Gaseous ozone to improve quality of corn as feedstuff

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Abstract. In the long term, fungi attacks will cause a decrease in the quality of corn during storage. In this case, Aspergillus flavus and Aspergillus parasiticus have become a serious concern related to food safety because of their ability to produce aflatoxins which are toxic to humans and animals. Nowadays, feed industries use fungicides to control fungal infections before the storage period due to their affordability, but the chemical residues are detrimental to our health. Therefore, an alternative method was needed to prevent and control fungi and aflatoxin formation in corn. One of the potential methods to apply is the gaseous ozonization. This research was conducted to investigate the potential of ozone in preventing fungal attacks and aflatoxin formation in corn, assuring that the corn quality can be maintained during the storage period. The objective of this study was to determine the effect of ozone on maintaining the quality of corn during storage, especially to reduce fungi colonies and aflatoxin formation. The experimental design used in this study was a completely randomized design with two treatment factors, namely temperature (20, 30, and 40°C) and exposure time (30 and 60 minutes). Corn quality parameters observed include moisture content, total fungal, moldy kernels, damaged kernels, and aflatoxin (AFB1, AFB2, AFG1, AFG2) contamination. The results showed that ozone treatment had an effect on reducing total fungal and aflatoxin contamination in corn, however, it had no effect on the moisture content, moldy kernels, and damaged kernels of corn. The optimum effect was obtained when the ozonization was conducted at a temperature of 20°C for 60 minutes exposure time, which results in the highest reduction in total fungal and total aflatoxin contamination of 36.77% and 92.45% respectively.

Keywords: aflatoxin, corn, fungi, ozone, temperature

1. Introduction
Corn is the second priority of cereal crop in Indonesia. Indonesia produces around 10.82 million tons of corn as feedstuff or 41.38% of Indonesian total production [1]. The high demand for corn by the feed mills industry is not followed by the improvement of its quality at the farmer level. Generally, the quality of corn at the farmer and middleman level was still low due to poor postharvest handling that does not meet the hygiene standards. Thus, it increases the risk of fungi contamination. Fungi, in the long term, will decrease the quality of corn during the storage period which will consequently affect its quality classification in the trading system. The presence of fungi in corn, especially Aspergillus flavus and Aspergillus parasiticus, is a serious threat to food safety because of its ability to produce aflatoxins. Aflatoxin is one of the mycotoxins produced by Aspergillus flavus and Aspergillus parasiticus in extreme conditions. Aflatoxins are toxic to animals because they induce immune disorders, weight loss, and affect their growth and productivity. Whereas for the human being, aflatoxins can cause poisoning, gene mutations, inhibit fetal growth, and some carcinogenic risks, especially liver cancer [2].

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1. Introduction
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Various methods have been used to prevent and control fungi infections in corn during the postharvest handling and storage period. Applying fungicide is one of the most common methods to control fungi in corn before storage, which has been widely practiced by the feed mills industry. This method is widely used because it is relatively inexpensive. However, its chemical residue has a negative effect on human and animal well-being. Another alternative method to reduce fungal contamination and simultaneously control aflatoxins in corn is the use of gaseous ozone. The efficacy of ozone to overcoming microbes in agricultural products has been reported, including bacteria, fungi, viruses, and fungal/bacterial spores. The application of ozone in fruits and vegetables was known to inactivate pathogens and rotting microorganisms. Besides that, ozone can eliminate insects and degrade mycotoxins [3]. Many studies have shown that gaseous ozone is quite effective to reduce the contamination of fungi and mycotoxins in grains. Ozone treatments are greatly influenced by some factors such as ozone concentration, temperature, and relative humidity. Furthermore, the penetration and adsorption of ozone on the seed surface depend on the concentration, exposure time, flow rate, temperature, characteristics of kernels, and the presence of insects and microbes on the seed surface [4]. Previous studies reported that ozone could inactivate 96% of fungal spores in barley [5], decrease 100% of Aspergillus sp. and Penicillium sp. in rice [6] inhibit 95.6% of Aspergillus flavus growth, reduce 86.75% and 96.66% of aflatoxin B1 with its initial level of 10 ppb and 20 ppb [7], and reduce 88% of aflatoxin B1 in corn [8].

