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Research article

# Evaluation of sweet orange essential oil on fermentation and aerobic stability of corn silage

Ismael Nacarati da Silva[1], Tadeu Silva de Oliveira[1]*, Elon Souza Aniceto[1], José Ribeiro Meirelles Júnior[1], Elvanio José Lopes Mozelli Filho[1], Alberto Magno Fernandes[1], Gonçalo Apolinário Souza Filho[2], Tanya Gressley[3]

[1]Universidade Estadual do Norte Fluminense – Lab. de Zootecnia, Av. Alberto Lamego, 2000 – 28013-602 – Campos dos Goytacazes, RJ – Brasil.
[2]Universidade Estadual do Norte Fluminense – Lab. de Alimentos, Av. Alberto Lamego, 2000 – 28013-602 – Campos dos Goytacazes, RJ – Brasil.
[3]University of Delaware – Dept. of Animal and Food Sciences – 19716 – Newark, NJ – USA.
*Corresponding author <tsoliveira@uenf.br>

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**ABSTRACT**: Corn silage is susceptible to losses through aerobic spoilage. Therefore, this study aimed to evaluate increasing sweet orange essential oil levels in chemical composition, *in vitro* degradability, losses, fermentation parameters, microbial count, and the aerobic stability of corn silage. The experiment was carried out in a completely randomized design with four treatment levels: Control (CON), ensiling corn without sweet orange essential oil; 200 mg $kg^{-1}$,400 mg $kg^{-1}$, and 600 mg $kg^{-1}$ of ensiling mass with four replicates per treatment. Sweet orange essential oil affected both the dry matter ($p$ = 0.035) and the organic matter ($p$ = 0.021), presenting quadratic behavior. Sweet orange essential oil did not affect ($p \geq 0.05$) *in vitro* dry matter degradability, *in vitro* neutral detergent fiber degradability, or gross energy. Sweet orange essential oil had a quadratic effect ($p$ = 0.022) on the acetic acid concentration in corn silage. There was a tendency ($p$ = 0.097) for sweet orange essential oil to reduce the ammoniacal nitrogen linearly. The levels of sweet orange essential oil did not influence losses by gases or effluents ($p \geq 0.05$). We observed no effect of sweet orange essential oil on Lactic acid-producing bacteria populations or fungi ($p \geq 0.05$). Furthermore, increasing sweet orange essential oil levels decreased temperature ($p$ = 0.02) over time but did not influence pH ($p$ = 0.404). Sweet orange essential oil does not affect *in vitro* degradability, fermentation parameters, nor microbial count in corn silage. However, the 600 mg of ensiling mass increases corn silage's acetic acid concentration and aerobic stability.

**Keywords**: antifungal, conservation, fermentative quality, secondary plant metabolites

## Introduction

Corn presents features for desirable fermentation, such as epiphytic microbiota, dry matter, water-soluble carbohydrates, and buffering capacity (BC). These characteristics are crucial to the ensiling process (Borreani et al., 2018). However, corn silage is particularly susceptible to losses through aerobic spoilage since well-fermented silages have greater substrate availability for spoilage microorganisms and contain a lower content of substances that inhibit such microorganisms (Wilkinson and Davies, 2012). Given this context, additives and/or inoculants can improve silage safety, fermentation, nutrient recovery, and quality. Many additives, such as chemicals and essential oils, have been evaluated to prevent aerobic deterioration and improve silage's fermentation parameters (Zhang et al., 2019; Li et al., 2021; Silva and Kung Jr., 2022).

Essential oils (EO) are secondary metabolites known to be active against various microorganisms, including Gram-negative bacteria, Gram-positive bacteria, and fungi (Lopez et al., 2007). There is a study that proved the antifungal effect of carvacrol essential oil extracted from oregano on food preservation, mainly against yeasts of the genera *Candida* sp., *Pichia* sp., and *Saccharomyces* sp. (Pahlow et al., 2003). The efficiency of EO of thymol, eugenol, vanillin, and limonene in antifungal control on corn silage was also proven (Lopez et al., 2007). Other studies have similarly demonstrated the potential for different EO to increase the aerobic stability of corn silage (Chaves et al., 2012; Foskolos et al., 2016; Turan and Soycan-Önenç, 2018; Cantoia Júnior et al., 2020).

