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Urochloabrizantha and corn or sorghum silage integrated production: agronomic evaluation, fermentation losses, and aerobic stability of silage

Tiago Antônio Del Valle^{2*} Francine Basso Facco² Mariana Campana¹ Rebeca Rodrigues Noernberg¹ Thainá Moreira Garcia¹ Estefani Capucho¹ Francisco Rafael da Silva Pereira³ Jozivaldo Prudêncio Gomes de Morais¹

ABSTRACT: The present study evaluated germination, production, and morphological composition of *Urochloabrizantha* intercropped with corn and sorghum; and silage fermentation losses and aerobic stability of intercrop silage using microbial inoculant. Twenty experimental parcels $(5.0 \times 3.6 \text{ m})$ were used in a blocked randomized design to evaluate four treatments obtained from a 2×2 factorial arrangements: I) crop material (corn vs. sorghum) and II) Brachiaria (U. brizantha) establishment (present vs. absent). Corn- and sorghum-brachiaria integrated systems showed similar brachiaria germination, forage yield, and morphological composition. There was no crop and brachiaria interaction effect on the variables related to corn and sorghum plants and the total productivity. Brachiaria decreased the stem diameter and increased the population of maize and sorghum plants. However, it did not affect systems productivity. Microbial inoculation did not affect corn silage effluent losses and reduced sorghum silage effluent losses. In corn silage, brachiaria did not affect gas losses, while in sorghum silage, brachiaria increased the gas losses. Total losses were higher in sorghum silage than in corn silage, which resulted in a lower DM recovery. The treatments did not affect the pH of the silage after aerobic exposure. However, brachiaria increased silage temperature evaluated at 32 and 40 hours after aerobic exposure. Thus, corn or sorghum consortium has similar brachiaria morphological composition and productivity. Moreover, in intercropped silage, brachiaria increases effluent losses and reduces silage aerobic stability.

Key words: brachiaria, crop production, intercrop silage, microbial inoculant.

Produção integrada de silagem de *Urochloabrizantha* e silagem de milho ou sorgo: avaliação agronômica, perdas de fermentação e estabilidade aeróbia da silagem

RESUMO: O presente estudo tem por objetivo avaliar a germinação, produção e composição morfológica de *Urochloabrizantha* consorciada com milho e sorgo, perdas de fermentação da silagem e estabilidade aeróbia da silagem consorciada com inoculante microbiano. Vinte parcelas experimentais (5.0 × 3.6 m) foram usadas em um delineamento em blocos casualizados para avaliar quatro tratamentos em arranjo fatorial 2 x 2: I) Cultura (milho vs. sorgo) e II) Estabelecimento da Brachiaria (*U. brizantha*) (presente vs. ausente). Os sistemas integrados milho e sorgo-brachiaria apresentaram germinação, produção de forragem e composição morfológica semelhante. Não houve efeito da interação cultura e braquiária sobre as variáveis relacionadas às plantas de milho e sorgo e a produtividade total. A brachiaria diminuiu o diâmetro do caule e aumentou a população de plantas de milho e sorgo. No entanto, não afetou a produtividade dos sistemas. A inoculação microbiana não afetou as perdas de efluente da silagem de milho, a brachiaria não afetou as perdas de gás, enquanto na silagem de sorgo, a brachiaria aumentou as perdas de gás. As perdas totais foram maiores na silagem de sorgo do que na silagem de milho, o que resultou em menor recuperação da MS. Os tratamentos não afetaram o pH da silagem pós exposição aeróbia. Contudo, a brachiaria aumentou a temperatura da silagem 32 e 40 horas após exposição aeróbia. Portanto, os consórcios milho e sorgo-brachiaria apresentam composição morfológica e produtividade semelhantes. Além disso, na silagem consorciada, a brachiaria aumenta as perdas por efluentes e reduz a estabilidade aeróbia.

Palavras-chave: brachiaria, silagem consorciada, inoculante microbiano, produção agrícola.

