Università degli Studi di Milano

GRADUATE SCHOOL OF VETERINARY SCIENCES
FOR ANIMAL HEALTH AND FOOD SAFETY
Director: Prof. Vittorio Dell'Orto

Doctoral Program in Animal Nutrition and Food Safety

Academic Year: 2009-2010

Application of ozone in food industries

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CHAPTER 1

Foreword

1. Foreword

1.1 Properties of ozone (O_3)

Ozone has been known as an accompaniment to electrical storms during all the history of mankind. Its first identification as a distinct chemical compound was due to Christian Friedrich Schönbein (Rubin, 2001), Professor of Chemistry at the University of Basel from 1828. To a considerable extent he dominated the study of ozone chemistry until his death in 1868. The molecular formula of ozone was determined in 1865 by Soret and confirmed by him in 1867, shortly before Schönbein's death. Schönbein, in 1839, noticed the emergence of a pungent gas with an "electric smell." According to the Greek language, he called it "ozone" and presented a lecture entitled "On the smell at the positive electrode during electrolysis of water" at the Basel Natural Science Society (Burns, 1997; Rubin, 2001). In nature ozone is continuously produced in the stratosphere (at 25–30 km from the Earth surface) by UV radiation (<183 nm) by splitting an atmospheric oxygen molecules into two highly reactive oxygen atoms. By an endothermic reaction, each of these atoms combines to intact oxygen to form the triatomic ozone. It is also produced during the electric discharge of lightning, which catalyzes the formation of ozone from atmospheric oxygen (Battino, 1981).
Ozone (O_3) is an allotropic form of oxygen. It has a molecular weight of 48, boiling point of -111.9°C, and melting point of -192.7°C at 1 atm (Merck Index, 1989). Ozone weighs ca. 0.135 lb/ft^3. The oxidation potential of ozone is high (-2.07 V) compared to that of hypochlorous acid (-1.49 V) or chlorine (-1.36 V) (Brady and Humiston, 1978).

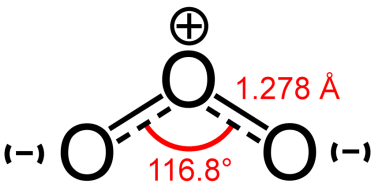


Figure 1: Ozone structure

Structurally, the three atoms of oxygen are in the form of an isosceles triangle with an angle of 116.8 degree between the 2 O-O bonds. The distance between the bond oxygen atoms is 1.278 Å. Ozone is a metastable gas with a temperature-dependent half-life, but it can be stored in liquid form at a temperature below -111.9°C with a specific weight of 1.571 g/mL.
The various physical properties of ozone are summarized in Tab 1.

Physical properties	Value
Boiling point, °C	- 111,9
Density, kg/m^3	2,14
Heat of formation, kJ/mole	144,7
Melting point, °C	-192,7
Molecular weight, g/mole	47,9982
Oxidation strength, V	2,075
Solubility in water, ppm (at 20°C)	3
Specific gravity	1,658

Table 1: Physical properties of ozone

Ozone has a cyclical structure assessed by the absorption at 253.7 nm and exists in several mesomeric states in dynamic equilibrium (Fig. 2) (Tanaka and Morino, 1970). Among oxidant agents, it is the third strongest ($E°$ = +2.076 V), after fluorine and persulphate. Molecular oxygen, by containing two unpaired electrons, is a diradical but it has not the reactivity of ozone and, by a stepwise reduction with four electrons, forms water. On the other hand, ozone having a paired number of electrons in the external orbit is not a radical molecule, but it is far more reactive than oxygen and generates some of the radical oxygen species (ROS) produced by oxygen during mitochondrial respiration. Phagocytes reacting with pathogens (Babior, 1978; Fialkow et al., 2007) produce anion superoxide (O^-_2), H_2O_2, and hypochlorous acid (HClO) catalyzed by mieloperoxidase. Interestingly, ozone, in the presence of inorganic and/or organic compounds immediately reacts and generates a great variety of oxidized molecules, disappearing in a matter of seconds (Heng et al., 2007).

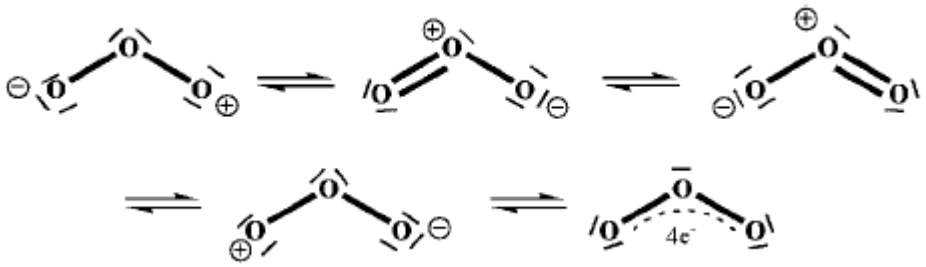


Figure 2: Mesomeric states of ozone.

Ozone is formed naturally in the stratosphere in small amounts (0.05 mg/liter) by the action of solar UV irradiation on oxygen. A small amount of ozone is also formed in the troposphere as a by-product of photochemical reactions between hydrocarbons, oxygen, and nitrogen that are released from automobile exhausts,

industries, forests, and volcanic action. However, the gas produced is very unstable and decomposes quickly in the air (Horwath et al., 1985). Ozone is a gas with a pungent, characteristic smell. At high concentrations in the air it becomes blue, whereas at low concentrations it is a colourless gas, lighter than the air. The half-life of ozone molecules in the air is relatively long and spans for *ca.* 12 hours; in aquatic solutions it depends on the content of organic matter. In other words: the lower the concentration of organic matter, the longer the ozone half-life (Graham, 1997). Ozone dissolves in water ten times better than oxygen and its solubility decreases with an increase in water temperature (Holcman and Domoradzki, 2003; Gordon, 1995). Ozone dissolves in water at pH below 7.0; at this level it does not react with water and it is present in the form of molecules. However, an increase in the pH value leads to a spontaneous decomposition of ozone, which results in producing highly reactive free radicals, such as hydroxyl ·OH. At pH value of 8, nearly half of the introduced ozone is decomposed to various intermediate forms and to oxygen within 10 minutes (Gordon, 1995) In the upper atmosphere, high-energy UV irradiation helps degrade ozone molecules. Ozone is converted to oxygen in the process and absorbs the UV energy before it reaches the earth's surface (Brady and Humiston, 1978). Levy (1971) postulated that the photolysis of ozone to oxygen atoms could lead to the generation of the hydroxyl radical ($^{\bullet}OH$), a key reactive species during the decomposition process. In addition to UV irradiation, high pH, hydrogen peroxide (H_2O_2), and activated carbon enhance the degradation of ozone (Jans and Hoigne, 1998).

Ozone decomposes in solution in a stepwise fashion, producing in turn hydroperoxyl ($^{\bullet}HO_2$), hydroxyl ($^{\bullet}OH$), and superoxide ($^{\bullet}O_2^-$) radicals (Adler and Hill, 1950; Grimes et al., 1983; Hoigne and Bader, 1975). The hydroxyl radical is an important transient species and chain-propagating radical. The reactivity of ozone is attributed to the great oxidizing power of these free radicals. According to Hoigne and Bader (1975), the rate constants for reactions of OH radical with many substrates are very high. Hence, these radicals are consumed preferentially by dissolved species before they encounter dispersed particles such as microorganisms. This occurs even when concentrations of molecular solutes are smaller than those of the particles. In many systems, however, OH radicals react with solutes to form secondary intermediates of lower reactivity (for example, peroxy radicals) that may survive until they encounter a dispersed particle. Decomposition of ozone is so rapid in the water phase of foods that its antimicrobial action may take place mainly at the surface (Hoigne and Bader, 1975).

1.2 Production of ozone

Ozone is generated by the exposure of air or another gas containing normal oxygen to a high-energy source, such as a high voltage electrical discharge or ultraviolet radiation, convert molecules of oxygen to molecules of ozone. Ozone must be manufactured on site for immediate use, since it is unstable and quickly decomposes to normal oxygen. The half-life of ozone in distilled water at 20°C is about 20 to 30 min (Khadre et al., 2001). Ozone production is predominantly achieved by one of three methods: electrical discharge, electrochemical, and ultraviolet (UV) radiation. Electrical discharge methods, which are the most widely used commercial methods, have relatively low efficiencies (2–10%) and consume large amounts of electricity. The other two methods (electrochemical and UV) are less cost effective. In addition to photochemical and electric discharge methods, ozone can be produced by chemical, thermal, chemonuclear, and electrolytic methods (Horwarth et al., 1985).

1.2.1 Electrical (Corona) Discharge Method

In the electrical discharge method, properly dried air or O_2 itself is passed through the space between two high-voltage electrodes separated by a dielectric material, which is usually glass. Air or concentrated O_2 passing through an ozonator must be free from particulate matter and dried to a dew point of at least -60°C to properly protect the corona discharge device. The ozone/gas mixture discharged from the ozonator normally contains from 1 to 3% ozone when using dry air and 3 to 6% ozone when using high purity oxygen as feed gas (Muthukumarappan et al., 2000). The electrodes are most often either concentric metallic tubes or flat, plate-like electrodes. When a voltage is supplied to the electrodes, a corona discharge forms between the two electrodes, and the O_2 in the discharge gap is converted to ozone. A corona discharge is a physical phenomenon characterized by a lowcurrent electrical discharge across a gas-containing gap at a voltage gradient, which exceeds a certain critical value (Taylor et al., 1996). First, oxygen molecules (O_2) are split into oxygen atoms (O), and then the individual oxygen atoms combine with remaining oxygen molecules to form ozone (O_3). Considerable electrical energy (5000 V) is required for the ozone producing electrical discharge field to be formed. An excess of 80% of the applied energy is converted to heat that, if not rapidly removed, causes the O_3 to decompose into oxygen atoms and molecules, particularly above 35°C. In order to prevent this decomposition, ozone generators utilizing the corona discharge method, must be equipped with a means of cooling the electrodes.
The temperature of the gas inside the discharge chamber must be maintained at a temperature between the temperature necessary for formation of O_3 to occur

and the temperature at which spontaneous decomposition of O_3 occurs (Miller et al., 2002). The cooling is usually accomplished by circulating a coolant, such as water or air, over one surface of the electrodes, so that the heat given off by the discharge is absorbed by the coolant. Dried gas is used to minimize the corrosion of metal surfaces due to nitric acid deposits produced from wet gas inside the generator (Rosen, 1972). The corona discharge method has been used most widely to produce large amounts of ozone (Fig. 3).

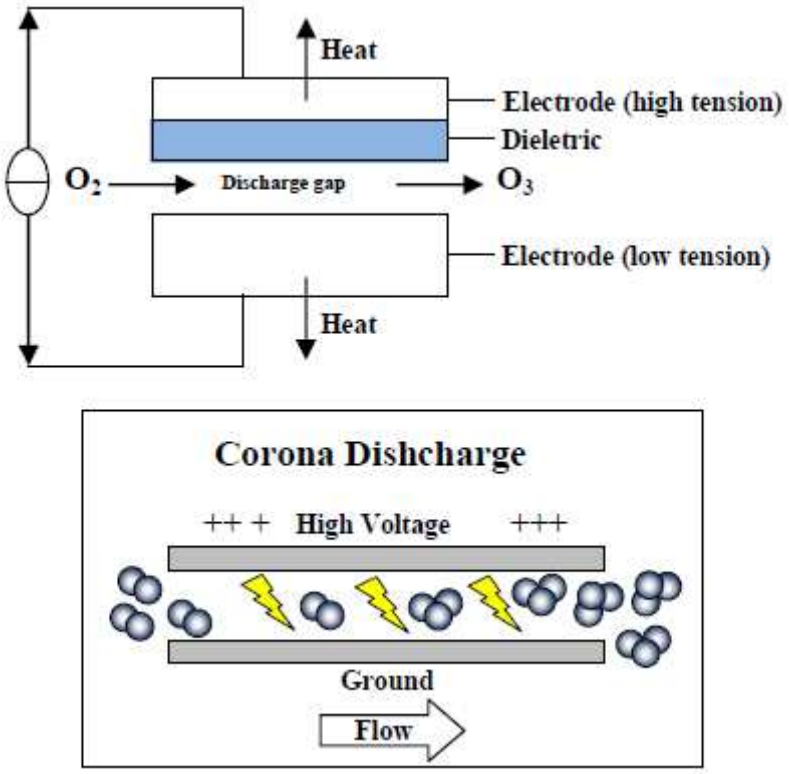


Figure 3: Scheme of Corona discharge method: Oxygen is forced between high voltage plates to simulate corona discharge. The oxygen is broken apart and recombines into ozone (Gonçalves, 2009).

1.2.2 Electrochemical (Cold Plasma) Method

A new approach in producing ozone has been implemented by Lynntech, Inc. (College Station, Tex.) (1998). This is an electrochemical procedure in which water is split into hydrogen and oxygen atoms by electrolysis. Hydrogen molecules are separated from the gas and water mixture and the oxygen atoms combine to form ozone and diatomic oxygen. The manufacturer claims their system produces ozone at concentrations that are three to four times higher (10 to 18% ozone in the gas-mixture output) than those attainable by corona discharge. The advantages associated with this method are use of low-voltage

DC current, no feed gas preparation, reduced equipment size, possible generation of ozone at high concentration, and generation in the water.

1.2.3 Ultraviolet (UV) Method

In ultraviolet method of O_3 generation, the ozone is formed when O_2 is exposed to UV light of 140–190 nm wavelength, which splits the oxygen molecules into oxygen atoms; then the oxygen atoms combine with other oxygen molecules to form O_3 (Muthukumarappan et al., 2000). The method has been reviewed thoroughly by Langlais et al. (1990). However, due to poor yields, this method has very limited uses. When used in industry, ozone is usually generated at the point of application and in closed systems. Ozone is produced at low concentrations (0.03 ppm) from oxygen in the air by radiation of 185 nm wavelength, emitted by high transmission UV lamps (Ewell, 1946).

1.3 Equipment

Ozonation systems have five basic components: the gas (air or pure oxygen) preparation unit; the ozone generator; the electric power source; the contactor (water) and the surplus gas elimination unit.

a) Preparation of the gas
The purpose of the gas preparation device is to dry and cool the gas containing the oxygen. Corona-discharge type generators use dry air or pure oxygen as the oxygen source for conversion into ozone. When air is used, it is vital to dry it to a point of condensation of -65°C to maximize the effect of the ozone and reduce to a minimum the formation of nitrogen oxides that accelerate electrode corrosion. The air should also be cooled because the ozone rapidly decomposes into oxygen at temperatures of over 30°C. Chemical driers can also be used instead of refrigeration to dry the air. The cost is a little higher and varies considerably from place to place. But in the case of small systems, the simplicity of their operation and maintenance can offset that cost. Zeolite towers that act like a molecular screen have been used successfully to produce pure oxygen by eliminating the nitrogen in the air.

b) Ozone generators (ozonizers)
The ozonation systems generate ozone at the application site and almost all of them do so by means of a corona discharge produced by the passage of oxygen or dry air between two dielectrics. (see 1.2.1-1.2.3).

c) Electric power source

The most commonly used electric power sources are low frequency (50 to 60 Hz) and high voltage (> 20.000 volts). Technological advances have produced devices that operate at high frequency (1.000 to 2.000 Hz) and 10.000 V, which are used for large water systems. The higher frequency power sources tend to be more effective.

d) Contactors

For water treatment, ozonation systems use contactors to transfer the ozone that has been generated to the water for disinfection. The type of contactor chosen depends on the specific objective of the ozonation.

There are two basic contactor designs: one with bubble diffuser chambers and the other containing a turbine-agitated reactor. In the former, there can be a series of chambers separated by deflectors or baffles or the chambers can be arrayed in parallel, in which case the device is called a "multicolumn" contactor. Studies have revealed that the multicolumn bubble diffuser produces the most efficient transfer. In water supply systems, ozone is frequently generated with a pressure of 1 kg/cm^2 and disperses in very fine bubbles that discharge in a 5-meter high water column in which the oxidation and disinfection take place.

Contact columns or chambers (usually filled with irregular pieces of plastic material to lengthen the exchange period and disperse the bubbles), static agitators and propeller or turbine diffusers can be used to accelerate the ozone gas solution and help to ensure mixing and contact.

All type of contacts use the counterflow, in which the water flows downward and the air bubbles rise to maximize the contact time.

e) Destruction of the surplus ozone

Surplus ozone is generally present in the escaping gas and should be destroyed or sufficiently diluted for safety reasons. In small treatment plants, ozone can be diluted with air, but large treatment plants use one of the following methods to destroy surplus ozone:

1) catalytic decomposition by making it flow through metal or metal oxides
2) absorption in moist granular activated carbon.

1.4 Measurement of ozone

The analytical methods for the determination of ozone can be grouped into physical, physicochemical, and chemical methods.
Physical methods are based on measuring particular ozone properties, such as the intensity of absorption in the UV, visible, or infrared region of the spectrum.
The physicochemical methods measure physical effects of ozone reaction with different reagents; such effects include chemiluminescence or heat of the reaction.
Chemical methods measure the quantity of the reaction products that are released when ozone reacts with an appropriate reagent (e.g., KI or HI) or the reduction in the molecular weight of a polymer.
These methods differ in sensitivity and accuracy (Adler and Hill, 1950). One of the chemical methods is the indigo colorimetric method (Bader and Hoigne, 1981) that was approved by the committee on standard methods for the examination of water and wastewater in 1988 (A.P.H.A., 1992*)*. In this method, ozone adds across the carbon– carbon double bond of sulfonated indigo dye and decolorizes it. The change in absorbance is determined spectrophotometrically. This method is subject to fewer interferences than most of the colorimetric methods and all iodometric procedures (Golden et al., 1988). For accurate determination of gaseous ozone, the UV spectrophotometric method should be used. Nowadays, a wide variety of ozone sensors are commercially available to monitor levels in the working environment. They are usually UV analysers, equipped with a cell that measures concentrations from 0.1 to 100 ppm v/v, that trigger an alarm as soon as the ozone concentration rises above 0.1 ppm. Safety aspects must always be taken into account, particularly when ozone is used in gas form in cold stores, rooms or closed spaces. In these situations, concentrations must be precisely monitored at different critical points and appropriate safety intervals before opening must be established in order to avoid personal health risks. When ozone is dissolved in water for use as a disinfectant it is accompanied by excess undissolved gas, as no ozone transfer system is 100% efficient. The excess ozone must therefore be destroyed or converted back into oxygen before being released into the atmosphere.