Some of the advantages of ozone are that it left no residue and does not cause nutritional changes in the product. The use of gaseous ozone shows no effects on changes in fatty acids and amino acids of soybeans, wheat, and corn after 30 days of its exposure. In addition, ozone treatment does not affect the polyphenols, acids, and peroxide of peanuts [9]. In 2001 the Food and Drug Association of United States declared ozone as a safe sanitizer for food because the decomposition product was in the form of oxygen and left no residue on the product. Additionally, the use of ozone has been proven to not cause any nutritional changes of grains or the formation of new compounds that can be harmful to humans and animals. Therefore, the ozone is considered to be safe to be widely used as a sanitizer or preservative in various food products [6]. The use of ozone in Indonesia is still limited to fruits and vegetables, hence further research on the use of gaseous ozone in corn is needed, more particularly to observe how it can reduce the fungal and aflatoxin contamination. The use of gaseous ozone in corn is expected to improve the quality as a raw material of feed. The purpose of this study is to determine the effect of ozone on improving the corn quality, especially the fungal colonies and aflatoxins.

2. Materials and methods

2.1. Materials

The main material used in this study was a hybrid type of corn intended for animal feed with a moisture content of less than 14%. The other materials used in this study were the culture of Aspergillus flavus, aflatoxin standards, aflatest column affinity, microfilter paper, distilled water, NaCl, methanol, alcohol, and potato dextrose agar (PDA) medium.

2.2. Sample preparation

The corn used in this study was inoculated using Aspergillus flavus culture. The inoculum was prepared by culturing Aspergillus flavus in 10 ml of nutrient broth (NB) and incubated for three days at room temperature. The culture was then re-inoculated into 100 ml of NB and incubated for three days at room temperature. The suspension was re-inoculated in 1000 ml of NB and incubated for three days at room temperature to obtain inoculum stock. Next, the inoculum was analyzed for total fungal colonies using the total plate count method to determine its initial population before it was used in corn. Then, the inoculum was added to the corn (dose of 50 ml kg⁻¹), and it was incubated for 14 days at room temperature. Finally, the corn was packaged and stored in the freezer.
2.3. Ozone treatment

The experimental design used was a completely randomized design with two factors, namely temperature (20, 30, and 40°C), and the exposure time of gaseous ozone (30 and 60 minutes). The ozone process was conducted by preparing 1 kg of corn and placing it on a ram wire that was installed 10 cm above the bottom surface of the cylinder tube. Next, the gaseous ozone produced by the ozone generator (0.15 ppm) was passed through the corn in the cylinder tube. The ozone treatment at the temperature of 20°C was conducted using a cool box. The treatment at the temperature of 30°C was conducted at room temperature. While the treatment at the temperature of 40°C was done using a water bath. The quality parameters of corn observed included total fungal, aflatoxin (aflatoxin B1=AFB1, aflatoxin B2=AFB2, aflatoxin G1=AFG2, aflatoxin G2=AFG2), moisture content, moldy kernels, and damaged kernels. The observation data were analyzed using analysis of variance (ANOVA) to determine the effect of treatment on the corn quality. The statistical analysis was continued using the Tukey test to determine the differences of each treatment at the confidence interval of 95% or at the level (α <0.05).

2.4. Analysis procedure

2.4.1. Aflatoxin. An amount of 25 g of corn was mixed with 5 g of NaCl and 125 ml of methanol 70% using a laboratory blender for 2 minutes. After that, the mixed solution was filtered using a fluted filter paper. Then, 15 ml of the extract was added to 30 ml of aquabidest, mixed well, and filtered using a microfilter paper. Next, 15 ml of the extract was taken and passed into the aflatest column affinity at a flow rate of 1 drop per second. The process was resumed with flushing by using 10 ml of aquabidest at the same flowrate. This process was executed two times. In this procedure, the final flushing was carried out using 1.0 ml of HPLC grade methanol until the filtration process was completed. Finally, the final extract from the aflatest column was ready to be injected into the HPLC. The data obtained were compared with the aflatoxin standards to calculate the final aflatoxin of the corn sample [10].

2.4.2. Total fungal. The analysis of fungal colonies refers to the total fungal method based on the Indonesian National Standard of SNI 01-2879:2008 [11]. First, 25 g of corn were added into 225 ml of buffer peptone water. The suspension was homogenized for 1-2 minutes. After that, 1 ml of the suspension was added into 9 ml of NaCl 0.85% solution to obtain 10⁻¹ dilution suspension. Next, 1 ml of the 10⁻¹ dilution suspension was transferred into 9 ml of NaCl solution to obtain 10⁻² suspension. The same dilution process was replicated to obtain the 10⁻³, 10⁻⁴ of suspension and so on as needed. Then, 1 ml of the final dilution suspension was added to a petri dish containing PDA media, and it was incubated at room temperature (±25°C) for 48-72 hours. Finally, the growing fungal colonies were counted and stated in log CFU g⁻¹.

