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A Preliminary Study of the Effects of Gaseous Ozone on the Microbiological and Chemical Characteristics of Whole-Plant Corn Silage

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Abstract: This study investigated the effect of gaseous ozone (O_3) on the chemical and microbiological properties of whole-plant corn silage. Conducted on a commercial dairy farm in Brazil, maize was ensiled in experimental bag silos and treated with varying levels of O_3 (0%, 1.25%, 3.12%, 4.15%, and 6.25%). The findings revealed minimal nutrient losses in starch, non-fiber carbohydrates, crude protein, and total digestible nutrients compared to untreated fresh maize. O_3-treated silages exhibited increased levels of ash, ether extract, calcium, and phosphorus. Notably, the application of 3.12% to 4.15% O_3 improved microbiological characteristics, significantly reducing mold and yeast populations, which are common issues in farm-produced silage. This study demonstrated that gaseous ozone is a promising additive for enhancing the microbiological quality of corn silage, offering an effective alternative to traditional chemical preservatives.

Keywords: bag silos; gaseous additive; feed quality; *Zea mays*

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1. Introduction

Forages play a crucial role as feed for dairy cows and other ruminants [1,2]. They serve as ruminants' primary source of fiber, which significantly affects the ruminal ecosystem and nutrient digestibility due to their physical and chemical characteristics [3]. The preservation of green forages through acidification is a natural process that occurs once these forages are ensiled. This method of conservation provides various benefits to farmers, with the most significant being a substantial reduction in forage harvesting times, especially when forced drying of the forage is not feasible. Among the various forages, corn is one of the most crucial forage sources for dairy cattle [4]. Maize is the most commonly ensiled crop because it possesses desirable characteristics, such as high levels of soluble carbohydrates and dry matter (DM) at maturity, which promote efficient fermentation processes and provide good nutritional value post-ensiling [5].

During the production of silage, issues such as poor fermentation, overheating, and mold formation can arise [6]. These problems are primarily influenced by the fermentation characteristics of the raw material [7]. The fermentation process can become complicated due to varying levels of proteins and sugars relative to different dry matter contents [8,9]. The nutritive value of silage is typically slightly lower than that of fresh forage due to nutrient consumption by homofermentative and heterofermentative bacteria, as well as other losses through leaching and volatilization during the ensilage process.

The activity of lactic acid bacteria (LAB) facilitates rapid pH reduction and anaerobic fermentation [10]. However, the initial aerobic phase of the fermentation process can lead to significant quality losses in silage, especially when *Clostridium* spp., yeasts, and molds utilize carbohydrates and proteins as substrates for their growth. These activities generate heat, making the temperature of the silage an important quality parameter to monitor [10].

It is crucial to shorten the aerobic phase to preserve as much of the nutrients and organic matter as possible. This requires preventing the proliferation of harmful microorganisms.

For these reasons, farmers have used a wide variety of silage additives to assist forage preservation [10]. The oldest and most common additives used to modulate silage fermentation are bacterial inoculants [11]. In recent years, the use of several chemical additives (e.g., propionic acid) has increased to prevent spoilage and improve silage quality by enhancing fiber digestibility, aerobic stability, and rapidly lowering pH [12,13]. However, chemical additives' high cost and hazardous nature [14] have limited their adoption, particularly among small producers [15].

Therefore, evaluating new additives to improve silage quality is important, not only nutritionally, but also for their positive effects on microbiological and sanitary quality. Gaseous ozone (O_3) is a promising alternative for feed preservation. It has gained considerable interest due to its rapid action, strong oxidative properties, and the fact that it decomposes into oxygen [16,17]. Most importantly, it leaves no residues after decomposition and reverts to oxygen [18]. Several authors [19–21] have already used ozone to efficiently mitigate issues caused by microorganism proliferation in cereal, vegetables, and fruits, including the inactivation of mycotoxins and pathogens. Ozone has also been used as a sanitizer for fish contaminated with *Salmonella* spp. [22]. The commercial use of O_3 in agriculture shows promise due to its ease of management and controlled environmental risks. Available apparatus is designed to prevent O_3 pollution and preserve food's sensory characteristics [23]. However, using O_3 as a silage additive is a novel approach in livestock and animal feeding. Several studies have assessed the effectiveness of O_3 for extending the shelf life of various vegetables and food products [16–18,24]. However, to the best of our knowledge, this was the first study to apply this technology to silage. Specifically, this study aimed to evaluate the impact of different O_3 levels on the chemical and microbiological properties of whole-plant maize silage.

