

Original Research Article

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## Comparative Effect of Ozone and Traditional Antimycotic Drugs on the Growth of Some Fungal Causes of Recurrent Mastitis in Egyptian Buffaloes

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### ABSTRACT

The antifungal potential of ozone fumigation was evaluated for growth inhibition of isolated fungi from recurrent mastitis in dairy buffaloes in comparison with some traditional chemical antifungal. One hundred samples were collected from private farm of dairy buffaloes suffering from repeated mastitis affection ( 20 samples of each of litters, swabs of workers hands and rations) and 40 mastitic milk samples from diseased mastitis animals. The predominant fungal isolates that recovered from samples were subjected for ozone fumigation. The effectiveness of ozone treatments on spore germination depended on ozone concentration and treatment duration. The gradual increase in ozone concentration induced progressive retardation of the spore viability and the required time for ozone exposure was decreased. Where, the growth inhibition of mould as *A.flavus* required exposure to 20 ppm of ozone for 20 minutes ; to 40 ppm for 15 minutes and exposure to 60 ppm of ozone for 10 minutes to be completely inhibited the fungal growth and no mycelia was observed. The ozone exposure of mould at different concentrations for time under 10 minutes not able to inhibit completely their growth and the inhibition was more pronounced with the extension of exposure time. On the other hand, the growth inhibition of yeast was more sensitive for ozone fumigation which required fewer time of exposure (5-10 min). When the treated fungi with ozone fumigation were subjected to scanning by electron microscopy (SEM), showed many destructive changes in morphology and loss of cell integrity and hence its viability and function were inhibited. When ozone concentrations and time of exposure increased, a serious and destructive changes to fungal structure would occur. The field application on selected highly contaminated commercial animal ration with different moulds, evidenced also higher potential antifungal effects of the ozone fumigation. Where, all used concentrations of ozone (20, 40 & 60 ppm) were effective in decontamination when contaminated rations exposed for 20 minutes and more. Therefore, fumigation with ozone gas can be used as a good method for achieving sanitation and decreasing initial microbial load in rations facilities and spoilage on a long term. Otherwise, the animal diseases could be prevented when healthy feed, disinfect storage, processing areas and other utensils which significantly reflected in improving animal health.

### Keywords

Ozone fumigation,  
Exposure time,  
Mastitis,  
Fungi,  
SEM,  
Buffaloes,  
Antifungal.

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## Introduction

Feeds beside other environmental factors suspected to play important role in animal infections. The dangers of mould and yeast besides causing animal mycosis, they produced fungal metabolites such as mycotoxins, such mycotoxins produced under adverse effect of environmental conditions. These mycotoxins residues in food and feed causes carcinogenic, teratogen, hemorrhagic and immunosuppression effect to human and animal health (Sayed El Ahl *et al.*, 2006 and Hassan *et al.*, 2007).

In spite of progressive advances in harvesting, storage and processing technologies, fungal spoilage still has a major economic impact on world food supplies. The most common and destructive food spoilage fungi belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*, although other genera are of significance in particular foods and feed (Refai and Hassan, 2013). Moreover, *Candida albicans* and other fungal infection are probably one of the most successful opportunistic pathogens in humans. Under conditions of a weakened immune system, colonizing *C. albicans* and mould can become pathogenic, causing recurrent mucosal infections and life-threatening contagious infections with high mortality rates (Refai *et al.*, 2014a). In addition, there are a number of fungal diseases which adversely affect the health of animal, as mastitis which is the major production-limiting disease causing staggering economic losses to the animal industry. The most important effects are related to economic losses due to decrease in milk yield (McDowell *et al.*, 1995), Several studies recovered various fungal causes of these diseases in man and animals as *C. albicans*, *Aspergillus sp.* and *Penicillium sp.* which are the dominant microbial isolates in cases of mastitis (Yuan *et al.*, 2012 and

Hassan *et al.*, 2014 and 2015 a). Some mould as members of *Aspergillus sp.*, *Penicillium sp.* and *Fusarium sp.* caused mycosis and mycotoxicosis in buffaloes (Hassan *et al.*, 2008, 2010 and 2014), in poultry (Hassan *et al.*, 2007) and in rabbits (Hassan *et al.*, 2016).

Recently, a rapid increase in microbial infections that are resistant to conventional antibiotics has been observed, especially, the frequency of infections provoked by opportunistic fungal strains has increased dramatically (Goffeau, 2008; Nabawy *et al.*, 2014 and Hassan *et al.*, 2015a and 2016 ). Furthermore, the number of known multidrug resistant fungi is increasing rapidly. Thus, the development of more effective antifungal therapies is of great importance.