2.4.3. Physical quality. The physical quality of the corn observed includes moisture content, damaged kernels, and moldy kernels. The moisture content was analyzed using the modification gravimetry method based on the SNI 01-2891-1992 [12]. The process was started by placing 10 g of corn into a cup and drying it using an oven at a temperature of 105°C for 3 hours. Afterward, the final weight was scaled, and the moisture content was calculated. Meanwhile, the analysis of damaged and moldy kernels refers to the SNI 8926:2020 [13]. First, 100 g of corn were observed visually, and the damaged and moldy kernels were separated manually from the healthy kernels. At the end, the results were compared with the total samples of corn to obtain the content of damaged and moldy kernel.

3. Result and discussion

3.1. Quality of corn

The quality of clean corn and corn inoculated by Aspergillus flavus is presented in Table 1. Generally, all values for quality parameters of corn significantly changed at the end of incubation, except for
the moldy kernels. The moisture content has increased almost 50% compared to the initial moisture content even though it was still below the standard required by the SNI 8926:2020. The increase of moisture content was caused by the water that was added from the isolates to the corn. In this case, the environmental conditions with minimal aeration during the incubation process caused the water added did not get to evaporate from the surface of kernels. Instead, it was absorbed into the corn kernels, and consequently, it increased the moisture.

**Table 1. Quality of corn inoculated by Aspergillus flavus.**

| Parameter              | Unit  | Non-inoculated corn | Inoculated corn |
|------------------------|-------|---------------------|-----------------|
| Moisture content       | %     | 8.86                | 13.27           |
| Damaged kernels        | %     | 0.06                | 9.60            |
| Moldy kernels          | %     | 0                   | 0.02            |
| Fungal colonies        | Log CFU g⁻¹ | 3.34              | 5.56            |
| Aflatoxin B1           | ppb   | 11.05               | 177.71          |
| Aflatoxin B2           | ppb   | 4.97                | 10.30           |
| Aflatoxin G1           | ppb   | Not detected        | 6.05            |
| Aflatoxin G2           | ppb   | Not detected        | 7.95            |
| Total aflatoxin        | ppb   | 16.02               | 202.01          |

The damaged kernels of inoculated corn also increased significantly to 9.58% compared to the initial value before the inoculation. The most common type of damage was the abnormal color change in which the corn turned greenish-yellow/brownish. This color change was caused by the high infection of *Aspergillus flavus* inside the corn kernels, making the corn color changed from yellow to greenish-yellow/brownish. The abnormal color seen from the damaged corn kernels was presumed to be caused by the presence of greenish-yellow conidia of *Aspergillus flavus* [14]. This assumption was strengthened by the significant increase of the total fungal infections after inoculation, which was 5.56 log CFU g⁻¹. The increase of the total fungal infections indicated that the *Aspergillus flavus* that was added to the corn can grow and develop very well during the 14-day incubation period, especially inside the corn kernels. The analysis of the non-damaged kernels of inoculated corn showed that it was categorized into the non-class quality because its presence exceeded the maximum damaged corn kernels required by the SNI 8926:2020. According to the Indonesian National Standard, the quality class of corn as an organic and non-organic feedstuff can be divided into three classes, namely premium, medium I, and medium II, with the maximum damaged kernels allowed was 3%, 5%, and 7% respectively [13].

The analysis on the moldy kernels of inoculated corn showed a slight increase of about 0.02%, although, on the other hand, the total fungal infections surged significantly. This phenomenon was presumably due to the fact that most of the fungal spores from the inoculum added could grow rapidly inside the corn kernels, resulting in serious damages inside the corn kernels. The effects can be seen from the corn color that turned brownish-yellow or blackish. Furthermore, the brownish-yellow or blackish color was presumably caused by the conidia of *Aspergillus flavus* [14]. Based on the SNI 8926:2020, moldy kernels were defined as corn kernels that appear to have mold on them when observed either directly or visually with ultraviolet light. The moldy kernels of inoculated corn were still below the moldy seed standard required by the SNI 8926:2020. The maximum standard of moldy kernels of corn as organic and non-organic feedstuff was 1% for premium class, 2% for medium I, and 4% for medium II [13].

