Animal Feed Science and Technology 302 (2023) 115667

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

# Effect of application rate of sodium nitrite and hexamine on the fermentation and the chemical composition of guinea grass silage harvested at different stages of maturity

A. Moraes [a], H.U. Auerbach [b], J.M. Bragatto [a], F.A. Piran Filho [a], S.M.S. Silva [a], L.G. Nussio [c], C.C. Jobim [a], J.L.P. Daniel [a,*]

[a] Department of Animal Science, State University of Maringa, 87020900, Maringa, Brazil
[b] International Silage Consultancy, Martha-Brautzsch-Straße 13, D-6108 Halle (Saale), Germany
[c] Department of Animal Science, University of Sao Paulo, Luiz de Queiroz College of Agriculture, 13418900, Piracicaba, Brazil

ARTICLE INFO

*Keywords:*
Chemical additive
*Clostridium*
Fermentation
Tropical grass
*Megathyrsus maximus*

ABSTRACT

Grass ensilability varies with maturity stage, mainly due to changing concentrations of dry matter and soluble carbohydrates with progressing forage maturity. Consequently, the required dose of silage additive to prevent the development of undesirable microorganisms may change with maturity stage. The objective of this study was to verify whether the application rate of an additive containing sodium nitrite and hexamine interacts with guinea grass maturity to alter silage fermentation and chemical composition. Four fields of guinea grass (0.5–0.7 ha each field) were mowed and divided into two plots per field. After 5 wk, one plot of each field was mowed again to establish differences in stage of maturity. Ten weeks after the first mowing, the grass plots with 5- and 10-wk regrowth were manually harvested and used for the trial. The grass from each plot (approx. 30 kg) was chopped and divided into 3 piles, totaling to 24 piles, as result of four fields, two maturities, and three additive treatments: control (without additive), low dose of sodium nitrite (0.5 g/kg) + hexamine (0.325 g/kg) (NHL), and high dose of sodium nitrite (1 g/kg) + hexamine (0.65 g/kg) (NHH). After 90 d of storage, the silos were opened and silages sampled to determine dry matter (DM) loss, microbial counts, fermentation end-products, aerobic stability, chemical composition, and in vitro DM digestibility. Guinea grass harvested at 10-wk regrowth had a lower content of crude protein ($P < 0.001$) and a greater content of cell wall components ($P < 0.001$), resulting in a more lignified ($P < 0.001$) and less digestible ($P < 0.001$) forage than that harvested at 5 wk. There were interactions between plant maturity and additive dose for several silage traits ($P < 0.05$), likely due to the slightly greater fermentability coefficient (+5.1 points) for the more mature grass ($P < 0.001$). Within each maturity stage, silage pH and fermentation end-products associated with clostridia metabolism (i.e., n-butyric acid, propionic acid, i-butyric

*Abbreviations:* ADF, acid detergent fiber expressed inclusive of residual ash; aNDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; BC, buffering capacity; CFU, colony-forming unit; CONN, control; CP, crude protein; DM, dry matter; FC, fermentability coefficient; FM, fresh matter; HEX, hexamine; iNDF, indigestible neutral detergent fiber; IVDMD, in vitro DM digestibility; LAB, lactic acid bacteria; $NH_3$-$N_{corr}$, ammonia nitrogen corrected for addition of nitrogen by additives; NHH, high dose of sodium nitrite plus hexamine; NHL, low dose of sodium nitrite plus hexamine; NIT, sodium nitrite; RDP, rumen degradable protein; RUP, rumen undegradable protein; SC, soluble carbohydrates; SEM, standard error of the mean; Sum BVA, sum of n-butyric, i-butyric, i-valeric and n-valeric acids; undVFA, undissociated volatile fatty acids; undVFA/SC+lactic acid, undissociated volatile fatty acids to soluble carbohydrates + lactic acid ratio; VFA, volatile fatty acids.

* Corresponding author.
*E-mail address:* jlpdaniel@uem.br (J.L.P. Daniel).

https://doi.org/10.1016/j.anifeedsci.2023.115667
Received 8 December 2022; Received in revised form 6 May 2023; Accepted 8 May 2023
Available online 9 May 2023

acid, i-valeric acid, n-valeric acid, ammonia, and 2,3-butanediol) linearly decreased ($P < 0.001$) with additive application rate, but the magnitude of improvement was slightly greater for 5-wk than 10-wk regrowth. Application of additive linearly decreased silage DM loss at both 5-wk (95.2, 46.7, and 20.6 g/kg DM, $P < 0.001$) and 10-wk (66.5, 31.7, and 13.6 g/kg DM, $P < 0.001$) regrowth stages, but only silages treated with NHH had n-butyric acid concentration < 3 g/kg DM. The proportion of rumen undegradable protein ($P < 0.001$), soluble carbohydrates concentration ($P < 0.001$), and in vitro DM digestibility ($P < 0.001$) were linearly increased with additive dose within each maturity stage. As treated silages were better conserved, silage aerobic stability was linearly reduced ($P < 0.001$) with additive dose, although all silages were aerobically stable for ≥ 4.7 d. In conclusion, the additive based on sodium nitrite and hexamine, applied at a regular dose, was able to largely restrict *Clostridium* development and DM losses during fermentation of guinea grass silage at both maturity stages. However, harvesting more mature grass markedly impaired its chemical composition and digestibility, rendering it no feasible strategy to reduce the additive application rate by half.

## 1. Introduction

The intensification of beef cattle backgrounding systems, and the possibility of using tropical grass silage as a low-starch forage source in dairy herds have led to renewed interest in tropical grass silages in Brazil. Furthermore, the increased risk of dry weather during the last growing seasons brought an additional motivation for planting crops with high drought tolerance. Therefore, tropical grasses have been cultivated as a complimentary forage to corn silage. However, tropical grasses are difficult to ensile, due to the low contents of dry matter (DM) and soluble carbohydrates, and the high buffering capacity (Tomaz et al., 2018).

Depending on the weather, late harvest frequently increases DM and soluble carbohydrates in tropical grasses (Wilkinson, 1983; Tomaz et al., 2018), which contribute to improving silage fermentability. However, advancing maturity consistently decreases the nutritive value of tropical grasses (Wilson et al., 1986; Daniel et al., 2016). Hence, producing tropical grass silage with high nutritive value requires strategies that ensure adequate fermentation, especially those that inhibit the development of undesired bacteria (e.g., enterobacteria, clostridia, and others; Pahlow et al., 2003).

