Evaluation of antifungal effect of gaseous ozone in a meat processing plant

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Abstract

Ozone is already known as effective food/environmental disinfection agent, thanks to its oxidative action towards microbial cell components. Bactericidal effect of ozone is well documented, while data concerning its inhibitory activity towards fungi are still ambiguous. Our study aims to evaluate the antimicrobial activity of gaseous ozone towards potentially contaminant fungi in a meat production plant in real working conditions. M_2 and M_5S_5 plates were inoculated with *Aspergillus niger*, *Penicillium roqueforti*, *Mucor racemosus*, *Saccharomyces cerevisiae* strains and positioned in a deboning room, where gaseous ozone was dispensed throughout the night (until a maximum concentration of 20 ppm). Nine different points were chosen, based on height and distance from the ozone dispenser. After the treatment, the presence of strains growth was evaluated. Gaseous ozone did not show any inhibitory activity against mould strains, as optimum growth during all the trials was observed. An appreciable and constant microbicidal effect against *S. cerevisiae* was evidenced, with a mean value of 2.8 Log reduction. Our results suggest the importance of the definition of environmental and technical use conditions in order to optimise the antimicrobial efficacy of ozone in real working situations in food industries.

Introduction

Ozone is a gas that is naturally present in the stratosphere; it is water-soluble and has a high oxidative power. The latter characteristic has been positively considered since ozone was discovered by Van Marum in 1783 and it has been used thanks to its bactericidal, fungicidal and virucidal activities. Ozone toxicity towards cells is due to its decomposition to oxygen; during this transformation, direct and indirect (via hydroxyl radicals formation) substrates' oxidation and peroxidation take place, leading to the alteration of both the structure and the functionality of biomolecules (Khadre *et al*., 2001; Laisk *et al*., 1989; Sarti *et al*., 2002; Staehelin and Hoigne, 1985).

Thanks to its useful properties, ozone is largely applied in several industrial processes. In particular, it is used for disinfection of water (bottled water, potable water networks and swimming pools), air, wood, several foodstuffs (in particular fruits and vegetables) and surfaces that are intended to be in direct contact with food. The application of ozone does not leave any residue in the environment or on treated substrates, thanks to its natural transformation into oxygen. It has been defined as a *generally recognised as safe* agent by the USA Food and Drug Administration, but the toxicity towards workers must be taken into account, so safety measures must be carefully applied.

The antimicrobial efficacy of ozone is influenced by environmental conditions, mainly temperature, humidity and pH values. The best microbicidal action is obtained in environments with low temperatures and presence of water or high relative humidity.

Sensitivity of bacteria to the action of ozone is variable: Gram positive bacteria are more susceptible than Gram negative bacteria, due to the different cell wall composition and the resulting sensitivity to lipid peroxidation, while bacterial spores are strongly resistant to the action of ozone (Khadre *et al*., 2001; Khadre and Yousef, 2001; Kim *et al*., 1999). The mechanism of virus inactivation is not completely cleared, but it is known that higher ozone concentrations are required to exert a virucidal effect (Kim *et al*., 1999). As regards fungi, several authors evidenced the efficacy of gaseous ozone as inactivating agent against spores. Since 1951, moulds growth prevention on cheese surfaces during ripening was demonstrated with a dose equal to 1 ppm (CNSA, 2010). A study conducted on Cheddar cheese in Canada (Gibson *et al*., 1960) showed that the exposure to an ozone concentration of 3-10 ppm resulted in moulds darkening on cheese surface, associated to a 96% reduction in air moulds concentration in ripening rooms. The application of lower ozone concentrations (0.2-0.3 ppm) was sufficient to obtain a significant reduction of moulds, with no modification of sensory characteristics of cheese. During the 70s, these findings were confirmed, identifying a 10 ppm ozone concentration as efficient for spore inactivation (CNSA, 2010). The inhibitory activity of ozone against yeasts has been evidenced by several authors, both *in vitro* and on different foods. Yeasts are naturally more resistant than bacteria to the action of ozone, thanks to their thicker cell wall, but they are clearly more susceptible than moulds (Kim *et al*., 1999; Moore *et al*., 2000). A different sensitivity has been observed among different yeast species; for example *Candida* spp. and *Saccharomyces* spp. are usually rapidly inhibited by ozone, while *Debaryomyces* spp. is strongly resistant (Naitoh, 1993; Pereira *et al*., 2011; Watanabe *et al*., 2010).

Considering the growing diffusion of ozone as disinfectant in food industries, this study aimed to evaluate the antimicrobial efficacy of gaseous ozone on potentially contaminating fungi and yeats in a meat deboning and processing plant, in real working conditions.

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Key words: Gaseous ozone, Mould, Raw meat, *Saccharomyces cerevisiae.*

Conflict of interests: the authors declare no potential conflict of interests.

Received for publication: 11 May 2013.
Revision received: 30 November 2013.
Accepted for publication: 1 December 2013.

