Atmospheric Pressure Cold Plasma and Ozone Technologies: Unconventional Treatments in the Dairy Field

Study of effects on milk proteins as model systems and relevant case studies

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To my mom, dad and sister;

to Giorgio, my rock

for always being there for me,

from near and from afar

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LIST OF ABBREVIATIONS

ACP: atmospheric pressure cold plasma
ALP: alkaline phosphatase
ANS: 8-anilino-1-naphthalene sulfonic acid
AOPs: advanced oxidation processes
CD: circular dichroism
CO: carbon monoxide
DBD: dielectric barrier discharge
DLS: dynamic light scattering
DNPH: 2,4-dinitrophenylhydrazine
DTNB: 5’5-dithiobis(2-nitrobenzoic acid)
FC/ FS: foam capacity/ foam stability
FDA: Food and Drug Administration
FTIR: fourier transform infrared
H_2O_2: hydrogen peroxide
HM: high moisture
$NO^{\bullet}$: nitric oxide
O_2/ O_3: oxygen/ ozone
$^{\bullet}OH$/ $HO_2^{\bullet}$/ $^{\bullet}O_2^-$: hydroxyl radical/ hydroperoxyl radical/superoxide anion radical
OES: optical emission spectroscopy
PdI: polydispersity index
PSI: protein solubility index
RA: residual activity
RNS: reactive nitrogen species
ROS: reactive oxygen species
SH: sulfhydryl
TBARS: thiobarbituric acid and reactive substances
TCA: trichloroacetic acid
TMC: total microbial count
WPC: whey protein concentrate
WPI: whey protein isolate
α-La/ β-Lg/ BSA: α-lactoalbumin/ β-lactoglobulin/ bovine serum albumin

Preface

Scientific research in Food Science is increasingly oriented towards the study of unconventional approaches and emerging technologies for the application in the food industry in order to find an alternative to conventional methods and strategies. Research is motivated by consumers' needs, and more and more oriented towards seeking safe, high quality nutritional and sensory properties, and minimally processed foods in addition to an ever-increasing focus on sustainability and respect of the environment.
Emerging or novel process technologies, to the contrary of conventional or well-established processes, includes new alternative preservation possibilities and other potential applications offered by the use of UV-light, pulsed light, pulsed electric fields, high pressure processing, ultrasound, ozone, cold plasma, dense phase and supercritical carbon dioxide.
Focusing on the dairy sector as one of the first Italian and global food divisions, the use of heat remains the primary technology throughout different dairy making processes, including milk, milk derivatives as well as cheese production. However, also in this field the studies and uses of the technologies mentioned above are becoming established. Among these, the non-thermal ones are the most interesting nowadays because they allow to reduce the microbial load, increase performance, functionality, and/or shelf-life of products without compromising the quality and creating defects due to thermal treatments.
In light of these considerations and taking into account the ongoing research trends, this PhD thesis aimed to study in-depth whether atmospheric pressure cold plasma and ozone technologies could be applied in the dairy field for different purposes.
Atmospheric pressure cold plasma (ACP) technology for food applications is a relatively new and rapidly growing field of research. Besides decontamination, ACP also provides opportunities for several other applications to food material processing. This includes the modification of food properties, improving their functionality and increasing product healthy value. To the best of our knowledge, only a few studies are focused on dairy matrices and the information available remains insufficient. To this regard, the interactions of plasma with whey proteins and milk enzyme model systems were studied to create the basis for a better understanding of what could happen if this technology was applied in a more complex dairy food product.
Ozone is a promising food processing agent, which is gaining interest due to its oxidant properties. These properties can generate overall effects on macromolecules. It has been exploited to tailored proteins for use in different formulated foods with the purpose of selectively modifying whey protein structure and functionality. However, the main effectiveness is related to the microbial inactivation, and the lack of studies on dairy products prompted us to explore its potentiality during different phases of the Mozzarella cheese making process in order to reduce surface microbial load and in brines of different types of cheeses in order to regenerate them by microbial contamination.

To sum up, this PhD thesis has been divided into six different activities, three of which implicate the ACP technology, and the other three use the ozone technology, as reported in Fig. A.

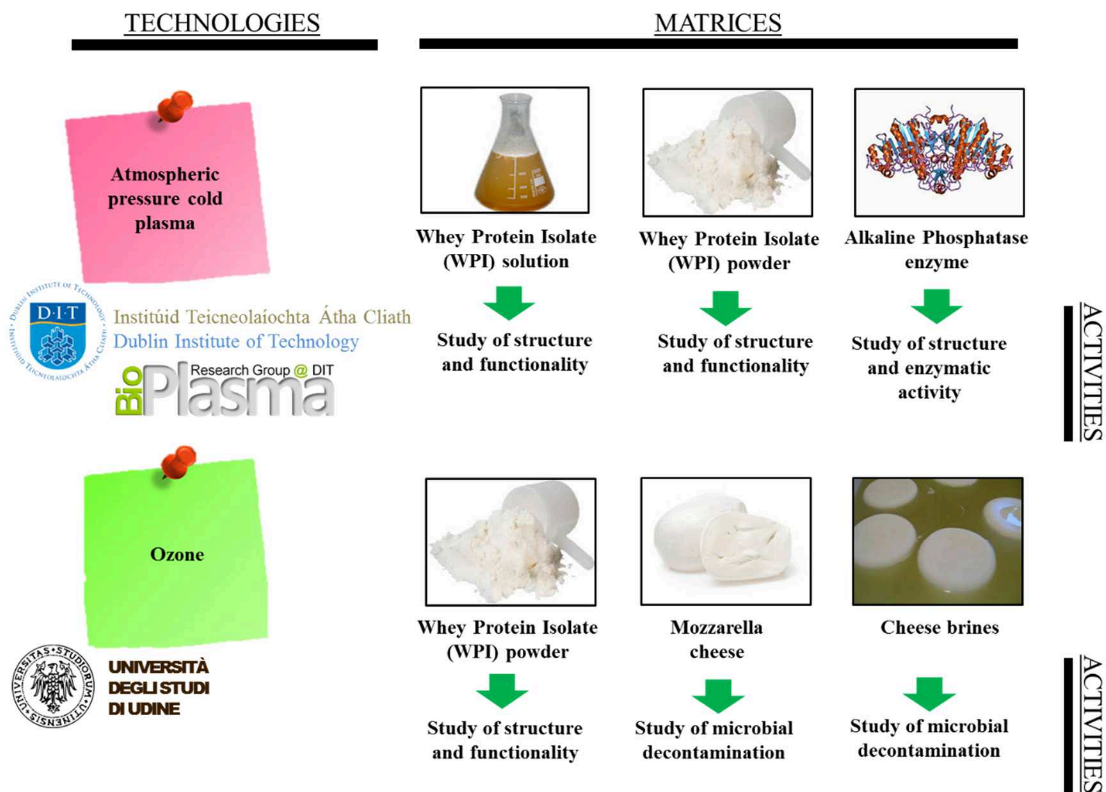


Figure A Schematic of the PhD activities

Summary

Among non-thermal technologies, atmospheric pressure cold plasma (ACP) and ozone treatments have gained enormous pace especially for their safety assurance and sustainability. Many studies and data regarding ACP inactivation of food-borne pathogens are already available in the literature. Most of them concern the decontamination by microorganisms in buffer or food matrices. However, ACP provides opportunities in several other applications including the modifications of food properties for improved functionality and healthy products.

The interaction between ACP and whey protein isolate (WPI) and alkaline phosphatase (ALP) enzyme model solutions were investigated in two different experiments. The aims were to study the effects on structure and functionality of the whey protein solutions and structure and residual enzymatic activity of the enzyme solution. In both cases, the findings covered a gap in the dairy field where the general information and research were insufficient.

In the first experiment, the results showed an increase in yellow colour and a minor reduction in the pH value in the whey protein solutions (2% w/v). These results were attributed to the reactions of reactive oxygen and nitrogen species of the plasma diffusing into the liquid. The carbonyl groups and the surface hydrophobicity increased, in addition to the reduction of free sulfhydryl (SH) groups, probably due to mild oxidation, which occurred in the proteins following the ACP treatments for 15 min. The protein structure modifications revealed a certain degree of unfolding, which improve foaming and emulsifying capacity. Upon extended treatment for 30 and 60 min, the changes were quite pronounced: the carbonyl and sulfhydryl groups reached equilibrium, the surface hydrophobicity remarkably increased and small aggregates were formed. Overall, the foaming and emulsifying capacity dramatically decreased against an increase of foam stability.

In the second experiment, the ALP subjected to the ACP was inactivated in a few seconds and the Weibull model was found to best describe the observed variance in residual activity for all the voltages applied. The results from the circular dichroism spectroscopy revealed a predominance of the α-helix structure, with a tendency to decrease with increasing treatment time and voltage. The ACP treatment did not affect the temperature and pH of the enzyme solution.

Since it is well known that protein functionality and protein structure are strictly connected, in the third experiment possible modifications in the secondary structure of WPI in powder treated with ACP at different voltages and times were studies, throughout the Fourier Transform-Infrared (FTIR) spectroscopy.

The second derivate of the amide I region of the spectra is obtained and the results show that the β-structures are relatively more stable compared to the α-helix. Changes in the α-helical structure are evident, and significantly affected by treatment time, at the lower voltage applied than the higher, for which no definite trend is recorded.

The study on model systems of whey proteins is also extended using solely the gaseous ozone at a high concentration in order to evaluate the changes in protein structure and their consequences on selected functional properties (fourth experiment). The results show a reduction of free SH groups and an increase in surface hydrophobicity, indicating a self-rearrangement in the protein structure following ozonation. Thus, ozonation allows for the creation of a more flexible structure without forming a strong network of disulphide bonds or aggregations (confirmed by the turbidity analysis and SDS-PAGE). Moreover, ozone processing induces modifications that improve foaming capacity and foam stability, however, a slight reduction in the solubility is encountered.

It is a well-known fact that ozone has a strong oxidant potential and the main applicability is related to the decontamination of foods, so the fifth and sixth experiments were focused on relevant case studies in the dairy field. The fourth one aimed to evaluate ozone effectiveness in reducing viable spoilage bacteria load throughout the high moisture (HM) Mozzarella cheese-making process. Many approaches in different phases of processing were tested but the ozone (gaseous and also solubilised in water) was not effective in surface microbiological decontamination of cheeses. However, it can be successfully applied in Mozzarella cheese processing only to decontaminate water contaminated by potential spoilage bacteria before coming into contact with the product. Finally, the last experiment aimed to reduce the microbial load of cheese brines contaminated by the reuse. The different ozone concentrations tested obtained a sufficient Log reduction. Moreover, considering the intermediate concentration tested (0.40 ± 0.2 ppm), the treatment time was extended up to 240 min to obtain a 4-Log reduction of the total microbial count (TMC) and other microorganisms considered. The longer the treatment time, the better the sanitisation effect. When the cheese brine samples with different protein contents (from 0.20 to 1.30 g/100 g) were treated, different levels of microbial inactivation were obtained: the higher the protein content, the lower the microbial reduction.

Introduction

Atmospheric pressure cold plasma (ACP) technology

Fundamentals of ACP technology

The term *plasma* is referred to a state of gas energized to such an extent that the molecules of the gas break down to release free electrons, radicals, positive and negative ions, and quanta of electromagnetic radiation, while some molecules may still remain neutral (incomplete ionization) (Misra et al., 2014). It can be produced when high energy is provided in order to ionize any neutral gas.

The plasma possesses a net neutral charge because the number of positive charge carriers is equal to the number of negative ones. Electrons and photons are considered as *light* species, and, on the contrary, the all others are *heavy* species (Misra et al., 2011). It is often considered the *fourth state* of the matter.

The plasma chemistry determines its behaviour, which could be very complex involving different constituent species (positive ions, negative ions, atoms, free radicals, electrons, photons, molecules, metastables). For example, for the plasma generated in the air, more than 75 species and almost 500 reactions are involved (Gordillo-Vàzquez, 2008). Optical emission spectroscopy (OES) is a non-intrusive technique that can monitor the concentrations of the plasma species (Chung et al., 2012). In OES, the spectrum of the radiation emitted by the plasma is grated and its intensity measured as function of the wavelength. An example of the emission spectra of the discharge (in arbitrary units) is presented in Figure B.

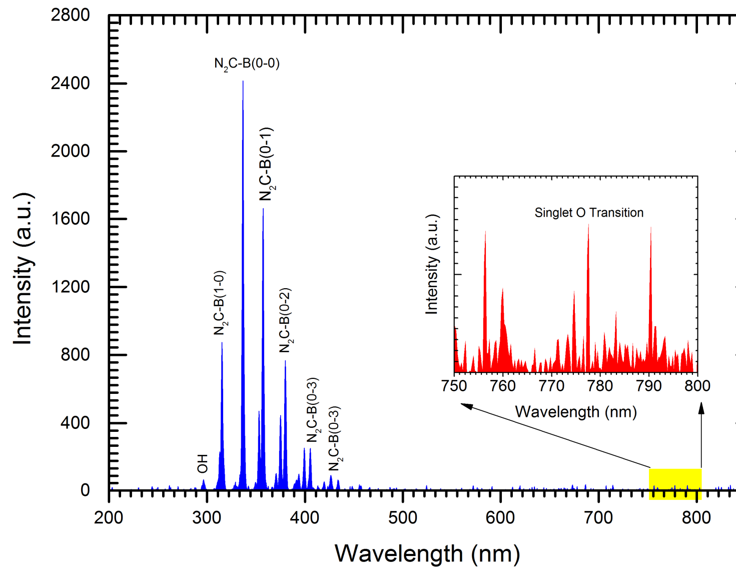


Figure B Optical Emission Spectra of the discharge inside an empty package 70 kV applied voltage (Misra et al., 2015)

Plasma can be classified in *thermal* and *nonthermal* plasma also called *cold plasma* based on the temperature and conditions it is generated (Nehra et al., 2008; Afshari and Hosseini, 2014; Tendero et al., 2006). The source of energy is very high temperatures (flames or arc) for thermal plasmas and electrical energy or electromagnetic waves for most cold plasmas (radiofrequency waves, capacitive and inductive coupling methods, dielectric barrier discharges). In addition to this, cold plasmas operating at atmospheric pressure (ACP) can be produced by means of high frequency microwave discharge (GHz range, 0.26-0.39 torr, power level 1 MW; Bárdos and Baránková, 2010), high or low radiofrequency electric field (13.56 MHz or <100 kHz); corona discharge (discharge gap in a large electric field, normally used in ozone generators) and dielectric barrier discharge (DBD, two electrodes, separated by one or more dielectric barriers). The latter, depicted in Figure C, can be employed with different gases or gas mixture such as helium and air for the in-packaging ionization and, recently, in the treatment of fresh produce.

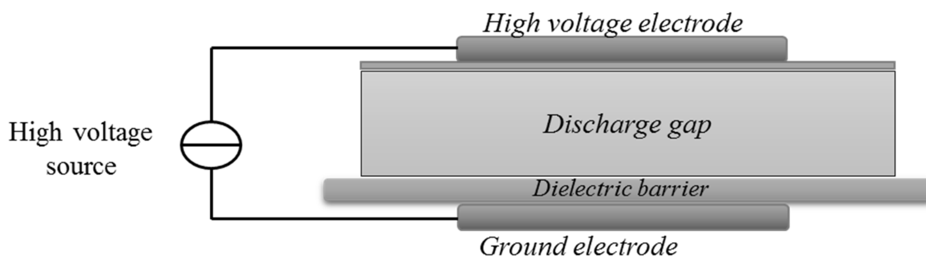


Figure C Dielectric barrier discharge (DBD) with a single dielectric barrier

Once the ACP is generated, it can be used with (1) electrode contact, (2) direct treatment or (3) remote treatment. In the first case, the sample is in contact with or between the electrodes. In the second operation mode, plasma is directly deposited on the sample and in the third, the plasma generated is moved to the sample.

The effect of ACP on microorganisms and macromolecules and the role of plasma species

ACP produced in a gas discharge, as previously reported, results in a mixture of active molecules that can continually and rapidly react with other molecules and particles present (Misra et al., 2014).

However, it is quite obvious that, the type of gas, the operating pressure, the food, and the microorganism characteristics can influence the effects of plasmas.

In particular, argon, helium and nitrogen/oxygen are the cheapest for ACP sterilization. In the presence of oxygen, hydroxyl radicals ($^{\bullet}OH$) and ozone (O_3) that are created, are the most aggressive among active chemical species, and in the presence of water (humidity or liquid in the sample), $^{\bullet}OH$, hydrogen peroxide (H_2O_2) and hydronium ions (H_3O^+) are created and all these species can exhibit different effects on different targets. Moreover, in addition to the gas composition, the operating pressure and the input power are important in determining the ionization potential that is also related to the density and

reactivity of the plasma. In ACP, which works in atmospheric pressure, the inactivation effects vary with increasing voltage and frequency that it is due to increased energy input and energy density (Deng et al., 2007). Furthermore, the different kind of contact between the sample/target microorganism and plasma (direct or indirect contact) generates different rates of inactivation, faster in direct then indirect contact in which, the short-lived charged particles do not play any significant role since they recombine before reaching the sample (Fridman et al., 2007). Physical state of food (i.e. the surface structure of solid food) and the duration of exposure must be considered in order to predict the efficiency of the treatment: higher treatment times, in general, lead to greater inactivation, even though in practical use, longer treatments can reduce or degrade the product quality and its sensitive compounds.

In addition to all the variables described above, the antimicrobial effect and the other effects on macromolecules or other food components can be due to the different plasma species included in the mix as ozone, atomic oxygen, superoxide, peroxide and hydroxyl radicals (reactive oxygen species -ROS- that could be generated in the air), ultraviolet (UV) radiation and charged particles; details of these species are discussed below however, the mechanism of action is very complex and it is probably the sum of synergistic effects of all of these species. It is also possible that a high number of different other low transient rate species, not well characterised, may be involved (Misra et al., 2011):

- *Ozone*. It is an unstable molecule that self-decompose in oxygen. The rate of decomposition depends on temperature, pH, oxidable compounds. Ozone can act on unsaturated aromatic and aliphatic compounds (Cataldo, 2003) in the microbial cell. It also reacts with unsaturated lipids, sulfhydryl groups, amino acids, nucleic acid bases (Khadre et al., 2001). An extensive discussion concerning ozone will be provided in the next chapter.
- *UV photons* production is involved in dimerization of thymine bases of DNA of microbial cell and spores as reported by Munakata et al. (1991) and Boudam et al. (2006). Roth et al. (2010) revealed that UV-C radiation was the most effective inactivation agent in plasma.
- *Carbon monoxide* (CO) effects during plasma application are not enough elucidated; however it can be hypothesised that plasma treatment of red meats may provide an added advantage of enhanced colour retention and extension of shelf-life (Misra et al., 2014; El-badawi et al., 1964; Jayasingh et al., 2001).
- *NOx species.* Nitric oxide and superoxide form peroxynitrite and other oxidants. Peroxynitrite is a potent oxidant that can act on sulfhydryl groups and nitration of tyrosine residues in cellular proteins (Burney et al., 1999).
- *Hydrogen peroxide* and hydroxyl radicals generated from its degradation have been suspected to act on spores and damage the vegetative bacteria DNA (Russel 1982; Setlow and Setlow, 1993; Imlay and Linn, 1988).

— *Singlet and atomic oxygen* are considered very effective sterilizing agents that can generate surface lesions of living cell (Singh et al., 2009).

— *Other free radicals and charged particles.* The presence of water vapour in a discharge feed gas results in the formation of $OH^{\bullet}$ by electron impact dissociation of H_2O and by reactions of electronically excited oxygen atoms and nitrogen molecules (Laroussi et al., 2004). Hydroxyl radicals are one of the most strongly oxidative species produced in water by plasmas and are also the building blocks of the formed H_2O_2 which is considered to be an important agent in the chemical reactivity of plasmas in contact with water (Bruggeman and Schram, 2010). When moist foods (such as fresh cut fruits and vegetables) are placed in a plasma discharge, the moisture on the surface of produce is likely to dissociate and yield hydroxyl radicals (Misra et al., 2014).

It still remains very difficult to describe univocally the inactivation pathways associated with active species with their effects on microbial cells and other components (Misra et al., 2014). However, in addition to these specific effects, ROS can oxidize the lipids in the cell membrane, which increases permeability and consequently can also cause the leakage of cytoplasm. According to Laroussi et al. (2003) and Mendis et al. (2002), the accumulation of charges at the outer surface of cell membrane and consequently the electrostatic forces applied can be the main cause for the cell wall rupture. Even though the mechanism of action remains controversial, one the possible bactericidal action of plasma was proposed as likely due to the diffusion of plasma-generated reactive species through the cell membrane into the cell where they react and possibly damage proteins and nucleic acids leading to cell death (Ziuzina et al., 2013). Moreover, bacteria, spores and fungi can be inactivated relatively easily by ACP and gram-negative bacteria are more resistant to plasma processing, because of their extra proteins and lipopolysaccharide walls.
However, the side effects have not always been studied, such as possible differences in colour, texture and overall quality of food matrices treated. Some information can be extrapolated from studies on microorganisms and can vary depending on the exposure time and the applied voltage. The chemical species of the plasma can also act in different ways on food lipids and proteins. Based on studies of protein model systems, such as BSA and lysozyme, we can also assume that plasma species alter the secondary and tertiary protein structure, modify some amino acids side chains and degrade the protein integrity (Takai et al., 2012; Deng et al., 2007; Bernard et al., 2006).
One possible mechanism of reaction between plasma generated reactive species and protein suggested by Takai et al. (2012) is that hydroxyl radicals ($OH^{\bullet}$), superoxide anion radicals ($O_2^{-\bullet}$), hydroperoxyl radicals ($HOO^{\bullet}$) and nitric oxide ($NO^{\bullet}$) leads to the chemical modifications of chemically reactive side-chains of the amino acids, such as cysteine, aromatic rings of phenylalanine, tyrosine, and tryptophan. A similar mechanism for decomposition of C–H, C–N and N–H bonds of protein bonds is also described by Hayashi et al. (2009). Moreover, it is a well-recognized fact that the resultant ionization that occurs during plasma discharge activity allows substantial levels of ozone to be

formed and in some cases to dissolve in liquids (Espie et al., 2001) and the ozone can act on proteins and oxidase lipids. The mechanism of action of the ozone molecule will be discussed later.
However, the information concerning the effects between plasma species and macromolecules and other food components remains insufficient, but on the other hand, the number of studies contributing to the literature is continuously growing.

Applications in dairy processing
ACP, as previously reported, has been proven effective for reducing microbial population in buffer solutions or in particular on the surface of materials, such as glass, metals, fabrics, and agar, with a wide range of microorganisms including vegetative forms, spores, fungi, viruses and parasites, etc. (Misra et al., 2011).
Several research studies have also been carried out in the application of cold plasma in the food industry. Scientific contributions concerning packaging sterilization (Laroussi, 2002; Deilmann et al., 2008), permeability reduction of polymers for carbon dioxide and oxygen (Basaran et al., 2008, Schneider et al., 2005), Fernández-Gutierrez et al. (2010), have demonstrated the applicability of cold gas plasma for deposition of vanillin film over red delicious apples. Other studies focus on the decontamination of grain and legumes infected by fungi (Selcuk et al., 2008), almond infected by *Escherichia coli* and salmonella (Deng et al., 2007; Niemira, 2012), inactivation of pathogens in poultry wash water (Rowan et al., 2007), decontamination of fruit pericarps from spoilage and pathogenic microorganisms (Perni et al., 2008) and vegetables (Critzer et al., 2007). Ragni et al. (2010) reported efficacy of plasma for decontamination of egg shell surfaces, Shi et al. (2011) employed a DBD to effectively kill *Staphylococcus aureus, Escherichia coli and Candida albicans* inoculated in orange juice.

Milk and milk products have long been associated with infectious diseases including campylobacteriosis, salmonellosis, yersinosis, listeriosis, tuberculosis, brucellosis, staphylococcal enterotoxin poisoning, streptococcal poisoning and *Escherichia coli* O157:H7 infection (Gurol et al., 2012; Sampedro et al., 2005). To this regard, nonthermal food processing could play an important role in improving food quality by maintaining its microbiological safety and quality profile while only minimally affecting its sensory and health-related properties (Sobrino-López and Martín-Belloso 2010). The first attempt to decontaminate milk using cold plasma generated from a corona discharge (9 kV of AC power supply) has been made by Gurol et al. (2012). This group has reported *E. coli* count reduction from 7.78 Log cfu/mL to 3.63 Log cfu/mL after 20 min of treatment in whole milk and an average 54% reduction after a 3-min treatment irrespective of fat content. Thus, it appears that cold plasma generated under controlled conditions has the potential to decontaminate milk. Milk products including sliced cheese are often associated with foodborne listeriosis. Recently the effects of atmospheric ACP on the inactivation of 3-strain cocktail of L. *monocytogenes* in sliced cheese have been examined by Song et al. (2009). This group reported c.a. a 6-Log reduction in the viable

population of L. monocytogenes after 120 sec of treatment at 125W power using radiofrequency plasma (13.65 MHz) operating in helium gas. Furthermore, the microbial Log reduction was enhanced with an increased input power and plasma exposure time (Misra et al., 2014).

Ozone technology

Fundamentals of ozone technology

Ozone (O_3) results from the rearrangements of atoms when oxygen molecules are subjected to high voltage electric discharge. Thus, molecular oxygen is split into two atoms of oxygen which are highly reactive moieties. When a free oxygen atom ($O^{\bullet}$) encounters molecular oxygen (O_2), it combines to form the highly unstable ozone molecule (O_3). Because ozone is unstable, it rapidly degrades back to molecular oxygen (O_2) with the released free oxygen atom ($O^{\bullet}$) combining with another free oxygen atom ($O^{\bullet}$) to form molecular oxygen (O_2) or combining with other chemical moieties to cause oxidation. Upon release of the third oxygen atom, ozone acts as a strong oxidizing agent. Ozone cannot be stored in gas cylinders, since it decomposes spontaneously, thus it must be produced on-site and the systems must be tailored to specific uses resulting in optimum efficacy. It can be produced by UV radiation (188nm wavelength) and corona discharge methods. However, for commercial purposes, the corona discharge method is commonly used (Guzel-Seydim et al., 2004).

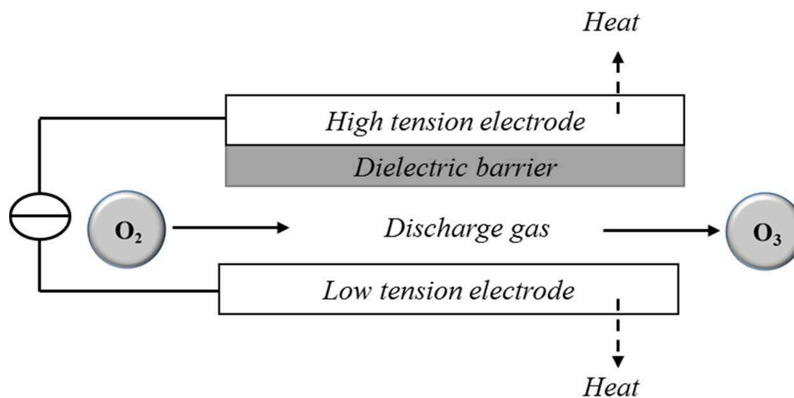


Figure D Schematic diagram of ozone generation by corona discharge method (Rice et al., 1981)

There are two electrodes in corona discharge, see Figure D, one of which is the high tension electrode and the other is the low tension electrode (ground electrode). Those are separated by a ceramic dielectric medium and narrow discharge gap is provided. When the electrons have sufficient kinetic energy (around 6–7 eV) to dissociate the oxygen molecule, a certain fraction of these collisions occur and an ozone molecule can be formed from each oxygen atom. If air is passed through the generator the rate of ozone is lower than that produced using pure oxygen (Rice et al., 1981).

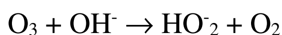
Once it is produced, ozone can be used in gaseous phase or solubilized in water. Normally, Venturi injection and fine bubble diffusion are used to mass transfer of ozone into water.

It is partially soluble in water and the dissolution follows Henry's law; the solubility ratio for ozone increases as the temperature of water decreases. The solubility in water is

greater for ozone than for nitrogen and oxygen, however it is less soluble in water than carbon dioxide and chlorine (Roth and Sullivan, 1981; Horvarh et al., 1985; Bablon et al., 1991). In addition to pressure and temperature, which directly affect the solubility, the bubble dimensions, purity and pH of water can influence the dissolution, as well. An optimum dissolution of ozone in water occurs when bubbles are small; the flow rate of ozone and contact time affect the transfer of the gas to water. Mixing and turbulence increase bubble contact and solubilization in water (Katzenelson et al., 1974). Details about ozone bubble columns and the interactions between gas and liquid are also elucidated in Cullen et al. (2009). Purity and pH of water affect the rate of ozone solubilisation, as well; in fact in tap water both for higher pH and organic matter, ozone destabilises faster and therefore the rate of solubilization decreases.
Ozone is relatively unstable in aqueous solution. It decomposes continuously and slowly in oxygen according to a first-order reaction (von Gunten, 2003). The half-life of ozone in distilled water at 20 °C is generally considered to be 20 to 30 min (Khadre et al., 2001). The stability of ozone in aqueous solutions depends on the source of water. The organic and inorganic substances presented in the water reduces the half- life of ozone as well as when the pH increases.
The ozone molecule acts as a dipole with electrophilic and nucleophilic properties and the organic substance in aqueous solution may react with it in two possible ways (Staehelin and Hoigné, 1985):
1) Direct reaction between a generic compound (M) and the molecular ozone:

$$O_3 + M \rightarrow M_{ox}$$

2) Decomposition of ozone in water in a radical (i.e. $^{\bullet}OH$) which reacts with M:



$$O_3 + OH^- \rightarrow HO^-_2 + O_2$$

$$O_3 + HO^-_2 \rightarrow {}^{\bullet}OH + O_2^{\bullet -} + O_2$$

$${}^{\bullet}OH + M \rightarrow M_{ox}$$

where O_3= ozone, OH^- = hydroxide ions, HO^-_2 = hydroperoxyl radicals, $^{\bullet}OH$ = hydroxyl radicals, $O_2^{\bullet -}$= superoxide anion radicals, O_2= oxygen, Mox =generic oxidized compound

The disintegration of ozone in water into $^{\bullet}OH$ radicals arises from the indirect reaction pathway (here, a simplify reaction). It is well known that $^{\bullet}OH$ resulting from this reaction have a very short life, with an even stronger oxidation potential than ozone because most radicals are highly unstable and immediately undergo a reaction with another molecule. Indirect reactions in an ozone oxidation process can be very complex and lead to the formation of several primary highly reactive species, Figure E.