Because the factors that contribute to fungi proliferation include environmental and ecological conditions that often are beyond human control, world food contamination by fungi represents a significant problem (Hussein and Brasel, 2001 and Scussel, 2005). Therefore, numerous studies investigated the development of methods for fungicide contamination as the use of modified atmospheres including carbon dioxide, nitrogen, oxygen and, more recently, ozone fumigation (Scussel *et al.*, 2011a; Giordano *et al.*, 2012 and Savi *et al.*, 2014a&b). One of the important applications of ozone in agriculture is the postharvest treatment of crops (Zorlugenic *et al.* 2008) and reducing or eliminating undesirable mycoflora from grains and their by-products (Tiwari *et al.*, 2010). Ozone acts through a progressive oxidation of vital cellular components on destroying microorganisms preventing the microbial growth, thus extending the shelf life of several foods (Guzel-Seydim *et al.*, 2004 and Aguayo *et al.*, 2006). These applications

include the surface decontamination, food storage, and preservation, as well as packaging sterilization (Desvignes *et al.*, 2008 and Cárdenas *et al.*, 2011).

Therefore, the present study was undertaken to investigate the fungal causes of mastitis in dairy buffaloes and the recovered fungi were subjected for ozone fumigation in comparison with some traditional chemical antifungal. In addition, the efficacy of ozone fumigation was evaluated by scanning electron microscope of the treated fungal spores and mycelia.

### **Materials and Methods**

**Samples:** One hundred samples were collected from private farm of dairy buffaloes at Giza governorate suffering from repeated mastitis affection, 20 samples of each of (litters, swabs of workers hand and feeds) and 40 milk samples from diseased mastitis animals. The samples and swabs were aseptically transferred into sterile polyethylene bags, swabs or sterile container without delay and transported to the laboratory for further investigation.

**Standards of antifungal agents:** A known antifungals as ketoconazole and Fluconazole were used in comparison with ozone gas fumigation. It were manufactured and purchased from by sigma chemical company.

**Ozone Gas Production:** Ozone gas was produced from air using ozone generator Model OZO 6 VTTL (OZO MAX LTD, shefford, Quebec, Canda) from purified extra dry oxygen fed gas. The amount of output from ozone was controlled by a monitor-controller having a plug-in. sensor on a board which is changed for different ranges of ozone concentration and a belt pan in the monitor controller that allows controlling the concentration in a selected range.

### **Isolation and identification of fungi from samples collected from dairy buffaloes farm had cases of animal mastitis**

One Ten grams of each sample or 1 ml of immerse swab of workers hand or milk were transferred aseptically into sterile blender jar, to which 90 ml of 1% peptone water were added and homogenized in a sterile warring blender for 2 minutes and tenfold serial dilutions of the homogenate were prepared (Refai, 1979). One milliliter quantities of the previously prepared serial dilutions were inoculated separately into Petri dish plates and mixed with Sabouraud dextrose agar medium. The plates were then left to solidify after mixing, and incubated at 25°C for 3-5 days. The counts of mould and yeast colonies were recorded. Individual suspected colonies were selected depending upon their morphological characters. Stock culture were made from each isolate and monitored on Czapek- Dox, malt extract and potato dextrose (PDA) agar slopes for further identification. The identification of different species was carried out by observation of their macroscopic and microscopic characteristics of mould colonies according to, Pitt and Hocking (2009) and Refai and Hassan (2013).

### **Preparation of spore suspension of isolated mould and yeast isolated from dairy farm had mastitic animals (Gupta and Kohli, 2003)**

At the end of incubation period (3-5 days), the mycelia spore mat was washed off with a 6 ml of sterile distilled water and by sterile loop, the outer most layer of growth (spores) was scraped. The mycelia were removed by filtration through a 500mm sieve. This spore suspension was counted in haemocytometer slide considering the dilution factor and the spores count was adjusted to the desired level. To each broth containing spore suspension a drop of tween 80 was added to keep the viability of the spore till used.

**Ozone treatment for controlling the fungal growth of isolates recovered from dairy buffaloes farm had mastitic animals (Ali, 2007)**

Ozone was generated via a controlled flow of oxygen through a corona discharge in the ozone generator (OZO-2000). Ten ml of spore suspension of each of the most dominant fungal species recovered from dairy buffaloes farm had mastitic animals were held in ozone chamber and exposed at 20, 40 and 60 ppm and the exposure times were for 0 (control), 5, 10, 15 and 20 min. Linear growth (cm) were measured after 7 days incubation at 28°C. For each type of isolate a plates were used for each treatment as a control. One ml of each treatment was subjected to colony count as recommended by Refai and Hassan (2013) to report the effect of ozone treatment.

**Scanning electron microscope (SEM) (Goldstein *et al.*, 1992)**

The samples of treated spores were coated by gold sputter coat (SPI Module) and examined by scanning SEM (JEOLJSM-5500 LV) by using high vacuum mode at the Regional Center of Mycology and Biotechnology, Cairo, Egypt to study the efficacy of treatment of fungi.