Meanwhile, during the incubation period, *Aspergillus flavus* was influenced by many factors such as moisture content, oxygen, light, temperature, humidity, and the presence of another fungus as well as macro elements (carbon, nitrogen, phosphorus, potassium, and magnesium) and also microelements (iron, zinc, copper, manganese, and molybdenum). *Aspergillus flavus* requires a higher temperature...
environment, but it has the ability to adapt to lower water activity (aw) with a minimum aw of 0.78 and to grow optimally at an aw of 0.99. Conidia *Aspergillus flavus* was reported to germinate at an aw of 0.2 and at a high temperature of 50-55 °C, with an optimal growth temperature coming close to 33°C. This fungus can live in a very wide pH range between 2.1 and 11.2, with growth tends to be slower at a pH below 3.5 [14]. At the beginning stages, the *Aspergillus flavus* colonies have a yellow color which will change to greenish-yellow or brown with an inversion color of golden brown or colorless. While in the final stages of its growth, the color of fungal colonies will turn dark green [15]. Another study affirmed that *Aspergillus flavus* can be easily distinguished from other *Aspergillus* species because it produces conidia with a bright yellow-green color, with a maximum growth rate at a temperature of 25°C and 37°C, and a slow growth rate at a temperature of 5°C. The growth of colonies tends to vary depends on the environment. Colonies usually consist of thin basal mycelium and a dense texture. The growth of the *Aspergillus flavus* colony showed a bright yellowish-orange color after 42-48 hours. The greenish-yellow color of the *Aspergillus flavus* fungi was presumably caused by the presence of the kojic acid, which was produced by the fungi and converted into fluorescent substances by the peroxidase of plant tissue [14].

*Aspergillus flavus* can survive in extreme conditions, which was not the ideal condition for its growth. In addition, these fungi also have the ability to produce aflatoxins in certain extreme conditions. Table 2 shows that the aflatoxin of inoculated corn has increased significantly. AFB1 has the highest percentage, which was followed by AFB2, AFG1, and AFG2. The increase of aflatoxin was similar to what some previous studies have discovered, confirming that the addition of *Aspergillus flavus* isolates has been proven to increase aflatoxin B1 of corn from 0.106 ppb to 349.04 ppb after the 15-day incubation period at a temperature of 25°C [16]. AFB1 was the most carcinogenic and it was usually produced in the highest amount among the others, while AFB2 and AFG2 were produced only one-tenth and one-third of the total number of the AFB1 and AFG1. The presence of double bonds at the furan ring terminal was the main factor determining the toxicity and carcinogenic level of aflatoxins [17]. The increase of aflatoxin was caused by some environmental factors which supported the *Aspergillus flavus* to produce aflatoxin. The temperature and humidity during the corn incubation were ranging from 29-33°C and 70% respectively. The best environment for aflatoxin formation by fungi was ranging from a temperature of 28-31°C and humidity of 60-80% [18]. Some other studies revealed that *Aspergillus flavus* produced aflatoxin best at a temperature of 12-41°C and humidity of 99%. The *Aspergillus* sp began to form aflatoxins on the ninth day of the incubation at a temperature of 25°C [19]. There were many factors affecting the properties and quantity of mycotoxins produced by fungi, including the type of substrate, moisture content, nutrients, temperature, humidity, maturity of fungal colonies, presence and competition with the other fungi or microorganisms, physical damage of substrate due to insect activity, and stress [20].

3.2. Efficacy of ozonization on quality of corn

The physical and microbiological quality of corn were observed to discover the effects of gaseous ozone exposure on the quality changes of the corn. The physical qualities observed included the moisture content, moldy kernels, and damaged kernels, while the microbiological quality observed included the total fungal infections and aflatoxin. These corn quality parameters are the main quality observed after gaseous ozone treatment because they are the main requirements on the selection process of corn by the feed mills industry, especially moisture content and aflatoxin. The physical qualities of corn treated with gaseous ozone were presented in Table 2, while the total fungal infections and aflatoxin of corn treated with ozone were presented in Table 3. The efficacy of gaseous ozone on the level of fungal infections and aflatoxin was presented in Table 4.

3.2.1. Moisture content. The analysis of variance on moisture content showed that the temperature, exposure time of ozone, and the interaction between both did not significantly affect the corn moisture content (p>0.05). This confirmed that gaseous ozone at various temperatures and exposure times could not reduce or increase the moisture content of corn. In this experiment, the moisture content of corn was
ranging from 13.20-13.42%, and there were no significant differences compared to the control setup (Table 2). The moisture content of corn was still below the benchmark required by the SNI 8926:2020 where the maximum moisture content standard of corn as feed had to be at least 14% (wet basis=wb) for premium quality and 16% (wb) for medium II [13]. As one of the physical properties of corn in this study, the moisture content discovered was relatively similar to the previous studies, most of which stated that ozone treatments had no effects on the physical qualities of grains, however, ozone treatments in a high intensity could remove mycotoxins and mold in grains. Whereas, excessive ozone exposure was reported to oxidize the chemical components of products [21].