The sweet orange or orange tree (*Citrus sinensis* (L.) Osbeck) belongs to the Rutaceae family. Sweet orange essential oil can have up to 300 different chemical compounds, and those in greatest abundance are limonene (94 %), α-pinene (0.54 %), sabinene (0.74 %), and β-myrcene (1.18 %). Limonene is primarily responsible for the oil's antifungal properties (Marriott et al., 2001), making sweet orange oil a promising candidate as a silage inoculant. However, few studies have analyzed the sweet orange oil's effect on silage (Chaves et al., 2012).

We hypothesized that increasing sweet orange essential oil levels could improve the fermentation parameters of corn silage and aerobic stability. Thus, the present study aimed to evaluate the effects of increasing sweet orange essential oil levels on chemical composition, *in vitro* degradability, losses, fermentation parameters, microbial count, and the aerobic stability of corn silage.

## Materials and Methods

### Location

The experiment was carried out between Feb and May 2022 in the municipality of Campos dos Goytacazes, Rio

de Janeiro state, Brazil (21°45′45″ S, 41°17′06″ W, 8 m). The climate of northern Rio de Janeiro is Aw, a humid tropical climate with rainy summers and dry winters according to the classification of Köppen-Geiger (Alvares et al., 2013), with an annual rainfall of 1,020 mm.

### Harvesting, ensiling, and treatments

Corn plants (*Zea mays* L.) cv. UENF MSV2210 were harvested manually, with an average dry matter of 323.17 g $kg^{-1}$ as fed. Next, the mass was chopped up in a stationary forage harvester (JF Maxxium, JF Agricultural Machinery Ltda.) with an average particle size of 1.5 cm.

Polyvinyl chloride (PVC) silos 150 mm in diameter × 50 cm in height were used with a Bunsen valve to expel gases. Approximately 600 g of dry sand was placed in the PVC silos separated by cotton fabric to determine the losses by effluents. The silos were packed to a 600 kg $m^3$ density (as fed), and then all silos were stored at 25 ± 2.3 °C for 60 days.

The experiment was carried out in a completely randomized design with four treatment levels and four replicates. The treatments were: Control (CON), ensiling corn without sweet orange essential oil; 200 (200 mg $kg^{-1}$ of ensiling mass [as fed]); 400 (400 mg $kg^{-1}$ of ensiling mass [as fed]); and 600 (600 mg $kg^{-1}$ of ensiling mass [as fed]).

The essential oil of the sweet orange was purchased commercially, and it was extracted by steam distillation. The specifications of the oil were: density (20 °C) = 0.83 –0.86 (g $mL^{-1}$); refractive index (20 °C) = 1.465 – 1.485 (g $cm^3$), and the main components were D-limonene (96 %), myrcene (1.8 %), α- pinene (0.5 %), and sabinene (0.3 %).

### Chemical composition

Plant and silage samples were dried in a forced air oven at 55 °C for 72 h. The samples were ground in a Wiley mill (Tecnal) fitted with a 1-mm-sieve. We analyzed dry matter (DM, AOAC method 967.03, AOAC, 2019), crude fat (CF, AOAC method 2003.06, AOAC, 2019), ash (method AOAC 942.05, method AOAC, 2019), and crude protein ([N × 6.25] CP, obtained by digesting the samples (0.25 g) in 100 mL tubes, using aluminum digestion blocks with 5 mL of $H_2SO_4$ and 1 g of a mixture with a 56:1 ratio of $Na_2SO_4$ and $Cu_2SO_4.5H_2O$, including N recovery with $NH_4H_2PO_4$ and Lysine-HCl according to methods AOAC 984.13, AOAC 2001.11, and AOAC, 2019). Neutral detergent fiber (NDF) was analyzed with sodium sulfite and two additions of a standardized heat-stable amylase solution, excluding ash (aNDF, Instituto Nacional de Ciência e Tecnologia de Ciência Animal/INCT-CA method F-001/1; Detmann et al., 2012), acid detergent fiber (ADF) according to INCT-CA F-003/1 as described by Detmann et al. (2012), and lignin (Lig) (INCT-CA method F-005/1; Detmann et al., 2012). Non-fiber carbohydrate (NFC) content was estimated as:

$$NFC\ (g\ kg^{-1}) = 1000 - CP - CF - ash - NDF$$

Hemicellulose was calculated by the difference between NDF and ADF, and cellulose by the difference between ADF and lignin, all expressed in g $kg^{-1}$ DM.

Gross energy (GE) was analyzed in samples using a PARR adiabatic bomb calorimeter (Model N° 2081).