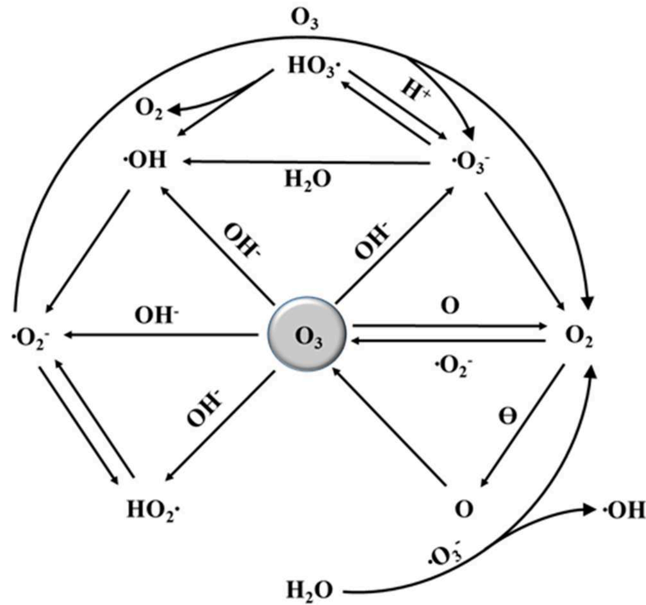


Figure E Schematic diagram of inter-transformations among primary high-reactive species. (Xue et al., 2008 reprinted in Ozone in Food Processing/ *edited by O'Donnell et al., 2012)*

Conversely, the molecular reactions are selective and limited. Moreover, the increasing temperature reduces the solubility and stability, but the reactivity of ozone with the substrate increases. Ozone is more stable at low than at high pH values. It decomposes at high pH values and the resulting radicals contribute to its efficacy.

Effect of ozone on microorganisms and macromolecules

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, as well as proteins and peptidoglycan in spore coats and virus capsids, damaging the DNA (Khadre et al., 2001; Bablon et al., 1991). The effects are generated by molecular ozone (1) as well as by-products of ozone decomposition (2) ($^{\bullet}OH$, $^{\bullet}O_2$, $HO^{\bullet}{}_3$). The relative importance of these two mechanisms may vary with the microorganism and treatment conditions. The presence of organic substances with high ozone demand may compete with microorganisms for ozone (Khadre et al., 2001), therefore the efficacy is demonstrated more readily when targeted microorganisms are suspended and treated in pure water or simple buffers (with low ozone demand) than in complex systems such as food. However, over the years, advanced oxidation processes (AOPs) have been used to produce OH radicals (by coupling ozone with high pH, hydrogen peroxide, or UV radiation) in order to successfully degrade target molecules, due to their higher powerful oxidants than molecular ozone and their nonselective reactions (Gottshalk et al., 2010).

The bactericidal effects of ozone have been documented on a wide variety of organisms, including Gram-positive and Gram-negative bacteria as well as spores and vegetative cells (Foegeding, 1985; Restaino et al., 1995). In general, gram-negative are more sensitive to the ozone than the gram positive, vegetative bacterial cells, bacterial and fungal spores, Despite the limited number of studies, viruses are most sensitive and the moulds have an intermediate resistance to ozone.

In the presence of organic compounds, ozone reacts in a variety of complex reactions, as previously reported. The reactions are selective and limited to unsaturated aromatic and aliphatic compounds, where ozone goes to break the double bonds (Gottschalk et al., 2010); in compounds containing polysaccharides it breaks the glycosidic linkages and form aliphatic acids and aldehydes (Bablon et al., 1991) while the reaction with primary and secondary aliphatic alcohols can lead to the formation of hydroperoxides which are precursors of the hydroxyl radical. Purines and pyrimidines are more resistant than other organic compounds (Adachi, 2001); as regards the saturated fats, the reaction is very slow, while unsaturated ones are easily oxidizable (Khadre et al., 2001) and their oxidation leads to the formation of peroxides (Adachi, 2001). The action of amino acids and peptides is significant especially at neutral and basic pH and affects the nitrogen atom or the group R, or both (Mustafa, 1990; Adachi, 2001), the carbon skeleton and the protein bonds (Kelly and Mudway, 2003; Uzun et al., 2012).
Several studies have been carried out to investigate the fundamental interaction between ozone and different proteins (Cataldo, 2003, 2004, 2007) and the interactions of specific amino acids in proteins such as methionine in glycophorin (Banerjee and Mudd, 1992), histidine in glutamine synthetase (Berlett et al., 1996), tryptophan in band III, serum albumin, and cyctochrome c (Mudd et al., 1997). Although all amino acids are potential targets for oxidation by reactive oxygen, the major aromatic amino acids like tyrosine, tryptophan, phenylalanine, the sulphur containing amino acids cysteine, methionine as well as the aliphatic amino acids arginine, lysine, proline and histidine, which appear especially sensitive to oxidation. The sequence of amino acids that are condensed in a chain by amide bonds, representing the main chain backbone of proteins exhibits negligible or very limited chain scission during ozonation. Helices, sheets, coils, and folded branches which define the secondary and tertiary structure of proteins seem to be modified by the reaction of ozone with proteins. The action of ozone converts the thiol group of cysteine into disulphides which denature the protein and change its solubility (Kelly and Mudway, 2003). As a result of ozone attack the protein molecules undergo changes in their visual folding and binding ability (Cataldo, 2003) which influence their functionality.

Application in dairy processing

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, colour, tastes, and odours 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 confirmed ozone as a GRAS substance (Graham, 1997) for broad food applications as a disinfectant or sanitizer for foods when used in accordance with good manufacturing practices. 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 gaseous or liquid phase (Kim et al., 2003) in the United States. These regulatory developments triggered interest in ozone applications among academic researchers and food processors. The use of ozone as an antimicrobial agent in food processing was reviewed by various researchers (Khadre et al., 2001; Kim et al., 1999; Xu, 1999). There is ample literature reporting successful applications of ozone in various food matrices such as lettuce, spinach, parsley, asparagus, carrots, eggs, apples, tomatoes, red bell peppers (Karaca and Velioglu, 2014; Alexopoulos et al., 2013; Huyskens-Keil et al., 2011; Donner and Keener, 2011; Hildebrand et al., 2008; Fuhrmann et al., 2010; Achen and Yousef, 2001; Aguayo et al., 2006). Possible applications of ozone in the food industry include reducing microbial populations in biofilms found on stainless-steel surfaces, treating and possibly recycling poultry chill waters, and depuration of shellfish (Greene et al., 1993; Schneider et al., 1991; Sheldon and Brown, 1986). Positive results have also been evaluated in the treatment of drinking water as well as washing water for fresh-cut vegetable, and lettuce (von Gunten, 2003; Selma et al., 2008; Selma et al., 2007).

It was by Greene et al. (1993), who compared the effectiveness of ozonated water and chlorinated sanitizer for the disinfection of stainless steel surfaces in the *dairy industry*, which had been incubated with UHT milk inoculated with either *Pseudomonas fluorescens* (ATCC 949) or Alcaligenes faecalis (ATCC 337) at 32 °C for 4–24 hours. They found that ozone was as effective as chlorination against dairy surface-attached bacteria, as both treatments reduced bacterial populations by 99%. However, while ozone is an effective sanitizer, it is also known for being corrosive. Therefore, in a subsequent study, Greene et al. (1994) investigated the effects of chlorine and ozone sanitizers on gasket appearance, tensile strength, elongation at failure and elastic modulus. They found that, with the exception of some slight discolouration, ozone does not cause damage to Buna, silicone and polyethylene gasket materials. Conversely, long-term ozone exposure affects PTFE (Teflon) by increasing the tensile strength and elongation.
In a later study Greene et al. (1999) found no corrosive effect of ozone on stainless steel when compared to a warm water control.
Güzel-Seydim et al. (2000) successfully studied the use of ozonated water as a pre-rinse technique in dairy equipment by soiling stainless steel and they found that the ozone containing water (10 °C) removed 84% of dairy soil when compared to warm water (40 °C), which removed 51%. These results were also confirmed by scanning electron

microscopy images. Other studies on removal of biofilms from dairy equipment with ozone and ozone in combination with ultrasounds were also conducted by Baumann et al. (2009) and Bott and Tianqing (2004); they found that when both ozone and ultrasound were used synchronously, the sanitisation was more effective than with their individual application. Moreover there is an enhancement of ozone biocidal efficiency through the application of ultrasound. Another study by, Dosti et al. (2005) looked into the efficacy of ozone (0.6 ppm for 10 minutes) and chlorine (100 ppm for 2 minutes) for the removal of bacterial biofilm. They reported no significant difference between ozone and chlorine inactivation of the bacteria, with the exception of *P. putida*, where ozone was found to be more effective compared to chlorine. This study shows that ozone can be a potential alternative to chlorine as a sanitising agent for dairy processing equipment.

When moulds occur in a food processing plant, they can result in spoilage of food products. As cheese ripening rooms are in an environment that encourages mould growth, it is likely that cheese will become mouldy if the room is contaminated with mould spores. The first study was done by Gibson at al. (1960) and they found that high concentrations (3-10 ppm) of ozone gave the appearance of destroying heavy mould growth on cheese. When the ozone treatment was discontinued a profuse growth of mould developed very rapidly on the cheese, indicating the moulds were not destroyed. Slight mould growth developed on the waxed cheese when exposed to high ozone concentrations. Low (0.2-0.3 ppm) ozone concentrations retarded the growth of mould on the sides of unwaxed cheese and aided in preventing mould from developing on the ends. The sides of the waxed cheese were protected from mould growth while it continued to grow on the ends. However, free mould spores were reduced in the ozone-treated rooms. The use of ozone did not cause any flavour defects in the cheese. Ozone concentrations of 0.1 and 10 µg/L in the atmosphere of a cheese-ripening room inactivated 80 to 90 and 99% of mould spores, respectively, 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/L ozone in air prevented growth of moulds on cheeses and packaging materials for 4 months without adversely affecting chemical and sensory properties of the cheese. Control cheese exhibited mould 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/L) during the ripening period. Other experiments conducted on cheddar cheese also indicated that the oxidizing action of ozone removes odours otherwise present in storage rooms. Shiler et al. (1983) described a method of ozonation for ripening and stored cheese, to inactivate contaminating microflora but to avoid damage to cheese-packaging materials and 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/L 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/L for 2 to 4 h.

Serra et al. (2003) indicated that it was necessary to wipe the surfaces with a commercial sanitizer in order to decrease the viable mould load on these surfaces. This finding was in correspondence with the report of Ingram and Barnes (1954), who concluded that ozone was able to destroy most aerial spores, but failed to prevent their germination when they were lodged in apple surface wounds (O'Donnell et al., 2012).
Cavalcante et al. (2013) evaluated ozone gas as a new preservation method for raw milk in combination with mild thermal treatment to reduce the microbial load and improve the shelf-life of milk during storage. The samples were collected before and after ozone gas bubbling at 1.5 mg/L for 5, 10 and 15 min. The ozone efficacy was evaluated by microbial reductions of *Enterobactereaceae*, total mesophilic aerobic, psychrotrophic bacteria, moulds and yeast and *Staphylococcus* sp counts. The ozonation for 5 minutes was not able to reduce the milk microbial counts but the treatment with ozone for 10 minutes caused significant reduction ($p < 0.05$) of *Enterobacteriaceae* (0.59 Log), moulds and yeast (0.25 Log) and *Staphylococcus* sp (0.59 Log). After 15 minutes of treatment, microbial inactivation was observed for the microorganisms evaluated, with reductions of 0.96; 0.60; 0.13; 0.48 and 1.02 Log cycles for *Enterobacteriaceae,* mesophilic aerobic, psychrotrophic bacteria, moulds and yeast and *Staphylococcus* sp, respectively. Moreover, no changes were observed in the physicochemical parameters of the milk.

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Part I – Atmospheric Pressure Cold Plasma Treatments in the Dairy Field

Chapter 1

Atmospheric pressure cold plasma treatment of whey protein isolate model solutions

Chapter 2

FTIR spectroscopic study of atmospheric pressure cold plasma treated whey protein isolates

Chapter 3

Effect of atmospheric pressure cold plasma on activity and structure of alkaline phosphatase

Chapter 1

Atmospheric pressure cold plasma treatment of whey protein isolate model solutions

ABSTRACT

The interaction between atmospheric pressure cold plasma (ACP) and whey protein isolate (WPI) model solutions was investigated as a function of treatment time (from 1 to 60 min). The effects on colour, pH, free sulfhydryl (SH) and carbonyl groups, surface hydrophobicity, protein structure and functionality were studied. The results showed an increase in yellow colour and a minor reduction in pH value, which was attributed to the reactions of reactive oxygen and nitrogen species of the plasma diffusing into the solution. Following ACP treatments for 15 min, mild oxidation occurred in the proteins. This was evident from an increase in carbonyl groups and the surface hydrophobicity, besides the reduction of free SH groups; these changes ultimately pointed to the effects of ACP on amino acid residues of the proteins. The protein structure modifications revealed a certain degree of unfolding, as confirmed by dynamic light scattering (DLS) and high performance liquid chromatography (HPLC) profiles, which improve foaming and emulsifying capacity. Upon extended treatment for 30 and 60 min, the changes were quite pronounced; the carbonyl and sulfhydryl groups reached equilibrium, the surface hydrophobicity remarkably increased and small aggregates were formed. Overall, the foaming and emulsifying capacity dramatically decreased; nevertheless the foam stability increased.

1.1. Introduction

Within recent years the efforts of food science researchers has largely focused on development and evaluation of novel non-thermal food technologies. The non-thermal technologies have gained enormous pace because in most cases these avoid the undesirable effects generated when heat treatment is applied to food matrices. Researchers are primarily motivated by the consumer demands for high quality and minimally processed foods, whilst ensuring microbiological and chemical safety. Atmospheric pressure cold plasma (ACP) is a relatively new technology and consists of highly energetic species in permanent interaction including photons, electrons, positive and negative ions, free radicals and excited or non-excited molecules and atoms (Fernández et al., 2013; Bárdos and Baránková, 2010; Laroussi, 2005). Cold plasma at atmospheric pressure can be obtained by exposing a gas/mixture of gases to an electric field, which in turn accelerates the charged particles, leading to collisions with the heavy species (e.g. ions and neutrals).

It is successfully studied mainly for food decontamination from microorganisms including spores and viruses. The antimicrobial effects are the result of a synergistic contribution of all the plasma species involved that can act damaging the outer surface of microbial cells and biological material such as lipids, proteins, DNA, cytoplasm (Misra et al., 2011; Laroussi 2005; Yu et al., 2006; Fernández and Thompson, 2012; Ziuzina et al., 2013) without altering the food characteristics (Ragni et al., 2010; Gurol et al., 2012; Kim et al., 2014; Misra et al., 2014b; Tappi et al., 2013; Misra et al., 2014a)

Although it is a promising technology for different fields in the food production, presently, the only commercial application of ACP technology for the food industries is limited to polymer processing used for food packaging applications, as reported by Pankaj et al., 2014. However, a fundamental understanding of the interactions between plasma and biomacromolecules has remained under researched.

In the past Deng et al. (2007) and Bernard et al. (2006) studied bovine serum albumin (BSA) and lysozyme as model proteins to assess the effects of gas plasma on modification and inactivation of proteins. More recently, different studies on enzymes allowed a mechanistic elucidation of the cold plasma-protein interactions (Surowsky et al., 2013; Pankaj et al., 2013; Tappi et al., 2013). Those authors mainly attributed the modifications on proteins to the action of the reactive oxygen species (ROS) and reactive nitrogen species (RNS) that in general can alter the secondary and tertiary structure of the enzyme and oxidase the most sensitive amino acid residues of the proteins.

The oxidation of proteins also involves a specific sector of the research that aims to selective modify proteins and their functionality (Liu et al., 2000; Chen et al., 2013; Cui et al., 2012; Kong et al., 2013). Among oxidising agents able to modify macromolecules, ozone, that is also included in the most reactive plasma species, has been recently applied on cassava starch (Klein et al., 2014), wheat starch (Sandhu et al., 2012), cocoyam and yam starches (Oladebeye et al., 2013), egg and whey proteins (Uzun et al., 2012; Segat et al., 2014). Misra et al. (2015) explored the possible effects of ACP generated in air

(which has many active species, including ozone) on wheat flours in order to evaluate also the effects on selected technological and chemical properties of wheat flour.
In light of these considerations, this study is focused towards the modification of whey proteins using ACP produced by a dielectric barrier discharge (DBD) source. Whey proteins are very important as a food ingredient in particular because they exerts different functionalities related to the solubility in water, the ability to create viscosity, form gels, emulsions, the improvement of flavour and texture in addition to the nutritional advantages (Innocente et al., 2009; Nicorescu et al., 2009; Venir et al., 2010; Innocente et al., 2011; Innocente et al., 2014; Matumoto-Pintro et al., 2011). Moreover, they often serve as ingredients in several foods including infant foods, beverages, bakery products, dairy foods, muscle foods, and snack bars (de Wit, 1998; Morr and Ha, 1993; Lucena et al., 2006).
Among whey proteins, the most used as ingredients are whey protein isolate (WPI) that have higher protein concentrations and less impurities compared to other whey proteins derivative ingredients (Foegeding et al., 2011; de Wit, 1998; Innocente et al., 2009).
Bases on these premises, the aim of the present study is to elucidate the main effects of ACP on model systems of whey protein isolate (WPI) solutions that can be employed as functional ingredient in different food formulations and therefore evaluate the industrial relevance of this technology as an emerging advanced oxidation process.

1.2. Materials and methods

1.2.1. Materials

Whey Protein Isolate (WPI) obtained from a commercial supplier was used for all the experiments. It was composed of c.a. 95% of proteins (on dry basis) including β-Lactoglobulin, α-Lactoalbumin and BSA at around 75, 23 and 2% respectively; humidity (4.9%), ash (0.8%) and fat content (not detected, <0.01) were also reported elsewhere (Segat et al., 2014). The lactose content was determined using an enzymatic assay kit for lactose/D-galactose (R-Biopharm, Darmstadt, Germany).
Solutions of WPI at 2% (w/v) in phosphate buffer (50 Mm, pH 6.8) were prepared and the plasma treatments were carried out using the solutions. All chemicals used in the study were purchased from Sigma-Aldrich, Ireland, unless explicitly specified.

1.2.2 Atmospheric pressure cold plasma (ACP) treatments

30 mL of WPI solutions were placed in petri dishes of 50 mm diameter, packaged inside polyethylene terephthalate (PET) trays and then subjected to ACP treatments. The plasma source comprised of two aluminium disc electrodes of 15 cm-diameter, over two polypropylene dielectrics (2 mm thick) between which the PET package with samples is placed. The tray with wall thickness of 270 μm also served as dielectric barrier. The distance between the two electrodes was 44 mm, equal to the height of the tray plus the thickness of the two dielectric layers. The voltage across the electrodes was monitored

on an InfiniVision 2000 X-Series Oscilloscope (Agilent Technologies Inc., Santa Clara, CA, USA) using high voltage probes coupled to voltage dividers (Figure 1.1). More information on experimental set-up has been described by Misra, et al. (2014b).
All experiments were performed at a discrete voltage of 70 kV applied for 1, 5, 10, 15, 30 and 60 minute using ambient air at atmospheric pressure conditions. Treatments were carried out in duplicate. After treatment, the samples were stored under refrigerated conditions (4 °C) for 12 h until further analysis.

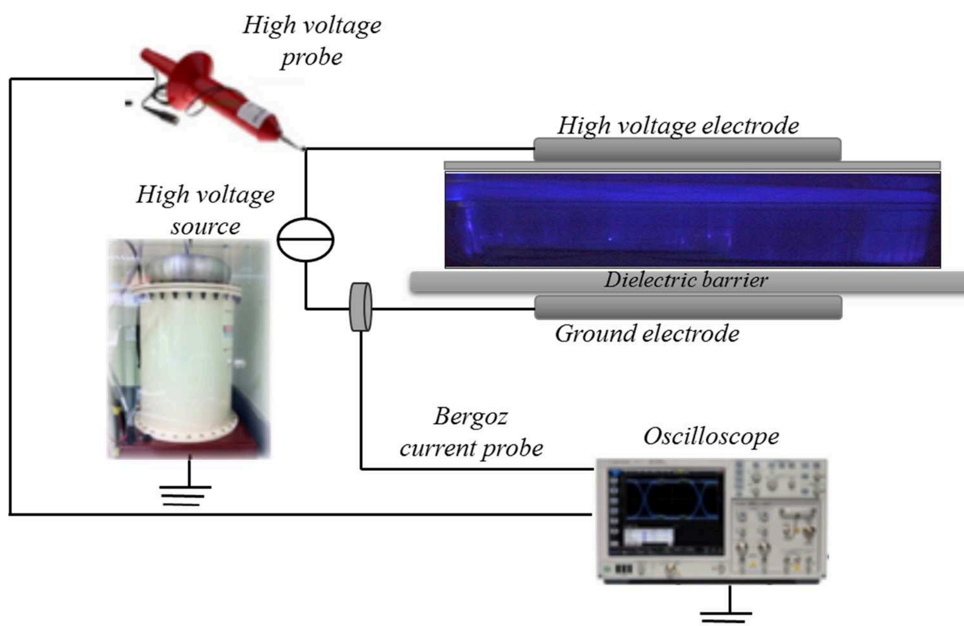

Figure 1. 1 Schematic of the DBD-ACP treatment of WPI solutions inside package. The package shown is filled with helium

1.2.3 Ozone concentration, temperature and pH measurements

Ozone concentration inside the package was measured immediately after treatments using Gastec ozone detection tubes (Gastec Corporation, Kanagawa, Japan). Further details regarding ozone measurement can be found in Misra et al. (2014c). Temperature of solutions was measured before and immediately after ACP treatments using an IR Thermometer with laser pointer (Maplin Electronics, UK). The pH of the solutions and all buffers used were measured before and after treatments using a pH meter (Thermo scientific Orion 2-star, Benchtop, Thermo Fisher, Waltham, MA, USA).

1.2.4 Colour measurement

The colour was quantified using a pre-calibrated L*, a*, b* colorimeter (Colour Quest XE Hunter Lab, Northants, UK). The colour values were reported as the average of at

least six measurements. Colour change was evaluated using the b* parameter which describes the yellow colour.

1.2.5 Protein carbonyls

The amount of protein carbonyls was determined in accordance with Levine et al. (1990). Briefly, 0.4 mL of 2,4-dinitrophenylhydrazine (DNPH, 10mM in 2M HCl, Lancaster Synthesis Ltd, UK) was added to 0.3 mL of protein solution. After 60 min of incubation, 0.7 mL Trichloroacetic acid (TCA) was added (10% final concentration). The samples were incubated on ice for 10 min and centrifuged (11000*g*, 3 min). The protein pellets were washed three times with 1mL of ethanol and ethyl acetate mixture (1:1, v/v) to remove the excess DNPH. The final pellet was dissolved in 0.5 mL of 6M guanidine hydrochloride and the absorbance at 370 nm was recorded using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

1.2.6 Determination of free sulfhydryl content

To determine the concentration of free sulfhydryl groups (SH) of the WPI samples, Ellman's reagent (5', 5-dithiobis (2-nitrobenzoic acid), DTNB) was used. 0.5 mL of WPI sample solution was added to 2.5 mL of 8M urea in Tris-Glycine buffer (10.4 g Tris, 6.9 g glycine, 1.2 g EDTA per litre, pH 8.0) and 0.02 mL of Ellman's reagent (4 mg mL^{-1} DTNB in Tris-glycine buffer) to develop colour. After 15 min, absorbance was measured at 412 nm on a UV-Vis spectrophotometer (UV-1800, Shimadzu Kyoto, Japan) (Beveridge et al., 1974). Concentration of the free sulfhydryl groups (μM SH/g) was calculated from the following equation:

$$\mu M\ SH/g = (73.53 \times A_{412} \times D)/C$$

where, A_{412} is the absorbance at 412 nm, C is the concentration of the WPI solution (mg/mL), D is the dilution factor, and the factor 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 being the molar absorptivity constant (Ellman, 1959).

1.2.7 Surface Hydrophobicity

The surface hydrophobicity of WPI samples was determined in accordance with Cui et al., 2012. A series of dilutions of the 2% (w/v) WPI were made with 50 mM phosphate-buffer (pH 6.8) to obtain a range of protein concentrations (0.02 – 2 mg/mL). Subsequently, 10 mL of each supernatant was reacted with 0.6 mL of ANS (8 mM in 50 mM phosphate buffer, pH 6.8) for 15 min. The relative fluorescent intensity (RFI) of each sample solution was measured using a fluorescence detector (Model FP-1520 Jasco, Intelligent Fluorescence Detector, USA). The settings were as follows: excitation at 390 nm, emission at 480 nm, gain = 1 and attenuation = 1. The surface hydrophobicity of solubilized WPI solutions was calculated from the initial slope of RFI versus protein concentration. The RFI was defined by $RFI = (F-F_0)/F_0$, where F is the fluorescence intensity of the protein-ANS conjugate, and F_0 is the intensity of the ANS solution without WPI.

1.2.8 Dynamic Light scattering (DLS)

The sample solutions were diluted by a factor of 1:100 in phosphate buffer (50mM, pH 6.8) prior to analysis. The analysis was performed using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 20 °C. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution and the polydispersity index. The viscosity of the solvent was measured before analysis and was assumed to be the same as water. Data were analysed using the Dispersion Technology Software (DTS; v 5.10, Malvern Instruments, UK).

1.2.9 HPLC analysis

WPI sample solutions were 10-fold diluted with Na-acetate buffer (0.1 M, pH 4.6) and centrifuged at 10000*g* for 15 min. 40 µL of supernatant was injected into the HPLC. Eluting solvents A and B were HPLC-grade water (VWR International, USA) and acetonitrile (Lab Scan, Analytical Sciences, Ireland), respectively, both containing 0.1% (v/v) trifluoroacetic acid. The analysis was carried out in accordance with the method described by De Noni et al. (2007). The HPLC system consisted of an Alliance 2695 pump system (Waters, Milford, MA, USA) combined with dual wavelength absorbance detector (Waters 2487, Waters, Milford, MA, USA). A PLRP-S column (4.6 mm i.d., 150 mm long, 5 µm particle size, 300 Å pore size) from Waters was used. β-lactoglobulin (β-Lg), α-lactalbumin (α-La) and BSA were injected as standard solutions. Chromatographic data were processed with Empower 2 software (Waters, MA, USA).

1.2.10 Foaming properties

10 mL of WPI solutions were homogenized at 16000 rpm for 2 min using an IKA Ultra Turrex homogeniser (T18 basic, IKA-Werke GmbH and Co., Staufen, Germany) in graduated tubes. Foaming capacity (FC) and foam stability (FS) were calculated according to the following equations:

$$FC\ (\%) = \frac{V_{F0} - V_L}{V_L} \times 100$$

$$FS\ (\%) = \frac{V_{F30}}{V_{F0}} \times 100$$

where V_L, V_{F0},and V_{F30} represent the volume of non-whipped WPI solution, the volume of the foam immediately after whipping (time = 0 min) and after standing at room temperature for 30 min, respectively.

1.2.11 Emulsifying capacity

Emulsifying capacity was evaluated according to the procedure described by Pearce and Kinsella (1978) with slight modifications. The emulsions were prepared by homogenizing a protein solution and 20% (v/v) soybean oil at room temperature using an IKA homogeniser (T18 basic, IKA-Werke GmbH and Co., Staufen, Germany) at 20000 rpm for 2 min. Aliquots of the emulsions were diluted with 1 gL^{-1} SDS to give

final dilution of 1:500. The absorbance of the diluted emulsions was then determined spectrophotometrically in a 10-mm path length cuvette at 500 nm immediately after preparation.

1.2.12 Statistical analysis

ACP treatments were independently performed twice and all the analyses were repeated three times. The results were averages of at least six measurements and reported as means ± standard deviation (S.D.). Analysis of variance (ANOVA) was performed with significance level set to $p<0.05$ using R software (cran.r-project.org, ver. 3.0.1 for Windows). The Tukey's HSD test was used to test differences between means.