Recently, Gomes et al. (2021) demonstrated that an additive based on sodium nitrite (NIT) at 1 g/kg fresh matter (FM) and hexamine (HEX) at 0.6 g/kg FM was highly efficient to inhibit clostridial development, reduce fermentative losses, and to improve the nutritional composition of guinea grass silage. While this sole study examined the effect of a nitrite-based additive in tropical grass silage, if the recommended dose of NIT and HEX can be changed by guinea grass maturity is unknown. In silages made from temperate forages, the application rate of this anticlostridial mixture can be reduced by increasing the DM level (Weissbach, 2011), either by wilting or by harvesting more mature material, which generally is higher in DM.

Thus, the objective of this study was to examine if the application rate of an additive based on NIT and HEX interacts with maturity at harvest to improve the fermentation and nutrient conservation of guinea grass. We hypothesized that the additive dose can be reduced for grass harvested at 10-wk, but not for grass harvested at 5-wk regrowth.

## 2. Material and methods

### 2.1. Ensiling and treatments

Four 0.5- to 0.7-ha fields of guinea grass [*Megathyrsus maximus* (Jacq.) B.K. Simon & S. W. L Jacobs (syn. *Panicum maximum* Jacq.)] cv. Mombaca, either at fourth- or fifth-year, at the Estancia Independente Farm (Mandaguari, PR, Brazil), were used for the trial. The fields were fertilized with nitrogen by urea (200 kg/ha per year) and liquid manure (30–40 $m^3$/ha per year) from a beef cattle feedlot.

In December 2019, the fields were mowed for standardization and divided into two plots per field. Five weeks later, a plot of each field, assigned randomly, was mowed again to establish a maturity gradient. We used this strategy primarily to induce differences in fermentability traits between the two forages. Ten weeks after the standardization cut, the grasses with 5- and 10-wk regrowth were cut and immediately picked up manually (approximately 30 kg/plot), with no wilting (i.e., direct cut). All plots were simultaneously harvested within 20 min. At harvest, the average canopy heights were 65 ± 5 cm and 98 ± 6 cm for the 5- and 10-wk grass plots, respectively. The grasses were cut at 15 cm stubble height.

Grass from each plot was chopped by a stationary forage chopper (10 mm of theoretical length of cut). Subsequently, the chopped forage from each plot was divided into 3 piles (6 kg/pile), totaling to 24 piles reflecting the combination of two maturities, three treatments with additives, and four field plots. Additive treatments were as follows: control (no additive), low dose of sodium nitrite (0.5 g/kg FM) + hexamine (0.325 g/kg FM) (NHL), and high dose of sodium nitrite (1 g/kg FM) + hexamine (0.65 g/kg FM) (NHH). The additives were diluted in distilled water (10 mL/kg FM) and applied with manual sprayers. The control treatment also received the same volume of distilled water (10 mL/kg FM). Then, 4.5 kg of treated forage was packed manually in 7.2-L plastic buckets (experimental silos). The buckets were sealed with plastic lids and the joint wrapped with six layers of self-adhesive tape. After sealing, the experimental silos were weighed and stored in a closed barn at room temperature (14–33 °C). At ensiling, composite samples of untreated chopped forages from each plot were collected in sterile bags to determine chemical composition, in vitro DM digestibility, pH, and microbial counts [lactic acid bacteria (LAB), clostridia, yeasts, and molds]. The pH and microbial analysis were initiated

immediately after sampling within 80 min after cutting and 40 min after chopping, respectively.

After 90 days of storage, the silos were weighed to determine the fermentation losses. The DM loss was calculated as the difference between the amount of DM ensiled and DM recovered as a proportion of DM ensiled. At silo opening, no visible mold was detected on the silage surface. Silage samples were collected for measuring microbial counts, pH, fermentation products, aerobic stability, chemical composition, and in vitro DM digestibility.

### 2.2. Aerobic stability test

Silage samples (4.0 kg) were transferred to 11-L plastic buckets with a temperature sensor placed in the center of the silage mass. Subsequently, the buckets were covered with perforated aluminum foil to reduce dehydration and prevent dirt entry. Temperature was recorded every 15 min for 10 d in a temperature-controlled room (25 ± 1.5 °C). Aerobic stability based on temperature was defined as the time elapsed until silage temperature reached 2 °C above the room temperature (O'Kiely, 1993). Additionally, during the 10-d aeration period, silage pH was recorded every morning. The pH was measured in aqueous extract prepared with 10 g of silage + 90 g of distilled water blended for 2 min and filtered through four layers of cheese cloth. Aerobic stability based on pH rise was defined as the time elapsed until silage pH increased by 0.5 unit (Gomes et al., 2021).

### 2.3. Laboratory analysis

Samples of fresh grasses and silages were dried in a forced ventilation oven at 55 °C for 72 h and ground in a Wiley mill with 1-mm screen, and analyzed for DM at 105 °C, ash, and crude protein (CP; AOAC, 1990), neutral detergent fiber (aNDF; assayed with a heat stable amylase and sodium sulfite and expressed inclusive of residual ash; Mertens, 2002), acid detergent fiber (ADF; assayed sequentially and expressed inclusive of residual ash), and lignin (sa) (Van Soest, 1973), indigestible neutral detergent fiber (iNDF; Huhtanen et al., 1994), ethanol-soluble carbohydrates (SC; Hall et al., 1999), soluble CP, acid-detergent insoluble N, and neutral-detergent insoluble N (Licitra et al., 1996). *In vitro* DM digestibility (IVDMD) was determined using a Daisy II incubator (Ankom Technology, Macedon, USA), with solutions prepared as described in Tilley and Terry (1963). The rumen fluid was obtained from two cannulated Holstein cows grazing Bermuda grass, 1 h after supplementation with 2 kg/d of concentrate based on ground corn grain, soybean meal and mineral-vitamin mix. Rumen-fluid donors were handled in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2020).

