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# Natamycin as a potential silage additive: A lab trial using sugarcane to assess greenhouse gas emissions

**Antonio Vinicius Iank Bueno[1], Gabriela Letícia Delai Vigne[2]*, Charles Ortiz Novinski[2], Cimélio Bayer[3], Clóves Cabreira Jobim[1], Patrick Schmidt[2]**

[1] Universidade Estadual de Maringá, Departamento de Zootecnia, Maringá, PR, Brasil.
[2] Universidade Federal do Paraná, Departamento de Zootecnia, Curitiba, PR, Brasil.
[3] Universidade Federal do Rio Grande do Sul, Departamento de Solos, Porto Alegre, RS, Brasil.

***Corresponding author:**
gabivigne@ufpr.br

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**ABSTRACT** - The objective of this study was to evaluate natamycin, *Lactobacillus buchneri* (LB), or their combination on the chemical composition, loss, fermentative profile, and aerobic stability as well as gas production and composition of sugarcane silages. The treatments were (wet basis): no additive (control), 10 g $t^{-1}$ of natamycin (N10), 5 × $10^4$ cfu $g^{-1}$ of LB, and the combination of 4 g $t^{-1}$ of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of LB (NLB). Sugarcane was chopped (10 mm), treated with the additives, and ensiled in experimental silos (four replicates). The silos remained stored for 51 days. The LB inoculation, alone or in combination with natamycin, increased the acetic acid content (by 105 and 78% respectively) and decreased ethanol content (by 83 and 71% respectively) when compared to N10 treatment and the control. A decrease in both dry matter and gas losses was observed in the LB (by 72 and 78%, respectively) and N10 (by 69 and 77%, respectively) silages compared with the control, but not the combination. The N10 treatment reduced greenhouse gas (GHG) emission by 86% compared with the control silage. Control and N10 silages deteriorated to the same extent with aerobic exposure, whereas LB and NLB presented higher aerobic stability. The use of natamycin alone is not recommended when ethanol and aerobic stability are concerns. However, natamycin may be considered for the composition of blend additives to decrease greenhouse gas emission and fermentative loss in silages. Further studies must be carried out to optimize doses of natamycin in blend additives.

**Keywords:** ethanol, gas production, inoculant, methane, yeast

## 1. Introduction

Crop and livestock production systems are claimed as important sources of greenhouse gases (GHG), accounting for approximately 10 and 14.5% of total GHG emissions worldwide (Gerber et al., 2013; Tubiello et al., 2013; Tubiello et al., 2015). However, silage production has been scarcely studied as a possible source of GHG (Grossi et al., 2019).

Ensiling is one of the most common methods for long-term forage storage for ruminants, and its fermentation can lead to on-farm GHG emission. According to Henriksson et al. (2012), higher dry matter (DM) losses during fermentation are linked to higher GHG emission from productive systems based on silage. However, fermentation gases were not assessed by those authors.

Several microorganisms are responsible for fermentation loss and gas emission from silage (e.g., heterofermentative lactic acid bacteria [LAB], clostridia, enterobacteria). Yeast activity in silages, for

instance, increases gas emission (mostly $CO_2$) as well as volatile organic compound synthesis (mainly ethanol), reducing air quality and greatly contributing to greenhouse effect (McDonald et al., 1991; Hafner et al., 2010; Borreani et al., 2018).

In Brazil, sugarcane (*Saccharum officinarum*) is one of the main crops studied for ensiling purposes. Since sugarcane is highly prone to ethanolic fermentation due to its high sugar availability and indigenous yeast population (Pedroso et al., 2005; Ávila et al., 2010a), many studies have been conducted to determine suitable additives to avoid yeast development and reduce gas production (Pedroso et al., 2005; Ávila et al., 2014). Thus, sugarcane silage can be considered a good model for assessing gas production and its control through additives.

*Lactobacillus buchneri* (LB) is an obligate heterofermentative bacterium that has been widely studied as a sugarcane additive to reduce yeast activity. It converts not only sugars but also lactic acid into acetic acid, which reduces yeast count, ethanol synthesis, and fermentative loss and increases aerobic stability compared with untreated silage (Holzer et al., 2003; Pedroso et al., 2008; Ávila et al., 2010b; Ávila et al., 2012). However, the results of LB inoculation in sugarcane silages are highly variable (Schmidt, 2009).