1.3. Results and discussion

1.3.1. Ozone concentration

The ozone is one of the gaseous component of plasma. Indeed, during ACP treatments the discharge generated when the high voltage is applied produces energetic electrons that dissociate oxygen molecules. The single O atom combines with oxygen molecule (O_2) to form ozone gas. Ozone is one of the most chemically stable and active species generated with ACP because of its relatively long halftime and high oxidation potential (2.07 V). For this reason, it was considered useful to assess the amount of ozone generated in our experimental conditions.
Table 1.1 reported the concentrations of ozone inside the package containing WPI solutions, measured immediately post- ACP treatments.

Table 1. 1 Ozone concentration (ppm) inside packages containing WPI solutions immediately after plasma treatment at 70 kV.

Treatment time (min)	Ozone (ppm)
0 (Control)	0
1	1200
5	1200
10	820
15	1200
30	1200
60	1800

As shown, the values were around 1200 ppm except for the longest treatment. The lower amount of ozone recorded after 10 min-treatment may be due to the generation and breakdown kinetics of ozone. However, the ozone concentrations remain very high.

1.3.2. Colour of WPI solutions

Whey protein isolate (WPI) solutions were treated with ACP for different times ranging from 1 up to 60 min. The foremost visually evident effect generated by the treatments was the change of colour, as can be seen in Figure 1.2. Accordingly, the b* value among the L*-a*-b* colour parameters, corresponding to yellow colour was significantly affected. Clearly, the increase in intensity of the yellow colour, is directly proportional to the duration of treatment and also significantly different compared to the control (p <0.05). After 60 min of treatment, the b* value is approximately thrice compared to untreated control sample. These changes clearly demonstrate the reactive formation of pigmented end product(s).

At first instance, the changes were suspected to be an outcome of limited non-enzymatic browning (NEB) reaction between amino acids and reducing sugars in the WPI solutions, which generated early-stage products of Maillard pathway, normally weakly yellowish or colourless (Hodge and Osman; 1976; Nursten, 2011). However, the initial pH of 6.8 at 15 °C, the negligible amount of lactose in the WPI (not detected via enzymatic assay), the maximum temperature of < 40 °C, all seemed to be conditions not favourable for the optimum development of Maillard's reaction (see Table 1.2 for details).

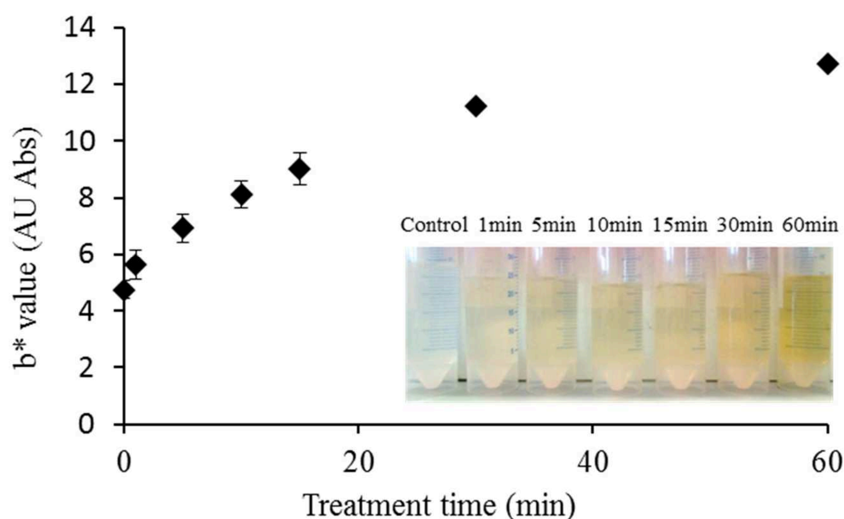

Figure 1. 2 Evolution of the b value of WPI solution as a function of the ACP treatment time*

The hypothesis of the Maillard reaction or any possible lipid peroxidation were excluded, the latter because the samples contained negligible amount of fat. Therefore, the observed change was solely attributed to the reaction between plasma species that diffused into the liquid and the WPI. It is suggested that ozone and other reactive oxygen species (ROS),

such as $^{\bullet}OH$, $HO_2^{\bullet}$, $^{\bullet}O_2^-$, $^{\bullet}O_3^-$, could act on aromatic rings of amino acid residues of whey proteins in the solutions.

Table 1. 2 Value of temperature (°C) of untreated and plasma treated WPI samples. Error values represent standard deviations.

Treatment time (min)	Final Temperature (°C)
0 (Control)	14.0 ± 1.5
1	22.5 ± 0.5
5	27.5 ± 1.0
10	30.0 ± 2.0
15	32.0 ± 2.5
30	35.0 ± 0.5
60	38.0 ± 2.0

Besides ozone, the plasma source employed has also been found to generate excited nitrogen species (NO and NO_X) (Misra et al., 2014a; Misra et al., 2015) which can diffuse into the liquid media to yield nitric and nitrous acids that react with proteins (Laroussi and Leipold, 2004). Finally, it is likely that the reaction(s) is/are favoured by a decrease in pH (see section 1.3.3), considering that the intensity of colour increased with treatment duration. Further experiments are desirable to obtain mechanistic insights into the actual reactions leading to the colour change.

1.3.3. Effects on pH

The pH of the control and ACP treated WPI solutions are tabulated in Table 1.3. From Table 1.3 it is clear that a slight decrease in pH occurs after plasma treatment, which is statistically significant ($p<0.05$), particularly after 60 min.

Table 1. 3 Value of pH of untreated and plasma treated samples at 70 kV. Error values represent standard deviations.

Treatment time (min)	pH
0 (Control)	6.8 ± 0.01[a]
1	6.8 ± 0.01[a]
5	6.7 ± 0.02[a,b]
10	6.7 ± 0.01[a,b]
15	6.6 ± 0.01[b]
30	6.4 ± 0.01[c]
60	6.2 ± 0.03[d]

a,b,c,d,e : means with the same letter in the column are not significantly different ($p > 0.05$).

As reported by Oehmigen et al. (2010) and Traylor et al. (2011) acidification could be explained as a consequence of the formation of nitrous acid (HNO_2) and nitric acid (HNO_3) from NO via NO_2, in addition to the generation of acidic H_3O^+ ions by the reactions of water molecules with hydrogen peroxide (H_2O_2) generated in air or liquid. Both Oehmigen et al. (2010) and Traylor et al. (2011) found a strong reduction in pH (from 7 to less than 4) in non-buffered physiological saline within 5 min of plasma treatment, followed by a slight decrease until stable pH values around 2-3 were reached within 30 min depending on sample volume (the decline in pH was higher for lower volumes). In this study the WPI was solubilised in a phosphate buffer (pH 6.8 and 50 mM) and 30 mL of the solution was subjected to ACP treatment which led to a drop in pH, although limited to 0.6 units. It may be noted that differences in acidity of plasma treated liquids may arise from several factors, including the volume treated, the buffering capacity and the type of plasma source and inducer gas employed.

1.3.4. Changes in carbonyl and free sulfhydryl groups

In general, protein oxidation could generate amino acid residue side-chain modifications and changes in protein polypeptide backbone, resulting in protein fragmentation, cross-linking, unfolding and conformational changes (Davies, 2005; Stadtman, 2006; Shacter, 2000; Chen et al., 2013; Segat et al., 2014).

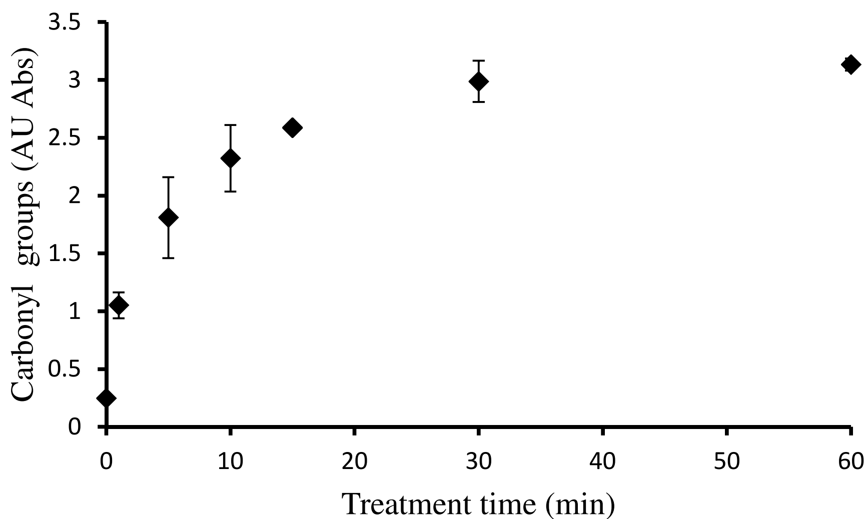

Figure 1. 3 Protein carbonyl groups as a function of the ACP treatment time

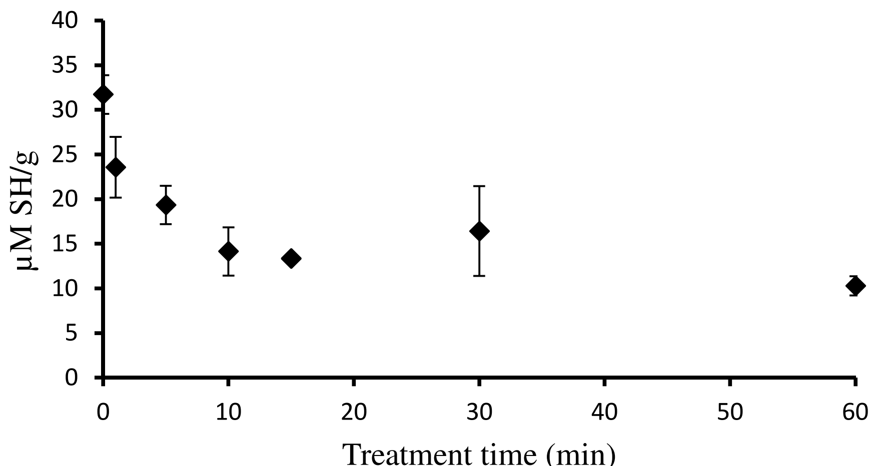

Figure 1. 4 Free sulfhydryl groups as a function of the ACP treatment time

In order to quantify the extent of protein oxidation, the amount of protein-bound carbonyl groups were assayed, the results for which are reported in the Figure 1.3. ACP treatment caused a substantial increase in protein carbonyl content. Non-treated samples (control) contained low amount of carbonyls. After plasma treatment the values dramatically increased; however, extended treatments (i.e. 30 and 60 min) did not lead to a significant increase ($p>0.05$) in the carbonyl content.

The formation of carbonyls could be attributed to the modifications of a number of amino acid side chain groups, especially with NH- or NH_2 or by peptide bond cleavages. Moreover, as recently reported by Cui et al. (2012), protein carbonyl formation is dependent on temperature, suggesting that in this case, the reactive species from plasma and the temperature rise have had a synergistic contribution to the release of carbonyls.

Plasma modification, furthermore, can trigger different changes in amino acid residue side-chains of which the sulfhydryl (-SH) groups are quite important. It has been well-recognised that the aromatic and sulphur-containing amino acid side chains are particularly vulnerable to oxidation and, cysteine seems to be very sensitive (Davies, 2005; Cataldo, 2003; Segat et al., 2014). Notably, the behaviour of this amino acid is also more important from a technological point of view since it is responsible for aggregation and most of the modifications occurring during food processing (Sava et al., 2005; Chandrapala et al., 2011; Arzeni et al., 2012; Shimada and Cheftel, 1988).

In Figure 1.4 the sulfhydryl group content is reported for proteins in untreated (control) and treated samples. Our results demonstrate a gradual reduction in the –SH group content during the first few minute of treatment, reaching a value of half that of initial after 10 min exposure to plasma. The trend clearly depicts that a near-equilibrium is reached after a steep decline and no further significant losses can be encountered. This also supports the previous observation made for carbonyls. In summary, it can be said that ACP induces the loss of –SH groups from amino acid cysteine present in the protein structure. The reduction of –SH groups implies the formation of disulphide (-S-S-) cross-

links, both intra or inter molecular, and/or an irreversible effect leading to formation of sulfinic and sulfonic acids (Eaton, 2006).

1.3.5. Changes in surface hydrophobicity exposure

The overall changes mentioned above lead to the modification of protein structure and to better elucidate all the ACP effects the Surface Hydrophobicity index was also calculated and reported in Figure 1.5.

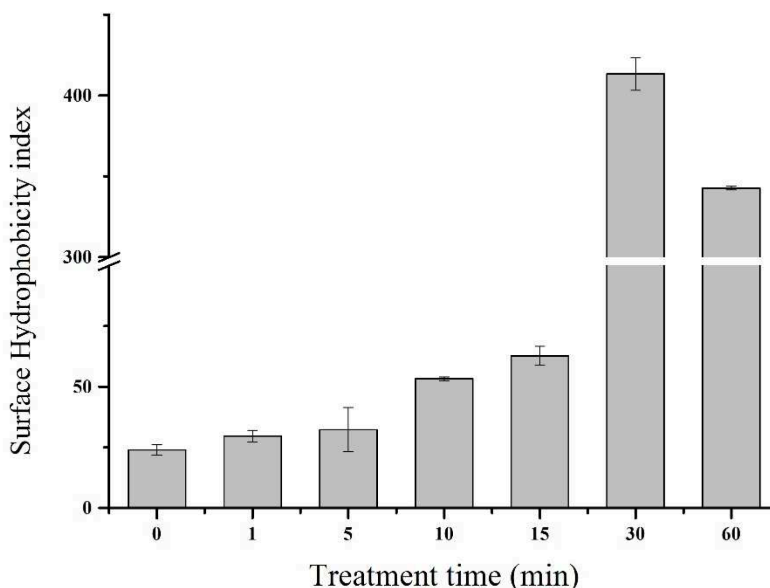

Figure 1. 5 Surface hydrophobicity index as a function of ACP treatment time

Data show a significant although slight increase of values until 15 min of treatment (minor structural changes) and a remarkable increasing for 30 and 60 min. It is noted that the surface hydrophobicity is an indicator of protein structural unfolding. Indeed, this test was conducted using a fluorescent probe, (8-anilino-1-naphthalene sulfonic acid – ANS) that is known to bind hydrophobic areas of proteins accessible to the aqueous solvent (Gaucheron et al., 1997; Ali et al., 1999; Kato and Nakai, 1980) resulting in a fluorescence increasing. We can then assume that the hydrophobic amino acid residues normally buried inside the protein structure during ACP treatments have exposed themselves and therefore they were more accessible to the binding. The strong impact of ACP processing for the longest treatments (30 and 60 min) was probably due to a general molecular unfolding that can also justify the lower value in 60 min than 30 min-treatment.

1.3.6 DLS and HPLC analysis

To confirm the hypothesis of structural changes, the polydispersity index (PdI) and mean particle size distribution were carried out on diluted control solution and ACP solutions. Results are summarized in Table 1.4.

Table 1. 4 PdI (polydispersity index) and mean particle size of WPI solutions processed with ACP at 70 kV for different time

Treatment time (min)	PdI	Size (nm)
0 (Control)	$0.313^{c} \pm 0.056$	$189.8^{b} \pm 0.003$
1	$0.398^{b} \pm 0.079$	$190.0^{b} \pm 0.320$
5	$0.294^{c} \pm 0.100$	$180.2^{b} \pm 0.250$
10	$0.310^{c} \pm 0.052$	$179.3^{b} \pm 0.103$
15	$0.273^{c} \pm 0.029$	$178.4^{b} \pm 0.120$
30	$0.965^{a} \pm 0.050$	$224.1^{a} \pm 0.001$
60	$0.883^{a} \pm 0.002$	$220.6^{a} \pm 0.083$

a,b,c,d,e : means with the same letter in the column are not significantly different ($p > 0.05$).

As reported, the changes in the PdI and mean particle size of WPI solutions treated by ACP did not differ significantly up to 15 min of treatment when compared to the control. Beyond that time, both size and polydispersity index changed. The first one slight increased. However, the PdI of 30 and 60 min-ACP treatment substantially increased; it may be due to a general unfolding of the protein solution and a possible network among proteins. In order to confirm this hypothesis, the HPLC profiles for control and ACP treatments were recorded and reported in Figure 1.6.

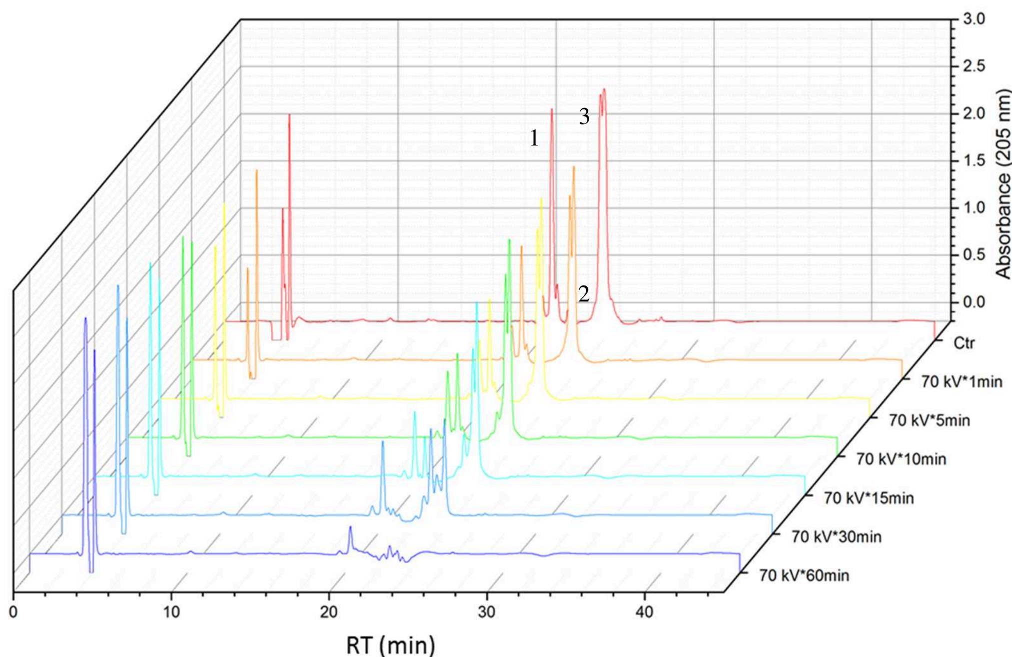

Figure 1. 6 RP-HPLC profiles of control and ACP treated samples as a function of treatment time. Numbers correspond to: 1) α-La, 2) BSA, 3) β-Lg.

The chromatographic profile for control solution reports the two large peaks eluting at c.a. 21 and c.a. 24 min represent α-La and β-Lg (with the variants B and A), respectively, and a small peak of BSA (c.a. 22 min), as confirmed by standards. Interestingly, the elution peaks showed major changes after ACP from 1 up to 15 min, to become quite impossible to recognise the main proteins in the solutions after 30 and 60 min of ACP treatment. Considering the findings, it can be assumed that ACP may generate aggregation among proteins (in agreement with particle size distribution results reported above) not clearly detected with the HPLC analysis parameters adopted here. In fact, the centrifugation prior HPLC test could have removed some soluble aggregates that lead to a different result.

1.3.7 Foaming and emulsifying properties changes

It is well established that the functionality of the whey proteins is strongly influenced by the processing conditions that can affect in different way both the structure and the chemical-physic parameters of proteins (Segat et al., 2014; Biasutti et al., 2010; Venir et al., 2010; George et al., 2013; De Wit, 1990; Arzeni et al., 2012; Xu et al., 2011). To this regard, the evaluation of the impact of ACP treatment on whey protein functionality takes

on a main importance for a technological point of view especially for a practical application in an industrial scale.
The foaming and emulsion abilities were carried out on the solutions (Figure 1.7).

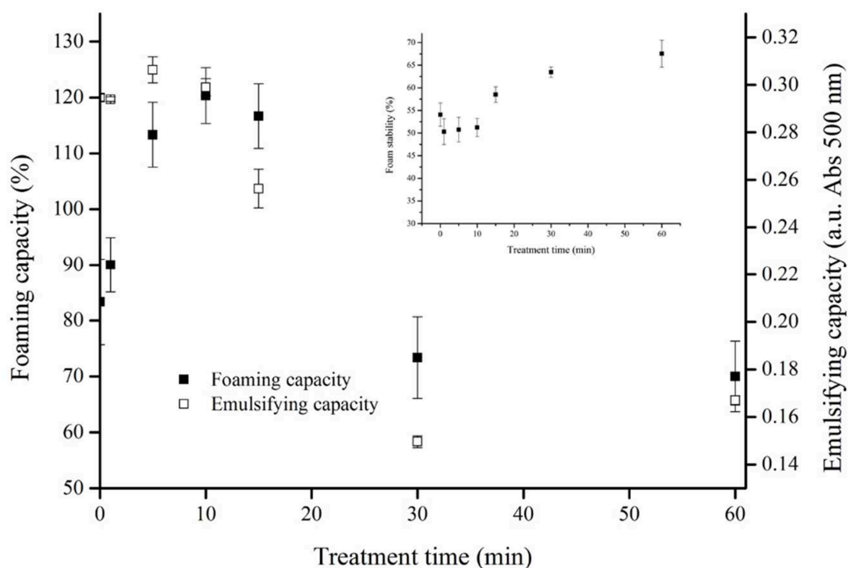

Figure 1. 7 Foaming and emulsion capacity as a function of ACP treatment time. Inset: foam stability as a function of ACP treatment time

As reported in the Figure, the ability to create foam improved during ACP treatment within 15 min. The general unfolding of the proteins created during the first minutes of treatment may have allowed generating a more flexible structure able to arrange itself at the air–water interface to form the overrun. In fact, according to Kinsella (1981), unfolding and partial surface denaturation involve a protein structure modifications followed by a rearrangement of molecules in the film to attain the lowest free energy conformation. This data were also in agreement with those reported in the previous work (Segat et al., 2014). However, for 30 and 60 min of treatment, the values dramatically reduced. This behaviour could be explained by the formation of aggregates among proteins, as confirmed by the free SH group analysis, size distribution test and HPLC profiles, that seems to play a negative role in the foam formation. The foam behaviour has been intensively studied by different Authors under different processing conditions and for some extent it is not enough clear (Kinsella, 1981; Zhu and Damodaran, 1994; Nicorescu et al., 2009; Fernández et al., 2012; Horozov, 2008; Croguennec et al., 2006; Bouaouina et al., 2006; Uzun et al., 2012). Many parameters are involved in the capacity to foam formations and in this study the first hand information obtained suggested that a relatively short time treatment was able to improve the functionality of the model system.

However, conversely to foam formation, the presence of aggregates and all the changes occurred can explain the more stable foams created with the solution of the longest ACP treatments (see insert plot, Figure 1.7). Rouimi et al. (2005) observed that there is a clear relationship between surface elasticity and foam stability. In particular, a high surface elasticity improves foam stability, interfacial films become more resistant and the protein network constitutes a mechanical barrier towards rupture of the bubbles and coalescence. Therefore, our results on stability could be related to the presence of the aggregates to the extent that the structure and protein network formed films with high rigidity due to the high packing density and strong intermolecular interaction (Dickinson, 1999; Foegeding and Davis, 2011). These results were also in agreement with Uzun et al. (2012) and their work regarding the effects of ozone on functional properties of whey proteins.
Similar trends were also obtained in the emulsion capacity although the differences among samples were very small. Probably the behaviour of these ACP- treated protein solutions exhibited more affinity in a water-air instead of water-oil system.

1.4. Conclusions

This work dealt with the interaction between a model system of whey protein isolate (WPI) solutions and the atmospheric pressure cold plasma (ACP). The treatments ranged from 1 to 60 min. Results showed a remarkable increase of yellow colour on the solutions and a slight decrease of pH for the action of ROS (reactive oxygen species) and RNS (reactive nitrogen species), respectively, released during the treatments.
Moreover, plasma generated overall changes within 15 min of treatment as a result of a "mild" oxidation occurred to the proteins. These modifications included the increase of carbonyls groups and the surface hydrophobicity that pointed out the effects on amino acid residues of the WPI. Furthermore, the reduction of free SH groups were correlated to the aggregation among proteins or a strong oxidative effect on cysteine.
Beyond this time, for the longest treatments (i.e. 30 and 60 min) the effects were more noticeable. In particular, the size distribution showed a slight increase and the polydispersity index suggested a general unfolding of the proteins due to a rate of aggregation. The changes in the structure via oxidative approaches is extremely relevant and of current interest because can affect the protein functionality. In this work the foaming and emulsifying capacity improved, however additional trials should be carried out to studies in depth all the consequences on functionality.
The results obtained from this work were important to understand the effects of plasma on biomolecules, to elucidate some mechanisms and create the basis to predict some behaviour when ACP is used for different purposes in a real food.

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Chapter 2

FTIR spectroscopic study of atmospheric pressure cold plasma treated whey protein isolates

ABSTRACT

Whey protein isolates (WPIs) were subjected to atmospheric pressure cold plasma (ACP) discharge. Two voltages (50 and 70 kV) and three discrete times (1, 5, and 10 min) were selected. FTIR spectroscopy was performed, in order to study the impact of ACP on secondary structure of WPI powder. The second derivate of amide I region of the spectra was obtained and the modifications in protein secondary structures were identified. The results show that the β-structures are relatively more stable compared to the α-helix. Changes in the α-helical structure were evident, which were significantly affected by treatment time ($p<0.05$) and the mean helical content steadily increased at 50 kV. However, no definite trend was recorded for samples treated at 70 kV.

2.1. Introduction

Fourier Transform Infrared (FTIR) spectroscopy is a very versatile technique, used for many years in biochemistry to investigate the molecular structure in different physical states (Lefèvre and Subirade, 2001). It is also useful to measure components in mixtures. It is rapid, non-destructive, and does not require the separation of the components before measurement (Mendenhall and Brown, 1991). That IR spectroscopy could provide information on the secondary structure of proteins was first demonstrated by Elliot and Ambrose (1950), Ambrose and Elliot (1951), and Elliot (1954). They showed the empirical correlation existing between the frequency of the so-called amide I and amide II absorptions of a protein and the predominant secondary structure within the protein as determined by X-ray diffraction studies (Jackson and Mantsch, 1995). Amide I, amide II as well as amide III are the modes most widely used in protein structural studies. The amide I band arises principally from the C=O stretching vibration of the peptide group. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations. The amide III absorption is normally very weak in the infrared, arising primarily from N-H bending and C-N stretching vibrations (Haris and Severcan, 1999).
Moreover, the common infrared data processing methods in proteins structure analysis are deconvolution and second-derivative analysis. These methods unravel peaks that are not readily visible in the original protein peaks but will also enhance other spectral features including background noise and water vapour absorbance (Haris, 2013)
Atmospheric pressure cold plasma (ACP) as a new method in food processing has been studied not only for the microbiological control of samples (Ziuzina et al., 2013; Schwabedissen et al., 2007; Eto et al., 2008; Klockow and Keener 2009; Chiper et al., 2011; Leipold et al., 2011; Rod et al., 2012; Wang et al., 2012) but also for other purposes that exploited the effects generated on biomolecules by all the plasma species produced when a gas (or a mixture of gases) is exposed to an electric field (Bogaerts et al., 2002; Laroussi and Leipold, 2004; Tendero et al., 2006; Wan et al., 2009; Ragni et al., 2010).
Asandulesa et al. (2013) for example, investigated the chemistry of polymerization mechanism related to the formation of plasma-polymerized film. In particular, these authors studied the plasma polymerization reactions of aromatic compounds using a cold atmospheric pressure glow discharge working in helium as initiator of the reaction. They revealed, using different spectroscopic techniques, reactions that could be possible during plasma polymerization process: activation and dissociation of organic compounds followed by poly-recombination of reactive species into randomly structured.
Pankaj et al. (2014) characterized biodegradable corn zein film. They studied the effects of atmospheric cold plasma treatments on surface, structural, thermal, and moisture sorption properties of this polymer. Plasma treatment increased the surface roughness and equilibrium moisture content of the zein film in a direct relationship with the applied voltage level. No significant difference in the thermal stability of the zein film is also observed after plasma treatment. Moreover, the application of the dielectric barrier

discharge (DBD) plasma treatments of zein film lead to a change in the protein conformation which is confirmed by X-ray diffraction and FTIR spectroscopy.
More recent, Misra et al. (2015) investigated the effect of ACP treatments on the properties of hard and soft wheat flour and the results indicated that ACP can be exploited as a mean to modulate functionality of wheat flour. In fact, the rheological properties of flours were studied using mixogram and oscillation rheometry. Mixographs revealed an improvement in the dough strength and optimum mixing time for both strong and weak wheat flours. The elastic and viscous moduli of strong wheat flour progressively increased with applied voltage and treatment time. A significant variation in the tan was not found. Changes in the secondary structure of proteins were evaluated using FTIR spectroscopy and revealed a decrease in β-sheets and increase in α-helix and β-turns, for both strong and weak wheat flour.
Based on these premises, the aim of this work was to investigate the effects of ACP on Whey Protein Isolate (WPI) powder, ingredient in several foods including infant foods, beverages, bakery products, dairy foods, muscle foods, and snack bars (de Wit, 1998; Morr and Ha 1993; Lucena et al., 2008), wherein it improves the nutritional value and functional properties of formulated foods. To this regard, to study the impact of ACP on secondary structure of WPIs, the FTIR spectroscopy was performed.