Recovery of digestible DM was computed as the mass of silage digestible DM as proportion of digestible DM ensiled. Nitrogen fractionation [fractions A1 (ammonia), A2 (soluble true protein), B1 (insoluble true protein), B2 (fiber-bound protein) and C (indigestible protein)] was determined according to CNCPS v.6.5 (Van Amburgh et al., 2015). From N fractionation, the concentrations of rumen degradable protein (RDP, g/kg DM) and rumen undegradable protein (RUP, g/kg DM) were calculated for growing cattle using first order kinetics [kd / (kd + kp)] (Van Amburgh et al., 2015). Fractional passage rates (liquid, concentrate and forage) were estimated (Tylutki et al., 2008) assuming 6.2 kg/d DM intake, 70% dietary forage level and 265 kg shrunk body weight.

Additionally, buffering capacity (BC; Weissbach, 1967) and nitrate content (Bezerra Neto and Barreto, 2011) were determined in forage samples. The fermentability coefficient (FC) was estimated as follows: FC = DM (g / 100 g) + 8 × SC / BC (Weissbach et al., 1974).

Forage and silage samples were also used to prepare an aqueous extract by blending 25 g of fresh sample and 225 g of sterile distilled water for 2 min and filtering through a funnel with gauze. The pH was recorded (Tec5, Tecnal®, Piracicaba, Brazil) and aliquots were serially diluted ($10^{-1}$ to $10^{-6}$) in sterile 0.1%-peptone water for microbial counts by pour-plating in selective media. The LAB were enumerated on de Man, Rogosa and Sharpe agar (7543 A, Acumedia, Lansing, Michigan, USA) supplemented with nystatin (400,000 IU/L). An overlayer was added after plate solidification to decrease the partial pressure of oxygen inside the medium. Yeasts and molds were enumerated on malt extract agar (M137, Himedia, Mumbai, India) acidified to pH 3.5 with lactic acid. After pasteurization (80 °C for 13 min), serial dilutions were also pour-plated on reinforced clostridium agar (M154, Himedia, Mumbai, India) supplemented with neutral red and D-cycloserine for enumeration of *Clostridium* spores (Jonsson, 1990). Agar plates were prepared in duplicates and incubated aerobically at 30 °C for 2, 3 and 4 d before enumeration of LAB, yeasts and molds, respectively. *Clostridium* colonies were counted after incubation in anaerobic jars at 37 °C for 5 d. The number of microorganisms were counted as colony-forming units (CFU) and expressed as log10.

For the silage samples, a portion of the undiluted aqueous extract was centrifuged at 10,000g for 15 min and the supernatant used for analysis of fermentation products. Lactic acid (Pryce, 1969) and ammonia (Chaney and Marbach, 1962) were determined by colorimetry. The $NH_3$-N concentration was corrected ($NH_3$-Ncorr) in the treatments containing NIT and HEX, considering that 90% of N released from hexamine and 50% of the N from added NIT was converted into $NH_3$ during fermentation. Volatile fatty acids (VFA), alcohols and esters were determined in a gas chromatograph (Nexis GC-2030, Shimadzu, Kyoto, Japan) with an autoinjector (AOC-20i Plus, Shimadzu, Kyoto, Japan), using a Stabilwax capillary column, Restek, Bellefonte, PA; 60 m, 0.25 mm ø, 0.25 μm polyethylene glycol crossbond carbowax. Compounds were identified based on their retention time and quantified with external standards. The silage DM content was corrected for volatiles loss during oven drying (Weissbach and Strubelt, 2008). The total concentration of undissociated volatile fatty acids (undVFA) was calculated by adding the concentrations of the undissociated forms of acetic, propionic, i-butyric, n-butyric, i-valeric and n-valeric acids. The proportion of the undissociated form of each VFA was calculated as $1/(1 + 10^{(pH - pka)})$ (Henderson-Hasselbalch equation). The sum of n-butyric, i-butyric, i-valeric and n-valeric acids (sum BVA) was also computed.

### 2.4. Statistical analysis

Data were evaluated for normality of residuals (Shapiro-Wilk test) and homogeneity of variance (Bartlett test). As the variables fulfilled normality and homoscedasticity, data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Institute, Cary, NC, USA), with the following model: $Y_{ijk} = \mu + F_i + M_j + A_k + MA_{jk} + e_{ijk}$, where $\mu$ = overall mean, $F_i$ = random effect of field (i = 1–4), $M_j$ = fixed effect of maturity (j = 5 or 10 wk), $A_k$ = fixed effect of additive (k = control, NHL, or NHH), $MA_{jk}$ = interaction between maturity and additive, and $e_{ijk}$, = residue, assumed independently and identically distributed in a normal distribution with mean zero and variance $\sigma^2$. Within maturity stage, means were compared by orthogonal contrasts (with one degree of freedom) to test linear and quadratic effects of additive application rate. Within each level of additive, means were compared by the Tukey-Kramer test. Significant differences were declared at $P \leq 0.05$.

## 3. Results

### 3.1. Forage composition

The maturity stage affected the composition of the fresh guinea grass, except the counts of LAB, yeasts, and molds, BC, and A2 fraction of N (Table 1). Harvesting guinea grass at 10-wk rather 5-wk regrowth increased the DM ($P < 0.001$), SC ($P = 0.009$), fermentability coefficient ($P < 0.001$), aNDF ($P < 0.001$), iNDF ($P < 0.001$), iNDF:aNDF ratio ($P < 0.001$), ADF ($P < 0.001$), lignin (sa) ($P < 0.001$), ash ($P = 0.008$), B2 ($P = 0.024$) and C fractions of N ($P < 0.001$), and proportion of RUP ($P < 0.001$), whereas it decreased the number of *Clostridium* spores ($P = 0.004$), pH ($P < 0.001$), nitrate ($P < 0.001$), CP ($P < 0.001$), B1 fraction of N ($P = 0.007$), proportion of RDP ($P < 0.001$), and IVDMD ($P < 0.001$).

