log cfu/g FF) of Mombaça grass silages with significant temperatures x treatments interaction.

Temperatures	Treatments[1]					
(ºC)	Control	hoBact	BACT	BACT2	BACT4	SEM[2]
26	8.09^{aA}	7.71^{cB}	8.08^{a}	7.89^{bA}	7.86^{bB}	0.05
18	7.89^{aB}	7.91^{aA}	8.03^{a}	7.57^{bB}	7.99^{aA}	0.05

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Student's T-Test ($p<0.05$).

[1] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1 x 10^5 cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5 x 10^3 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4 x 10^4 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2 x 10^5 cfu/g FF), amylase, xylanase, cellulase and glucanase.

[2] Standard error of the mean.

Apparently, warm ambient harms homofermentative LAB since the count in the hoBACT and BACT4 silages (with higher proportion of homofermentative LAB) was about 2.5% and 1.6% lower, respectively, than those maintained at 18ºC. The opposite occurred with the Control and BACT2 treatments, in which the storage temperature of 26ºC increased on average 2.5% and 4% the LAB population, respectively. The contrast of responses between temperatures also changed the ordering of treatments, e.g., the hoBACT silages kept at 18 ºC had one of the highest LAB counts (+4.5%) when compared to BACT2 treatment, whereas in the warm ambient this treatment had the lowest absolute value (-3.4% in relation to general means). Similarly, the BACT4 treatment also had one of the highest colonies counts in the cool ambient, but in 26ºC it presented intermediate values.

Table 26 shows how the previous grass wilted was efficient to control fermentative losses and DML_{AE} (average 2.56%) because in general they were low. Only the gas production was influenced by silage storage temperature, which was favored in the cool ambient. It is possible to describe the following ratio: for each 1 mL/kg DM of gas produced by silages at 26ºC, 2 mL/kg DM of gas were produced by silages at 18ºC.

Table 26. Gas production, fermentative losses and total dry matter losses during air exposure of Mombaça grass silages, with or without additives, after 107 days of storage at different temperatures.

Variables[1]	Treatments[2]					SEM[3]	Temperatures (ºC)		SEM[3]	Interaction Effects[4]		
	Control	hoBact	BACT	BACT2	BACT4		26	18		Temp	Treat	Temp x Treat
Total DML (%)	6.32	1.81	5.93	3.93	2.66	1.69	5.38	2.88	1.05	0.094	0.222	0.610
Gas (% DM)	6.19	1.87	5.81	3.86	2.55	1.71	5.28	2.83	1.07	0.110	0.284	0.617
Effluent (kg/t FF)	1.54	0.84	1.26	0.84	1.12	0.23	1.12	1.12	0.15	0.996	0.198	0.485
GP (mL/kgDM)	103.81	51.62	75.11	116.50	47.98	20.87	52.09^b	105.92^a	13.20	0.009	0.112	0.0003
DML_{AE} (%)	2.73	2.56	2.46	2.40	2.65	0.24	2.52	2.60	0.15	0.704	0.861	0.877

Means followed by different letters in rows differ statistically between treatments and temperatures by Student's T-Test ($p<0.05$).

[1] DML: dry matter losses; DM: dry matter; FF: fresh forage; GP: gas production; DML_{AE}: total dry matter losses during air exposure.

[2] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1 x 10^5 cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5 x 10^3 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4 x 10^4 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2 x 10^5 cfu/g FF), amylase, xylanase, cellulase and glucanase.

[3] Standard error of the mean.

[4] Temp: temperatures (26 ± 2ºC, 18 ± 2ºC); Treat: treatments (control, BACT4, hoBact, BACT, BACT2).

Gas production was also the only loss variable influenced by the treatment x temperature interaction (Table 27).

Table 27. Gas production (mL/kg DM) of Mombaça grass silages with significant temperatures x treatments interaction.

Temperatures (ºC)	Treatments[1]					
	Control	hoBact	BACT	BACT2	BACT4	SEM[2]
26	165.38^{aA}	20.90^{b}	26.19^{bB}	9.23^{bB}	38.76^{b}	29.51
18	42.25^{bB}	82.34^{b}	124.04^{bA}	223.78^{aA}	57.19^{b}	29.51

Means followed by lowercase letters in rows and uppercase letters in columns differ statistically by Student's T-Test ($p<0.05$).