### *In vitro* degradability

The institutional Ethics Committee on the Use of Experimental Animals approved all experimental procedures, protocol 419/2019. For *in vitro* incubations, ruminal fluid inoculum came from three sheep with rumen cannulas and 55 ± 4.8 kg body mass. The animals were fed a mixed ration with 180 g crude protein kg $DM^{-1}$ and 440 g neutral detergent fiber kg $DM^{-1}$. Ruminal contents (1 L per sheep) were collected from the anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral regions. The collections were performed moments before the diurnal feeding, according to Yáñez-Ruiz et al. (2016). The rumen fluid pH was measured (6.9 ± 0.09) and was then strained through four layers of cotton cloth into an insulated thermos bottle and immediately transported to the laboratory. The strained ruminal fluid was continuously purged under oxygen-free carbon dioxide and kept at 39 °C in a water bath before the *in vitro* incubations. The ruminal fluid was mixed with two volumes of pre-warmed (39 °C) buffer solution of McDougall (1948) to prepare the inoculum.

*In vitro* degradability was focused on one stage of digestion in ruminal fluid, omitting the stage with pepsin as recommended by Tilley and Terry (1963). Each sample was performed in triplicate, with approximately 200 mg of the sample being weighed and packed into 100 mL amber vials (fitted with rubber stoppers to prevent the escape of fermentation gases) with 20 mL of the buffer solution and inoculum. The free space in the vials was immediately saturated with $CO_2$, and then they were sealed and taken to the water bath previously heated to 39 °C. Triplicate vials with no substrate (buffer plus inoculum) were prepared to serve as blank controls for each time point.

After 48 h of incubation, the vials were taken out of the water bath and immediately washed with hot distilled water (higher than 90 °C). The incubated material was filtered through quantitative filter paper (55 L $s^{-1}$ $m^2$ of air permeability). After washing, these materials were dried (55 °C for 24 $h^{-1}$ followed by 105 °C for 16 $h^{-1}$) and weighed, resulting in the undigested residue of dry matter (DM). Next, this material was analyzed for NDF content, resulting in the undigested residue of NDF. The methodology used to evaluate the *in vitro* degradability of NDF was described by Detmann et al. (2012).

The degradability (D) of DM and NDF was calculated according to the Eq. (1):

$$D = (M - [R - B] / M) \times 1{,}000 \quad (1)$$

where: M = incubated mass (g) of DM or NDF; R =

residue of DM or NDF from incubation (g); B = residue of DM or NDF from blanks (g).

**Gas and effluent losses and dry matter recovery**

Losses were calculated according to the equations proposed by Jobim et al. (2007):

Gas losses were calculated using Eq. (2):

$$GL = (SMA - SMB) / (EFM \times EDM) \times 100 \quad (2)$$

where: GL = gas losses (% dry matter); SMB = silo mass before ensiling (kg); SMA = silo mass after opening (kg); EFM = forage mass before ensiling (kg); and EDM = dry matter of ensiled forage (% dry matter).

Effluent losses were calculated according to Eq. (3):

$$EL = \{[(MESo - M) - (MESc - M)] / EFM\} \quad (3)$$

where: EL = effluent losses (kg $t^{-1}$ fresh material); MESc = mass of empty silo + mass of sand at closure (kg); M = mass of empty silo (kg); MESo = mass of empty silo + mass of sand after opening (kg); and EFM = ensiled forage mass (kg).

Dry matter recovery was calculated using Eq. (4):

$$DMR = (FMO \times DMO) / (EFM \times EDM) \times 100) \quad (4)$$

where: DMR = dry matter recovery (% DM); FMO = forage mass at opening (kg); DMO = dry matter at opening (%); EFM = ensiled forage mass (kg); and EDM = ensiled dry matter (% dry matter).

Flieg's Score was calculated through the determination of dry matter (DM) and pH values of silages according to Kilic (1986):