INTRODUCTION

Silage production has been one of the most used strategies by farmers to meet the forage requirements of ruminants during the dry periods (ARAÚJO et al., 2020). However, every day it becomes more difficult for the producer to get good harvests due to rapid intensification of climate change. Pasture and annual crops integrated production have been used to improve area utilization and reduce costs of pasture establishment (FREITAS et al., 2005). This strategy could benefit smallholdings too.

According to FREITAS et al. (2005) after harvesting and ensiling of crops, the soil becomes unprotected and physical characteristics are altered by machine traffic. Intercrop silage accelerates forage production and reduces the negative effects

¹Departamentode Biotecnologia Vegetal e Produção Animal, Centro de Ciências Agrícolas, Universidade Federal de São Carlos (UFSCAR), Araras, SP, Brasil.

²Departamento de Zootecnia, CCR, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, 97.105-900, Brasil. E-mail: tiago.valle@ufsm.br.
*Corresponding author.

³Instituto Federal de Alagoas (UFAL), Satuba, Alagoas, Brasil.

Del Valle et al.

of ensiling on soil structure (COSTA et al., 2012). Brachiaria (*Urochloa spp.*) can produce large root systems (CHIODEROLI et al., 2010), which favor soil aeration and infiltration. OLIVEIRA et al. (2019) evaluated different Brachiaria species and observed higher biomass production and nutrient cycling using U. brizantha. Conversely, corn and sorghum are the most widely grown crop for silage production (BERNARDES & RÊGO, 2014). According to TSUMANUMA et al (2012), corn has a higher initial growth rate, which potentially affects brachiaria establishment. Therefore, we hypothesized that brachiaria germination, crop and forage production, and leaves content of forage would be lower when corn was used instead of sorghum in intercropping.

Although, brachiaria intercrop increases feed production per area (COSTA et al., 2016), it could affect silage fermentation profile because grass has lower water-soluble carbohydrates (WSC) (HAIGH, 1990) and higher buffering capacity (PALUDO et al., 2020) than whole-plant sorghum and corn silages. Therefore, homofermentative lactic acid bacteria (LAB) inoculants have been used to inhibit grass silage fermentation losses; although, LAB can reduce the aerobic stability of silage (OLIVEIRA et al., 2017). Our second hypothesis is that microbial inoculants could inhibit fermentation losses, especially in brachiariacontaining silages, whereas inoculation increased silage pH and temperature after aerobic exposure regardless of crop and brachiaria levels. The present study aimed to evaluate: 1) crop (corn vs. sorghum) effect on brachiaria germination, growth, and morphological composition; 2) crop and brachiaria addition effects on crop characteristics and systems production; 3) crop, brachiaria, and homofermentative LAB inoculant effects on silage fermentation losses and aerobic stability.

MATERIALS AND METHODS

Trial was performed from January until April 2019 at the Agrarian Sciences Center of Universidade Federal de São Carlos (UFSCar), Araras, Brazil: 22°18' S latitude, 47°22' W longitude, and 665 m asl.

Agronomic management and treatments

Twenty experimental parcels $(5.0 \times 3.6 \text{ m})$ were used in a blocked randomized design to evaluate four treatments obtained from a 2 \times 2 factorial arrangements: I) crop material (corn

[4285VYHR®, Pioneer, Des Moines, USA] vs. sorghum [BM-515, SementesBiomatrix, Rio Claro, Brazil]) and II) Brachiaria (*Urochloabrizantha*, c.v. MG-4) establishment (present vs. absent). Soil was traditionally prepared and seeded on January 10, 2019. It used 13 corn seeds/m, 25 sorghum seeds/m, and 2.22 g/m² of brachiaria seeds. Row space was 90 cm. Fertilization was performed using 400 kg/ha of a commercial formulation (40 g/kg of N, 140 g/kg of P, and 80 g/kg of K) at seeding and 300 kg/ha of another commercial formulation (200 g/kg of N, 50 g/kg of P, and 200 g/kg of K), twenty days after seeding.