Table 2. Physical qualities of corn exposed with gaseous ozone.

| Temperature (°C) | Exposure time (minutes) | Moisture content (%wb) | Damaged kernels (%) | Moldy kernels (%) |
|-----------------|-------------------------|------------------------|---------------------|------------------|
| 20              | 30                      | 13.20^a                | 9.50^a              | 0.01^a           |
| 20              | 60                      | 13.42^a                | 9.49^a              | 0.01^a           |
| 30              | 30                      | 13.21^a                | 9.50^a              | 0.02^a           |
| 30              | 60                      | 13.25^a                | 9.49^a              | 0.01^a           |
| 40              | 30                      | 13.21^a                | 9.62^a              | 0.01^a           |
| 40              | 60                      | 13.41^a                | 9.58^a              | 0.01^a           |
| Control         |                         | 13.27^a                | 9.60^a              | 0.02^a           |

Note: numbers followed by the same letter show no significant difference at the level of 0.05.

3.2.2. Damaged kernels. The variance analysis on the damaged kernels showed that temperature and exposure time had a significant effect on the damaged corn kernels. However, the interaction between them did not have a significant effect on the damaged kernels after treatment with a p-value>0.05. Overall, the damaged kernels of the ozonized corn were ranging from 9.49 to 9.62% (Table 2). Based on the SNI 8926:2020, damaged kernels are corn kernels that are damaged and/or become abnormal. It could damages due to physical damages, germinating processes, burning processes, and insect pests including abnormal colors. The results showed that the damaged corn kernels were above the standards required by the SNI 8926:2020. The standard of damaged corn kernels as a feed and food ingredient was a maximum of 3% for the premium quality, and a maximum of 7% for the medium II [13].

The most damaged corn kernels were those with abnormal colors. It could be brownish/greenish-yellow with a softer texture than the healthy corn kernels. These damages were caused by fungi that grew inside the corn kernels. While the color changes in the damaged corn were caused by the conidia color of the many fungal colonies that may infect the corn kernels. Conidia of Aspergillus sp. and Penicillium sp. had greenish and black characteristics, while the genus Fusarium was characterized by blackish conidia [22]. Confirming these findings, Previous studies found that the growth of Aspergillus flavus inside the corn kernels occurred because of the ability of micelles to penetrate cell walls and take advantage of nutritional components within the kernels. The colonization of Aspergillus flavus tissue inside the corn kernels was closely related to changes in salicylic acid and jasmonic acid in corn. The interaction between corn as a host and Aspergillus flavus as a pathogenic fungus has been widely studied where proteomic-based techniques have identified several virulence proteins produced by these fungi, most of which were turned into hydrolytic enzymes. The presence of this enzyme was in accordance with the biological properties and classification of Aspergillus flavus as a saprophyte which generally catabolizes decaying plant material as a nutrition source. This was the reason how corn kernels that were infected with the fungus, especially Aspergillus flavus, were damaged and why the texture became softer and even cracked[23]. The high ambient temperature and stress of kernels as a host were the main factors closely related to the infection rate and growth of Aspergillus flavus [24].

3.2.3. Moldy kernels. The analysis of variance on moldy kernels showed that exposure time of ozone had an effect on moldy kernels with a p-value<0.05, while temperature and the interaction between
temperature and ozone exposure time did not have a significant effect on moldy kernels with a p-value > 0.05. Table 2 showed that the moldy kernels were relatively small with a range of 0.01-0.02%. It was because most of the molds were infected and the infections went through inside the corn kernels, making the texture on the inside was softer compared to the surface. According to the SNI 8926:2020, the maximum standard for moldy corn kernels corn as feedstuff was 1% for the premium quality, 2% for the medium I, and 4% for the medium II. Corn with moldy kernels was corn with molds that grew on its surface and can be visually observed either directly or using ultraviolet lights [13]. The fungi on the corn surface have a yellow-green glow where most of the luminescence appears on the base of corn kernels. In addition, the fungi on the corn surface can also be identified with the presence of thin white mycelia or greenish/blackish conidia. Furthermore, the Aspergillus niger was reported to have black sporulation with white mycelium, while the Aspergillus flavus had yellowish-green colonies with white mycelium on the edge of the colony, and its subsurface colony had a yellowish-orange color [22]. Yellowish orange pigmentation on the subsurface colony was the result of the reaction between ferric ions from ferric ammonium citrate and the aspergillaric acid molecules in the aflatoxin biosynthesis pathway produced by the Aspergillus flavus [25].

