
Lactic Acid Bacteria in Tropical Grass Silages

Edson Mauro Santos, Thiago Carvalho da Silva,
Carlos Henrique Oliveira Macedo and Fleming Sena Campos

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1. Introduction

Lactic Acid Bacteria (LAB) have applications in many industrial areas and play an important role in the preservation process of moist forages for animal feeding (silage).

The basic principle silage is to store the surplus forage keeping its stability and nutritional value until it is required to feed the animals. This process takes place in anaerobic conditions, where the lactic acid produced by the LAB inhibits the proliferation of spoilage microorganisms, which are less tolerant to acidic conditions. Thus, as the pH values decline, the silage losses decline as well due to the greater conversion of plant soluble carbohydrates (the main substrate for LAB) in lactic acid, with 96.9% rate of energy recovery (Mc Donald et al., 1991). The major soluble carbohydrates present in forage crops are fructose, glucose, sucrose and fructosanas, according to Woolford, (1984), sucrose and fructosanas are rapidly hydrolyzed in their monomers at forage harvest.

Lactic fermentation produces lactic acid as the main product. Therefore, homofermentative bacteria such as *Lactobacillus plantarum* are desirable in the silage fermentation process, once 87% of their metabolites become lactic acid. On the other hand, in the heterofermentative process, additional substances like ethanol, acetate and CO₂, are formed. Microbial inoculants used as additives include homofermentative LAB, heterofermentative LAB, or both combined. The specificity between the forage specie and its epiphytic micro flora implicates the need for studies related with isolation and identification of the main microorganism groups present in the forage used for silage.

In this chapter we will discuss the characteristics of tropical grasses, the main LAB species found in these grasses and how the LAB's are used to improve the quality of tropical grass' silages.

2. Tropical grass characteristics

The forage characteristics that contribute to a good fermentation are: dry matter content, autochthonous plant microbiota and, most importantly, the quantity of soluble carbohydrates. Corn and sorghum are the most appropriate grasses to make silages due to their high soluble carbohydrate contents and dry matter production. However, some studies have shown that different grasses can be utilized if they are ensilage at the right developmental stage or if appropriate additives are used (Zanine et al., 2010).

The decline in pH values inhibit the spoilage microorganism proliferation, which allows the silage nutritive values to be preserved. Thus, the best silage forages are the ones with high soluble carbohydrates contents, which should be sufficient to promote the fermentation and produce enough acid to preserve the silage. According to Ferreira (2002), the minimum soluble carbohydrates contents recommended to ensure adequate fermentation of good silage, varies between 6% and 12% of the dry mass. McDonald et al. (1991) found that, since the soluble sugar level is adequate, dry mass contents higher than 25% are sufficient to ensure a good silage production. The buffering capacity is another factor affecting the silage final product. It reflects the capacity to resist change in the pH values, determined by buffering substances, represented in plants by inorganic bases such as potassium (K) and calcium (Ca), protein, ammonia (N-NH₃), organic salts (malate, citrate).

Several factors affect the fermentation pattern and consequently the silage quality, including dry matter content, amount of soluble carbohydrates readily available and initial LAB population (Pereira et al., 2006). These inherent plant characteristics may vary according to species and maturity stage. Corn (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench), followed by millet (*Pennisetum glaucum*) and sunflower (*Helianthus annuus*) seems to be the most adapted species for silage due to the high soluble carbohydrates content, low buffering capacity, satisfactory dry matter productivity and quality of the silage produced. Although, sorghum silage nutritional value is considered lower than that of corn, it has shown an important role in forage production in Brazil and in the world as well, standing out as a resistant species to adverse environmental factors, such as drought stress (Miranda et al., 2010). This grass provides silage at low costs and the plant regrowth can be used (Rezende et al., 2011), because they keep the root system active.

As corn and sorghum have ideal characteristics for silage, a factor that drew the researcher's attention was the ideal harvest moment, considering the maturity stage and silage quality. Faria Júnior et al. (2011), working with the effect of seven grain maturity stages on the quality of sorghum BRS 610 silage, observed that the most appropriate stage for ensiling is the milk and soft dough stages, due to its higher silage fermentation quality and nutritional value.

Pearl millet silage presents high crude protein content as an intrinsic characteristic, when compared with corn and sorghum silage. Crude protein values varying from 8.51% to 10.68% were observed by Amaral et al. (2008). The storage system efficiency must not be defined only by the silage nutritional value, but also include the losses that occur from the plant harvest to the animal feeding (Neumann et al., 2007).

Sugarcane (*Saccharum officinarum* L.) is an important grass due to its tolerance to drought periods and high production potential of dry matter and soluble carbohydrates per hectare. The sugarcane silage confection has been unusual, being used more for animal feeding in its natural form, after cutting and chopping, but it can be recommended when desires to store the sugarcane in its higher nutritional value stage (the dry season) for use throughout the year (Molina et al., 2002). However, according to Santos et al. (2006), sugar cane silage becomes justifiable only when there is a surplus or when accidental burning of sugar cane fields happen, always taking into account the difficulty of achieving a good fermentation pattern due to intense alcoholic fermentation (8% to 17% of dry matter of ethanol) caused by yeast (Kung Jr. & Stanley, 1982), leading to losses of up to 30% of dry matter (Ferreira et al., 2007), accumulation of cell wall components and reduction in the *in vitro* dry matter digestibility. Furthermore, sugar cane silage has low aerobic stability, as result of high residual carbohydrate and lactic acid contents (McDonald et al., 1991). On the other hand, the adoption of the silage method represents a chance to keep the sugarcane nutritional value and allows better logistics for their manufacture and use, what implies the hand labor rationalization, concentrating the sugar cane harvest process in a particular time of year or time period, resulting in easier daily farm handling and maximizing the machinery use.

Thus, there has been a growing number of research projects, especially in Brazil, seeking additives that inhibit yeast growth in sugar cane silages (Valeriano et al., 2009). Nevertheless, some studies have shown that grasses can also be stored if they are ensiled at the ideal stage of development, or if the suitable additives are applied (Zanine et al., 2010).