Agronomic evaluations

Brachiaria germination was evaluated 15 days after seeding using two areas of 0.25 m² in every experimental unit. Ninety-one days after seeding (April 11), crops found 300 to 350 g/kg of dry matter content and harvest was performed. It was evaluated brachiaria morphological composition after oven dried, as described by RODRIGUES et al. (2020). Crop height, height of 1st spike, and stem diameter were evaluated using ten plants. The crop plant stand was evaluated considering the usefularea of parcels 3.0 × 2.7 m. The harvest was performed at a 5-cm height from soil. After harvesting, it was weighed crops and brachiaria, and samples were obtained to perform chemical analysis.

Crops processing and ensiling process

Crops were processed in a stationary mill (TRF300, Trapp, Jaraguá do Sul, Brazil), and two experimental silos were prepared from each parcel, according to a split plot design, to evaluate microbial inoculation of silages. Silos were prepared in PVC tubes 28 cm in diameter, 25 cm high and equipped with a Bunsen valve to allow gas to escape. Silage specific density was standardized as 650 kg/m³, and fresh material for each silos was weighed before inoculants supplied. It was evaluated the following levels of microbial inoculation: -INO: non-inoculated silages; and +INO: inoculation with 160,000 colony-forming units (CFU)/g fresh matter of Lactobacillus plantarum and 160,000 CFU/g fresh matter of Pediococcusacidilactici. Inoculation was performed using 4 g/tof a commercial inoculant (Kera SIL, KeraNutrição Animal, Bento Gonçalves, Brazil). Inoculation level was defined according to manufacturer recommendation.

Fermentation losses and aerobic stability evaluation

Silos were stored in room temperature and opened 60 days after ensiling. Five kilograms of sand were placed at the bottom of silos. The weight of empty and sealed silos at ensiling and at opening was recorded. Fermentation losses were calculated according to JOBIM et al. (2007): gas losses (GL) were obtained by difference between whole silo weight at ensiling and opening. The difference between weight of empty silo before ensiling and after opening was considered effluent losses (EL). Total losses were calculated by sum of gas and effluent losses. Dry matter recovery (DMR) was estimated by the ratio between DM at silos after silos opening and ensiled DM.

Aerobic stability evaluation performed using 3 kg of silage. It was placed in a plastic bucket and maintained in a controlled temperature room (17.8 \pm 1.59; mean \pm S.D.) for seven days. Temperature of the silage center was recorded every 8 h using an infrared digital (DT-8380, thermometer Tianjin Cheerman Technology Co. Ltd., Tianjin, China) and silage pH was evaluated after dilution in distilled water (15 g:150 mL; KUNG JR. et al., 1984).

Statistical analysis

using PROC MIXED of SAS (Version 9.4) and the following model:

$$Y_{ij} = \mu + C_i + b_j + e_{ij}$$

With $b_j \sim N(0, \sigma_b^2)$ and $e_{ij} \sim N(0, \sigma_e^2)$; where: Y_{ij} is observed value of dependent variable;

Data of brachiaria germination, production and chemical composition were analyzed

 μ is overall mean; C_i is the fixed effect of crop (i = 1 and 2); b_j is the random effect of block (j = 1 to 4); e_{ij} is the random residue; N stands for Gaussian distribution; σ_b^2 and σ_e^2 are the variances associated with block and residue random effects. Spike characteristics were evaluated using a similar model considering brachiaria (B) effect instead of C_i effect; where B_i effect is the fixed effect of brachiaria addition (i = 1 and 2).