3.2.4. Total fungal. The analysis of variance on the total fungal infections showed that the temperature, exposure time of ozone, and their interaction had a significant effect on the total fungal with a p-value < 0.05. The results of Tukey's test showed that there were significant differences in every treatment (Table 3). This showed that the total fungal infections decreased along with the drop in temperature and the increase in exposure time. An ozone treatment at a temperature of 20°C and an exposure time of 60 minutes showed that the highest reduction of total fungal infections could reach 36.77% (Table 4). The reduction level was closely related to the effect of temperature on the half-life of the ozone where the ozone has a longer half-life at low temperatures than at higher temperatures. In this study, the half-life of ozone is defined as the moment when the ozone naturally breaks down into oxygen. The longer the half-life of ozone impacts, the higher the availability of ozone for the oxidation process. Thus, the fungal spore inactivation may take longer intensively. Besides that, exposure time also affected the reduction of total fungal infections because it was related to the oxidation intensity between the ozone and the fungi. The longer the exposure time of ozone took, the longer the oxidation process between the ozone and the fungi took place. Consequently, the reduction of the total fungal infections also became higher. This was in accordance with many previous studies reporting that the efficacy of ozone in degrading fungal infections was influenced by the exposure time and temperature among others [26]. The ozone exposure at a temperature of 0-20°C recorded a higher rate of inactivation of fungal spores than the one at a temperature of 20-40°C [5]. The half-life of ozone was significantly reduced about 38% when the ozone temperature increased around 4°C to 24°C and would decrease by 48% when the ozone temperature increased around 24°C to 40°C [27].

The reduction of the total fungal infections occurred due to the oxidation process carried out by the ozone. Ozone was an oxidant with higher oxidation potential than chlorine which has been widely used to reduce microbial contamination and to prevent any damages caused by fungi in postharvest handling of many agricultural products [4][28]. The mechanism of ozone in reducing the total fungal infections occurs through the progressive oxidation of vital cellular components. Ozone could react to a protective protein layer of genetic material from microorganisms so that it could rapidly inhibit growth and reduce the microbial population in the product. Ozone was reported to oxidize polyunsaturated fatty acids or sulphhydryl groups and amino acids from enzymes, peptides, and proteins into shorter molecular fragments. In addition, ozone also could degrade cell walls resulting in cell disruption and further leakage of cell content components from microorganisms [7]. Another opinion stated that ozone can reduce germination of conidia and cause changes in the morphology of hyphae, resulting in impaired growth and even cause the mortality of fungi [29].

Many studies reported that ozone effectively controlling the growth of *Aspergillus flavus* and *Aspergillus parasiticus* in many grains such as paddy, rice, barley, corn, wheat, and peanuts [30][31]. The highest reduction of total fungal infections in ozonized corn in this research was about 36.77%. This
was slightly different from the previous studies, most of which reported that the fungal reduction could reach 63% for *Aspergillus parasiticus*, 70% for *Aspergillus* sp., and even 100% for *Aspergillus flavus* [32][29][6]. This was due to the difference in the concentration and exposure time of ozone. Another opinion stated that the initial fungal contamination during ozone treatment affected the fungal reduction of grains. Ozone exposure of 31 ppm for 5 hours was reported to be able to reduce the population of *Aspergillus parasiticus* and *Aspergillus flavus* with an initial total fungal infection of 4.83 log CFU g⁻¹ [29][6]. Furthermore, existing studies found that ozone exposure of 47800 ppm for 5.4 minutes in corn led to a reduction in *Aspergillus flavus* by about 4 log CFU g⁻¹ [33], meanwhile, ozone exposure of 0.987 ppm for 138.56 minutes in corn was known to reduce *Aspergillus sp.* and *Penicillium* sp. by 78.5% and 98.0% respectively, at a temperature of 25°C [34]. Another study stated that ozone exposure of 60 ppm for 480 minutes in corn could reduce the total colonies of *Aspergillus* sp. about 3 log CFU g⁻¹[35].