The design of new additives might be helpful to better decrease DM loss as well as GHG emissions from ensiling (Muck et al., 2018), and their combination with chemicals can improve the results of microbial additive inoculation.

Natamycin is a polyene macrolide (bacteriocin) produced by *Streptomyces natalensis, Streptomyces chattanoogensis,* or *Streptomyces gilvosporeus* and is commonly used as a food additive (e.g., in hard cheeses, sausages, wine, fermented olive) to avoid yeast spoilage (Hondrodimou et al., 2011; Dalhoff and Levy, 2015; Wang et al., 2016). Natamycin binds ergosterol (fatty acid present in the cell wall of molds and yeasts), thereby impairing the selective permeability of the cell (Welscher et al., 2008). It is poorly absorbed in the digestive system of mammals and totally excreted in the feces, making it safe for use in human food and feed (EAEMP, 1998). Recently, Shah et al. (2020) reported a low yeast count in elephant grass silage treated with natamycin. Pinto et al. (2020) observed the synergistic effect of combining natamycin and LB in corn silages. However, information about the use of natamycin as an additive in sugarcane silage as well as its combination with LB has never been reported. Thus, we hypothesized that the use of natamycin, alone or combined with a commonly used additive (LB), would be an important tool for mitigating GHG emission from sugarcane silage. Our objective was to evaluate gas production, chemical composition, aerobic stability, and fermentative loss resulting from sugarcane silages dosed with natamycin, with or without *L. buchneri*.

## 2. Material and Methods

### 2.1. Ensiling

The research was performed in Curitiba (25°25'42" S and 49°16'24" W), located in the state of Paraná, Brazil. Sugarcane was grown and harvested in Paranavaí (23°4'26" S and 52°27'55" W).

The sugarcane variety RB 72-454 (12 months regrowth, second harvest) was manually harvested (no chopping) in July 2012 (Table 1), and forage was carried to Curitiba the same day. The next day, the forage was processed in a stationary forage chopper adjusted to a theoretical length of 10 mm. Some moisture loss may have occurred between the harvest and processing (~20 h) stages; however, it was not measured.

The processed forage was split into small piles (four piles per treatment), and additives were manually sprayed and mixed into the forage. Four treatments were tested: control with no additive (distilled water only); 10 g $t^{-1}$ (wet basis) of natamycin (N10); 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri* NCIMB 40788 (LB – Lalsil Cana – Lallemand Animal Nutrition); and a combination of 4 g $t^{-1}$ of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of LB (NLB). The doses for treatments N10 and NLB were established to present

similar costs to the LB treatment. All the additives were diluted with distilled water and applied at the rate of 2 L $t^{-1}$. After spraying and mixing, a laboratory silo (20 L plastic bucket) was manually filled up with fresh forage from each pile (14.8 kg) and compacted (density of 220 kg DM $m^{-3}$).

The gas volume (GV) produced was recorded daily using a 1-L low-density polypropylene cylinder, as described by Restellato et al. (2019) (Figure 1). The silo lid was equipped with silicone hose that passed through a three-way stopcock for gas measurement and sampling. One cylinder (Ø-6.5 cm; 43 cm long) was connected to the stopcock of each silo by a silicone hose. In the bottom of the cylinder, a 2-mm hole was made and a hollow metal pin inserted to connect the hose. These cylinders were placed with the mouth facing down and immersed in water to avoid any gas leakage. The lid and all the connections were sealed airtight. Direct GV measurements were taken several times per day, and the gas was released after recording the volume. The total GV was the sum of all measurements of each silo during the fermentation period. To record the effluent production, the lab silos were previously filled in the bottom with dry sand (1.0 kg), covered with a thin plastic screen and two layers of cheesecloth. The silos remained stored for 51 days.