Tropical weather grasses have high production in favorable seasons and a sharp decline in the less favorable ones. In this context, the surplus silage can be an option to increase the dry matter supply to the animals in unfavorable times. Such examples of tropical forages with a potential for silage are: *Brachiaria brizantha* (cv. Marandu), *Brachiaria decumbens* (cv. Basilisk), *Brachiaria humidicula*, *Panicum maximum* Jacq. (Cv. Colônião, Tobiata, Tanzânia, Mombaça, Vencedor, Centauro, Massai), *Pennisetum purpureum* Schum. (Cv. Napier, Taiwan, Merker, Porto Rico, Cameroon, Mott), *Cynodon dactylon* (Tifton) and the hybrid of *Cynodon dactylon* x *C. nlemfuensis* (Coastcross). (Patrizi et al., 2004; Santos et al., 2006; Ribeiro et al., 2008; Oliveira et al., 2007; Zopollatto et al., 2009; Lopes & Evangelista, 2010). When compared to the others, elephant grass stands out in silages researches because of present high productivity and higher soluble carbohydrates concentration.

According to Evangelista et al. (2004), the tropical grasses present low dry matter contents, high buffering capacity and low soluble carbohydrates in growth stages in which they present good nutritive values, endangering the conservation through ensilage, once secondary fermentations are possible to occur. Bacteria from the *Clostridium* genus are favored by humid environments with high pH values and temperature. These bacteria are responsible for large losses because they produce CO₂ and butyric acid instead of lactic acid.

The grasses are colonized by a large number of LAB. In the most of the cases different species occur simultaneously in the same culture (Daeschel et al., 1987). According to Pahlow et al. (2003), in literature review studies, the species more commonly found in plants

are *Lactobacillus plantarum*, *Lactobacillus casei*, *Pediococcus acidilactici*, *Enterococcus faecium*. Some heterofermentative lactic bacteria species can also be found in plants.

The lactic acid bacteria from the autochthonous microbiota are essential for the silage fermentation. However, no bacteria group varies as much as this one regarding number, with a detection limit of 10^1 to 10^5 CFU g^{-1} in alfalfa forage, 10^6 in perennial grasses and 10^7 in corn and sorghum (Pahlow et al., 2003).

The Table 1 shows contents of dry matter, crude protein, soluble carbohydrates and LAB number of mombaça grass (*Panicum maximum*) and *Brachiaria decumbens* with different regrowth ages. It is observed that in none of regrowth ages, neither grass showed dry matter content exceeding 30% and only the grasses cut over 50 days after regrowth presented LAB population greater than 5 log CFU/g. On the other hand, there is a sharp drop in crude protein content with increasing regrowth age.

Signal grass (<i>Brachiaria decumbens</i> .)				
AGE (days)	DM (%)	CP (%)	SC (%)	LAB (log CFU/g)
30	20.99	9.65	2.62	3.93
40	21.23	6.97	2.92	4.81
50	21.94	5.86	3.13	5.37
60	22.35	5.30	2.73	5.32
70	23.67	4.37	2.53	5.51
Mombaça grass(<i>Panicum maximum</i> Jacq. cv. Mombaça)				
AGE (days)	DM (%)	CP (%)	SC (%)	LAB (log CFU/g)
30	17.75	7.43	3.34	4.35
40	19.63	7.30	4.12	4.56
50	21.50	6.47	4.18	5.16
60	23.38	4.94	5.43	5.55

Table 1. Dry matter (DM), crude protein (CP) and soluble carbohydrates (SC) and number of lactic acid bacteria (LAB) in signal grass and mombaça grass silage with different regrowth ages (Sousa et al., 2006).

Santos et al. (2011) studying the regrowth age influence in the LAB population observed that silages made with older plants presented LAB populations higher than the silages made with younger plants. According to Knicky (2005), it can be attributed to the increase in soluble carbohydrates and dry matter content, as well as to the decrease of anionic substances such as salts of organic acids, nitrate, sulfates, and so on. Pereira et al. (2005) found an increase in LAB population in elephant grass with the increase in regrowth age.

Meeske et al. (1999) found population of approximately 1 log CFU/g of fresh forage in *Digitaria eriantha*. Cai et al. (1998), analyzing Guinea grass (*Panicum maximum*) indigenous

microbiota, found values lower than 3 log CFU/g of fresh forage. Pereira et al. (2007) reported initial LAB population of 4.92 log CFU/g in elephant grass plants.

Table 2 presents a data compilation of chemical composition and other parameters considered determinants of tropical grass silages quality, such as buffering capacity, soluble carbohydrates and pH values.

	Corn	Sorghum	Pearl millet	Sugar Cane	Elephant grass	Buffel grass	<i>Brachiaria brizantha</i>	<i>Brachiaria decumbens</i>
n*	6	6	6	7	5	4	6	6
DM	30.68	30.20	31.21	25.25	20.75	37.15	38.36	30.9
OM	96.91	92.79	90.9	97.45	90.91	90.60	92.89	92.25
CP	7.22	8.04	11.09	2.80	7.81	5.03	9.67	7.01
MM	5.81	4.45	9.1	2.68	9.53	9.92	5.29	7.53
EE	2.16	-	-	0.82	3.33	1.8	1.16	2.51
NDF	50.32	61.36	60.64	46.88	72.44	73.94	70.05	75.47
ADF	26.57	37.27	35.68	28.24	44.11	50.60	38.64	38.26
NFC	32.49	-	-	44.21	9.99	14.05	8.74	14.12
LIGNIN	4.72	6.2	4.24	4.72	6.24	8.4	4.67	5.9
IVDMD	59.19	52.87	-	53.87	60.90	37.4	58.77	51.61
pH	5.60	5.93	3.62	4.76	5.6	-	-	-
N-NH ₃	0.785	-	1.28	1.20	-	-	-	-
ETHANOL	-	-	-	2.12	-	-	-	-
YEASTS	5.30	-	-	2.71	-	-	-	-
BUFFERING CAPACITY	-	19.98	-	10.80	-	-	-	-
STARCH	21.31	-	-	5.50	-	-	-	-

Table 2. Chemical characterization of tropical grass used for silage. *Number of researches; DM = dry matter (%); OM = organic matter (%); CP = crude protein (%); EE = ether extract (%); NDF = neutral detergent fiber (%); NFC = non-fibrous carbohydrates (%); IVDMD = *in vitro* dry matter digestibility (%); N-NH₃ = ammonia nitrogen (% TN); ADF = acid detergent fiber (%); MM = mineral matter (%).