**Determination the minimal inhibitory concentration (MIC) of some traditional antimycotic drugs (Gupta and Kohli, 2003)**

One ml spore suspension from each tested isolate was poured in sterile Petri dishes and were over layered with Sabouraud's dextrose agar containing 1 ml of different concentrations of the tested antimycotics (ketoconazole and Fluconazole) which are (0.50,0.75,1.0,2.0,3.0,and 4.0 µg/ml for ketoconazole and 5, 10, 15, 20 µg/ml for Fluconazole). The tested antifungals were

prepared in 2 replicates. The plates were rotated to mix the content and allowed to solidify, at room temperature, then they were incubated at 25°C for 2-15 days before reporting the MIC which was the concentration that prevented growth of fungus completely. All experiment were reported at least 3 times to obtain average data.

**Evaluation the efficacy of ozone fumigation in control the fungal contamination in commercial animal feed (Ali, 2007 and Refai and Hassan, 2013)**

Twenty of commercial animal feed that contaminated with fungi and related to the current mastitis cases of the present study were selected. Twenty five grams of each sample were exposed to ozone fumigation at 20, 40 and 60 ppm and the exposure times were 0 (control), 5, 10, 15 and 20 min. The total colony count of fungi was detected before and after treatment for evaluation the effective dose of ozone.

Statistical analysis (SPSS 14, 2006):The obtained data were computerized and analyzed for significance. Calculation of standard error and variance according to.

**Results and Discussion**

Buffaloes are an economically important source of milk and meat, there are about 3.98 million head in Egypt and several serious problem facing animals health including respiratory disorders, mastitis and diarrhea (Arab agriculture statistics year book (A.A.S., 2011). The mastitis is one of important infections of buffaloes is the mastitis which a frustrating, costly and extremely complex disease resulted in a marked reduction in the quality and quantity of milk (Akhtar *et al.*, 2012). The pathogens that cause mastitis can be divided into different groups of organisms depending on

the source of the organism involved; these include contagious and, environmental pathogens (Philpot and Nickerson, 1999). Moulds and yeasts are found on a wide variety of environmental factors such as feed, litters, air, soil, plants, water and animals discharges. The previous literatures recorded that the environmental pollution affect upon the growth rate and health of human being and animals and may cause several diseases as thrush, disseminated candidiasis, aspergillosis, dermatophytosis and mastitis, anemia, stunted growth, carcinogenic, tremor-genic, hemorrhagic, dermatitis, pulmonary edema, immunosuppressive and hormonal effects (Hassan, 2003 and Asfour *et al.*, 2009; Hassan *et al.*, 2009, 2014; 2015a and 2016 and El-Hamaky *et al.*, 2016). While, the mycotoxigenic fungi can induce both toxicological and immunologic effects in a variety of cell systems and animal species as cytotoxic effect to reticulocytes, fibroblasts and lymphocytes and the cellular toxicity appears to be mediated by the inhibition of protein synthesis as reported by (Mogeda *et al.*, 2002; Hassan *et al.*, 2015 b&c and 2016). Whereas, other fungi as *C. albicans* is considered a commensal organism for humans and animals and when host defenses are compromised *C. albicans* can transform into a tissue invasive pathogen (Palmer, 2008) resulting in several affections of the oral cavity, gastrointestinal tract, animal abortion and skin diseases (Shawky *et al.*, 2014; Hassan *et al.*, 2015 and 2016). However, *Candida sp.* is recognized as the fourth most common cause of blood stream infection, with a high attributable mortality rate, while, *C.albicans* remains the most common pathogen (Marr, 2004). The intestinal tract provides an important reservoir for many nosocomial pathogens, including *Candida* species and some bacterial species. Disruption of normal barriers, such as gastric acidity and endogenous microflora

of the colon, facilitates the overgrowth of pathogens (Donskey, 2004). The reproductive tracts of different animals are the major reservoir of yeasts such as *C. albicans* and *C. neoformans* (Radwan *et al.*, 2008; Hassan *et al.*, 2014 and Shawky *et al.*, 2014). Several studies indicated that there was significant prevalence of fungal infection in cattle and buffaloes that infested with ticks. This may be due to the fact that toxins present in saliva of ticks result in immunosuppression of the animals. In cattle, it causes high economic losses as body weight loss, decreased meat and milk yield and the acceptance of live animals in the market would also be reduced (Hassan *et al.*, 2015 a and 2016).