**Table 1**
Microbial counts and chemical composition of fresh guinea grass harvested at 5-wk or 10-wk regrowth (n = 4).

| Item | 5 wk | 10 wk | SEM[a] | P-value |
|---|---|---|---|---|
| Lactic acid bacteria, log CFU[b]/g FM[c] | 4.46 | 4.60 | 0.214 | 0.656 |
| Clostridia, log CFU/g FM | 3.36 | 2.46 | 0.162 | 0.004 |
| Yeasts, log CFU/g FM | 1.60 | 1.68 | 0.569 | 0.923 |
| Molds, log CFU/g FM | 3.70 | 3.98 | 0.108 | 0.106 |
| pH | 6.10 | 5.96 | 0.017 | < 0.001 |
| DM[d], g/kg FM | 232 | 281 | 1.1 | < 0.001 |
| Soluble carbohydrates, g/kg DM | 17.5 | 19.0 | 0.3 | 0.009 |
| Buffering capacity, g/kg DM | 43.1 | 44.0 | 1.02 | 0.560 |
| Fermentability coefficient | 26.4 | 31.5 | 0.12 | < 0.001 |
| Nitrate, g/kg DM | 2.53 | 1.69 | 0.045 | < 0.001 |
| aNDF[e], g/kg DM | 678 | 724 | 3.0 | < 0.001 |
| iNDF[f], g/kg DM | 249 | 423 | 3.2 | < 0.001 |
| iNDF:aNDF ratio | 0.367 | 0.584 | 0.0046 | < 0.001 |
| ADF[g], g/kg DM | 368 | 418 | 2.6 | < 0.001 |
| Lignin (sa), g/kg DM | 51.6 | 83.6 | 0.77 | < 0.001 |
| iNDF:lignin (sa) ratio | 4.83 | 5.06 | 0.068 | 0.059 |
| Ash, g/kg DM | 81.8 | 86.9 | 1.02 | 0.008 |
| Crude protein (CP), g/kg DM | 128 | 71.9 | 0.51 | < 0.001 |
| N fractionation, g/kg N | | | | |
| A1 | 0 | 0 | - | - |
| A2 | 287 | 288 | 8.3 | 0.974 |
| B1 | 272 | 202 | 12.2 | 0.007 |
| B2 | 373 | 402 | 7.0 | 0.024 |
| C | 68.2 | 108 | 1.0 | < 0.001 |
| RDP[h], g/kg CP | 673 | 635 | 1.8 | < 0.001 |
| RUP[i], g/kg CP | 327 | 365 | 1.8 | < 0.001 |
| IVDMD[j] | 0.655 | 0.519 | 0.002 | < 0.001 |

[a] Standard error of the mean.
[b] Colony-forming unit.
[c] Fresh matter.
[d] Dry matter.
[e] Neutral detergent fiber.
[f] Indigestible neutral detergent fiber.
[g] Acid detergent fiber.
[h] Rumen degradable protein.
[i] Rumen undegradable protein.
[j] *In vitro* DM digestibility.

### 3.2. Silage fermentation and aerobic stability

Microbial counts and fermentation profile of guinea grass silages are shown in Table 2. There was an interaction ($P < 0.018$) between plant maturity and additive dose for several traits. The lactic acid concentration linearly increased with additive dose ($P < 0.001$) for both maturity stages, but the increment was slightly greater for silage harvested at 10-wk regrowth. The pH, propionic, n-butyric, i-butyric, i-valeric and n-valeric acids, 2,3-butanediol, and the sum BVA linearly decreased with additive dose ($P < 0.001$) for both maturity stages, but the decline was slightly magnified for grass silage harvested at 5-wk regrowth. The acetic acid and ethanol concentrations were linearly reduced with additive dose for silage harvested at 5-wk regrowth ($P < 0.001$), but they were unchanged by additive at 10-wk regrowth. The undVFA/(SC+lactic acid) ratio linearly decreased as the additive dose increased ($P < 0.001$) for both maturity stages, but the decline was slightly amplified for silage harvested at 10-wk regrowth.

The LAB, clostridia, yeasts, and $NH_3$-$N_{corr}$ were affected by the main effects of additive and maturity. The *Clostridium* counts and $NH_3$-$N_{corr}$ concentration were steeply reduced by increasing additive application rate ($P < 0.001$) and plant maturity ($P < 0.001$). The LAB population quadratically increased as the additive dose increased for both silages harvested at 5-wk ($P = 0.002$) and 10-wk ($P = 0.016$) regrowth. Yeast counts were low in all treatments, and they were below the detection limit of 1 CFU/g in CONN and NHL silages. Mold count was not affected by treatment.

Dry matter loss and aerobic stability of guinea grass silages were affected by the main effects of additive and maturity (Table 3). Dry

**Table 2**
Microbial counts and fermentation profile of guinea grass silage harvested at 5-wk or 10-wk regrowth (n = 4).