(Pariz, C.M. et al., 2011; Silva, T.C. et al., 2011; Viana, M.C.M. et al., 2011; Hu, W. et al., 2009; Martinez, J.C. et al., 2009; Valeriano, A.R., 2009; Bennett, C.G.S. 2008; Reis, J.A.G. et al., 2008; Ribeiro, J.L. et al., 2008; Moreira, J.N. et al., 2007; Pedroso, A.F. et al., 2007; Velho, J.P. et al., 2007; Valadares Filho, S.C. et al., 2006; Velho, J.P. et al., 2006; Kollet, J.L. et al., 2006; Aroeira, L.J.M. et al., 2005; Bernardino, F.S. 2005; Moraes, E.H.B.K. et al., 2005; Santos, G.R.A. et al., 2005; Silva, A.V. et al., 2005; Patrizi, W.L. et al., 2004; Dairy, J. et al., 2003; Santos, M.V.F. et al., 2003; Landell, M.G.A. et al., 2002; Neumann, M. et al., 2002; Rodrigues, P.H.M. et al., 2002).

It is observed that tropical grasses have characteristics influenced by several factors, ranging from species choice to maturity stage at harvest. These factors are primordial in silage confection, because if handled properly, they will favor the LAB development, resulting in higher quality silage.

To understand how the factors related to the grass management will influence the LAB population dynamics consequently the fermentation, it is necessary to know the characteristics related to metabolism and the main tropical grass species.

3. Characteristics of lactic acid bacteria (LAB) present in tropical grasses

Lactic acid bacteria are gram-positive. They are negative catalase, do not present motility and do not produce spores. The final fermentation product is lactic acid, however, some groups produce considerable amount of CO₂, ethanol and other metabolites, these being called heterofermentative. Particularly, *Lactobacillus plantarum* are the larger silage fermentative bacteria (Ohmomo et al., 2002). *Lactococcus*, *Streptococcus* and *Enterococcus* are very important in the fermentation initial stage, because they keep an acidic environment, which then becomes, predominantly colonized by *Lactobacillus*.

Fermentation can be considered the anaerobic decomposition of organic compounds to organic products, which may be metabolized by the cells without the oxygen intervention. Under anaerobiosis conditions, phosphorylation occurs at the substrate level in which an organic acid donates electrons to a NAD⁺, so that in microorganisms the NAD⁺ needs to be regenerated and it occurs through various oxidation-reduction pathways, involving pyruvate or its derivatives, like acetyl-CoA. Pyruvate is a key molecule of fermenting microorganisms, from that, it can be formed by several compounds such as: acetaldehyde (ethanol), acetyl-CoA, lactate, acetoacetate (butyrate, isopropanol), acetoin (2, 3-butanediol, diacetyl), acetate, oxaloacetate, succinate, and propionate.

The homofermentative LAB are characterized by a faster fermentation rate, reduced proteolysis, higher lactic acid concentrations, lower acetic and butyric acids contents, lower ethanol content, and higher energy and dry matter recovery. Heterofermentative bacteria utilize pentoses as substrate for acetic and propionic acids production, which are effective at controlling fungi, at low pH values. The facultative heterofermentative use the same hexoses pathway of homofermentative, but they are able to ferment pentoses, as they have aldolase and fosfocetolase enzymes. The facultative heterofermentative may produce lactic and acetic acids when the substrate is a pentose, or lactic acid, ethanol and CO₂ when hexose is the substrate, due to the need of oxidation of two NAD molecules produced in the glycolytic pathway (White, 2000).

Table 3 summarizes the main lactic acid bacteria found in silages including some *Lactobacillus* with heterofermentative metabolism and some *Leuconostoc* species which have heterofermentative metabolism also.

For species of *Lactobacillus* genus were defined three groups based on the presence or absence of aldolase and fosfocetolase enzymes (Kandler and Weiss, 1986). These groups are as follows:

<i>Lactobacillus</i>		<i>Enterococcus</i>	<i>Leuconostoc</i>	<i>Pediococcus</i>
<i>L. plantarum</i>	<i>L. brevis</i>	<i>E. faecalis</i>	<i>L. dextranicum</i>	<i>P. acidilactici</i>
<i>L. casei</i>	<i>L. buchneri</i>	<i>E. faecium</i>	<i>L. citrovorum</i>	<i>P. pentosaceus</i>
<i>L. curvatus</i>	<i>L. fermentum</i>	<i>E. lactis</i>	<i>L. mesenteroides</i>	<i>P. cerevisae</i>
<i>L. acidophilus</i>	<i>L. viridescens</i>			

Table 3. Main lactic acid bacteria found in silages. (Woolford, 1984)

Group 1: Homofermentative, which ferment hexoses homolactically almost exclusively to lactic acid (>85%), however, they are unable to ferment pentoses, due to the fosfocetolase enzyme lack;

Homofermentative <i>Lactobacillus</i>	
1A. <i>Lactobacillus delbrueckii</i> subsp. <i>Delbrueckii</i>	9. <i>L. helveticus</i>
1B. <i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	10. <i>L. jensenii</i>
1C. <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	11. <i>L. ruminis</i>
2. <i>L. acidophilus</i>	12. <i>L. salivarius</i>
3. <i>L. amylophilus</i>	13. <i>L. sharpeae</i>
4. <i>L. amylovorus</i>	14. <i>L. vitulinus</i>
5. <i>L. animalis</i>	15. <i>L. yamanashiensis</i>
6. <i>L. crispatus</i>	
7. <i>L. farciminis</i>	
8. <i>L. gasseri</i>	

Group 2: Facultative heterofermentative that use the same hexoses pathway as the one of group 1, but are able to ferment pentoses, since they have aldolase and fosfocetolase enzymes;

Facultative heterofermentative <i>Lactobacillus</i>	
16. <i>L. agilis</i>	20b. <i>L. coryniformis</i> subsp. <i>Torquens</i>
17. <i>L. alimentarius</i>	21. <i>L. curvatus</i>
18. <i>L. bavaricus</i>	22. <i>L. homohiochii</i>
19a. <i>L. casei</i> subsp. <i>Casei</i>	23. <i>L. maltaromicus</i>
19b. <i>L. casei</i> subsp. <i>pseudo-plantarum</i>	24. <i>L. murinus</i>
19c. <i>L. casei</i> subsp. <i>rhamnosus</i>	25. <i>L. plantarum</i>
19d. <i>L. casei</i> subsp. <i>tolerans</i>	26. <i>L. sake</i>
20a. <i>L. coryniformis</i> subsp. <i>coryniformis</i>	

Group 3: Obligately heterofermentative, which ferment hexoses, forming lactic acid, ethanol (or acetic acid) and CO₂, being able to still ferment pentose to form lactic and acetic acids.