1.5 Safety requirements

The toxicity of ozone varies, depending on its concentration and the length of exposure. Ozone is a toxic gas and can cause severe illness, and even death, if inhaled in high quantity. It is one of the high active oxidants with strong toxicity to animals, plants and living organisms. Toxicity symptoms, such as sharp irritation to the nose and throat could, result instantly at 0.1 ppm dose. Loss of vision could arise from 0.1–0.5 ppm after exposure for 3–6 h. Ozone toxicity of 1–2 ppm could cause distinct irritation on the upperpart of throat, headache, pain in the chest, cough and drying of the throat. Higher levels of ozone (5–10 ppm) could cause increase pulse, and edema of lungs. Ozone level of 50 ppm or more is potentially fatal (Muthukumarappan et al., 2000). The ozone exposure levels as recommended by the Occupational Safety and Health Administration (OSHA) of U.S. have been shown in Table 2.

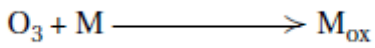
Exposure	Ozone level, ppm
Detectable odor	0.01-0.05
OSHA 8h limit	0.1
OSHA 15 min limit	0.3
Lethal in few minutes	>1700

Table 2: Approved levels of ozone application (Muthukumarappan et al., 2002)

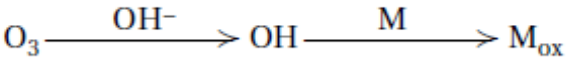
In the United States, the current permissible level for ozone exposure in the workplace environment is 0.1 ppm, as adopted by the Occupational Safety and Health Administration (OSHA). This is the concentration at which a susceptible individual may be continuously exposed to ozone under normal working conditions for 8 h a day or 40 h a week without adverse effects. The short-term exposure limit is 0.3 ppm: short-term means exposure for less than 15 min not more than 4 times a day, with intervals of at least 1 h between each short-term exposure (Prior and Rice, 2000). Ozone is, therefore, a toxic gas that must be monitored in the workplace when it is used to disinfect equipment and installations.

1.6 Reactivity of ozone

The ozone molecule acts as dipole with electrophilic and nucleophilic properties. Organic and inorganic compounds in aqueous solutions react with ozone in one of two pathways (Staehelin and Hoigné, 1985):
(a) Direct reaction of organic compound (M) with molecular ozone.



(b) Decomposition of ozone in water into a radical (for example OH) which reacts with the compound (M).



Molecular ozone reactions are selective and limited to unsaturated aromatic and aliphatic compounds. Ozone oxidizes these compounds through cycle-addition to double bonds (Bablon et al, 1991a). Oxidation of sulfhydryl groups, which are abundant in microbial enzymes, may explain rapid inactivation of microorganisms and bacterial spores by ozone. Ozone reacts with polysaccharides slowly, leading to breakage of glycosidic bonds and formation of aliphatic acids and aldehydes (Bablon et al., 1991a). Reaction of ozone with primary and secondary aliphatic alcohols may lead to formation of hydroxy-hydroperoxides, precursors to hydroxyl radicals, which in turn react strongly with the hydrocarbons (Anbar and Neta, 1967). Perez et al. (1995) showed that N-acetyl glucosamine, a compound present in the peptidoglycan of bacterial cell walls and in viral capsids, was resistant to the action of ozone in aqueous solution at pH 3 to 7. Glucosamine reacted relatively fast with ozone but glucose was relatively resistant to degradation. This observation may explain, at least in part, the higher resistance of gram-positive bacteria compared to gram negative ones; the former contains greater amounts of peptidoglycan in their cell walls. The action of ozone on amino acids and peptides is significant especially at neutral and basic pH. Ozone attacks the nitrogen atom or the R group or both. Ozone reacts slowly with saturated fatty acids. Unsaturated fatty acids are readily oxidized with ozone and cycle-addition products are formed. Ozone reacts quickly with nucleobases, especially thymine, guanine, and uracil. Reaction of ozone with the nucleotides releases the carbohydrate and phosphate ions (Ishizaki et al., 1981).

1.7 Mechanism of microbicidal action of ozone

Inactivation of bacteria by ozone is a complex process because ozone attacks numerous cellular constituents including proteins, unsaturated lipids and respiratory enzymes in cell membranes, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm, and proteins and peptidoglycan in spore coats and virus capsids. Some authors concluded that molecular ozone is the main inactivator of microorganisms, while others emphasize the antimicrobial activity of the reactive by-products of ozone decomposition such as $^{\bullet}OH$, $^{\bullet}O_2^-$ and $HO^{\bullet}_3$ (Chang, 1971; Bablon et al., 1991b; Glaze and Kang, 1989; Harakeh and Butler, 1985; Hunt and Marinas, 1997).

a) Cell envelopes
Ozone may oxidize various components of cell envelope including polyunsaturated fatty acids, membrane-bound enzymes, glycoproteins and glycolipids leading to leakage of cell contents and eventually causing lysis (Scott and Lesher, 1963; Murray et al., 1965). When the double bonds of unsaturated lipids and the sulfhydryl groups of enzymes are oxidized by ozone, disruption of normal cellular activity including cell permeability and rapid death ensues. Dave (1999) found that treatment of *Salmonella enteritidis* with aqueous ozone disrupted the cell membranes as seen in transmission electron micrographs. However, Komanapalli and Lau (1996) found that short-term exposures of *E. coli* K-12 to ozone gas compromised the membrane permeability but did not affect viability, which progressively decreased with longer exposure.

b) Bacterial spore coats
Foegeding (1985) found that *Bacillus cereus* spores with coat proteins removed were rapidly inactivated by ozone, compared to intact spores. The researcher concluded that the spore coat is a primary protective barrier against ozone. Recently, Khadre and Yousef (2001) found that spores of *Bacillus subtilis* treated with aqueous ozone showed heavily disrupted outer spore coats.

c) Enzymes
Several authors referred to enzyme inactivation as an important mechanism by which ozone kills cells. Sykes (1965) reported that chlorine selectively destroyed certain enzymes, whereas ozone acted as a general protoplasmic oxidant. Ingram and Haines (1949), in view of their finding general destruction of the dehydrogenating enzyme systems in the cell, proposed that ozone kills *E. coli* by interfering with the respiratory system. Takamoto et al. (1992) observed that ozone decreased enzyme activity in *E. coli* at a greater degree in case of cytoplasmic α-galactosidase than in case of the periplasmic alkaline phosphatase.

Inactivation of enzymes by ozone is probably due to oxidation of sulfhydryl groups in Cysteine residues (Chang, 1971).

d) Nucleic material
Reaction of aqueous ozone with nucleic acids in vitro supports the notion that it may damage nucleic material inside the cell. Ozone modified nucleic acids in vitro, with thymine being more sensitive than cytosine and uracil (Scott, 1975; Ishizaki et al., 1981). In another study, ozone opened the circular plasmid DNA and reduced its transforming ability, produced single- and double-strand breaks in plasmid DNA (Hamelin, 1985), and decreased transcription activity (Mura and Chung, 1990). Studying *E. coli*, l'Herault and Chung (1984) found that ozone may induce mutations. However, other investigators did not detect any mutagenic effect of ozone on *Salmonella* spp. (Victorin and Stahlberg, 1988). Compared to other known mutagens, ozone was found to be a weak mutagen on *Saccharomyces cerevisiae* (Dubeau and Chung, 1982).

1.8 Inhibitory spectrum

a) Bacteria
Ozone inactivates numerous bacteria that include gram-negative and gram-positive and both vegetative cells and spore forms. It is not feasible to compare the sensitivity of bacteria to ozone using results from different sources; effectiveness of ozone varies appreciably with minor changes in experimental variables. Selected studies, however, are presented to illustrate the effectiveness of ozone against various bacterial species.
Finch et al. (1988) determined the extent of inactivation of *E. coli* using ozone doses of 4.4 to 800 μg/liter at contact times of 30 to 120 s. They reported 0.5- to 6.5-log decreases in counts of *E. coli,* depending on the ozone dose and contact time. *Pseudomonas putrefaciens* was added to a pilot-scale water recycling system where ozone was maintained at 1.5 ppm (Montecalvo et al., 1995). The population of *P. putrefaciens* decreased 3 log after 5 min and 6 log after 20 min of exposure. Bactericidal action of ozone depends on the medium into which bacteria are present. Dave et al. (1998) showed that a *Salmonella* Enteritidis population, in distilled water, decreased 6 log at a low concentration of ozone (1.5 ppm). However, when broiler skin was inoculated with *Salmonella* Enteritidis and exposed to an ozone–air mixture (8%, wt/wt) for 15 s, approximately 1 log reduction in population of the pathogen was observed (Ramirez et al., 1994). Antimicrobial effects of ozonated water in a recirculating concurrent reactor, against different bacterial species, were evaluated (Reistano et al., 1995). Death rates among the gram-negative bacteria (*Salmonella* Typhimurium, *E. coli, P.*

aeruginosa, and *Yersinia enterocolitica*) were not significantly different, whereas among gram-positive bacteria, *L. monocytogenes* was significantly more sensitive than either *Staphylococcus aureus* or *Enterococcus faecalis*. Kim (1998) determined the effectiveness of ozone against foodborne microorganisms such as *P. fluorescens, Leuconostoc mesenteroides, L. monocytogenes,* and *E. coli* O157:H7 in a batch-type reaction system. He found that all tested microorganisms were inactivated by 1.5 to 5 log at 1 to 1.5 ppm of ozone within 15 s. Among these microorganisms, *L. monocytogenes* was the least resistant and *L. mesenteroides* was the most resistant to ozone.

When compared to vegetative cells, bacterial spores have greater resistance to ozone. Broadwater et al. (1973) reported that the lethal threshold concentration for *Bacillus cereus* was 0.12 mg/liter while that for *E. coli* and *B. megaterium* was 0.19 mg/liter. The threshold concentration for the spores of *B. cereus* and *B. megaterium* was 2.3 mg/liter. When ozone treatment was combined with other deleterious factors, greater inactivation rates of bacterial spores were observed. Foegeding (1985) found that acidic pH enhanced the lethality of ozone against the spores of *Bacillus* and *Clostridium*. The author also suggested that the spore coat is a primary protective barrier against ozone. Naitoh (1992a, 1992b) found that the addition of metallozeolites, ascorbic acid, and isoascorbic acid improved the inactivation of *B. subtilis* spores by ozone treatment at 5 to 50 ppm for 1 to 6 h. Naitoh (1992a) also investigated synergistic sporicidal activities of gaseous ozone and UV irradiation. The author reported that combined treatment reduced the contact time required for the inactivation.

b) Fungi

Ozone is an effective fungicidal agent. Ewell (1938) stated that depending on the cleanliness, minimum continuous concentrations of 0.6 to 1.5 ppm ozone were necessary to prevent mold growth on eggs kept at 0.6°C and 90% relative humidity (RH), whereas 2.5 to 3.0 ppm ozone were required to control molds on beef that was stored under similar conditions. According to Farooq and Akhlaque (1983), ozone also inactivated yeast. The population of *Candida parapsilosis* decreased by 2 log in 1.67 min when the yeast was exposed to 0.23 to 0.26 mg/liter ozone. Counts of *C. tropicalis* decreased by 2 log when the yeast cells were exposed to ozone at 0.02 mg/liter for 20 s or at 1 mg/liter for 5 s (Kawamura et al., 1986). Yeasts appear more sensitive than molds to ozone treatments. More than 4.5 log of *C. albicans* and *Zygosaccharomyces bailii* populations were killed instantaneously in ozonated water in a recirculating concurrent reactor, whereas less than 1 log of *Aspergillus niger* spores were killed after a 5-min exposure (Restaino et al., 1995). The average ozone output levels in the deionized water was 0.188 mg/liter. Naitoh and Shiga (1982) found that the threshold of microbicidal activity of aqueous ozone (0.3–0.5 mg/liter) against

spores of *Aspergillus, Penicillium,* and *C. paracreus* was 90 to 180, 45 to 60, and 5 to 10 min of exposure, respectively. Yeasts vary in sensitivity to ozone. Naitoh (1992c) treated *Hansenula anomala, Saccharomyces rosei, Pichia farinosa, C. parapsilosis, Kluyveromyces marxianus,* and *Debaryomyces hansenii* var. *hansenii* with gaseous ozone at 4 to 5 ppm for 1 to 5 h at 30 to 60°C and 25 to 90% RH. At lower temperature and 5 h exposure, counts of *C. parapsilosis* and *K. marxianus* decreased more than 1 log; however, counts of the other yeasts did not decrease appreciably. The antimicrobial effect increased with increasing temperature, RH, and treatment time. Ozone increased lag and exponential phases of *H. anomala* and *K. marxianus* by 1.5 to 4 and 1.4 to 6.7 h, respectively.

c) Viruses

Ozone is potentially an effective virucidal agent. Relatively low concentration of ozone and short contact time are sufficient to inactivate viruses. However, inactivation of viruses in wastewater requires longer contact time and larger ozone concentration than inactivation in ozone demand-free systems because of oxidizable materials present in the medium. Majumdar et al. (1973) reported a rapid decrease in virus survival at ca. 1 mg/liter initial ozone concentration after a 2-min contact period. Katzenelson et al. (1974) demonstrated the potent virucidal effect of ozone and suggested that ozone alone or in combination with chlorine be used in treating water and wastewater.

Herbold et al. (1989) tested the resistance of viruses and bacteria to ozone in steadily flowing water at 20°C and pH 7. The order of resistance was poliovirus 1 < *E. coli* < hepatitis A virus < *Legionella pneumophila* serogroup 6 < *B. subtilis* spores. For the complete inactivation of poliovirus 1 and hepatitis A virus (ca. 10^4 $TCID_{50}$/ml), 0.13 and 0.25 to 0.38 mg/liter ozone was needed, respectively. Emerson et al. (1982) found that viruses associated with cells or cell fragments are protected from inactivation by ozone at concentrations that readily inactivate purified virus. The authors tested ozone to disinfect human epithelial cells infected with poliovirus (Sabin type) or coxsackievirus A9. In a continuous-flow ozonation system, the cell-associated poliovirus and coxsackievirus samples demonstrated survival at applied ozone dosages of 4.06 and 4.68 mg/liter, respectively for 30 s. Unassociated viruses in the control treatment were inactivated by 0.081 mg/liter for 10 s. Ultrasonic treatment did not increase inactivation of the cellassociated enteric viruses. In a batch reactor, inactivation of cell-associated viruses required 2 min contact with 6.82 mg/liter and ozone residual of 4.7 mg/liter, whereas unassociated viruses were completely inactivated after 5 min with 4.82 mg/liter and ozone residual of 2.18 mg/liter.

d) Protozoa

Wickramanayake et al. (1984a) reported the effect of aqueous ozone on the inactivation of cysts of *Naegleria gruberi* and *Giardia muris*. The *N. gruberi* cysts were more resistant to ozone than *G. muris*. A 2-log decrease of population was observed with 0.2 mg/liter ozone at 25°C and pH 7 in 7.5 min for *N. gruberi* compared to 1.05 min for *G. muris*. The intestinal parasite, *Cryptosporidium parvum,* that can cause gastroenteric disease was exposed to ozone that inactivated >90% of the parasite population within 1 min at 1 mg/liter ozone in ozone demand-free water (Korich et al., 1990).

1.9 Factors altering reactivity and antimicrobial efficacy

Although microorganisms inherently vary in sensitivity to ozone, the physiological state and environmental factors affect greatly the degree of inactivation of these microorganisms by ozone. Susceptibility of microorganisms to ozone vary according to the pH of the medium, temperature, humidity, additives (e.g., acids, surfactants, and sugars), and the amount of organic matter surrounding the cells.

a) Temperature

The rate of destruction of microorganisms by a disinfectant generally increases with increasing temperature. According to the van't Hoff-Arrhenius theory (Fair et al., 1968), temperature partly determines the rate at which the disinfectant diffuses through the surfaces of microorganisms and its rate of reaction with the substrate. At constant reagent concentration, increasing the temperature by 10°C increases the reaction rate with the substrate by a factor of 2 or 3. In the case of ozone, however, as temperature increases ozone becomes less soluble and less stable, but the ozone reaction rate with the substrate increases. As the temperature increased from 0°C to 30°C, the rate of inactivating *Giardia* cysts increased (Wickramanayake et al., 1984b). However, Kinman (1975) reported that when bacteria were treated with ozone at 0°C to 30°C, treatment temperature had virtually no effect on the disinfection rate. The researcher related this observation to the decrease in solubility and increase in the decomposition and reactivity of ozone as temperature increases. Achen and Yousef (2001) treated *Escherichia coli*-contaminated apples with ozone at 4, 22, and 45°C, and observed that counts of the bacterium on the surface decreased 3.3, 3.7, and 3.4 $\log_{10}$-units, respectively. Statistical analysis showed no significant differences among the three treatments ($P > 0.05$). The residual ozone concentration was greatest at the lowest temperature (4°C) and decreased with increasing temperature. It appears that when treatment temperature increased,

the increase in ozone reactivity compensated for the decrease in its stability, and thus no appreciable change in efficacy was observed. On the contrary, J.-G. Kim (1998) observed that ozone reduced more microbial contaminants when it was applied at higher than the refrigeration temperatures.

b) pH value

Under constant residual ozone concentrations, the degree of microbial inactivation remained virtually unchanged for pH's in the range of 5.7 to 10.1 (Farooq et al., 1977). However, efficacy of ozone seems to decrease at alkaline pH for rotaviruses (Vaughn et al., 1987) and poliovirus type 1 (Harakeh and Butler, 1985). Ozone is more stable at low than at high pH values, as indicated earlier. Inactivation of microorganisms is mostly through reaction with molecular ozone when the pH is low. Ozone decomposes at high pH values and the resulting radicals contribute to its efficacy. The relative importance of these two inactivation mechanisms may vary with the microorganism and treatment conditions (for example, presence of ozone-demanding contaminants).

Leiguarda et al. (1949) reported that bactericidal efficiency of ozone on *E. coli* and *C. perfringens* was slightly greater at pH 6.0 than at pH 8.0. Farooq et al. (1977) noted higher a survival rate of *Mycobacterium fortuitum* during ozone treatment when pH was increased. The authors attributed this increased survival to a smaller ozone residual as the pH of water increased. Foegeding (1985) studied ozone inactivation of *Bacillus* and *Clostridium* spores at different pH values and found that acidic pH values enhanced the lethality of ozone.

c) Humidity

Elford and Ende (1942) used low ozone concentrations and long exposures at variable RHs to disinfect airborne microorganisms. At RH <45%, the germicidal power of ozone was negligible. Inactivation was substantial even at concentrations far below 0.1 mg/liter when high humidities were used. Ewell (1946) demonstrated that microorganisms were killed more readily by ozone in an atmosphere having a high rather than low RH. The need for moisture in a cell for it to be inactivated by ozone was elucidated by Guerin (1963). The author indicated that not only were desiccated microorganisms more resistant than hydrated cells to sterilization by ozone, but once desiccated, some cells were difficult to rehydrate sufficiently to be susceptible to ozone sterilization. Guerin concluded that ozone was an effective inhibitor only for nondehydrated microorganisms.

d) Ozone-consuming compounds

Presence of organic substances with high ozone demand may compete with microorganisms for ozone. Viruses and bacteria associated with cells, cell debris,

or feces are resistant to ozone, but purified viruses are readily inactivated with the sanitizer (Emerson et al., 1982). Hence, the presence of organic matter in water intended for use in ozone-associated food processing is highly undesirable. Furthermore, unwanted by-products from ozone action on organic compounds may shorten the shelf-life, change the organoleptic quality, or jeopardize the safety of the final product.