| | | Additive[a] | | | | P-value[b] | | | Contrast[c] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Maturity | CONN | NHL | NHH | SEM[d] | M | A | M×A | L | Q |
| Lactic acid bacteria, log CFU[e]/g FM[f] | 5 wk | 7.87 | 8.35 | 8.11 | 0.086 | 0.123 | < 0.001 | 0.151 | 0.050 | 0.002 |
| | 10 wk | 7.56 | 8.15 | 8.19 | | | | | < 0.001 | 0.016 |
| Clostridia, log CFU/g FM | 5 wk | 5.55 | 5.09 | 3.50 | 0.153 | 0.041 | < 0.001 | 0.807 | < 0.001 | 0.006 |
| | 10 wk | 5.20 | 4.93 | 3.22 | | | | | < 0.001 | < 0.001 |
| Yeasts, log CFU/g FM | 5 wk | < 1 | < 1 | 2.75 | 0.475 | 0.235 | < 0.001 | 0.257 | < 0.001 | 0.026 |
| | 10 wk | < 1 | < 1 | 1.37 | | | | | 0.044 | 0.245 |
| Molds, log CFU/g FM | 5 wk | 3.35 | 3.22 | 3.85 | 0.251 | 0.884 | 0.138 | 0.846 | 0.157 | 0.218 |
| | 10 wk | 3.36 | 3.31 | 3.66 | | | | | 0.390 | 0.512 |
| pH | 5 wk | 5.55[a] | 4.94[a] | 4.74[a] | 0.027 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 5.28[b] | 4.87[a] | 4.68[a] | | | | | < 0.001 | 0.004 |
| $NH_3$-$N_{corr}$[g], g/kg N | 5 wk | 403 | 144 | 48.3 | 8.7 | < 0.001 | < 0.001 | 0.715 | < 0.001 | < 0.001 |
| | 10 wk | 363 | 101 | 19.1 | | | | | < 0.001 | < 0.001 |
| Lactic acid, g/kg DM[h] | 5 wk | 0.07[a] | 4.93[b] | 27.2[b] | 0.966 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 0.12[a] | 20.2[a] | 33.5[a] | | | | | < 0.001 | 0.008 |
| Acetic acid, g/kg DM | 5 wk | 14.4[a] | 8.71[a] | 5.74[a] | 0.743 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.139 |
| | 10 wk | 8.11[b] | 6.60[a] | 7.15[a] | | | | | 0.345 | 0.264 |
| Propionic acid, g/kg DM | 5 wk | 3.87[a] | 1.24[a] | 0.858[a] | 0.186 | < 0.001 | < 0.001 | 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 2.28[b] | 1.10[a] | 0.355[a] | | | | | < 0.001 | 0.346 |
| n-Butyric acid, g/kg DM | 5 wk | 10.6[a] | 4.64[a] | 1.94[a] | 0.395 | < 0.001 | < 0.001 | 0.001 | < 0.001 | 0.003 |
| | 10 wk | 6.95[b] | 3.57[a] | 1.19[a] | | | | | < 0.001 | 0.309 |
| i-Butyric acid, g/kg DM | 5 wk | 1.53[a] | 0.484[a] | 0.321[a] | 0.084 | < 0.001 | < 0.001 | 0.018 | < 0.001 | < 0.001 |
| | 10 wk | 0.953[b] | 0.389[a] | 0.095[a] | | | | | < 0.001 | 0.198 |
| i-Valeric acid, g/kg DM | 5 wk | 0.677[a] | 0.237[a] | 0.112[a] | 0.038 | < 0.001 | < 0.001 | 0.001 | < 0.001 | 0.003 |
| | 10 wk | 0.317[b] | 0.149[a] | 0.025[a] | | | | | < 0.001 | 0.644 |
| n-Valeric acid, g/kg DM | 5 wk | 0.983[a] | 0.352[a] | 0.199[a] | 0.049 | < 0.001 | < 0.001 | 0.004 | < 0.001 | < 0.001 |
| | 10 wk | 0.574[b] | 0.244[a] | 0.091[a] | | | | | < 0.001 | 0.152 |
| Ethanol, g/kg DM | 5 wk | 4.45[a] | 0.865[a] | 0.765[a] | 0.372 | 0.290 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 1.96[b] | 1.66[a] | 1.51[a] | | | | | 0.375 | 0.875 |
| 2,3-Butanediol, g/kg DM | 5 wk | 3.76[a] | 1.17[a] | 0.803[a] | 0.194 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 2.02[b] | 1.06[a] | 0.312[a] | | | | | < 0.001 | 0.640 |
| Sum BVA[i], g/kg DM | 5 wk | 13.8[a] | 5.71[a] | 2.58[a] | 0.534 | < 0.001 | < 0.001 | 0.002 | < 0.001 | 0.002 |
| | 10 wk | 8.79[b] | 4.35[a] | 1.40[a] | | | | | < 0.001 | 0.278 |
| undVFA/(SC+lactic acid)[j] | 5 wk | 1.37[a] | 0.785[a] | 0.155[a] | 0.086 | 0.010 | < 0.001 | 0.008 | < 0.001 | 0.829 |
| | 10 wk | 1.35[a] | 0.235[b] | 0.128[a] | | | | | < 0.001 | < 0.001 |

[a,b]Means within each level of additive bearing unlike superscripts differ (Tukey-Kramer test, α = 0.05).
[a] CONN: without additive, NHL: sodium nitrite at 0.5 g/kg + hexamine at 0.325 g/kg, NHH: sodium nitrite at 1 g/kg + hexamine at 0.65 g/kg.
[b] M: effect of maturity; A: effect of additive; M×A: interaction between maturity and additive.
[c] L: linear effect of additive dose, Q: quadratic effect of additive dose.
[d] Standard error of the mean.
[e] Colony-forming unit.
[f] Fresh matter.
[g] $NH_3$-N corrected for addition of nitrogen by additives.
[h] Dry matter.
[i] Sum of n-butyric, i-butyric, i-valeric and n-valeric acids.
[j] Undissociated volatile fatty acids to soluble carbohydrates + lactic acid ratio.

**Table 3**
Dry matter loss during fermentation and aerobic stability of guinea grass silage harvested at 5-wk or 10-wk regrowth (n = 4).

| | | Additive[a] | | | | P-value[b] | | | Contrast[c] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Maturity | CONN | NHL | NHH | SEM[d] | M | A | M×A | L | Q |
| DM[e] loss, g/kg DM | 5 wk | 95.2 | 46.7 | 20.6 | 4.49 | < 0.001 | < 0.001 | 0.054 | < 0.001 | 0.052 |
| | 10 wk | 66.5 | 31.7 | 13.6 | | | | | < 0.001 | 0.136 |
| Aerobic stability pH[f], d | 5 wk | 10.0 | 9.00 | 6.25 | 0.295 | 0.050 | < 0.001 | 0.336 | < 0.001 | 0.028 |
| | 10 wk | 10.0 | 9.75 | 7.00 | | | | | < 0.001 | 0.003 |
| Aerobic stability Temp.[g], d | 5 wk | 10.0 | 9.39 | 4.68 | 0.319 | 0.006 | < 0.001 | 0.105 | < 0.001 | < 0.001 |
| | 10 wk | 10.0 | 10.0 | 6.49 | | | | | < 0.001 | < 0.001 |

[a] CONN: without additive, NHL: sodium nitrite at 0.5 g/kg + hexamine at 0.325 g/kg, NHH: sodium nitrite at 1 g/kg + hexamine at 0.65 g/kg.
[b] M: effect of maturity; A: effect of additive; M×A: interaction between maturity and additive.
[c] L: linear effect of additive dose, Q: quadratic effect of additive dose.
[d] Standard error of the mean.
[e] Dry matter.
[f] Aerobic stability based on pH rise (+0.5).
[g] Aerobic stability based on temperature rise (+2 ∘C).

matter loss was steeply depressed by increasing additive application rate ($P < 0.001$) and plant maturity ($P < 0.001$). No pH or temperature change was observed in CONN silages during the 10 d of aeration test, but aerobic stability based on pH or temperature increase was shortened in silages by increasing additive dose ($P < 0.001$), and the effect was stronger in silage from 5-wk than from 10-wk regrowth ($P < 0.050$).