[1] Control: without additive; hoBACT: *L. plantarum, L. salivarius, P. acidilactici, E. faecium* (2.1 x 10^5 cfu/g FF), amylase, xylanase, cellulase and hemicellulase; BACT: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (5 x 10^3 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT2: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (4 x 10^4 cfu/g FF), amylase, xylanase, cellulase and glucanase; BACT4: *L. plantarum, P. acidilactici, P. pentosaceus, P. acidipropionici* (2 x 10^5 cfu/g FF), amylase, xylanase, cellulase and glucanase.

[2] Standard error of the mean.

Due to gas production of BACT2 silages be 147 mL/kg DM higher than average of the others, that is, an increase of 193%, this was the only different treatment at 18ºC. Unlike what occurred in the warm ambient, where the Control silages was 596% higher than the average production of others.

These two treatments (BACT2 and Control) plus BACT differed between temperatures. The untreated silages produced 4 times more gas at 26ºC, while the additivated silages BACT and BACT2 produced about 5 and 24 times more at 18ºC, respectively.

4.4 DISCUSSION

At both temperatures, untreated silages were among the highest LAB counts (Table 25); then, it is possible to assume that most of the epiphytic microflora was composed by homofermentative LAB because, despite the low DM, there was high lactic acid, low pH and lower acetic, propionic and butyric acids concentration (Table 22). The higher counts in the Control silages do not mean inefficiency of the inoculated bacteria since the additives composition exceeds the minimum amount of 10^5 to 10^6 recommended in the meta-analysis made by Oliveira et al. (2017), as well as the silages fermentation profiles corresponded to what was expected for the additive used, e.g., the high lactic acid concentration and low pH of the hoBACT silages.

The hoBACT and BACT4 additives, composed largely by homofermentative LAB, had higher count at 18ºC than at 26ºC (Table 25). However, Control silages that also presented homofermentative profile had a lower count in the cool ambient. As Mombaça grass was cultivated under environmental temperature generally above 20ºC, it may be that until the harvesting time there was no opportunity for the epiphytic microflora (native LAB) to adapt to long periods (107 days of storage) at 18ºC. This can occur due to the bacteria acclimatization because longer periods of time in the exponential phase in a specific condition contribute to the development of bacterial resistance to environmental stressors (Puntillo et al., 2020). Although some studies (Liu et al., 2012, 2016) reported that lower storage temperature favors the homofermentative LAB growth, as seen in our study, this may be related to bacteria adaptation and acclimation since the previous contact with stressors agents will increase thermotolerance and survival of bacterial cultures (Hao et al., 2021). Therefore, the challenge history of commercial strains during its development will impact the responses in the field and may, e.g., explain the divergent results for the same inoculant in different countries.

Warm ambient favors heterolactic fermentation because the butyric, acetic and propionic acids were higher in silages maintained in these conditions (Table 22). The lactic acid: acetic acid ratio in silages under 18ºC was about 1:3, while under 26ºC this ratio was about 1:2. Considering that lactic acid must represent 65-70% of total acids (Kung and Shaver, 2001) in good silages, it can be said that the Mombaça grass silages stored at 18ºC (71.6% of lactic acid) had better fermentation (based on this premise) than those kept at 26ºC (63.6% of lactic acid).

The average acetic acid concentration (17.4 g/kg DM) is within the standards for tropical grass silages (Rodrigues et al., 2002), mainly due to long period of ensilage. Lactic acid and pH values also corresponded to expectations for the DM content ensiled; however, propionic and butyric acids are far from ideal of the <0.8 and 4 g/kg DM, respectively, especially in BACT and BACT4 silages (Kung and Shaver, 2001). An interesting finding refers to BACT4 treatment, with a lower proportion of heterofermentative LAB and high enzyme concentration, which presented a fermentative profile similar to BACT silages (additive with a higher proportion of heterofermentative LAB), even with lower lactic acid content at 26ºC. Wang et al. (2019a), studying the inclusion of *L. plantarum* (6 log cfu/g FF), fibrolytic enzymes (0.1% FF, cellulase and xylanase), and their combinations in mixed silages of hulless-barley straw and tall fescue, observed that those added exclusively with enzymes had lower lactic acid content in all storage periods (7, 24, 45 and 60 days) and higher acetic (after 24 days of storage) and butyric acid concentrations. What may have occurred in our work is that the action of a large amount of enzymes present in the BACT4 additive may have stood out in relation to bacteria and resulted in silages with a fermentative profile similar to those added only with fibrolytic enzymes, as occurred with Wang et al. (2019a).