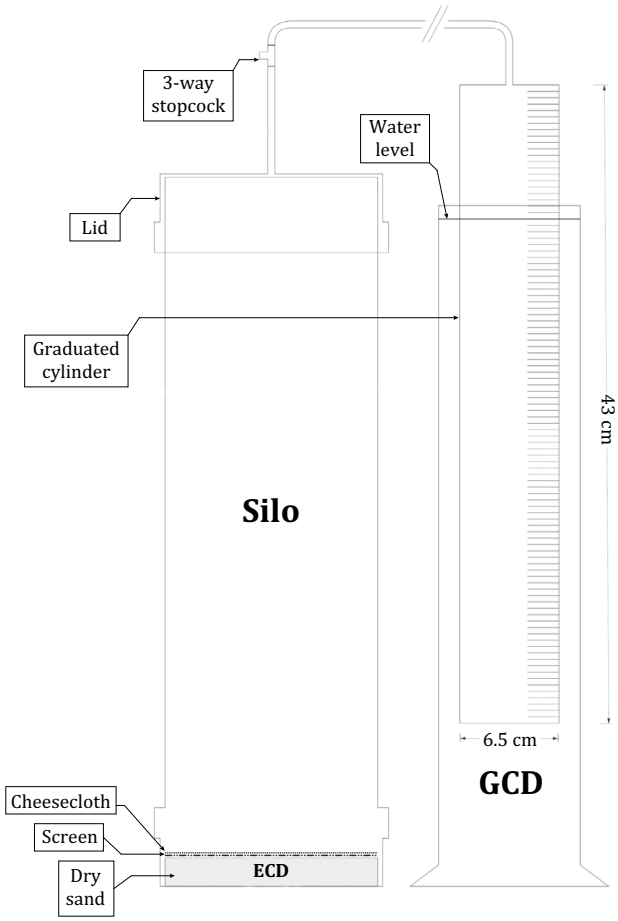



**Figure 1 -** Schematic illustration of the gas collector device (GCD) and effluent collector device (ECD). Adapted from Souza (2015).

## 2.2. Laboratory analyses

Fresh samples were taken from each treatment before ensiling to determine the dry matter content (55 °C during 72 h) in a forced-air- oven as well as Brix grade by using a refractometer (Table 1). From another sub-sample of each treatment, an aqueous extract (Kung Jr. et al., 2000) was prepared, and its pH value was measured using a potentiometer (model WTW 330i).

**Table 1 -** Dry matter content, Brix grade, and pH in fresh sugarcane before ensiling

| Item | Additive[1] | | | | SD |
|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | |
| Dry matter (g kg$^{-1}$) | 357 | 327 | 332 | 344 | 1.3 |
| Brix (°) | 20.6 | 22.4 | 20.1 | 21.4 | 1.0 |
| pH | 6.50 | 6.00 | 6.30 | 6.10 | 0.2 |

FM - fresh matter; cfu - colony-forming units; SD - standard deviation.
[1] Control - no additive; N10 - 10 g t$^{-1}$ FM of natamycin; LB - 5 × 10$^4$ cfu g$^{-1}$ of *L. buchneri*; NLB - 4 g t$^{-1}$ FM of natamycin and 2.5 × 10$^4$ cfu g$^{-1}$ of *L. buchneri.*

Gas samples were taken from each silo over two consecutive days (14 and 15 days after closing the silos) using a 20 mL polypropylene disposable syringe equipped with a stopcock. The syringe was connected to the system, and 10 movements of suction and expulsion were performed to homogenize the gas content before collecting the gas sample. The syringes containing the samples were packed with ice and immediately sent to the laboratory. Concentrations of $CO_2$, $N_2O$, and $CH_4$ were determined using a gas chromatograph (Shimadzu 14-A). The average gas composition of each silo was applied to the total gas produced by the same silo throughout the trial to calculate the total $CO_2$ equivalent (GHG) produced per ton of ensiled DM, according to Houghton et al. (2001). To reach those values, the gas composition (ppm for $CO_2$ and $CH_4$, and ppb for $N_2O$) was transformed to mg/m$^3$ using the molar mass of the molecules and the ideal gas equation. The result was applied to the total volume of gas produced per kg of forage to obtain the weight of the produced molecules (mg/kg of forage). The $CO_2$-equivalent production was estimated by applying correction factor 25 to $CH_4$ and 298 to $N_2O$.