Data of crop production and plants measures were analyzed using the model:

$$Y_{ijk} = \mu + C_i + B_j + C \times B_{ij} + b_k + e_{ijk}$$

With $b_j \sim N(0, \sigma_b^2)$ and $e_{ijk} \sim N(0, \sigma_e^2)$ where: Y_{iik} is observed value of dependent variable; $C \times B_{ii}$ is the fixed interaction effect between crop and brachiaria; and e_{iik} is the random residue. Data of fermentation losses and DM recovery of silage were

analyzed using the following model:
$$Y_{ijkl} = \mu + C_i + B_j + C \times B_{ij} + b_k + \omega_{ijk} + I_l + C \times I_{il} + B \times I_{jl} \\ + C \times B \times I_{ijl} + e_{ijkl}$$

With
$$b_j \sim N(0, \sigma_b^2)$$
, $\omega_{ijk} \sim N(0, \sigma_\omega^2)$,

and $e_{ijkl} \sim N(0, \sigma_e^2)$; where: Y_{ijkl} is observed value of dependent variable; ω_{ijk} is the random residue associated with parcels effect; I_{i} is the fixed effect of microbial inoculant (l = 1 and 2); $C \times I_{il}$, $B \times I_{il}$, and $C \times B \times I_{iii}$ are interaction between previously defined fixed effects; and σ_{ω}^2 is the variance associated with parcels effect. Data of silage temperature and pH after aerobic exposure were analyzed as repeated measures considering previously defined model and time effect, as well as its interaction with other fixed effects of the model. Matrix of variance and covariance were chosen base on Bayesian method.

Table 1 - Brachiaria germination, production, and morphological composition in corn or sorghum intercrop.

Item	Cı	rops	SEM ¹	Probabilities ²		
	Corn	Sorghum				
Germination, plants/m2	46.8	47.2	3.68	0.956		
Forage yield						
ton. as-fed/ha	3.58	4.02	0.358	0.621		
ton. DM/ha	0.711	0.829	0.0723	0.433		
Leaves	0.494	0.468	0.0221	0.562		
Stalk	0.409	0.434	0.0297	0.684		
Deadmatter	0.097	0.099	0.0157	0.952		
Leaves: stalkratio	1.21	1.08	0.181	0.981		

¹Standard error of mean.

²Probabilities (corn vs. sorghum).

Table 2 - Corn and sorghum production and measurements in brachiaria intercrop.

Item	Co	rn	Sorg	ghum	SEM ²	Probabilities ³				
	- B ¹	$+B^1$	-B ¹	$+B^1$	1.2	Crop	В	B×Crop		
Plantheight, cm	218	221	226	225	0.023	0.007	0.531	0.266		
Stemdiameter, cm	2.00	1.94	1.20	1.02	2.2	< 0.001	0.012	0.213		
Height of 1st spike, cm	112	120	-	-	0.075	-	0.134	=		
Spike/plant	2.20	2.08	-	-	0.171	-	0.446	-		
Stand plants/m ²	2.36	2.68	1.94	2.29		0.016	0.040	0.933		
Cropyield					1.08					
ton. as-fed/ha	36.6	32.8	31.5	30.0	0.39	0.028	0.115	0.482		
ton. DM/ha	12.4	11.1	11.9	11.3		0.776	0.128	0.544		
Total yield					1.10					
ton. as-fed/ha	36.6	36.4	31.5	34.0	0.39	0.042	0.498	0.420		
ton. DM/ha	12.4	11.8	11.9	12.1	1.2	0.858	0.783	0.488		

¹Brachiaria seeding during crop establishment (-B: absence and +B: presence).

RESULTS

Corn and sorghum-brachiaria integrated systems showed similar brachiaria germination (P = 0.956), forage yield (P \geq 0.433) and morphological composition (P \geq 0.562) (Table 1). In addition, there was no interaction between crop and brachiaria on the variables related to corn and sorghum plants and the total production of the areas (Table 2; P \geq 0.213).

Sorghum had taller plants (P=0.007) and of smaller stem diameter (P<0.001) than corn. Corn had a higher yield per unit of area (P=0.028) and higher total natural matter production (crop + brachiaria as-fed; P=0.042) than sorghum. The presence of brachiaria has decreased the stem diameter (P=0.012) and increased the population of maize and sorghum plants (P=0.040). However, it did not affect production by area ($P\ge0.115$).