In relation to propionic and butyric acids, although the presence is undesirable, silages with high concentrations of these acids present better aerobic stability after opening the silo due to antifungal properties (Kung, 2010), and perhaps for this reason the losses observed were low for this material (Zopollatto et al., 2009). The results found mainly in BACT and BACT4 silages at 26ºC suggest the presence of clostridia (> 0.5 g/kg DM of butyric acid; Kung, 2010) and this may have contributed to DM reduction of these silages, despite the deleterious effects on fermentative losses have not been observed.

Despite the low relation between lactic acid and acids from heterolactic fermentation has occurred at 18ºC, yeast and mold counts were lower in silages stored in the warm ambient (Table 24). Higher fungi count in the cool ambient opposes the theory that low pH reduces the proliferation of undesirable microorganisms (McDonald et al., 1991). Similarly, although the pH was higher at 26ºC, this variable was not related to lactic acid production because only BACT and BACT2 treatments were influenced by temperature resulting in about 2 times less and more g/kg DM of lactic acid, respectively, in silages stored in the warm ambient. Even so, pH and lactic acid concentration (4.5 and 43.8 g/kg DM, respectively) of the present study were similar to the average of 4.6 and 47.2 g/kg DM, respectively, found by Nishino et al. (2012) in wilted guinea grass silage with molasses added, which presented 3.8 log cfu/g of yeasts. Two important facts may be related to this result: first, the speed of the pH drop, which probably took a long time to happen at cool ambient; and second, the lactic acid final concentration that, in this case, did not differ between temperatures.

All of these factors together in the cool ambient (possible delay in pH drop, high fungi count, low concentration of acids from heterofermentation) may have contributed to the gas production, being 2 times higher in the silages at 18ºC (Table 26). The two most probable pathways for this result are fermentation of lactate by clostridia or fermentation of glucose by yeast (McDonald et al., 1991). In both, other by-products such as, e.g., water, are generated, and in our study there were no differences in the effluent production, which makes the results unclear.

The higher gas production of the silages in the cool ambient was caused by the high volume measured mainly in BACT2 silages (Table 27). Generally the greater gas production is justified by heterolactic fermentation that produces CO_2 as a by-product (Muck, 2010). However, in our study this statement cannot be made with certainty, since propionic and butyric acids only differed at 26ºC (the same should be seen in the gas production). In addition, the highest percentage of lactic acid in relation to other acids was observed exactly at 18ºC, which makes us think that beyond to pathways proposed in the previous paragraph, malate fermentation (malate → lactate and CO_2; McDonald et al., 1991) it can also be an explanation for the results.

The choice of the additive used in the ensiling of this culture according to the ambient temperature of storage can improve fermentation. As it is known, the epiphytic microflora influences the fermentation process and the performance of the additives. However, the magnitude of this influence and the altered variables in grass silages are shown. In addition, new effects of ambient temperature and acclimatization of bacteria (commercial and native) were discussed in order to demonstrate how research on this topic is still incipient and how silages production on large scale can be harmed (or not be better) for not considering the importance of temperature where fermentation will occur.

4.5 CONCLUSION

The Mombaça grass silage stored at 18ºC had better fermentation than those at 26ºC, and in addition this condition favored homolactic LAB growth from additives. In turn, heterolactic fermentation predominated at 26ºC, especially in silages with *P. acidipropionici* inoculated, which results in higher propionic and butyric acids concentrations. Although it did not cause significant fermentative losses in this study, it is potentially detrimental to good fermentation.

Acknowledgments

To Lallemand Animal Nutrition.

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5 GENERAL CONSIDERATIONS

The storage temperature affects silage fermentation with and without additives, in both tropical grasses (*S. officinarum* and *P. maximum*). At lower temperature (18ºC), sugarcane silages at beginning of the maturation phase have heterolactic fermentative profile and no yeast growth, which from a practical point of view represents a major step towards reducing losses and better nutritional quality of the preserved forage, which can also increase economic return for farmers. In turn, Mombaça grass silages have heterolactic profile at high temperatures (26ºC) due to the optimization of heterofermenters LAB growth from additives. This result can support decision-making on which additive to use (according to climate) to achieve an objective, e.g., when the intention is to obtain silages with greater resistance to aerobic degradation.

Thus, again the benefits and importance of acquired knowledge can provide for science, companies, and the farm are highlighted.

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