Mandatory heterofermentative <i>Lactobacillus</i>	
27. <i>L. bif fermentans</i>	36. <i>L. halotolerans</i>
28. <i>L. brevis</i>	37. <i>L. hilgardii</i>
29. <i>L. buchneri</i>	38. <i>L. kandleri</i>
30. <i>L. collinoides</i>	39. <i>L. kefir</i>
31. <i>L. confusus</i>	40. <i>L. minor</i>
32. <i>L. divergens</i>	41. <i>L. reuteri</i>
33. <i>L. fermentum</i>	42. <i>L. sanfrancisco</i>
34. <i>L. fructivorans</i>	43. <i>L. vaccinostercus</i>
35. <i>L. fructosus</i>	44. <i>L. viridescens</i>

The homofermentative LAB presence in silage is extremely necessary. CO₂ generation results in carbon loss, ie, nutrient losses in plant materials. Therefore, homofermentative bacteria such as *Lactobacillus plantarum*, are desirable in the fermentation of silage.

Several lactic acid bacteria have antimicrobial peptides known as bacteriocins which are responsible for inhibiting the growth of or related species which have similar nutritional requirements. The bacteriocins action mechanism involves interaction with specific receptors on the cell membrane to its insertion resulting in proton-motive force dissipation and pores formation, which may cause cell viability loss (Montville and Chen, 1998; Ennahar et al., 2000).

According Lücke (2000), gram-negative bacteria are less susceptible to the action of bacteriocins from lactic acid bacteria due to the presence of outer membrane, which limits the access of peptides to the target site. In addition, the gram-negative bacteria are more sensitive to organic acid produced by LAB compared with the gram-positive bacteria (Ennahar et al., 2000).

Table 4 presents the lactic acid bacteria percentages isolated from sorghum plant in a study conducted by Tjandraatmadja et al. (1991). Likewise, *Lactobacillus plantarum* was the predominant specie and it kept 100 days after ensiling. It was observed the presence of *Lactobacillus fermentum* and *Lactobacillus brevis* heterofermentative bacteria in large quantities at the end of the ensiling process. It demonstrates that these bacteria are active during the fermentation process.

Evaluating the microbiological composition of silages obtained from three different grass species, Tjandraatmadja et al. (1994) found that *Lactobacillus plantarum* and *Pediococcus spp.* are the predominant species, observing one more time the presence of significant amounts of *Lactobacillus brevis* and *Lactobacillus fermentum* (Table 5). Santos et al. (2006) observed that

Lactobacillus plantarum was the predominant species in mombaça grass (*Panicum maximum*) and signal grass (*Brachiaria decumbens*).

Species	Days after ensiling			
	0	4	8	100
<i>Lactobacillus plantarum</i>	35	84	87	44
<i>Leuconostoc spp.</i>	59	0	0	0
<i>Lactobacillus fermentum</i>	6	6	4	7
<i>Lactobacillus brevis</i>	0	10	9	49

Table 4. Percentage of lactic acid bacteria species isolated from sorghum silage (Tjandraatmadja et al., 1991).

Species	Days after ensiling		
	<i>P. maximum</i>	<i>D. decumbens</i>	<i>S. sphacelata</i>
<i>Lactobacillus plantarum</i>	21	39	47
<i>Lactobacillus coryneformis</i>	6	21	0
<i>Leuconostoc spp.</i>	27	12	0
<i>Enterococcus faecium</i>	0	10	4
<i>Enterococcus faecalis</i>	3	0	3
<i>Pediococcus spp.</i>	30	12	31
<i>Lactobacillus brevis</i>	7	6	11
<i>Lactobacillus fermentum</i>	6	0	4

Table 5. Main lactic acid bacteria (%) isolated from grasses (*Panicum maximum* cv Hami; *Digitaria decumbens*; *Setaria sphacelata* cv Kazungula) (Tjandraatmadja et al., 1994).

It is evident that *Lactobacillus plantarum* and the species from the *Pediococcus* genus are prevalent in forage plants. The species from *Leuconostoc* genus are present in plants. However, according to Chunjian et al. (1992) and Tjandraatmadja et al. (1991) they disappear early in the ensiling process.

According Lücke (2000), gram-negative bacteria are less susceptible to the action of bacteriocins from lactic acid bacteria due to the presence of outer membrane, which limits the access of peptides to the target site. In addition, the gram-negative bacteria are more sensitive to organic acid produced by LAB compared with the gram-positive bacteria (Ennahar et al., 2000).

Santos et al. (2011) conducted a study aiming to characterize and quantify microbial populations in signal grass harvested at different regrowth ages. The six lactic acid bacteria strains isolated from signal grass were characterized according Gram staining, catalase enzyme reaction, and bacilli form, submitted to growth and identification tests. The microbial isolates identification was performed by carbohydrates fermentation in API 50 CH kit (BioMérieux - France).

Regarding the predominant bacteria identification in signal grass plants, it is observed in Table 6 that all isolates had the form of short bacilli with rounded ends, arranged in pairs or in short chains (3-4 cells). All of them showed negative reaction to the catalase enzyme test and were gram-positive. None of the strains grew at pH 9.6 and 6.5% NaCl, but all grew at pH 7.2 and 4% NaCl at 45°C.

	Isolated strain						
	EB1	EB2	EB3	EB4	EB5	EB6	<i>Lactobacillus plantarum</i>
Test							
form	bacillus	bacillus	bacillus	bacillus	bacillus	bacillus	bacillus
Arrangement	DB*	DB	DB	DB	DB	DB	DB
Gram	+	+	+	+	+	+	+
Catalasis	-	-	-	-	-	-	-
	Growth at different pH						
7,2	+	+	+	+	+	+	+
9,6	-	-	-	-	-	-	-
	Growth at different salt concentration (NaCl)						
NaCl 4%	+	+	+	+	+	+	+
NaCl 6,5%	-	-	-	-	-	-	-
	Growth at different temperatures (T °C)						
15°C	+	+	+	+	+	+	+
45°C	+	+	+	+	+	+	+

Table 6. Morphology and biochemical characteristics of the isolates EB1, EB2, EB3, EB4, EB5, EB6, signal grass plant (*Brachiaria decumbens* cv. Basiliski). *DB: diplobacillus. (Santos et al., 2011).