### Table 3. Total fungal and aflatoxin of corn treated with gaseous ozone.

| Temperature (°C) | Exposure time (minutes) | Total fungal (log CFUg⁻¹) | AFB1 (ppb) | AFB2 (ppb) | AFG1 (ppb) | AFG2 (ppb) | Total aflatoxin (ppb) |
|------------------|-------------------------|---------------------------|------------|------------|------------|------------|----------------------|
| 20               | 30                      | 3.66<sup>c</sup>          | 25.62<sup>e</sup> | 6.56<sup>c</sup> | Not detected | Not detected | 32.18<sup>c</sup>   |
| 20               | 60                      | 3.52<sup>d</sup>          | 13.56<sup>d</sup> | 1.69<sup>e</sup> | Not detected | Not detected | 15.25<sup>f</sup>   |
| 30               | 30                      | 3.84<sup>d</sup>          | 40.77<sup>d</sup> | 6.75<sup>c</sup> | Not detected | Not detected | 47.51<sup>d</sup>   |
| 30               | 60                      | 3.74<sup>e</sup>          | 37.10<sup>d</sup> | 6.54<sup>ad</sup> | Not detected | Not detected | 43.64<sup>d</sup>   |
| 40               | 30                      | 4.03<sup>b</sup>          | 76.27<sup>b</sup> | 8.48<sup>bc</sup> | 2.04<sup>b</sup> | 2.64<sup>b</sup> | 89.44<sup>b</sup>   |
| 40               | 60                      | 3.92<sup>c</sup>          | 58.24<sup>c</sup> | 5.63<sup>d</sup> | Not detected | Not detected | 63.87<sup>c</sup>   |
| Control          |                         | 5.56<sup>a</sup>          | 177.71<sup>a</sup> | 10.30<sup>a</sup> | 6.05<sup>a</sup> | 7.95<sup>a</sup> | 202.01<sup>a</sup> |

Note: numbers followed by the same letter show no significant difference at the level of 0.05.

### Table 4. Efficacy of ozonization on total fungal and aflatoxin level of corn (%).

| Temperature (°C) | Exposure time (minutes) | Total fungal | AFB1 | AFB2 | AFG1 | AFG2 | Total aflatoxin |
|------------------|-------------------------|--------------|------|------|------|------|----------------|
| 20               | 30                      | 34.13        | 85.59 | 36.31 | 100  | 100  | 84.07          |
| 20               | 60                      | 36.77        | 92.37 | 83.60 | 100  | 100  | 92.45          |
| 30               | 30                      | 30.92        | 77.06 | 34.52 | 100  | 100  | 76.48          |
| 30               | 60                      | 32.74        | 79.12 | 36.53 | 100  | 100  | 78.40          |
| 40               | 30                      | 27.51        | 57.08 | 17.66 | 66.19| 66.77| 55.72          |
| 40               | 60                      | 29.46        | 67.23 | 45.41 | 100  | 100  | 68.38          |

3.2.5. Aflatoxin. The analysis of variance on aflatoxins showed that temperature, ozone exposure time, and their interaction had a significant effect on AFB1, AFB2, AFG1, AFG2, and total aflatoxins with a p-value <0.05. The results of Tukey’s test showed significant differences among the different treatments. This showed that the aflatoxin of corn decreased along with the drop in temperature and the increase in exposure time. The lowest aflatoxin was shown by ozone treatment at a temperature of 20°C and an exposure time of 60 minutes (Table 3). An ozone treatment at a temperature of 20°C and an exposure time of 60 minutes recorded the highest aflatoxin reduction: 92.37% for AFB1, 83.60% for AFB2, 100% for AFG1 and AFG2, so it made the total aflatoxin 92.45% (Table 4). These showed that gaseous ozone could effectively reduce the aflatoxin of corn, especially at low temperatures and longer exposure time. Ozone exposure at lower temperatures was more effective in reducing aflatoxin levels compared to the higher temperatures. It was related to the half-life of ozone. The half-life of ozone itself is defined as the effective life of ozone until it breaks down naturally into oxygen. The longer the ozone half-life was, the higher the availability of ozone compounds was needed for the oxidation process in aflatoxins, making the aflatoxin degradation process occurred more intensively. The half-life of ozone was greatly influenced by the temperature, where a lower temperature would increase the half-life of ozone, whereas
a higher temperature will decrease the half-life of ozone. The half-life of ozone at a temperature of 35°C was around 8 to 10 minutes [36]. The exposure time of ozone was related to the intensity of the oxidation reaction between the ozone and aflatoxin. The longer the exposure time took, the longer the oxidized aflatoxins took place, and it also made the aflatoxin reduction higher [5]. This result was in accordance with many previous studies stating that the higher aflatoxin reduction was induced by ozone treatments at lower temperatures. Ten-minute Ozone exposure at a temperature of 25°C was proven to have a higher reduction of AFB1, AFG1, AFB2, and AFG2. It was higher compared to ozone exposure at temperatures of 50°C and 75°C [37]. The exposure time of ozone also showed a positive correlation with aflatoxin reduction of corn. The longer the exposure time, the higher the aflatoxin reduction [9].