### 3.3. Chemical composition and digestibility of silages

Chemical composition and IVDMD of guinea grass silages are shown in Table 4. There was an interaction ($P < 0.048$) between additive dose and plant maturity for SC, iNDF, B2 fraction of N, RDP, RUP, IVDMD, and recovery of digestible DM. The SC concentration quadratically increased ($P < 0.001$) with additive application rate for both maturity stages, but the benefit was slightly greater for silage made from 10-wk regrowth. The iNDF and proportion of RDP linearly decreased ($P < 0.001$) whereas B2 fraction of N and proportion of RUP linearly increased ($P < 0.001$) with additive dose for both maturity stages, but the improvement was slightly magnified for silage harvested at 10-wk regrowth. The IVDMD and recovery of digestible DM were steeply increased ($P < 0.001$) with additive dose for both maturity stages, but the increment was slightly greater for silage harvested at 5-wk regrowth.

Other nutritional traits were affected by the main effects of maturity and additive dose (Table 4). Harvesting at 10-wk regrowth increased silage DM ($P < 0.001$), aNDF ($P < 0.001$), ADF ($P < 0.001$), lignin (sa) ($P < 0.001$), ash ($P = 0.017$), and C fraction of N ($P < 0.01$), whereas decreased CP ($P < 0.001$), A1 ($P = 0.006$) and A2 fractions of N ($P = 0.033$). Silage DM ($P < 0.001$), CP ($P < 0.001$), A2 ($P < 0.001$), and C fractions of N ($P < 0.001$) linearly increased whereas aNDF ($P = 0.020$), ADF ($P < 0.001$), ash ($P = 0.005$), and A1 fraction of N ($P < 0.001$) largely decreased as the additive dose increased. The B1 fraction of N remained unaffected by treatment.

## 4. Discussion

### 4.1. Forage composition

Chemical and microbiological traits of the fresh forages were typical of guinea grass harvested at such maturity stages based on sward heights or regrowing periods (Vasconcelos et al., 2009; Santos et al., 2014; Tomaz et al., 2018). Late harvest, however, decreased silage digestibility, which is caused by cell wall thickening and lignification, along with progressive decrease in the proportion of leaves (Jung and Vogel, 1992; Jung and Allen, 1995). The iNDF:lignin (sa) ratio found in our guinea grass (~5) was as high as that found in sugarcane (~4.6; Daniel et al., 2017), and much higher than that reported for alfalfa (~2.5), whole plant corn silage or temperate grass (~3.5; Raffrenato et al., 2019). This suggests that the negative impact of lignin (sa) on fiber digestibility in tropical grasses is more pronounced than in other grasses, and that a universal equation to estimate the iNDF fraction (e.g., iNDF = 2.4 × lignin (sa); Sniffen et al., 1992) is not valid for tropical grasses. Furthermore, if the grass silage is intended to supply nutrients in the diet (i.e., not only supply physically effective fiber) the practice of late harvest should be discouraged.

Although our data confirm previous findings on the changes in DM and SC concentrations with progressing maturity in tropical grasses (Wilkinson, 1983; Santos et al., 2014; Tomaz et al., 2018), the magnitude of the effect on fermentability (5.1 points of FC) was not as pronounced as expected, which was mainly due to a lack of response of SC:BC ratio to the stage of maturity at harvest. As predicted by the fermentation pattern anticipation models (Weissbach et al., 1974; Kaiser et al., 2002), this increase in FC by delaying harvest was not sufficient to prevent the clostridial activity, as will be discussed below.

Beyond the FC, forage epiphytic LAB and nitrate concentrations play an important role in inhibiting clostridia although it is not known if this applies to tropical grass too. According to Kaiser et al. (2002), 4.4 g/kg DM of nitrate in temperate forages are required to consistently prevent butyric acid formation, which is above the value we detected in untreated forage. However, based on a large set of forage samples from mainly temperate grasses and legumes (n = 244) with a FC > 35, Weissbach and Honig (1996) suggested a minimum nitrate concentration of 1 g/kg and also highlighted the effect of LAB alone (minimum $10^5$ CFU/g) and the combination of