Before opening, the silos were weighed again, and the gravimetric gas losses and DM loss were estimated using the initial and final weight of each silo and the DM contents of the fresh sugarcane and silages (Jobim et al., 2007). Upon opening, the first 5 cm were discharged, and the remaining silage was homogenized (in plastic bags) and sampled for further analyses.

A sub-sample from each silo (500 g) was dried in a forced-air oven at 55 °C for 72 h and milled (1-mm sieve) for laboratory analyses. The absolute dry matter (105 °C), ash, and crude protein (CP) contents were determined (AOAC, 2000). The neutral detergent fiber with thermostable amylase and ash-inclusive (aNDF) and acid detergent fiber (ADF) concentrations were sequentially assessed in accordance with Mertens (2002) and Van Soest (1963), respectively.

The fermentative profile was assessed for the silage extract obtained by hydraulic pressing the wet samples of each silo. The samples were prepared in accordance with Palmquist and Conrad (1971) and frozen for further analyses. The concentration of volatile fatty acids, lactic acid, and ethanol was performed by gas chromatography (Palmquist and Conrad, 1971).

The DM content, fermentative profile, and chemical composition variables of the silages were corrected for volatiles (Weissbach, 2011).

## 2.3. Aerobic stability

For assessing the aerobic stability, silage from each replicate was unpacked and homogenized, and 3 kg of silage was transferred to 20-L plastic buckets (no pressing) and exposed to air in a temperature-controlled room at 25 °C for 120 h. A mercury-in-glass thermometer was positioned in the center of the forage mass in each bucket, and the temperature was recorded twice daily (at 08:00 and 15:00 h). A second set of buckets was used to take daily samples of the silage for pH measurement. Samples were taken from a central point of each bucket. Aerobic stability was defined as the time (h) taken for the silage temperature to rise 2 °C above room temperature (Kung Jr. et al., 2000). The maximum temperature, time (h) to reach maximum temperature, and cumulative temperature were also assessed to evaluate aerobic deterioration intensity (Novinski et al., 2012).

### 2.4. Statistical analysis

Statistical analysis was performed using the MIXED procedure of SAS (Statistical Analysis System, version 9.0) as a completely randomized design with four treatments and four replicates, totalizing 16 experimental units (silos). The model included the fixed effect of additives. The results were analyzed with ANOVA, and the means were compared using the Tukey test at a 5% significance level.

Silage pH during aerobic exposure was analyzed using repeated measurements over time. The model included the fixed effects of additive, length of aerobic exposure, and their interactions. A covariance structure was chosen by considering the lowest Akaike Information Criterion (Littel et al., 1998). The structures of covariance tested included variance compounds (VC), compound symmetry (CS), first-order autoregressive (AR 1), and unstructured (UN).

## 3. Results

Additives did not change the pH, lactic acid, and butyric acid values of the silages (Table 2). The control and N10 silages presented higher concentrations of propionic acid and ethanol but a lower concentration of acetic acid than the LB and NLB silages.

The control presented higher GV and DM losses than the LB and N10 silages. However, the combination of additives was not capable of reducing the DM and gas losses (Table 3). Effluent production was not affected by the treatments. All the treated silages presented lower GV production than the control. The control silage had the highest GHG value compared with the additive-treated silages. However, the additive combination led to higher GHG emissions than the N10 silage.

**Table 2 -** Fermentative profile of sugarcane silages treated with natamycin, *Lactobacillus buchneri*, or their combination

| Item | Additive[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | | |
| pH | 3.61 | 3.54 | 3.59 | 3.52 | 0.02 | 0.23 |
| Lactic acid (g $kg^{-1}$ DM) | 55.1 | 47.3 | 37.0 | 47.3 | 4.88 | 0.32 |
| Acetic acid (g $kg^{-1}$ DM) | 14.6c | 14.7c | 29.9a | 26.0b | 0.50 | <0.01 |
| Propionic acid (g $kg^{-1}$ DM) | 1.08a | 0.93a | 0.33b | 0.50b | 0.05 | <0.01 |
| Butyric acid (g $kg^{-1}$ DM) | 0.41 | 0.26 | 0.35 | 0.36 | 0.09 | 0.77 |
| Ethanol (g $kg^{-1}$ DM) | 51.9a | 40.2a | 8.7b | 15.0b | 3.32 | <0.01 |

DM - dry matter; SEM - standard error of the mean.
[1] Control - no additive; N10 - 10 g $t^{-1}$ FM of natamycin; LB - 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*; NLB - 4 g $t^{-1}$ FM of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*.
Means followed by a different letter, in rows, are different (P≤0.05).