According with the carbohydrate fermentation pattern (Table 7), the isolates EB1, EB2, EB5 e EB6 were identified as *Lactobacillus plantarum* with 99.9% of similarity.

The *Lactobacillus plantarum* specie, identified as dominant in signal grass plants (*Brachiaria decumbens* cv. Basiliski) (Santos et al., 2011) has been isolated and characterized as major species in several cultures. Lin et al. (1992) evaluated the corn and alfalfa autochthonous microbiota and found that from the total lactic acid bacteria isolated, over 90% were homofermentative lactic bacteria, being *Lactobacillus plantarum* the predominant specie. Tjandraatmadja et al. (1994), in studies on tropical grasses silage, found *Lactobacillus plantarum* and *Pediococcus spp.* as the predominant species.

	Isolated strain				Lactobacillus plantarum
	EB1	EB2	EB5	EB6	
Glycerol	-	-	-	-	-
Erythritol	(+)	(+)	(+)	(+)	-
D-arabinose	-	-	-	-	-
L-arabinose	+	+	+	+	+
Ribose	+	+	+	+	+
D-xylose	-	-	-	-	-
L-xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β -methyl D-xyloside	-	-	-	-	-
Galactose	+	+	+	+	+
D-glucose	+	+	+	+	+
D-fructose	+	+	+	+	+
D-mannose	+	+	+	+	+
L-sorbose	-	-	-	+	-
Rhamnose	(+)	(+)	(+)	(+)	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Mannitol	+	+	+	+	+
Sorbitol	+	+	+	+	+
α -methyl D-mannose	-	-	-	-	+
α -methyl D-glycoside	-	-	-	-	-
N-acetyl-glucosamine	+	+	+	+	+
Amygdaline	+	+	+	+	+
Arbutin	+	+	+	+	+
Esculin	+	+	+	+	+
Salicin	+	+	+	+	+
Cellobiose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Saccharose	+	+	+	+	+
Trehalose	+	+	+	+	+
Inulin	-	-	-	-	-
Melezitose	+	+	+	+	+
D-raffinose	+	+	+	+	+
Amidon	-	-	-	-	-
Glycogene	-	-	-	-	-
Xylitol	-	-	-	-	-
β -gentibiose	+	+	+	+	+
D-turanose	+	+	+	+	+

	Isolated strain				Lactobacillus plantarum
	EB1	EB2	EB5	EB6	
L-lyxose	-	-	-	-	-
D-tagatose	-	-	-	-	-
D-fucose	-	-	-	-	-
L-fucose	-	-	-	-	-
D-arabitol	(+)	(+)	(+)	(+)	-
L-arabitol	-	-	-	-	-
Gluconate	+	+	+	+	+
2 Cetogluconate	-	-	-	-	-
5 Cetogluconate	-	-	-	-	-

Table 7. Carbohydrate fermentation pattern of the isolates EB1, EB2, EB5, and EB6, signal grass plants (*Brachiaria decumbens* cv. Basiliski). + Intense fermentation, - no fermentation; (+) less intense fermentation (Santos et al., 2011).

In another study, Rocha (2003), evaluating the lactic acid bacteria populations in elephant grass plants cv. Cameroon (*Pennisetum purpureum* Schum) identified the isolates as *Lactobacillus casei* ssp. *Pseudoplantarum*, using the carbohydrate fermentation profile as an identification criterion. Santos et al. (2011) observed the *Lactobacillus plantarum* as LAB predominant specie in signal grass (*Brachiaria decumbens* Stapf). Based on the reported above, it is observed that there were differences between the LAB dominant species among the cultures evaluated, however *Lactobacillus plantarum* has been identified as the predominant specie for most plants.

4. Lactic acid bacteria and their effects on silage fermentation

A suitable acidification is essential for the silage successful preservation, especially when the crop moisture is relatively high, condition which favors the proliferation of spoilage microorganisms. The acidity prevents the development of spoilage microorganisms because they are less tolerant to the acidic conditions than lactic acid bacteria (Woolford, 1984; McDonald et al., 1991).

Among the fermentation stages, aerobic remains during the filling and some hours after the silage closing. The growth of aerobic microorganisms such as yeasts, fungi and bacteria, favored by high concentrations of oxygen (O₂) with the plant respiration process, promotes the O₂ reduction, initiating the active fermentation process. Thus, occurs a sharp drop in silage pH due to the formation of organic acids from sugars, in which initially actuate the heterofermentative bacteria and enterobacteriaceae, that becomes, then, dominated by homofermentative until the pH falls to below 5.0.

In the stability phase, when only the lactic acid bacteria are active, the anaerobic and acidic pH conditions preserve the silage until the opening time. When the silo is opened, it typically happen the molds and yeasts growth. The inhibition of the fungi multiplication through the contact with O₂ is called aerobic stability (Santos et al., 2006).

According to Ohmomo et al. (2002) in the early fermentation stage, *Lactococcus* species, such as *Lactococcus lactis*, *Enterococcus faecalis*, *Pediococcus acidilactici*, *Leuconostoc mesenteroides*, and *Lactobacillus* species such as *Lactobacillus plantarum*, *Lactobacillus cellobioses* grow together with aerobic microorganisms like yeasts, molds and aerobic bacteria, due to the presence of air between the plant particles. At the same time, it is the plant respiration process. To promote the fermentation, an anaerobic environment is formed making the population to become predominantly composed by LAB, basically *Lactococcus* and *Lactobacillus*.

At the final fermentation stage, *Lactobacillus* becomes prevalent, due to their tolerance to the acidity. However, the silage LAB is pretty well diversified, depending on plant material properties, silage technology and silo type. The LAB predominance change from *Lactococcus* to *Lactobacillus* usually occurs in the final fermentation stage. According to Langston et al. (1960), these chemical changes is resulted from bacterial or plant enzymes action making the conversion of carbohydrates into other components such as gas and organic acids, as well as the partial protein breakage resulting in formation of non-protein structures.

The LAB use as microbial inoculants have been widely documented in research (Penteado et al., 2007; Ávila et al., 2009a; Ávila et al., 2009b; Jalč et al., 2009; Reich & Kung Jr., 2010).