2.2. Materials and Methods

2.2.1. Materials

Whey protein isolate was obtained from a commercial supplier (see Chapter 1 for details on the chemical composition). FTIR grade potassium bromide (KBr) was obtained from Sigma-Aldrich, Ireland.

2.2.2. ACP treatments

The WPI samples (2 g) were placed in the Petri dishes of 50 mm diameter, packaged inside polyethylene terephthalate (PET) trays and then subjected to ACP treatments. The plasma source and experimental set-up has been described elsewhere (Misra et al. 2014a). Briefly, it is comprised of to two aluminium disc electrodes of 15 cm-diameter, over two polypropylene dielectrics (2 mm thick) between which the PET package with samples is placed. It may be noted that the trays, with wall thickness of 270 µm, also served as dielectric barrier. The distance between the two dielectrics was 44 mm, equal to the height of the trays. The voltage across the electrodes was monitored on an InfiniVision 2000 X-Series Oscilloscope (Agilent Technologies Inc., Santa Clara, CA, USA) using high voltage probes coupled to voltage dividers. All experiments were performed at 50 and 70 kV for 1, 5 and 10 minutes using ambient air and atmospheric pressure conditions. After treatment, the samples were stored at room temperature for 12 h before further analysis. The powder samples were directly analysed by FTIR spectroscopy.

2.2.3. Ozone concentration measurement

Ozone concentration inside the package was measured immediately after treatments using Gastec ozone detection tubes (Gastec Corporation, Kanagawa, Japan). Further details regarding ozone measurement can be found in Misra et al. (2014b).

2.2.4. FTIR spectroscopy

A 3% w/w dilution of the powder samples was prepared by mixing 5 mg of the sample with 295 mg of dry potassium bromide (KBr). KBr pellets were prepared by exerting a pressure of 100 kg/cm^2 for approximately 2 minute in a pellet press (Specac, United Kingdom). Different pellets were prepared from the same sample and their spectra were recorded (spectra were nearly identical). The IR spectra were collected in absorbance mode using a Nicolet Avatar 360 FTIR E.S.P. (Thermo Scientific, Waltham, MA, USA) over the frequency range 4000-400 cm^{-1}. Three pellets were prepared for each sample, and two scans of each pellet were collected at 2 cm^{-1} resolution at room temperature using OMNIC software (version ESP 5.2). The spectra collected were the average of 64 measurements and were also corrected for background noise. All spectra were baseline corrected using OMNIC software (version ESP 5.2).

2.2.7. Statistical Analysis

Every ACP treatment was performed twice and all the analyses were repeated three times. The Tukey's HSD test was used to test differences between means. Statistical analysis was performed with significance level set to $p<0.05$ using R software (cran.r-project.org, ver 3.0.1 for Windows).

2.3. Results and Discussion

2.3.1. Functional group assignments

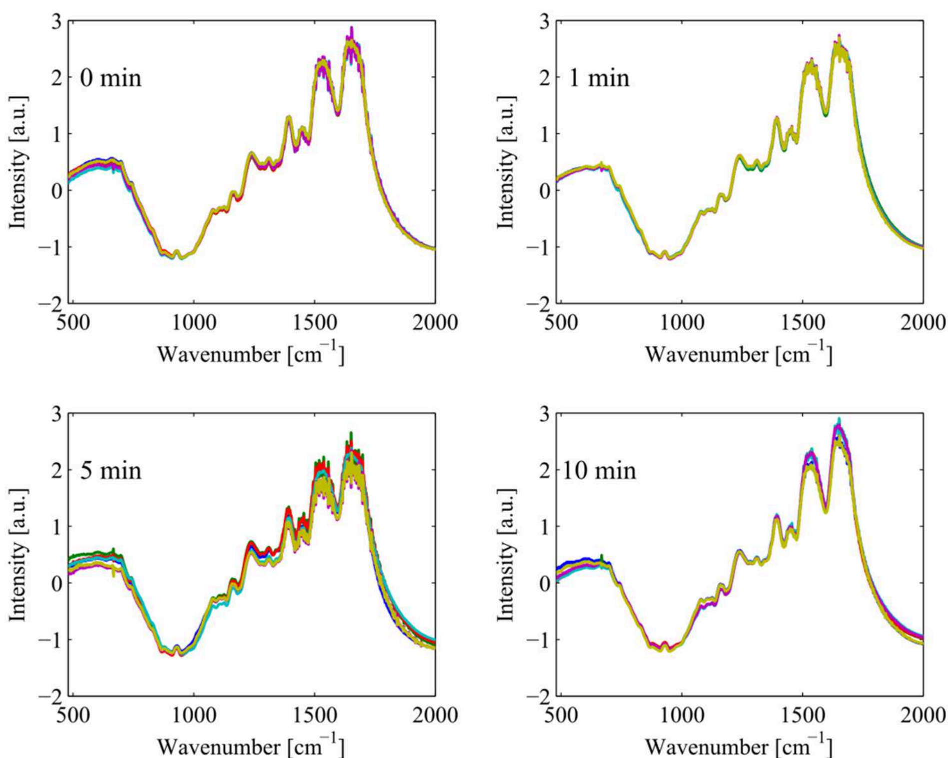

Figure 2. 1 Representative FTIR spectra of the control and ACP treated (50 kV) WPI powder.

Figure 2.1 provides representative FTIR spectrum of ACP treated and control (0 min) whey protein (only 50 kV shown here). The vibrational bands in the region 1605 to 1650 cm^{-1} originated from C=C stretching, while the 1415-1460 cm^{-1}, 1390-1425 cm^{-1} and 2855-3000 cm^{-1} bands from C-H bending of alkyl groups. The peaks in the region 1690-1730 cm^{-1} were assigned to C=O stretching. The 690-750 cm^{-1}, 1500-1530 cm^{-1} and 3060-3120 cm^{-1} windows were characterised by multiple peaks from N-H stretch of amino acids. The 1310-1350 cm^{-1} band originated from C-N vibrations. The strong vibrations of the carboxylate groups are evident from the C-O stretch vibrational peaks in the region 1360-1440 cm^{-1} and 1560-1605 cm^{-1}. The bands from amino acid side chain vibrations due to tyrosine at about 1515 cm^{-1}, phenylalanine at about 1499 cm^{-1}, proline at about 1452 and 1437 cm^{-1} were also notable. The carbonyl vibrations of the protein backbone were also noticeable between 1700 cm^{-1} and 1600 cm^{-1} (amide I region) and were further analysed for probing changes in the secondary structure of the proteins. The amide I band of proteins consisted of many overlapping component bands that

represented different structural elements such as α-helices, β-sheets, turns and non-ordered or irregular structures (Haris and Severcan 1999).

2.3.2. Protein secondary structure

The most commonly used infrared spectral data processing methods in proteins structure analysis are deconvolution and second-derivative analysis. These methods help identifying peaks that are not readily visible in the original spectrum. In the present work, no attempt was made to deconvolute the spectra considering the presence of highly overlapped regions. However, the second derivative spectrum was used for calculating the relative content of protein secondary structures. Figure 2.2 provides a snapshot of the changes in second derivative spectrum of ACP treated whey protein within the amide I region. The shaded regions across the spectrum indicate the upper and lower bounds of the variation over eight repetitions of individual measurements. While differences among the spectra are evident in terms of the relative intensity, the position of the peaks and troughs do not change among the samples.

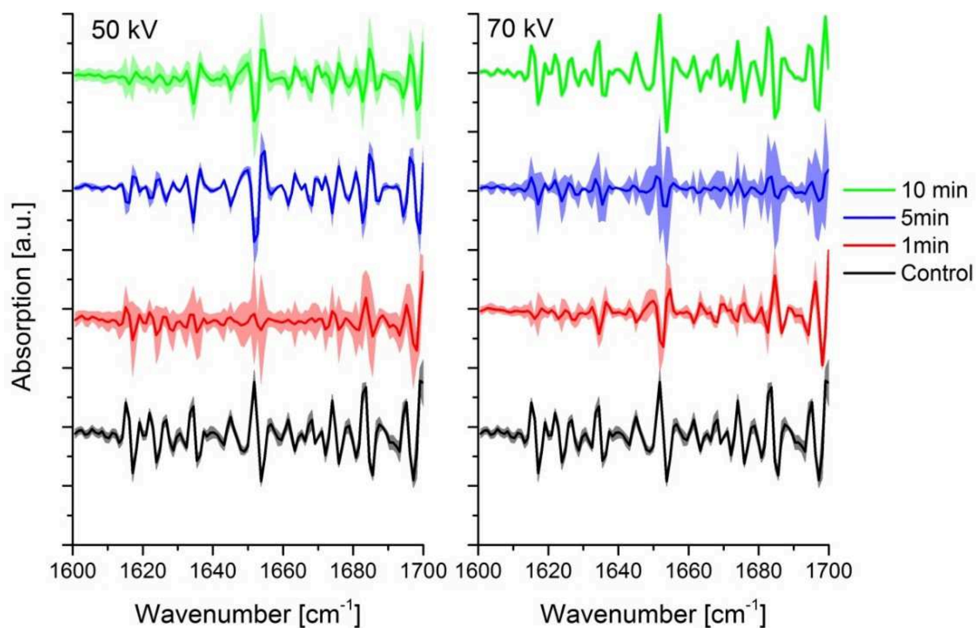

Figure 2. 2 Effect of plasma treatments on second derivative spectrum of whey protein isolate in the amide I region.

The modes most widely used in protein structural studies are amide I, amide II and amide III. The amide II band is primarily N-H bending with a contribution from C-N stretching vibrations. The amide III absorption is normally very weak in the infrared, arising primarily from N-H bending and C-N stretching vibrations (Haris and Severcan, 1999). The amide I band arises principally from the C=O stretching vibration of the peptide

group (ca. 80%) and widely adapted for analysis of the changes in secondary structure content, as also in the present study. The results of quantitative analysis based on integration of the second derivative spectrum are summarised in Figure 2.3. The FTIR data revealed no significant changes in the content of β-sheet and β-turn structures of proteins. This indicated that the ACP treatments of WPI did not affect the intermolecular hydrogen bonding of the β-sheets. This result is in agreement with previous studies regarding ozone treatment of WPI powder (Segat et al., 2014). It may be noted that the plasma source employed here is also a source of ozone (1200 ppm ± 10%), along with nitrogen oxides (Patil et al., 2014).

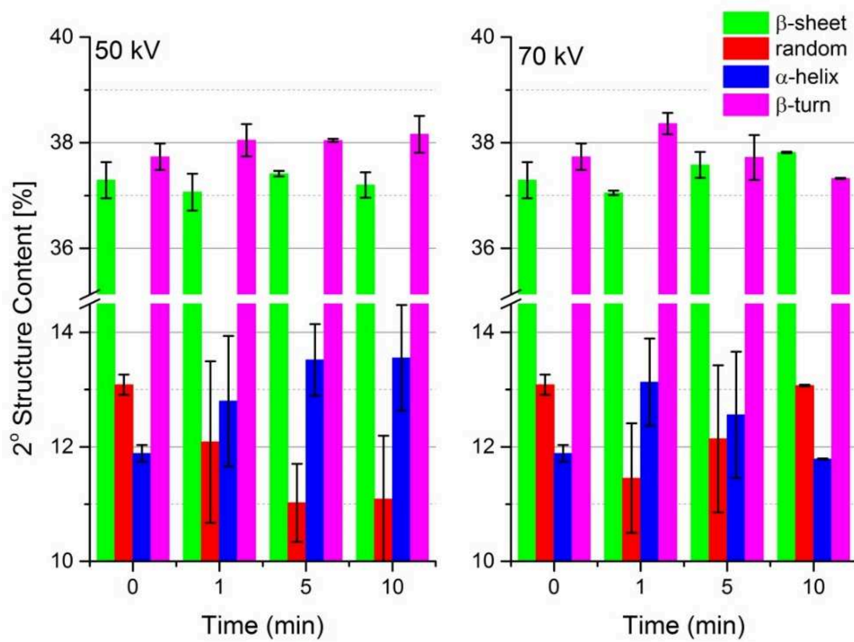

Figure 2. 3 Secondary structure content of ACP treated whey protein.

The relatively stable structure of the β-structures compared to the α-helix can be attributed to the less intermolecular hydrogen bonding in the former. Changes in the α-helical structure were evident, which was significantly affected by treatment time ($p<0.05$) and the mean helical content steadily increased at 50 kV. However, no definite trend was recorded for samples treated at 70 kV, and is expected to be an outcome of the complex changes in plasma chemistry. The WPI used in this study is largely constituted of β-lactoglobulin, whose secondary structure contains a helix located on the surface of the molecule, as can be observed in Figure 2.4. This topological arrangement by itself explains the relative instability of the helical structure which is more prone to attack by the chemically active species of ACP.

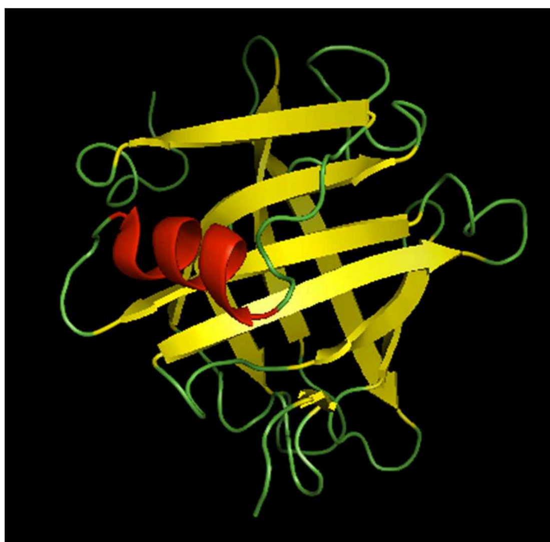

Figure 2. 4 Cartoon of bovine β-lactoglobulin protein structure (drawn using PyMol; file retrived from protein database, http://www.rcsb.org/pdb/home/home.do).

The red regions correspond to the α-helical structure, the yellow region indicate the parallel and anti-parallel β-sheet structures, whereas the green represent the random coil structures.

2.4. Conclusions

The structure modifications of whey protein isolate (WPI) in powder subjected to the atmospheric pressure cold plasma (ACP) at different voltages (50 and 70 kV) and times (1, 5 and 10 min) were investigated by FTIR spectroscopic study. After the functional group assignments, secondary structures of proteins were evaluated. The data showed that ACP treatments did not affect the intermolecular hydrogen bonding of the β-sheets that resulted relatively stable compared to the α-helix. It can be attributed to less intermolecular hydrogen bonding in the former. Changes in the α-helical structure were evident, which were significantly affected by the treatment time ($p<0.05$), and the mean helical content steadily increased at 50 kV. However, no definite trend was recorded for samples treated at 70 kV.

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Chapter 3

Effect of atmospheric pressure cold plasma on activity and structure of alkaline phosphatase

ABSTRACT

Alkaline phosphatase (ALP) enzyme in solution was treated with atmospheric cold plasma (ACP) at three discreet high voltages (40, 50 and 60 kV) for durations ranging between 15 sec to 5 min. The residual activity kinetics and the circular dichroism spectroscopy analyses were performed, while the temperature and pH measurements on the enzyme solutions were also carried out. Results demonstrated that the dielectric barrier discharge based plasma technology was able to inactivate the enzyme within a few seconds. Kinetic models, namely first order, Weibull and logistic models were fitted to the experimentally observed data and the model parameters were determined. The Weibull model was found to best describe the observed variance in residual activity for all the voltages applied. The dichroic spectra suggested that the enzyme was characterized by a predominance of α-helix structure, and the helical content showed a tendency to decrease with increase in treatment time and voltage. The maximum temperature recorded for most intense treatments was in the order of only 30 °C and no change in pH was noticed.

3.1. Introduction

Within recent years, nonthermal technologies have been widely studied regarding their efficacy for inactivating enzymes, either singularly or in combination with mild heat treatments. Particularly, several researchers have suggested that high pressure technology, ultrasound, ultraviolet (UV), pulsed electric field (PEF) and pulsed light technology are capable of inactivating enzymes, both in model systems and in real food matrices (Ho et al., 1997; Huang et al., 2012; Manzocco et al., 2009; Navarro et al., 2014; O'Donnell et al., 2010).

Atmospheric pressure cold plasma (ACP) is relatively a new nonthermal technology which has been reported to efficiently inactivate microorganisms including bacteria, bacterial spores, fungi, and biofilms in model media and food systems (Hashizume et al., 2013; Hashizume et al., 2015; Ishikawa et al., 2012; Jahid et al., 2013; Misra et al., 2014d). ACP consists of highly energetic species in permanent interaction including photons, electrons, positive and negative ions, neutrals, free radicals and excited or non-excited molecules and atoms (Bárdos and Baránková, 2010; Misra et al., 2011; Pankaj et al., 2014). In ACP, cooling of ions and uncharged molecules is more effective than energy transfer from electrons and the gas remains at low temperature (Fridman et al., 2008; Misra et al., 2014a). ACP may be induced in a gas by subjecting the gas (or gas mixture) to an electric field, which in turn accelerates the charged particles leading to collisions with the heavy species (e.g. ions and neutrals).

In a recent study, ACP was investigated for *E. coli* inactivation efficacy in milk samples with different fat contents and it is was found that the treatment (from 1 to 20 min) significantly reduced the number of colonies in different types of milk (whole, semi-skimmed, skimmed) without inducing negative effects on pH and colour (Gurol et al., 2012). In yet another recent study, Cavalcante et al. (2013) evaluated ozone gas as a new preservation method for raw milk in combination with mild thermal treatment to reduce the microbial load and improve the shelf-life of milk during storage. Observing other parallel developments, ACP has also been found to reduce the activity or inactivate a range of enzymes such as lysozyme (Takai et al., 2012), polyphenol oxidase and peroxidase (Pankaj et al., 2013; Surowsky et al., 2013; Tappi et al., 2014). Their findings suggested that hydroxyl radicals (OH), superoxide anion radicals ($^{\bullet}O_2$), hydroperoxyl radicals ($HOO^{\bullet}$) and nitric oxide (NO) chemically react with the side-chains of the amino acids, including cysteine, aromatic rings of phenylalanine, tyrosine and tryptophan to induce structural changes, ultimately leading to the inactivation of the protein. Moreover, the atomic oxygen and particularly $^{\bullet}OH$ radicals could also cleave peptide bonds, oxidize amino-acid side chains and form protein-protein cross-linkages.

However, there have been no studies to evaluate the effect of ACP on enzymes in milk. Milk contains about 70 indigenous enzymes, which are minor but very important members of the milk protein system (Fox and Kelly, 2006). Some of them are significant for several reasons including technological relevance. Alkaline phosphatase (ALP, orthophosphoric mono-ester phosphohydrolase, EC 3.1.3.1) is an enzyme naturally

present in blood and milk of all mammals (Fox and McSweeney, 1998). It is well-known that ALP enzyme has z-value similar to heat-resistant pathogens and its activity is routinely evaluated to measure the efficacy of pasteurization process in industrial milk processing (Rankin et al., 2010). ALP activity in pasteurized milk generally indicates inadequate pasteurization, and the presence of ALP activity may be due to the contamination with raw milk or post-bacterial contamination. Therefore, ALP represents a good model system for evaluating the efficacy of ACP against inactivation of enzymes endogenous to milk.

Based on these premises, the goal of the present study was to evaluate the effects of ACP process variables upon the activity of alkaline phosphatase (ALP). The specific objectives of this study were to: (1) mathematically model the ACP mediated inactivation kinetics of ALP; (2) obtain mechanistic insights into the process by CD spectroscopy and determine the structural changes to the enzyme.

3.2. Materials and Methods

3.2.1 Materials

The alkaline phosphatase (ALP) enzyme used was a commercial lyophilized powder from bovine intestinal mucosa (P7640, Sigma-Aldrich, Italy). The lyophilized powder was dissolved in phosphate buffer (50 mM, pH 6.8) at a concentration of 250 mg/L and it can be stored at 5 °C for up to five days. For the experiments, an aliquot of this solution was diluted in phosphate buffer to give a final concentration of 0.38 mg/L; see c.f. Aguiar et al. (2012). All chemicals used were obtained from Sigma-Aldrich, Italy, unless explicitly specified.

3.2.2 Atmospheric Cold Plasma (ACP) treatment

The ACP source was based on a DBD plasma source, further details regarding which can be found elsewhere (Misra et al., 2014b; Misra et al., 2014c). Briefly, it comprised of two circular aluminium plate electrodes (outer diameter = 158 mm) over polypropylene (PP) dielectric layers (2 mm thickness) between which a PP package, containing 15 mL of ALP enzyme solution in a petri plate was placed, see Figure 3.1. ACP treatment was carried out at 40, 50, 60 kV for durations ranging from 15 sec to 5 min, at room temperature. Immediately after the treatments, the residual activity of enzyme was evaluated and the circular dichroism (CD) spectroscopy was performed.

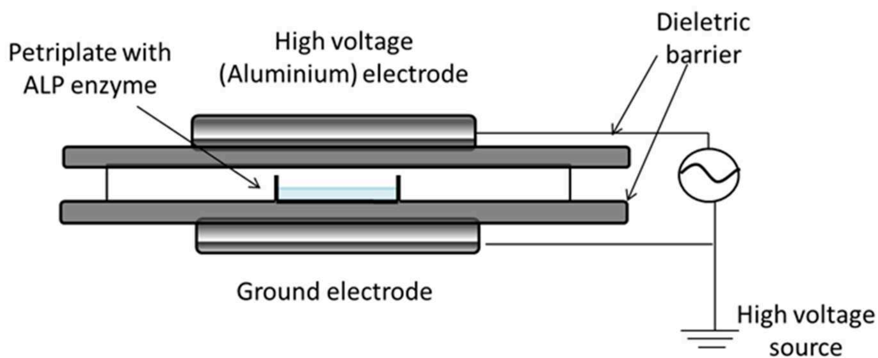

Figure 3. 1 Schematic of the experimental setup for DBD plasma system.

3.2.3 Activity assay for alkaline phosphatase

The activity of ALP was determined according to FIL-IDF 82A, 1987 and Ludikhuyze et al., (2000). Untreated enzyme was used as control. 300 µL of control and ACP enzyme solution was added to 3 ml of substrate solution (4 mM of p-nitrophenyl di-sodiumphosphate in sodium carbonate buffer) and incubated for 2 h at 37 °C to develop the reaction leading to yellow colour. Subsequently, 0.1 ml of zinc sulphate (1.05 M) was added to the test tube, shaken and allowed to stand for 3 min. After addition of 0.1 ml of potassium hexacyanoferrate (0.36 M), the solution was filtered and the absorption was determined using a spectrophotometer at 425 nm (Shimadzu UV 1800, Shimadzu Scientific Instruments). The residual activity was calculated according to the following equation-

$$Residual\ Activity\ (\%RA) = \frac{A_t}{A_0}.100 \quad (1)$$

where, A_t is the residual activity of ALP solution at time t and A_0 is the residual activity of ALP solution at time 0 s (control). A_t and A_0 were determined immediately after plasma treatment to avoid any effect of storage time.

3.2.4 ALP inactivation kinetics

Experimental data was modelled using three different inactivation kinetics equations viz. the first-order, Weibull and logistic equation. The first-order inactivation model is given by the following equation-

$$RA = RA_0 * e^{-Kp*t} \quad (2)$$

where, the inactivation rate constant K_p was obtained by least squares non-linear regression.

The Weibull model fitted to the data is given by-

$$RA_t = RA_0 * e^{-(\frac{t}{\alpha})^{\beta}} \quad (3)$$

where, RA_t is the percentage of residual activity of ALP at time t, RA_0 (%) is the residual activity without any treatment (100%), t is the treatment time (min), α is the scale factor (min) and β is the shape parameter (dimensionless) which indicates the shape of the curve.

The logistic model was specifically evaluated considering the visual shape of experimental data-

$$RA = \frac{(100 - A_{min})}{1+(\frac{t}{t_{50}})^{P}} \quad (4)$$

where, A_{min} (≥ 0) is the minimum value attained by the logistic function, t_{50} is the time for half maximal activity and P is the power term. This equation represents a sigmoidal type of inactivation curve.

3.2.5 CD spectroscopy

Far-UV CD spectrum (200 to 250 nm) of the ALP was recorded using a Jasco Model J-810 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan). The samples were analysed at room temperature with a constant nitrogen flow in a 1.0 mm optical path length quartz cell. The enzyme solutions were analysed without further dilutions. Each CD spectrum was the accumulation of 5 scans at 50 nm/min with a 0.5 nm slit width. The spectrum of phosphate buffer was used as a blank and subtracted from the average of two spectra to obtain a corrected spectrum expressed as CD (mdeg) for each sample. Spectra were recorded in duplicate. The spectrum was used for estimating changes in the secondary structure of the enzyme and analysed using chemometric approaches.

3.2.6 Measurement of pH and temperature

Temperature of solutions was measured before and immediately after ACP treatments using a handheld infrared thermometer with laser pointer (Maplin Electronics, UK). The pH of the solutions and all buffers used were measured before and after treatments using a pH meter (Thermo scientific Orion 2-star, Benchtop, Thermo Fisher, Waltham, MA, USA).

3.2.7 Statistical and multivariate chemometric analysis

The results are averages of at least three measurements taken from different samples and are reported as means ± SD. The Tukey's HSD test was used to test differences between means using R software (cran.r-project.org, ver 3.0.1 for Windows). The effect of treatment time and treatment voltage on residual activity of ALP was statistically explored by Tukey’s test in open-source R software (R Foundation for Statistical Computing, Vienna, Austria).

The model parameters for all equations were estimated by non-linear least squares regression using the *lsqcurvefit* numerical routine available in Matlab® (The MathWorks, MA, USA). The goodness of fit was assessed based on the adjusted coefficient of determination, R_{adj}^2 given in equation (5) along with an analysis of residuals.

$$R_{adj}^2 = 1 - \left(\frac{n_t - 1}{n_t - n_p}\right) \cdot \frac{SSE}{SSTO} \tag{5}$$

Herein, SSTO is the total sum of squared errors $\sum(y_i - \bar{y})^2$ and SSE the sum of squared errors, $\sum\left(y_{exp}(t_i) - y(t_i, p_{ls})\right)$. The root mean squared error (*RMSE*) was used as a criterion to evaluate the adequacy of model fittings; see equation (6).

$$RMSE = \sqrt{\sum_{i=1}^{n_t} \frac{\left(y_{exp,i} - y_{pred}\right)^2}{n_t - n_p}} \tag{6}$$

where $y_{exp,i}$ are experimental observations, y_{pred} are model predictions, n_t is the number of experimental data points and n_p is the number of estimated model parameters. Principal Component Analysis (PCA) and hierarchical clustering in principal component space were performed using *FactoMineR* package (Lê et al., 2008) for R software, for the CD spectral data matrix to assess the inherent grouping in the data.

3.3. Results and discussion

3.3.1 Residual inactivation

Following ACP treatments, a rapid inactivation of the enzyme was noticed at all the three applied voltages (Figure 3.2). After 120 sec of treatment, the activity losses were found around 45-50% for all the voltages applied, and beyond this time the differences were further minimized ($p>0.05$). However, independent of the voltage used, after 180 sec of treatment the activity of the enzyme was found to below 10%. Both treatment duration and applied voltage had a significant effect ($p<0.05$) on the inactivation of ALP. The interaction between voltage and time was also found to be significant ($p \leq 0.05$).

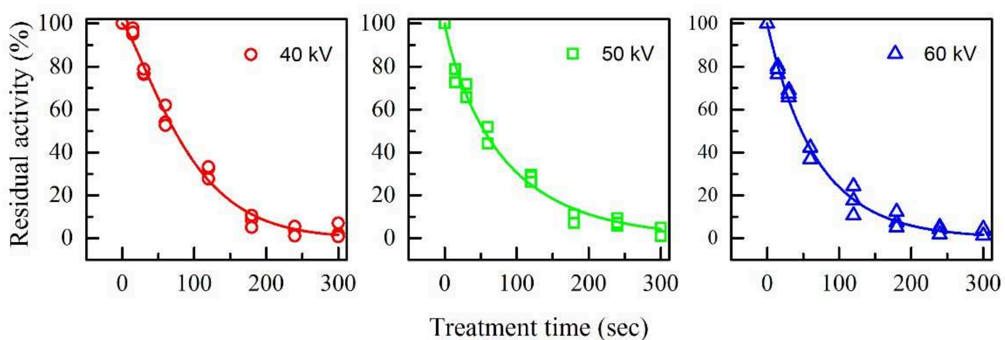

Figure 3. 2 Residual activity (%) of ALP and the Weibull model curve fitting at different voltage levels.

3.3.2. Kinetic modelling of inactivation

In order to study the interactions between the ACP treatments at 40, 50 and 60 kV and the phosphatase enzyme, the residual activity was evaluated and the experimental data was modelled using the first-order inactivation kinetics, Weibull and logistic model. The results of the model fitting, including the parameter values, the coefficient regression and RMSE are summarised in Table 3.1.

Table 3. 1 Results on the parameters of the models fitted to inactivation kinetics of ALP enzyme.