Table 3 - Fermentation losses, pH, and DM content at opening of sorghum or corn and brachiaria intercrop silage treated with homolactic inoculants.

Item	Crop		B ¹		SEM		Ino ²		SEM ³		Probabilities ⁴				
	Corn	Sorghum		-	-	Crop	+	-		В	Ino	Crop×B	Crop× Ino	B×Ino	Crop× B×Ino
Effluentlosses															
g/kg as-fed	10.5	25.2	0.89	19.5	20.4	< 0.001	15.3	19.5	0.85	0.045	0.047	0.061	0.031	0.844	0.772
g/kg DM	31.4	68.1	2.53	54.3	55.5	< 0.001	44.0	54.3	2.39	0.102	0.048	0.098	0.031	0.802	0.738
Gaslosses															
g/kg as-fed	5.35	6.67	0.197	5.26	5.57	0.024	6.45	5.26	0.201	0.117	0.005	0.051	0.598	0.871	0.530
g/kg DM	16.1	18.2	0.58	15.0	15.6	0.188	18.7	15.0	0.59	0.060	0.005	0.055	0.693	0.829	0.519
Total losses															
g/kg as-fed	15.9	31.9	0.78	24.7	26.0	< 0.001	21.9	24.7	0.79	0.061	0.321	0.109	0.065	0.961	0.969
g/kg DM	47.8	86.7	2.22	69.4	71.3	< 0.001	63.3	69.4	2.22	0.184	0.356	0.187	0.066	0.979	0.944
DM recovery g/kg	893	818	5.6	857	829	< 0.001	883	857	5.3	0.003	0.806	0.211	0.633	0.869	0.343
Silage pH	3.97	4.02	0.016	3.95	3.97	0.353	4.02	3.95	0.014	0.276	0.017	0.178	0.453	0.722	0.225
Silage DM g/kg	300	307	2.3	304	303	0.219	304	304	2.2	0.800	0.875	0.792	0.486	0.964	0.651

¹Brachiaria seeding during crop establishment (-B: absence and +B: presence).

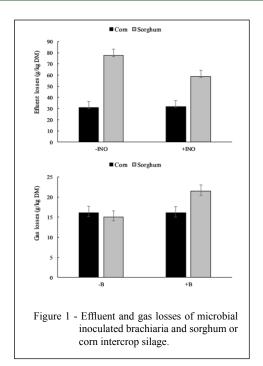
²Standard error of mean.

³Probabilities: Crop (corn vs. sorghum); B: brachiaria (-B vs. +B); B×Crop: brachiaria and crop interaction effect.

²Inoculation: absence or presence: 160,000 colony-forming units (CFU)/g of *Lactobacillus plantarum* and 160,000 CFU/g of *Pediococcusacidilactici*.

³Standard error of mean.

⁴Probabilities: Crop (corn vs. sorghum); B: brachiaria (-B vs. +B); INO (presence vs. absence).



There was an interaction between the effects of crop and inoculant on effluent losses (Table 3; P = 0.031). The addition of inoculant did not affect corn silage effluent losses (P > 0.05) and reduced sorghum silage effluent losses (Figure 1; $P \le 0.05$). In general, effluent losses (P < 0.001) from sorghum silage were greater than from corn, and both brachiaria and inoculant reduced effluent losses (P \leq 0.045). There was an interaction between the effects of crop and brachiaria on gas losses (P \leq 0.055): in corn silage, brachiaria did not affect gas losses (P > 0.05), while in sorghum silage, brachiaria increased the gas losses (P \leq 0.05). There was no interaction between the factors evaluated in the present study (crop, brachiaria, and inoculant) on total losses, dry matter recovery, pH, and DM content of the silage at the opening (P \geq 0.065). Total losses (P < 0.001) were greater in sorghum silage than in corn silage, which resulted in a lower DM recovery (P < 0.001).