**Table 3 -** Gas volume produced, fermentative losses, and greenhouse gases equivalent (GHG) in sugarcane silages treated with natamycin, *Lactobacillus buchneri*, or their combination

| Item | Additive[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | | |
| Gas volume (L $t^{-1}$ DM) | 7282a | 2804b | 1050b | 3053b | 716.8 | <0.01 |
| Gas losses (g $kg^{-1}$ initial DM) | 117a | 27.1b | 25.7b | 86.2a | 22.8 | <0.01 |
| Effluent (kg $t^{-1}$ fresh forage) | 14.0 | 13.1 | 11.0 | 12.5 | 3.72 | 0.90 |
| DML (g $kg^{-1}$ DM) | 130a | 40.1b | 36.4b | 97.9ab | 23.8 | <0.01 |
| GHG[2] (g $CO_2$ eq $t^{-1}$ fresh forage) | 311a | 43.7c | 85.5bc | 162b | 28.3 | <0.01 |

DM - dry matter; SEM - standard error of the mean.
[1] Control - no additive; N10 - 10 g $t^{-1}$ FM of natamycin; LB - 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*; NLB - 4 g $t^{-1}$ FM of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*.
[2] Calculated according to Houghton et al. (2001).
Means followed by a different letter, in rows, are different (P≤0.05).

The untreated silage presented higher $CO_2$ emissions than the treated silages (Table 4). Of the inoculated silages, natamycin reduced $CO_2$ emission compared with NLB treatment. The control silage presented higher contents of $CH_4$ and $N_2O$ in its gas than the N10 treatment.

Silage treated with LB presented the highest aerobic stability, followed by NLB, N10, and control (Table 5). In addition, LB inoculation increased the time taken to reach the maximum temperature and reduced the accumulated temperature during aerobic exposure compared with the other treatments.

The pH values were similar among the treatments up to 48 h of aerobic exposure (Figure 2). However, in the N10 silages, pH increased after 72 h and was higher than in all other the treatments, remaining

**Table 4 -** Gas composition of sugarcane silages treated with natamycin, *Lactobacillus buchneri*, or their combination

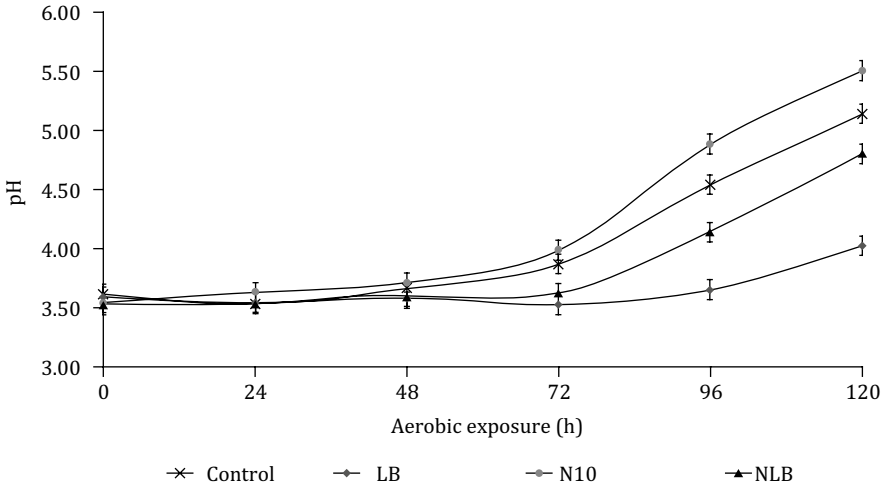
| Gas | Additive[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | | |
| $CH_4$ (g $t^{-1}$ DM) | 0.005a | 0.0007b | 0.001ab | 0.003ab | 0.0006 | 0.04 |
| $CO_2$ (g $t^{-1}$ DM) | 129a | 25.3c | 49.6bc | 68.8b | 10.5 | <0.01 |
| $N_2O$ (g $t^{-1}$ DM) | 0.002a | 0.0002b | 0.0005ab | 0.001ab | 0.0003 | 0.03 |