Yang and Chen (1979) reported that the bactericidal effects of ozone were lower in Ringer solution, 5% NaCl solution, and in the presence of egg albumin than in distilled water. Restaino et al. (1995) reported that in the presence of organic material, death rates of some gram-positive microorganisms (e.g., *S. aureus* and *L. monocytogenes*), and gram-negatives, *E. coli* and *Salmonella* Typhimurium, in ozonated water were not significantly affected by 20 ppm of soluble starch but were significantly reduced by addition of 20 ppm of bovine serum albumin. Residual ozone in water containing bovine serum albumin was significantly lower than in deionized water and water with soluble starch. When microorganisms are suspended in an ozone demand-free medium, the only source of ozone demand is the seeded organisms. In water, ozone may react directly with dissolved substances, or it may decompose to form secondary oxidants that immediately react with solutes. These different pathways of reactions lead to different oxidation products, and they are controlled by different types of kinetics (Staehelin and Hoigne, 1985). The solutes present in water influence appreciably the rate of the radical-type chain reaction leading to the decomposition of ozone. This reaction is promoted by solutes, such as formic acid and methanol, that convert the nonselective hydroxyl ($^{\bullet}OH$) into a superoxide ($^{\bullet}O_2^-$) radical that is a more efficient chain carrier. Such promoters counteract the inhibiting effects of OH radical scavengers that generally terminate the chain reaction. Acetic acid and acetate are known to terminate the reaction by scavenging $^{\bullet}OH$, thus stabilizing ozone in aqueous solutions (Forni et al., 1982; Hoigne and Bader, 1976; Sehested et al., 1987). Schuchmann and Sonntag (1989) explained ozone effectiveness in reducing the load of organic matter (added D-glucose) in raw water purification. They found that direct mode of reaction by ozone predominated at high glucose concentration; however, the $^{\bullet}OH$ pathway predominated at low glucose concentration, especially at higher pH (e.g., 9.0).

1.10 Interaction with materials

Ozone interaction with the equipment and surfaces to be cleaned and disinfected is a key factor that must be taken into consideration, essentially because of the corrosion it may cause, but also because the ozone loses its effectiveness. The corrosive effect of ozone depends on the concentration employed. At high concentrations it may corrode equipment, but such high concentrations only occur within the ozone generator or in the system that injects the ozone into the water. Most materials are compatible with ozone at moderate concentrations of 1-3 ppm ozone. The plastics most frequently employed in the food industry perform well in the presence of ozone and their resistance to corrosion by ozone is considered good or excellent: PTFE (Teflon), PVDF (Kynar), PVC (rigid and flexible) and ECTFE (Halar) are mentioned in various publications. Other materials that show resistance are 316L and 304L stainless steel, particularly the former, which stands up better to corrosion by ozone than by chlorine according to some authors (Green et al., 1999; Singh and Singh, 1999). However, natural rubber is highly sensitive to contact with ozone, leading to total disintegration (Kim et al., 2003). Silicone is resistant in the short-term but oxidises on extended exposure to ozone. Consequently, it is good practice to identify all the materials that could come into contact with ozone and check their potential resistance.

1.11 The use of ozone in the food processing industry

1.11.1 Disinfecting production areas and equipment

A high reactivity and leaving no harmful residues make ozone an effective disinfectant in assuring the quality and microbiological safety of food (Kim et al., 1999). It is important in food processing plants that the areas which are in contact with food should be as microbiologically pure as possible. Therefore, it should be remembered that surfaces that visually seem clean may still be contaminated with a large number of microorganisms which can further contaminate food (Moore et al., 2000). To prevent this, after proper washing various cleaning agents are used. They include chlorine compounds, acids, iodine and four-valent ammonium compounds. Some food processing plants use thermal sanitization and/or radiation. Thermal sanitization is an effective method for destroying microorganisms contaminating food, however, steam and hot water are expensive to generate and excessive heat may damage equipment.
Radiation methods are practically not used in food processing plants due to inherent risks related to the presence of radioactive materials. The application of chemical sanitizing agents is the most common method used in the food

processing industry. Chlorine-containing agents are widely used to disinfect water, waste and food processing plant equipment. However, these chlorine compounds have some drawbacks: harmfulness and irritating action at high concentrations, a tendency to form carcinogenic compounds and a toxic effect on the environment. Despite these disadvantages, chlorine has been used as a disinfectant for water and food for many years as it deactivates all types of vegetative cells. Although chlorination effectively reduces the frequency of food poisoning, chlorine reacts with many organic compounds and forms toxic intermediate products which adversely affect the public health and environment (Güzel-Seydim et al., 2004a). As a result, the interest in additional or alternative disinfectants has been observed to increase (Moore et al., 2000).

Ozone dissolved in water was employed in a newly developed line for washing plastic containers used for storing and transporting meat. Water enriched with ozone is used instead of traditional washing agents. Ozone obtained from the atmospheric oxygen is introduced under controlled conditions to the circulating water under lowered pressure, allowing colder water – between 15 and 30°C – to be used for washing. Although colder water can be used as a result of applying ozone, this method stands out among other such methods as highly effective and having numerous advantages. It is not necessary to use aggressive chemicals or to heat water to 80°C at the final stage of the process. As a result, the pollution of the environment is not as high as in other methods.

Ozone causes also the coagulation of proteins and fats. As a result, fat "catchers" work more effectively and the degree of waste contamination is lower. Being the strongest oxidiser and disinfectant, ozone is able to oxidise remnants of fat and protein and remove them from the surface of containers.

The reaction between ozone and the contaminated surfaces lasts several seconds when the concentration of contaminations is about 10%. Compared to traditional chemicals, this time is considerably shorter. Detailed microbiological tests have proven that at all stages of washing ozone caused the total inactivation of bacteria (Steigert and Franke, 2000).

Using gaseous ozone is a method which ensures the full sterility and can be applied in slaughterhouse to sterilize slaughtering tools, particularly knives, which can become a source of contamination during the slaughter, post-slaughter processing and partition of carcasses. Bacteriocidal properties of ozone have been confirmed in the study by Uradziński et al. (2005) with knives contaminated earlier with *Escherichia coli*, *Salmonella Enteritidis* and *Staphyloccocus aureus*. The knives were exposed to ozone in 20-minute intervals until they had become fully sterile. In the mentioned study, the total time needed to eliminate the bacteria was found to be 120 minutes, as this is how much it took to deactivate all the *Salmonella Enteritidis* and *Staphylococcus aureus*, although *Escherichia coli* was inactivated after 80 minutes of ozonation. This results from the stronger

oxidising ability towards Gram-negative bacteria. In the current study, small tools were exposed to ozone in a device equipped with a UV lamp, emitting 185 nm of radiation, that was enclosed within a leak-proof ozone generator which produced ozone at a dose 0.03 mg/m^3 air. This device was made of stainless steel to enable its long-term use. Holah et al. (1995) evaluated different air disinfection systems and found that ozone was effective and reproducible in its effect on airborne microorganisms. Ozone also can be applied for preventing secondary contamination during bread manufacturing (Staszewska, 1994). The interior environment in a factory that manufactures plastic films was exposed to 0.02 to 0.16 ppm ozone for 10 h per day and 1 to 1.5 years (Naitoh, 1993). Aerial contaminants such as *Bacillus* spp. and *Micrococcus* spp. in the plastic film processes were reduced. Chun et al. (1993) developed a UV air cleaner for the sterilization and deodorization of the air in refrigerators. The authors reported that ozone production reached 0.082 ppm in the holding section at 25°C and 0.06 ppm at 3°C. The bactericidal action of activated oxygen (O_2, O_3, and O) destroyed or reduced organisms on food preparation surfaces and inhibited development of cold-tolerant bacteria and *Pseudomonadaceae* on foods (Anonymous, 1994). Decupper (1992) obtained a patent to use ozone and UV sterilization unit for cold storage of foods.

1.11.2 The use of ozone in food production

Apart from sterilising equipment and production area, ozone is used in the food processing industry to eliminate microorganism from the surface of meat from slaughtered animals and poultry carcasses as well as in the preservation of food and extension of its shelf life. Reduction of microflora, destruction of pathogens, disinfection and extension of the shelf life of a product can all be achieved by using ultraviolet radiation (Smith and Pillai, 2004). It has been found that the inactivation of microorganisms by UV radiation results primarily from the damage to their DNA structure. However, some authors see the use of UV radiation in disinfection as problematic since some microorganisms are able to repair the damaged DNA (Morita et al., 2002).
Contemporary studies have shown the effective bactericidal action of ozone towards microorganisms inducing food decay (*P. aeruginosa* and *Z. bailii*), faecal contaminants (*E. coli* and *E. faecalis*) and pathogens causing food poisoning, *e.g. L. monocytogenes*, *B. cereus*, *S. typhimurium* (Restaino et al., 1995). Despite its effectiveness against spores and vegetative forms of bacterial cells, it is hardly possible that ozone could be used directly to treat food, the reason being the presence of organic matter. Under these conditions, ozone is less effective in reducing the number of bacteria, as it first oxidises the components of the substrate and then the bacterial cells, which results in decreasing the

concentration of free ozone with its bactericidal action. In order to achieve the desired reduction in the bacteria number, a higher concentration of ozone would be needed, which in turn might decrease the sensory and health-promoting properties of food (Moore et al., 2000; Unal et al., 2001).
Although the direct application of ozone in food preservation seems hardly probable, at present the food processing industry commonly uses a technique of food preservation which consists in the simultaneous use of methods with additional or synergistic action against pathogens. For example, exposing beef surface to ozone at a dose of 5 mg/L water increases the effectiveness of subsequent treatment at a temperature of 45-75°C against strains of *Clostridium perfringens* which produce enterotoxins. The resistance of both spores and vegetative cells is reduced as a result of exposure to ozone before thermal treatment in which the temperatures might prove ineffective, and applying higher temperatures might reduce the quality of meat, *i.e.* meat surface browning. This, in turn, is another argument for the synergistic application of ozone and moderate temperature in order to reduce the number of *Cl. perfringens* (Novak and Yuan, 2004). However, the technique of food preservation needs to be carefully evaluated to ensure that the cells which retain their vital functions do not pose a threat of food poisoning or infection. The pathogens which survive the exposure to ozone are less dangerous during the consumption of food than those which survive sublethal thermal treatment (Novak and Yuan, 2003).
Williams et al. (2004) argues that applying ozone is a potential alternative method for thermal pasteurisation in controlling the population of *E. coli* O157:H7 and *Salmonella* sp. in apple and orange juice. Considerable reduction of this bacteria is caused by heat and ozone. Similar results are caused by the simultaneous application of ozone and electric fields (pulsed electric field). Due to the simultaneous use of these two methods, microorganisms which cause damage to food, *i.e. L. leichmanii*, pathogens which cause food poisoning, *i.e. E. coli* O157:H7 and *L. monocytogenes*, are deactivated more effectively because the lethal effect of the electrical field is synergistically enhanced by the prior application of ozone (Unal et al., 2001).

Meat. The feasibility of using ozone in meat processing has been the focus of several studies. Kaess and Weidemann (1968) reported that the count of *Pseudomonas* spp. and *C. scottii* on contaminated beef decreased significantly at >2 μg/liter gaseous ozone and the lag phase of *Thamnidium* spp. and *Penicillium* spp. increased, but their growth rate did not change. The color of the muscle surface treated with <0.6 μg/liter ozone did not differ from that of the control treatment. Ozone has been tested in the process of tenderizing meats to control surface microflora (*Pseudomonas* spp., spores, *Salmonellae* spp., *Staphylococcus* spp.). Ozone in a gas mixture at 0.1 mg/liter and RH of 60 to 90% were required in

the tenderizing room to inactivate bacteria, but higher concentrations of ozone were required to inhibit molds. Kaess and Weidemann (1973) also reported that simultaneous use of UV (0.2 μW/cm^2) and ozone (0.5 μg/liter) produced a synergistic inhibitory effect against *Thamnidium* spp. and *Penicillium* spp. This inhibition was manifested by an increase in the lag phase and a decrease in the growth rate. Contrary to these findings, Fournaud and Lauret (1972) detected little reduction in counts of *Microbacterium thermosphactum, Lactobacillus, P. fluorescens,* and *Leuconostoc* on a beef surface as a result of gaseous ozone treatment (100 ppm) for 30 min. The authors concluded that low activity and side effects such as discoloration and odor development rendered ozone use unacceptable.

Spraying beef brisket fat with hydrogen peroxide (50 g/liter) solution and ozonated water (5 g/liter) was effective in reducing bacterial contamination, when compared to treatments with trisodium phosphate (120 g/liter), acetic acid (20 g/liter) and a commercial sanitizer (3 g/liter) (Gorman et al., 1995). Reagan et al. (1996) conducted a study to compare procedures and interventions for eliminating physical and bacterial contamination from beef carcasses. Rinsing with ozonated water (0.3 to 2.3 mg/liter) reduced aerobic plate counts by 1.3 log CFU/cm^2 that was approximately equivalent to conventional washing in reducing bacterial populations on beef.

Ozone treatment decreased the counts of aerobic mesophiles, coliforms, and sulfite-reducing clostridia in the meat-transport vehicles (Billion, 1978). The author reported that ozone treatment also improved the storage quality and decreased counts of mesophilic aerobes and sulfite-reducing anaerobes on meat. Other investigators (Kolodyaznaya and Suponina, 1975) found that ozone at 10 to 20 μg/liter inhibited microbial growth on beef that was kept at 0.4°C and 85-90% RH and extended the permissible storage period by 30 to 40%.

Rusch and Kraemer (1989) used ozone for the treatment of airborne microorganisms on the surface of meat stored at 2.5 to 6°C and 92 to 95% RH. The treatment halted growth of several *Enterobacteriaceae* but not that of *Pseudomonas* spp. When beef carcasses were continuously ozonated (0.03 ppm) at 1.6°C and 95% RH for up to 9 days of ageing, ozone prevented bacterial growth on carcass surfaces; however, it did not increase the retail case life (as judged by odor and appearance) nor did it reduce bacterial growth on retail steaks (Greer and Jones, 1989). Dondo et al. (1992) evaluated ozone usage for beef kept in a refrigerator. Ozone stopped the growth of surface contaminants during several days of storage, improved the sensory quality, and decreased the formation of total volatile N compounds. Horvath et al. (1985) indicated that in the presence of ozone, growth of microflora on meat surfaces decreased at refrigeration temperatures; however, no inhibitory effect was observed if the meat was heavily contaminated.

Poultry. Ozone has been tested for disinfecting hatchery, hatching eggs, poultry chiller water, poultry carcass, and contaminated eggs. Cultures of *Staphylococcus, Streptococcus,* and *Bacillus* species previously isolated from poultry hatcheries and culture collections of *E. coli, P. fluorescens,* and *Salmonella* Typhimurium, *Proteus* species, and *A. fumigatus* were spread-plated on open petri plates and exposed to ozone gas in a prototype laboratory poultry setter (Whistler and Sheldon, 1989b). Ozone treatment (1.5 to 1.65%, wt/wt) decreased microbial populations by >4 to 7 logs for bacteria and >4 logs in the case of fungi. Whistler and Sheldon (1989a) also evaluated ozone as a disinfectant against natural contaminants on hatching eggs. Microbial counts significantly decreased (>2.5 logs) on the shell of eggs that were misted with water and ozonated (ozone in gas mixture was 2.83%, wt/wt) for 2 h. However, hatchability was significantly reduced (26.5 to 37.5%) following ozonation using 3.03% ozone (wt/wt) for 2 h. Bailey et al. (1996) reported that ozone decreased the aerobic plate counts and *Salmonella* in hatching cabinet air samples by 75 to 99%.

Sheldon and Brown (1986) evaluated the effects of ozone on the quality of poultry chiller water and broiler carcasses. Carcasses, chilled in tap water containing ozone at 3.0 to 4.5 ppm for 45 min, were consistently lower in microbial count during storage when compared with nontreated ones. Ozonation of chiller water decreased microbial load >2 logs and chemical oxygen demand by ca. 33% and increased light transmission (at 500 nm) without significantly changing the sensory quality of poultry meat. Yang and Chen (1979) treated broiler parts in ice-cold water with gaseous ozone at 3.88 mg/liter for 20 min and also treated microbial suspension obtained from fresh and spoiled chicken necks with gaseous ozone at 2.48 mg/liter for 5 to 9 min, respectively. According to these authors, the total microbial counts of broiler and microbial suspensions from fresh and spoiled parts decreased 1, 0.6, 3 logs, accordingly. They also noticed that ozone treatment preferentially destroyed gram-negative rods. In another study, ozone was used to disinfect microorganisms on poultry meat (Kim and Kim, 1991). All microbial contaminants were inactivated when meat was flushed for 50 min with a gas mixture containing ozone flowing at 1500 ppm/min.

Rudavskaya and Tishchenko (1978) evaluated the quality and keeping characteristics of retail eggs after ozonation. Eggs were treated with ozone gas (10 to 12 μg/liter air) for 6 h and then stored for 6 months at 21°C with 86% RH and 29°C with 75% RH. Eggs were analyzed for sensory quality, changes in acid, peroxide and thiobarbituric acid values of the yolk, white and yolk indices, and variations in quality grading. All quality parameters had better values in the ozone-treated samples than in the controls, and the lower storage temperature had an additional beneficial effect on quality. Krivopishin et al. (1977) suggested a method for preservation of eggs using ozone. Eggs were dipped in paraffin

wax at 40 to 45°C and treated for 10 to 30 min in air containing 1 to 3 mg/liter ozone. Cox et al. (1995) patented a hyperpasteurization process that involves treatment of washed egg shell with heat (59.4°C) and ozone in a vacuum chamber. The treated eggs have extended shelf-life and reduced microbial load.