Italian Journal of Food Safety 2014; 3:1680
doi:10.4081/ijfs.2014.1680

Materials and Methods

The efficacy of gaseous ozone was tested in a deboning and processing industry in which vacuum packaged beef meats are produced. The plant is equipped with a centralised, medium frequency corona discharge ozone generator, with a production capacity of 2-20 g/h. Our evaluation was performed in the deboning room, with a surface of 220 m^2 and 4 m high. Gaseous ozone was supplied by the refrigeration sock ducts for 4 h during the night (from 11.00 p.m. to 03.00 a.m.); ozone amount was settled to obtain a constant environmental concentration of 20 ppm, revealed by a fixed probe.

With the aim of detecting the eventual presence of autochthonous moulds in the environment, the contamination of air was evaluated by keeping open Petri plates with M_2 (composition: malt extract=20 g/L, yeast extract=3 g/L, agar=15 g/L) and M_5S_5 (composition: malt extract=50 g/L, NaCl=50 g/L, agar=15 g/L) culture media (Dragoni *et al*., 1997) in different points of the room for 2 h before every test session. After the exposition, the plates were collected, transferred to the laboratory and then

incubated at 25 and 35°C for 7-10 days. For inactivation tests, three moulds strains, namely *Mucor racemosus*, *Penicillium roqueforti* and *Aspergillus niger*, and one yeast strain, *i.e. Saccharomyces cerevisiae*, from the collection of the Laboratory of Mycology, Department of Health, Animal Science and Food Safety, University of Milan, Italy, were selected, in view of their wide diffusion in meat production and storage plants (Dragoni *et al*., 1997).

Each strain was grown on M_2 (*M. racemosus*, *P. roqueforti* and *S. cerevisiae*) or M_5S_5 (*A. niger*) Petri plates, incubated at 30°C for 2 days (*S. cerevisiae*), 25°C for 7-10 days (*M. racemosus* and *P. roqueforti*) and 35°C for 7-10 days (*A. niger*). Then, a portion of mycelium from each strain was collected by sterile loop and inoculated onto Petri plates with their specific culture. For *S. cerevisiae*, a suspension with a known concentration (settled by optical density determination with spectrophotometer) was prepared in a diluent solution (NaCl/triptone, 0.85%); decimal dilutions were inoculated onto M_2 Petri plates.

The inactivation of selected fungal strains, inoculated onto culture media and submitted to gaseous ozone was used as index to evaluate the efficacy of ozonation treatment.

After the usual cleaning/disinfection procedures, plates were placed with opened covers, in different points of the deboning room. Three positions (identified as 1, 2 and 3) were chosen, based on their distance from the refrigeration sock ducts, and three points at different heights (identified as L=10 cm; M=110 cm; H=270 cm) for each position, in order to evaluate the vertical stratification of ozone, as it is characterised by a high molecular weight and tends to accumulate in lower layers.

After the overnight ozonation, the plates were collected, transported to the laboratory and incubated as described previously. For each selected strain, the same inoculation procedures were performed by Petri plates used as control; after the inoculum process, such plates were refrigerated at +2°C up to collect all exposed test plates, then they were incubated in the same conditions. A total of 6 analytical sessions were performed, during different working weeks; for each session, the test was performed twice.

The inhibitory activity of ozone was evaluated as follows: for moulds, any slow growth, or its absence, was evidenced as difference in mycelium diameter in treated plates when compared with controls. Considering *S. cerevisiae*, the logarithmic decrease of microbial count in treated plates compared with controls was determined.

These data were submitted to a mixed ANOVA by SAS/STAT package version 8.0 (SAS Inst. Inc., Cary, NC, USA). Position and height of sampling points were identified as fixed factors, while analytical session was considered as random factor. A value of $P<0.05$ was considered statistically significant.

Results

The evaluation of the concentration of autochthonous moulds in the environment revealed a mean count of 5.6 CFU/plate; moulds belonged to the genera *Aspergillus*, *Penicillium*, *Alternaria* and *Mucor*; this presence was expected as these genera are extremely common and are widespread in food production environments.

The night distribution of gaseous ozone did not exert any detectable inhibitory action against the selected mould strains in the usual production conditions. An optimal moulds growth on the treated plates was observed, if compared with the control plates in all the analytical sessions. No difference was revealed among the Petri plates placed in different positions or at different heights.

Unlike moulds, an evident microbicide effect against *S. cerevisiae* was observed in all the analytical sessions (Table 1); the statistical analysis revealed a highly significant ($P<0.01$) decrease in treated plates against the control. In 96.3% of the replicates a fall in the counts >2 Log was detected with a mean value of 2.8 Log (standard deviation=0.6). The inhibitory action was evidenced in all the selected points of the room (Figure 1); no significant difference was detected among the three positions (1-2: P=0.91; 1-3: P=0.72; 2-3: P=0.81), or the different heights (H-M: P=0.91; H-L: P=0.64; M-L: P=0.57), revealing the uniform distribution of gaseous ozone within the treated environment. Thus, we can suppose that the vertical stratification of ozone, due to its high molecular weight, could be contrasted by constant air flow supplied by the sock ducts placed near the ceiling.