2. Materials and Methods

2.1. Plant Material, Site, and Condition

The experiment was conducted using a maize variety sown in an experimental plot close to a commercial farm in the rural area of Santo Antônio do Palma municipality, 28°49′41″ S, 51°96′51″ W, at an altitude of 746 m in the state of Rio Grande do Sul, Brazil. According to the Köppen classification, the region's climate is classified as Cfa [25], with an annual rainfall of 1944 mm and an average temperature of 15.2 °C. The soil in the experimental field was sandy loam, containing 2.9% organic matter, 27.1 mg/dm^3 of available phosphorus, and 183 mg/dm^3 of available potassium in the 0–20 cm soil layer. The field's previous harvest was wheat (*Triticum aestivum*).

Plants were sown on 22 January 2022, with the goal of achieving a plant density of 76,000 plants per hectare, at a row spacing of 0.55 m. This resulted in a sowing density of 4.18 grains per linear meter. The maize crop was fertilized with 81 kg ha^{-1} of nitrogen at the V4 phenological stage (four-leaf development stage) using urea as the nitrogen source. Insecticide and herbicide applications were performed according to the manufacturers' recommendations. Harvesting occurred on 30 June 2022, at the R5 phenological stage (farinaceous grain stage).

2.2. Experimental Design and Silage Preparation

For this study, 20 polyethylene silage bags (four repetitions per treatment) sized 0.51 m^2 (110 cm × 52 cm), 200 µm thick, with 40 kg/60 L volume capacity (Gobi Brasil LTDA., São José do Rio Preto, Brazil) were used. The maize was chopped into 2–3 cm particles using milling equipment (TRF800, Trapp®, Jaraguá do Sul, Brazil) and placed in a collector bucket on a scale, until it reached 30 kg. To fill each silo bag, the O_3 equipment's (O_3 Air model, Philozon®, Balneário Camboriú, Brazil) hose was inserted into the bag until it reached the bottom, then 30 kg of chopped maize was gradually placed and compacted inside the bag (to a density of approximately 128.42 kg/m^3). Full bag capacity was not reached, with the intent of guaranteeing maximum exposure of the bag's content to the gas, without rupturing it. After the full silage content was placed, and before the O_3 equipment was operational, the opening of the bag was partially closed. Therefore, as O_3 was inserted, some gas exited the bag, and the people responsible for these procedures wore personal protective equipment (PPE) to ensure their safety.

Based on the fresh matter and ozone equipment regulation (10 g/h), O_3 levels were 0, 1.25, 3.12, 4.15, and 6.25%. These percentage inclusion levels were controlled by application times:

- 0% was the control treatment.
- 1.25% was achieved by applying 0.5 g of O_3 for three minutes.
- 3.12% was achieved by applying 1.25 g of O_3 for seven minutes and thirty seconds.
- 4.15% was achieved by applying 1.66 g of O_3 for ten minutes.
- 6.25% was achieved by applying 2.5 g of O_3 for fifteen minutes.

After O_3 inclusion, silo bags were closed using a cable tie and allocated to a brick-tiled storage room for 96 days before opening.

2.3. Sampling Operations and Analysis

The chemical composition of whole-plant maize was analyzed before (D0) and after (D96) ensilage (Table 1). For that purpose, a 1 kg aliquot was separated from fresh maize before ensiling and from made silages. Concentrations of dry matter (DM), organic matter (OM), ash, starch, neutral detergent fiber (NDF), acid detergent fiber (ADF), non-fiber carbohydrates (NFC), crude protein (CP), ether extract (EE), total digestible nutrients (TDN), calcium, and total phosphorous content were analyzed for both fresh forage and the ozone-treated silage (that with a 3.12% concentration), according to the methodologies of Cavallini et al. [2] and Felini et al. [26].