$$Flieg's\ Score = 200 + (2 \times \%\ DM - 15) - 40 \times pH \quad (5)$$

**Fermentation parameters**

At the opening of each silo, the material was homogenized, and a 25 g sample of fresh silage was taken. Next, the sample was processed with 225 mL of saline solution (8.5 g NaCl $L^{-1}$ distilled water) for 1 min in a blender. The material was filtered, and three aliquots were collected, two to determine the fermentation parameters and one to determine the microbial count. In the first aliquot, the pH was measured, and 0.036 N sulfuric acid was added to determine the ammoniacal nitrogen content ($NH_3$-N) through distillation with magnesium oxide, as described by Fenner (1965). The second aliquot was used to quantify short-chain fatty acids (SCFAs). To this aliquot, 0.5 mL of sulfuric acid solution (50 %) was added, according to Kung Jr. and Ranjit (2001), and it was stored at –18 °C until analysis. The short-chain fatty acid concentrations were determined by High-Performance Liquid Chromatography (HPLC; YL9100 HPLC System [Young Lin]), equipped with a REZEX RCM-Monosaccharide $Ca^{+2}$ (8 %) column. Ultra-pure water was used as the mobile phase with a 0.7 mL $min^{-1}$ flow, the column was kept at 60 °C, and a refractive index detector was used. Previously, a calibration curve had been drawn with a linearity interval of the analyzed compounds between 0.5 and 1 g $L^{-1}$ for butyric and acetic acids and 1 to 2 g $L^{-1}$ for propionic acid.

**Microbial count**

The third aliquot of the aqueous silage extract was filtered, 9 mL was added in a sterile falcon tube and was subjected to serial dilutions ($10^{-1}$ to $10^{-6}$). For the Enterobacteria count, the culture medium Violet Red Bile (VRB) and incubation of 24 h at 37 °C were used. For the fungi count, the Potato Dextrose Agar (PDA) was used with incubation of four days at 30 °C, and for lactic acid bacteria, the De Man, Rogosa, Sharpe (MRS) was used for 48 h at 37 °C. Microbial counts were expressed as colony-forming units per gram (CFU $g^{-1}$). Thus, the microbial count was transformed to $\log_{10}$ to obtain the lognormal distribution.

**Aerobic stability**

After opening the silos, 2.0 kg of silage was packed in plastic buckets of 5.0 kg capacity, remaining there for seven days to evaluate aerobic stability. These samples were kept at ambient temperature and monitored every 6 h with data loggers (Log 110 EXF Inconterm) inserted 10 cm deep in the center of the silage mass. Additionally, samples (200 g) from silos housing each treatment were collected every 24 h to assess pH after silo oxygen exposure; aerobic stability was calculated as the time in hours when the silage temperature exceeded the ambient temperature by 2 °C after exposure to the air (Kung Jr. and Ranjit, 2001).

**Statistical analysis**

Data on chemical composition, gas and effluent losses, microbial count, fermentation parameters, *in vitro* degradability, gross energy, and Flieg's Score were analyzed through linear regression analysis with a significance level of 0.05, using the mixed package from SAS (SAS University Edition, SAS Institute Inc.). A tendency was considered when $0.10 > p > 0.05$. Data normality was verified using the Shapiro-Wilk test (PROC UNIVARIATE).

The following statistical model was used:

$$Y_{ij} = \mu + \alpha_i + e_{ij}$$

where: $Y_{ij}$ is the value observed for the variable under

study referring to *the j-th* replicate of the *i-th* factor level α; μ, the mean of all experimental units for the variable under study; $\alpha_i$, the levels of sweet orange essential oil in silages with i = 0, 200, 400, 600; and $e_{ij}$, the error associated with the observation $Y_{ij}$.

The aerobic stability and pH data were analyzed as repeated measures over time through regression analysis with a significance level of 0.05, using the MIXED package from SAS (SAS University Edition, SAS Institute Inc.).

The following statistical model was used:

$$Y_{ijk} = \mu + \alpha_i + \tau_j + \alpha\tau_{ij} + e_{ijk}$$

where: $Y_{ijk}$ is the value observed for the variable under study referring to *the k-th* replicate of the *i-th* factor level α in the *j-th* h; μ, the mean of all experimental units for the variable under study; $\alpha_i$ the levels of sweet orange essential oil in silages (fixed effect), with i = 0, 200, 400, 600; $\tau_j$, the random effect of the evaluation h with j = 0.24,..., 144 for pH and 0,8,16,..., 162 for temperature; $\alpha\tau$, the interaction between sweet orange essential oil and evaluation h; and $e_{ijk}$, the error associated with observation $Y_{ijk}$.

## Results

Sweet orange essential oil affected DM (Linear $p$ = 0.043 and Quadratic $p$ = 0.035) and organic matter (OM) (Linear $p$ = 0.011 and $p$ = 0.021), presenting quadratic behavior. The other variables were not affected ($p \geq 0.05$) (Table 1). However, there was a tendency ($p$ = 0.086) for sweet orange essential oil to increase quadratically lignin (Table 1). When analyzing *in vitro* degradability, the sweet orange essential oil did not affect *in vitro* dry matter degradability (IVDMD), *in vitro* neutral detergent fiber degradability (IVNDFD), or gross energy (GE) ($p \geq$ 0.05) (Table 2).