DM - dry matter; $CH_4$ - methane; $CO_2$ - carbon dioxide; $N_2O$ - nitrous oxide; SEM - standard error of the mean.
[1] Control - no additive; N10 - 10 g $t^{-1}$ FM of natamycin; LB - 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*; NLB - 4 g $t^{-1}$ FM of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of *L. buchneri.*
Means followed by a different letter, in rows, are different (P≤0.05).

**Table 5 -** Aerobic stability (AS) of sugarcane silages treated with natamycin, *Lactobacillus buchneri*, or their combination

| Item | Additive[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | | |
| AS[2] | 37.5c | 39.0c | 82.5a | 54.0b | 4.71 | <0.01 |
| MT (°C) | 40.0 | 40.5 | 40.7 | 41.5 | 0.52 | 0.66 |
| TMT | 67.5b | 61.5b | 111.0a | 81.0b | 5.52 | <0.01 |
| AT (°C) | 178a | 176a | 91b | 159a | 10.52 | <0.01 |

MT - maximum temperature; TMT - time (h) to reach the maximum temperature; AT -cumulated temperature.
[1] Control - no additive; N10 - 10 g $t^{-1}$ FM of natamycin; LB - 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*; NLB - 4 g $t^{-1}$ FM of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of *L. buchneri.*
[2] Time (h) to exceed 2 °C in the room temperature.
Means followed by a different letter, in rows, are different (P≤0.05).




SEM - standard error of the mean.
SEM = 0.0836; P<0.01 for the interaction additive × aerobic exposure.

**Figure 2 -** pH values during aerobic exposure of sugarcane silages treated with natamycin (N10), *L. buchneri* (LB), or their combination (NLB).

high until the end of aerobic exposure (120 h). The LB silage had the lowest pH until the end of the trial, followed by the NLB silage.

Regarding the chemical composition, the DM, ash, NDF, and ADF contents were similar for all the treatments; however, the N10 silages presented high CP content compared with the control and NLB silages, and similar to the LB-treated silages (Table 6).

**Table 6 -** Chemical composition (g $kg^{-1}$ DM unless stated) of sugarcane silages treated with natamycin, *Lactobacillus buchneri*, or their combination

| Item | Additive[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | Control | N10 | LB | NLB | | |
| Dry matter[2] (g $kg^{-1}$) | 322 | 324 | 327 | 318 | 5.47 | 0.68 |
| Ash | 34.5 | 40.6 | 38.3 | 36.2 | 1.42 | 0.06 |
| Neutral detergent fiber | 527 | 533 | 532 | 516 | 11.6 | 0.73 |
| Acid detergent fiber | 332 | 352 | 347 | 343 | 5.01 | 0.09 |
| Crude protein | 19.4b | 23.6a | 21.3ab | 19.9b | 0.69 | <0.01 |

[1] Control - no additive; N10 - 10 g $t^{-1}$ FM of natamycin; LB - 5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*; NLB - 4 g $t^{-1}$ FM of natamycin and 2.5 × $10^4$ cfu $g^{-1}$ of *L. buchneri*.
[2] Corrected for volatiles.
Means followed by a different letter, in rows, are different (P≤0.05).

## 4. Discussion

In sugarcane silages, for each 1 g $kg^{-1}$ DM of ethanol accumulated, an extra DM loss of 0.65 g $kg^{-1}$ DM is expected (Rabelo et al., 2019). This effect was also detected in our trial. Ethanol is highly volatile and may worsen air conditions in silage-based livestock systems (Hafner et al., 2013). Moreover, high ethanol synthesis is also linked to $CO_2$ synthesis (McDonald et al., 1991), which contributes to GHG emission.