In the present study, the current results in Table (1) illustrated that the most predominant members of *Aspergillus* species in samples of litters, feed, workers hand and mastitis milk that collected from mastitis buffaloes were belonging to *A.flavus* which recovered from (90%, 60%, 40%, 25%), *A.ochraceus* from (50%, 60%, 25%, 25%), *A.niger* from (60%, 40%, 50%, 30%) and *A.fumigates* recovered from (10%, 25%, 0%, 40%) of samples, respectively. While, *Penicillium sp.* and *Fusarium sp.* were recovered from (20% and 25 %) (25% and 15%) (0% and 0%) (25% and 0%) of litters, feed, workers hand and mastitis milk samples, respectively. On the other hand, *C.albicans* and *Rhodotorula sp.* were isolated at the rates of (70% and 30%) (40, 20%) (50%, 0%) (37.5%, 20%), respectively. Similar results were detected by Mosherf (2005), who reported that various *Candida* species particularly, *C. albicans* were the most common yeasts recovered from milk of clinically and sub-clinically mastitis milk. While, Hassan *et al.* (2012a) reported that yeast of *C. albicans* was recovered from 24% of mastitis cases. In other study, many mould isolates were

recovered from mastitis milk of sheep and cattle (Hassan *et al.*, 2010), who isolated *Aspergillus flavus* from animal feeds, mastitis milk and vaginal swabs at the rates of (80%, 50% and 50%), respectively, while the rates of isolation for *A. parasiticus* were (35%, 24% and 10%), respectively. However, Shawky *et al.* (2014) recovered *C. albicans* from cases of buffalo's abortion, where it was isolated from 20 % of milk samples, 7.5% of placenta and 20% of fetal stomach contents, respectively.

Hence, up to date the active search for new pharmacologically active agents of natural sources was led to the discovery of many useful drugs which could play important role in treatment of many fungal and mycotoxin diseases with neither environmental pollution nor development of fungicide resistance pathogens.

In addition to, the continued evolution of drug resistance, which has already invalidated many routinely used antibiotics, has reached a fevered pitch and is a serious public health threat, with some even warning of the possibility of the 21st century becoming the "post-antibiotic" era (Kåhrström *et al.*, 2013).

Recently, various studies evaluated different methods in control the fungal growth as the use of ozone fumigation (Scussel *et al.*, 2011a; Giordano *et al.*, 2012 and Savi *et al.*, 2014a&b). One of the important applications of ozone in agriculture is the postharvest treatment of crops (Zorlugenic *et al.*, 2008), reducing or eliminating undesirable mycoflora from grains and their by-products (Tiwari *et al.*, 2010). Although commercial ozone application for grain management is not well documented, there are numerous studies that describe the potential benefits of that technology (McDonough *et al.*, 2011). Ozone efficacy depends on several

factors that include its concentration applied, the characteristics of each food, and environmental factors such as temperature and humidity.

Currently, the predominant isolates that recovered from samples related to mastitis were subjected for ozone fumigation in parallel with traditional chemical antifungals. The effectiveness of ozone treatments on spore germination was a function of ozone concentration and treatment duration. The gradual increase in ozone concentration induced progressive retardation of the spore viability and the required time for exposure was decreased. Where, the growth inhibition of mould as *A. flavus* that recovered from cases of mastitis required exposure to 20 ppm for 20 minutes ; to 40 ppm for 15 minutes and exposure to 60 ppm for 10 minutes to be completely inhibited their growth and no mycelial growth was observed (Tables, 2,3,4&5). The ozone exposure of mould for time under 10 minutes not able to inhibit completely their growth and the inhibition was more pronounced with the extension of exposure time. Whereas, any increase in the exposure time at any concentration of gaseous ozone resulted in a significant gradual retardation in growth of mould and accordingly the inhibition efficiency increased with the increase in ozone concentration.

On the other hand, the growth inhibition of yeast was more sensitive for ozone fumigation which required fewer time of exposure (5-10 min). This is related the differences in structures and the presence of profuse mycelial mass in case of mould which require elongated time of exposure than the cellular structure of yeast (Guzel-Seydim *et al.*, (2004). In addition, during food storage, spores are the major source of inoculums. Hence reduction or inhibition of

spore production is very advantageous in food storage facilities. Such inhibition/reduction of spore production has been previously observed in fungi cultured under ozone rich environment (Antony-Babu and Singleton, 2009). Similarly, the ozone gas is known to possess antimicrobial activity and thereby at higher concentrations could be used to reduce the initial load of contaminants on feed and food and to disinfect storage and processing areas (Najafi and Khodaparast, 2009 and Korzun *et al.*, 2008).

Regarding the use of traditional antifungal agents, it has been reported that the potency ketoconazole and fluconazole against fungi isolated from cases of buffaloes mastitis showed that the effective inhibitory concentrations that inhibited all fungi by ketoconazole was 4ug/ml and fluconazole was 20 ug/ml. The obtained data confirm data reported by Hassan (1994); Hassan and El Sharnou by (1997); Neil *et al.* (1998); Okeke *et al.* (2000) and Nakashima *et al.* (2002). Whereas, Various studies in different laboratories showed that antifungals as Azoles inhibit sterol formation and polyenes that bind to mature membrane sterols have been regarded antifungal therapy for several decades (Kullberg and Pauw, 1999; and Sheehan *et al.*, 1999). On the other hand, Amphotericin B and fluconazole were used as a positive control toward fungi; amphotericin B is a fungicidal agent widely used in treating serious systemic infections (Hartsel and Bolard. 1996) and fluconazole is used in the treatment of superficial skin infections caused by dermatophytes and *Candida* species (Boaz and Marcelo, 1998).