Table 1. Chemical composition (g/kg) of whole-plant maize before and after ensiling with gaseous ozone.

	Before	After
Dry matter	257	256
Organic matter	966	951
Crude protein	84	79
Starch	340	297
Ether extract	13	16
Neutral detergent fiber	445	444
Acid detergent fiber	219	291
Non-fiber carbohydrates	424	414
Total digestible nutrients	703	693
Ash	34	49
Calcium	1.3	2.1
Total phosphorus	1.0	1.6

Silages' pHs were analyzed [27] and their temperatures were recorded with TP101 electronic digital thermometers (Elecrow, Limassol, Cyprus) allocated into the bags prior to opening them.

For microbial counts, 25 silage samples were transferred to a sterile homogenization bag, suspended 1:10 *w*/*v* in a peptone salt solution (1 g of bacteriological peptone and 9 g of sodium chloride per liter), and homogenized for 4 min in a laboratory Stomacher blender (Seward Ltd., London, UK). Serial dilutions were prepared, and yeast and mold numbers were determined using the pour-plate technique, with 40.0 g/L of yeast extract glucose chloramphenicol agar (YGC agar, DIFCO, West Molesey, Surrey, UK) after incubation at 25 °C for 3 and 5 d for yeasts and molds, respectively. Yeast and mold colony-forming units (CFUs) were enumerated separately on plates that yielded 1–100 CFUs according to their macromorphological features.

2.4. Statistical Analysis

Data were subjected to the normality test [28], analyses of variance, and orthogonal polynomial contrasts [29] at 5% probability ($p < 0.05$). Data were analyzed with the aid of SAS® (SAS Institute Inc., Cary, NC, USA) OnDemand for Academics, following the statistical model:

$$Yi = \mu + \alpha i + \varepsilon i,$$

where Yi is the observed value, μ is the population average, αi is the effect of ozone level application (1 to 4), and εi is the residual error.

Variables were compared between groups using non-parametrical ANOVA, with the Kruskal–Wallis test for multiple comparisons.

3. Results and Discussion

The use of additives aims to retain as many nutrients as possible in silage compared to fresh forage. It is essential to note that the nutritional content of silage also depends on several factors related to the proper management of the process, particularly the quality of the crop before harvest and the management of silage from field to feed trough. No silage additive can turn poor-quality forage into good silage, but they can help produce excellent silage from high-quality forage.

Table 1 presents the chemical characteristics of maize both at and after (pool sampling of control group bags) the ensiling procedure. Results observed in this study regarding the DM content (257 g/kg) are lower compared to those reported by Silva et al. [30], who analyzed the quality of forages from 40 dairy farms located in southern Brazil and observed a DM content ranging from 304.30 to 344.23 g/kg. However, as reported by Johnson et al. [31] and by Der Bedrosian et al. [32], the DM content of silages is influenced by several factors, such as plant maturity, agronomical practices, and mechanical processing. According to Johnson et al. [31], the optimum time for harvesting plants to make corn silage is when the whole plant reaches a DM content of about 320 to 350 g/kg. However, the authors specify that this recommendation applies only to corn silage intended for horizontal silos.

In practice, farmers often harvest corn at different stages of maturity, resulting in varying DM contents. Early harvesting occurs when plants are less mature and have lower DM, often due to limited harvesting capacity to handle large amounts of forage in a short period. Conversely, mature plants with a higher DM content are harvested when equipment cannot keep up with plant maturity or when custom harvesting equipment is unavailable at the optimal time. Another common issue is the lack of monitoring of whole-plant DM, leading to harvests outside the recommended DM range. Corn plants harvested with low DM contents typically yield less DM and starch, while overly mature plants yield higher amounts of both [33]. Despite the apparent benefits of harvesting corn at higher DM concentrations due to increased yields and starch content, this material is more difficult to pack, and the resulting silage is prone to rapid spoilage when exposed to air [34]. Moreover, according to Yan et al. [35], the DM content of silage is also affected by the length of storage, with a general tendency for DM content to increase with longer storage periods. Therefore, it is difficult to compare data from our study with those in the literature due to different ensiling periods used in other studies (e.g., 150 days for Der Bedrosian et al. [32]).