As expected, LB inoculation, alone or in combination with natamycin, increased acetic acid content (by 105 and 78%, respectively) and decreased propionic acid (by 69 and 54%, respectively) and ethanol content (by 83 and 71%, respectively) in sugarcane silage due to a probable decrease in yeast activity (Pedroso et al., 2008; Ávila et al., 2010b; Ávila et al., 2012). In addition, LB use also led to lower DM and gas losses (72 and 78%, respectively). No effect was reported for the combination of additives, possibly because the NLB treatment was a result of a combination of smaller doses of both the additives. Our results contrast with those of Pinto et al. (2020), who reported low yeast counts and dry matter losses in maize silage treated with a combination of natamycin and LB compared with the control silage; however, no decrease in the yeast counts and dry matter losses was reported when additives were used alone. Those authors used higher doses in combination with the additives than we did. Thus, the effectiveness of natamycin combined with LB may be dependent on the target silage (e.g., chemical composition, initial microbial counts) as well as the dose.

Interestingly, we did not observe any differences in the lactic acid content and pH values among the silages. Besides soluble sugars, LB also uses lactic acid as a substrate, which may result in decreased lactic acid content (Oude Elferink et al., 2001). Contrary to expectations, the ethanol concentration of the N10 silage was similar (P>0.05) to that of the control silage. Our hypothesis is that the natamycin additive had a certain amount of effectiveness against yeast during the initial stages of sugarcane fermentation (hours or days) but lost its efficacy over the course of fermentation when used alone or in combination with LB. Different from acetic acid, which needs to be synthetized in silage to start acting against fungi, natamycin is readily available to interact with ergosterol in yeast membranes and decrease its activity (Welscher et al., 2008). Indeed, a decrease in DM and gas losses was observed in N10 silages (69.15 and 76.83%, respectively) compared with the control.

Gas production, the main source of DM losses in silages, achieves its peak in the first few days of fermentation (Schmidt et al., 2012).

We supposed that natamycin activity might be curtailed by low pH in silage. Under acidic conditions, natamycin is broken down, producing mycosamine and other inactive degradation products (amphoteric aponatamycin, acidic di-natamycinolidediol, and nonionic di-decarboxy-anhydronatamycinolidediol) (Brik, 1976; Brik, 1981; Dalhoff and Levy, 2015), which seems to reduce natamycin effectiveness in the advanced stages of fermentation and after opening the silo. On the other hand, acetic acid is not negatively affected by acidic conditions. In our trial, the average pH was 3.9, an unfavorable condition for natamycin action (Stark and Tan, 2003; Delves-Broughton et al., 2005; Hanušová et al., 2012). In addition, sugarcane presents high lactic acid production and a fast pH drop (Custódio et al., 2016), which also result in lowered natamycin effectiveness during fermentation. Shah et al. (2020) reported lower yeast counts in Napier grass silage treated with natamycin for 60 days than in untreated silage. Additionally, the authors reported lower lactic acid levels than those of our silages. Indeed, Napier grass presents a low level of soluble carbohydrates to draw on slow pH drop rate (Desta et al., 2016), reinforcing the above hypothesis. Nonetheless, trials evaluating the effects of different natamycin doses on silage fermentation and microbial counts over time are required to draw conclusions about this topic in sugarcane silages.

Normally, untreated sugarcane silage presents a high predominance of yeast fermentation, leading to higher gas yield and, consequently, GHG emission (as observed in our study). The use of additives, in general, reduced GV as well GHG emission (g $CO_2$ eq $t^{-1}$ fresh forage) by 68% compared with untreated silage, probably because of the lowered $CO_2$ emissions. Natamycin decreased total GV production by 61% compared with the control, whereas the GHG emission was reduced by 86% in natamycin-treated silages in comparison with the non-treated silage. Nitrous oxide, methane, and carbon dioxide were less representative in the gas composition of natamycin silage compared with the control. Those three gases are the main generators of global warming, and methane and nitrous oxide are, respectively, 23 and 298 times more harmful than carbon dioxide (Houghton et al., 2001). Despite the relatively high amount of GHG emission from untreated sugarcane silage (311 g $CO_2$ eq $t^{-1}$ fresh forage), those values are small when compared with the estimates of GHG emission from feedlot cattle (5.6 kg $CO_2$ eq per kg of live weight gain) or dairy cattle (1.1 kg $CO_2$ eq per kg of milk) (Phetteplace et al., 2001).