**Table 4**
Chemical composition of guinea grass silage harvested at 5-wk or 10-wk regrowth (n = 4).

| | | Additive[a] | | | | *P*-value[b] | | | Contrast[c] | |
|---|---|---|---|---|---|---|---|---|---|---|
| Item | Maturity | CONN | NHL | NHH | SEM[d] | M | A | M×A | L | Q |
| DM[e], g/kg FM | 5 wk | 215 | 228 | 233 | 1.1 | < 0.001 | < 0.001 | 0.117 | < 0.001 | 0.005 |
| | 10 wk | 267 | 282 | 290 | | | | | < 0.001 | 0.029 |
| Soluble carbohydrates, g/kg DM | 5 wk | 3.51[a] | 3.39[a] | 3.95[b] | 0.21 | < 0.001 | < 0.001 | < 0.001 | 0.020 | 0.040 |
| | 10 wk | 3.49[a] | 3.25[a] | 5.32[a] | | | | | < 0.001 | < 0.001 |
| aNDF[f], g/kg DM | 5 wk | 649 | 647 | 643 | 2.9 | < 0.001 | 0.020 | 0.429 | 0.219 | 0.750 |
| | 10 wk | 718 | 717 | 705 | | | | | 0.011 | 0.151 |
| iNDF[g], g/kg DM | 5 wk | 220[b] | 216[b] | 190[b] | 2.1 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | 10 wk | 370[a] | 340[a] | 319[a] | | | | | < 0.001 | 0.077 |
| ADF[h], g/kg DM | 5 wk | 398 | 364 | 359 | 3.0 | < 0.001 | < 0.001 | 0.116 | < 0.001 | 0.002 |
| | 10 wk | 432 | 423 | 415 | | | | | 0.002 | 0.840 |
| Lignin (sa), g/kg DM | 5 wk | 56.1 | 53.4 | 52.8 | 1.36 | < 0.001 | 0.097 | 0.952 | 0.100 | 0.517 |
| | 10 wk | 86.9 | 85.8 | 84.5 | | | | | 0.214 | 0.590 |
| Ash, g/kg DM | 5 wk | 93.7 | 91.0 | 90.5 | 1.54 | 0.017 | 0.005 | 0.451 | 0.130 | 0.548 |
| | 10 wk | 99.0 | 93.3 | 92.4 | | | | | 0.004 | 0.206 |
| Crude protein, g/kg DM | 5 wk | 127 | 133 | 139 | 0.99 | < 0.001 | < 0.001 | 0.182 | < 0.001 | 0.723 |
| | 10 wk | 68.2 | 70.3 | 78.5 | | | | | < 0.001 | 0.022 |
| N fractionation, g/kg N | | | | | | | | | | |
| A1 | 5 wk | 403 | 179 | 114 | 8.6 | 0.006 | < 0.001 | 0.097 | < 0.001 | < 0.001 |
| | 10 wk | 363 | 153 | 113 | | | | | < 0.001 | < 0.001 |
| A2 | 5 wk | 121 | 268 | 312 | 12.3 | 0.033 | < 0.001 | 0.320 | < 0.001 | 0.003 |
| | 10 wk | 100 | 263 | 269 | | | | | < 0.001 | < 0.001 |
| B1 | 5 wk | 211 | 235 | 228 | 9.8 | 0.947 | 0.061 | 0.090 | 0.235 | 0.207 |
| | 10 wk | 230 | 243 | 203 | | | | | 0.063 | 0.039 |
| B2 | 5 wk | 214[a] | 258[a] | 282[b] | 4.6 | 0.004 | < 0.001 | 0.019 | < 0.001 | 0.086 |
| | 10 wk | 226[a] | 256[a] | 309[a] | | | | | < 0.001 | 0.055 |
| C | 5 wk | 51.2 | 59.5 | 64.3 | 2.45 | < 0.001 | < 0.001 | 0.125 | 0.001 | 0.561 |
| | 10 wk | 80.6 | 84.2 | 107 | | | | | < 0.001 | 0.005 |
| RDP[i], g/kg CP | 5 wk | 783[a] | 733[a] | 714[a] | 2.6 | < 0.001 | < 0.001 | 0.012 | < 0.001 | < 0.001 |
| | 10 wk | 750[b] | 710[b] | 673[b] | | | | | < 0.001 | 0.051 |
| RUP[j], g/kg CP | 5 wk | 217[b] | 267[b] | 286[b] | 2.6 | < 0.001 | < 0.001 | 0.012 | < 0.001 | < 0.001 |
| | 10 wk | 250[a] | 290[a] | 327[a] | | | | | < 0.001 | 0.525 |
| IVDMD[k] | 5 wk | 0.601[a] | 0.647[a] | 0.659[a] | 0.005 | < 0.001 | < 0.001 | 0.022 | < 0.001 | 0.008 |
| | 10 wk | 0.460[b] | 0.480[b] | 0.494[b] | | | | | < 0.001 | 0.565 |
| Recovery of digestible DM, g/kg | 5 wk | 827[a] | 941[a] | 985[a] | 9.6 | < 0.001 | < 0.001 | 0.048 | < 0.001 | 0.008 |
| | 10 wk | 824[a] | 895[b] | 937[b] | | | | | < 0.001 | 0.251 |

[a,b]Means within each level of additive bearing unlike superscripts differ (Tukey-Kramer test, α = 0.05).
[a] CONN: without additive, NHL: sodium nitrite at 0.5 g/kg + hexamine at 0.325 g/kg, NHH: sodium nitrite at 1 g/kg + hexamine at 0.65 g/kg.
[b] M: effect of maturity; A: effect of additive; M×A: interaction between maturity and additive.
[c] L: linear effect of additive dose, Q: quadratic effect of additive dose.
[d] Standard error of the mean.
[e] Dry matter.
[f] Neutral detergent fiber.
[g] Indigestible neutral detergent fiber.
[h] Acid detergent fiber.
[i] Rumen degradable protein.
[j] Rumen undegradable protein.
[k] *In vitro* DM digestibility.

nitrate and LAB. On the contrary, a recent study by Gomes et al. (2021) showed that guinea grass with low nitrate content (~0.2 g/kg DM), even with epiphytic LAB count in excess of $10^6$ CFU/g, had characteristics typical for clostridial fermentations when ensiled without additive. Thus, there is no conclusive evidence on which of the two traits – nitrate and LAB numbers – has a more pronounced effect on the outcome of the fermentation process of tropical grasses. Although nitrate accumulation in guinea grass heavily depends on N fertilization rate (Namihira et al., 2010), in our study, nitrate concentration decreased with advancing plant maturity, which likely alleviated the effect of a slight increase in FC.

### 4.2. Silage fermentation

Apart from the mold count at silo opening, all silage fermentation traits were affected by additive dose and maturity stage or their interaction. Although the interaction between additive dose and forage maturity was statistically significant, the relevance of such interaction was biologically neglectable for most fermentation outcomes. Depending on the variable, the response was slightly more favorable at 5-wk regrowth, but the opposite also occurred. Furthermore, within each forage maturity, the concentrations of fermentation end-products were always steeply changed with additive application rate, reflecting a clear dose-response relationship.

Exceptions for this pattern were ethanol and acetic acid, which steeply decreased in grass silage harvested at 5-wk but not at 10-wk regrowth.

In silages, ethanol and acetic acid are produced by different groups of microorganisms. Ethanol is mainly produced by yeasts, enterobacteria, clostridia, heterofermentative LAB, and bacilli, whereas acetic acid is mainly produced by heterofermentative LAB, enterobacteria, clostridia, bacillus, and propionic acid bacteria (McDonald et al., 1991). As the concentrations of ethanol and acetic acid were lower and unchanged by additive dose at 10-wk regrowth, those fermentation end-products likely originated from mixed bacterial metabolic pathways. Meanwhile, clostridia might have had a significant role in ethanol and acetic acid production in silage harvested at 5-wk regrowth, as *Clostridium* development was inhibited by additive application.