Model	**Parameter**	**Estimated Value**		
		40kV	50kV	60kV
First Order	K_p (sec^{-1})	0.0102 ± 0.0005	0.0118 ± 0.0004	0.0145 ± 0.0004
	R^2_{adj}	0.97	0.98	0.99
	RMSE	5.85	3.97	3.12
Weibull	α (sec)^a	97.15± 2.89	82.99± 3.14	69.23±2.18
	β^a	1.28 ± 0.06	0.89± 0.04	0.98±0.04
	R^2_{adj}	0.99	0.99	0.99
	RMSE	3.85	3.56	3.17
Logistic	A^a_{min}	0.0 ± 3.9	0.0 ± 6.6	0.0 ± 4.6
	t^a_{50}	66.5 ± 4.8	51.7 ± 7.8	47.1 ± 5.2
	P^a	1.9 ± 0.2	1.3 ± 0.2	1.4 ± 0.2
	R^2_{adj}	0.98	0.97	0.98
	RMSE	4.8	5.6	4.9
R^2_{adj} = regression coefficients; a = Value ± Standard Error				

The first-order kinetics is normally used to describe the inactivation rate of both microorganisms and enzymes. Many studies regarding enzyme activity in different food matrices and model solutions have approved this equation especially when thermal treatment is applied for denaturation (Claeys et al., 2002). However, the first-order model was found to be unsatisfactory for ALP inactivation by ACP, as reflected by the higher RMSE values. In view of the complexity of the structure of an enzyme and the possible variety of different phenomena involved in the inactivation, the assumption that the

disruption of a single structural element of the protein is sufficient to inactivate the enzyme, as proposed in first-order kinetics seems to be exceedingly simple (Adams, 1991; Pankaj et al., 2013). In fact, the inactivation of ALP seems to occur due to a two phase (or higher) structural change in the enzyme.

In previous reports related to inactivation of peroxidase with plasma a logistic sigmoidal function was found suitable (Pankaj et al., 2013). The visual shape of the inactivation data suggested a similar trend here. Therefore, the logistic model was also tried. However, based on the fact that higher the R^2_{adj} and lower the RMSE, better the model fits to experimental data, the Logistic model was also found inappropriate for the modelling ALP kinetics.

Figure 3.2 shows the Weibull model curve fitted to the experimental data at various voltages. The Weibull model exhibited a strong fit to predict residual ALP activity after ACP treatments as indicated by the high coefficients ($R^2_{adj} \geq 0.99$) and relatively low RMSE obtained for all voltage levels studied in this experiment. The Weibull equation was also found suitable in earlier studies of enzyme inactivation by various non-thermal technologies (Elez-Martínez et al., 2006; Giner et al., 2005; Odriozola-Serrano et al., 2008). In Weibull equation, if the shape parameter, $\beta > 1$ the curve is convex and forms shoulders, if $\beta < 1$ the curve is concave and forms tails and if $\beta = 1$ the curve is a straight line. The shape parameter (β) ranged from 0.89 to 1.28 for ALP. Only for the lowest voltage, the β value is higher than 1 (1.28 ± 0.06) indicating that the curve had a shoulder. It is most likely due to a lag phase in which ALP exhibits a resistance during the first 30 sec. To the contrary, β values for 50 and 60 kV treatments were 0.89 ± 0.04 and 0.98 ± 0.04 respectively and the curves had no shoulder; it may be noted that the value closer to 1 for the 60 kV indicates that the treatment effect could be estimated through a linear model for longer treatment durations (beyond 120 sec). The scale parameter (α) ranged from 69.2 to 97.2 and exhibited an inverse dependency on voltage levels i.e. the higher the applied voltage level, the lower was the scale parameter obtained.

3.3.3. Circular dichroism analysis

Circular dichroism (CD) is a commonly used technique for protein structure and dynamics studies (Hebbar and Raghavarao, 2007; Kong and Yu, 2007; Sreerama et al., 2000), which provides information about structural changes in proteins at a mesoscopic scale. Figure 3.3 depicts the CD spectra of control and ACP treated ALP for various ACP process variables employed. The CD spectrum of ALP showed distinct regions typical to common proteins. There was a negative band near 222 nm, followed by another negative band at 208 nm and a crossover at 202 nm. These characteristics of the spectrum for ALP is in close agreement with that reported in earlier literature for ALP in neutral pH conditions, where the two negative bands are observed at 220 nm and 209 nm respectively (Applebury and Coleman, 1969). The two negative peaks at 208 and 222 nm observed in the CD spectra are hallmark features of α-helical secondary structural elements, and the intensity of the peaks reflected the amount of α-helix structure in the proteins.

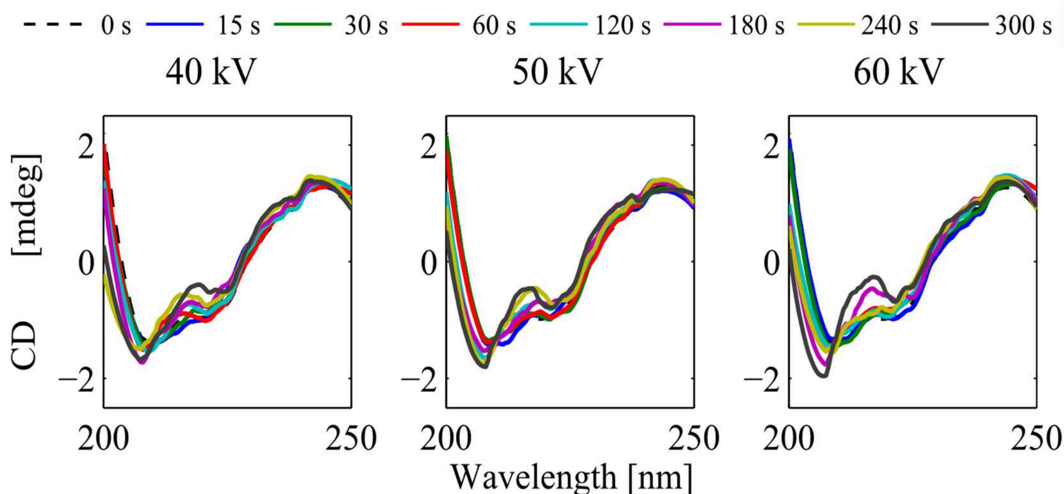

Figure 3. 3 Circular dichroic spectra of control and ACP treated ALP.

In Figure 3.3, differences among the CD spectral fingerprint can be visualised for various treatments. This in turn confirms that the secondary structure of ALP is modified after plasma treatment. Observing the control spectrum, the α-helix is the predominant structural element. The higher content of α-helix reflects upon the greater stability of the enzyme despite the complexity of its structure. The band with the negative dichroic peak with a minimum in the 215 nm region is typical for proteins with β sheet configuration, besides the two positive peaks around 225 and 250 also indicate the percentage of β-sheet (Chen et al., 1972; Kasinos et al., 2013; Kelly and Price, 2000).

A shift in the spectrum towards lower wavelength is also clearly noticeable, especially in samples treated for 5 min. Considering that UV absorption spectrum of phosphatases is due chiefly to the presence of tyrosine and tryptophan, the shift indicates the action of strong oxidants; see c.f. Sizer (1942). This enzyme is stabilised by weak non- covalent forces, such as hydrogen bonds and hydrophobic interactions and after plasma treatment the globular shape of ALP is modified. These results confirm that ACP led to modification of the secondary structure and damaged the internal bonds.

3.3.3. Multivariate chemometric analysis

For obtaining information about the pattern and extent to which ACP treatments cause changes in the enzymes structure, multivariate chemometric methods were applied. A hierarchical clustering on the principal component space was conducted for the CD spectra. The algorithm detected three distinct clusters, which showed that samples treated for up to 30 sec, irrespective of the applied voltage, were closely related to the untreated control samples (see Figure 3.4). In addition, the samples treated for 300 sec were very distinct compared to all samples treated for relatively shorter durations. However, samples treated at different voltages for 300 sec were closely related, indicating that treatment time had a stronger effect than voltage for extended treatments durations.

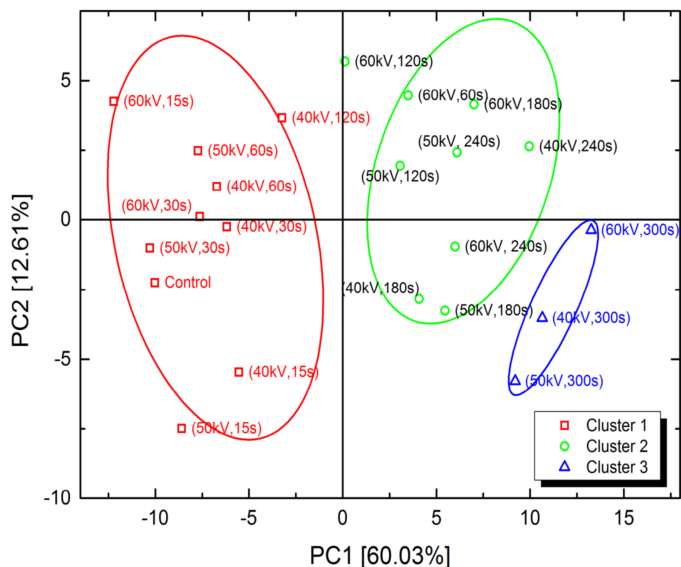

Figure 3. 4 Hierarchical clustering in the principal component space of the CD spectral data generated three clusters. The treatment conditions are provided as data labels

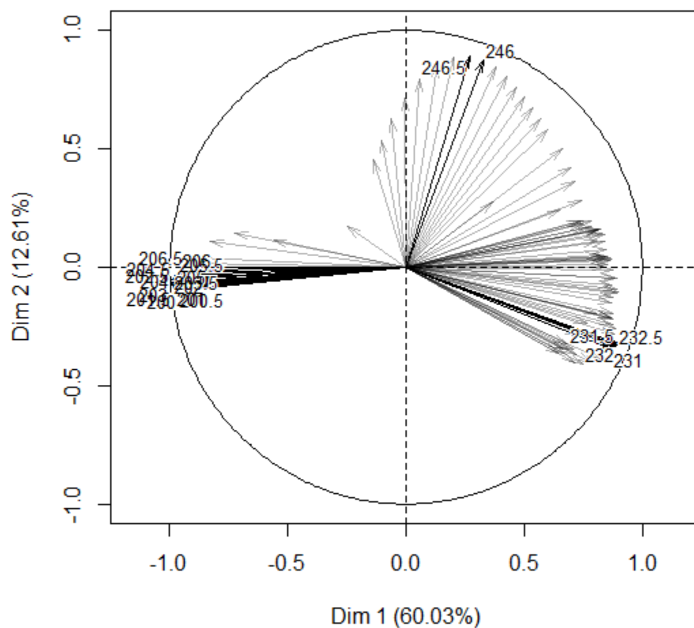

Figure 3. 5 Loadings plot for the PCA variable factors conducted on the CD spectroscopy dataset. Only 25 most important variables are labelled to impart readability to the plot.

The loadings plot (Figure 3.5) was used to identify the important variable range (i.e. the wavelength region) which was contributed to the grouping. This can be observed from

Figure 2.5, where the 25 most important variables are plotted. Clearly, the groups 1, 2, and 3 correlate with wavelength regions centred about 200-205 nm, 246 nm, and 231 nm respectively. The first two wavelength regions correspond primarily to the α-helical structure and the latter to β-sheet conformation (Chen et al., 1974). Therefore, the changes to β-sheet structure of protein increase with increase in treatment time. Overall, it can be concluded that ACP treatment leads to decrease of α-helix and β-sheet structures. Surowsky et al. (2013) have shown that a simultaneous reduction of α-helix structure and increase of β-sheet content leads to the inactivation of peroxidase and polyphenol oxidase in a model food system, subjected to cold plasma treatment.

3.3.4. Changes of temperature and pH

During plasma treatments, a minor increase in temperature was noticed; however, the temperature never reached more than 30 °C even for the highest treatment (60 kV for 300 sec). This temperature is not high enough to inactivate this enzyme (Fox and McSweeney, 1998; Rankin et al., 2010). The pH measured immediately after the ACP exposure was not affected by the treatment and remained constant at 6.8. Since temperatures and pH that could have caused thermal inactivation or instability of the enzyme respectively were never reached, the observed inactivation was attributed solely to the action of plasma species.

The enzyme inactivation can be attributed to the action of reactive oxygen species (ROS), including ozone and ultraviolet radiation (Innocente et al., 2014; Pankaj et al., 2013; Surowsky et al., 2013; Takai et al., 2012). It is worth mentioning that the dielectric barrier discharge used in this study is also a source of high concentrations of ozone and reactive nitrogen species (Misra et al., 2015; Misra et al., 2013). The influence of ozone on the secondary structure of proteins was also confirmed and quantified in our recent study (Segat et al., 2014).

3.4. Conclusions

The results of this study indicated that dielectric barrier discharge (DBD) plasma treatments were significantly effective in inactivating alkaline phosphatase enzyme. Treatment conditions were found to influence the inactivation rates and the inactivation curve exhibited a shoulder for the lowest voltage. The enzyme inactivation kinetics was found to be best described by the Weibull model. The inactivation was attributed to the loss of α-helical and β -sheet secondary structures of the protein, as observed through circular dichroism studies and chemometric analysis. The correlation between plasma chemical kinetics and enzyme inactivation kinetics requires further elucidation and extensive studies. Such correlations in turn will provide better mechanistic insights at a molecular scale (plasma-amino acid interactions). The fact that enzymes are inactivated by ACP also points to the fact that changes in biochemical pathways would follow in ACP treated foods during post-treatment storage.

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Part II – Ozone Treatments in the Dairy Field

Chapter 4
Effects of ozone processing on chemical, structural and functional properties of whey protein isolate

Chapter 5
Use of ozone in production chain of high moisture Mozzarella cheese

Chapter 6
Preliminary data on reducing microbial load of cheese brines by means of ozone

Chapter 4

Effects of ozone processing on chemical, structural and functional properties of whey protein isolate

ABSTRACT

High concentration of gaseous ozone was used to treat whey protein isolate (WPI) powder for different times ranging from 30 to 480 min. The aim of the present work was to study the changes in protein structure and evaluate their consequences on selected functional properties. Surface hydrophobicity, free sulfhydryl groups, turbidity, FTIR, SDS-PAGE, HPLC analysis are performed to evaluate the chemical and structural effects of ozone.

The effect of ozonation on the solubility and the foaming properties of proteins are also determined. Results show a reduction of free sulfhydryl groups and an increase of surface hydrophobicity, indicating a self-rearrangement in the protein structure following ozonation. Thus, ozonation allows creation of a more flexible structure without formation of a strong network of disulphide bond or aggregations, which is corroborated by the turbidity analysis and SDS-PAGE. Ozone processing induces modifications that improve the foaming capacity and foam stability, however, a slight reduction in the solubility is encountered.

4.1. Introduction

In addition to their nutritional quality, proteins in food should possess specific functional properties to facilitate processing. The functional properties of proteins, therefore, serve as a basis for product performance.

Functional properties of proteins are dictated by their physicochemical properties, which broadly govern the behaviour of proteins in foods (Kinsella and Melachouris, 1976; Luyten et al., 2004). These properties can be broadly classified into two different groups: hydrodynamic or hydration-related properties, which include water absorption, solubility, viscosity, gelation and aroma retention capacity, and surface-active properties, such as emulsification, foaming and film formation capacity (Kilara and Vaghela, 2000).

Dairy whey is an important source of protein, whose functionality can be exploited commercially in the manufacture of numerous complex foods to increase viscosity, form gels, stabilize emulsions or foams, or serve as a water-holding agent (Innocente et al., 2002; Kinsella and Whitehead, 1989; Luyten et al., 2004). In fact, whey proteins, owing to their high percentage of β-lactoglobulin and α-lactalbumin protein fractions and due to the presence of other important minor components such as proteose peptone fraction, are responsible for hydration, gelation, emulsifying and foaming properties (Cayot and Lorient, 1997; Innocente et al., 1998a,b).

Whey can be processed into a food ingredient by simple drying or the protein content can be further increased by removing lipid, minerals and lactose. Different techniques such as ultrafiltration, microfiltration, reverse osmosis, and ion-exchange have resulted in the development of several different final whey products, such as whey protein concentrates (WPC) and whey protein isolates (WPI) (Foegeding et al., 2002; Li et al., 2005).

The properties of these whey products vary as a function of different environmental parameters such as pH, temperature, ionic strength and concentration of calcium and other polyvalent ions, sugars and hydrocolloids, as well as with processing operation conditions (De Wit, 1989; Innocente et al., 2011). All these factors affect protein's structure and consequently their behaviour in a food formulation. Indeed, it is well known that a protein's structure is closely related to their functionality (Creighton, 1993).

There is ample literature focusing on protein structural modifications induced by different processes, primarily heat treatments (Dissanayake et al., 2009; Nicorescu et al., 2009; Tosi et al., 2007) and more recently, nonthermal technologies such as high pressure processing, ultrasound, pulsed electric field, UV and pulsed light (Arzeni et al., 2012; Biasutti et al., 2010; Bouaouina et al., 2006; Chandrapala et al., 2011; Kristo et al., 2012; Liu et al., 2005; Venir et al., 2010; Xiang et al., 2009). These technologies can be employed to selectively modify functional properties of proteins in order to obtain protein derivatives with targeted functionality appropriate for different formulated foods.

Besides these studies, a limited number of works with similar aims have focused on treatments that take advantage of oxidative processes. In particular, for milk proteins, Liu et al. (2000) studied the effect of oxidation with $FeCl_3/H_2O_2$/ascorbate on whey protein isolates (WPI) and β-lactoglobulin purified fractions. It was reported that the amino-acid

side chains of WPI were modified during oxidation, but the thermal stability of whey proteins was not significantly altered. Results obtained on β-lactoglobulin fraction reveal a marked increase in carbonyl groups but a slight increase in amines and disulphide bonds.
Cui et al. (2012) reported that the WPI exposed to $FeCl_3/H_2O_2$ hydroxyl radical-generating systems (HRGS) at room temperature (20 °C) showed an increase in carbonyl content, a reduction of total SH groups, higher dityrosine content, surface hydrophobicity, and turbidity than non-oxidised WPI. This treatment promoted protein polymer formation coincidental to β-lactoglobulin, α-lactalbumin, and bovine serum albumin losses. These oxidation-induced changes demonstrated high susceptibility of WPI to oxidative stress at room temperature and partially explained variations in functionality of whey proteins often observed in formulated foods.
Kong et al. (2013) examined the WPI sensitivity to oxidizing radicals generated by ascorbic acid, $FeCl_3$ and different concentration of H_2O_2. Results showed a general impairment of hydrodynamic properties including solubility and gelation; however, the surface-active properties like emulsification and foaming were found to improve.
In light of these results, it is interesting to evaluate the effect of other oxidizing treatments, particularly, ozone processing on whey proteins.
The ozone molecule is a powerful oxidizing agent, which finds applications in different fields, including water and wastewater treatment, medical application and food processing operations. Within recent years, many studies have been carried out to evaluate the effects of ozone processing on different food products such as fruits, vegetables, meat, fish, cheese and other dairy products (Aguayo et al., 2006; Alexandre et al., 2011; Crowe et al., 2012; Kim et al., 1998; Manousaridis et al., 2005; Segat et al., 2014). Most of these studies focused upon the evaluation of the antimicrobial activity of ozone treatments (Khadre et al., 2001). However, ozone processing also provides scope to modulate the structure of proteins and their functionality, thanks to the molecule's strong oxidative potential. Cataldo (2003, 2004, 2007) extensively studied the reactivity of ozone with different proteins such as invertase, pectinase, papain, trypsine, gelatine and methaemoglobin. Recently, the effects of ozone on the functional properties of whey proteins were reported by Uzun et al. (2012). In particular the authors investigated the action of ozone on the solubility, emulsifying and foaming properties of proteins as well as rheological behaviour of emulsions. However, this study lacked mechanistic insights explaining the protein chemistry and structure modifications that occur during treatments.
The aim of the present work was to evaluate the effect of gaseous ozone treatments of WPI on the chemical, physic-chemical and structural changes, and their functional consequences. Herein, WPI powders are directly subjected to high concentrations of gaseous ozone for different times.

4.2. Materials and methods

4.2.1 Materials

Whey protein isolate (WPI) was obtained from a commercial supplier. The WPI content in dry matter, total nitrogen, ash and fat were determined by standard analytical procedures (IDF 20B, 1993; AOAC, 1997).
All chemicals used in the study were purchased from Sigma-Aldrich, Italy, unless explicitly specified.

4.2.2 Ozone treatment

5 g of WPI was placed in Petri dishes located inside a container connected to an ozone generator (AirNow OG-36AN2K, O3 technology, Brescia, Italy) and an ozone analyser (BMT 964, Messtechnik GMBH, Germany). The WPI samples were treated for durations ranging from 30 to 480 min, at a temperature of 20 °C and relative humidity of 70%, which was monitored by an external device connected to the apparatus. The ozone production rate was 36 g/h and the concentration inside the box was c.a. 20000 ppm for all treatments. A schematic of the experimental setup is provided in Figure 4.1.
After treatments, the samples were stored in under vacuum pouches until further analysis. All the analysis (except infrared spectroscopy) were carried out after solubilization of WPI at 2% (w/v) in phosphate buffer (50 mM, pH 6.8), stirred at medium speed for 60 min at room temperature and left overnight at 4 °C to allow protein hydration (Innocente et al., 1998).

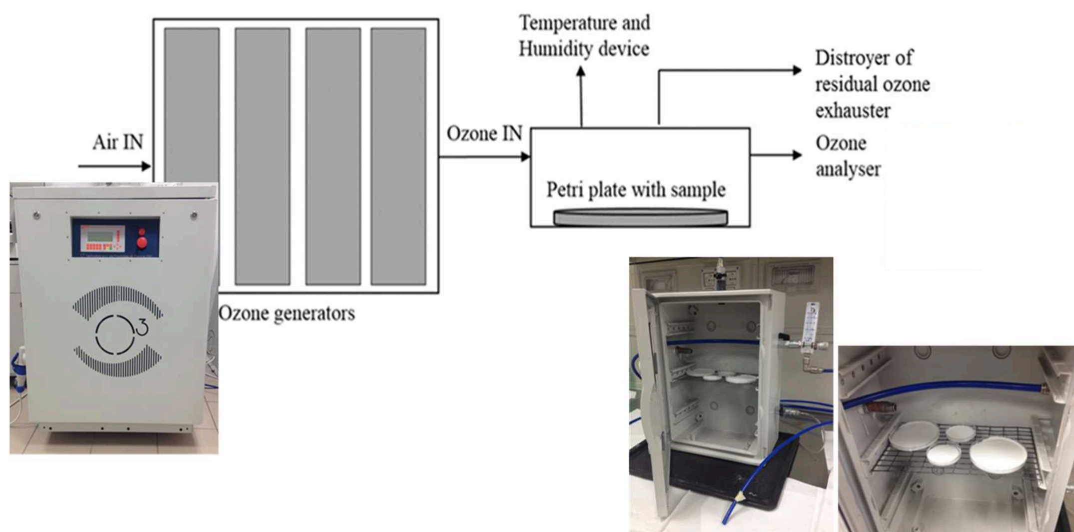

Figure 4. 1 Schematic of the experimental setup for ozone system

4.2.3 Turbidometry

The turbidity of the solutions of control and ozone-treated WPI samples was measured at room temperature by determination of absorbance at 600 nm by a UV-Vis spectrophotometer (UV-2501 PC, Shimadzu Kyoto, Japan) at 25 °C with a 1 cm path length cuvette (Liu et al., 1994).

4.2.4 Surface Hydrophobicity

The surface hydrophobicity of WPI samples was determined in accordance with Cui et al. (2012). A series of dilutions of 2% (w/v) WPI solutions were made with the phosphate buffer (50 mM, pH 6.8) to obtain a range of protein concentrations (0.02 – 2 mg/mL). Then 10 mL of each dilution were reacted with 0.6 mL of 8-anilino-1-naphthalene sulfonic acid, ANS (8 mM in 50 mM phosphate buffer, pH 6.8) for 15 min. The fluorescence of each sample was measured using a fluorescence detector (Intelligent Fluorescence Detector, Jasco FP-1520, Jasco Corporation, Tokyo, Japan). The analysis conditions were excitation at 390 nm, emission at 480 nm, gain 1 and attenuation 1. The surface hydrophobicity index (SHI) of solutions was calculated from the initial slope of relative fluorescent intensity (RFI) *versus* protein concentration of serial dilutions. The RFI was defined by $RFI = (F-F_0)/F_0$, where F is the fluorescence reading of the protein-ANS conjugate, and F_0 is the reading of the ANS solution without WPI.

4.2.5 Determination of free sulfhydryl content

The concentration of free sulfhydryl groups (SH) of the WPI samples was determined using Ellman's reagent (5',5-dithiobis (2-nitrobenzoic acid), DTNB) as reported by Beveridge et al. (1974). Briefly, 0.5 mL of WPI sample was added to 2.5 mL of 8M urea in Tris-Glycine buffer (10.4 g Tris, 6.9 g glycine, 1.2 g EDTA per liter, pH 8.0) and 0.02 mL of Ellman's reagent (4 mg mL-1 DTNB in Tris-glycine buffer) to develop color. After 15 min, absorbance was measured at 412 nm by a UV-Vis spectrophotometer (UV-1800, Shimadzu Kyoto, Japan). Concentration of free sulfhydryl groups (μM SH/g) was calculated from the following equation:

$$\mu M\ SH/g = (73.53 \times A_{412} \times D)/C$$

where A_{412} is the absorbance at 412 nm; C is the WPI solution concentration (mg/mL), D is the dilution factor, and the factor 73.53 is derived from $10^6/(1.36 \times 10^4)$; 1.36×10^4 is the molar absorptivity constant (Ellman, 1959).

4.2.6 FT-IR analysis

A 3% w/w dilution of the powder samples was prepared by mixing 5 mg of the sample with 295 mg of dry potassium bromide (KBr). KBr pellets were prepared by exerting a pressure of 100 kg/cm^2 for approximately 2 minute in a pellet press (Specac, United Kingdom). The IR spectra of the pellets were collected in absorbance mode using a Nicolet Avatar 360 FTIR E.S.P. (Thermo Scientific, Waltham, MA, USA) over the frequency range 4000-400 cm^{-1}, at 2 cm^{-1} resolution at room temperature using OMNIC software (version ESP 5.2). Six pellets were prepared for each sample and their spectra

recorded. Each spectrum was the average of 64 measurements and also corrected for background noise and baseline drifts. The quantitative estimation of protein secondary structure was obtained from the second-derivative spectra in the amide I region. Details on infrared absorption frequencies of the protein secondary structures can be found in Dong et al., (1990). For this study, no attempts were made to deconvolute the highly overlapping regions.

4.2.7 HPLC analysis

WPI sample solutions were 10-fold diluted with Na-acetate buffer (0.1M, pH 4.6) and centrifuged at 10000*g* for 15 min. 40 µL of supernatant was injected into the HPLC apparatus. Eluting solvents A and B were HPLC-grade water (VWR International, USA) and acetonitrile (Lab Scan, Analytical Sciences, Ireland), respectively, both containing 0.1% (v/v) trifluoroacetic acid, according to De Noni et al. (2007). The HPLC apparatus consisted of an Alliance 2695 pump system (Waters, Milford, MA, USA) combined with dual λ absorbance detector (Waters 2487, Waters, Milford, MA, USA). A PLRP-S column (4.6 mm i.d. 150 mm, 5 mm 300 Å) from Waters was used. β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) were injected as standard solutions. Chromatographic data were processed with Empower 2 software (Waters). The percentage of individual whey protein fractions (α-La, BSA, and β-Lg) was calculated using the area under the peak of each component (A) divided by the total area (A_{tot}).

4.2.8 SDS-PAGE

WPI solutions before and after ozone treatments were examined by SDS-PAGE (15% polyacrylamide) under reducing and non-reducing conditions (Laemmli, 1970) using the Mini Gel 2D (Bio Rad, Richmond, California, USA) electrophoresis equipment. WPI solutions (1% w/v) were added with Laemmli sample buffer in presence or absence of 2% β-mercaptoethanol and heated at 98 °C for 5 min before loading into the gel. Commercial purified BSA, β-Lg and molecular weight markers were used as protein standards. The separated proteins were subsequently stained with NOVEX® Colloidal Blue Staining (Invitrogen) overnight and then washed in deionized water. Bands were identified by densitometry using ImageQuant software (Amhersham). Estimation of the apparent masses of the protein bands was obtained by the plotting of the migration distances of high range standard proteins (Precision Plus Protein™ Standards Biorad) versus their known molecular masses.

4.2.9 Nitrogen solubility

The Nitrogen solubility index (NSI) of non-treated and treated solutions of 2% (w/v) WPI were determined according to Biasutti et al. (2010). Solutions were centrifuged at 3000*g* for 15 min at 25 °C and the nitrogen content in the supernatant was determined by Kjeldhal method. The value of total nitrogen content in the supernatant of each sample was compared to that in the bulk suspension to give the mass fraction of dissolved

material. The NSI was expressed as the % reduction in solubility of solutions relative to the control.

4.2.10 Foaming properties

10 mL of WPI solutions were homogenized at 16000 rpm for 2 min using an IKA Ultra Turrex homogeniser (T18 basic, IKA-Werke GmbH and Co., Staufen, Germany) in graduated tubes. Foaming capacity (FC) and foam stability (FS) were calculated according to the following equations:

$$FC\ (\%) = \frac{V_{F0} - V_L}{V_L} \times 100$$

$$FS\ (\%) = \frac{V_{F30}}{V_{F0}} \times 100$$

where V_L, V_{F0}, and V_{F30} represent the volume of non-whipped WPI solution and the volumes of the foam immediately after whipping (time 0) or after standing at room temperature for 30 min, respectively.