Discussion

Although the antimicrobial efficacy of ozone has been evidenced by several authors, various studies have already shown a lack of this activity against moulds. Perez *et al*. (1999) reported an increase in *Botrytis cinerea* prevalence on

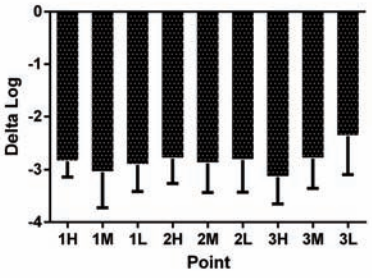

Figure 1. Numbers of *S. cerevisiae* in different points of the deboning room after ozone treatment. Different positions (1-2-3) and heights (H=270 cm, M=110 cm, L=10 cm) are shown. A value of 0 Log indicates the value of control plates.

Table 1. Logarithmic decrease of *S. cerevisiae* counts after ozonation treatment throughout the analytical sessions.

Height	Session					
	1	2	3	4	5	6
1H	-2.49±0.10	-3.00±0.21	-2.66±0.34	-3.10±0.00	-3.21±0.11	-2.59±0.03
1M	-2.35±0.11	-3.43±0.00	-2.42±0.09	-3.75±0.00	-3.74±0.43	-2.56±0.19
1L	-2.49±0.17	-3.47±0.21	-2.26±0.43	-2.99±0.10	-3.49±0.07	-2.74±0.09
2H	-2.44±0.04	-3.17±0.43	-2.31±0.38	-2.89±0.00	-3.49±0.07	-2.43±0.05
2M	-2.34±0.01	-2.69±0.21	-3.5±0.00	-2.96±0.07	-3.54±0.16	-2.24±0.01
2L	-2.46±0.07	-2.69±0.12	-2.06±0.16	-2.68±0.10	-3.84±0.03	-3.15±0.15
3H	-2.58±0.03	-3.17±0.00	-2.66±0.34	-3.05±0.00	-3.96±0.21	-3.41±0.04
3M	-2.58±0.04	-2.87±0.00	-2.15±0.05	-2.70±0.00	-3.84±0.00	-2.59±0.03
3L	-2.77±0.11	-2.69±0.21	-1.16±0.09	-1.81±0.10	-2.95±0.10	-2.85±0.17

H, 270 cm; M, 110 cm; L, 10 cm. Values are indicated as mean±standard deviation.

strawberries treated with 0.35 ppm of ozone for 4 days; the development of *Botrytis cinerea*, *Mucor piriformis* and *Rhizopus stolonifer* on grapes was not reduced by a 0.3 ppm ozone treatment for 7 days (Palou *et al*., 2002) and similar results were obtained with similar tests on apples, blueberries, cranberries and melons (Rice *et al*., 1982).

These differences in experimental results can be ascribed to the different methodological approaches and to the variable functionality of different ozone generators and distribution systems (De Alencar *et al*., 2012; Kim *et al*., 1999, 2003; Serra *et al*., 2003).

Furthermore, it has to be considered the critical role played by environmental conditions, such as humidity and temperature, on antimicrobial efficacy of ozone, as it is known that also little variations in these parameters can significantly modify its activity.

The natural moulds resistance, due to their cellular constituents, must also be considered in evaluating ozone application; the structure of mould cell wall, unlike bacterial cells, is characterised by a very low lipid content (<5%) and it is consequently less susceptible to oxidising agents (Dragoni *et al*., 1997). In many cases, high doses of ozone are required; for example, Ozkan *et al*. (2011) observed that the dose needed to remove *Penicillium digitatum*, *Penicillium italicum* and *Botrytis cinerea* from table grapes was also dangerous for workers. Italian legislation allows a maximum exposition dose of 0.1 ppm of ozone within a 8 h working period, or a 0.3 ppm dose for max 15 min, twice a day (Lgs. Decree n° 81/2008; Italian Republic, 2008).

Considering yeasts, many authors indicated a higher sensitivity to ozone treatments than moulds (Naitoh and Shiga, 1982; Kim *et al*., 1999), but evident evident variability in ozone susceptibility has been observed among different yeast species (Kim *et al*., 1999). The efficacy of ozonisation assessed in our study towards *Saccharomyces cerevisiae* confirmed the results of previous studies and it indicated a marked susceptibility of *Saccharomyces* spp. to such treatments (Guzzon *et al*., 2010; Watanabe *et al*., 2010).

Conclusions

The results obtained confirm the absolute need for an accurate definition of the treatment conditions with ozone in order to maximise its antimicrobial effect in real working situations, considering the treatment parameters (ozone dose and supply modality) in combination with environmental parameters, such as temperature, humidity and air flow.

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