In the present study, the treated fungi with ozone fumigation were subjected to

scanning by electron microscopy (SEM) to detect the changes in the morphological structures of fungal elements which resulted from treatment. The results revealed that all treated fungi showed many destructive changes in morphology and loss of cell integrity and hence its viability and function inhibited. When ozone concentrations and time of exposure increased, a serious and destructive changes to fungal structure would occur (Photo 1-4).

Similar findings to our results were detected by several studies as that illustrated by Guzel-Seydim *et al.* (2004) and Ali (2006), who stated that there are two major mechanisms identified in ozone destruction of the target organisms; the first mechanism is that ozone oxidize sulfhydryl groups and amino acids of enzymes, peptides and proteins to shorter peptides. While, the second mechanism is that ozone oxidizes polyunsaturated fatty acids to acid peroxides. The differential activity of ozone against the test fungi might be due to the variation in their organic matter content which may accelerate or reduce the toxicity of ozone. Similarly, Ali (2006), found that the specific interaction of sucrose or polysaccharides with ozone affects the ozone activity. Other study suggested that the photolysis of ozone to oxygen atoms could lead to the generation of the hydroxyl radical (OH), a key reactive species during the decomposition process (Jans and Hiogne, 1998) and the inhibition of mycelial growth and sporulation of fungal cell due to oxidizing action of ozone as detected by Liew and Prange (1994).

Recently, it is suggested that ozone penetrates cell membrane and reacts with cytoplasmic substances and chromosomal DNA which affected by ozone degradation and its damage occur which may be one of the factors responsible for the cell killing (Todar, 2009 and El-Desouky *et al.*, 2012).

Also, ozone concentrations at ground levels modulate oxidative DNA damage (Palli *et al.*, 2009).

Currently, as the animal feeds are the main sources of infections in animal diseases, the field application of the present results on selected highly contaminated commercial animal feed with different moulds,

evidenced also higher potential antifungal effects of the ozone fumigation. Where, all used concentrations of ozone (20, 40 & 60 ppm) were effective in decontamination when contaminated feed exposed for 20 minutes and more (Table, 6). These results were in agreement with other studies in this respect (Antony-Babu and Singleton (2009); Gabler *et al.*, 2010 and Alencar *et al.*, 2012).

**Table.1** Prevalence of fungi in samples collected from dairy buffaloes farm had cases of recurrent mastitis in buffaloes

| Genera of isolated fungi      | Samples collected from cases of mastitis |    |             |    |                   |    |                    |      |
|-------------------------------|--|----|-------------|----|-------------------|----|--------------------|------|
|                               | litters (20)                             |    | Ration (20) |    | Workers Hand (20) |    | Mastitis Milk (40) |      |
|                               | No.                                      | %  | No.         | %  | No.               | %  | No.                | %    |
| <i>Aspergillus fluves</i>     | 16                                       | 90 | 12          | 60 | 8                 | 40 | 10                 | 25   |
| <i>Aspergillus ochraceus</i>  | 10                                       | 50 | 12          | 60 | 5                 | 25 | 10                 | 25   |
| <i>A.fumigatus</i>            | 2  | 10 | 5           | 25 | -                 | -- | 16                 | 40   |
| <i>Aspergillus niger</i>      | 12                                       | 60 | 8           | 40 | 10                | 50 | 12                 | 30   |
| <i>Penicillium species</i>    | 4  | 20 | 5           | 25 | -                 | -  | 10                 | 25   |
| <i>Fusarium species</i>       | 5  | 25 | 3           | 15 | -                 | -  | -                  | -    |
| <i>Cladosporium species</i>   | 4  | 20 | 6           | 30 | 10                | 50 | -                  | -    |
| <i>Scopulariopsis species</i> | 2  | 10 | -           | -  | -                 | -  | 4                  | 10   |
| <i>Mucor species</i>          | -  | -  | -           | -  | 8                 | 40 | 6                  | 15   |
| <i>Candida species</i>        | 14                                       | 70 | 8           | 40 | 10                | 50 | 15                 | 37.5 |
| <i>Rhodotorula species</i>    | 6  | 30 | 4           | 20 | -                 | -  | 8                  | 20   |