The treatments did not affect the pH of the silage after aerobic exposure ($P \ge 0.402$), regardless of the evaluation time (Table 4; $P \ge 0.194$). There was an interaction between the effects of brachiaria and time on silage temperature after aerobic exposure (P = 0.026; Figure 2). Brachiaria increased the temperature of the silage evaluated at 32 and 40 hours after aerobic exposure ($P \le 0.05$) and decreased the temperature in the evaluations performed 72, 80, 88 e 96 hours after aerobic exposure ($P \le 0.05$).

DISCUSSION

Although, we had hypothesized that crop species could affect brachiaria germination, forage production, and leaves content, no differences among species were observed on these variables. Sorghum silage had higher fermentation losses and lower DM recovery compared to corn silage. Microbial inoculation reduced sorghum silage effluent losses, but had no other effects on fermentation losses and aerobic stability. Brachiaria addition in the intercrop silage production increased sorghum silage gas losses and decreased silage aerobic stability.

Corn and sorghum-brachiaria consortia showed similar brachiaria germination, production, and morphological composition. As reported by PUGH et al. (2018), corn and sorghum has a logarithmic height up to the flowering. Although, crops had small differences in growth curves, initial growth of crops was small and had no effect on brachiaria characteristics in the present study. Therefore, different from our hypothesis, crops (corn and sorghum) did not affect brachiaria effects on system productivity.

Evaluating corn and Brachiaria intercrop, OLIVEIRA et al. (2019) observed higher nitrogen cycling and biomass accumulation when Urochloabrizantha was associated with corn. These improved nutrients cycling could explain absence of brachiaria presence effect on crop

Del Valle et al.

Table 4 - Temperature above environmental temperature and silage pH after aerobic exposure of sorghum or corn and brachiaria intercrop silage treated with homolactic inoculants.

Item	C	rop	B ¹		B ¹ S		SEM	Ino ² SEM ³		Probabilities ⁴								
	Corn	Sorghum	-	+			-		+		Crop	В	Ino	Crop×B	Crop× Ino	B×Ino	Crop× B×Ino	
Temperature, °C	1.32	1.36	1.43	1.25	0.109	1.47	1.47	0.117	1.21	0.117	0.814	0.313	0.092	0.207	0.075	0.892	0.584	
pH	4.54	4.54	4.55	4.54	0.057	4.54	4.54	0.062	4.54	0.062	0.972	0.944	0.971	0.402	0.444	0.711	0.935	

¹Brachiaria seeding during crop establishment (-B: absence and +B: presence).

Other non-reported P-values: Time: P < 0.001; Time×crop: $P \ge 0.194$; Time×B: $P \ge 0.026$; Time×Ino: $P \ge 0.191$; Time×Crop×B: $P \ge 0.373$; Time×Crop×Ino: $P \ge 0.667$; Time×B×Ino: $P \ge 0.290$; Time×Crop×B×Ino: $P \ge 0.663$.

production. FREITAS et al. (2008), evaluating corn-brachiaria intercropping, also reported that forage presence did not affect corn growth. This author associated this result with the low initial development rate of plants (FREITAS et al., 2008). SODRÉ (2021) also observed no effects of brachiaria addition of crop production when he evaluated the sorghum-grass intercrop.

According to SANTOS et al. (2019), sorghum easily adapts to direct competition with other species. Among physiological responses to competition for light and physical space, stem elongation is one of most known (OLIVEIRA et al., 2020). In the present study, sorghum showed 2.7% increased height and 43.7% decreased stem diameter compared to corn. According to SANGOI (2001), greater stands positively affect grain yield. However, it was not evaluated in the current study. Conversely, brachiaria decreased 7.5% of crops stem diameter, and increased crops stand. Studying alfalfa and corn intercrop production, BERTI et al. (2021) observed reduced biomass production compared to corn single establishment. According to these authors, intercrop production is largely affected by rainfall during critical months of corn growth. Reduced stem diameter observed when brachiaria was added to the intercrop system could increase lodging and stembreakage risks (CARNEIRO JÚNIOR et al., 2021).