4.2.11 Statistical analysis

Every ozone treatment was performed twice and the all analysis were repeated three times. The results were averages of at least six measurements and are reported as means ± SD. Analyses of variance (ANOVA) was performed with significance level set to $p<0.05$ using R software (cran.r-project.org, ver 3.0.1 for Windows). The Tukey's HSD test was used to test differences between means. Hierarchical clustering in principal component space was performed using *FactoMineR* package (for R software) (Lê et al., 2008) for the FTIR spectral data matrix to assess the inherent grouping in the data.

4.3. Results and discussion

4.3.1 WPI chemical composition

The WPI product used in this work was analysed for dry matter, protein, ash and fat. These results are reported in Table 4.1.

The protein samples were composed of beta-lactoglobulin (β-Lg), alpha-lactoalbumin (α-La) and bovine serum albumin (BSA) at around 75, 23 and 2% content of total protein respectively, as determined through HPLC analysis (see Figure 4.2 for the corresponding peaks).

Table 4. 1 Chemical composition of WPI

Chemical composition	
Humidity (g/100g)	4.90 ± 0.12
Total protein (g/100g $_{\text{dry matter}}$)	94.70 ± 0.12
Ash (g/100g $_{\text{dry matter}}$)	0.80 ± 0.08
Fat (g/100g $_{\text{dry matter}}$)	n.d.

Values are the average of three determinations ± SD
n.d.: not detected (< 0.01%)

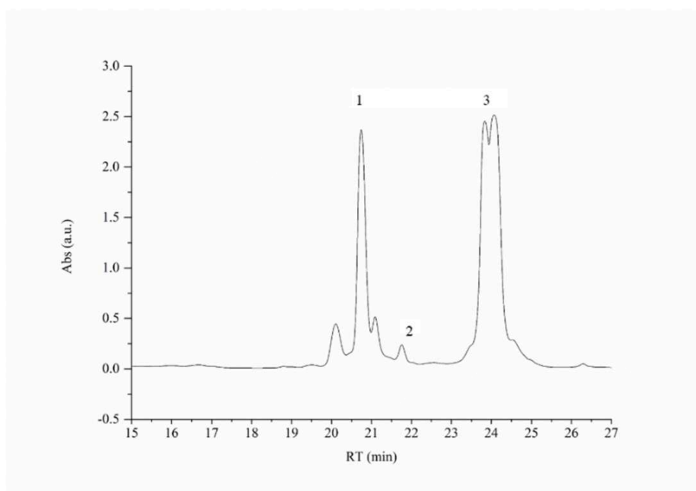

Figure 4. 2 HPLC chromatogram of control WPI samples. Numbers correspond to: 1) α-La, 2) BSA, 3) β-Lg.

4.3.2 Structural modifications

FTIR spectroscopy is a useful tool for the characterization of protein secondary structure with a precision lying between that of the purely predictive and the molecular coordinate approaches (Jackson and Mantsch, 1995). To evaluate the effect of high concentration of gaseous ozone on the secondary structure of proteins, the FTIR spectra of WPI treated for different times was carried out before and after the ozone treatments. The overlaid FTIR spectra of the control and plasma treated WPI samples is shown in Figure 4.3.

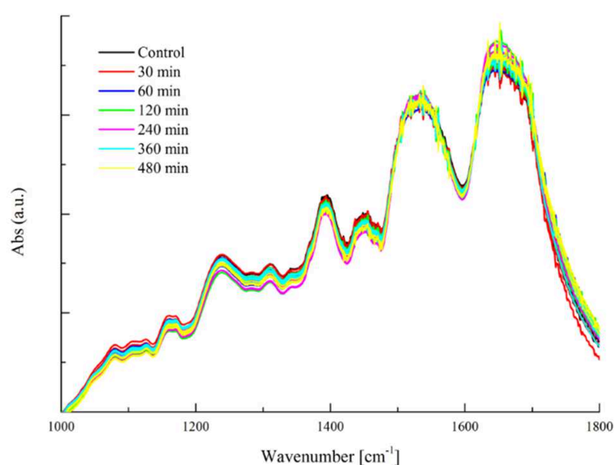

Figure 4. 3 Overlaid FTIR spectrum of control and ozone treated WPI.

For obtaining first-hand information about whether and how ozone treatment causes changes in the protein, a hierarchical clustering on the principal component space was conducted for the fingerprint region of the FTIR spectra (400-1800 cm^{-1}). The algorithm detected three clusters, which showed that ozone treatments up to 30 minute and control are quite similar, as shown in Figure 4.4. However, there were differences among samples treated beyond this time with the formation of a cluster centred around samples treated for 480 minutes. To determine the extent of changes in the secondary structure, integration of the second derivative spectrum was employed.

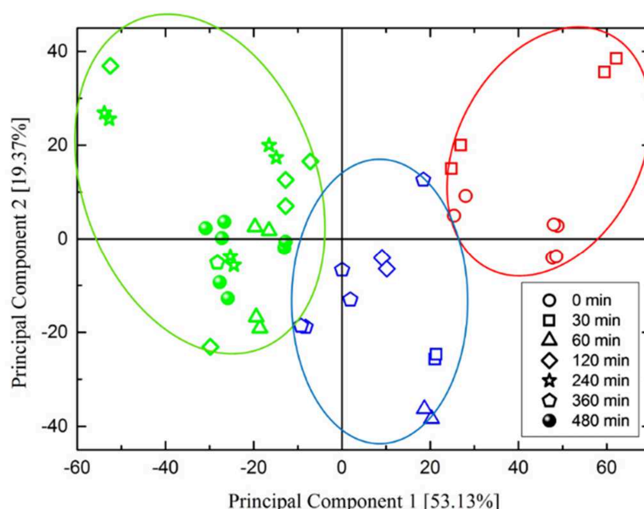

Figure 4. 4 Hierarchical clusters in the principal component space for the FTIR spectra in the fingerprint region. Green, blue and red symbols are the three clusters.

Quantitative analysis of protein secondary structure is based on the assumption that protein can be considered as a linear sum of a few fundamental secondary structural elements. The α-helix, β-sheet, β-turn, and aperiodic structures, when considered together, constitute the secondary structure of the protein backbone (Bock and Damodaran, 2013). Each of these secondary structures is associated with characteristic hydrogen bonding patterns between amide C=O and N-H groups on the polypeptides. Absorption associated with the amide I band originates from stretching vibrations of the C=O bond of the amide, while absorption associated with the amide II band derives primarily from bending vibrations of the N-H bond. The most sensitive spectral region to the protein secondary structures is the amide I band (1700-1600 cm^{-1}), which is due almost entirely to the C=O stretch vibrations of the peptide linkages (approximately 80%) (Kong and Yu, 2007). Therefore this region was selected for quantifying the relative content of the secondary structures. The relative contents of the β-sheet, random coil structure, α-helix and β-turn obtained from the second derivative spectrum are summarised in Table 4.2.

Table 4. 2 Secondary structures (%) of control and ozone treated whey protein.

Sample	β-Sheet	Random coil	α-helix	β-turn
Control	$29.54^{a} \pm 0.26$	$18.51^{a} \pm 0.26$	$12.87^{c} \pm 0.17$	$39.08^{a} \pm 0.46$
30 min	$25.07^{a} \pm 0.66$	$21.48^{a} \pm 0.59$	$16.55^{ca} \pm 0.70$	$36.89^{a} \pm 0.26$
60 min	$28.98^{a} \pm 1.12$	$19.92^{a} \pm 0.64$	$14.43^{cb} \pm 0.38$	$36.66^{ba} \pm 0.25$
120 min	$27.18^{a} \pm 2.09$	$21.31^{a} \pm 0.56$	$15.65^{ca} \pm 0.29$	$35.85^{ba} \pm 1.33$
240 min	$31.70^{a} \pm 1.98$	$18.12^{a} \pm 1.44$	$14.65^{ca} \pm 0.62$	$35.53^{ba} \pm 0.45$
360 min	$30.29^{a} \pm 1.46$	$20.43^{a} \pm 1.50$	$18.33^{a} \pm 1.37$	$30.95^{b} \pm 2.01$
480 min	$28.02^{a} \pm 1.62$	$21.07^{a} \pm 0.66$	$16.99^{ab} \pm 0.88$	$33.91^{ba} \pm 1.67$

a, b, c and d: means with the same letter in the column are not significantly different ($p > 0.05$)

The FTIR data revealed no changes in the content of β-sheet and random coil structures of proteins. This indicated that the ozone treatments did not affect the intermolecular hydrogen bonding of the β-sheets (Parris et al., 1991). As such β-sheet has less intermolecular hydrogen bonds, which results in a less stable secondary structure than the α-helix (Jackson and Mantsch, 1995). In addition, α-helix being a tight structure also explains its greater stability. However, a small increase in α-helix form was recorded, which was statistically significant ($p<0.05$). It may be noted that WPI is largely comprised of β-lactoglobulin, whose secondary structure contains a lone helix located on the surface of the molecule (Kilara and Vaghela, 2000) and could explain the modification of this structure. This to some extent also indicates that the surface hydrophobicity of the ozone treated WPI should increase, which is also observed here (discussed later). Also Safonova et al. (2011), using FTIR spectroscopy found that ozone treatments promote ordering in the structural elements of protein. A decrease in the β-turn structure was also recorded. This could be attributed to the possible oxidation of amino acid residues or their cross-linkage.

4.3.3 Chemical changes

The Surface Hydrophobicity Index (SHI) is an often used method for characterizing/detecting partially folded states of proteins and detecting the "effective hydrophobicity" of the protein (Ali et al., 1999; Kato and Nakai, 1980). The analysis is based on the hydrophobic interaction between a fluorescent probe (8-anilino-1-naphthalene sulfonic acid –ANS) and the protein. Particularly, the partially folded states of proteins have groups of hydrophobic side chains that are not fully embedded in the native core structure and provide binding sites for ANS.

The SHI of WPI solutions as a function of ozone treatment times are reported in Figure 4.5.

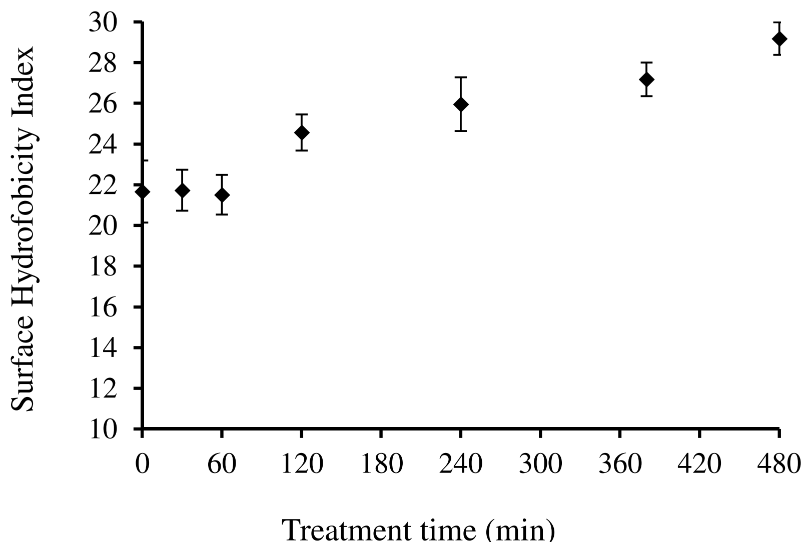

Figure 4. 5 Surface Hydrophobicity Index of WPI solutions as a function of treatment time.

The Figure 4.5 depicts an increase in SHI during ozone exposure, especially after 120 and 240 min. However, when longer treatment times beyond 240 min are considered, the SHI values do not differ significantly from their immediate preceding values (p>0.05). In other words, this data reveals that the nonpolar amino acid residues of WPI, which are located in the interior of protein structure, to avoid contact with the aqueous space (Kato and Nakai, 1980), needed high free energy for long time to expose themselves. Thus, longer treatment times beyond 240 min did not induce any further increase in hydrophobic surface exposure. These preliminary data suggest that the gaseous ozone plays an important role in surface amino acid chain modifications and to better understand how the gaseous ozone affects the protein changes, the free sulfhydryl (SH) groups exposure was monitored as function of treatment time (see Table 4.3).

Table 4. 3 Free SH groups (µM SH/g) as a function of ozone treatment time (min).

Treatment Time (min)	**µM SH/g**
0	$33.73^{a} \pm 0.19$
30	$30.04^{b} \pm 0.02$
60	$29.46^{cb} \pm 0.88$
120	$29.89^{cb} \pm 0.37$
240	$27.10^{c} \pm 0.37$
360	$24.40^{d} \pm 0.89$
480	$22.93^{d} \pm 0.78$

a,b,c,d means with the same letter in the column are not significantly different (p>0.05)

As reported in Table 4.3, the free SH groups decreased during ozone exposure. In particular, the changes were significant after 30, 240 and 360 min of treatment.

Prolonging the ozone treatment until 480 min did not generate statistically significant effects in the WPI samples, when compared to ozone exposure time of 360 min ($p>0.05$). The decrease in free SH groups could indicate amino acid oxidation or cross-linking to form S-S bond. In fact, cysteine is one of the most sensitive amino acid residues to oxygen radicals, perhaps one of the first ones to be oxidised giving rise to different oxidation products, as reported by Liu and Xiong (2000).

It is well known that β-Lg, the major component of this mix, contains a unique free thiol group and two disulphide bonds. In the native state, the free sulfhydryl is buried in the hydrophobic interior (Croguennec et al., 2006; Sava et al., 2005) and therefore weakly reactive to ozone and its reactive species, suggesting that it needs long ozone exposure times to be oxidized compared to other proteins that have more exposed sulfhydryl groups (Liu and Xiong, 2000). In our experiment, the reduction of free SH group content was evident only after 480 min of treatment and was about 30% compared to the initial value of free SH content. This result is in agreement with a previous work carried out by Liu and Xiong (2000) on β-Lg protein exposed to oxidative stress.

Generally, treatments that destabilize native whey protein structure, promote unfolding or polymerization could lead to protein-protein interactions between whey proteins in solution and lead to intra- or intermolecular thiol/disulphide (SH/S-S) interchange or thiol/thiol (SH/SH) oxidation reactions (Monahan et al., 1995).

Turbidimetric analysis has been reported as a means of determining if aggregates are created in the solutions after treatments or processing operations. Our data reveals very low values of absorbance in all cases (0.073 ± 0.015 nm) including 480 min of ozone treatment. The differences among the samples were not significant ($p > 0.05$). These absorbance values were too low compared to literature data to prove aggregations among proteins due to covalent and non-covalent bond protein-protein associations (values greater than 1; Cui et al., 2011).

In the left side (a) of Figure 4.6 the electrophoretic pattern of samples without β-mercaptoethanol is reported. Based on their relative mobility, it is plausible to deduce that the bands with the apparent molecular masses of 14, 18 and 66 kDa contain α-La, β-Lg and BSA, respectively. The thin bands around 35 and 150 kDa were partly attributed to β-Lg dimer and octamer (Qin et al., 1999). The patterns clearly show that no differences exist among the samples even after 480 min of treatment suggesting that gaseous ozone and its reactive substances did not generate protein fragmentation or aggregation in WPI samples, in spite of the oxidation of sulfhydryl groups.

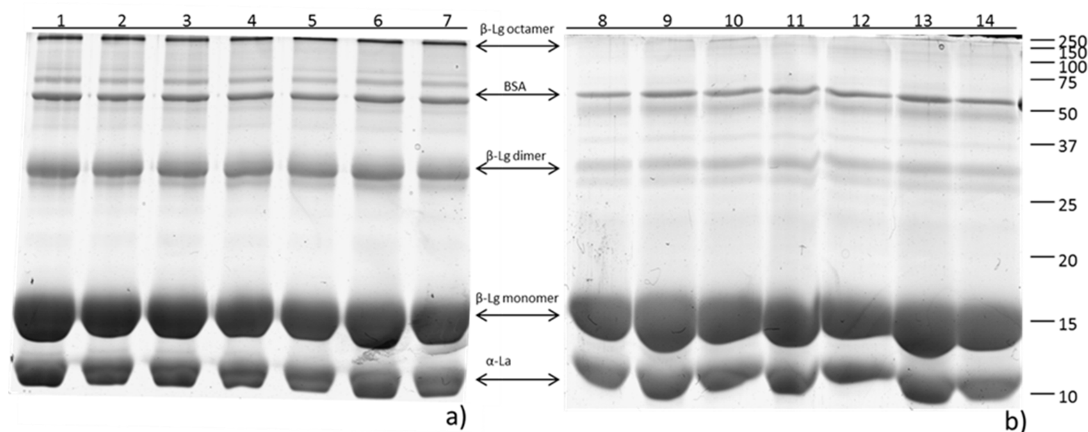

Figure 4. 6 SDS-PAGE patterns of WPI solutions analysed in absence (a) or presence of (b) 2% β-mercaptoethanol during the electrophoresis. Untreated WPI was loaded as control (lane 1 and lane 8), and treated WPIs were loaded as follows: 30 min (lane 2 and lane 9), 60 min (lane 3 and lane 10), 120min (lane 4 and lane 11), 240 min (lane 5 and lane 12), 360min (lane 6 and lane 1) and 480 min (lane 7 and lane 14).

In the right side (b) of Figure 4.6 the electrophoretic patterns of WPI samples pre-incubated with the reducing agent β-mercaptoethanol before SDS-PAGE are reported. Herein, a reduction of band intensity of the presumed β-Lg dimer and octamer is noticeable, as compared to Figure 4.6 (a) due to β-mercaptoethanol effect. Most importantly, no differences among untreated samples and ozone treated samples were detected. This confirms that no protein aggregates due to the formation of disulphide bonds were generated during ozone treatments, suggesting that SH-content reduction was caused by oxidation of cysteine residues to sulfinic/sulfonic acid. Moreover, this finding is in agreement with previous observations on turbidity.
In conclusion, these results show that after ozone treatments, some degree of unfolding occurred to the WPI proteins that generated the reduction of SH group content and an increase of exposed hydrophobic groups. As a consequence, the protein structures could have lost their native and globular shapes and become more flexible; however, these changes did not cause an overall aggregation among proteins by S-S bridges.

4.3.4 Protein Functionality

In order to investigate how the effects of ozone on the protein structure can affect their binding ability and functionality, the solubility, the foaming capacity and foam stability (measured after 30 min) were studied. The WPI samples subjected to the ozone treatments were characterized by a reduction of solubility values and Table 4.4 reports the percentage reduction of nitrogen solubility index (NSI) relative to the control for all the treated samples.

Table 4. 4 NSI reduction percentage of WPI ozone treated solution from control as a function of treatment time

Treatment Time (min)	Reduction of NSI (%)
30	3.42 [a] ± 0.12
60	4.40 [b] ± 0.09
120	5.93 [b] ± 0.23
240	11.95 [c] ± 0.08
360	14.58 [d] ± 0.10
480	15.63 [d] ± 0.01

a,b,c,d means with the same letter in the column are not significantly different ($p>0.05$)

In the present study, the solubility value was observed to gradually decrease as a function of ozone treatment duration. This data was in agreement with the results of tests for SH groups and surface hydrophobicity exposure. It should be noted that the action of ozone converts the thiol group of cysteine into disulfides which change its solubility (Cataldo, 2003; Kelly and Mudway, 2003). Following ozone treatments, as the protein denaturation proceeds, the exposition of a major number of hydrophobic groups at the molecular surface occurs. This reduces the ability of protein to bind water because of changes in the polarity. However, WPI exibits a high water solubility index, estimated around 97% before ozone treatments. The solubility value observed after ozone tratments, particularly longer treatment time (around 82%) was still comparable to the solubility value of similar milk-based products such as whey protein concentrates (WPC) (Qin et al., 1999). Thus, it is suggested that the solubility reduction due to ozone treatment would not limit the applicability of WPI in food formulations such as ice cream, mousse, dairy cream, frothed drinks or other products in which WPI plays an important role owing to its functional properties (Foegeding et al., 2002).

Figure 4.7 reports the foaming properties of WPI solutions as a function of treatment time.

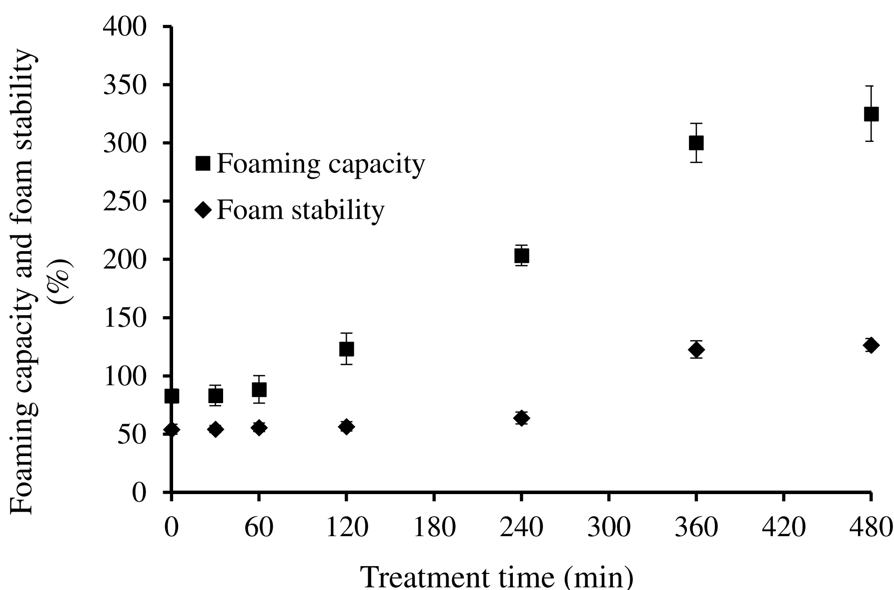

Figure 4. 7 Foaming capacity and foam stability (%) of WPI solutions as a function of treatment time.

As shown in Figure 4.7, the foaming properties were improved by ozone treatments. Both the capacity to form foam and its stability increased in ozone treated WPI ($p<0.05$). Generally, the foam formation is thermodynamically favourable due to the simultaneous dehydration of the hydrophobic interface and the exposition of hydrophobic portions of the protein (Dickinson, 1989). The foam formation involves a large number of factors including the physical and chemical properties of the proteins as well as the environmental factors like ionic strength or pH. In addition, steric and electrostatic repulsions during the adsorption processes play an important role in foam formation. Ozone-treated WPI exhibited high values of foam capacity and stability compared to the untreated WPI and this data correlated well with the surface hydrophobicity aspect previously discussed and also as reported in literature (Kato and Nakai, 1980; Foegeding et al., 2006). It is worthwhile noting that the exposed hydrophobic groups of the molecules at the foam (bubble) surface point towards the air, the hydrophilic groups towards aqueous solution and the proportion between these dictate the foam stability. In addition to the overall changes in the WPI, ozone treatment could lead to a more flexible protein structure. This flexible structure would be capable of arranging itself at the air-water interface, to reduce the interfacial tension and this explains the significant increase in foam stability. According to Nakai (1983) our data concerning solubility and foamability are correlated to those on hydrophobicity demonstrating a relationship between structure and functionality of whey proteins.

4.4. Conclusions

The present work demonstrated that high concentrations of gaseous ozone generated mild oxidative effects in WPI, depending on the duration of treatments. From the FTIR analysis, data revealed no changes in the content of β-sheet and random coil structures of proteins, however, a significant increase in α-helix form was recorded. Moreover, the interactions of whey proteins with ozone led to an increase in surface hydrophobicity, a negligible difference in turbidity and a decrease in free sulfhydryl groups. These changes reduced the solubility, which remained high; however, the treatments improved the foaming properties. This was suspected to be an outcome of the molecular self-arrangement within the protein molecules that become more flexible due to the overall chemical and structural changes induced by ozone. The findings reported in this work are important for describing the structure-function relationships of control and ozone-treated WPI and provide an overall idea about the feasibility of using ozone modified products. Exploiting the structural changes and the resulting functionality in real food systems could be quite challenging, however, these preliminary results indicate the feasibility of ozone processing as a new method to develop tailored whey proteins with specific functionality.

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Chapter 5

Use of ozone in production chain of high moisture Mozzarella cheese

ABSTRACT

This work aimed to evaluate ozone effectiveness in reducing viable spoilage bacteria load throughout high moisture (HM) Mozzarella cheese-making process. At first, Mozzarella cheese samples were packaged with ozonated water (2 mg/L), stored at low temperature and monitored during shelf life. In a following phase cheese samples were put, before packaging, in direct contact with ozonated water at ozone concentrations of 2, 5 and 10 ppm for 60 min. Then, gaseous ozone at concentrations of 10, 20 and 30 ppm for different times were tested. In these experiments, ozone was not effective in surface microbiological decontamination of cheeses. Ozone has high oxidant potential and lipid oxidation on Mozzarella cheese fat was evaluated. In all cases, there was no increase in the formation of primary and secondary lipid oxidation products.

Finally, we treated only the cooling water, artificially contaminated with *Pseudomonas* spp. strains, obtaining a reduction of about four Log cfu/mL in the microbial load. HM Mozzarella cheeses cooled with pre-treated water was characterized by a low microbial load and an increased shelf life.

We concluded that ozone can be successfully applied in Mozzarella cheese processing only to decontaminate water contaminated by potential spoilage bacteria before that it comes into contact with the product.

5.1. Introduction

High moisture (HM) Mozzarella cheese is a widely known fresh dairy product obtained by a particular process of plasticization and stretching of the acid curd in hot water. The stretched curd is usually shaped, cooled under running water and then packaged in bags containing diluted brine solution (preserving liquid). This solution preserves the soft-springy texture and high amounts of expressible serum during storage at 4 °C (Kindstedt et al., 2004).

Although HM Mozzarella cheese receives heat treatment during curd stretching that reduces dramatically its microbial load (Morea et al., 1998; Morea et al., 1999), post-processing contamination by microorganisms may occur, causing cheese spoilage and reduction of shelf life, or resulting in a possible risk for consumers' health (De Felip and Toti, 1984; Buazzi et al., 1992; Spano et al., 2003). The short shelf life of traditional HM Mozzarella cheese is caused by high level of non-pathogenic non-lactic acid bacteria population that, during storage, can cause cheese defects and alterations by hydrolyzing different casein fractions (Baruzzi et al., 2012). They are mainly coliforms, *Pseudomonas* spp. and other psychrotrophic bacteria that can release lipases and proteases, and consequently influence the textural properties of the product, and also cause the appearance of off-colours (Soncini et al., 1998; Cantoni et al., 2003), putrid smell and a bitter flavour (Oommen et al., 2002). Not all of the microorganisms that are responsible of Mozzarella cheese alterations originate from milk. Different Authors reported that often the main source of contamination during Mozzarella cheese processing is water employed in cooling phase (Cabrini and Neviani, 1983; Cantoni et al., 2003). Particularly, Greene et al. (1999) found that coliforms and *Pseudomonas* spp. frequently contaminate recirculating chilling water employed for dairy operations.

Recent studies have proposed different approaches to increase HM Mozzarella cheese shelf life by controlling spoilage microorganisms. Sinigaglia et al. (2008) evaluated the effectiveness of lysozyme and Na_2-EDTA against the spoilage microorganisms of Mozzarella cheese. Conte et al. (2009) and Del Nobile et al. (2009) studied the combined use of active coating and modified-atmosphere packaging and chitosan, coating and modified-atmosphere packaging for preserving this cheese. The use of silver nanoparticles in bio-based nanocomposite coating have been receiving considerable attention also for this product because of their attractive physic-chemical properties and strong toxicity against a wide range of microorganisms (Sondi and Salopek-Sondi, 2004; Gammariello et al., 2011). Gammariello et al. (2008) proposed the use of plant essential oil as natural food preservative in Mozzarella cheese. The innovative application of lactoferrin and its peptide digested hydrolysate, recently demonstrates the possibility to delay the growth of both *Pseudomonas* spp. and coliforms growing in the preserving liquid (Quintieri et al., 2012).

An alternative method to reduce the microbial load of HM Mozzarella cheese and extend its shelf life could be the use of ozone. The FDA's approval of ozone as a direct additive in food in 2001 triggered an increasing interest in ozone applications.

Ozone or triatomic oxygen (O_3), is an unstable allotrope of oxygen. It is an alternative sanitizer to chlorine compounds in the food industry, with the advantage that ozone decomposes to a number of free radicals but no residual components are left on product when decomposition is complete, liberating the main product of oxygen (Novak and Yuan, 2007). Solubility in water, stability and reactivity with organic and inorganic compounds of ozone are closely related to its antimicrobial efficacy. This multifunctionality of ozone makes it a promising food processing agent (O'Donnell et al., 2012). There is ample literature reporting successful applications of ozone in various food matrices such as asparagus, carrots, eggs, apples, tomatoes (Huyskens-Keil et al., 2011; Hildebrand et al., 2008; Fuhrmann et al., 2010; Achen and Yousef, 2001; Aguayo et al., 2006). Positive results have also been evaluated in the treatment of drinking water, washing water for fresh-cut vegetable, and lettuce (von Gunten, 2003; Selma et al., 2008; Selma et al., 2007). Other recent examples reported that spraying aqueous ozone in salmon fillets at a concentrations up to 1.5 mg/L can be effective in reducing the initial count of aerobic bacterial populations and significantly reducing the count of *Listeria innocua* without causing significant increases in lipid oxidation values (Crowe et al., 2012). Patil et al. (2009) demonstrated that the use of gaseous ozone at concentration of 75-78 μg/mL is useful to inactivate *Escherichia coli* in orange juice and the auto-decomposition of ozone makes its use safe for fruit juice processing. The ozone is also an alternative method of grain disinfestation without toxic residues (Tiwari et al., 2010). In dairy industry, there are only few studies related to the use of ozone, in particular to decontaminate surfaces of equipment or to reduce the growth of mould during cheese ripening (Serra et al., 2003; Moore et al., 2000; Greene et al., 1999; Gibson et al., 1960; Morandi et al., 2009). The aim of this work was to evaluate the possibility of using ozone at different stages of the Mozzarella cheese-making process, in particular to remove surface contamination of the Mozzarella cheese and to decontaminate curd's cooling water and preserving liquid by *Pseudomonas* spp. and coliforms.