With the yeast population being the major cause of gas losses in sugarcane silages, other ensiled crops (e.g., corn or grass) present significantly lower ethanol synthesis and gas emission rates. Schmidt et al. (2012) observed GV production in corn silages lower than the untreated sugarcane silage from our trial (424 vs 7282 L $t^{-1}$ DM); this was also the case with GHG emission (14.5 vs 311 g $CO_2$ eq $t^{-1}$ fresh forage). Contrary to our data, Gomes et al. (2019) reported higher GV (27640 vs 10880 L $t^{-1}$ DM) as well as ethanol synthesis (5.60 vs 1.43 g $kg^{-1}$ DM) in wilted oat silage treated with LB than in untreated silage. Those authors indirectly estimated GV, which may have led to much higher values than ours.

The large yeast population typically found in sugarcane silage (Ávila et al., 2010b; Santos et al., 2015) makes it prone to deterioration after exposure to air. The deterioration rate is also related to the amount of substrate (e.g., sucrose, lactic acid) and the presence, or lack, of protective substances (e.g., short-chain fatty acids) (Ávila et al., 2012; Wilkinson and Davies, 2013). As observed in our trial, the acetic acid formed during LB development reduced spoilage after silo opening (Table 5; Figure 2). The weakness of acetic acid ($pk_a$ 4.76) contributes to its effectiveness against yeast under the typical acidic conditions found in silages (Danner et al., 2003). Different from our data, a meta-analysis performed by Rabelo et al. (2019) did not show positive effects related to aerobic stability of LB-treated sugarcane silage despite the higher acetic acid content in those silages.

Natamycin alone had no effect on aerobic stability, indicating a lack of action of this compound to inhibit yeast growth after opening the silos. This effect can be related to the short half-life of that compound after being applied to low pH media, such as silages (Brik, 1976; Brik, 1981; Stark and Tan, 2003; Delves-Broughton et al., 2005; Hanušová et al., 2012; Dalhoff and Levy, 2015). In contrast,

Pinto et al. (2020) did not find positive effects of natamycin or LB alone, but their combination increased aerobic stability.

In general, the additives used in our trial did not cause any changes in the DM content or chemical composition of the silages. Unexpectedly, the silage treated with natamycin showed higher CP values than the control and NLB silages, while LB-treated silages presented intermediate CP values. According to Rosi et al. (1987), yeasts are capable of producing extracellular proteases that consume part of the CP. In addition, yeasts are involved in the nitrogen cycle due to nitrous oxide ($N_2O$) reductase production (Shoun et al., 2012). As observed, silage treated with natamycin showed the lowest $N_2O$ production as well as the highest protein content. Shah et al. (2020) reported lower protein degradation in elephant grass silage treated with natamycin than in untreated silage. It is possible that the presence of natamycin and acetic acid impaired protein metabolization by yeasts.

As expected, LB was effective in controlling ethanol synthesis as well as reducing fermentation losses and improving aerobic stability in sugarcane silages. In contrast, natamycin was effective only with GV-related variables and did not present a synergistic effect when combined with LB.

## 5. Conclusions

Natamycin alone as well as its combination with *Lactobacillus buchneri* decrease the volume of gas and greenhouse gases emitted by sugarcane silages. Due to the lack of effectiveness in reducing ethanol content or improving the aerobic stability, natamycin seems to be effective only in the early stages of ensiling process. Further studies must be carried out for natamycin to be recommended for composing blend additives with microbial or other chemicals.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

Conceptualization: C.C. Jobim and P. Schmidt. Data curation: A.V.I. Bueno. Formal analysis: A.V.I. Bueno. Investigation: A.V.I. Bueno, C.O. Novinski, C. Bayer, C.C. Jobim and P. Schmidt. Methodology: A.V.I. Bueno, C.O. Novinski, C. Bayer, C.C. Jobim and P. Schmidt. Project administration: A.V.I. Bueno, C.O. Novinski, C.C. Jobim and P. Schmidt. Supervision: P. Schmidt. Visualization: A.V.I. Bueno and P. Schmidt. Writing-original draft: A.V.I. Bueno, G.L.D. Vigne and P. Schmidt. Writing-review & editing: A.V.I. Bueno, G.L.D. Vigne and P. Schmidt.

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