Zopollatto et al. (2009) in a meta-analysis study (1999-2009) found a data limitation on the effect of microbial additives in silage quality. They observed that the number of conducted studies is not enough to provide conclusive positions regarding the effects of additives, emphasizing also the data scarcity in certain areas, such as dairy cattle performance. The results documented by these authors show that the magnitude of the response, especially on animal performance, is low. Thus, the justification for the use of additives should be evaluated considering the losses reduction in silage and the higher plant nutritional value preservation. Furthermore, they found that the response intensity varies with plant species and microorganism studied, suggesting a specificity between these components.

However, studies conducted in the 1980s and 1990s had already shown that the fermentation responses differ between strains of the same species (Woofford & Sawczyk 1984, Hill, 1989; Fitzsimons et al., 1992). Hill (1989) found that inoculating corn silage with two *Lactobacillus plantarum* strains isolated from corn and grass, the dominant strain after ensiling was the isolated from corn. The same was observed for the grass silage, where the dominant lactic bacteria strain of were the one isolated from grass.

Many inconclusive results observed in silage fermentation studies may be related to this principle, which must have been overlooked. The specificity between the forage specie and its epiphytic microflora implicates in the need for studies related with isolation and identification of the main microorganism groups present in the forage used for silage. Ávila et al. (2009b) isolated *Lactobacillus buchneri* strains from sugar cane (*Saccharum officinarum* L.) and found that *L. buchneri* UFLA SIL 72 addition reduced the fungi population and the ethanol concentration in silages. Santos et al. (2007) observed reduction in ammonia concentration and enterobacteria population in mombaça grass silage (*Panicum maximum*) inoculated with *Lactobacillus plantarum*, which were isolated from the epiphytic microflora.

Thereby the silage inoculants can facilitate or accelerate the ensiling process, but they do not replace the fundamental factors (plant maturity, dry matter content, oxygen exclusion), which are essential for producing good quality silage. Among these factors the regrowth age is the one that influences all the silage characteristics, from fermentation to the nutritional value, considering the losses.

Meeske & Basson (1998) evaluated the effect of inoculant containing *Lactobacillus acidophilus*, *Lactobacillus delbruekii ssp. bulgaricus* and *Lactobacillus plantarum* on corn silage and found no inoculants effect on pH values and the lactic acid production. According to the authors, the high LAB concentrations present in the plant before ensiling led to such results. Furthermore, the amount of bacteria from *Clostridium* genus present in greater numbers in the treatment without inoculants had no effect on the protein content decrease of the untreated silage. It was not detected the butyric acid formation.

The high residual soluble carbohydrates content in silage, mainly the ones made of corn, sorghum and sugarcane, favors the aerobic deterioration process by fungi and yeasts, causing losses after the silo opening. However, the organic acids produced by fermentation, mainly acetic acid, have fungicidal effect and can mitigate the deterioration, increasing silage aerobic stability (Ranjit & Kung Jr. 2000; Kung Jr. & Ranjit, 2001). Therefore, inoculants containing heterofermentative LAB (e.g. *Lactobacillus buchneri*) have been used to increase the silage aerobic stability.

Ávila et al. (2009a) evaluated the aerobic stability of mombaça grass silage (*Panicum maximum* Jacq. cv. Mombaça) inoculated with two *Lactobacillus buchneri* strains, one provinient from a commercial inoculant and another isolated from sugarcane (*Saccharum officinarum* L.) silage. It was observed an increase in dry matter content after silo opening, while the carbohydrate ratio did not change due to the low residual concentration, characteristic of grass silage. The ammonia (NH₃) concentrations were above the 12% of the total-N recommended by Molina et al. (2002) for good quality silage, indicating high proteolysis during fermentation, due to low soluble carbohydrates supply, what makes possible a rapid decline of pH values.

Table 8 present few studies evaluating the effect of LAB on the silage fermentation. It is observed that there is a pattern of responses, as discussed previously, and its effect depends of the crop used, the microorganism strain and its concentration at the inoculation time. Although significant, the effects are of low magnitude, which leads to reflect about the use of inoculants without the microbiological principles and characteristics of forage plants knowledge.

Kleinschmit and Kung Jr. (2006), in a meta-analysis study (43 experiments), evaluated the *Lactobacillus buchneri* effect on fermentation and aerobic stability of corn, grasses and small grains silages. In general, the inoculation reduced pH, lactic acid concentration and mold counts. At the same time increases in acetic acid concentrations and aerobic stability were detected in all silage types. The increase in aerobic stability was more pronounced in corn silage. Furthermore, it was observed an increase in the propionic acid and ethanol concentrations, on the other hand decreases in soluble carbohydrates concentrations were

found in grass and small grains silages. It was observed correlation between acetic acid concentration and fungi population reduction.

Crop	Microorganism	pH ¹	NH ₃ ²	LA ³	AA ⁴	PA ⁵	BA ⁶	ET ⁷	AE ⁸	DML ⁹	DMR ¹⁰
				% total N		% DM				hours	%
Grass	LP	--	--	++	--		--	ns	ns	--	++
Corn	LB	++	ns	--	++	ns		ns	+		ns
Grass		--	ns	--	++	++		++	+		--
Corn	PA/LP	--		--	--	--	--				++
Wheat	LB	++	ns	--	++				++	++	
	LP	ns	--	++	ns				ns	--	
	LP/LB	ns	--	ns	++				++	--	
Sorghum	LB	ns	ns	--	++				++	++	
	LP	ns	--	ns	ns				--	--	
	LP/LB	ns	--	--	++				++	--	
Wheat	LP/EF	--		++	ns		ns		ns		
	L.Pe	--		++	ns		ns		ns		
Wheat	LB	ns		ns	++				+	++	
	LP	ns		--	++				+	--	
Sugar cane	LB	ns		ns	++	++	ns	--	+		
Sunflowerl	SF/PA/LP	ns	ns	ns	ns	ns	ns		ns	ns	
	LP/L.	ns	ns	ns	ns	ns	ns		ns	ns	
	SF/LP	--	--	ns	--	++	ns		ns	ns	
Potato + WB*	LB	--	--	++	++	ns	--		++		
	LPa/LL/PA	--	--	++	--	--	--		--		

Table 8. Effect of inoculants with lactic acid bacteria on the fermentation of the silage. *Potato by-product + 30% of wheat bran; ¹lactic acid, ²acetic acid, ³propionic acid, ⁴butyric acid, ⁵ethanol, ⁶aerobic stability, ⁷dry matter losses, ⁸dry matter recovery. ns = not significant, + = numerical increase, - = decreasing numbers; ++ = significant increase (P < 0.05) / - = significant decrease (P < 0.05). (Filya et al., 2000; Rodrigues et al., 2001; Weinberg et al., 2002; Filya, 2003; Kleinschmit & Kung Jr., 2006; Rowghani & Zamiri, 2009; Ávila et al., 2009b; Nkosi et al., 2010; Santos et al., 2011). LP = *Lactobacillus plantarum*, EF = *Enterococcus faecium*, LPe = *Lactobacillus pentosus*, SF = *Streptococcus faecium*, PA = *Pediococcus acidilacti*, L = *Lactobacillus sp.*, LB = *Lactobacillus buchneri*; Pac = *Propionibacterium acidipropionici*; LPa = *Lactobacillus paracasei paracasei* LL = *Lactococcus lactis*.