5.2. Materials and methods

5.2.1 Experimental design and ozonation conditions

The research focused on High Moisture (HM) Mozzarella cheese purchased from a local dairy plant, made from cow milk and produced through biological acidification using 1% of direct-to-vat powder commercial starter culture (Mofin Alce Group, Novara, Italy). Figure 5.1 displays HM Mozzarella cheese processing after milk standardization, acidification and curding. In this flow chart the principal points, at which levels it is very important to control viable spoilage bacteria that can contaminate the cheese, were also indicated.

The research activities were therefore divided into different experimental phases considering the possibility to employ the ozone treatments in these various critical processing points. In the different experimental phases cheeses were made with the same

technology but processed on different days from different production batches. In each batch from 4000 Kg of milk approximately 560 kg of cheese were produced (about 2200 pieces of HM Mozzarella). Then, HM Mozzarella cheeses have been subdivided into three parts and all treatments considered was then replicated three times in the same conditions.
In the 1st experimental phase HM Mozzarella cheese samples were taken at the end of cooling phase and packaged in our laboratory (polylaminate PET-PE/EVOH/PE plastic film) with ozonated preserving liquid, consisting of sterilised water added with 1 g/L of NaCl and 2 ppm of ozone. Ozonated water was produced by WaterNow OX-0203WN2K, ozone generator 15 g/h, equipped with an inline electrochemical device monitoring continuously ozone concentration (by O3 technology, Brescia, Italy). Cheese samples were analysed after packaging and after 7, 15 and 21 days of storage under refrigerated conditions (see Figure 5.2 for details).
In the 2nd experimental phase HM Mozzarella cheeses were sampled after the shaping phase and inoculated on the surface with a suspension of *Pseudomonas fluorescens* and *P. putida* to achieve a final microbial concentration of 10^7 cfu per g of cheese. The inoculated samples were then placed into a tank in direct contact with cooling ozonated water. The ozonated water, at 2, 5 and 10 ppm of concentration, was continuously recirculated for 60 minutes to ensure a constant concentration of ozone throughout the tests. The water temperature was 15 °C.

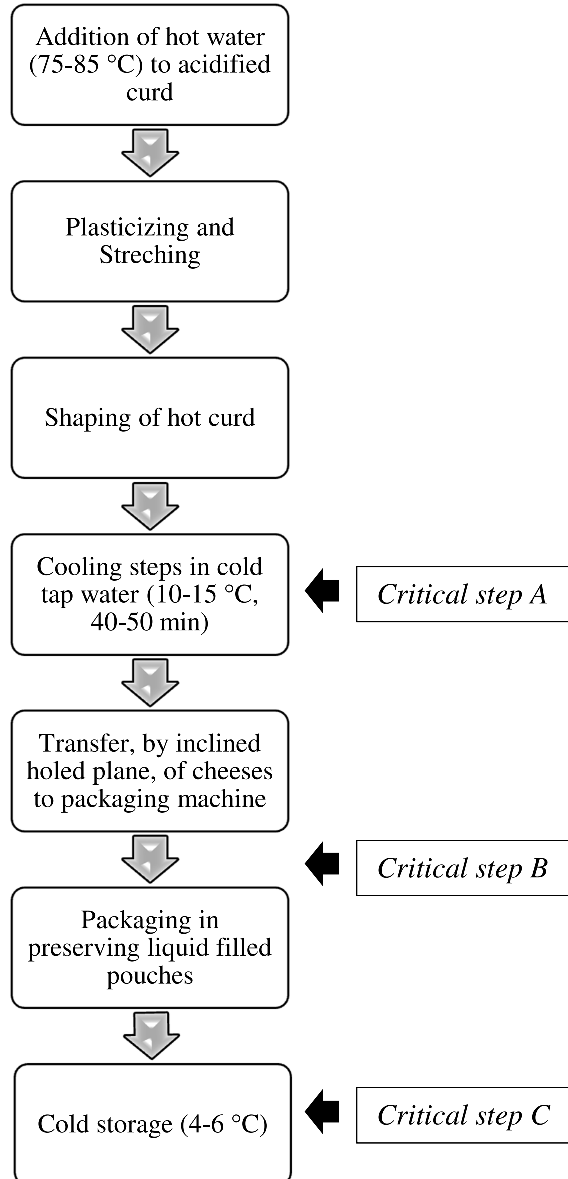

Figure 5. 1 Flow chart of HM Mozzarella cheese processing after milk standardization, acidification and curdling. Letters indicate principal points at which level ozonation could control microbial contamination after the stretching step: A) tap water used for cooling; B) reduction of viable cell count of surface contaminated HM Mozzarella cheese before packaging; C) control of microbial contamination during cold storage

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In the 3rd experimental phase, samples of HM Mozzarella cheeses were sampled at the end of cooling phase and placed on Petri dishes within a box connected to the ozone generator. Different gaseous concentrations (10, 20 and 30 ppm) for 30 minutes were tested; additional trials were performed at 30 ppm for 60 and 120 minutes. AirNow OG-36AN2K, ozone generator 36 g/h, was employed to produce gaseous ozone (O3 technology, Brescia, Italy). The value of relative humidity (90 ± 5%), temperature (4.0 ± 2.0 °C), and concentration of ozone (Ozone analyser, BMT 964, Messtechnik GMBH, Germany) were constantly monitored during the tests.
In the 4th experimental phase, the effect of ozone was evaluated by treating only the cooling water, without HM Mozzarella. Sterilised water was inoculated with *P. fluorescens* and *P. putida* (10^4 cfu/mL) and treated with ozone at concentrations of 2, 4, 6, 8, 10 ppm (ramp at 2 ppm/5 min). The temperature of water was 15.0 ± 0.5 °C. The water treated with ozone at concentration of 2 ppm was employed in the cooling step of mozzarella cheeses. Cheese samples were then packaged and analysed after 7, 15 and 21 days of storage under refrigerated conditions.

A)

B)

C)

D)

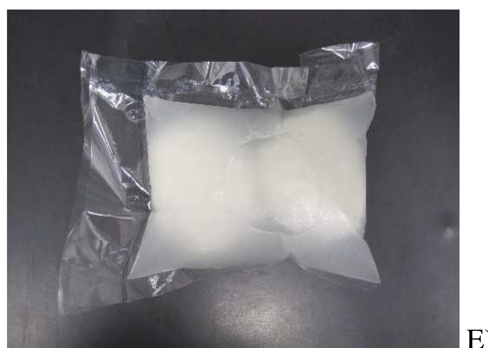
E)

Figure 5. 2 Imagines concerning the different experimental phases
A),B),C)Ozone generator and pilot plants for gaseous ozone treatments; D)Pilot plant for aqueous ozone treatments; E)Packaging of Mozzarella cheese with ozonated water

5.2.2 Composition of HM Mozzarella cheese samples

Dry matter, total nitrogen, sodium chloride and fat contents were determined respectively by AOAC methods (AOAC, 1980), Kjeldahl method (FIL-IDF 25, 1964), Vohlard method (FIL-IDF 17A, 1971) and modified Schmid-Bondzynski-Ratzlaff method (FIL-IDF 5B, 1986). The protein content was obtained by multiplying the percent Tot. N by 6.38 (Innocente and Corradini, 1996; Innocente et al., 2002; Innocente et al., 2002).

5.2.3 Decomposition of ozone in aqueous solution with and without HM Mozzarella cheese samples

To evaluate the kinetics of decomposition of ozone in polylaminate PET-PE/EVOH/PE plastic film, 200 mL of ozonated water (2 ppm, temperature 15.0 ± 2.0 °C) were put in pouches, closed with an automatic packaging machine and stored at low temperature. After 15, 30, 60, 90, 120, 180, 240, 300 minutes the residual ozone concentration was determined using a photometer (Hanna Instrument HI 83226-02, Villafranca Padovana, Padova, Italy) and a colorimetric kit for ozone test method with DPD (N,N-diethyl-1,4 phenylene-diamine sulphate) reagent (Hanna instrument HI 93757 Ozone Reagent, Villafranca Padovana, Padova, Italy). The same test was performed with the HM Mozzarella cheese sample inside the pouches.

5.2.4 Inoculum preparation of *Pseudomonas fluorescens* and *P. putida* strains (2nd phase)

P. fluorescens (DSA 12) and P. putida (DSA 13), previously isolated from HM Mozzarella cheese and identified by sequencing of 16S rRNA and alignment in GenBank with the Blast program (Altschul et al., 1997) to determine the closest known relatives (Accession Numbers FJ652606.1 and JX010782.1, respectively), were grown for 48 hours at 30 °C in test tubes containing Brain Heart Infusion broth (BHI, Oxoid, Italy). The cell suspensions were then standardized to an optical density at 600 nm [OD600] of 0.1 (Nanodrop, Thermo Scientific, Wilmington, DE, USA), corresponding to a cell

concentration of 10^7 colony forming units cfu/mL, as confirmed by plating count of the serial diluted suspensions (saline - peptone water, 9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy) on Pseudomonas Agar base (Oxoid, Milan, Italy) supplemented with CFC supplement (Oxoid, Milan, Italy) incubated at 30 °C for 48 h. The two standardized suspensions of *P. putida* and *P. fluorescens* were mixed and used to inoculate the samples as mentioned in paragraph 5.2.1; in particular 10^7 cfu/g and 10^4 cfu/mL for cheese and cooling water respectively. The decision to use a lower concentration for cooling water contamination was based on the fact that this is the frequent level of contamination in real making process.

5.2.5 Microbiological analysis of Mozzarella cheeses and water

Cheese samples were analysed by traditional microbiological methods (*1st, 2nd, 3rd* experimental phases). 10 g of each HM Mozzarella sample were transferred into a sterile stomacher bag, 90 ml of saline-peptone water (9 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy) were added and mixed for 1.5 min in a Stomacher machine (PBI International, Milan, Italy). Further decimal dilutions were made in the same saline – peptone water and the following microbiological analyses were performed in duplicate agar plates: i) total aerobic bacterial count on Gelisate agar (Oxoid, Milan, Italy) incubated for 48–72 h at 30 °C; ii) mesophilic lactic acid bacteria (LAB) on double layer MRS agar (Oxoid, Milan, Italy), incubated at 30 °C for 48 h; iii) *Pseudomonas* spp. on Pseudomonas Agar base (Oxoid, Milan, Italy) supplemented with CFC supplement (Oxoid, Milan, Italy) incubated at 30 °C for 48 h; iv) coliforms and Escherichia coli on ColiID medium (Bio-Merieux, Marcy d'Etoile, France), incubated at 37 °C for 48 h.
Preserving liquid (*1st* experimental phase) and water samples (*4th* experimental phase) were analysed following the same protocol described above; serial dilutions were performed without the need of the first 1:10 dilution in saline-peptone water. Each analysis was conducted in triplicate. After counting means and standard deviations were calculated.

5.2.6 Peroxide value determination

The HM Mozzarella cheese fat was extracted in accordance with the Rose Gottlieb fat extraction method (FIL-IDF 1D, 1986). The determination of peroxide value was then performed on lipid fraction according to the ISO 3976:2006-IDF 74:2006 method. A 0.1-0.2 g test portion of cheese fat was dissolved in a mixture of methanol/1-decanol/n-hexane, then iron (II) chloride and ammonium thiocyanate are added. The peroxides oxidize the iron(II) which forms a red iron (III) complex with the ammonium thiocyanate. The amount of substance is calculated from a photometric determination of the red iron (III) complex at 510 nm, after 10 minutes of reaction, using a UV-VIS spectrophotometer (Shimadzu, UV-Vis Spectro Photometer UV-2501 PC, Kyoto, Japan). The peroxide value is expressed as millimoles of oxygen per kilogram of sample.

5.2.7 Thiobarbituric acid test

The thiobarbituric acid test (TBARS) measures malondialdehyde and other reactive substances in the sample and was performed according to Shan et al. (2011). 10 g of sample was blended with 50 mL of deionised water, followed by addition of 10 mL of 15% trichloroacetic acid using a Stomacher machine (PBI International, Milan, Italy) for 2 minutes. The homogenate was centrifuged at 1500*g* for 5 minutes, and the supernatant was filtered through a Durapore 0.45 µm (pore size) HV membrane filter (Millipore Corporation, Bedford, MA 01730 USA). Two millilitres of the 60 mM thiobarbituric acid reagent was added to 8 mL of the clear filtrate. The solution was vortex-mixed for 15 seconds and then heated in a boiling water bath for 10 minutes to develop a pink colour. After cooling on ice, the supernatant was kept at room temperature and the absorbance was measured spectrophotometrically at 532 nm using a UV-VIS spectrophotometer (Shimadzu, UV-Vis Spectro Photometer UV-2501 PC, Kyoto, Japan). The TBARS value was expressed as mg of malondialdehyde (MDA) per kilogram of sample and the concentration of MDA was calculated on the basis of standard curve obtained using serial dilutions of 1,1,3,3–tetramethoxypropane solution (Sigma Aldrich, Milan, Italy).

5.2.8 Statistical analysis

All analysis were performed twice on three replicates for each experimental phase. The data shown are therefore the averages of the six values obtained. One-way analysis of variance was carried out and differences among means were assessed by using the Tukey's multiple comparison test (STATISTICA for Windows, 5.1, Statsoft Inc, Cary, NC, USA). Means were significantly different at $p \leq 0.05$.

5.3. Results and discussion

5.3.1 Antimicrobial effectiveness of ozone

In the *1st* experimental phase, HM Mozzarella cheese samples were packed in ozonated preserving liquid using a plastic film to high barrier to gas exchanges (polylaminate PET-PE/EVOH/PE).

As shown in Table 5.1, where the microbiological load of untreated and ozone-treated samples as a function of storage time is reported, untreated samples (control samples) at the time of packaging (0 days) resulted already contaminated with *Pseudomonas* spp. bacteria that during storage multiplies to achieve the concentration of 7.85 Log cfu/g after 21 days. Also coliforms increased during cold storage, reaching, at the end of the storage, values of unmarketable food.

Table 5. 1 Effect of the storage time in ozonated water on the reduction of bacteria population of HM Mozzarella cheese (*1st* experimental phase).

	Sample	**Bacteria Population in HM Mozzarella cheese (Log cfu/g)**			
		Storage time (days)			
		0	7	15	21
TMC	Untreated water	4.65 ± 0.12[a]	6.84 ± 0.02[a]	8.08 ± 0.32[a]	8.82 ± 0.09[a]
	Ozone-treated water	4.85 ± 0.17[a]	7.04 ± 0.22[a]	7.73 ± 0.15[a]	8.83 ± 0.32[a]
***Pseudomonas* spp.**	Untreated water	2.00 ± 0.12[a]	7.08 ± 0.80[a]	7.43 ± 0.22[a]	7.85 ± 0.23[a]
	Ozone-treated water	2.00 ± 0.11[a]	7.18 ± 0.45[a]	7.65 ± 0.38[a]	7.76 ± 0.09[a]
Lactic acid bacteria	Untreated water	3.97 ± 0.72[a]	6.78 ± 0.52[a]	8.15 ± 0.42[a]	8.73 ± 0.05[a]
	Ozone-treated water	4.26 ± 0.62[a]	7.08 ± 0.12[a]	7.43 ± 0.75[a]	7.85 ± 0.10[a]
Coliforms	Untreated water	<1	4.00 ± 0.19[a]	5.37 ± 0.61[a]	7.36 ± 0.18[a]
	Ozone-treated water	<1	4.85 ± 0.32[a]	5.67 ± 0.12[a]	6.84 ± 0.38[a]
E. coli	Untreated water	<1	<1	<1	<1
	Ozone-treated water	<1	<1	<1	<1

a,b Different letters within the same column for the same bacteria and the same time of storage refer to statistically significant differences (Student t test, $p \leq 0.05$)

Furthermore, it is evident that the experimental samples packaged in ozonated water were not significantly different from the control untreated samples (Student t test, $p>0.05$), at each one of the considered sampling points (0, 7, 15 and 21 days of storage). This result may be due to the high content of organic compounds that limits the effectiveness of ozone on microorganisms that are present on the surface of the HM Mozzarella cheese. In fact, presence of organic substances with high ozone demand may compete with microorganism for ozone (Khadre et al., 2001) and the type of organic material, rather than its amount in food, can influence the effectiveness of the treatment with ozone (Restaino et al., 1995; Patil et al., 2009). Guzel-Seydim et al. (2004) affirmed that the presence of food components such as caseinate in whipping cream provided a high level of protection to the bacterial populations against ozone treatment. Then, the poor effectiveness of packaging of the HM Mozzarella cheeses with ozonated preserving liquid is probably linked to the rapid decay of ozone inside the package, which would not have the time to act on the microbial cell. In order to support this hypothesis, the residual ozone concentration in water packaged with and without cheese samples was evaluated (Figure 5.3).

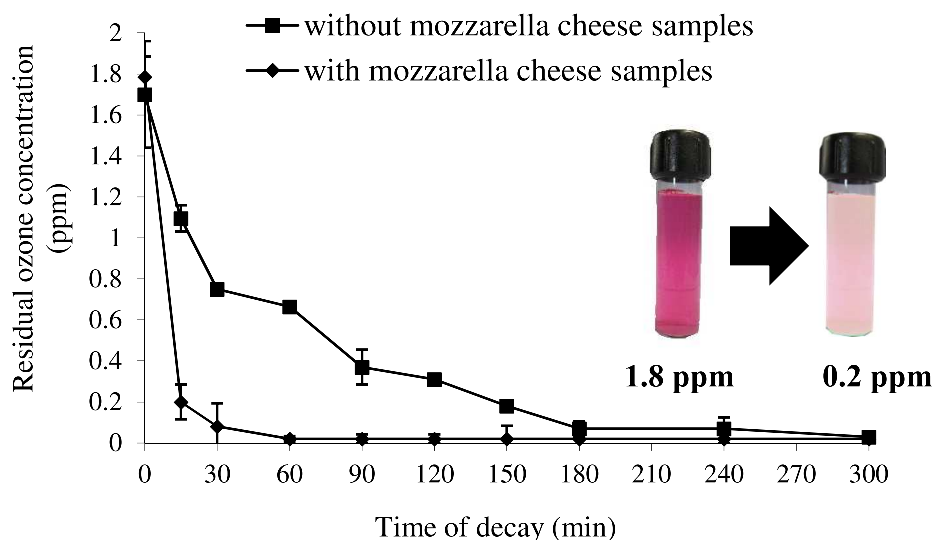

Figure 5. 3 Residual ozone concentration in polylaminate PET-PE/EVOH/PE packaging with and without Mozzarella cheese sample as a function of time

In absence of cheese ozone concentration inside the package was reduced by half after about 30 minutes and under 0.2 ppm after 3 hours. Decomposition of ozone in presence of HM Mozzarella cheese was even fast, in agreement with data previously reported by Kim et al. (2003).

It is known that very long exposures to high concentrations of ozone can lead to changes of the structure and barrier properties of the packaging materials (Ozen et al., 2002; Karaca and Smilanick, 2011). However, in our experimental conditions the concentration of the solubilised ozone in water was too low and the time of contact was too short to influence the properties of packaging and modify the kinetics of decay of ozone in water (Ozen et al., 2002). Thus, we believe that the decay of ozone was not due to its passage in a gaseous phase through the plastic film, but only to its chemical decomposition in water, that is really fast in presence of organic material (Kilpatrick et al., 1955; Staehelin and Hoigné, 1985; von Gunten, 2003). The action of ozone on organic and inorganic compounds in aqueous solution is expressed in two pathways: the direct reaction of ozone on the organic compounds and the reaction of radicals that are formed by the decomposition of ozone, which acts on the organic matrix (Staehelin and Hoigné, 1985; Khadre et al., 2001).

In our experimental conditions the concentration of the solubilised ozone in water was too low and the time of contact was too short to reduce the growth of microorganisms during storage.

For this reason, in the 2*nd* experimental phase, the ozone in the aqueous solution normally employed to cool the curd at the end of HM Mozzarella cheese process was applied. HM Mozzarella cheeses sampled at the end of the shaping phase were first inoculated on the

surface with *Pseudomonas* spp. Untreated samples were compared to those that were treated with ozonated water at concentration of 2, 5, and 10 ppm for 60 minutes (Table 5.2). The treatment duration was chosen taking into account that the cooling phase has an average term of about 40 minutes; therefore, it was considered that prolonging this stage for more than 60 minutes is incompatible with the real production process of this product. From the data reported in Table 5.2, it is clearly evident that none of the used experimental conditions were significantly able to reduce the microbiological load of the Mozzarella's surface.

In *3rd* experimental phase*,* samples of HM Mozzarella cheese were taken at the end of cooling phase and treated with gaseous ozone at concentrations of 10, 20, and 30 ppm for 30 minutes (Table 5.3), simulating a possible treatment before packaging.

Table 5. 2 Effect of ozone concentration of the cooling water on the reduction of bacteria population of HM Mozzarella cheese sampled after forming step and inoculated with *Pseudomonas* spp (*2nd* experimental phase).

	Bacteria population in HM Mozzarella cheese (Log cfu/g)				
	Untreated Non-inoculated sample	**Untreated inoculated sample**	**Ozone-treated inoculated sample**		
			2 ppm	**5 ppm**	**10 ppm**
TMC	3.64 ± 0.39	7.62 ± 0.32^{a}	7.49 ± 0.15^{a}	6.95 ± 0.32^{a}	7.92 ± 0.23^{a}
***Pseudomonas* spp.**	2.00 ± 0.05	7.38 ± 0.35^{ab}	7.79 ± 0.20^{a}	6.82 ± 0.21^{b}	6.85 ± 0.21^{b}
Lactic acid bacteria	5.10 ± 0.29	5.42 ± 0.28^{a}	5.50 ± 0.13^{a}	5.47 ± 0.29^{a}	5.55 ± 0.01^{a}
Coliforms	< 1	< 1	< 1	< 1	< 1
E. coli	< 1	< 1	< 1	< 1	< 1

a,b Different letters within the same row refer to statistically significant differences (Tukey test, p ≤0.05)

Table 5. 3 Effect of gaseous ozone treatment for 30 minutes at different concentrations on the reduction of bacteria population of HM Mozzarella cheese (*3rd* experimental phase).

	Bacteria population in HM Mozzarella cheese (Log cfu/g)			
	Untreated sample	**Ozone-treated sample**		
		10 ppm	**20 ppm**	**30 ppm**
TMC	7.20 ± 0.32^{a}	7.49 ± 0.45^{a}	6.90 ± 0.35^{a}	7.92 ± 0.85^{a}
***Pseudomonas* spp.**	6.38 ± 0.37^{a}	6.79 ± 0.22^{a}	6.82 ± 0.31^{a}	6.52 ± 0.25^{a}
Lactic acid bacteria	4.42 ± 0.28^{a}	4.60 ± 0.13^{a}	4.47 ± 0.19^{a}	4.55 ± 0.08^{a}
Coliforms	< 1	< 1	< 1	< 1
E. coli	< 1	< 1	< 1	< 1

a,b Different letters within the same row refer to statistically significant differences (Tukey test, p ≤0.05)

The control sample was characterized by the presence of a high total microbial load and by a strong contamination whit *Pseudomonas* spp. that probably can be attributed to an improper control of critical points of contamination during the process in this specific batch production. Also the treatment with gaseous ozone is not proved to be suitable to reduce the present microorganisms, even at the highest concentrations of treatment. Considering the maximum concentration of 30 ppm, the test was repeated extending the treatment duration at 60 and 120 minutes. Also in these conditions the desired microbiological effect was not reached (Table 5.4).

Table 5. 4 Effect of gaseous ozone treatment at a concentration of 30 ppm at different times on the reduction of bacteria population of HM Mozzarella cheese (*3rd* experimental phase).

	Bacteria population in HM Mozzarella cheese (Log cfu/g)		
	Untreated sample	**Ozone-treated sample**	
		60 min	**120 min**
TMC	6.80 ± 0.32[a]	6.59 ± 0.25[a]	6.01 ± 0.39[a]
***Pseudomonas* spp.**	5.98 ± 0.37[a]	5.79 ± 0.22[a]	6.10 ± 0.41[a]
Lactic acid bacteria	4.52 ± 0.28[a]	3.98 ± 0.13[a]	4.47 ± 0.39[a]
Coliforms	< 1	< 1	< 1
E. coli	< 1	< 1	< 1

a,b Different letters within the same row refer to statistically significant differences (Tukey test, $p \leq 0.05$)

As far as the microbiological counts of the control samples are concerned, it can be noticed that the HM Mozzarella cheese samples resulted heavily contaminated after the cooling phases in each one of the experimental phases (Tables 5.1, 5.3 and 5.4). Most probably, this contamination, especially by *Pseudomonas* spp., comes from the water employed for the cooling of the shaped HM Mozzarella cheese that in small and local dairy farms very often is an important source of product contamination. The microorganisms initially present in the water found then in Mozzarella cheese an ideal environment for their growth. Preliminary microbiological evaluation on tap water showed average values of 3 Log cfu/mL of both total mesophilic count and *Pseudomonas* spp. At the contrary, coliforms and *E. coli* were never found. Moreover, the European legislation on the quality of the water intended for human consumption (Council Directive 98/83/EC) does not set specific limits for the presence of *Pseudomonas* spp. in water used in any food-production undertaking for the manufacture, processing, preservation or marketing of products. The evaluation of *Pseudomonas aeruginosa* is necessary only in the case of water offered for sale in bottles or containers. On the other hand, *E coli* and enterococci should always be absent in tap water.

In the light of these considerations, in the 4*th* experimental phase the treatment of the sole water destined to contact with food was considered. The water was previously

contaminated with *Pseudomonas* spp. (10^4 cfu/mL). At an ozone concentration of 2 ppm achieved in less than 5 minutes it was possible to obtain a reduction of 4 Log of the inoculated *Pseudomonas* spp. The positive effects obtained with water are supported by previous works carried out on water, waste water and drinking water (Selma et al., 2007; Selma et al., 2008; Restaino et al., 1995). Furthermore, efficacy of ozone is demonstrated more readily when microorganisms are suspended and treated in pure water or simply in buffers (which lows ozone demand) than in complex systems such as food (Khadre et al., 2001).

The pre-treated water was used in the cooling phase of HM Mozzarella. In Table 5.5 the microbiological data of the Mozzarella cheeses cooled with pre-treated water were compared with those of Mozzarella cheeses cooled in untreated water. As can be noted, the control cheeses cooled with untreated water were already contaminated with *Pseudomonas* spp after the packaging (0 days). Then, during storage this spoilage microorganisms increase causing a reduction in the quality of the product. This contamination was not present in experimental Mozzarella cheeses cooled in water pre-treated with ozone. In addition, even after 21 days of storage these cheeses had low microbial load and a good state of preservation.

Table 5. 5 Effect of storage time on bacteria population of HM Mozzarella cheese (*4th* experimental phase) cooled in water pre-treated with ozone at concentration of 2 ppm.

	Sample	Bacteria Population in HM Mozzarella cheese (Log cfu/g)			
		Storage time (days)			
		0	7	15	21
TMC	Untreated water	3.44 ± 0.10[a]	5.83 ± 0.06[a]	7.36 ± 0.24[a]	8.24 ± 0.15[a]
	Ozone-treated water	3.85 ± 0.17[a]	3.78 ± 0.32[b]	4.62 ± 0.12[b]	4.66 ± 0.32[b]
Pseudomonas spp.	Untreated water	2.29 ± 0.14[c]	4.08 ± 0.80[b]	7.43 ± 0.22[a]	7.85 ± 0.23[a]
	Ozone-treated water	< 1	< 1	1.65 ± 0.28[b]	1.76 ± 0.18[b]
Lactic acid bacteria	Untreated water	3.96 ± 0.72[a]	5.28 ± 0.42[a]	7.95 ± 0.32[a]	8.45 ± 0.22[a]
	Ozone-treated water	3.33 ± 0.62[a]	3.28 ± 0.22[b]	5.43 ± 0.25[b]	5.99 ± 0.12[b]
Coliforms	Untreated water	<1	<1	<1	1.64 ± 0.13
	Ozone-treated water	<1	<1	<1	<1
E. coli	Untreated water	<1	<1	<1	<1
	Ozone-treated water	<1	<1	<1	<1

a,b Different letters within the same column for the same bacteria and the same time of storage refer to statistically significant differences (Student t test, $p \leq 0.05$)

5.3.2 Influence of ozone on lipid oxidation products

Ozone is characterized by high oxidant potential (2.07 V), a value greater than chlorine, and one of the consequence of its use is a possible oxidative process to lipid fraction, especially in a long time treatment and high level of ozone. The HM Mozzarella cheese samples considered in this study showed the following compositional characteristics: dry matter 35.40 g/100 g of cheese; protein 46.89 g/100 g of dry matter; fat 39.15 g/100 g of dry matter. As can be seen, HM Mozzarella is a cheese characterized by a relatively high fat content. For this reason, the amount of primary and secondary lipid oxidation products in HM Mozzarella cheeses was evaluated.