Cheese. Ozone concentrations of 0.1 and 10 μg/liter in the atmosphere of a cheese-ripening room inactivated 80 to 90 and 99%, respectively, of mold spores without affecting the sensory qualities of cheeses (Shiler et al., 1978). Batches of Rossiiskii, Poshekhonskii, Kostroma, and Swiss-type cheeses were stored at 2 to 4°C and 85 to 90% RH with ozone generated in the atmosphere of the storage area (Gabriel'yants' et al., 1980). Researchers found that periodical ozonation for at least 4 h at 2 to 3-day intervals with 5 to 7 μg/liter ozone in air prevented growth of molds on cheeses and packaging materials for 4 months without adversely affecting chemical and sensory properties of the cheese. Control cheese exhibited mold growth as early as 1 month. Horvath et al. (1985) noted that storage life of cheese increased to 11 weeks by the application of ozone at low concentrations (0.02 mg/liter) during the ripening period. Other experiments conducted on cheddar cheese also indicated that the oxidizing action of ozone removes odors otherwise present in storage rooms. Shiler et al. (1983) described a method of ozonation for ripening and storing cheese to inactivate contaminating microflora but to avoid damage to cheese-packaging materials and to improve hygiene. For optimum results, ozonation was carried out for 1 to 3 h/day at an ozone concentration in the air of 0.08 to 0.1 μg/liter with intervals of 2 to 12 h, and every 10 to 30 days the chambers were treated with ozone at a concentration of 8 to 12 μg/liter for 2 to 4 h.

Fish. In the fishery industry, ozone was tested to disinfect fishery products and to improve sensory qualities. Haraguchi et al. (1969) studied the preserving effect of ozone on fresh jack mackerel (*Trachurus trachurus*) and shimaaji (*Caranx mertensi*). Treatment of the skin of the gutted fish with 3% NaCl solution containing 0.6 ppm of ozone for 30 to 60 min decreased the viable bacterial count by 2 to 3 logs. The storage life of the fish increased 20 to 60% when the ozone treatment was applied every 2 days. Chen et al. (1987) studied ozone for in-plant sterilization of frozen fishery products. They found that ozone was effective in distilled water and 3% NaCl solution for the inactivation of microorganisms such as *Vibrio cholera, E. coli, Salmonella* Typhimurium, *V. parahaemolyticus,* and *S. aureus.* Ozone treatment of shrimp decreased *E. coli* count by 98.5%. Coudrain and Starck (1988) applied 10 to 15 mg/liter gaseous ozone in the air for 4 to 6 min to remove odor and color from fish flesh. Dondo et al. (1992) reported that ozone decreased surface contaminants of fish during several days of refrigerated storage. Ozone treatment improved the sensory quality of

fish by decreasing the formation of trimethylamine. A beneficial decoloration effect of horse mackerel (*T. japonicus*) mince resulted from washing with ozonated water for 10 to 20 min (Chen et al., 1997). However, a marked decrease in pH and undesirable gel strength of mince, as well as oxidation of the fish oil, occurred during this ozone treatment. Ozone promoted detachment of the surface slime of redfish aboard fishing vessels and ozonation during transport reduced bacterial count and extended the shelf life of the fish by ca. 1.5 days (Köetters et al., 1997). Simulation trials in the laboratory indicated that bacterial counts were higher on fish held in ozonated water than on control fish. The author attributed this difference to the lower freshness of redfish used in the laboratory. Therefore, it was recommended that fish should be treated with ozone when it is fresh. Ozone was tested to improve the washing process that is applied during the manufacture of dark-fleshed fish surimi (Chen and Lao, 1997). The investigators found that ozone washing treatment minimized the washing time and improved color; however, undesirable gel strength and a decrease in the pH of the minces were observed.

Fruits and vegetables. Ozone treatments increased the shelf life of some fruits. Bazarova (1982) stored apples in a specially constructed stainless steel chamber at 0 to 1°C and 90 to 95% RH with ozone gas being admitted daily for 4 h at 5 to 6 mg/liter. The author concluded that ozone treatment reduced weight loss and spoilage incidence in apples. Ozone at 0.1 to 0.3 ppm in atmosphere during blackberry storage suppressed fungal development for 12 days at 2°C and did not cause observable injury or defects (Barth et al., 1995). Grapes exposed for 20 min to ozone (8 mg/liter) had considerably reduced counts of bacteria, fungi, and yeasts (Sarig et al., 1996). Fungal decay following cold storage of the grapes was reduced and shelf life increased by the ozone treatment. Horvath et al. (1985) attributed the increase of the shelf life of apples and oranges to the oxidation of ethylene and to the removal of other metabolic products by ozone. However, inactivation of spoilage microorganisms on fruits, without a doubt, contributed to this shelf life extension. In vegetables, the advantages of ozone were similar to those experienced in fruit processing. Onions and potatoes were stored in wooden chambers covered with polyethylene film in which ozone (0.2 μg/liter) was produced for 8 h/day on 5 days/week (Faitel'berg-Blank et al., 1979). Ozone treatment decreased chemiluminescence, oxygen uptake, catalase, and peroxidase activities and had a marked inhibitory effect on the growth of surface microorganisms. Losses due to spoilage at the end of storage were 1 and 0.8%, respectively, for treated onions and potatoes versus 9.7 and 6.7% for controls. Baranovskaya et al. (1979) used ozone in the industrial storage of potatoes, onions, and sugar beets. They maintained ozone concentration at 3 mg/liter with temperature within 6 to 14°C and RH at 93 to 97%. Their analysis

showed that bacteria and mold counts were very low for treated samples, whereas chemical composition and sensory quality did not change appreciably. Ozone was presented to be an alternative to chlorpropham (isopropyl-*N*-[3-chlorophenyl] carbamate) as a sprout control agent for Russet Burbank potatoes in Canada (Prange et al., 1997).

Kim et al. (1998) treated shredded lettuce with ozone under different mechanical actions such as sonication, stirring, and stomaching. Bubbling ozone gas (4.9%, vol/vol; 0.5 liter/min) in a lettuce–water mixture decreased the natural microbial load by 1.5 to 1.9 logs in 5 min. These authors concluded that bubbling gaseous ozone was the most effective ozonation method. For efficient ozone delivery to microorganisms on lettuce, ozone bubbling should be combined with high-speed stir.

Dry foods. *Bacillus* and *Micrococcus* are dominant bacterial genera of cereal grains, peas, beans, and spices. Counts of these microorganisms decreased 1 to 3 logs by < 50 mg/liter ozone (Naitoh et al., 1988). Naitoh et al. (1987, 1988) studied the effects of ozone concentration (0.5 to 50 mg/liter), exposure time (1 to 6 h), and temperature (5 to 50°C) on several cereal grains, cereal grain powders, peas, beans, and whole spices. With few exceptions, longer exposure time and lower temperature resulted in higher microbicidal activity in these dry foods. The authors found that oxidation of lipids in these commodities rarely occurred at <5 ppm but was considerable at higher concentrations. Naitoh et al. (1989) reported the treatment of wheat flour with 0.5 to 50 ppm ozone for 6 h. This treatment inhibited microbial growth in namamen product and increased storage life two to fivefold. During the storage time, thiamine content decreased 4 to 17%, but sensory quality of namamen did not change. In a microbial decontamination study of spices by gaseous ozone, several samples showed only a slight (<1 log) microbial inactivation with 30 to 145 mg/liter residual ozone but white pepper showed a 4.4-log reduction (Zagon et al., 1992).

Ozone was applied in heating peanut meal to destroy aflatoxins or to greatly reduce their levels (Dollear et al., 1968). Weight gains for ducklings and rats receiving treated meals were essentially comparable to control animals, however, treated meals had reduced protein efficiency ratios. Rayner et al. (1971) reported that ozone reduced aflatoxin in cottonseed meal and peanut meal. Contaminated cottonseed and peanut meals were hydrated and brought into contact with ozone at 75 to 100°C to achieve substantial lowering of the aflatoxin content. With 15 mg/liter for 30 min, ozone effectively decreased the *A. flavus* population and its aflatoxin in dried soup (Paulina et al., 1984). The destruction and detoxification of aflatoxins B1, G1, B2, and G2 (50 μg/ml in 4% dimethyl sulfoxide) with ozone were confirmed by Maeba et al. (1988). Aflatoxins B1 and G1 were degraded with 1.1 mg/liter ozone within 5 min; however, B2 and G2 required

34.3 mg/liter ozone and 50 to 60 min treatment time for comparable degradation. Chatterjee and Mukherjee (1993) studied the impact of ozone on the immunity-impairing activity of aflatoxin B1. The phagocytosis-suppressing activity of aflatoxin B1 was destroyed with gaseous ozone treatment (1.2 mg/liter) for 6 min at a flow rate of 40 ml/min.

Water and fluid food. Sander (1985) developed an ozone treatment for fruit juices and liquid dairy products that minimizes possible quality deterioration. Rojek et al. (1995) attempted to use pressurized ozone to decrease the microbial population of skim milk. In this study, ozone gas concentration was 5 to 35 mg/liter and treatment time was 5 to 25 min. Their results showed that pressurized ozone was effective in decreasing psychrotrophic counts by 2.4 logs. Treatment of whey and apple juice also produced favorable microbial reduction. Greene et al. (1993) proved effectiveness of ozone against biofilms of milk spoilage bacteria, such as *P. fluorescens* and *Alcaligenes faecalis,* on stainless steel plates. Greater than 99% of the population was eliminated by ozone treatment at 0.5 ppm for 10 min.

Franz and Gagnaux (1971) investigated an ozone treatment to sterilize contaminated spring water for use in the food industry. They found that coliforms and spore-forming bacteria were inactivated during 8 min treatment at 0.1 to 0.2 mg/liter and 1.6 to 3.2 mg/liter ozone, respectively. However, in an industrial installation, only 80% sterilization was achieved within 14 min and ozone concentrations of 1.12 to 2.18 mg/liter. Ozone consumption increased with increases in suspended matter and the pH. They also reported that preliminary flocculation decreased ozone consumption and produced completely germ-free water. Possible applications of ozone in the brewery industry were suggested (Tenney, 1973). These include yeast washing, selective removal of bacterial contaminants and final rinses of bottles, cans, fillers, pipelines, and tanks.

Ozone is considered one of the means of ensuring water quality in the beverage industry (Fritsch, 1994). Hargesheimer and Watson (1996) reported that ozone altered the fishy odor associated with some phytoplankton blooms in drinking water sources to an undesirable plastic-like odor. They suggested a combination of granulated activated carbon with ozonation for removal of particulates, color, taste, and odor compounds.

Process water and effluents. Woerner et al. (1970) examined direct ozonization to disinfect protein-containing fluid synthetic media, household effluent, and slaughterhouse effluent. They found that 5 to 10 mg/liter gaseous ozone was adequate to eliminate bacteria according to the degree of contamination. *Salmonella* were eliminated after a contact time of 7 min and

Bacillus anthracis spores after 30 min. While describing possible methods for sterilization of slaughterhouse effluents, Boehm (1989) suggested ozone treatment as the best chemical method. Hurst (1991) patented a method by which ozone is bubbled through the food process water to remove fat, bacteria, solids and other impurities before recycling this water. Postprocess spoilage of canned food decreased by using ozonated water for cooling cans (Ito and Seeger, 1980). Loorits et al. (1975) explored the possibility of using ozone for oxidizing major milk components. Ozone reduced the fat content in condensates (80 to 230 mg/liter) by 96 to 98% and completely eliminated turbidity. The authors concluded that ozone treatment could be applied to the purification of lightly polluted dairy effluent for subsequent reuse in water supply systems. The chemical oxidation of olive mill effluents by ozone was developed to reduce chemical oxygen demand, aromatic content, and phenolic content (Benitez et al., 1997).

Using ozone during food storage. The success of most methods of food preservation depends on how the processed food is protected during storage from adverse environmental conditions. Food can be protected mainly by packaging. However it is essential that during storage the exposure to different conditions does not cause changes of physical and/or chemical properties of packing materials. On the other hand, efforts should be made to avoid any modification of containers which might affect the quality of food contained in them (Ozen and Floros, 2001). It has been observed that treating plastic containers with ozone-enriched water reduces the number of bacteria stuck to their surface by 5 orders of magnitude (Khadre and Yousef, 1989). Ozone is used in food storage for yet another purpose – for controlling odour. The presence of ozone at low concentrations of 0.01–0.04 mg/m^3 air in cold store and store rooms increases the feeling of air freshness. Moreover, ozone enhances the flavour of fresh unstable products, such as fruit and vegetables, by oxidising pesticides and neutralising ammonia and ethylene produced during the ripening process. Reducing the ethylene level prolongs the permissible storage period and reduces the shrinking of fruit and vegetables (Jaksch et al., 2004). Some research has been conducted to examine the effect of ozone released continuously to a cold store room at doses of 0.3 and 1.0 mg/m^3 air, on the development of major diseases of grapes and citrus fruit. Ozone had an inhibiting effect on mycelium and considerably reduced the sporulation of *Penicillium digitatum* and *Penicillium italicum*. It should be emphasized that the ozonated room was closed throughout the storage period (Palou et al., 2003).

1.12 Ozone as an alternative sanitizer to chlorine

Chlorine in various forms, specially hypochlorite salts, has been successfully used to sanitize utensils and equipment in dairy and other food-processing industries. Hypochlorites are considered generally recognized as safe (GRAS) substances and thus are permitted in various food application in the United States. Chlorine compounds are effective and inexpensive disinfectants. For example, use of hypochlorite dip or spray is effective in controlling bacterial contamination of fruits and vegetables. In the egg industry, chlorine compounds are used in the wash water to decrease the load of spoilage and pathogenic microorganisms. Chlorine compounds have a few drawbacks that increasingly limit their use in the food industry. Chlorination may lead to the formation of toxic or carcinogenic chlorinated organic compounds in water (Brungs, 1973; Page et al., 1976), and food or on food contact surfaces (Wei et al., 1985). Collins and Deaner (1973) reported that chlorine residues >0.1 mg/liter may be excessive with respect to toxicity and that in critical areas of biological significance, it may be necessary to provide dechlorination facilities to reduce chlorine concentration.

Much information attesting to the superiority of ozone over other chemical disinfectants has been accumulated. Gomella (1972) reported that ozone, compared to chlorine, showed stronger and more rapid antimicrobial action against spores, fecal and pathogenic microorganisms, and viruses, mainly in an environment with a high organic-matter content. Kessel et al. (1943) showed that free ozone residues of 0.05 to 0.45 mg/liter were sufficient to inactivate poliovirus within 2 min, while free chlorine residues of 0.5 to 1.0 mg/liter at pH 6.0 required 1.5 to 2.0 h for similar degree of virus inactivation. Another study by Scarpino and his colleagues (1972) also confirmed that ozone was superior to chlorine in the rate of disinfection of poliovirus. With 0.3 mg/liter of disinfectant, ozone reduced virus particle count by 2 logs within 10 s, while chlorine reduced the count by 2 logs in 100 s. Korich et al. (1990) reported that chlorine dioxide and ozone were more effective than chlorine and monochloramine against *C. parvum* oocysts. Greater than 90% inactivation of oocysts was achieved with exposure to 1 mg/liter ozone for 5 min. Exposure to 1.3 mg/liter chlorine dioxide yielded 90% inactivation after 1 h, while 80 mg/liter chlorine and 80 mg/liter monochloramine required approximately 90 min for 90% inactivation. Forsythe and Waldroup (1994) reported the economic benefits of ozone usage in poultry-processing plants such as reduced water purchase, reduced sewage treatment costs, and savings in electrical energy from recycling ozonated water.

In the past, application of ozone in the food industry in the United States was limited. It had been used primarily for the removal of iron, manganese, color,

tastes, and odors in water (O'Donovan, 1965). In 1982, the U.S. Food and Drug Administration affirmed that ozone is generally recognized as safe (GRAS), with specific limitations, for use as a disinfectant in bottled water (FDA, 1982). The U.S. Department of Agriculture permitted recycling of reconditioned water in poultry chillers (U.S.D.A, 1984). Recently, an expert panel in the United States affirmed ozone as a GRAS substance (Graham, 1997) for broad food applications. Because the U.S. Food and Drug Administration had no objection to this affirmation, ozone now can be used as a disinfectant or a sanitizer in food processing in the United States. These regulatory developments triggered interest in ozone applications among academic researchers and food processors.

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CHAPTER 2

Objectives

2. Objectives

Ozone (O_3) is a strong antimicrobial agent with several potential applications in the food industry. High reactivity, penetrability and spontaneous decomposition to a nontoxic product (O_2) make ozone a viable disinfectant for ensuring the microbiological safety of food products. Ozone has been used for decades in many countries and recently, the generally recognized as safe (GRAS) status of this gas has been reaffirmed in the United States. Ozone, in the gaseous or aqueous phases, is effective against the majority of microorganisms tested by numerous research groups. Relatively low concentrations of ozone and short contact time are sufficient to inactivate bacteria, molds, yeasts, parasites and viruses. Ozone applications in the food industry are mostly related to decontamination of environments and water treatment. Moreover, ozone has been used with success to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits, vegetables and dry foods.
The aim of this work was to investigate the real potentiality of gaseous ozone in food industries. Ozone was tested:
- as a disinfectant in meat industry;
- as an alternative method of mite control on meat products;
- to reduce extraneous molds on the surface of meat products.
It was also conducted a preliminary investigation on the possible surface oxidation of stored products treated with low concentration of ozone.

CHAPTER 3

Ozone efficacy on different species of *Listeria*, *Salmonella* and *Bacillus*

3. Ozone efficacy on different species of *Listeria*, *Salmonella* and *Bacillus*

3.1 Abstract

Effective cleaning and sanitation of food processing equipment are major concerns in the food industry. Soiled food processing equipment provides a suitable environment for miccroorganisms to proliferate. Ozone is one of the more effective disinfectants; it does not leave any harmful residues in food or on the surfaces which are in contact with it. In addition, compared to chlorine and other disinfectants, it is more effective against resistant viruses and spores.
The aim of this study was to evaluate, under laboratory conditions, the effect of gaseus ozone on different species of *Listeria*, *Salmonella* and *Bacillus*. Fresh 24h bacterial cultures of *L. monocytogenes* (ATCC 19111), *L. innocua* (ATCC 33090), *L. grayi* (ATCC 25401), *S.* Enteritidis (food isolate), *S.* Typhimurium (food isolate), *S.* Derby (food isolate), *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 21228), *B. thuringiensis* (ATCC 10792) were exposed to ozone (1 ppm) for 10, 30, 60 and 180 minutes. *Listeria* spp. showed a mean reduction over all species of 2.1, 2.7, 3.5, 5.1 log units rispectively. *Salmonella* spp. showed a mean reduction of 1.1, 1.5, 2.7, 3.8 log units. *Bacillus* spp. showed a mean reduction of 2.5, 3.0, 3.2, 3.5 log units. Different species of the same genus showed similar sensitivity to ozone.