Sweet orange essential oil had a quadratic effect ($p$ = 0.022) on acetic acid concentration in corn silage (Table 3). Using 600 mg of essential oil increased (Linear $p$ = 0.027 and Quadratic $p$ = 0.022) acetic acid by 72.72 % compared to CON (28.03 mmol $mL^{-1}$), again presenting quadratic behavior. The other parameters were not affected ($p \geq 0.05$) (Table 3). There was a tendency ($p$ = 0.097) for sweet orange essential oil to reduce the $NH_3$-N linearly (Table 3).

The levels of sweet orange essential oil did not influence losses by gases ($p \geq 0.05$) and effluents ($p \geq$ 0.05) (Table 4). However, the DM recovery showed quadratic behavior (Linear $p$ = 0.006 and Quadratic $p$ = 0.016), with greater recovery at the 400 and 600 levels compared to 0 and 200. Flieg's Score increased linearly ($p$ = 0.016) with sweet orange essential oil levels. When analyzing the microbial count, there was no effect of sweet orange essential oil on lactic acid bacteria (LAB) populations ($p \geq 0.05$) or fungi ($p \geq 0.05$) (Table 5). No enterobacteria were detected.

**Table 1** – Effects of sweet orange essential oil levels on the chemical composition of corn silage.

| Variables | Corn | Levels | | | | SEM | *p*-value | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 200 | 400 | 600 | | L | Q |
| DM | 323.17 | 293.43 | 300.26 | 310.32 | 303.68 | 1.901 | 0.043 | 0.035* |
| OM | 261.44 | 228.10 | 237.00 | 245.75 | 236.12 | 1.838 | 0.011 | 0.021* |
| CP | 54.70 | 60.50 | 61.15 | 61.00 | 62.67 | 0.374 | 0.205 | 0.416 |
| CF | 23.40 | 32.57 | 32.74 | 33.16 | 33.00 | 0.422 | 0.714 | 0.286 |
| NDF | 553.98 | 546.63 | 556.78 | 543.55 | 535.17 | 6.007 | 0.524 | 0.707 |
| ADF | 266.93 | 297.97 | 299.74 | 292.45 | 279.27 | 3.412 | 0.164 | 0.296 |
| Lig | 15.99 | 9.15 | 12.54 | 11.46 | 11.16 | 0.359 | 0.277 | 0.086 |
| NFC | 306.19 | 294.97 | 286.08 | 297.72 | 301.59 | 5.639 | 0.660 | 0.843 |
| Hem | 287.04 | 248.67 | 257.04 | 251.10 | 255.90 | 3.077 | 0.647 | 0.881 |
| Cell | 250.94 | 288.82 | 287.20 | 280.99 | 268.11 | 3.365 | 0.132 | 0.286 |

Corn = corn plant before ensiling; 0 = control (0 mg); 200 = 200 mg $kg^{-1}$ of mass forage; 400 = 400 mg $kg^{-1}$ of mass forage; 600 = 600 mg $kg^{-1}$ of mass forage; SEM = standard error of the mean; L = linear; Q = quadratic; DM = dry matter; OM = organic matter; CP = crude protein; CF = crude fat; NDF = neutral detergent fiber; ADF = acid detergent fiber; Lig = lignin; NFC = non-fibrous carbohydrate; Hem = hemicellulose; and Cell = cellulose, all expressed as g $kg^{-1}$, except DM expressed as as-fed. Means followed by an asterisk (*) differ significantly by regression analysis ($p < 0.05$). *DM = 274.88 (10.65) + 20.91 (9.72) × Levels – 3.36 (1.91) × $Levels^2$; *OM = 205.36 (10.15) + 2.45 (9.26) × Levels – 4.63 (1.82) × $Levels^2$.