In concluded studies, the inoculation with *Lactobacillus buchneri* changed silages fermentation pattern, decreasing the lactate/acetate ratio, without compromising the processes efficiency, because the dry matter values recovery remained above 90%, as the minimum value recommended for this variable in these plants. The authors also suggest the existence of culture-specific effect.

Evaluating barley silage inoculated with *Lactobacillus buchneri*, Taylor et al. (2002) observed a decrease in yeasts and molds number, contrasting with an increase in aerobic stability. Changes in dry matter consumption and milk production were not affected.

The homofermentative LAB are used in order to improve the fermentation of the silage by increasing the concentration of lactic acid, which reduces the ammonia and the loss of dry matter. The heterofermentative LAB, for its turn, promote improvements, especially after the opening of the silo, increasing the aerobic stability of silage by inhibiting the growth of molds and yeasts. Thus, many research papers have recommended the use of inoculant combining the above two groups of LAB, due to its greater efficiency compared to the isolated use.

5. Use of additives and management practices aimed at the development of lactic bacteria in tropical grass silages

For an appropriate fermentation process with lactic acid predominance, it is necessary to provide ideal conditions for the LAB to develop and predominate in the silage environment. In order to attend these conditions it is used some additives, which can absorb moisture or provide soluble carbohydrates, making this way a more propitious environment to the LAB growth. Some management practices may also be employed with the same purpose.

The key point in the management of grass for silage is undoubtedly the harvest time. Grass harvested in advanced maturity stage present high LAB population, however high tissues lignification is an intrinsic characteristic also, what reduces its nutritional value. In contrast, young grasses have good nutritional value, however it also have unfavorable characteristics to the fermentation process, such as high humidity, low LAB population and high buffering capacity. In case of young grasses it can be used various additives. In case of mature grasses it can be settled a point in which the dry matter content and the LAB populations are suitable and the nutritive value is not compromised.

Research conducted with tropical grasses, evaluating the addition of a wide variety of additives, show that the increase in forage dry matter content or soluble carbohydrates supply favors lactic fermentation and, in most cases, reduces the silage losses. Among many, it has been used wheat bran, corn, fruit pulp and biodiesel industry by-products, sugar cane molasses and even tropical fruits such as jackfruit (Zanine et al., 2006; Pardo et al., 2008; Santos et al., 2008; Rêgo et al., 2010; Andrade & Melotti, 2004; Zanine et al., 2010; Silva et al., 2011). It is important to remind that these additives should be used respecting the level recommended by the authors, otherwise the effects can endanger the fermentative process.

Andrade & Melotti (2004) evaluated the effect of 20 additives on the silage quality made of elephant grass with 80 days (Tables 9 and 10).

In this study, it is observed that cotton fiber, sweeping residue, corn meal, elephant grass hay and guandu hay were used as additives, absorbing moisture (90.91% of dry matter) .The sweeping residue and molasses were used to supply carbohydrates (97.65%).

Looking at N-NH₃ results, it seems that the use of urea, cotton fiber, elephant grass hay, guandu hay, corn meal and molasses with urea, resulted in increased protein degradation during fermentation process. However, no changes were observed in the lactic acid concentration.

Treatment	DM	pH	N-NH ₃	Lactic acid	Acetic acid	Butyric acid
	%		% total N	%DM		
Control (without additive)	15.58f	4.15b	12.39d	2.40a	0.30b	0.00b
Urea 0.5 %	15.49f	5.36a	35.76abc	1.05a	1.81a	0.57a
Cotton fiber (10%)	23.25b	5.33a	36.07ab	1.8a	0.66b	1.73a
Elephant grass hay (10%)	25.88a	4.26b	25.63bcd	2.48a	0.46b	0.12b
Guandu hay(10%)	25.78a	4.21b	8.33d	1.38a	0.58b	0.14b
Drying for 6 hours	19.84cd	4.08b	15.17d	1.81a	0.30b	0.02b
Sugar waste (2%)	16.50de	4.09b	13.68d	4.69a	0.66b	0.00b
Corn Meal (2%)	16.90de	4.00b	13.68d	2.47a	0.28b	0.00b
Corn Meal (4%)	20.39c	4.00b	12.94d	4.96a	1.15a	0.08b
Corn Meal (6%)	21.60c	4.04b	12.01d	4.41a	0.33b	0.00b
Corn Meal (2%) / Urea (0.5%)	17.96de	4.19b	36.67ab	5.31a	0.53b	0.04b
Corn Meal (4%)/ Urea (0.5%)	20.26c	4.29b	49.36a	1.96a	0.85b	0.05b
Corn Meal (6%) / Urea (0.5%)	20.43c	4.20b	46.86a	2.25a	0.38b	0.01b
Dried Molasses (1%)	16.95de	4.04b	10.52d	3.60a	0.22b	0.00b
Dried Molasses (2%)	17.58de	3.92b	10.27d	3.29a	0.23b	0.00b
Dried Molasses (3%)	16.67de	3.89b	9.43d	3.98a	0.35b	0.00b
Dried Molasses (1%) Urea (0.5%)	17.20de	4.18b	34.93abc	1.25a	0.46b	0.04b
Dried Molasses (2%) Urea (0.5%)	18.20de	4.09b	32.43abc	5.24a	0.44b	0.04b
Dried Molasses (3%) Urea (0.5%)	17.55ed	3.97b	11.50d	4.84a	0.36b	0.00b
Biosilo inoculant	15.88f	4.06b	15.24d	2.61a	0.50b	0.03b
CV (%)	7.04	5.55	34.87	50.62	62.54	137.65

Table 9. Dry matter (DM) content and fermentation pattern of elephant grass, Napier, ensiled with different additives (Andrade & Melotti, 2004). DM = dry matter (%), CP = crude protein (% DM), N-NH₃ = ammonia nitrogen/total nitrogen (%), lactic acids, acetic and butyric acids: values in % of the silage DM. Equal means in column do not differ (P>0.05): CV = coefficient of variation.