**Table.2** Influence of Ozone fumigation (20 PPM) and time of exposure on the growth fungal isolates that recovered from cases of mastitis in buffaloes

| Fungal isolates           | Dose of ozone exposure | C.C. Before Ozone treatment | C.C. of fungi at different exposure times of ozone fumigation |                 |                 |         |
|---------------------------|------------------------|-----------------------------|---|-----------------|-----------------|---------|
|                           |                        |                             | 5 min.  | 10 min.         | 15 min.         | 20 min. |
| <i>Aspergillus flavus</i> | 20 ppm                 | 1x10 <sup>7</sup>           | 10 <sup>5</sup>   | 10 <sup>3</sup> | 10 <sup>1</sup> | 0       |
| <i>A. ochraceus</i>       | 20 ppm                 | 3x10 <sup>6</sup>           | 10 <sup>4</sup>   | 10 <sup>3</sup> | 10 <sup>1</sup> | 0       |
| <i>A.niger</i>            | 20ppm                  |                             | 10 <sup>3</sup>   | 0               | 0               | 0       |
| <i>A.fumigatus</i>        | 20ppm                  | 2x10 <sup>5</sup>           | 10 <sup>3</sup>   | 10 <sup>2</sup> | 0               | 0       |
| <i>Penicillium sp.</i>    | 20ppm                  | 1x10 <sup>6</sup>           | 10 <sup>3</sup>   | 10 <sup>2</sup> | 10              | 0       |
| <i>Fusarium sp.</i>       | 20ppm                  | 4x10 <sup>4</sup>           | 10 <sup>3</sup>   | 10 <sup>2</sup> | 10              | 0       |
| <i>Mucor sp.</i>          | 20ppm                  | 2x10 <sup>6</sup>           | 10 <sup>2</sup>   | 10              | 0               | 0       |
| <i>Rhizopus sp.</i>       | 20ppm                  | 3x10 <sup>5</sup>           | 10 <sup>2</sup>   | 10              | 0               | 0       |
| <i>Candida sp.</i>        | 20ppm                  | 2x10 <sup>5</sup>           | 10  | 0               | 0               | 0       |
| <i>Rhodotorula sp</i>     | 20ppm                  | 1x10 <sup>5</sup>           | 10  | 0               | 0               | 0       |

The inhibitory Concentration for all fungi for ketoconazole was 4 ug/ml and fluconazole was 20 ug/ml.



**Table.3** Influence of Ozone fumigation (40 PPM) and time of exposure on the growth fungal isolates that recovered from cases of mastitis in buffaloes

| Fungal isolates           | Dose of ozone exposure | C.C. Before Ozone treatment | C.C. of fungi at different exposure times of ozone fumigation |         |         |         |
|---------------------------|------------------------|-----------------------------|---|---------|---------|---------|
|                           |                        |                             | 5 min.  | 10 min. | 15 min. | 20 min. |
| <i>Aspergillus flavus</i> | 40 ppm                 | $1 \times 10^7$             | $10^3$  | 10      | 0       | 0       |
| <i>A. ochraceus</i>       | 40 ppm                 | $3 \times 10^6$             | $10^3$  | $10^2$  | 0       | 0       |
| <i>A.niger</i>            | 40 ppm                 |                             | 10  | 0       | 0       | 0       |
| <i>A.fumigatus</i>        | 40 ppm                 | $2 \times 10^5$             | $10^2$  | 10      | 0       | 0       |
| <i>Penicillium sp.</i>    | 40 ppm                 | $1 \times 10^6$             | $10^3$  | $10^2$  | 0       | 0       |
| <i>Fusarium sp.</i>       | 40 ppm                 | $4 \times 10^4$             | $10^3$  | $10^2$  | 0       | 0       |
| <i>Mucor sp.</i>          | 40 ppm                 | $2 \times 10^6$             | $10^2$  | 10      | 0       | 0       |
| <i>Rhizopus sp.</i>       | 40 ppm                 | $3 \times 10^5$             | $10^2$  | 10      | 0       | 0       |
| <i>Candida sp.</i>        | 40 ppm                 | $2 \times 10^5$             | 10  | 0       | 0       | 0       |
| <i>Rhodotorula sp</i>     | 40 ppm                 | $1 \times 10^5$             | 10  | 0       | 0       | 0       |

The inhibitory Concentration for all fungi by ketoconazole was 4 ug/ml and fluconazole was 20 ug/ml.

**Table.4** Influence of Ozone fumigation (60 ppm) and time of exposure on the growth fungal isolates that recovered from cases of mastitis in buffaloes