**Table 2** – Effects of sweet orange essential oil levels on *in vitro* degradability and gross energy of corn silage.

| Variables | Corn | Levels | | | | SEM | *p*-value | |
|---|---|---|---|---|---|---|---|---|
| | | 0 | 200 | 400 | 600 | | L | Q |
| IVDMD, g $kg^{-1}$ | 502.17 | 525.50 | 489.85 | 487.52 | 509.07 | 16.317 | 0.772 | 0.75 |
| IVNDFD, g $kg^{-1}$ | 283.54 | 294.75 | 267.19 | 275.18 | 279.48 | 6.706 | 0.616 | 0.571 |
| GE, MJ $kg^{-1}$ DM | 16.74 | 16.63 | 16.67 | 16.62 | 16.63 | 0.030 | 0.928 | 0.982 |

Corn = corn plant before ensiling; 0 = control (0 mg); 200 = 200 mg $kg^{-1}$ of mass forage; 400 = 400 mg $kg^{-1}$ of mass forage; 600 = 600 mg $kg^{-1}$ of mass forage; SEM = standard error of the mean; L = linear; Q = quadratic; IVDMD = *in vitro* dry matter degradability; IVNDFD = *in vitro* neutral detergent fiber degradability; GE = gross energy; DM = dry matter.

As regards aerobic stability, no interaction was observed between levels and h on silage temperature ($p$ = 0.997) and pH ($p$ = 0.996) (Figure 1A and B, respectively). Increasing levels of sweet orange essential oil decreased temperature ($p$ = 0.02) over the days (Figure 1A), but it did not influence pH ($p$ = 0.404) (Figure 1B).

## Discussion

Four hundred mg of sweet orange essential oil increased DM recovery, consequently increasing the DM content in corn silage (Tables 1 and 4). The increased DM content using lemongrass essential oil be due to citral α, citral

**Table 3** – Effects sweet orange essential oil levels on fermentative profile of corn silage.

| Variables | Levels | | | | SEM | *p*-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 200 | 400 | 600 | | L | Q |
| T, °C after opening the silo | 22.43 | 22.65 | 22.60 | 22.63 | 0.201 | 0.824 | 0.96 |
| pH after opening the silo | 3.62 | 3.55 | 3.59 | 3.54 | 0.028 | 0.642 | 0.898 |
| $NH_3$-N, g kg$^{-1}$ CP | 0.94 | 0.91 | 0.87 | 0.84 | 0.018 | 0.097 | 0.284 |
| Acetic acid, g kg$^{-1}$ | 28.03 | 50.19 | 20.09 | 105.76 | 6.12 | 0.027 | 0.022* |
| Propionic acid, g kg$^{-1}$ | 0.12 | 0.09 | 0.12 | 0.25 | 0.02 | 0.534 | 0.567 |
| Butyric acid, g kg$^{-1}$ | 0.03 | 0.02 | 0.05 | 0.04 | 0.001 | 0.145 | 0.199 |

0 = control (0 mg); 200 = 200 mg kg$^{-1}$ of mass forage; 400 = 400 mg kg$^{-1}$ of mass forage; 600 = 600 mg kg$^{-1}$ of mass forage; SEM = standard error of the mean; L = linear; Q = quadratic; T = temperature; $NH_3$-N = ammoniacal nitrogen; CP = crude protein. Means followed by an asterisk (*) differ significantly by regression analysis ($p$ < 0.05). *Acetic acid = 36.43 (3.68) – 0.14 (0.01) × Levels + 0.004 (0.002) × Levels$^2$.

**Table 4** – Effects of sweet orange essential oil levels on losses, dry matter recovery, and Flieg's corn silage score.

| Variables | Levels | | | | SEM | *p*-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 200 | 400 | 600 | | L | Q |
| Gas losses, g kg$^{-1}$ DM | 23.96 | 93.80 | 36.34 | 28.20 | 7.061 | 0.769 | 0.494 |
| Effluent losses, g kg$^{-1}$ DM | 12.90 | 12.51 | 25.23 | 21.48 | 2.05 | 0.149 | 0.336 |
| Dry matter recovery, g kg$^{-1}$ DM | 897.5 | 896.2 | 941.4 | 925.0 | 0.458 | 0.006 | 0.016* |
| Flieg's Score | 60.98 | 63.60 | 64.68 | 66.27 | 1.130 | 0.016* | 0.118 |

0 = control (0 mg); 200 = 200 mg kg$^{-1}$ of mass forage; 400 = 400 mg kg$^{-1}$ of mass forage; 600 = 600 mg kg$^{-1}$ of mass forage; SEM = standard error of the mean; L = linear; Q = quadratic; DM = dry matter. Means followed by an asterisk (*) differ significantly by regression analysis ($p$ < 0.05). *Dry matter recovery = 860.7 (23.7) + 37.0 (22.0) × Levels – 4.9 (0.04) × Levels$^2$; *Flieg's Score = 61.34 (0.40) + 0.008 (0.001) × Levels.