The mechanism of ozone in degrading AFB1 and AFG1 involves an electrophilic reaction in the C8-C9 double bond of the furan ring which caused formation of ozonide compounds. The compound was then rearranged into monozonide derivatives such as aldehydes, ketones, acids, and carbon dioxide where the new compounds were not toxic. Besides that, ozone was known to directly attack the aflatoxin double bonds and cause them to break down into organic acids, aldehydes, ketones, and carbon dioxide [37]. Another opinion stated that ozone could destroy anthraquinones, intermediate pigments produced by the fungus that may help in the conversion of aflatoxin [7]. In general, gaseous ozone could significantly prevent the growth of fungi on grains so that it would consequently reduce the production of aflatoxins [38].

The reduction of aflatoxin in this study was quite high at about 57.08-92.37% for AFB1, 17.66-83.60% for AFB2, and 55.72-92.45% for the total aflatoxins. According to the total aflatoxin standard of corn as feedstuff regulated by the SNI 8926:2020, ozonized corn was included in the premium quality class with the total aflatoxin under 20 ppb [13]. In general, the reduction of AFB1 was higher than AFB2. AFB1 of corn was shown more easily degraded by ozone than other aflatoxins at the same ozone concentration and exposure time [8]. This was due to the difference of double bond in the molecular structure of them which affected the resistance to ozone oxidation. This was consistent with the previous studies which stated that AFB2 was more difficult to degrade by ozone. Moreover, the AFB2 and AFG2 were reported to be more resistant to the ozone process than the AFB1 and AFG1 because there were no C8-C9 double bonds in their structures [9].

Many previous studies stated that ozone could significantly reduce the aflatoxin of grains. Ozone exposure in peanuts for 10 minutes at a temperature of 75°C could reduce the AFB1 by 77%, AFG1 by 8%, AFB2 and AFG2 by 52% [37]. Whereas exposure ozone of 40 ppm for 20 minutes on whole-wheat kernels can reduce the AFB1 by 86.75% and 96.66% with initial aflatoxin of 10 ppb and 20 ppb [7]. Another study stated that exposure of ozone of 21 ppm for 96 hours in peanuts had been proven to reduce the total aflatoxin by 30% and the AFB1 by 25% with aflatoxin initial contamination of 20 ppb [30]. Some recent studies also reported that ozone of 6 ppm with exposure of 30 minutes in peanuts reduced the total aflatoxin by 65.8% and the by AFB1 65.9% [9]. Ozone also showed a significant reduction by 88% for the AFB1 of corn with an exposure time of 40 minutes from 83 ppb to 9.9 ppb [8]. Another study stated that exposure of ozone of 60 ppm for 480 minutes in corn was proven to reduce the AFB1 by 57.0%, AFB2 by 30.0%, AFG1 by 54.6%, and AFG2 by 36.1% with the initial total aflatoxin of 50 ppb [35]. In conclusion, the use of ozone in grains with longer exposure times has no effect on their nutritional quality, chemical composition, and functional properties, so it is safe to be used in grains such as corn, peanuts, soybeans, rice, and wheat [9].

4. Conclusion
In conclusion, the gaseous ozone has an effect on reducing the fungal contamination and aflatoxin in corn, however, it has no effect on the moisture content, moldy kernels, and damaged corn kernels. The optimum gaseous ozone treatment is at a temperature of 20°C and exposure time of 60 minutes which showed the highest reduction of the total fungal colonies and total aflatoxins, 36.77% and 92.45% respectively with the initial total fungal infections of 5.56 log CFUg⁻¹ and total aflatoxins of 202.01 ppb. The final result of the total fungal infections and total aflatoxins of ozonized corn were 3.52 log CFUg⁻¹.
1, and 15.25 ppb respectively, which consist of AFB1 13.52 ppb, AFB2 1.69 ppb, AFG1, and AFG2 were not detected.

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Acknowledgments
NH is grateful to the Indonesian Agency for Agricultural Research and Development for the magister scholarship and this research project.