In general, sorghum had higher fermentation losses and lower DM recovery compared to corn silage. Sorghum has reduced levels of WSC compared to corn (SANTOS & ZANINE, 2007). The content of WSC is essential as a substrate for lactic

acid bacteria development and acid silage stability (MCDONALD et al., 1991). Delayed pH drop is associated with increased losses and reduced DM recovery due to CO₂ and H₂O production during the ensiling process (MUCK, 2010). SUCU et al. (2016) have already documented higher fermentation losses of sorghum compared to corn silage.

Besides the higher fermentation losses observed in sorghum silage, it was also observed a reduction on sorghum silage effluent losses by microbial inoculation. Although effluent losses are mainly affected by the dry matter content of the ensiled forage (BUXTON et al., 2003; OLIVEIRA et al., 2010), JUNGES et al. (2013) stated that homolactic inoculants could reduce effluent losses due to fast silage pH decrease and reduction of cell ruption by plant enzymes (MCDONALD et al., 1991). However, microbial inoculants had no effect on corn effluent losses in the present study. Corn silage naturally has an adequate biochemical profile, showing no advantage when inoculated (SANTOS & ZANINE, 2007).

One of the main factors that affect fermentation during the ensiling process is the dry matter values reported in this study are within the range of values from 26% to 38% recommended by MCDONALD et al. (1991). Grasses in the growth stage, despite having good nutritive values, have a low content of soluble carbohydrates, high buffering power, and low dry matter content, putting the conservation process through ensiling at risk (EVANGELISTA et al., 2004). SANTOS & ZANINE (2007) reported that pre-wilting is a technique that makes it possible to increase the dry matter content

²Inoculation: absence or presence: 160,000 colony-forming units (CFU)/g of *Lactobacillus plantarum* and 160,000 CFU/g of *Pediococcusacidilactici*.

³Standard error of mean.

⁴Probabilities: Crop (corn vs. sorghum); B: brachiaria (-B vs. +B); INO (presence vs. absence).

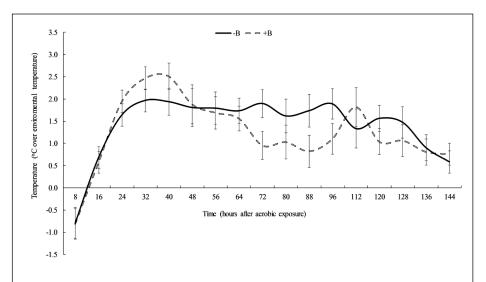


Figure 2 - Temperature after aerobic exposure of sorghum or corn and brachiaria intercrop silage treated with homolactic inoculants.

of grasses before ensiling, avoiding undesirable fermentation. In the present study, increased moisture content provided by brachiaria addition increased gas losses, especially in sorghum silage.

Although, there was no treatment effect on silage pH after aerobic exposure, brachiaria increased the temperature of the silage evaluated at 32 and 40 hours after aerobic exposure. Increased temperature is the result of aerobic bacteria, filamentous fungi, and yeast growth. We agreed that increased water activity observed in brachiaria-containing silages improved aerobic degradation microorganism's growth (MUCK, 2010), even with no effects of brachiaria on intercrop silages DM content.

CONCLUSION

Corn or sorghum consortium has similar brachiaria morphological composition and productively. Brachiaria increases silage effluent losses and reduces the aerobic stability of intercrop silage, but has no negative effect on intercrop silage production.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTIONS

JPGM, MC and FRSP conceived and designed experiments. RRN, TMG and EC performed the experiments and laboratory analysis. TAD and FBF performed statistical analyzes of experimental data and prepared the draft manuscript. All authors critically reviewed the manuscript and approved the final version.

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