**Table 5** – Effects of sweet orange essential oil levels on microbial populations of corn silage.

| Variables | Levels | | | | SEM | *p*-value | |
|---|---|---|---|---|---|---|---|
| | 0 | 200 | 400 | 600 | | L | Q |
| LAB, $log_{10}$ g$^{-1}$ fresh silage | 2.29 | 1.61 | 2.20 | 1.34 | 0.175 | 0.567 | 0.851 |
| Fungi, $log_{10}$ g$^{-1}$ fresh silage | 2.55 | 4.67 | 2.87 | 1.22 | 0.393 | 0.114 | 0.105 |

0 = control (0 mg); 200 = 200 mg kg$^{-1}$ of mass forage; 400 = 400 mg kg$^{-1}$ of mass forage; 600 = 600 mg kg$^{-1}$ of mass forage; SEM = standard error of the mean; L = linear; Q = quadratic. LAB = lactic acid bacteria. The presence of enterobacteria on bacterial counts was not observed.

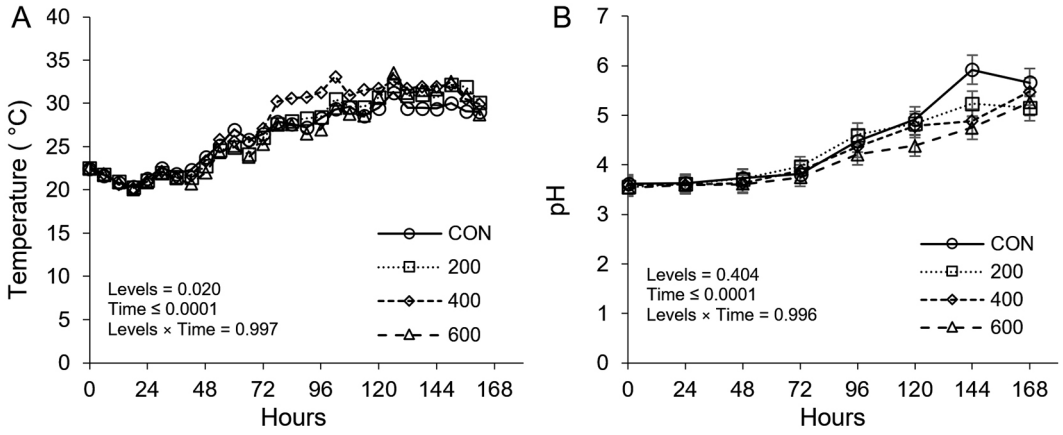


**Figure 1** – Temperature and pH values of corn ensiled for 60 days with different sweet orange essential oil levels and subjected to aerobic exposure for seven days. CON = Control (0 mg); 200 = 200 mg kg$^{-1}$ of mass forage; 400 = 400 mg kg$^{-1}$ of mass forage; 600 = 600 mg kg$^{-1}$ of mass forage. On the panel A = Temperature; and B = pH. Temperature above the environmental temperature (2 °C): CON (2.4 °C in 36 h); 200 (2.0 °C in 36 h); 400 (2.9 °C in 42 h); 600 (4.1 °C in 60 h).

β, nerolgeraniol, citronellal, terpinolene, or geranyl methylheptenone, which are responsible for biological activities such as antibacterial and antifungal (Cantoia Jr et al., 2020). The addition of essential oils can restrict the growth of undesirable microorganisms, reducing DM losses (Li et al., 2022). The OM content behaved similarly to the DM content (Table 1).

The *in vitro* degradabilities of DM and NDF were not influenced by sweet orange essential oil levels (Table 2). Although it did not have a direct effect ($p > 0.05$) 200 and 400 mg levels reduced IVDMD by an average of 7 %. The inhibition is likely provoked by antibacterial activity on rumen fluid Archaea, protozoa, and cellulolytic bacteria (*Fibrobacter succinogenes* (Hungate 1950), *Ruminococcus flavefaciens* (Sijpesteijn 1948), and *Ruminococcus albus* (Hungate 1957) (Patra and Yu, 2012). However, the effect of essential oils on *in vitro* degradability of DM and NDF was inconsistent (Di Pasqua et al., 2007). For these authors, the composition, functional groups, and synergistic interactions between components can affect degradability. For example, the hydroxyl group in the structure of phenolic compounds confers antimicrobial activity, and its relative position is very crucial for the effectiveness of these natural components (Di Pasqua et al., 2007). Furthermore, *in vitro* studies of the headspace gas composition and media bicarbonate concentrations can affect digestibility (Patra and Yu, 2013). Other factors, including source and collection time of inoculum, inoculum size and preparation, apparatus design, incubation length, headspace gas pressure, and medium composition, including buffer components, trace elements, nitrogen concentrations, and reducing agents (Cone et al., 1996; Rymer et al., 2005) can also affect digestibility.