| Fungal isolates           | Dose of ozone exposure | C.C. Before Ozone treatment | C.C. of fungi at different exposure times of ozone fumigation |         |         |         |
|---------------------------|------------------------|-----------------------------|---|---------|---------|---------|
|                           |                        |                             | 5 min.  | 10 min. | 15 min. | 20 min. |
| <i>Aspergillus flavus</i> | 60 ppm                 | $1 \times 10^7$             | $10^2$  | 0       | 0       | 0       |
| <i>A. ochraceus</i>       | 60 ppm                 | $3 \times 10^6$             | $10^2$  | 0       | 0       | 0       |
| <i>A.niger</i>            | 60 ppm                 |                             | 10  | 0       | 0       | 0       |
| <i>A.fumigatus</i>        | 60 ppm                 | $2 \times 10^5$             | $10^1$  | 0       | 0       | 0       |
| <i>Penicillium sp.</i>    | 60 ppm                 | $1 \times 10^6$             | $10^2$  | 0       | 0       | 0       |
| <i>Fusarium sp.</i>       | 60 ppm                 | $4 \times 10^4$             | $10^2$  | 0       | 0       | 0       |
| <i>Mucor sp.</i>          | 60 ppm                 | $2 \times 10^6$             | $10^1$  | 0       | 0       | 0       |
| <i>Rhizopus sp.</i>       | 60 ppm                 | $3 \times 10^5$             | $10^1$  | 0       | 0       | 0       |
| <i>Candida sp.</i>        | 60 ppm                 | $2 \times 10^5$             | 0   | 0       | 0       | 0       |
| <i>Rhodotorula sp</i>     | 60 ppm                 | $1 \times 10^5$             | 0   | 0       | 0       | 0       |

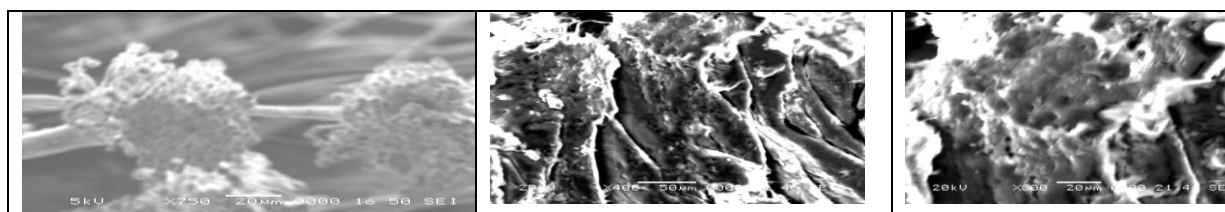
The inhibitory Concentration for all fungi by ketoconazole was 4 ug/ml and fluconazole was 20 ug/ml.

**Table.5** Comparison between effective inhibitory times of different doses of ozone fumigation on the growth fungal isolates that recovered from cases of mastitis in buffaloes

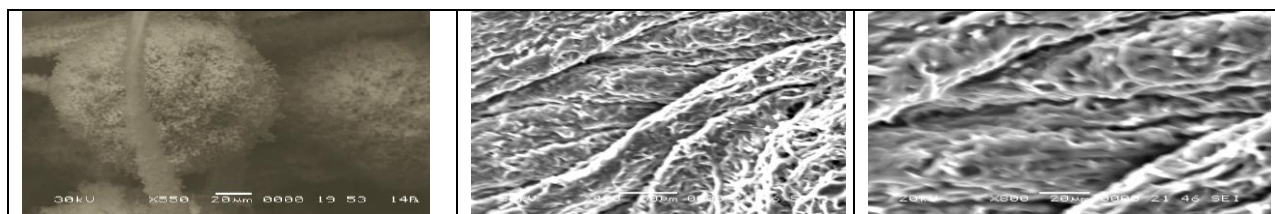
| Fungal isolates           | Maximum effective exposure time of ozone fumigation at 20 ppm (minutes) | Maximum effective exposure times of ozone fumigation at 40 ppm(minutes) | Maximum effective exposure time of ozone fumigation at 60 ppm (minutes) |
|---------------------------|---|---|---|
| <i>Aspergillus flavus</i> | 20  | 15  | 10  |
| <i>A. ochraceus</i>       | 20  | 15  | 10  |
| <i>A.niger</i>            | 15  | 10  | 10  |
| <i>A.fumigatus</i>        | 15  | 15  | 10  |
| <i>Penicillium sp.</i>    | 20  | 15  | 10  |
| <i>Fusarium sp.</i>       | 20  | 15  | 10  |
| <i>Mucor sp.</i>          | 15  | 15  | 10  |
| <i>Rhizopus sp.</i>       | 15  | 15  | 10  |
| <i>Candida sp.</i>        | 10  | 10  | 5   |
| <i>Rhodotorula sp</i>     | 10  | 10  | 5   |