3.2 Introduction

Salmonella enteritidis, *Listeria monocytogenes* and *Bacillus cereus* are food-borne pathogens of major public health concern. A variety of foods, including poultry, eggs, meat, milk, fruits and vegetables, have been implicated as vehicles of one or more of these pathogens in outbreaks of food-borne illness (Beuchat, 1995; D'aoust, 1997). Effective methods of reducing or eliminating pathogens in foods are important to the successful implementation of Hazard Analysis and Critical Control Point (HACCP) programs by the food industry and for the establishment of critical control points in restaurants, homes, and other food service units.
Bacteria of the genus Listeria are widely distributed in our environment and they have been isolated from soil, vegetation, sewage, water, animal feed, fresh and frozen poultry, slaughter house waste and human and animal carries (Welshimer, 1981). *Listeria monocytogenes* poses significant threat to the food industry because it is pathogenic to humans, it is able to grow over a wide range of temperature (from 0-45°C, with an optimum of 30-37°C) and it can grow and multiply on

most nonacidic foods. Although not all *Listeria* species are pathogenic, their occurrence in food is generally indicative of unsanitary conditions. Because of its high-phenotypic similarity to *L. monocytogenes, L. innocua* is often used as a nonpathogenic indicator for *L. monocytogenes* microorganism (Fairchild and Foegeding, 1993).

Bacteria of the genus Salmonella are Gram-negative, facultatively anaerobic, non-spore forming, usually motile rods belonging to the family *Enterobacteriaceae* and primarily associated with animals. The genus currently contains just two species, *Salmonella enterica* (including six subspecies) and *Salmonella bongori*. Most of the Salmonella isolates from cases of human infection belong to *Salmonella enterica* subspecies *enterica*. The genus is also further subdivided into approximately 2500 serovars (or serotypes), characterised on the basis of their somatic (O) and flagellar (H) antigens. All species share a high degree of DNA homology (WHO, 1980). By using cross-absorption and antiserum reactions to differentiate O (somatic or polysaccharide) and H (flagellar) antigens, workers have defined over 2000 Salmonella serotypes (Crosa et al., 1973).

Bacillus cereus is a spore-forming bacterium that can be frequently isolated from soil and some food (Kramer and Gilbert, 1989). It can form spores that are resistant to heating and dehydration and can therefore survive cooking and dry storage. When foods containing *B. cereus* spores are in the 'temperature danger zone' the spores may germinate, and the bacteria may grow, produce toxins, and make people sick. Such illness is frequently linked with starchy foods of plant origin such as rice, pasta, potatoes, pastry and noodles.

In any food processing environment, bacterial growth is encouraged by the presence of food residues not only on production surface themselves but also within crevices, joints or unions (Bott, 1991; Gibson et al., 1999). Surface that appear clean visually can still be contamined by large numbers of viable microorganisms that could contaminate food (Adams and Moss, 1997). Therefore, after removal of food residues, additional measures may be needed to reduce the number of present microorganisms. Such measure, known as terminal disinfection or microbiological cleaning, are especially important in food handling environments. Within the food industry, disinfection is traditionally achieved by means of heat in the form of hot water or steam or liquid chemicals such as chlorine. Sanitizers such as hypochlorite solutions and quaternary ammonium compounds have been used in food-processing facilities to control contaminant microorganisms, particularly those causing foodborne diseases.

Use of some sanitizers has been limited or banned (e.g., formaldehyde) because of the potential health hazards. The presence of chemical by-product (Richardson et al., 1998) and the increasing of bacterial resistence to conventional biocides (Russell, 1997) have prompted an increasing interest in the use of alternative disinfectants. Ozone has been used for many years in water

disinfection and its bacteridical effects, mainly due to its powerful oxidizing properties, have been well documented (Bott, 1991; Broadwater et al.,1973; Graham, 1997; Greene et al., 1993; Ingram and Haines, 1949; Kowalski et al., 1998, Lezcano et al., 1999; Restiano et al., 1995).
Many advantages could derive from using ozone as a potent oxidizing agent in food industries and other. It is potentially useful in decreasing the microbial load, the level of toxic organic compounds, the chemical oxygen demand, and the biological oxygen demand in the environment. Ozone converts many nonbiodegradable organic materials into biodegradable forms. Its molecule decomposes spontaneously to oxygen; thus, its use minimizes the accumulation of inorganic waste in the environment (Horvath et al., 1985). The high oxidizing power and spontaneous decomposition also make ozone a viable disinfectant for ensuring the microbiological safety and quality of food products.
The aim of this study was to evaluate, under laboratory conditions, the effect of gaseus ozone on different species of *Listeria*, *Salmonella* and *Bacillus*.

3.3 Materials and methods

3.3.1 Treatment chamber

The treatment chamber consist in an aluminium box (60x60x75 cm) hermetically sealed with polyethylene film, which was operated at 20°C and 60-70% relative humidity (RH). A small air-circulating fan was istalled in the treatment chamber. Ozone was produce by an ozone generator (Model Coccinella - C.G.C. - Bergamo - Italy) using atmospheric air as source of oxygen. The ozonated air produced at a costant flow rate by the apparatus was inflated into the chamber via a silicone pipe . Ozone concentration was monitored continuously using an ozone analyzer (O.E.C. - Milano - Italy). Ozone concentration within the treatment chamber was manteined at 1 ppm (1.96 mg/m^3) during the experiments.

3.3.2 Preparation of bacteria for ozone treatment

Bacterial cultures used for the present study were:
- *Listeria monocytogenes* ATCC 19111
- *Listeria innocua* ATCC 33090
- *Listeria grayi* ATCC 25401
- *Salmonella* Enteritidis (food isolate)
- *Salmonella* Typhimurium (food isolate)
- *Salmonella* Derby (food isolate)
- *Bacillus cereus* ATCC 11778
- *Bacillus subtilis* ATCC 21228
- *Bacillus thuringiensis* ATCC 10792

Stock cultures were suspended in 10 ml of Tryptic Soy Broth (TSB, Merck) and incubated at 30-37°C for 24h. The initial suspension of each test strain was inoculated on Petri dishes by the surface spread technique and cultured as shown in Table 1. Immediately after inoculation, the plates were exposed to an ozone treatment (10', 30', 1h and 3h) with their lids removed. Duplicate plates were inoculated at the same time in order to confirm the count of the initial suspension.

Microorganism	Growth medium	Incubation temperature	Incubation time
Listeria spp.	Rapid' L. mono **	37°C	24 h
Salmonella spp.	Xylose Lysine Deoxycholate agar *	37°C	24 h
Bacillus spp.	Tryptic Soy Agar *	30°C	48 h

Table 1: Growth medium and incubation parameters used for the tested microorganisms.
* Merck medium
** BIO-RAD medium

The efficacy of the treatments were expressed as logarithmic difference in colonies count (CFU/ml) between the initial suspension and plates after ozone treatment.

3.3.3 Statistical analysis

Each experiment was repeated five times for each microorganism at each ozone treatment. Data analysis was performed using Microsoft Excel 2003. Statistical significance was determined by use of *t* tests. Significant differences were determined at $P \leq 0.05$ and $P \leq 0.01$.

3.4 Results and Discussion

The numbers of bacteria on the control samples were compared with the numbers of organisms surviving the ozone treatments. Tables 2, 3 and 4 show the mean results for the ozone inactivation of different species of *Listeria*. The mean reduction of *Listeria* spp. resulting from the ozone treatments (1 ppm) were 2.1, 2.7, 3.5 and 5.1 Log CFU/ml after 10, 30, 60 and 180 minutes respectively. All the treatments showed significant difference in Log reduction from non exposed control ($P < 0.01$). Microbial reduction curves of *Listeria* spp. are given in Figure 1. During exposure to ozone, the numbers of colonies decrease in a time-dependent manner.

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	4.9×10^8	3.6×10^7	//
10	3.6×10^6	4.0×10^5	2.1*
30	9.7×10^5	6.0×10^4	2.7*
60	1.2×10^5	5.9×10^4	3.6*
180	4.6×10^3	3.6×10^2	5.0*

Table 2: Mean Log reduction of *L. monocytogenes* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	7.3×10^7	3.6×10^6	//
10	5.0×10^5	4.0×10^4	2.2*
30	1.4×10^5	6.0×10^4	2.7*
60	2.2×10^4	5.9×10^3	3.5*
180	6.2×10^2	3.6×10^1	5.1*

Table 3: Mean Log reduction of *L. innocua* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	2.0×10^8	3.6×10^7	//
10	1.2×10^6	4.0×10^5	2.1*
30	3.3×10^5	6.0×10^4	2.8*
60	6.2×10^4	5.9×10^3	3.5*
180	1.5×10^3	3.6×10^2	5.1*

Table 4: Mean Log reduction of *L. grayi* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control (P <0.01).

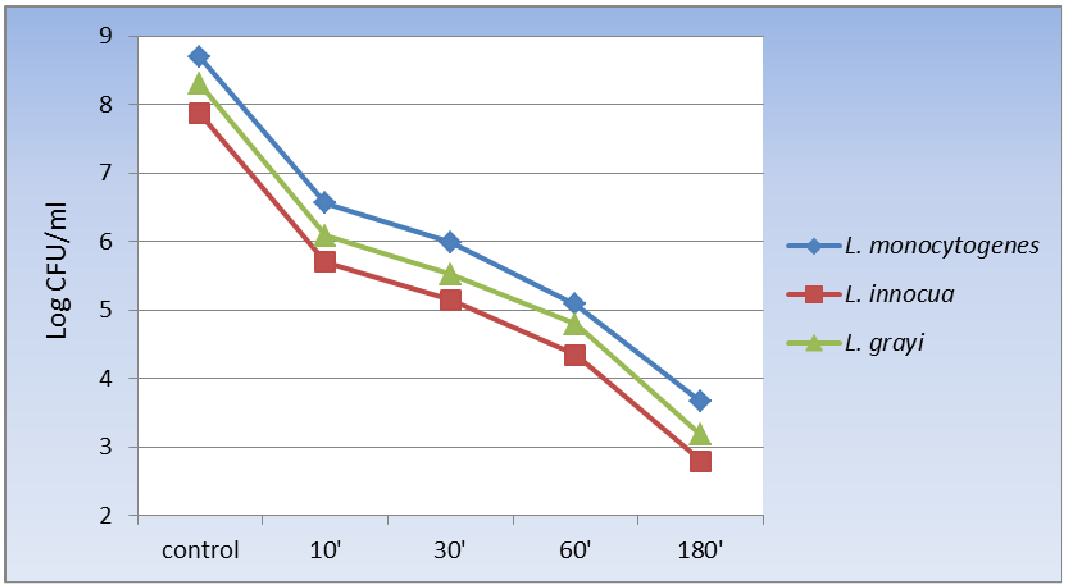

Figure 1: Log reduction of *L. monocytogene, L. innocua, L. grayi* after 10 to 180 min of exposure to 1 ppm of ozone.

Tables 5, 6 and 7 show the mean results for the ozone inactivation of different species of *Salmonella*. The mean reduction of *Salmonella* spp. resulting from the ozone treatments (1 ppm) were 1.1, 1.5, 2.7 and 3.8 Log CFU/ml after 10, 30, 60 and 180 minutes respectively. All the treatments showed significant difference in Log reduction from non exposed control (P <0.01). Microbial reduction curves of *Salmonella* spp. are given in Figure 2.

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	2.6×10^9	3.6×10^8	//
10	2.1×10^8	4.0×10^7	1.2*
30	8.7×10^7	6.0×10^6	1.5*
60	5.8×10^6	5.9×10^5	2.7*
180	3.2×10^5	3.6×10^4	3.8*

Table 5: Mean Log reduction of *S*. Typhimurium after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	1.8×10^9	3.6×10^8	//
10	1.7×10^8	4.0×10^7	1.0*
30	7.6×10^7	6.0×10^6	1.4*
60	4.9×10^6	5.9×10^5	2.6*
180	2.5×10^5	3.6×10^4	3.9*

Table 6: Mean Log reduction of *S*. Derby after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

Ozone treatment (min)	CFU/ml	SD	(Δ Log)
0	1.9×10^9	3.6×10^8	//
10	1.6×10^8	4.0×10^7	1.1*
30	6.4×10^7	6.0×10^6	1.5*
60	4.1×10^6	5.9×10^5	2.7*
180	3.9×10^5	3.6×10^4	3.7*

Table 7: Mean Log reduction of S. Enteritidis after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

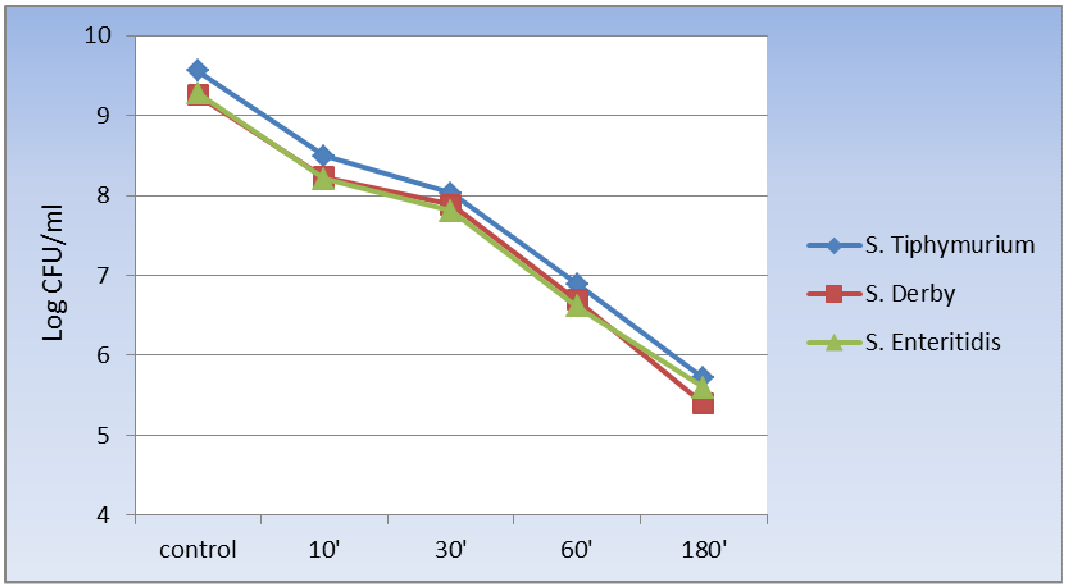

Figure 2: Log reduction of *S*. Tiphymurium, *S*. Derby, *S*. Enteritidis after 10 to 180 min of exposure to 1 ppm of ozone.

Tables 8, 9 and 10 show the mean results for the ozone inactivation of different species of *Bacillus*. The mean reduction of *Bacillus* spp. resulting from the ozone treatments (1 ppm) were 2.5, 3.0, 3.2 and 3.5 Log CFU/ml after 10, 30, 60 and 180 minutes respectively. All the treatments showed significant difference in Log reduction from non exposed control ($P < 0.01$). Microbial reduction curves of *Listeria* spp. are given in Figure 3.

Ozone treatment (min)	CFU/ml	SD	Δ Log
0	4.7×10^8	3.6×10^7	//
10	1.6×10^6	4.0×10^5	2.5*
30	6.5×10^5	6.0×10^4	2.9*
60	4.1×10^5	5.9×10^4	3.1*
180	1.4×10^5	3.6×10^4	3.5*

Table 8: Mean Log reduction of *Bacillus cereus* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control ($P < 0.01$).

Ozone traetment (min)	CFU/ml	SD	Δ Log
0	3.8×10^7	6.6×10^6	//
10	1.5×10^5	6.8×10^4	2.4*
30	3.9×10^4	5.5×10^3	3.0*
60	2.5×10^4	9.7×10^3	3.2*
180	1.7×10^4	3.2×10^3	3.4*

Table 9: Mean Log reduction of *Bacillus subtilis* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control (P <0.01).

Ozone treatment (min)	CFU/ml	SD	Δ Log
0	4.4×10^7	2.5×10^6	//
10	1.1×10^5	5.5×10^4	2.6*
30	2.8×10^4	6.0×10^3	3.2*
60	2.0×10^4	4.2×10^3	3.3*
180	1.4×10^4	4.6×10^3	3.6*

Table 10: Mean Log reduction of *B. thuringiensis* after 10 to 180 min of exposure to 1 ppm of ozone.
* Significant difference in Log reduction from non exposed control (P <0.01).

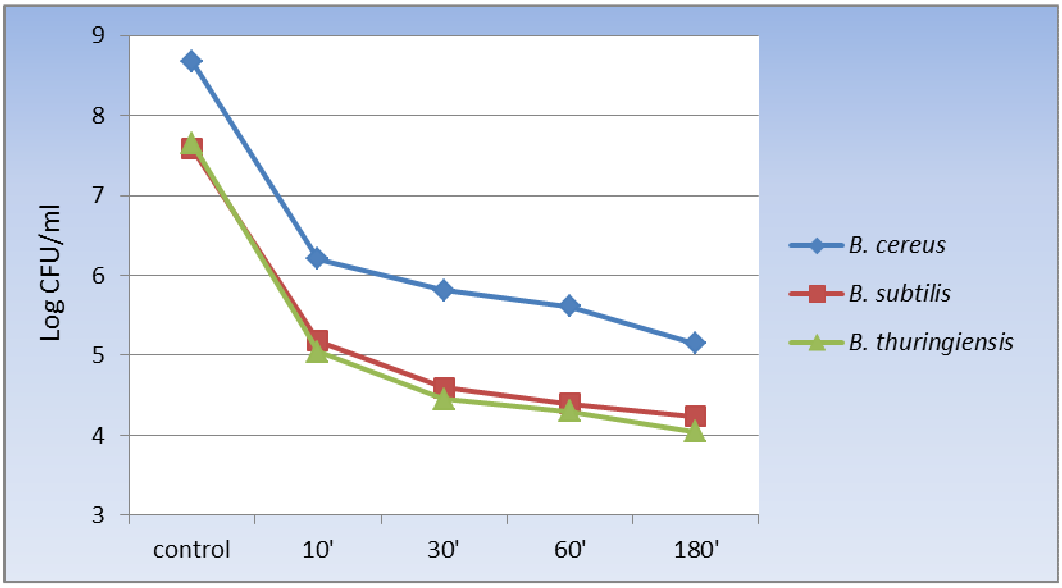

Figure 3: Log reduction of *Bacillus cereus*, *B. subtilis* and *B. thuringiensis* after 10 to 180 min of exposure to 1 ppm of ozone.

Results obtained in the present study indicate that gaseous ozone is highly effective in reducing both gram-positive and gram-negative food-associated bacteria. *Salmonella* spp. seams to be less sensitive to brief ozone treatment (1.1 Log CFU/ml reduction) compared to *Listeria* spp. and *Bacillus* spp. (2.1 and 2.5 Log CFU/ml reduction respectively). Longer treatment (1 ppm for 60 min.) shows more effective reduction (3.5, 2.7 and 3.2 Log CFU/ml) for all tested species (*Listeria* spp., *Salmonella* spp. and *Bacillus* spp. respectively).
The last treatment (1 ppm for 180 min.) shows that the most sensitive microorganism to ozone is *Listeria* spp. (5.1 Log CFU/ml reduction) followed by *Salmonella* spp. (3.8 Log CFU/ml) and *Bacillus* spp. (3.5 Log CFU/ml).