Starch levels observed in this study are consistent with those reported in other studies [32,34,36]. Starch is one of the main fractions in a maize crop, and under good management, maize silage with a higher starch content is nutritionally desirable. However, the feeding value of maize silage is affected by kernel processing and fermentation patterns [37].

The average NDF content observed in the present research (445 g/kg) reflects the advanced maturity of the plants at the time of harvest. However, our results are similar to findings reported by Horst and Neumann [38], who observed an average NDF content of 445 g/kg^{-1} in 63 beef cattle farms located in Paraná, South Brazil. The same authors noted substantial variations in NDF levels over time, with values ranging from 432 to 517 g/kg.

Daniel et al. [37] found that in Brazil, maize, and consequently silage, typically has higher NDF levels compared to those in North America. This difference is likely due to several factors, including maize hybrids, altitude, location, soil type and nutrient status, agronomic and ensiling practices, and various climatic conditions, all of which significantly influence the final quality of the silage.

The OM content of silages observed in this research (966 g/kg) was similar to those reported in the scientific literature [36,39,40]. CP values observed (84 g/kg) were within the range (75–86 g/kg) reported by Von Pinho et al. [41] as indicative of good quality corn silage. Similar levels of CP were also observed by Silva et al. [30] in corn silages from Southern Brazil, which ranged from 75 to 86 g/kg. However, as noted by Terler et al. [42], OM and CP content can be significantly affected by variety and harvest date. For this reason, some differences observed may be due to comparisons between different varieties.

Minimal losses in starch, NFC, CP, and TDN concentrations were observed in maize silages treated with O_3 compared to those of the fresh maize roughage (Table 1). Also, there were increases in ash, EE, calcium, and phosphorous concentrations. Such results were related to the excellent ensilage management adopted in this study, especially the adequate silage density (625 kg/ha), the maize maturity stage at harvesting, proper bags for ensiling, and silage allocation in a brick-tiled storage room [43]. Observed losses were unavoidable, indicating mainly nutrient consumption by homofermentative and heterofermentative bacteria [37]. Increased ash, calcium, and phosphorous contents were primarily related to OM consumption by microorganisms (e.g., LAB, clostridium, and yeasts) over the fermentation processes. Such increases may represent gas and effluent losses [44]. However, the silages' chemical traits remained acceptable after ensiling [45].

Concentrations of DM and moisture were not affected by O_3 levels; neither were pH, humidity, or yeast's CFUs results (Table 2; Figure 1).

Table 2. Dry matter content, pH, and microbiological characteristics of whole-plant maize silage with ozone (O_3) levels added and sealed over 96 days.

Variable	Ozone Level (% as Feed)					SEM	*p*-Value [1]			
	0.00	1.25	3.12	4.15	6.25		L	Q	C	Qr
pH	3.64	3.65	3.63	3.64	3.63	0.01	0.189	0.263	0.267	0.140
DM, g/kg	257	256	257	256	257	0.02	0.674	0.396	0.064	0.244
Temperature, °C	15.3	15.0	15.1	15.2	15.4	0.05	0.392	0.770	0.206	0.001
Molds CFUs/g	12.0	3.0	7.0	3.3	1.7	0.87	<0.001	<0.001	<0.001	<0.001
Yeasts CFUs/g	8.5	6.7	6.2	5.1	3.7	0.42	<0.001	0.215	0.094	0.705

[1] L: linear effect; Q: quadratic effect; C: cubic effect; Qr: quartic effect; SEM: standard error of mean.

The final pH observed in all silages was in line with that suggested by Kung et al. (2018) for well-preserved corn silages. According to Weinberg and Ashbell [46], the pH value is one of the most important quality parameters used to evaluate silage. This parameter is strongly influenced by the extent of lactic fermentations and helps inhibit the growth of microorganisms that can deteriorate the product [47], thereby contributing to the proper preservation of the silage [48]. The DM content of all silages was over 250 g/kg, and the pH varied from 3.63 to 3.65 (Table 2). A silage DM content over 250 g/kg and a pH between 3.8 and 4.2 suggested that fermentation was suitable, with more lactic acid production than secondary fermentations, such as propionic or butyric [49], although analyses were not conducted in the current research.