**Table.6** Total colony count of commercial feed (20 samples) after treatment with ozone fumigation

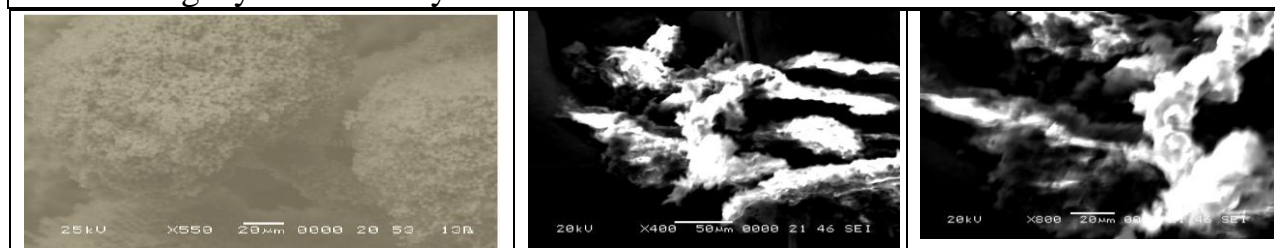
| Doses of ozone | C.C. at different times of exposure for ozone |                 |                 |                 |
|----------------|---|-----------------|-----------------|-----------------|
|                | 5 min.  | 10 min.         | 15 min          | 20 min.         |
| 0 ppm          | $1 \times 10^7$                               | $1 \times 10^7$ | $1 \times 10^7$ | $1 \times 10^7$ |
| 20 ppm         | $1 \times 10^6$                               | $1 \times 10^5$ | $1 \times 10^2$ | 0               |
| 40 ppm         | $1 \times 10^5$                               | $1 \times 10^4$ | $1 \times 10^1$ | 0               |
| 60 ppm         | $1 \times 10^4$                               | $1 \times 10^1$ | 0               | 0               |



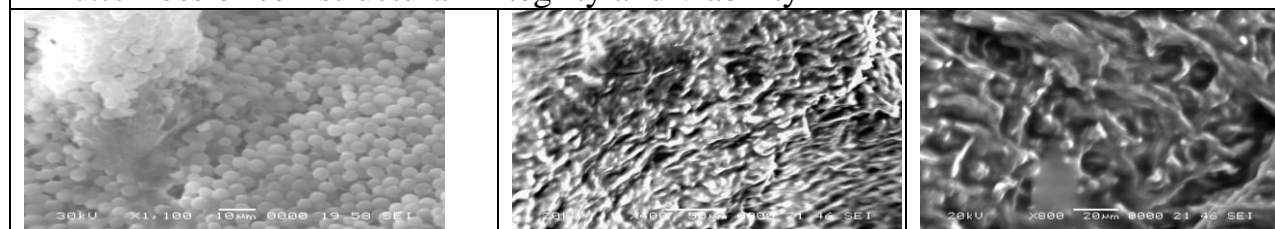
**Photo (1) *A.flavus*** : (A) Scanning electron micrographs of before exposure to ozone. Showed intact conidial head and conidiophore of fungus (B) After exposure to lower concentration of ozone therapy for 10 min- clumps of conidia. (C) after exposure to ozone for 20 minutes – loss of cell integrity and viability



**Photo (2) *A. niger*** : (A) Scanning electron micrographs of before exposure to ozone. Showed intact conidial head and conidiophore of fungus (B) After exposure to lower concentration of ozone therapy for 10 min- clumps of conidia. (C) after exposure to ozone for 20 minutes- loss of cell integrity and viability



**Photo (3) *A. ochraceus*** : (A) Scanning electron micrographs of before exposure to ozone. Showed intact conidial head and conidiophore of fungus (B) After exposure to lower concentration of ozone therapy for 10 min- clumps of conidia. (C) after exposure to ozone for 20 minutes- loss of cell structural integrity and viability



**Photo (4) *C. albicans*** : (A) Scanning electron micrographs of before exposure to ozone. Showed large clusters of yeast cells (B) After exposure to lower concentration of ozone therapy for 10 min- small clumps of cells. (C) after exposure to ozone for 20 minutes- loss of cell integrity and viability

Furthermore, this approach using ozone gas could be put into practice, where higher concentrations can impart surface sanitation of storage facilities and food handling equipment and storage and preservation (El-Desouky *et al.*, 2012).

Therefore, fumigation with ozone gas can be a good method for achieving sanitation and decreasing initial microbial load in feed facilities and spoilage on a long term. Otherwise, the animal diseases could be prevented when healthy feed, disinfect

storage, processing areas and other utensils which significantly reflected in improving animal health.

In conclusion, several fungal isolates that recovered from recurrent mastitis in buffalo were reported as potential pathogens and can cause other diseases conditions, particularly after prolonged exposure to adverse environmental conditions. The dangers of fungi besides caused animal diseases, they may produce fungal mycotoxins which are carcinogenic for animal and human.

Therefore, the essential significance of this study is the indication that ozone fumigation can be used as inhibitor for the growth of fungi and could be used in the field of veterinary medicine as a fungicide in successful treatment of fungal diseases. The antifungal potential of ozone is due to the damage of the fungal cell wall of the microbial cells leading to leakage of the cell contents and finally cell death. In addition to the treatment of the serious fungal diseases as mycotic mastitis by traditional Chemical and mechanical sterilization methods adversely affect public health as well as the environment. Ozone fumigation can be successfully used for reducing the microbial loads. Hence, for this reason ozone may replace chemical antifungal agent.

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