The lowest *in vitro* dry matter digestibility was obtained with the use of guandu hay. On the other hand the highest one was obtained using corn meal and urea (Table 10). Compared to the control treatment, only the urea and cotton fiber had higher dry matter loss (11.0 and 10.5%, respectively).

According to the authors, it is not recommended the inclusion of urea, hay and cotton fiber in elephant grass silage. Additives rich in nonstructural carbohydrates, such as corn meal and molasses can be used, however, further studies are required to establish suitable levels

for better fermentation. The microbial inoculant 'Biosilo' does not benefit the elephant grass silage.

Treatment	IVDMD (%DM)	DML (%)
Control (without additive)	41.62abcde	6.80b
Urea 0.5 %	34.47abcde	11.00a
Cotton fiber (10%)	27.62de	10.50a
Elephant grass hay (10%)	34.12abcde	9.80b
Guandu hay(10%)	26.36e	7.00b
Drying for 6 hours	41.71abcde	6.70b
Sugar waste (2%)	42.89abcd	6.85b
Corn Meal (2%)	41.36abcde	6.70b
Corn Meal (4%)	45.68abc	7.20b
Corn Meal (6%)	41.81abcde	5.70b
Corn Meal (2%) /Urea (0.5%)	50.30ab	6.60b
Corn Meal (4%)/ Urea (0.5%)	51.31a	7.10b
Corn Meal (6%) /Urea (0.5%)	41.82abcde	7.10b
Dried Molasses (1%)	40.03abcde	6.80b
Dried Molasses (2%)	46.84abc	6.65b
Dried Molasses (3%)	45.25abc	6.80b
Dried Molasses (1%) Urea (0.5%)	43.73abc	6.90b
Dried Molasses (2%) Urea (0.5%)	47.15bc	7.10b
Dried Molasses (3%) Urea (0.5%)	49.65ab	6.85b
Biosilo inoculant	32.52de	7.00b
CV (%)	13.70	18.5

Table 10. *In vitro* dry matter digestibility (IVDMD) and dry matter losses (DML) of elephant grass, Napier, ensiled with different additives (Andrade and Melotti, 2004). Equal means in column do not differ ($P > 0.05$), CV = coefficient of variation.

In more recent studies, evaluating the effect of four additives in sugar cane silage (sugarcane with 1.5% of urea; 0.5% of urea + 4% of corn; 0.5% of urea + 4% of dried cassava, 1.5% of starea and sugar cane control), Lopes & Evangelista (2010) concluded that the additive 0.5% urea + 4% corn, provides better results to the sugar cane silage.

Ávila et al. (2006), using combinations of different additives types (citrus pulp, wheat bran, and corn meal) with various doses (3, 6, 9 and 12%), found that Tanzania grass has low soluble carbohydrates contents and citrus pulp was the additive which contributed to increase the forage carbohydrate concentration and to reduce the buffering capacity. It provides an increase in the relation soluble carbohydrate x buffering capacity and better conditions for the fermentation process, resulting in better quality silages.

Besides the additives, some management practices from the harvest time to the silo sealing can influence the LAB development. When the grass is chopped at harvest time, the LAB population tends to increase due to reactivation of dormant and non-culturable cells. Thus,

as faster the time between cutting the grass and sealing the silo, better will be the fermentation conditions.

The well done compaction and sealing is one of the secrets for good silage. It serves to expel the air from inside the forage mass, considering that air presence affects the fermentation process, implicating in losses caused by undesirable microorganisms. According to Senger et al. (2005) the original material must present compression level exceeding 650 kg/m^3 of green matter, reducing the quality losses of the ensiled material.

Furthermore, the particle size influences the compression and consequently the silo density. Igarasi (2002) observed an inverse relationship between particle size and silage density, suggesting that as smaller the particle size greater the density, and thus there will be more oxygen remaining among the plant particles.

Neumann et al. (2007) evaluating the effect of particle size (small: 0.2 to 0.6 cm or large: 1.0 to 2.0 cm) and cutting height of corn plants (low: 15 cm or higher: 39 cm) on silage fermentation dynamics and opening period, found that small sized particles provide greater compression efficiency and consequently reduces temperature and pH gradients in the silo opening time. The temperature differential between silage and environment is greater on the top, what is related with the time that the silo remain opened and exposed to the external environment and also the lower compression efficiency. It causes an increase in ammoniac nitrogen content and elevation of silage pH values, indicating changes in silage nutritional value.

The plant moisture content and the particle size after chopping are directly related to the compression. Excessively wet forage provides favorable conditions for butyric fermentation and, favors nutrients losses through leaching, and proteins degradation. On the other hand, forage with high dry matter content hinders compaction and air expulsion in the ensiling process. Amaral et al. (2007) found that increase in compression of 100 to 160 kg MS/m^3 increased effluent production from 2.2 to 9.8 kg/t of green matter.

Summarizing, as faster and more efficient the process of harvest, chopping, compaction and sealing, greater is the amount of LAB present in silage, and thus lower the losses.

6. Conclusions

The increase in lactic acid fermentation is a big challenge for tropical grass silages confection, determining the success of this technology. It is really important to know the species of lactic acid bacteria prevalent in tropical grasses as well as their metabolism in order to obtain maximum use with its utilization.

The use of lactic acid bacteria as microbial inoculants in tropical grasses silage still shows some inconsistency in the results obtained in research works. More research that evaluates their effects on the fermentation parameters, dry matter losses and mainly on the quality, regarding nutrient intake and animal performance is required.

However, tropical grass silages represent a promising technology for livestock in areas threatened by periodic droughts. Furthermore, in tropical countries like Brazil, this practice has been quite taken by the producers.

Author details

Edson Mauro Santos and Carlos Henrique Oliveira Macedo
Department of Animal Science, Federal University of Paraíba, Areia, PB, Brazil

Thiago Carvalho da Silva
Department of Animal Science, Federal University of Viçosa, Viçosa, MG, Brazil

Fleming Sena Campos
Department of Animal Science, Federal University of Bahia, Salvador, BA, Brazil

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