3.5 Conclusions

The present study demonstrates that gaseous ozone can significantly decrease the viability of food-borne pathogens as *Listeria* spp., *Salmonella* spp. and *Bacillu*s spp. In fact, a significant reduction was observed after a 10 minutes treatment with 1 ppm of ozone, and a substantial reduction in microorganisms concentration was observed after 180 minutes. The different species of *Listeria*, *Salmonella* and *Bacillus* used in this trial showed similar sensitivity to ozone treatment. This fact will allow us to choose, when possible, non pathogenic species for future experimentation in meat industry. This result is important for future studies of the application of gaseous ozone in the food industry.
Although pure cultures were used in this investigation, ozone sensitivity data obtained on the species used in this study can provide guidelines for further studies on ozone applications in the food industries. It can be concluded that gaseous ozone treatment is a promising alternative to the use of chemical disinfectant for the sanitation of the industrial food environments.

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CHAPTER 4

Use of gaseous ozone as a disinfectant in meat industry

Published in:
IXVIII Convegno Nazionale AIVI
11-13 giugno 2008, Sabaudia (LT), Italy – 3.1/09 pag. 31-34

4. Use of gaseous ozone as a disinfectant in meat industry

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4.1 Abstract

The aim of this study was to investigate the possible use of gaseous ozone as a disinfectant for meat industry environments. Firstly microbial inactivation trials were conducted in laboratory conditions on Petri plates inoculated with some microorganisms of importance to the food industry. The treatment with 1 ppm of ozone resulted to be effective in 1 h on the strains in use. Then similar trials were conducted in a meat industry, 1 ppm of ozone was supplied for 3 h. The results confirmed the antimicrobial efficacy of ozone even if it was less active than in laboratory conditions. A different sensibility among the microorganisms was observed, the most resistant being *P. fluorescens* and *B. thuringiensis*.
Our results confirm the suitability of gaseous ozone as a disinfectant for meat industry environments and underline the need to calibrate ozone treatment parameters on the real environmental conditions of work-room.

4.2 Introduction

The consumer preference for less processed and preservative-free foods together with the identification of new pathogens in food products pose the need to find other means of ensuring health and microbiological quality of food. The processes of sanitation play a central role in preventing and limiting contamination by pathogens and alterants microorganisms: in fact, North American FDA has entered the environmental sanitation among the *top 10* issues related to food (ERG, 2004). Among the various causes of contamination three different critical points have been identified: lack of efficacy of disinfectants in use, corrosion of materials in contact with food, contamination of food with residues of sanitation. Recently, in addition to disinfection methods already in use, such as the application of thermal or chemical compounds, ozone has been proposed in various production units as an alternative disinfection agent.

Ozone is an unstable gas composed of three oxygen atoms (O_3) which occurs naturally in the atmosphere. It is one of the most powerful disinfectants known and has the highest oxidant power (10 times the chlorine); it is very effective even at low concentrations against a wide range of microorganisms (Khadre and Yousef, 2001) and leaves no residue or toxic by-product. Restaino et al. (1995) tested the antimicrobial action of water treated with ozone against bacteria contaminating food and have actually found that this compound is able to destroy Gram positive and Gram negative bacteria, yeasts and fungal spores. In addition, several studies showed the antibacterial and antifungal effectiveness of gaseous ozone as disinfecting agent in food processing environments (Moore et al., 2000; Robbins et al., 2004).

The employment of ozone can be beneficial also from an economic point of view if one considers that the costs for the purchase and maintenance of the ozone supply units are lower than the cost for the purveyance of disinfectants (Greene et al., 1993). It should also be stressed that the usage of chemicals for disinfection in food industry poses a problem of pollution due to increasing accumulation in the environment, whereas ozone leaves no residue. Its molecule spontaneously decomposes to O_2 thus minimizing risks to human health related to inhalation of large quantities of ozone (Greenberg, 1980; Horvart et al., 1985). Ozone residue in the environment can be eliminated by placing fans in the premises or using special tools which can destroy it (Rice et al., 1982).

Ozone is suggested for several applications in food industry: to wash food products before they are packaged and sent to supermarkets, restaurants and shops; to treat surfaces hygienically; to disinfect both equipment in contact with food and material used for its wrapping and packaging; to recycle waste water (Majchrowicz, 1998; Rice et al., 1982).

Our study is part of a research project that aims to investigate the possible use of ozone in various fields related to the production of food of animal origin. The purpose of this study was to evaluate air ozonization as a disinfecting treatment for surfaces and plants in meat industry. Experimental microbial inactivation was carried out in laboratory conditions to determine the parameters for the use of ozone in industry premises. Such parameters were subsequently applied in a production facility to evaluate their effectiveness under normal processing conditions.

4.3 Materials and methods

4.3.1 Laboratory tests

Laboratory tests for microbial inactivation were conducted setting culture mediums at given concentration of the following microorganisms: *Listeria monocytogenes* (ATCC 19115), *Staphylococcus aureus* (ATCC 6358), *Bacillus thuringiensis* (internal collection), *Escherichia coli* (ATCC 25988), *Pseudomonas fluorescens* (internal collection) and *Saccharomyces cerevisiae* (internal collection). They were then seeded on non selective mediums with a spatula. Petri plates were subsequently subjected to ozonization for 1 hour by placing them in an airtight container 60x60x75 cm in size, connected to an ozone generator (corona discharge). The average concentration of ozone inside the chamber, continuously monitored by a portable detector, was 1 ppm, equal to 1.96 mg/m^3, at a temperature of about 20°C with relative humidity of 60%. For *L. monocytogenes* additional tests were carried out using scalar time of exposure to gas, up to a maximum of 2 hours. At the end of the treatment, the microorganisms were placed in an incubator at optimal growth temperatures/time. After incubation, the colonies were counted. The microbial reduction was expressed as logarithmic difference between the initial titre (plates not subjected to ozonization) and the titre detected after treatment.

4.3.2 Tests in meat industry

The bacteria used for testing in meat industry, were the same as described above, with the exception of *L. monocytogenes*, which was replaced with a strain of *L. innocua* (ATCC 33090). The preparation of microbial suspensions and plates was carried out following the same procedures used in laboratory tests. The inoculated plates kept at 4°C were transported to the industry premises within 3 hours. A 60 m^2 area within the workroom devoted to meat cutting was chosen to perform the experiment. The plates were placed, opened, on processing tables and ozone at a concentration of 1.1 ppm for 3 hours was supplied in the night. After the treatment, the plates were brought to laboratory, and incubated at growth temperatures/time optimal for the organisms under test. The microbial reduction was expressed as logarithmic difference between the original titre and the titre detected at the end of the test. The experiments conducted both in laboratory conditions and in industry premises were made in triplicate.

4.4 Results

The following tables show the results obtained from laboratory (Tables 1 and 2) and industry premises (Table 3) experiments. The data reported show the average value obtained from our tests.

Ozone treatment (min)	CFU/ml	S.D.	Δ Log
0	2.1×10^8	3.4×10^7	//
10	3.2×10^6	4.4×10^5	1.8
20	4.6×10^5	5.4×10^4	2.7
30	3.3×10^5	4.4×10^4	2.8
45	1.6×10^5	2.9×10^4	3.1
60	1.2×10^5	2.5×10^4	3.3
120	5.7×10^4	6.1×10^3	3.6

Table 1: Reduction of the titre of *Listeria monocytogenes* in laboratory condition after treatment with 1 ppm ozone at different application time.

Microorganism	Initial CFU/ml	S.D.	Final CFU/ml	S.D.	Δ Log
L. monocytogenes	6.3×10^8	6.4×10^7	2.2×10^5	3.3×10^4	3.5
S. aureus	4.5×10^9	5.2×10^8	3.9×10^6	4.7×10^5	3.1
B. thuringiensis	1.1×10^8	2.4×10^7	3.7×10^5	4.5×10^4	2.5
E. coli	1.8×10^9	3.0×10^8	2.9×10^3	3.9×10^2	5.8
P. fluorescens	2.4×10^9	3.6×10^8	5.6×10^7	5.9×10^6	1.6
S. cerevisiae	3.8×10^7	4.6×10^6	3.3×10^3	4.2×10^2	4.1

Table 2: Reduction of the titre of microorganisms tested in laboratory conditions after treatment with 1 ppm ozone for 1 hour.

Microorganism	Initial CFU/ml	S.D.	Final CFU/ml	S.D.	Δ Log
L. innocua	9.8×10^7	8.6×10^6	7.0×10^5	1.9×10^5	2.1
S. aureus	7.0×10^9	7.4×10^8	3.0×10^6	1.2×10^6	3.4
B. thuringiensis	2.2×10^8	3.6×10^7	2.8×10^6	4.1×10^5	1.9
E. coli	1.1×10^9	9.2×10^7	8.9×10^5	8.3×10^4	3.1
P. fluorescens	2.2×10^9	1.6×10^8	3.5×10^8	4.5×10^7	0.8
S. cerevisiae	1.1×10^7	9.1×10^5	9.2×10^3	8.3×10^2	3.1

Table 3: Reduction of microorganisms tested in industry premises after treatment with 1.1 ppm ozone for 3h.

4.5 Discussion and conclusions

Efficacy of ozone-based sanitizing treatments on the microorganisms we tested was demonstrated by experiments performed so far. Laboratory experiments on *L. monocytogenes* were carried out to obtain a curve of efficacy under controlled conditions. It was noted that gaseous ozone at 1 ppm proves effective after just 10 minutes and leads to a sharp decrease of the titre after 2 hours (3.6 Log). A good antimicrobial action of the gas on other tested bacteria was also shown by laboratory data obtained after providing 1 ppm ozone for 1 hour. Data indicate a different sensitivity among species, though; *B. thuringiensis* and in particular *P. fluorescens* proved to be the most resistant organisms to the action of ozone. Considering the results obtained in the first phase and the strong influence of environmental conditions (temperature/humidity) on susceptibility of microorganisms to ozone (Kim et al., 1999), treatment performed in the industry premises was prolonged up to 3 hours. Seeding with spatula allowed to simulate bacterial contamination of processing surfaces and proved to be an easy method to perform testing experiments within a production facility. Tests were accomplished in the night as the amount of ozone in the environment, due to safety reasons for workers, must be less than 0.03 ppm, which also influenced maximum time allowed for erogation.
Even when applied in industry premises, treatment resulted to be effective. As expected, reductions were lower than those in laboratory testing, owing to different environmental conditions. Different sensitivity of microbial species, such as a greater resistance from *B. thuringiensis* and *P. fluorescens*, was reconfirmed.

In conclusion, ozone appears to be an effective method of disinfection in meat processing environments, having a good antimicrobial activity against major pathogens and alterants.
Results from the tests in this study suggest the importance of calibrating the parameters of ozone treatment to different environmental conditions in the production units. Moreover, additional variables, such as the presence of biofilm on processing surfaces, must be considered when defining proper ozone dosage. In fact, as already outlined in previous investigations (Robbins et al., 2004), biofilm can increase significantly microbial resistance to ozone, which results in its further dose increase.

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CHAPTER 5

Gaseous ozone as an alternative method of mite control on meat products

5. Gaseous ozone as an alternative method of mite control on meat products

5.1 Abstract

Mite infestations determine a relevant problem associated to the storage of foodstuffs. Mites develop and feed on stored food, which can yield high losses due to food weight reduction and degradation as a consequence of their activity. This study was aimed at the development of an alternative method of integrated mite control in the industrial production of an Italian product, *speck*. The investigation was carried out on the premises of five factories in the north-eastern Italy. *Tyrophagus putrescentiae* and *T. longior* were the predominant species. Gaseous ozone treatment performed at low level (0.4 ppm) was able to kill mites within 1 - 2 months. The characteristic layer of mould on the product surface reappears within 1 month after the reduction of mites presence on the products. As positively observed in all treated plants under our investigation, the use of ozone can represent a valid alternative method in the control of mite infestations on meat products.

5.2 Introduction

Speck is a smoked and cured meat specialty produced in alpine regions, especially in South Tyrol (Italy) and North Tyrol (Austria). Following traditional methods, pork hind quarters are trimmed to ensue in the typical whole portions. Then, pork is brined with curing salts and left in the resulting pickling solution for about 3-4 weeks at 8°C. Meat is periodically cold-smoked with wood smoke at a temperature of about 20°C for 3 to 6 weeks. Frequency and respective duration of smoking during this period could vary strongly. After this, Speck is ripened in well-ventilated ripening chamber at 10-15°C for an average of 5 to 8 month.
The colonization of speck occurs spontaneously by the mycobiota from the production environment or by contamination due to addedspices (Hadlok, 1969). One of the most important problems associated to the storage of foodstuffs are mite infestations. Mite species infesting stored food mainly belong to order Astigmata, family Acaridae that include several species frequently found in a wide variety of food products, like those belonging to genus *Tyrophagus* and *Acarus* (Hughes, 1976). Mites develop and feed on storage food yielding high losses due to weight reduction and degradation of stored food derived from their

activity (Zdarkova, 1991). In addition, mite infestations produce great health and hygienic problems (Zdarkova, 1991).
Meat stored products are attractive for mites because of the layer of mould on their surface and their rich composition in fatty acids. Mites enter curing factories by phoresy on humans or insects or by floating on air currents. Their proliferation is related to an increase in relative humidity. Mites cause problems to factories not only on account of possible direct losses, but also because of their hard and costly removal. In fact, common practise is manual cleaning of the products by removing the mould on the surface. (Ottoboni et al., 1989). Mites are also highly allergenic and may pose serious health risks to workers (Stengard Hansen et al., 1996), as well as being implicated in the transmission of microorganisms and prions (Griffiths et al., 1959; Sigrianskii, 1940; Wisniewski et al., 1996).
The control of arthropods associated to strored products is usually accomplished through the use or organophosphates and pyrethroid insecticides (Collins, 2006; White and Leesch, 1996; Zettler and Arthur, 2000). Insect pest management in different animal products involves conventional methods such as fumigations and residual spray treatment of the products or of the premises. In fumigations the choise is between phosphine and methyl bromide. Methyl bromide, an important and highly effective fumigant, is being phased-out on a global basis due to its ozone deplting potential under the Montreal Protocol (TEAP, 2000). Organophosphates (OP) compounds are widely used; however, increasing concerns over the use of OPs linked to health (Stephens et al., 1996) and environmental fears, as well as the development of resistant mite populations, has fuelled the need to find effective alternative compounds. Pyretroid insecticides are recommended as an alternative to some of the traditional OP due to their quick action, low odor and low toxicity to humans. Nonetheless, stored-product mites have been reported to be fairly tolerant to pyrethroids (Zdarkova and Horak, 1974; Zdarkova, 1994). Potential alternatives to the use of OP for the control of mite pests in stored commodities has benn recently reviewed by Collins (2006). Substances including insect groth regulators, inert dusts, botanicals, pyrethroids and other novel materials are discussed as to their efficacy against important storage mite pests. Any chemical or physical methods to be used should not pose any risk to human health nor modify the organoleptic characteristics of the treated products.
The present study was aimed at developing an alternative method based on the use of gaseous oxone for integrated mite control in the industrial production of a typical Italian speck.
Ozone is a generally recognized as safe (GRAS) disinfectant that can be used to sanitize food storage rooms and packaging materials, that may help prevent insect infestations during storage of foods (Beachat, 1991). Ozone can eliminate

insects in grain storage facilities without affecting food quality or environment. Ozone is an unstable gas and decay naturally into diatomic oxygen, thus leaving no residues (Finch and Fairbairn, 1991; Larson, 1988). It is effective against viruses, bacteria, spores and stored grain insects (Bonjour et al., 2008; Finch and Fairbairn, 1991; Kells et al., 2001; Korich et al.,1990; Restaino et al., 1995).

5.3 Materials and methods

5.3.1 Ozone treatment

Ozone was produced by an ozone generator (C.G.C. - Bergamo - Italy) using atmospheric air as the source of oxygen. The ozonated air produced at a costant flow rate by the apparatus was inflated into the ripening rooms from the ceiling via silicone pipes.
Ozone concentration within the treatment rooms was monitored continuously using an ozone analyzer (O.E.C. – Milano - Italy) and manteined at 0.4 ppm automatically during the experiments.
The ozone treatment began in the third month of ripening when rooms presented a high level of mites infestation. For the first two months the rooms were treated 8 hours per day (overnight). Given a high decrease in the infestation level, the treatment was then reduced to two days every 15 days (8h/day). Once a week the ripening rooms were cleaned and all mites present on the floor removed.

5.3.2 Mite investigation

Mite abundance on speck surface was determined by visual inspection using a hand magnifying glass (10x). The following scale for infestation levels was used:
0: no living mites detected;
1: mite colonies occupy less than 25% of the product surface;
2: mite colonies occupy 25-50% of the product surface;
3: mite colonies occupy 50-75% of the product surface;
4: mite colonies occupy 75-100% of the product surface.
When no living mites were detected, superficial scraping was taken for stereomicroscope confirmation and for species identification. Mite prevalence, that is the percentage of sampled products infested by mites, was assessed after estimation of mite abundance or after observation of individual surface scraping. The species were identified (based on males) following keys by Hughes (1976) and Fain (1992).

5.3.3 Mite sampling at the factories

Mite population dynamics on speck during the curing process was studied for about three months in 5 factories located in the north-eastern Italy. The prevalence and mite abundance on selected products (20 speck) were determined at intervals of 2 weeks initially, and then of 4 weeks.

5.4 Results and discussion

Mites identification (Fig. 1) was performed by surface scraping of infested products at the beginning of the treatment period.
Factory 1 and 4 showed a massive infestation due to *Tyrophagus longior*. Factory 3 presented a high infestation by *Tyrophagus putrescentiae*. Factory 2 and 4 had a simultaneous infestation by *T. putrescentiae* and *T. longior* (75/25% and 84/16% respectively).
The acaricidal activities of gaseous ozone treatment was estimated with multiple observation, after 15, 30, 60 and 90 days, of twenty randomly selected products in each factory.For each factory, the percentage of products that showed vital mites on the surface and the mean infestation level (0-4 scale) were calculated, as summarized in Table 1.
At the beginning of the treatment, all the products from every factory presented more than 75% of their surface covered with mites (Fig. 2). Tested speck were chosen randomlyamong the products present in the ripening rooms.
After 15 days of treatment, the presence of mites decreased to level 2 (less then 50% of surface) in almost all factories. In one case the mean infestation lowered at level 1.
After 1 month of treatment, products presented a mean infestation level of 1 (less then 25% of surface covered with mites). Thanks to positive results obtained, the treatment with gaseous ozone was reduced to 2 day every 15 days (8h/d).
After 2 months of treatment, almost all the factories presented very few infestation or no infestation at all (one case only still showed 35% of products at level 1 of infestation).
At the end of the studies (after 3 months of treatment) only one factory presented very few products with some mites on the surface.
Colonization by the tipical molds appeared on Speck about one month after the reduction of the treatment with gaseous ozone.