The moderate increase of DM and SC for the more mature grass was capable of slightly improving fermentation quality, as noted by lower $NH_3$-$N_{corr}$ concentration, *Clostridium* count, and DM loss at 10-wk regrowth. However, guinea grass directly ensiled without additive underwent butyric fermentation (>3 g/kg DM; Kaiser et al., 2002), regardless of plant maturity, and must be considered unfit for feeding purposes (Weiss et al., 2003). On the other hand, the additive based on NIT and HEX was efficient in controlling clostridial fermentation for an extended storage period in both maturity stages, confirming our previous findings in tropical grass silage (Gomes et al., 2021) and those by Weissbach et al. (1989) and Reuter and Weissbach (1991) in temperate forages. In our study, *Clostridium* metabolites (i.e., n-butyric acid, propionic acid, i-butyric acid, i-valeric acid, n-valeric acid, $NH_3$-$N_{corr}$, and 2,3-butanediol; McDonald et al., 1991; Pahlow et al., 2003) were markedly suppressed with additive application rate in both maturity stages. The LAB count was higher in treated silages, suggesting that the additive containing NIT and HEX may have protected sugars from utilization by undesired microorganism leading to a better nutrient supply for beneficial LAB. Greater lactic acid concentrations and lower pH values were observed in treated silages, indicating that there was greater production of lactic acid and/or less degradation of lactic acid (Pahlow et al., 2003). In line with observations by Weissbach et al. (1989) and Reuter and Weissbach (1991), NIT and HEX not only reduced the population size but also the activity of saccharolytic and proteolytic clostridia (McDonald et al., 1991). Metabolic pathways by clostridia ultimately lead to gaseous losses, mainly $CO_2$, resulting in greater DM loss and lower efficiency of the fermentation process (Rooke and Hatfield, 2003).

Although applying half dose of additive (NHL) improved the fermentation pattern to some extent and reduced DM loss, the magnitude of the effect was smaller, and only silages treated with NHH were free of n-butyric acid (i.e., <3 g/kg DM). In silages made from temperate forages, Auerbach and Nadeau (2019) reported that lower application rates (e.g., 600 g/t NIT and 400 g/t HEX) already improved silage fermentation, but using the higher rate consistently resulted in greater reduction in butyric acid concentration and the frequency of butyric acid-free silages in 21 trials. Therefore, a regular dose of additive based on NIT and HEX (i.e., NHH) is recommended for direct-cut tropical grass silage, regardless of maturity stage.

### 4.3. Silage aerobic stability

At silo opening, the numbers of molds and yeasts were relatively low, which was expected considering the presence of a relatively high VFA concentration (Moon, 1983). Consequently, all silages showed relatively long aerobic stability (i.e., ≥4.7 d based on temperature or ≥6.3 d based on pH increase). Only silages treated with NHH showed temperature and pH increases before the end of the 10-days aeration period, which can likely be attributed to the lower undVFA/(SC+lactic acid) ratio. Gomes et al. (2021), who first suggested this index (i.e., the amount of antifungal compounds per unit of utilizable substrates) to evaluate the risk of aerobic instability, showed a positive correlation between these two parameters in guinea grass silage. Collectively, these findings suggest that molds may have played a central role on the aerobic deterioration of tropical grass silage, due to its slower development and greater resistance to undVFA compared with yeasts (Woolford, 1975). As the additive based on NIT and HEX is effective in preserving utilizable substrates (i.e., lactic acid and SC) and restrict the formation of antifungal compounds (i.e., VFA), integrated management strategies should be adopted to decrease the risk of aerobic deterioration in well-preserved tropical grass silage.

### 4.4. Chemical composition and digestibility of silages

Apart from the B1 fraction of N, silage composition traits were affected by maturity stage and additive dose or their interaction. Compared with fermentation traits, the interaction between additive dose and forage maturity was even less relevant biologically. Additionally, within each forage maturity, the concentrations of nutrients and recovery of digestible DM were always markedly improved with increasing additive application rate, which strongly suggests a close dose-response relationship.

As late harvest markedly depressed silage IVDMD (−0.141 to −0.167), the farm goal of making tropical grass silage of sufficiently high nutritive value is likely to be achieved only with early-harvested grasses that are strategically managed for good fermentation quality. Although wilting is a widely adopted strategy to increase forage DM content in temperate and sub-tropical areas, it has been a challenge in the tropics. The lack of appropriate machinery and the high risk of field losses may lead to nutrient losses that exceed those incurred by ensiling direct-cut forages with rather low DM content, especially during the rainy season (Muck et al., 2003; Borreani et al., 2018). Thus, ensiling direct-cut tropical forages and using an effective anticlostridial additive is a suitable approach to produce well-fermented silage with high nutritive value.

Within each maturity stage, changes in chemical composition and digestibility of silages corresponded well with the fermentation pattern. The higher DM content observed for treated silages was certainly due to the lower DM loss during fermentation (McDonald et al., 1991). Regardless of maturity stage and despite the observed differences in the magnitude of the effect between additive application rates, more CP (+2 to +12 g/kg DM) and RUP (+40 to +77 g/kg CP) were preserved and IVDMD was enhanced (+0.020 to +0.058), which can be exploited in diet formulations. The much higher recovery of digestible DM after long storage in treated silages

supports the similar results reported by Gomes et al. (2021) in guinea grass silage treated with NHH. Reducing nutrient losses during fermentation and improving the nutritional composition of treated silages offer opportunities to increase the efficiency and the utilization of silage from tropical grasses and has the potential to improve animal performance and, likely, to save more feed cost than the cost of the additive. However, more silage fermentation studies and in vivo feeding trials are warranted to confirm this hypothesis.

## 5. Conclusion

Regardless of maturity, the additive based on NIT and HEX, applied at a regular dose, was able to largely restrict *Clostridium* development and dry matter loss during fermentation of guinea grass silage. Late harvest markedly impaired silage composition and in vitro digestibility, and - contrary to our hypothesis - did not prove to be a feasible strategy to reduce the additive application rate by half.

## CRediT authorship contribution statement

**A. Moraes:** Investigation, Formal analysis, Data curation, Writing – original draft. **H. U. Auerbach:** Conceptualization, Writing – review & editing. **J. M. Bragatto:** Investigation, Formal analysis. **F. A. Piran Filho:** Investigation, Formal analysis. **S. M. S. Silva:** Investigation, Formal analysis. **L. G. Nussio:** Methodology, Resources, Visualization. **C. C. Jobim:** Methodology, Resources, Visualization. **J. L. P. Daniel:** Conceptualization, Funding acquisition, Project administration, Methodology, Resources, Formal analysis, Writing – review & editing.

## Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from jlpdaniel@uem.br (João Daniel, corresponding author).

## Acknowledgements

We thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brasilia-DF, Brazil, for the scholarships provided to the authors.

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