In the *1st* experimental phase, the high barrier plastic film was used also to avoid further oxidation processes of samples due to the transfer of oxygen inside the pouches. In this way it was possible to evaluate the sole effect of ozone on lipid component in packaged HM Mozzarella cheese.

As shown in Figure 5.4, the peroxide values of ozone-treated HM Mozzarella cheese samples, when compared with the untreated samples, showed no significant differences during storage. However, low peroxide values (primary oxidation products) could mean

high values of secondary products (aldehydes, ketones). For this reason also the thiobarbituric acid and reactive substances (TBARS) test was carried out. In all cases TBARS value remained constant, in accordance with the rapid decay of ozone previously reported.

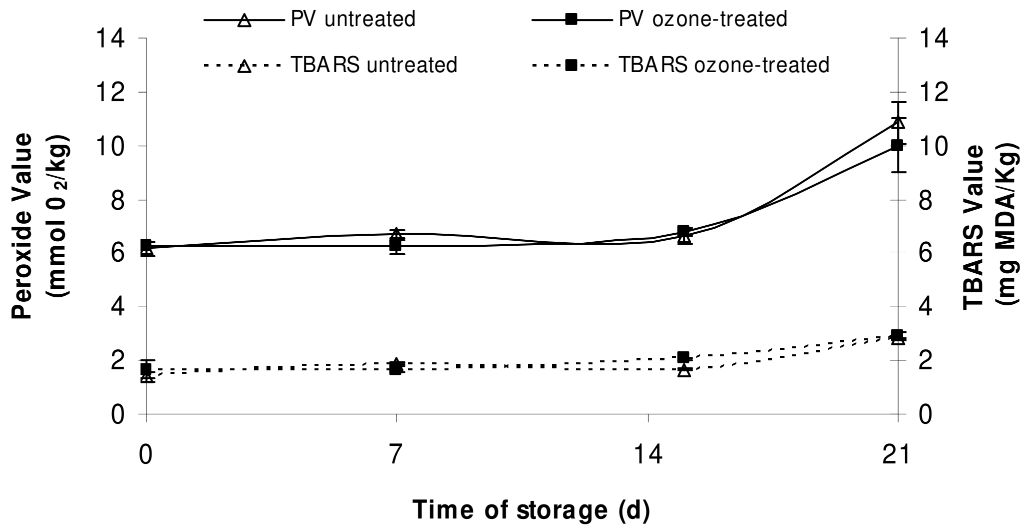

Figure 5. 4 Peroxide and TBARS values of untreated and ozone-treated samples as a function of ozone storage time

According to Crowe et al. (2012) it is reasonable that at the tested ozone concentration there was no significant effect of treatment. These Authors found that the aqueous ozone spray concentration (1 and 1.5 mg/L) and number of passes under the spray nozzle (1, 2 and 3) on salmon fillets did not achieve high levels of TBARS values.

Increasing the concentration of ozone in aqueous solution and maintaining it constant for a long time (*2nd* experimental phase), you might expect an increase of oxidative processes. The effect of aqueous ozone treatments at different concentration on peroxide and TBARS values of HM Mozzarella cheese samples were reported in Table 5.6. No statistically significant differences between untreated and treated samples (with 2, 5 and 10 ppm ozone) were obtained. Also observing the values of peroxides and TBARS obtained for the untreated and gas ozone-treated samples at maximum concentration of 30 mg/m^3 for different treatment time (*3rd* experimental phase) no statistically significant differences were found in all cases (Table 5.6).

Table 5. 6 Effects of aqueous ozone treatments (60 min) at different concentrations on Peroxide value (mmol 0_2/Kg) and TBARS value (mg MDA/Kg) of HM Mozzarella cheese samples (*2nd* experimental phase).

	Ozone concentration (ppm)	**Peroxide value mmol 0_2/Kg**	**TBARS value mg MDA/Kg**
Untreated sample	-	5.99 ± 0.29^a	2.35 ± 0.30^a
Ozone-treated sample	2	6.28 ± 0.34^a	2.45 ± 0.12^a
	5	5.94 ± 0.01^a	2.73 ± 0.40^a
	10	6.32 ± 0.24^a	2.35 ± 0.29^a

a,b Different letters within the same column refer to statistically significant differences (Tukey test, $p \leq 0.05$)

Table 5. 7 Effect of maximum gaseous ozone concentration (30 ppm) at different treatment times on Peroxide value (mmol 0_2/Kg) and TBARS value (mg MDA/Kg) of HM Mozzarella cheese samples (*3rd* experimental phase).

	Treatment time (min)	**Peroxide value mmol 0_2/Kg**	**TBARS value mg MDA/Kg**
Untreated sample	-	5.34 ± 0.07^a	2.51 ± 0.32^a
Ozone-treated sample	30	5.02 ± 0.84^a	2.39 ± 0.45^a
	60	5.50 ± 0.49^a	2.01 ± 0.18^a
	120	5.90 ± 0.54^a	2.26 ± 0.58^a

a,b Different letters within the same column refer to statistically significant differences (Tukey test, $p \leq 0.05$)

Gaseous ozone has better penetrability especially when applied under pressure and low temperatures (Kim et al., 2003). Fuhrmann et al. (2010) evaluated the effect of gaseous ozone treatment (50 mL/L per 60 minutes) on egg components. They obtained significant alterations of egg components and also at lower ozone doses (10 and 25 mL/L) the oxidative processes occurred mainly at the egg surface. Differently from these Authors, our data demonstrated that high level of gaseous ozone (30 ppm) for a time maximum of 120 minutes did not lead to a considerable increase in oxidative processes in HM Mozzarella cheese samples.

5.4. Conclusions

For the first time in literature, this work aimed to investigate the use of ozone, as a gas or aqueous solution, throughout the HM Mozzarella cheese processing in order to find a better step where it can express the higher bactericidal activity against spoilage microorganisms.
Results show that ozone cannot be applied for the recovery of the product if it already has high microbial load or when HM Mozzarella cheese floats in water containing microorganisms. However, in these cases ozone oxidant activity was ineffective in causing damage to lipids.
Ozone was found to reduce dramatically microbial contamination of tap water that is, after the stretching step, the first and most important source of HM Mozzarella cheese contamination. Since tap water is used in many steps of HM Mozzarella cheese processing, the ozonation of water used in dairies could be useful in reducing the contamination of this cheese with spoilage bacteria in several steps of the manufacturing process.

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Chapter 6

Preliminary data on reducing microbial load of cheese brines by means of ozone

ABSTRACT

This work aimed to reduce microbial load of cheese brine contaminated by the reuse. Different ozone concentrations were tested: 0.20 ± 0.02, 0.40 ± 0.02 and 2.00 ± 0.20 ppm and the samples were treated for 30 and 60 minutes to obtain a 1-Log reduction for the first and second concentrations and a 5-Log reduction for the third one. Considering the intermediate concentration (0.40 ± 0.2 ppm), the treatment time was extended up to 240 min to obtain a 4-Log reduction of total microbial count (TMC) and other microorganisms considered. The longer the treatment time, the better the sanitisation effect.
Finally, cheese brine samples with different protein contents (from 0.20 to 1.30 g/100 g) were treated with 0.40 ± 0.02 ppm for 240 minutes obtaining different levels of microbial inactivation: the higher the protein content, the lower the microbial reduction .

6.1. Introduction

Salting by immersion in brine is used for many varieties of cheese and is a common way to achieve uniform salt penetration and brining time. The difference in osmotic pressure between brine and cheese causes the expulsion of water and other components from cheese, such as whey proteins, lactic acid and minerals in exchange for sodium chloride (Guinee, 2004). Previous researches have shown that cheese brine can contain different bacteria populations (Alessandria et al., 2010; Ingham et al., 2000; Ingham et al., 1997; Larson et al., 1999; Seiler and Busse, 1990). The microbial load of brine must be kept under control to avoid quality defects in cheese, e.g. slimy surface, formation of pigments and surface discolouration (Guinee and Fox, 2004; Brendel-Thimmel et al., 2010).

The brine contamination can mainly occur via cheese made from unpasteurized milk, improperly pasteurized milk, or milk contaminated after pasteurization. Furthermore, the leaching of proteins and other nitrogenous compounds from cheese into the cheese brine may enhance microbial survival. Once cheese brine is contaminated, subsequent batches of brined cheese may also become contaminated (Ingham et al., 2000). The amount of organic compounds as well as the microbial load depend on the type of cheese and the length of time the brine is used. When cheese brines are used for many batches of cheese, they need to be replaced or regenerated in order to remove the present organic compounds, as well as decontaminated the brine through microorganisms, prevent post contamination of cheese during salting and ensure correct balance among salt, pH, acidity, minerals and whey proteins affected by the reuse (Aboyaa and Auboisb, 2000; Brendel-Thimmel et al., 2010).

In practical terms, regeneration is more common than the total removal of cheese brine. First of all, it is well known that the dairy industry produces significant amounts of liquid wastes and has to move, pasteurise or work off large volumes; therefore it makes easier to replace only one part of the brine and add water and salt according to use (Carvalho et al., 2013). Furthermore, a new cheese brine with tap water and salt could lead to quality defects on cheese surfaces (Innocente and Sensidoni, 1999). It seems to be more aggressive and less effective in salting of cheese.

Heat treatment, filtration and microfiltration are still the most used technologies for brine treatment, but all three present many disadvantages: heat treatment requires expensive non corroding heat exchanger materials and the heat can cause protein and calcium phosphate precipitation, while filtration and microfiltration need manual handling and filter regeneration. Other ways to reduce the microbial load are UV light treatment combined with filtration or the addition of chemicals (sodium hypochlorite, sodium or potassium sorbate), but their use is regulated by national laws (Bintsis, 2000; Aboyaa and Auboisb, 2000). In light of these considerations, new approaches should be tested. The FDA's approval of ozone as GRAS as a direct additive in food triggered its use in the food industry (FDA 2003). Furthermore, decomposition to non- toxic products makes it an environmentally safe agent. Ozone has a high oxidizing potential and for this reason, it is a useful disinfecting agent against a broad range of microorganisms, mycotoxins and

pesticides (Restaino et al., 1995; Kim and Yousef, 2000; Graham, 2000; Diao et al., 2013; Ikeura et al., 2011; Tiwari et al., 2010).
Many authors have suggested that ozone solubilized in a liquid media with low organic demand exerts a powerful germicide effect in a few minutes (Segat et al., 2014; Alexandre et al., 2011; Selma et al., 2007; Greene et al., 1999). With these previous studies in mind, the aim of the present work is to test a new method of cheese brine regeneration applying different ozone concentrations directly solubilised into them. Moreover, the germicidal effect of ozone solubilized in cheese brine was evaluated in the presence of different amounts of organic compounds.

6.2. Materials and methods

6.2.1 Sample preparation

Cheese brines were obtained from local dairies. At the time of retrieval, these products had been used for salting different cheeses and, therefore, they had been used for different period of salting (from 1 to 12 months). The cheese brines were kept refrigerated (4 °C) until use.

6.2.2 Composition analysis of cheese brine samples

Total nitrogen, sodium chloride, fat and acidity contents were determined respectively by Kjeldahl method (FIL-IDF 25, 1964), Vohlard method (FIL-IDF 17A, 1972), Röse-Gottlieb method (FIL-IDF 1B, 1983) and by titration with NaOH. The protein content was obtained by multiplying the percent Tot. N by 6.38. For the trials with different protein content, milk proteins were artificially added and solubilised in brines before the ozone treatment (WPC 8000, Natural World srl, Ravenna, Italy). The final protein content in brines was 1.00 and 1.32 g/100 g determined by Kjeldahl method. The pH value was measured with pHmeter at a controlled temperature (Hanna Instruments, mod. pH 301, Villafranca Padovana, Italy).

6.2.3 Ozonation conditions

The samples (approximately 30 L) were thrown into a 100 L- tank that was part of a pilot plant (WaterNow OX-0203WN2K by O3 technology, Brescia, Italy) equipped with ozone generators (total ozone production: 15 g/h) and an inline electrochemical device, monitoring continuously ozone concentration. The gaseous ozone was directly solubilised in cheese brines with a bubble diffuser located at the bottom of the tank. The pilot plant was connected with a chiller to maintain the brine temperature at 12 ± 3 °C during the treatments. The samples for analysis were taken in sterilised conditions through a faucet. A schematic representation of the pilot plat is shown in Figure 6.1 and Figure 6.2.
The ozone concentrations tested were 0.20 ± 0.02, 0.40 ± 0.02 ppm and 2.00 ± 0.20 ppm for 30, 60, 120, 180, 240 minutes of treatment.

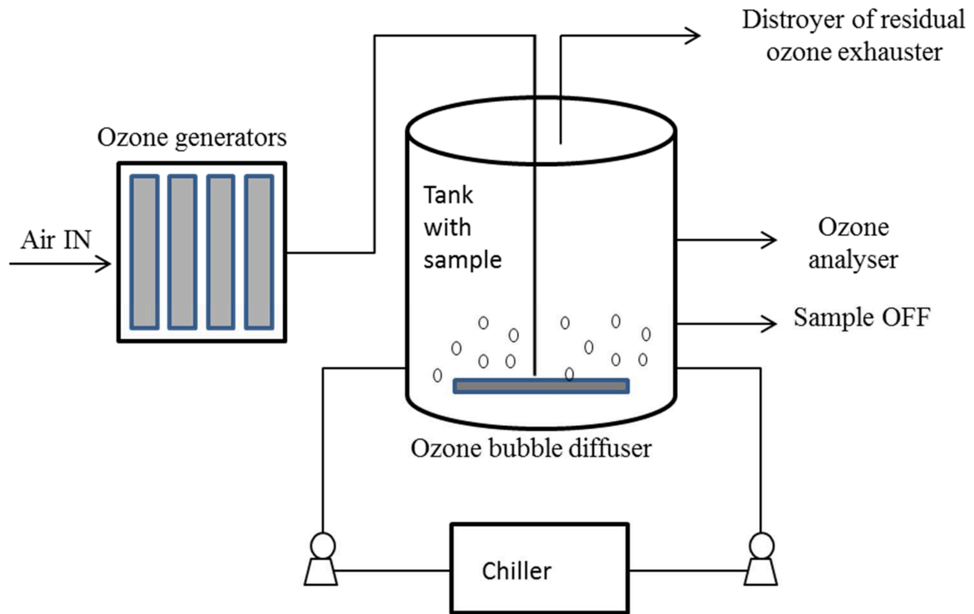


Figure 6. 1 Schematic diagram of the ozone pilot plant for cheese brine treatments

Figure 6. 2 Ozone analyser and tank with sample, from the bottom to the top respectively

6.2.4 Microbiological analysis of cheese brine samples

0.1 mL of each brine sample were transferred into a sterile stomacher bag, 9 ml of saline-peptone water (8.5 g/L NaCl, 1 g/L bacteriological peptone, Oxoid, Milan, Italy) were added and mixed. Further decimal dilutions were made and the following microbiological analyses were performed in duplicate: total mesophilic count on PCA (Plate Count Agar, Oxoid, Milan, Italy), moulds and yeasts on OGYE (Oxytetracycline Glucose Yeast Extract, Oxoid, Milan, Italy) and staphylococcus and micrococcus on MSA (Mannitol Salt Agar, Oxoid, Milan, Italy) incubated at 30 °C for 48 - 72 hours. 10% w/v of NaCl was added on PCA and OGYE to confirm the maximum recovery of halophilic and halotolerant microorganisms.

6.2.5 Statistical analysis

All the analysis were performed twice on two replicates for each treatment. The data shown are therefore the means of the four values obtained. One-way analysis of variance was carried out and differences among means were assessed by using Tukey's multiple comparison test (STATISTICA for Windows, 5.1, Statsoft Inc, Cary, NC, USA). The means were significantly different at $p<0.05$.

6.3. Results and discussion

6.3.1 Effect of different ozone concentrations

Gaseous ozone was directly solubilised in cheese brine to reach an ozone concentration of 0.20 ± 0.02, 0.40 ± 0.02 and 2.00 ± 0.20 ppm. The samples were treated for 30 and 60 minutes. The cheese brines considered in this test showed the following compositional characteristics: 0.20 ± 0.001, 0.05 ± 0.012 and 12.73 ± 3.87 g/100 g respectively of proteins, fat and sodium chloride, and 9.86 ± 0.15 °SH/50 mL of acidity and 5.25 pH at 15 °C.
The microbial loads before and after the ozone treatments were reported in table 6.1.

Table 6. 1 Bacteria populations of cheese brine samples (Log cfu/mL) treated with ozone at 0.20 ± 0.02, 0.40 ± 0.02 and 2.00 ± 0.20 ppm for 30 e 60 minutes.

	Bacteria population in cheese brine samples (Log cfu/mL)						
		Ozone concentration (ppm)					
	Control	0.20 ± 0.02		0.40 ± 0.02		2.00 ± 0.20	
	0 min	30 min	60 min	30 min	60 min	30 min	60 min
TMC	5.86 ± 0.01^{a}	4.98 ± 0.17^{b}	4.25 ± 0.36^{b}	4.89 ± 0.26^{b}	4.77 ± 0.09^{b}	2.54 ± 0.02^{c}	< 1
Yeast	3.85 ± 0.12^{a}	3.77 ± 0.10^{a}	2.24 ± 1.75^{b}	3.30 ± 0.26^{a}	2.51 ± 0.03^{b}	< 10	< 1
Micrococcus	3.00 ± 0.38^{a}	3.49 ± 1.54^{a}	2.35 ± 0.25^{b}	3.79 ± 0.19^{a}	2.26 ± 0.12^{b}	< 10	< 1
Staphylococcus	4.34 ± 0.07^{a}	4.04 ± 0.45^{a}	2.48 ± 0.62^{b}	4.24 ± 0.90^{a}	3.54 ± 0.19^{b}	< 10	<1

a,b,c,d Different letters for 0, 30 and 60 min in the same row for the same microorganism and for the same ozone concentration refer to statistically significant differences (Tukey test, $p \leq 0.05$)

As shown, considering two different concentrations (0.20 and 0.40 ppm), one 2-fold the other, 60 minutes allowed to obtain a 1-Log reduction for all the microorganisms considered, and in our case where the ozone concentration was kept low, there are no differences among the microbial results ($p<0.05$) between 0.20 and 0.40 ppm. Probably, considering the composition and the initial microbial load of the considered matrix, the time and concentrations were not enough to ensure higher microbial reduction. In fact, it is well-known that organic compounds, temperature, humidity, residual ozone, and type of microorganisms could affect the effectiveness of the treatment (Khadre et al., 2001; Cullen et al., 2009).
There are only few studies relating to the use of ozone in the dairy industry, in particular to decontaminate surfaces of equipment, reduce the growth of mould during cheese ripening (Serra et al., 2003; Moore et al., 2000; Greene et al., 1999; Gibson et al., 1960; Morandi et al., 2009) and more recently to improve the microbiological quality of raw milk (Cavalcante et al., 2013) and decontaminate tap water, which is the first and most important source of high moisture Mozzarella cheese contamination during making process (Segat et al., 2014).
Therefore, to the best of our knowledge, no data are available concerning the decontamination of cheese brine by means of ozone and only some research reports that ozone could be applied to reduce microbial load in dairy waste water (Greene et al., 1999;

Guzel-Seydim et al., 2000). In these works the ozone was as effective as chlorine reducing dairy surface bacteria by 99%, and the ozonated water at 10 °C as the pre-rinse technique for dairy equipment removed 84% of dairy soil and 51% in warm water (40 °C). However, differently from these authors, our data showed a weak effect at the applied conditions.
Considering the intermediate concentration (0.40 ± 0.02 ppm), increasing the treatment time up to 240 min led to a 4-Log reduction of TMC. Similar results were also obtained for yeast, micrococcus and staphylococcus. Moulds were not present in any case, according to Kure et al. (2004); their results indicated that the cleaning and disinfection of the equipment and the mycological quality of brine were sufficient to avoid mould development.
Increasing ozone concentration by about 10-fold (2.00 ± 0.20 ppm) the germicidal effect was reached in 30 minutes (see Table 6.1). This could mean that to recover contaminated cheese brine, it is possible to both increase time and ozone concentration, depending on the microbial load and organic matter present.

6.3.2 Effect of different protein concentrations

It is well-known that cheese brine may have different compositions depending on the type of cheese that is immersed for salting, brining time and reuse. The germicidal effect of ozone on cheese brines with different composition, in particular different rates of protein has been also evaluated. The cheese brines considered in these tests showed the following compositional characteristics: 0.091 ± 0.031 and 11.25 ± 2.83 g/100 g of fat and sodium chloride respectively, 9.85 ± 1.00 °SH/50 mL of acidity, 5.19 ± 0.20 pH value (at 15 °C). All the samples differed only for their protein content ($p<0.05$), which was 0.20, 0.46, 1.00 and 1.32 g/100 g, naturally present or artificially added prior to the treatments (see paragraph 2.2). The microbial results on TMC after keeping low the ozone concentration (0.40 ± 0.02 ppm) and prolonging time up to 240 min, are reported in Table 6.2.

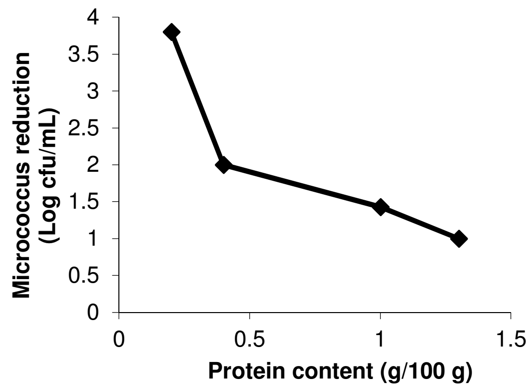
Table 6. 2 Total microbial count of cheese brines at different protein contents as a function of treatment time (ozone concentration: 0.40 ± 0.02 ppm)

Protein Content (g/100 g)	Total Microbial Count (Log cfu/mL)					
	Time of treatment (min)					
	0	30	60	120	180	240
0.20	5.69 ± 0.55^{a}	4.89 ± 0.26^{b}	4.77 ± 0.09^{b}	3.42 ± 0.05^{c}	2.71 ± 0.05^{d}	1.47 ± 0.18^{e}
0.46	5.08 ± 0.04^{a}	4.41 ± 0.37^{b}	4.18 ± 0.06^{b}	3.13 ± 0.29^{c}	2.52 ± 0.01^{d}	2.04 ± 0.03^{e}
1.00	5.72 ± 0.05^{a}	5.10 ± 0.05^{a}	5.25 ± 0.54^{a}	4.65 ± 0.10^{b}	4.01 ± 0.70^{b}	4.22 ± 0.01^{b}
1.30	6.76 ± 0.02^{a}	6.71± 0.13^{a}	6.88 ± 0.04^{a}	4.95 ± 0.22^{b}	4.93 ±0.07^{b}	4.92 ± 0.01^{b}

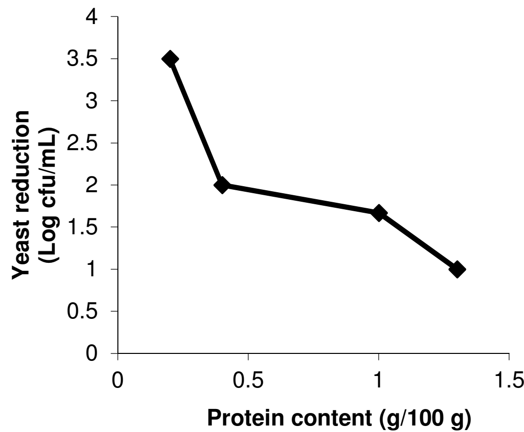
a,b,c,d Different letters within the same row refer to statistically significant differences (Tukey test, $p \leq 0.05$)

As shown, the addition of proteins to cheese brines reduced the effect of the ozone treatment, according to Guzel-Seydim et al., 2004). The microbial reduction became progressively slow with increasing protein content. The 240-min treatment achieved only a 1.5-Log reduction of TMB in brine containing 1.00 and 1.30 g/100 g of proteins. Similar trends were also observed for the other microorganisms considered.

Ozone inactivates microorganisms by oxidizing essential cell components such as enzymes, proteins, nucleic acids, and then causes cell lysis (Kim and Yousef, 2000; Restaino et al., 1995). Yeast, staphylococcus and micrococcus showed different sensitivity to ozone. Considering the maximum treatment time (240 min),the microbial Log reduction for the microorganisms considered is presented in Figure 6.2.



A)



B)

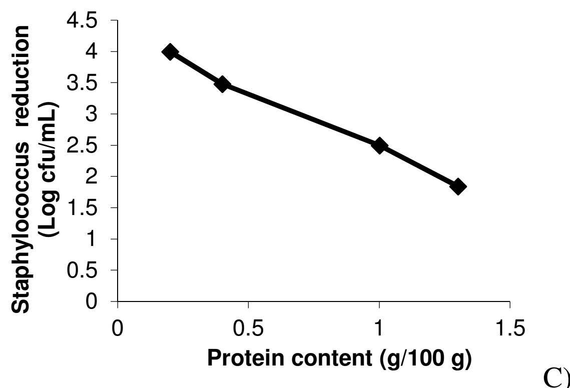


Figure 6. 3 Microbial Log reduction of A) yeast B) micrococcus and C) staphylococcus in cheese brine treated at 0.40 ± 0.02 ppm of ozone for 240 minutes.

The Figures shows how the *staphylococcus* and micrococcus are more sensitive to ozone than the yeast. This could be attributed to a longer lag phase of yeast or its different morphology when compared with others. Moreover, it seems that the staphylococcus population reduction was well correlated with the applied ozone concentration (R^2=0.9944, $p<0.05$), and the higher susceptibility to ozone of this microorganism could be due to its different level of adaptation to the environment. .
However, further studies are necessary in order to better investigate what happens in the presence of other pathogenic microorganisms, very often able to contaminate cheese brines, but these preliminary data suggest that it is possible to reduce the bacterial load of cheese brine in a few hours by applying a very low ozone concentration.

6.4. Conclusions

These preliminary data show how ozone could be applied to decontaminate cheese brines populated by mesophilic, micrococcus, staphylococcus bacteria and yeast. For the first time it is reported in the literature that gaseous ozone has been directly solubilised in cheese brine at three concentrations achieving different decontamination levels. It is well-known that cheese brines can be full of organic compounds such as whey proteins, fat, acid lactic, minerals. The amount can depend on the type of brined cheese and the time of use, and this work shows how even in the presence of a high protein amount (>1.0 g/100 g), low ozone concentration (0.40 ± 0.02 ppm) was enough to obtain a 1.5-Log reduction of TMC in 240 minutes.
Further studies will be developed in order to find the best treatment conditions to achieve the chemical regeneration of brines, as well.

6.5. References

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General conclusions and future trends

Plasma generation at atmospheric pressure and ozone technology brings both commercial and technical relevance to the food industry because they do not require extreme processing conditions.
The findings of this PhD thesis confirmed that, as emerging novel food processes, ACP and ozone in the dairy field could be applied to the development of gentle and targeted effects in order to improve the quality and safety of foods as well as to provide new process options.
For the first time in the scientific literature, changes in milk proteins were explored by means of ACP and a high concentration of ozone with satisfactory results in functionality improvement and enzyme activity inactivation. Moreover, the applicability of ozone solubilized in water contaminated by microorganisms in different steps of cheese production has been confirmed to also reduce the microbial load within few minutes.
Time and voltage/concentration must be kept under control and modulated according to the different aims and products, especially when the food is likely to undergo oxidation. Furthermore, the negative effects can be avoided by modifying the composition of gas in which the plasma is induced or changing the physical state (gas or liquid) in the case of ozone.
Both technologies can be considered versatile, and as a general remark, future studies should be oriented towards real foods or more complex matrices, delving further into the effects and interactions with different gases. Also, exploring the hurdle technology concept for a synergistic effect with other technologies is recommended.

Publications inherent to the PhD activities

- **Segat A**., Biasutti M., Iacumin L., Comi G., Baruzzi F., Carboni C., Innocente N. (2014). Use of ozone in production chain of high moisture Mozzarella cheese. *LWT-Food Science and Technology,* 55, 513-520
- **Segat A**., Misra N.N., Fabbro A., Buchini F., Lippe G., Cullen PJ., Innocente N (2014). Effects of ozone processing on chemical, structural and functional properties of whey protein isolate. *Food Research International,* 66, 365-372
- Innocente N., **Segat A**., Manzocco L., Marino M., Maifreni M., Bartolomeoli I., Ignat A., Nicoli M.C. (2014). Effect of pulsed light on total microbial count and alkaline phosphatase activity of raw milk. *International Dairy Journal,* 39, 108-112
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- **Segat A**., Marino M., Bartolomeoli I., Sepulcri C., Maifreni M., Innocente N. (2015). Antimicrobial effect of ozone in used brines for cheesemaking. *Food Research International,* in submission

Vita

Annalisa Segat was born on October 14th 1986 in Latisana (Udine - IT). After the high school diploma (Technic of Tourism) in 2005, she started the University of Udine studying Food Science and Technology. She had her Bachelor's and Master's degrees in 2009 and 2011, respectively. Shortly afterwards, she won the PhD position for the XXVII Cycle in Food Science that started in January 2012 and ended with this PhD thesis. During the PhD, she spent six month in Dublin as visiting student at Dublin Institute of Technology to develop a part of the PhD project. She is fist author and co-author of many scientific articles and book chapters.

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Annalisa