Nutritional characteristics of forage are naturally maintained under anaerobic conditions due to bacteria present on the plants. The type and efficiency of fermentation vary depending on the number and type of lactic acid bacteria on the plants [50]. The rate at which pH decreases influences the amount of sugar utilized by bacteria, the preservation of proteins, and levels of lactic acid, acetic acid, and ethanol, thereby affecting the final quantity of silage [51]. Whole-plant maize often provides good-quality silages due to its content of water-soluble and non-fiber carbohydrates, mainly because of the grains in the silage composition [4].

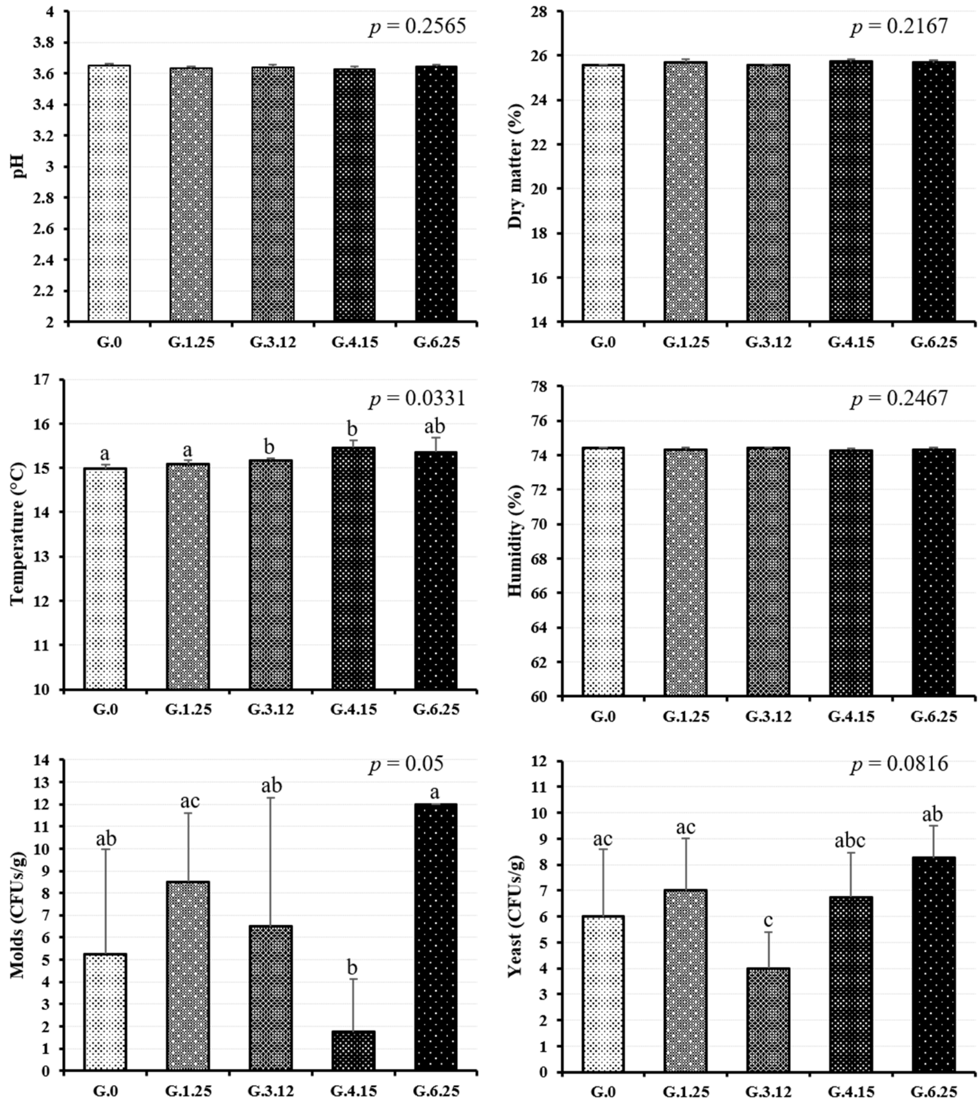

Figure 1. Chemical and microbiological variables for different levels of O_3 inclusion (0, 1.25, 3.12, 4.15, and 6.25%) in maize silage bales. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