Table 1: Prevalence of mites and mean infestation level in five factories treated with gaseous ozone (0.4 ppm).

Observation Time*	**12 weeks**		**14 weeks**		**16 weeks**		**20 weeks**		**24 weeks**	
	%	**L**	**%**	**L**	**%**	**L**	**%**	**L**	**%**	**L**
Factory 1	100	4	100	2	15	0	0	0	0	0
Factory 2	100	4	100	2	95	1	35	0	15	0
Factory 3	100	4	100	2	65	1	10	0	0	0
Factory 4	100	4	100	1	5	0	0	0	0	0
Factory 5	100	4	100	2	55	1	5	0	0	0
L mean		4		2		1		0		0
O_3 Treatment	8h/every day						8h for 2 days every 15 days			

* time from the beginning of curing period
% = percentage of samples infested (mite presence)
L = mean infestation level of samples

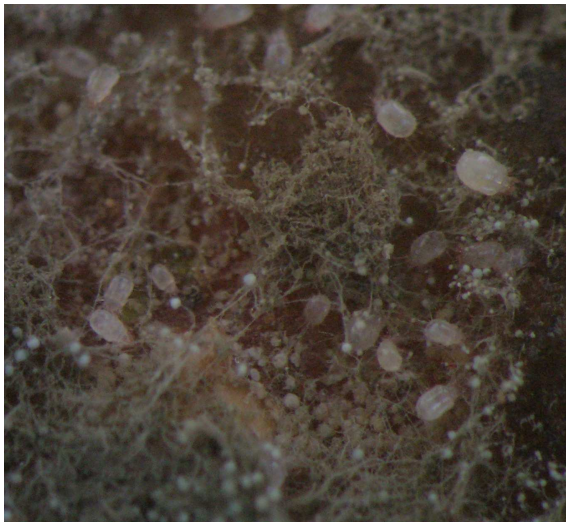

Figure 1: microscope observation of mites present on the surface of speck.

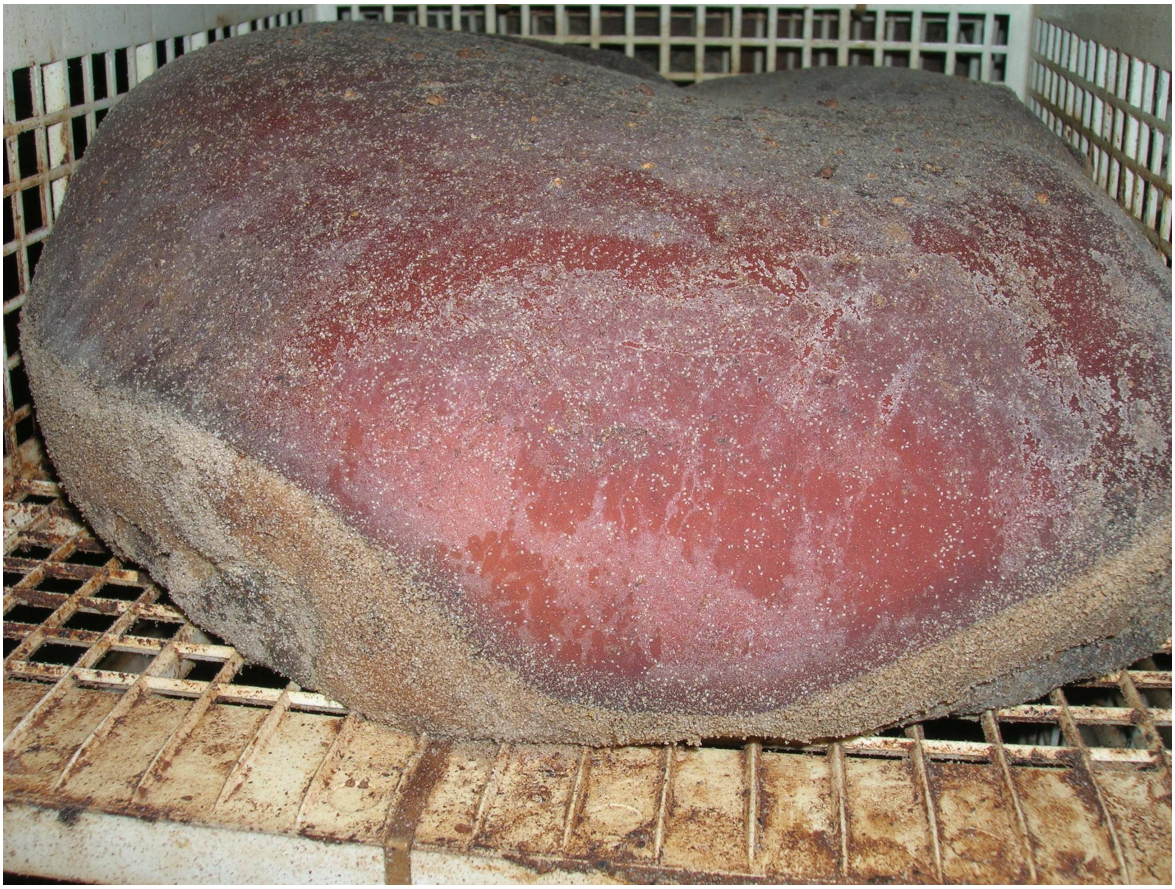

Figure 2: Speck superficial infestation of level 4. All the surface of the products is covered by mites.

5.5 Conclusions

Astigmatid mites, *Tyrophagus putrescentiae* and *T. longior*, are cosmopolitan species commonly infesting a variety of stored food and food grains with a high fat and protein content (Hughes, 1976). Mites normally live on the external surface of these products, although they sometimes penetrate internally, causing serious economic losses (Zdarkova, 1991). The physical conditions (high humidity and temperature) required during the maturing and storage processes of dry-cured products favour the development of mites.
In the present study, gaseous ozone was applied to the curing rooms during night hours so that at the resumption of work, the environmental O_3 concentration was below 0.03 ppm (safety limit for workers).
All the factories showed a remarkable decrease in mite infestation after a period of treatment ranging from 15 days to 1 month and an almost complete elimination of the problem after 1-2 months. It was interesting that the tipical molds colonization of the products reappeared in about a month after decresing the initial ozone treatment so that, at the end of the ripening period, the products were complitely covered by molds. Moreover, thanks to the absence of mites on the surface of treated speck, the long and expensive labour required to remove the mites, usually made by manual brushing of the speck surface, was no longer necessary.
The use of gaseous ozone for the removal of mites by cured products seems to be very interesting as it is produced when necessary, thus eliminating the problem of storage of hazardous substances (insecticides, disinfectants). It also decomposes spontaneously into oxygen, reducing toxic by-products that can cause environmental pollution.
In conclusion, the advantageous effects observed in all treated plants in this investigation make the use of gaseous ozone a likely alternative method efficient in the control of mite infestations.

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CHAPTER 6

Gaseous ozone as an alternative method of mold control on meat products

6. Gaseous ozone as an alternative method for molds control on meat products

6.1 Abstract

Penicillium nalgiovense is the main starter culture used in the production of sausage in Italy; though, spontaneous mycoflora sometimes can sometimes predominates over starter cultures. The use of a starter culture based on *P. nalgiovense* does not limit the growth of heterogeneous molds. The growth on the casings of *P. stoloniferum* and *P. lanoso-griseum* could lead to the appearance of grey-black spot on the surface of fermented sausages that are nonacceptable to most consumers. This cause a damage to the producer that have to wash sausages and to brush away the molds before selling them to markets. Molds strains growing on casings depends on production area and on technological parameters such as A_W, humidity, temperature and relative humidity of the drying and ripening rooms. If good manufacturing practices are not followed properly or control of the relative humidity and temperature in the drying and ripening rooms fails, a significant increase of problems associated with colour and mycelium appearance may often occur.
The aim of this work was to study the use of gaseous ozone at low concentration to reduce or eliminate the development of black spots, caused by heterogeneous molds on the surface of fermented sausages which limits theis marketing.

6.2 Introduction

The use of mould on sausage surfaces can lead to both desirable and undesirable effects. The pursued effects consist in: typical flavour and taste mediated by lactate oxidation, proteolysis, degradation of amino acids, lipolysis, β-oxidation (Grazia et al., 1986; Leistner, 1984; Lücke, 1997), protection against spontaneous colonisation with unwanted moulds, yeasts and bacteria (Lücke and Hechelmann, 1987), delay in rancidity and stabilisation of colour through catalase activity, oxygen consumption and protection against light (Bacus, 1986; Bruna et al., 2001; Lücke and Hechelmann, 1987), characteristic white or greyish appearance of the mycelium and conidia (Lücke, 1997), reduced risk of development of a dry edge and reduced water loss due to slower water evaporation (Lücke, 1997), easy skin peeling (Grazia et al., 1986). The undesirable effects are usually connected to growth of undesirable moulds. It is important to highlight that moulds can produce highly-toxic secondary

metabolites, mycotoxins, that – apart from their acute toxic effects - could often have long-termed toxinogenic, carcinogenic, haemorrhagic or liver degenerative effects (Samson et al., 1995). Moreover, moulds can originate green, brown or black spots that are nonacceptable to most consumers, other moulds may also have negative impact on flavour and taste.

The traditional source of molds on raw dry sausages is the natural house mycoflora. This often consists of heterogeneous molds composed of representative of different genera and species (Berwal and Dinchev, 1991; Dinchev, 1981). Much of these molds are undesiderable and may lead to serious problems for both consumer and producer. Quality and safety requirements in dry meat sausages are achieved by using starter cultures. These molds should be well adapted to the sausage environment. This give them an advantage in the competition with other molds (Andersen, 1995).

Several authors have studied the mycoflora of fermented sausages manufactured in Italy (Cantoni et al., 1982a,b, 2007; Dragoni and Cantoni, 1979; Dragoni et al., 1991; Grazia et al., 1986; Leistner and Eckardt, 1979; Mutti et al., 1988). Mycoflora is primarily heterogeneous, and although there are a variety of mycoflora, the predominant genus is *Penicillium*. In particular, *Penicillium nalgiovense*, and, to a lesser extent, *Penicillium chrysogenum* (Leistner, 1990), appear to be responsible for the commercial covering and the seasoning of sausages. Both strains are used as starter cultures in sausage production (Leistner, 1986; Sunesen and Stahnke, 2003) being spread on casings to improve and to standardize the quality of the product. For this reason, strains used during fermenting process are carefully selected and added during the processing to ensure the safety of the final product (Comi et al., 2004; Lopez-Diaz et al., 2001). However, it is well known that other moulds can grow during sausage production.

Penicillium nalgiovense is the typical species used as a starter culture in the casing of dry fermented sausages. Growth of this mold gives the casing a white, velvety appearance that consumers prefer and allows standardization of the manufacturing process (Fink-Gremmels et al., 1988).

However, spontaneous mycoflora sometimes predominates on starter culture. The prevalence of molds strains growing on casings depends on the type of sausage, on area of production and on technological parameters of production. These parameters include water activity (A_W), humidity, temperature and the relative humidity of drying and ripening rooms (Cantoni et al., 2007; Comi et al., 2004; Dragoni and Cantoni, 1979; Grazia et al., 1986; Lucke, 1997; Mizakovà et al., 2002; Mutti et al., 1988; Sunesen and Stahnke, 2003). If good manufacturing practices are not followed properly or mistakes occur in the control of the relative humidity and temperature of the drying and ripening rooms, a significant increase of problems associated with color and mycelium appearance is often

observed (Dragoni et al., 1986; Dragoni et al., 1988; Dragoni et al., 2000; Vallone et al., 1995).
Few studies have been focused on the use of ozone in filamentous fungi inactivation. Most of the applications of ozone deal with the direct application of gaseous ozone and evaluate the sporulation, germination, or growth of the fungi. Hibben and Stotzky (1969), after exposing fungal spores to 0.0002–0.002 g/m^3 of gaseous ozone for 6 hours, found that the sensitivity of fungal species varies within species. They also observed the suppression of aerial hyphae growth and the occasional stimulation of sporulation of colonies maintained in sublethal doses of O_3. Palou et al. (2001) studied the development of molds in decaying citrus fruits. They observed, in *in vitro* tests, that gaseous ozone, at a level of 0.00006 g/m^3, neither killed all the spores nor adversely affected their germination ability. However, the sensitivity of species to ozone was different. While *Penicillium italicum* radial growth was inhibit by ozone, *Penicillium digitatum* was resistant. At this ozone dosage, sporulation was not affected. Vijayanandraj et al. (2006), studying *in vitro* the effect on *Aspergillus niger* causing black-rot disease in onions, found that an exposure to ozone, at a level of 4.8 g/m^3 of ozone for 5 minutes, induced the spore germination and few spores showed rapid swelling compared to control. However, although inducing germination, the colony morphology was not uniform and the formation of sterile mycelia was induced. When using ozone with mycelia, these researchers have also found that aerial mycelia growth was more sensitive to ozone than spores.
The aim of this work was to study the use of of gaseous ozone at low concentration to reduce or to stop the development of grey-black spots, caused by heterogeneous molds, on the surface of fermented sausages that are nonacceptable to most consumers.

6.3 Materials and methods

6.3.1 Preparation of the sausages

The experiments for the present study were conducted in an industrial plan producing fermented sausages named Milano type. These sausages were prepared using: pork meat (70%), pork fat (30%), NaCl (2.5%), KNO_3 and $NaNO_2$ (200 ppm), black pepper. The ingredients were processed in a mincer equipped whit an adjustable plate set at a hole diameter of 3 mm. The mixtures was stuffed into synthetic sausage casings (60 mm in diameter). *Penicillium nalgiovense* was used as starter culture. The sausages were fermented at 23°C and 90% relative humidity (RH) for 12h. Then, the temperature and RH were slowly reduced to 20°C and 60% respectively in 18h. Finally, the sausages were dried at

11-12°C and 85% RH to the end of the ripening process (4 months). For our analysis, sausages were collected before last step in the production process, which entails washing and brushing the casings to remove the moulds.

6.3.2. Mould identification

Three sausages from every production batch were randomly collected for mycological analysis. Mycological samples were taken from by swabbing the surface of each sausages with a cotton swab soaked in sterile, physiological saline solution (0.7% NaCl, 0.05% Tween 80). The cotton swabs were then streaked onto Malt Extract Agar plates (Oxoid) and incubated at 25°C for 3–5 days. The colonies of the moulds grown on Malt Agar (Oxoid) were inoculated onto three different agars: Czapek Dox Agar (Biolife); Malt Agar (Oxoid); and salt–malt agar pH 6.2, containing 5% malt extract (Oxoid), 5% NaCl, and 2% agar (Oxoid). The moulds were identified basing on morphology and growth characteristics according to Ainsworth et al. (1973), Pitt and Hocking (1997), Samson and Pitt (2000), and Samson et al. (2004).

6.3.3 Ozone treatment

Ozone was produced by an ozone generator (C.G.C. - Bergamo - Italy) using atmospheric air as the source of oxygen. The ozonated air produced at a costant flow rate by the apparatus was inflated into the ripening rooms from the ceiling via silicone pipes.
Ozone concentration within the treatment rooms was monitored continuously using an ozone analyzer (O.E.C. – Milano - Italy) and automatically manteined at 0.5 ppm during the experiments. The ozone treatment was conducted 8 hours per day overnight for all the ripening period.

6.4 Results and Discussion

A total of 97 strains were isolated from the casings of the sausages (3) taken from each produced batch. One batch was ripened without ozone treatment and three batchs were treated during the ripening period.
Sausages produced in the tested plant without ozone treatment presented diffuse growth of grey-black molds (Fig. 1). The species detected on casings of these sausages were *Penicillium nalgiovense* (52%), *P. stoloniferum* (13%) and *P. lanoso-griseum* (35%).

Figure 1: Grey-black molds on the casing of sausages ripened without ozone treatment.

Sausages ripened with gaseous ozone treatment presented a uniform growth of molds with a white, velvety appearance (Fig. 2). The species detected on casings of these sausages resulted to be 100% *Penicillium nalgiovense.*

Figure 2: Casing appearance of sausages ripened with ozone treatment.

6.5 Conclusions

Penicillium nalgiovense is the main starter culture used in the production of sausages manufactured in Italy, though, spontaneous mycoflora can sometimes predominates over starter cultures. As observed in the tested plant, the use of a starter culture based on *P. nalgiovense* does not limit the growth of heterogeneous molds. The growth on the casings of *P. stoloniferum* and *P. lanoso-griseum* lead to the appearance of grey-black spot on the surface of fermented sausages that are nonacceptable to most consumers. This cause a damage to the producer that have to wash sausages and to brush away the molds before their marketing.
As considered by many authors, molds strains growing on casings depends on production area and on technological parameters of production such as A_W, humidity, temperature and the relative humidity of drying and ripening rooms (Cantoni et al., 2007; Comi et al., 2004; Dragoni and Cantoni, 1979; Grazia et al., 1986; Lucke, 1997; Mizakovà et al., 2002; Mutti et al., 1988; Sunesen and Stahnke, 2003).
According to the present study, treatment with gaseous ozone at low concentration during ripening period inhibited the growth of anomalous mold

strains, and allowed the growth of the starter culture used, *P. nalgiovense*, resulting in a uniform growth of a white, velvety mold covering on the sausage surfaces. In conclusion, the achievement of our investigation ca be considered as a good starting point for future studies on the application of gaseous ozone in food industry as an alternative method of mold control on meat products.

6.6 References

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CHAPTER 7

Preliminary investigation on the possible surface oxidation of meat products treated with gaseous ozone

7. Preliminary investigation on the possible surface oxidation of meat products treated with gaseous ozone

7.1 Abstract

Several volatile and nonvolatile substances are formed during lipid oxidation. These can change the taste and odour of foods. In many products these modifications are not desirable because they produce a rancid flavor.
Ozone is a strong oxidizing agent with a redox potential of +2.07 V. Its use is suggested for several applications in food industry also directly on food. Thus, the aim of this study was to determine the effects induced by a low ozone concentration treatment on the lipid oxidation of Milano sausages in order to evaluate the applicability of gaseous ozone on cured meat products.