The essential oil probably inhibits protein degradation by interacting with proteolytic bacteria through hydrogen bridges and ionic or hydrophobic interactions, resulting in proteolytic inhibition (Foskolos et al., 2016). These results corroborate the findings of Turan and Önenç (2018). When analyzing acetic acid, we observed that including 600 mg of sweet orange essential oil increased the concentration of this acid (Table 3). The antifungal activity of acetic acid can suppress the growth of lactate-assimilating fungi and yeasts that start aerobic deterioration (Kung Jr. et al., 2018; Muck et al., 2018). In this study, we observed that the silage that received 600 mg of sweet orange essential oil presented better aerobic stability (Figure 1A and B). Temperature (Figure 1A) was stable for up to 60 h. In addition, during the 168 h of aerobic stability evaluation, the temperature did not exceed 30 °C (max 27.8 °C and min 18.5 °C). For proper silage fermentation process, the temperature must be between 20 and 30 °C as higher temperatures (> 37 °C) can accelerate deterioration, and lower temperatures result in low concentrations of lactic acid and ammonia and increased levels of pH (Weinberg et al., 2001; Zhou et al., 2016). Although there was no direct effect ($p \geq 0.05$), using 600 mg of sweet orange essential oil increased by 54.43 % the concentration of propionic acid compared to CON (Table 3). This acid is linked to improved aerobic stability due to the inhibition of mold and yeast, which prevents silage heating (Muck et al., 2018).

The losses associated with fermentation in the silo are primarily from $CO_2$ production; these losses typically are in the range of 2 to 4 % (Borreani et al., 2018). Dry matter losses from fermentation depend on the dominant microbial species and fermented substrates (Wróbel et al., 2023). On the other hand, effluent losses are mainly organic compounds, such as proteins, sugars, and organic acids, representing a loss of nutritional value during silage conservation (McDonald et al., 1991). In the present study, although there is no statistical difference ($p \geq 0.05$) in the EO on gas and effluent losses (Table 4), the 400 mg sweet orange essential oil decreased gas loss by 33.33 % compared to CON. Losses (gases and effluents) are related to dry matter recovery (DMR) (Zanine et al., 2020), while the increased DMR in response to orange oil appears to have been driven by other mechanisms.

Lactic acid-producing bacteria (LAB) are primarily responsible for improving fermentation. These microorganisms produce lactic acid ($pK_a = 3.86$), which reduces the silage's pH and helps preserve the forage mass (Kung Jr et al., 2018). In this study, we observed that sweet orange essential oil levels did not impact LABs levels measured following 60 days of ensiling (Table 5). This fact may be linked to the great antimicrobial action of the EO, corroborating the findings of Foskolos et al. (2016). However, LAB plays a fundamental role in the ensiling process. In addition, the 600 mg level numerically reduced the fungal population by 52.15 % compared to CON (Table 5), demonstrating greater antifungal efficiency. Essential oils can promote bacterial cell wall degradation, causing membrane weakening and changing its permeability, compromising intracellular components (Nazzaro et al., 2013).

Overall, sweet orange essential oil does not change *in vitro* degradability, fermentation parameters, and microbial count in corn silage. However, 600 mg increases corn silage's acetic acid concentration and aerobic stability.

However, knowledge of the action of essential oils as silage additives still needs to be discovered. Therefore, further in-depth studies are needed to elucidate the mechanisms of action of these compounds as additive to control the preservation of silage quality.

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## Authors' Contributions

**Conceptualization:** Oliveira TS. **Data curation:** Silva IN, Fernandes AM. **Formal analysis:** Oliveira TS. **Investigation:** Silva IN, Meirelles Júnior JR, Mozelli Filho EJL. **Methodology:** Silva IN, Meirelles Júnior JR, Mozelli Filho EJL. **Project administration:** Oliveira TS, Fernandes AM. **Resources:** Oliveira TS. **Supervision:** Oliveira TS. **Writing-original draft:** Silva IN, Oliveira TS. **Writing-review & editing:** Oliveira TS, Aniceto ES, Souza GA, Gressley T.

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