A quartic effect was observed on the maize silage temperature ($p < 0.05$), with the lowest value at 1.25% and the highest at 6.25% O_3 inclusion (Table 2). Linear, quadratic, cubic, and quartic effects of the O_3 level were found in the mold population, with a considerable reduction from 12.0 to 1.7 CFUs/g. Control the mold, and yeast populations can serve as a useful indicator of silage quality. According to Gotlieb [52], the total number of molds can be used as an indicator of mycotoxins, but high numbers (>6 CFUs/g of wet silage) are also usually associated with aerobically spoiled silages [49]. Elevated yeast counts in silage typically correlate with increased ethanol concentrations, and their abundance is often inversely proportional to aerobic stability, particularly in corn silages. Regarding the yeast population, a linear effect was recorded. Adding 6.25% O_3 into whole-plant maize silage mass led to 3.7 CFUs/g, and the control silage displayed the highest value (8.5 CFUs/g).

The presence of molds, yeasts, mycotoxins, and high temperatures are ordinary in maize silages made in farm conditions in Brazil. Carvalho et al. [53] evaluated the microbiological aspects of maize silage produced at 36 dairy farms in Minas Gerais state, Brazil. The authors found mycotoxins in 77% of these silages, yeast populations ranging from 2.0 to 7.0 CFUs/g, and mold populations from 2.0 to 4.6 CFUs/g. Moreover, the average silage temperature was 26.5 °C. Results recorded in silages of the present study were considerably below those reported by Carvalho et al. [53], probably because of low temperatures at the site (an average of 15.2 °C). Environmental conditions such as temperature, pH, and water activity influence microbiological silage populations and mycotoxin production. O_3 has a redox potential of 2.07 V. Thus, it is one of the most potent oxidant disinfectants against bacteria, viruses, algae, and fungi [20]. Controlling these microorganism populations is essential for silage quality, as molds, yeasts, and undesirable bacteria can consume carbohydrates and proteins during the fermentation process and after the opening of the silo [49,54].

The future of ozone treatment in enhancing the microbiological quality of whole-plant corn silage is promising, with several key avenues for further research and application. Long-term studies are essential to evaluate the sustained impacts of ozone treatment on silage quality and livestock health, while economic analyses will help determine the cost–benefit ratio for farmers, particularly those operating on a smaller scale. Additionally, exploring the environmental implications of widespread ozone use will ensure that its benefits outweigh any potential risks. Broader applications of this technology across different types of forage and feed can further expand its utility in agriculture. Technological advancements aimed at developing more efficient and user-friendly ozone application methods will facilitate adoption among farmers. To disseminate these findings effectively, innovative tools, such as social media, interactive websites, and mobile apps, will be utilized [55]. Educational initiatives, including virtual reality simulations, gamification, blended learning approaches, and hands-on workshops, will integrate the research into agricultural science curricula, ensuring that both current and future generations of farmers and researchers can benefit from these advancements [56].

4. Conclusions

Gaseous ozone has proven to be an effective additive for preventing undesirable microbiological proliferation in whole-plant maize silage during fermentation while maintaining its chemical and bromatological composition. The inclusion of ozone at concentrations between 3.12% and 4.15% significantly improved the microbiological characteristics of silage. Utilizing gaseous ozone as an additive in corn silage stored in bag silos offers a promising alternative for farmers. Economically, this technology may potentially reduce spoilage and enhance feed quality, leading to lower feed costs and improved livestock productivity.

It is worth noting that this study was preliminary, and further research is necessary to fully evaluate the efficacy, economic benefits, and potential of this technology. Future studies may explore the use of ozone-treated silage in animal feed to assess its impact on the productive performance of livestock. This research may pave the way for new advancements in animal nutrition and farm management, ultimately contributing to more efficient and sustainable agricultural practices.

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