7.2 Introduction

Several volatile and nonvolatile substances are formed during lipid oxidation. These can change the taste and odour of foods. In many products these modifications are not desirable because they produce a rancid flavor.
Nevertheless, a certain degree of oxidation can give an important contribution to the peculiar taste and odour of some products such as of dry-fermented sausages and dry ham. The substrates of these reactions are mainly unsaturated fatty acids. Generally, these are oxidised more rapidly in their free form than when they are part of triglycerides or phospholipids.
Lipid peroxides, also called hydroperoxides, formed during the propagation phase are the primary products of oxidation. These compounds have no odour or taste and therefore do not participate in the flavour or odour of food products. However, being unstable they are rapidly broken down into by-products responsible for the organoleptic changes induced by phenomena of autoxidation (De Man, 1992; Keeney, 1962). Each unsaturated fatty acid produces specific hydroperoxides that decompose to also form specific aldehydes. The final products of lipid oxidation (aldehydes, alcohols, ketones, furanes, etc.) are highly volatile and have a low olfactory threshold and therefore acquire an important role in the development of the flavour and odour of food products in which they are present. Their spectra and their amounts in food can determine whether they generate acceptable or unacceptable flavour and, as a consequence, the degree of acceptance of the product by the consumer.

Intensity of the autoxidative reactions that take place in the meat depends on several factors, particularly on the level of polyunsaturated fatty acids present in a muscular system (Allen and Foegeding, 1981). Phospholipid content in the meat is relatively small compared with the triglyceride fraction, although phospholipids are especially important because of their susceptibility to oxidation. This is due to their high unsaturated fatty acid content (especially linoleic and arichidonic acids) and to their increased contact with tissular oxidation catalysts such as trace metals or metaloporphyrins (Hornstein and Crowe, 1963; Love and Pearson, 1971). Phospholipids are the main constituent of cellular and intracellular membranes, and therefore present a large area that is susceptible to attack. However, as triglycerides are located inside adipocytes, only a very reduced area is exposed to oxidation.

In fact, lipid oxidation in dry-fermented sausages is influenced by a wide range of factors: composition of their mixture, degree of meat mincing, pH, addition of ingredients such as NaCl, nitrites, spices, antioxidants, etc. When meat cells are damaged as a consequence of chopping and mincing, the phospholipids and triglycerides are exposed to oxygen, enzymes, heme pigments, and metallic ions, which facilitates their rapid oxidation (Asghar et al., 1988; Pearson et al., 1977). The rate of oxidation increases with pH values lower than 7.0 (Tichivangana et al., 1985; Yasosky et al., 1984). In fresh meat, lipid oxidation is inhibited by enzymic reductor systems in the mitochondria (Kwoh, 1971). However, because of the low pH present in the dry-fermented sausage of approximately 4.8 to 5.0, these enzymic systems show little activity (Rhee et al., 1984a; Rhee et al., 1984b). The increased NaCl concentration during ripening as a consequence of dehydration also contributes to lipid oxidation. The prooxidant effect of NaCl is partially attributed to its ability to displace the iron ions from the macromolecules so that these can participate in the oxidation reactions. This hypothesis is supported by the finding that this action is inhibited by EDTA (Kanner et al., 1992). The addition of nitrite also influences oxidation reactions, due to its antioxidant effects. Other ingredients with antioxidant properties include certain spices, tocopherols and ascorbates.

Ozone is a strong oxidizing agent with a redox potential of +2.07 V (Criegee, 1975). Ozone reacts readily with olefins such as polyunsaturated fatty acids (PUFA). The reaction of ozone with olefins is complex and controversial; however, it probably occurs as proposed in Figure 1 (Menzel, 1976). Apparently the initial adduct [1] decomposes rapidly to the Criegee zwitterion [2] which combines readily with the resultant aldehyde [3] to form the final ozonide [4]. If excess external aldehyde is added to the system, it will tend to react predominantly in place of [3]. Ozonides decompose to aldehydes and acids (Gunstone, 1975) and may contribute to off-flavors in foods. The Criegee zwitterion can react with active hydrogen compounds such as alcohols or acids

to yield the corresponding hydroperoxides (Criegee, 1962). These peroxides might decompose by hemolytic scission to initiate free radical peroxidation of lipids. Menzel (Menzel, 1976) suggests that the reaction of O_3 with olefins is a mixture of an initial ionic reaction of O_3 with the olefin to yield the Criegee zwitterion, and perhaps ozonide, and a second reaction involving the peroxidation of remaining olefin. The ozonides and peroxides may arise in two competing reaction pathways.

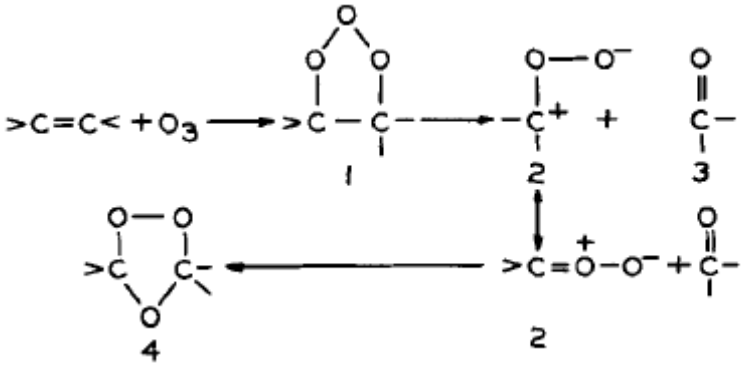

Figure 1: Reaction of ozone with olefinic compounds.

Ozone is suggested for several applications in food industry: to wash food products before they are packaged and sent to supermarkets, restaurants and shops; to treat surfaces hygienically; to disinfect both equipment in contact with food and material used for its wrapping and packaging; to recycle waste water (Majchrowicz, 1998; Rice et al., 1982). In addition, several studies showed the antibacterial and antifungal effectiveness of gaseous ozone as disinfecting agent in food processing environments (Moore et al., 2000; Robbins et al., 2004).
The aim of this study was to determine the effects induced by low ozone concentration treatment on the lipid oxidation of Milano sausages in order to evaluate the applicability of gaseous ozone on cured meat products.

7.3 Materials and methods

7.3.1 Preparation of the sausages

The experiments for the present study were conducted in an industrial plan that producing fermented sausages named Milano type. The sausages were prepared using: pork meat (70%), pork fat (30%), NaCl (2.5%), KNO_3 and $NaNO_2$ (200 ppm), black pepper. The ingredient were processed in a mincer equipped whit an adjustable plate set at a hole diameter of 3 mm. The mixtures was stuffed into

synthetic sausage casings (60 mm in diameter). The sausages were fermented at 23°C and 90% relative humidity (RH) for 12h. Then, the temperature and RH were slowly reduced to 20°C and 60% respectively in 18h. Finally, the sausages were dried at 11-12°C and 85% RH until the end of the ripening process (4 months).

7.3.2 Ozone treatment

Ozone was produced by an ozone generator (C.G.C. - Bergamo - Italy) using atmospheric air as the source of oxygen. The ozonated air produced at a costant flow rate by the apparatus was introduced in the ripening from the ceiling rooms via silicone pipes.
Ozone concentration within the treatment rooms was monitored continuously using an ozone analyzer (O.E.C.) and automatically manteined at 0.5 ppm during the experiments. The ozone treatment were conducted for all the ripening period 8 hours per day (overnight).

7.3.3 Peroxide determination

10-20 g of sample were extracted with chloroform for 2-3h at room temperature, then 2-3 g of anhydrous sodium sulphate were added and filtered through paper in a 200 mL volumetric flask. 25 mL of this solution were transferred into a stoppered conical flask and then 37 mL of glacial acetic acid and 1 mL of freshly made satured potassium iodide solution was added. The solution was allowed to stand exactly 1 min, then 30 mL of water were added and titrate with 0.01N sodium thiosulphate using starch as indicator.
For determin the fat content, 50 mL of chloroformic solution were transferred in a weighted flask and the solvent was removed by rotavapor. The flask was cooled and weighted. Fat weight was used to calculate the peroxide value.

$$\text{Peroxide value} = \frac{\text{mL titration x N x 1000}}{\text{grams of fat}} = meq \text{ / kg}$$

where N= normality of sodium thiosulfate.

7.3.4 Statistical analysis

Data analysis was performed using Microsoft Excel 2003. Statistical significance was determined by use of *t* tests. Significant differences were determined at $P \leq 0.05$ and $P \leq 0.01$.

7.4 Results and Discussion

In this trial two different batches of sausages were treated with gaseous ozone. The analysis was performed on ten samples per batch.
Table 1 and 2 show the results obtained.

	Peroxide values	
Sample	**Control**	**Ozone treated**
1	3.4	7.3
2	3.9	5.3
3	4.7	3.9
4	3.8	4.8
5	3.1	5.2
6	4.2	3.3
7	5.5	5.0
8	5.9	5.8
9	2.7	6.9
10	3.2	4.2
M ± SD	4.0 ± 1.1	5.2 ± 1.3

Table 1: Peroxide values of sausages cured with and without (control) ozone treatment
M = mean value; SD = standard deviation.

	Peroxide values	
Sample	**Control**	**Ozone treated**
1	3.0	4.8
2	3.6	5.2
3	4.9	5.3
4	5.3	7.3
5	2.9	5.0
6	5.1	3.9
7	4.9	4.2
8	2.8	5.8
9	3.8	6.9
10	5.5	4.1
M ± SD	4.2 ± 1.1	5.3 ± 1.1

Table 2: Peroxide values of sausages cured with and without (control) ozone treatment
M = mean value; SD = standard deviation.

In both tests the mean peroxide value was slightly higher in treated samples compared to untreated samples (4.0 vs. 5.2, 4.2 vs. 5.3 respectively); however,

the difference was not significative in both experiments ($P > 0.05$). The difference may be due to variability of samples and not derectly related to the performed treatment.

7.5 Conclusions

Since ozone is a strong oxidizing agent with a redox potential of +2.07 V (Criegee, 1975), it reacts with olefins such as polyunsaturated fatty acids (PUFA). Ozone treatment is suggested for several applications in food industry also directly on food. For this reason it is important to study the possible effects of ozone on food components. This preliminary study show no effect of ozone on the peroxide value of treated sausage samples. Noticeably, in the presence of inorganic and/or organic compounds ozone reacts immediately to generate a wide variety of oxidized molecules, just disappearing in few seconds (Heng et al., 2007). Probably the presence of moulds on the casings and the casings itself limit the effect of ozone on lipid compounds. It would be interesting to proceed in future study with a panel-test to consider if the slight difference in mean peroxide values noticed in the present study is perceptible even at sensory level.

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CHAPTER 8

General Discussion

8. General discussion

It is beyond doubt that food processing plants must assure customers about the safety of their products. To this end, a number of disinfection methods and agents are used which are aimed at inhibiting the growth of pathogenic microflora, restricting food decay and prolonging its storage time. It is also important that they should not leave any harmful residues, but this condition is not always met.

Sanitizers such as hypochlorite solutions and quaternary ammonium compounds have been used in food-processing facilities to control contaminant microorganisms, particularly those causing foodborne diseases but the use of some sanitizers has been limited or banned (e.g., formaldehyde) because of the potential health hazards. Accumulation of chemicals in the environment has increased the international focus on the safe use of sanitizers, bleaching agents, and other chemicals in industrial processing, food, feed and other areas.

On the other hand, the need for potent antimicrobial agents has increased in recent years, due to increasing disease outbreaks and emergence of new foodborne pathogens. The food industry is in search of disinfectants that are effective against common and emerging pathogens and safe to use in many specific applications of food processing.

It is therefore important that alternative and effective disinfectants should be sought (Güzel-Seydim et al., 2004; Khadre et al., 2001). Such expectations can be met by ozone that has been utilized as a sanitizer in many European water treatment plants since the beginning of this century (Gomella, 1972).

Ozone is a strong oxidizer that acts, preferentially, against unsaturated compounds by what may be classified as an electrophilic attack mechanism. This is the primary cause for its antimicrobial activity, since molecules with exposed double bonds are found to be more susceptible to degradation.

Ozone is one of the more effective disinfectants; it does not leave any harmful residues in food or on the surfaces which are in contact with it. In addition, compared to chlorine and other disinfectants, it is more effective against resistant viruses and spores. Exposing some products (*e.g.* fruit or vegetables) to ozone during their storage period extends their shelf life without affecting their sensory value. The use of ozone does not require high temperature, hence it offers the possibility of energy saving. Ozone must be produced on the spot, which leads to savings on transport and storage costs of disinfectants. The cost of an ozone generator may raise concerns in a small businesses; however, such fears are unfounded because the purchase of such a generator may prove economical in the long run (Moore et al., 2000; Steigert and Franke, 2000).

An often-cited disadvantage of using ozone as a disinfectant is that, unlike chlorine, it is extremely unstable (Meddows-Taylor, 1947). It is difficult to

predict how ozone reacts in the presence of organic matter. It can oxidize or ionize the compound, or spontaneously decompose to oxygen and free radicals. Therefore, it may be difficult to generalize that a particular concentration of ozone at a given rate will always be effective in inhibiting a definite concentration of microorganisms in a food product. Furthermore, surface oxidation of food may result from excessive use of ozone (Rice et al., 1982). Some authors stressed that ozone is not universally beneficial and, in some cases, may promote oxidative spoilage. Fournaud and Lauret (1972) detected discoloration and undesirable odours in ozone-treated meat. However, other researchers reported that ozone treatment improved the sensory quality in beef and eggs (Bailey et al., 1996, Dondo et al., 1992) and it did not alter the sensory quality of some fruits and vegetables significantly (Baranovskaya et al., 1979; Kute et al., 1995; Lewis et al.,1996). Therefore, alterations in the sensory attributes depend on the chemical composition of food, ozone dose, and treatment condition.

In spite of ozone's pleasant odour at low concentrations, 0.1 mg/liter is objectionable to all normal humans because of irritation to the nose, throat, and eyes (Witheridge and Yaglou, 1939). In practical application of ozone in the food industry, safety-of-use is an important issue. Ozone detection and destruction systems and are needed for the safety of workers in food-processing facilities. Good manufacturing practice and hazard analysis and critical control point systems are also needed to control high ozonedemand materials in food processing. In the United States, ozone in the work environment is limited to a maximum of 0.1 ppm (vol/vol) on an 8-h/day basis, of a 40-h work week (CFR, 1997).

On the other hand, there are many advantages using ozone, as a potent oxidizing agent, in food and other industries. It is potentially useful in decreasing the microbial load, the level of toxic organic compounds, the chemical oxygen demand and the biological oxygen demand in the environment. Ozone converts many nonbiodegradable organic materials into biodegradable forms. The molecule decomposes spontaneously to oxygen; thus, using ozone minimizes the accumulation of inorganic waste in the environment (Horvath et al., 1985). The high oxidizing power and spontaneous decomposition also make ozone a viable disinfectant for ensuring the microbiological safety and quality of food products.

Ozone in food and feed offers negligible loss of nutrients or sensory qualities in food, as it does not substantially raise the temperature of the food during processing (Cullen et al., 2009). On the other hand, ozone exposure may inhibit growth, germination and sporulation, but its effects are very dependent on species, growth stage, ozone concentration and exposure time.

Due to the strong desire to reduce the use of chemicals applied in the food and feed chains, and considering the nonresidual ozone feature as an important

advantage, the application of ozone technology in food has been considered safe and effective by the World Health Organization (WHO), the Food and Agriculture Organization (FAO), and the U.S. Food and Drug administration (WHO, 2007; FDA, 2008).
The FDA approval of ozone as a direct additive to food has triggered the use of ozone in food-processing applications. Ozone application has given promising results for important problems of the food industry.
Improvements and innovations in ozone generation and application systems will facilitate enhanced control of both quality and safety parameters of ozonized foods. For effective and safe use in food processing, optimum ozone concentration, contact time and other treatment conditions should be defined for all products. A pilot test must be conducted for each case, before starting commercial application, since every ozone application is unique.
Increasing the effectiveness of ozonation processes while optimizing ozone use is an urgent issue for successful applications in food processing. With acquired knowledge and experience, operating specifications and protocols can be developed to use ozone at the most efficient and safe level.

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CHAPTER 9

Summary

9. Summary

Ozone (O_3) is a strong antimicrobial agent with several potential applications in the food industry. High reactivity, penetrability and spontaneous decomposition to a nontoxic product (O_2) make ozone a viable disinfectant for ensuring the microbiological safety of food products. Ozone has been used for decades in many countries and recently, the generally recognized as safe (GRAS) status of this gas has been reaffirmed in the United States. Ozone, in the gaseous or aqueous phases, is effective against the majority of microorganisms tested by numerous research groups. Relatively low concentrations of ozone and short contact time are sufficient to inactivate bacteria, molds, yeasts, parasites and viruses. Ozone applications in the food industry are mostly related to decontamination of environments and water treatment. Moreover, ozone has been used with success to inactivate contaminant microflora on meat, poultry, eggs, fish, fruits, vegetables and dry foods. The gas also is useful in detoxification and elimination of mycotoxins and pesticide residues from some agricultural products. Excessive use of ozone, however, may cause oxidation of some ingredients on food surface.

The aim of this work was to investigate the real potentiality of gaseous ozone in food industries. Ozone was tested:

- as a disinfectant in meat industry;
- as an alternative method of mite control on meat products;
- to reduce extraneous molds on the surface of meat products.

It was also conducted a preliminary investigation on the possible surface oxidation of stored products treated with low concentration of ozone.

The present study shows that gaseous ozone can effectively reduce spoilage microorganisms (*Ps. fluorescens*), fecal contaminants (*E. coli*) and food-borne pathogens (*L. monocytogenes, B. cereus, S. aureus*). It can be used for the disinfection of processing equipment and environment in food industries. Additionally, gaseous ozone might be effective in controlling mites or extraneous molds on meat stored products. Preliminary study show that low ozone treatments of meat stored products did not effect surface oxidation. The studies have shown that ozone can be used with good results both for the sanitation of the environment both for prevention and decontamination of stored product surface. Many people are critical of the use of ozone as it is an irritating substance; it can be felt at low concentrations and can even be poisonous at higher concentrations. Despite such reservations, it must be said that when used under controlled conditions, it is an effective and totally safe disinfectant.

CHAPTER 10

Acknowledgements

10. Acknowledgements

Foremost, I would like to gratefully acknowledge Professor Carla Bersani for the opportunity to have this experience.

I would like to thank the enthusiastic supervision of Professor Carlo Cantoni during this work.

I especially want to thank Professor Maria Antonietta Paleari for her support and guidance. She has been actively interested in my work and has always been available to advise me.

Thanks to the irreplaceable Giuseppe Beretta, for having tolerated / supported me and my doctoral work, far beyond its responsibility. I am very grateful for his help, patience, motivation, enthusiasm, and immense knowledge.

I am grateful to all my friends for their continued moral support during these years.

Finally, I am greatly indebted to my parents and Luca for their understanding, endless patience and encouragement when it was most required.

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January 2011